Jembrana Disease and the Bovine Lentiviruses

Proceedings of a workshop 10–13 June 1996 Bali, Indonesia

Editors: G.E. Wilcox, S. Soeharsono, D.M.N. Dharma and J.W. Copland

Hosted by: Direktorat Jenderal Petermakan and the Bali Cattle Disease Investigation Unit

Sponsored by: Australian Centre for International Agricultural Research in association with Direktorat Jenderal Petermakan (Indonesia)

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Wilcox, G.E., Soeharsono, S., Dharma, D.M.N. and Copland, J.W. 1997.
Jembrana Disease and the Bovine Lentiviruses.
Proceedings of workshop 10–13 June 1996 Bali, Indonesia. ACIAR Proceedings No. 75, 191 p.

ISBN 1 86320 197 1

Editorial management: P.W. Lynch
Production editor: P.J. Knight
Typesetting and page layout: Sun Photoset Pty Ltd, Brisbane, Australia
Printed by: Watson Ferguson & Co., Brisbane, Australia

Contents

Welcome and opening address by the Director General of Livestock Services
Ir. Erwin Soetirto **1**

The Occurrence and History of Jembrana Disease in Indonesia
S. Soeharsono and I.G.N. Teken Temadja **2**

Early Observations and Research on Jembrana Disease in Bali and Other Indonesian Islands
S. Ramachandran **5**

Clinical Changes in Bali Cattle and Other Ruminants Following Infection with Jembrana Disease Virus
S. Soeharsono, A. Budiantono, K. Sulistyana, M. Tenaya, N. Hartaningsih, D.M.N. Dharma, M. Soesanto and G.E. Wilcox **10**

The Pathology of Jembrana Disease
D.M.N. Dharma **26**

Bali Cattle: Origins in Indonesia
John Copland **29**

Bali Cattle — Their Economic Importance in Indonesia
S. Wiryosuhanto **34**

The Physicochemical Characteristics of a Virus Associated with Jembrana Disease
G. Kertayadnya, S. Soeharsono, N. Hartaningsih and G.E. Wilcox **43**

Nucleotide Sequence Analysis of Jembrana Disease Virus: A New Bovine Lentivirus
B.J. Chadwick, R.J. Coelen, G.E. Wilcox, L.M. Sammels and G. Kertayadnya **49**

Anti-JDV Monoclonal Antibodies and Immunocytochemical Tests
G. Kertayadnya, K. Sulistyana and A. Budiantono **61**

Detection of Jembrana Disease Virus in Paraffin-Embedded Tissue Sections by In Situ Hybridisation
B.J. Chadwick, M. Desport, D.M.N. Dharma, J. Brownlie and G.E. Wilcox **66**

Current Information on Jembrana Disease Distribution in Indonesia
S. Soeharsono **72**

The Transmission and Persistence of Jembrana Disease Virus in Cattle
S. Soeharsono, A.A. Putra, N. Hartaningsih, K. Sulistyana, M. Tenaya and G.E. Wilcox **76**

Serological Tests for JDV Antibodies and Antibody Response of Infected Cattle
N. Hartaningsih, K. Sulistyana and G.E. Wilcox **79**

Investigation of the Cell-Mediated Immune Response to Jembrana Disease Virus Proteins in Cattle
S. Wareing **83**

The Development and Use of a Vaccine for the Control of Jembrana Disease
N. Hartaningsih, S. Soeharsono, D.M.N. Dharma, G. Kertayadnya, K. Sulistyana, A. Budiantono and G.E. Wilcox **85**

Epidemiological Observations of Jembrana Disease in Bali
A.A. Putra and K. Sulistyana **90**

Jembrana Disease in Lampung
H. Prabowo **96**

Jembrana Disease in West Sumatra
Tazril Tembok and Erinaldi **100**

Biology of the Bovine Immunodeficiency Virus
M.A. Gonda, J.K. Battles, S.E. Fong, D.G. Luther, T.G. Snider, III, K.J. Garvey, L. Feigenbaum and G.J. Tobin **103**

Regulation of Gene Expression in Bovine Lentiviruses
S. Carpenter, E.M. Vaughn and Y. Wannemuehler **110**

The Comparative Pathology of the Lentiviruses
R.S.F. Campbell **115**

Pathogenesis of Bovine Immunodeficiency Virus
S. Carpenter and L.D. Miller **124**

Bovine Lentivirus Infection in Bali Cattle (*Bos javanicus*) Following Inoculation with BIV
C.A. Whetstone, D.L. Suarez, B.A. Pesch, J.A. Harp, J.M. Miller, M.J. Van Der Maaten, B. Read and G.E. Wilcox **128**

Studies of Bovine Lentivirus Infection in Sheep
R.M. Jacobs **133**

Immunological Responses in Cattle (*Bos taurus*) After Inoculation with BIV
C.A. Whetstone, D.L. Suarez, J.M. Miller, B.A. Pesch and J.A. Harp **138**

Molecular Studies on BIV Infection and its Interaction with Other Bovine Viruses
Charles Wood, Harish Minocha and Yunqi Geng **144**

Expression of Recombinant Jembrana Disease Virus Env Protein in *Escherichia coli*
E. Burkala, I. Narayani and G.E. Wilcox **150**

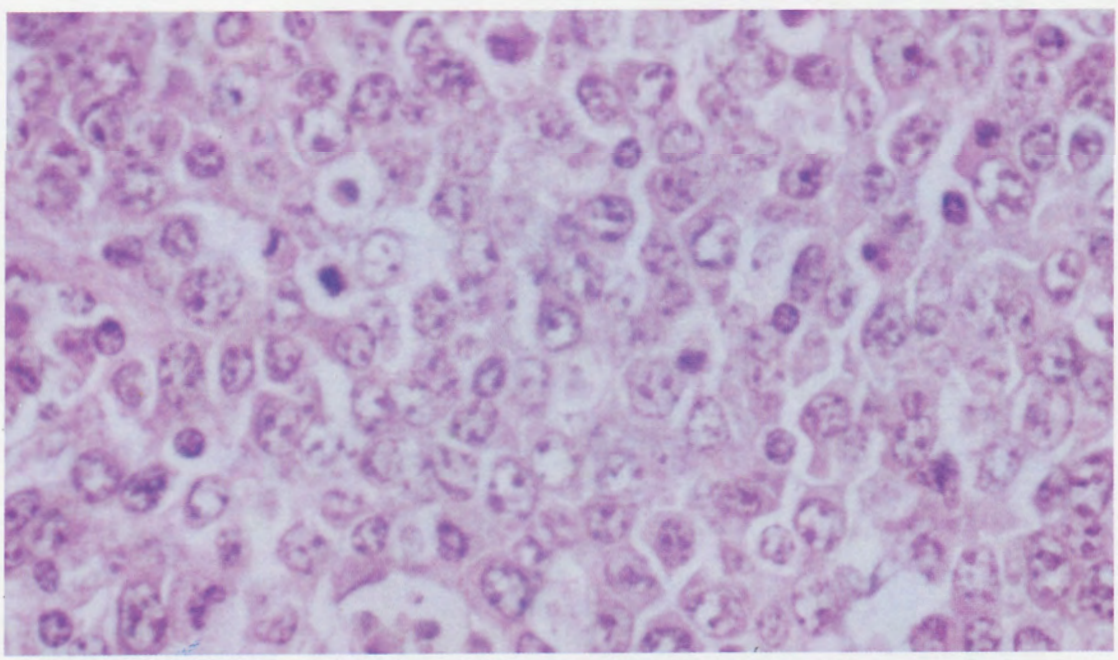
Cloning and Expression of Jembrana Disease Virus (JDV) in Yeast *Pichia pastoris*
Inna Narayani, E. Burkala and G.E. Wilcox **152**

Studies on bovine lentivirus infections in the U.K.
J. Brownlie **154**

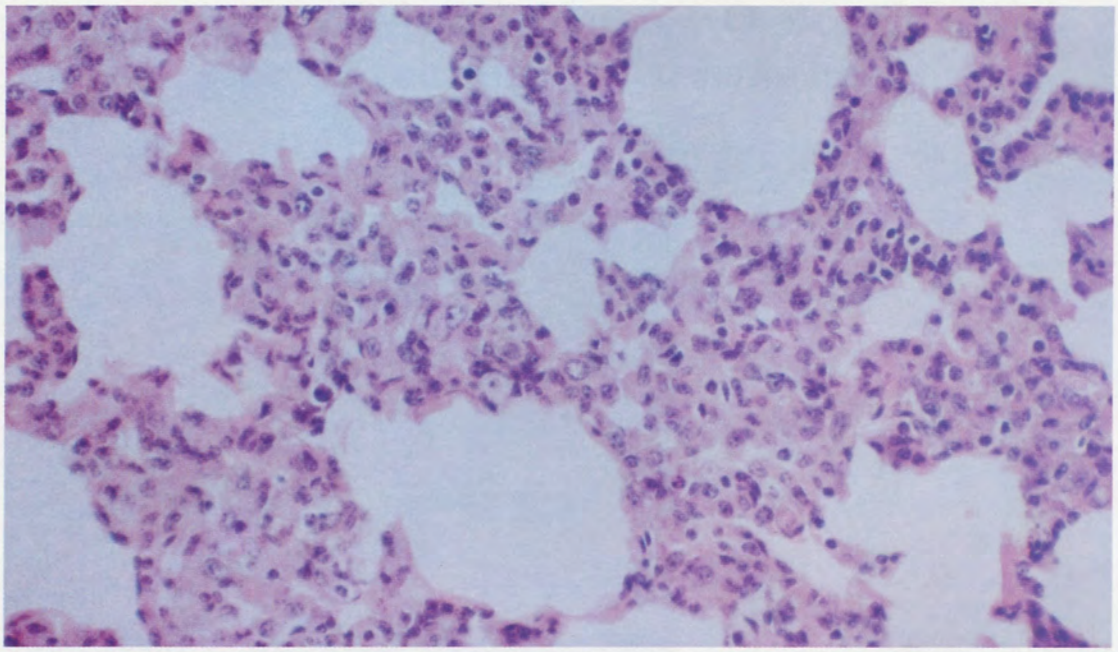
Jembrana Disease and Jembrana Disease Virus Refereed Papers 159
Bovine Immunodeficiency Virus Publications 166

Classic Organ and Tissue Changes Associated with Jembrana Disease

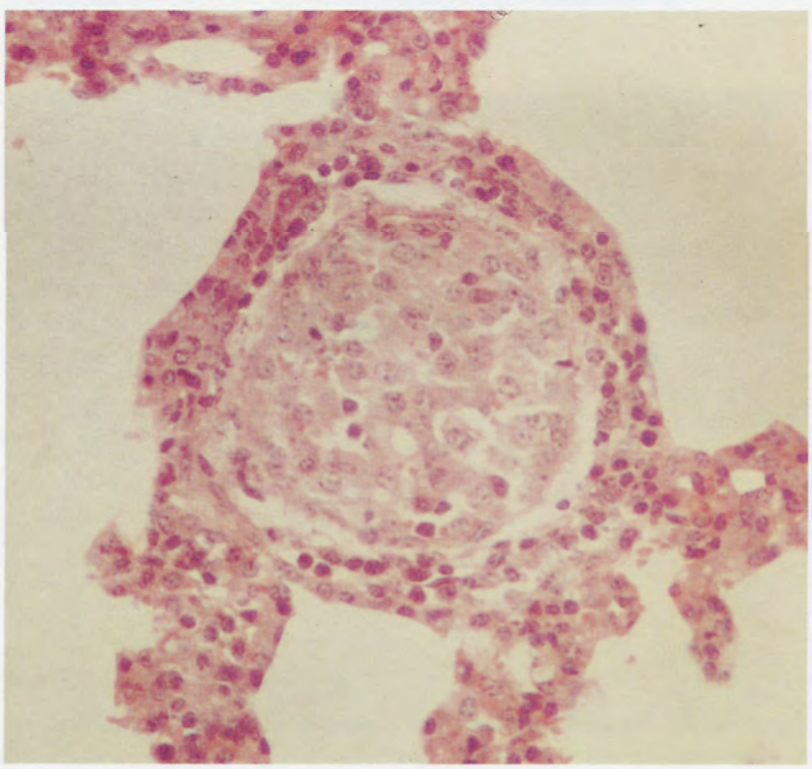
Marked enlargement of the prescapular lymph node. Enlargement of the subcutaneous lymph nodes is a consistent clinical feature of Jembrana disease in Bali cattle.



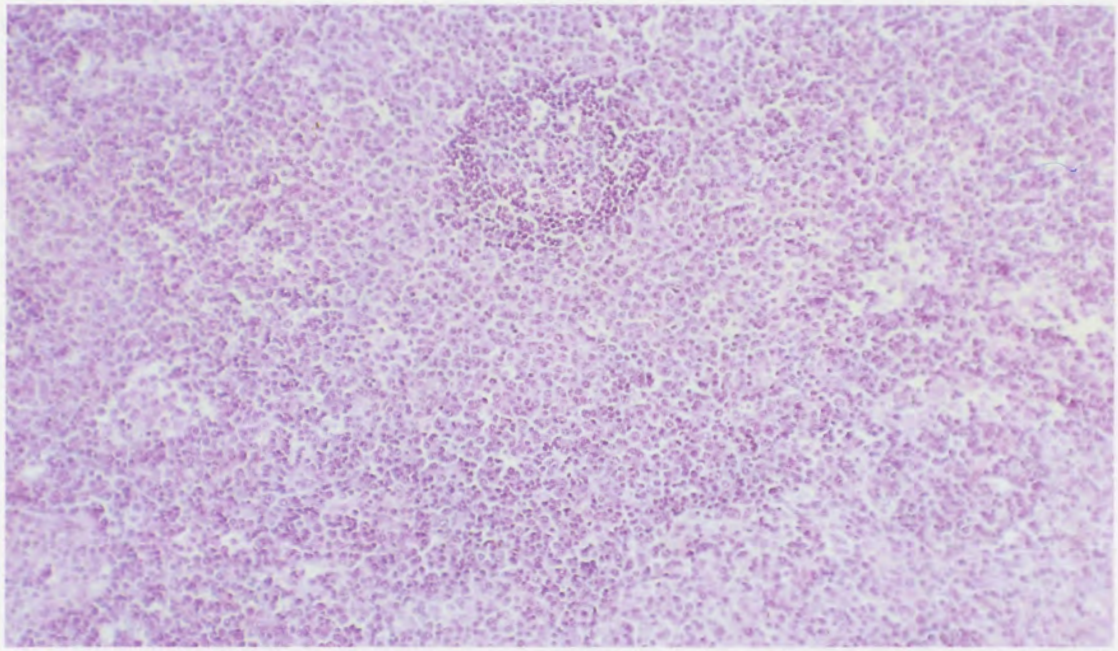
Lymph node. Parafollicular reaction. Large lymphoid and reticular (dendritic) cells.



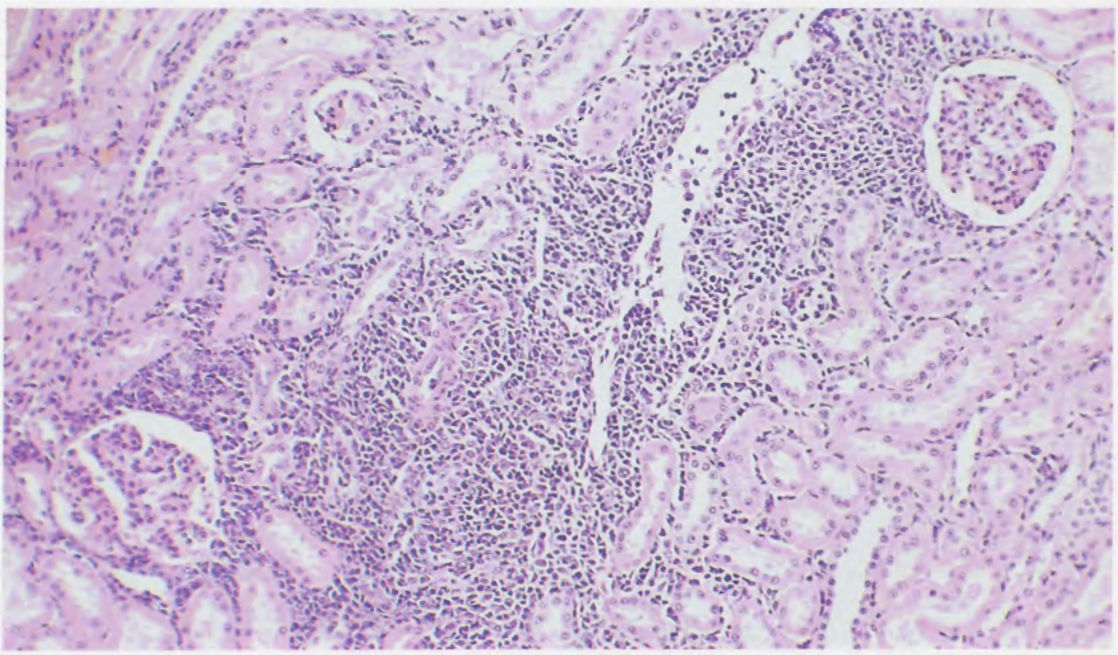
Acute alveolar pneumonia. Partial alveolar collapse. No exudation.



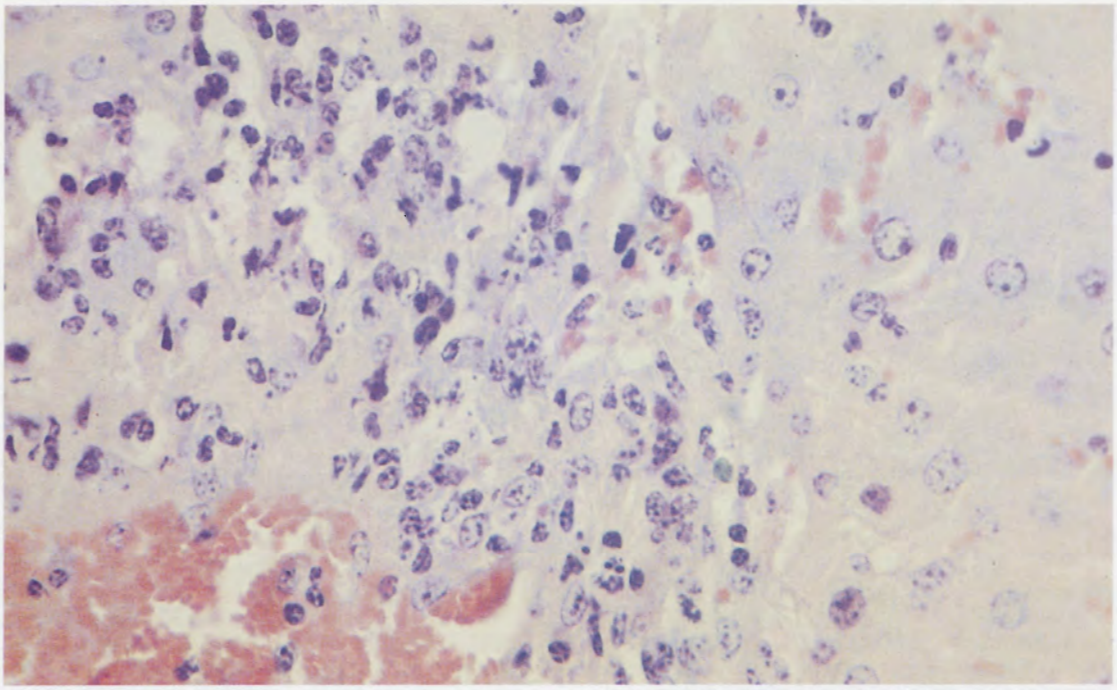
Section of lung from an animal with acute Jembrana disease, showing leucostasis (accumulation of mononuclear cells in the lumen of small and medium sized pulmonary blood vessels), a consistent pathological lesion in Bali cattle with Jembrana disease.



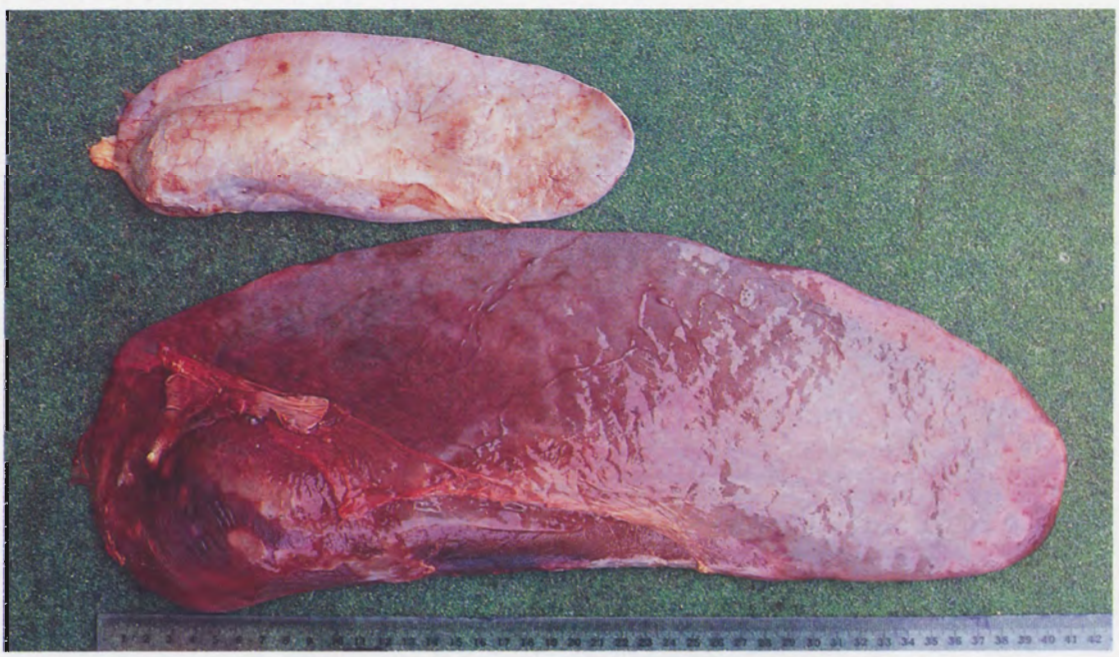
Section of lymph node, showing follicular atrophy and marked parafollicular hypertrophy, consistently present in Bali cattle during the acute clinical phase of Jembrana disease.



Section of kidney from an animal with acute Jembrana disease showing proliferative lymphoid infiltrate that is consistently detected in affected cattle.



Section of liver from an animal with acute Jembrana disease showing the proliferative lymphoid infiltrate that is consistently detected in affected cattle.



Enlargement of the spleen, often 4-6 times normal size, a consistent pathological change in cattle with Jembrana disease. A spleen from an unaffected animal has been included for comparison.

Welcome and opening address by the Director General of Livestock Services

Distinguished participants,
Ladies and Gentlemen.

First of all, let us give thanks that we could meet together in this place to attend the opening of the Workshop on Jembrana Disease in Indonesia.

On behalf of the Indonesian Government, allow me to welcome you all to Bali, Indonesia, and thank you for attending this workshop. It is my great pleasure to meet so many outstanding experts in veterinary sciences, especially those who work with retroviruses. I am sure that in the next four days each of you will share many experiences in this very important workshop.

The Government of Indonesia pays attention to the development of Bali Cattle, especially for the following reason:

Bali cattle, known as *Bos Sondaicus*, is a domesticated Banteng (*Bibos banteng*) considered as an Indonesian original breed of cattle. Nowadays, Bali cattle are distributed throughout Indonesia from Aceh to Irian Jaya, and they have proved to have high adaptability to various environments and can act as pioneer animals in new areas.

Bali cattle have good production and reproduction performances compared to other Indonesian cattle breeds, but they are most susceptible to certain diseases, i.e., Jembrana Diseases, Malignant Catarrhal Fever (MCF) and Bali Ziekte (BZ).

For these reasons, the GOI conducted two important projects. The first related to improvement of the genetic quality of Bali cattle, known as the Bali Cattle Breeding Project, which was started in 1978 and assisted by the New Zealand Government. The second related to the investigation of Jembrana disease by the Bali Cattle Disease Investigation Unit (BCDIU), which was funded by IFAD under the Smallholder Cattle Development Project (Loan No. 396 — ID).

This project actually finished last year, but in the interests of developing Jembrana vaccine quality and the method of Jembrana disease eradication, it will be continued this year through IFAD Project Phase III (1996/1997–1999/2000).

Since the First International Seminar on Jembrana Disease held in Bali, 22–24 September 1975, magnificent progress has been achieved. Thanks to the hard work of the BCDIU Team, and with the collaboration of scientists from all over the world, the most crucial question about the causal agent of Jembrana disease has been answered. It is a retrovirus.

In this workshop, all participants will be informed of the considerable progress which has been made by the BCDIU Team concerning their efforts to control Jembrana disease, work to establish the future research priorities, and efforts to promote the control of Jembrana disease in Indonesia. Your contributions to this workshop will be beneficial, not only to Indonesia, but also to the various countries where various species are affected by retroviruses.

On behalf of the Government of Indonesia, I would like to extend my gratitude to the Australian Centre for International Agricultural Research (ACIAR) for its kind sponsorship of this workshop. I would like also to thank especially Dr John Copland, Animal Science Coordinator of ACIAR and Dr Graham Wilcox, Associate Professor (Virology), School of Veterinary Studies, Murdoch University, for their efforts to make this workshop happen.

Although the time available for the preparation of this workshop was very short, I think the preparation has been very thorough thanks to the hard work of the Organising Committee. I appreciate that very much.

Finally, with your permission, I declare the Workshop on Jembrana Disease in Indonesia officially open. Thank you for your kind attention and I wish you luck and success in your deliberations.

Director General of Livestock Services
Ir. Erwin Soetirto

The Occurrence and History of Jembrana Disease in Indonesia

S. Soeharsono and I.G.N. Teken Temadja[1]

Abstract

An outbreak of a new disease was reported in the Jembrana district of Bali province, Indonesia, in 1964. At first, it was reported to affect Bali cattle (*Bos javanicus* syn *Bos sondaicus*) and buffaloes (*Bubalis bubalis*), but only Bali cattle were reported in the later outbreaks in Bali and elsewhere in Indonesia. The disease was diagnosed firstly as a rinderpest-like disease and subsequently named Jembrana disease (JD).

Due to strict regulation of the exportation of Bali cattle from Bali island to other islands in Indonesia, the disease was confined to Bali island for more than 12 years. The first outbreaks of JD outside Bali island were reported in Lampung Tengah district, Sumatra island, in 1976. Illegal introduction of JD Bali cattle from Bali island was the suspected cause of the outbreak. From Lampung, JD spread northwards to West Sumatra in 1992, Bengkulu in 1994, and South Sumatra in 1995. The disease also occurred in the Banyuwangi district of Java island in 1978. More recently the disease was reported in the South Kalimantan and Central Kalimantan Tengah provinces of Kalimantan island.

Introduction

BALI island, located in the eastern part of Indonesia, consists of eight districts, i.e. (in alphabetical order) Badung, Bangli, Buleleng, Gianyar, Jembrana, Karangasem, Klungkung and Tabanan. Its main product is agricultural, and in general, the soil is very fertile.

Bali cattle are indigenous to Indonesia (Payne and Rollinson 1973) and were domesticated directly from wild banteng (*Bos javanicus* syn. *Bos sondaicus* syn. *Bibos banteng*) (Porter 1991). The economic importance of Bali cattle in Indonesia is presented elsewhere (these Proceedings). Individual farmers in Bali on average own only 3.3 head of Bali cattle (Soeharsono et al. 1983). In Bali and most other parts of Indonesia, Bali cattle are used as draught animals in the rice fields, young animals may be sold if the farmer needs cash for various purposes, and older animals are sold for beef production.

Before Jembrana disease (JD) occurred, the trade of Bali cattle from Bali to other Indonesian islands, or even for export to Hong Kong and Singapore for slaughter, was unrestricted, but the inter-island trade in Bali cattle from areas where JD is endemic is now banned. An exception is that Bali cattle from Bali may be trucked to Jakarta for slaughter.

This paper describes the occurrence and history of JD in Indonesia, based on available papers and reports, and the field observations and experiences of veterinarians.

History of the Outbreak

Origin

Information concerning the origin of the outbreak is scarce. The only written information before the outbreak concerned the presence of a foreign vessel in Buleleng harbour, about 100 km from the village where the disease was first reported. The ship was reported to contain a number of cattle, but there was no further information about the origin of the ship nor about deaths among the animals. No reports of a similar disease outbreak were received from any neighbouring countries.

Two other events preceded the outbreak of JD. The first was a mass vaccination against foot and

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mouth disease using an inactivated vaccine produced in Surabaya in 1963. The vaccine had caused severe post-vaccinal reactions. As a number of Bali cattle collapsed and farmers were upset with the implementation of the vaccination program, it was temporarily stopped. A decision was made to import an inactivated monovalent type-O standard vaccine prepared in cell culture in England. The mass vaccination was continued using the imported vaccine. The question arises: did the inactivated vaccine contain a virus which was non-pathogenic in *Bos taurus* and *Bos indicus*, but pathogenic in *Bos sondaicus*? Jembrana disease appears to produce a marked clinical disease in Bali cattle only, and a subclinical disease in other species of cattle (Soeharsono et al. 1990a, b).

The second event was the eruption of Mount Agung volcano in 1964 when more than 1000 people died. Dust from the eruption covered the grass and leaves of plants used for cattle consumption, so farmers had to wash the grass and plants before feeding the cattle. This condition lasted for weeks and might have caused severe stress in Bali cattle, but it is unknown if there was an indirect relation between the JD outbreak and the eruption of Mount Agung.

An additional point worthy of note is that West Bali National Park is situated in the western part of the Jembrana district. Wildlife, including wild banteng and deer, are present in this protected park and indirect contact between deer and Bali cattle grazing nearby is likely to occur. There have not been any studies to determine the possible presence of the infectious agent associated with JD in wildlife.

The first outbreak in Bali

Sangkaragung, a village in the Jembrana district of Bali province, was the first place where an outbreak of a previously unrecognised disease was reported in December 1964 (Pranoto and Pudjiastono 1967). The disease was reported to affect Bali cattle (*Bos javanicus* syn. *Bos sondaicus*) and buffaloes (*Bubalus bubalis*) (Adiwinata 1968). The common disease affecting Bali cattle and buffalo in this region is haemorrhagic septicaemia (HS); in order to control the disease HS specific antiserum and vaccine were used by the local veterinary service. The disease did not seem to cease after the application of HS antiserum and vaccine. By August 1965, the disease had spread to all eight districts of the island and high mortality figures were reported (Pranoto and Pudjiastono 1967).

In April 1965, the first specimens for laboratory diagnosis were submitted to the Animal Disease Research Institute in Bogor (West Java); no potentially pathogenic bacteria or blood parasites were detected.

In June 1965, a team from the Institute for Animal Virus Disease (Lembaga Virologi Kehewanan, LVK), Surabaya (East Java), was sent to carry out a field investigation and to collect specimens for laboratory examination. After consideration of epidemiological, clinical and pathological findings, this group suspected the disease was rinderpest or a rinderpest-like disease (Adiwinata 1968).

In order to control the disease, the Directorate of Animal Health of the Directorate General of Livestock Services conducted a mass vaccination program using lapinised-avianised attenuated rinderpest vaccine imported from Japan. Coincidentally or not, for a couple of years after the mass rinderpest vaccination program, the disease seemed to disappear. A second approach was to ban the export of cattle from Bali to other islands within the Indonesian archipelago, with the exception that cattle were permitted to be exported from Bali to Jakarta for slaughter.

A second outbreak of JD occurred in 1972, in the Tabanan district (adjacent to the Jembrana district) of Bali island. The disease that occurred there was similar to that reported during the initial 1964 outbreak (Hardjosworo and Budiarso 1973). A third outbreak of JD occurred in 1981 in the Karangasem district in the Eastern part of Bali island (Putra et al. 1983). There were no reports of the occurrence of Jembrana disease between these three outbreaks but the disease is now endemic in Bali cattle on Bali island.

Outbreaks of JD on other Indonesian islands

In June 1976, a disease similar to JD was reported in Bali cattle in Lampung, South Sumatra (Soeharsono and Darmadi 1976; Ramachandran 1981). Severe clinical and pathological changes with high morbidity and mortality rates similar to those in the first outbreak in Bali were reported. Bali cattle, introduced by a Balinese transmigrant, were suspected of being the source of the outbreak.

In November 1978, JD was diagnosed in the district of Banyuwangi in East Java province, on the route that Bali cattle may be legally trucked from Bali to Jakarta. These cattle were a possible source of the outbreak. There is only a short distance between Bali and Banyuwangi, and the smuggling of live Bali cattle from Bali to Java island by small boats was a common practice at that time and difficult to prevent. Some JD cases were confused with malignant catarrhal fever, also endemic to this region (Tranggono 1988).

In April 1992, an outbreak of JD was reported in Sawahlunto–Sijunjung district of West Sumatra

province (Tembok 1992). The diagnosis of the disease was confirmed by histopathological examinations and serological tests (ELISA) for antibody to Jembrana disease virus at BCDIU, Denpasar. It is possible that JD carrier Bali cattle from Lampung were introduced into this area through the Solok cattle market in West Sumatra. Control of the movement of animals in Sumatra island is very difficult and therefore the spread of JD to other provinces is likely to occur. In 1995, JD was reported in Bengkulu province of Sumatra island.

In 1993, by histopathological examination of tissues from Bali cattle, JD was also diagnosed in South Kalimantan province (Kalianda 1993, pers. comm.). The morbidity and mortality rates were low, possibly because the population of Bali cattle was low and mixed with other breeds. Further confirmation of the disease in this area was made by histopathological examination and serological tests conducted at the BCDIU. The cause of this outbreak is unknown but importation of cattle, especially Ongole cattle, from East Java to South Kalimantan has long been practiced (Kalianda 1993, pers. comm.) and these cattle, when infected, develop viraemia. Bali cattle from an infected area in East Java (Banyuwangi) might also have been included in the shipment and caused the outbreak.

Conclusion

The source of the infectious agent responsible for the first cases of the disease in Bali cattle in the Jembrana district of Bali island has never been determined. The restriction of cattle movement has proved effective to control the spread of the disease. Introduction of live carrier animals from a JD-infected area to uninfected areas has contributed to the spread of JD.

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Early Observations and Research on Jembrana Disease in Bali and Other Indonesian Islands

S. Ramachandran[1]

Abstract

Jembrana disease, an enigmatic persistent virus disease of banteng (*Bos sondaicus*), is restricted to some Indonesian islands. Discovered in Jembrana, a district in West Bali, in 1964, it has several perplexing epidemiological and clinicopathological features.

The causal agent, a medium-sized (100–200 nm) virus, induces clinical disease only in banteng. Infections do occur naturally in the buffalo and banteng non-descript cattle crosses and can also be artificially induced in sheep and goats. Such infections result in virus persistence in blood and spleen for short periods.

Clinical disease in banteng is heralded by tell-tale features such as high fever, lymphopenia, thrombocytopenia, buccal erosions and haemorrhages in the skin ('blood sweating') and soft tissues, lymphadenopathy and diarrhoea. Clinical recovery is associated with a marked lymphocytosis. It is, however, transient as relapses are common.

Jembrana disease virus is thermolabile, pH sensitive (pH 3.0 and 11.0), ether and deoxycholate resistant. It has no immunising antigens and convalescent sera do not contain virus-neutralising antibodies. There is a significant hypogammaglobulinaemia which is in fact an IgG deficiency.

The disease is non-contagious. There is preliminary evidence suggesting that it spreads through *Boophilus microplus* ticks in which there is transovarian transmission of the agent.

IN December 1964, the Desa Sankaragung in the Kabupaten Jembrana, Bali, witnessed a colossal bovine disaster. A virgin epizootic had erupted on an unprecedented scale and decimated almost the entire banteng (*Bos sondaicus*) population in a few days. More than three decades have passed since the recognition of this new murrain called Jembrana disease (JD), named after the locality. Although several salient features of this malady have been brought to light and doubts regarding its viral aetiology laid at rest, several questions still remain unanswered, particularly its mode of spread and the natural history of the causal agent.

Historical Aspects

The disease in Bali

Jembrana disease was thoroughly investigated in three distinct periods namely, 1965–67 (Adiwinata 1967, Pranoto and Pudjiastono 1967), 1972–73 (Budiarso and Harjosworo 1976) and 1974–80 (Ramachandran et al. 1975, Ramachandran 1981).

In the virgin epizootic of 1964, an estimated 60% of the Bali cattle and buffalo populations was attacked. The rate of spread was cataclysmic. Total mortalities were 98.9% by December 1964. By August 1965, all the eight kabupatens (districts) were affected. Mean case fatalities in 1965, 1966 and 1967 were 71.6 ± 6.0, 31.3 ± 12.0 and 38.6 ± 10.0 respectively (Sonoda 1969).

The mistaken diagnosis that JD was an outbreak of a virulent form of rinderpest resulted in the mass anti-rinderpest vaccination by Sonoda. This was despite Ishitani's (1968) finding that histopathological changes in JD were distinctly different from those documented for rinderpest. Also, in Prof. Tanjung Adiwinata's transmission studies in Surabaya (Adiwinata, 1967), the causal agent of JD was found to be non-infective to Zebu cattle and pigs — species that are highly susceptible to rinderpest. The specificity of the JD agent to banteng was a

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novel finding and was Adiwinata's singular contribution to research on JD epidemiology.

The next outbreak of JD was in 1971 in Kabupaten Tabanan, and was called 'Tabanan Disease'. Although acute and peracute cases were observed on occasions, the disease was relatively mild with the total mortality in 1971–72 at 22.0%. Buffaloes were spared. A rickettesial aetiology was proposed by Budiarso and Hardjosworo (1976) on the basis of laboratory studies in Bogor, but unfortunately, these were not followed by experimental reproduction of the disease in banteng.

An FAO team comprising M. Lobry, H.P. Harding, E. Teuscher, H. Dennig and S. Ramachandran pursued investigations on the rickettsial hypothesis, but, by the end of 1975, reached the conclusion that the causal agent was a virus. This was based on several findings.

1) Prolonged treatment of infective tissue suspensions with bactericidal drugs did not alter infectivity to banteng, the only species in which the clinical disease could be readily reproduced.

2) Infectivity of suspensions was unaffected after filtration through a 200 nm membrane filter.

3) There were no significant differences in clinical parameters, mortality rates and severity of pathological changes in groups of banteng experimentally infected with filtered and unfiltered suspensions.

4) The suspensions were not infective to guinea pigs, rabbits or mice.

5) Sera of banteng recovering from natural or experimentally-induced JD did not develop anti-rickettsial or anti-ehrlichial antibodies readily detectable in Weil–Felix and CF tests (CFT). Infective tissue suspensions also failed to react in the CFT with reference to sera containing antibodies to *Cowdria ruminantium*, *Coxiella burneti* and *Ehrlichia phagocytophila*.

6) In spite of a careful research, rickettsiae were not detected in electron micrographs (EMs) of peripheral blood and tissue lymphocytes and monocytes.

Moreover, in the detailed histopathological studies of Teuscher et al. (1981) on 47 natural cases of JD, there was substantial evidence that tissue changes in JD were akin to those commonly observed in virus diseases. In particular, the microscopic picture was reminiscent of that in bovine malignant catarrh except that there was no encephalitis and that the vasculitis was not of the necrotising type. There was a significant endotheliosis in several tissues and organs. A similar opinion was offered by Dr R.J. Brown of NAMRU-2, Jakarta Detachment (R.J. Brown 1975, pers. comm.).

Rama Dewa disease

This was first recognised in the Balinese transmigration settlements in Lampung, south Sumatra in May 1976. The disease bore all the hallmarks of a virulent form of JD. Although there was free mixing of banteng with large numbers of Ongole cattle and other non-descript breeds, the disease was confined to the former. Attack rates in nine desa with a total population of 2596 banteng ranged from 2.4% to 71.6% (mean 36.5%). Case fatality rates were 12.5% to 72.0% (mean 50.6%).

In a controlled experimental study, rickettsiacidal drugs such as chloramphenicol, terramycin and

Table 1. Comparison of clinical responses of antibiotic-treated and untreated Bali cattle in Lampung.

Parameter	Treated	Not-treated	Significance of differences		
			t	df	p
1. Fever (per cent)	100	100	—	—	—
(a) Incubation period (days)	3.4 ± 0.2	3.0 ± 0.3	0.901	24	>0.10
(b) No. of fever days	6.2 ± 0.3	6.6 ± 0.9	0.587	24	>0.10
(c) Peak temp. (°C)	41.0 ± 0.1	40.7 ± 0.3	1.644	24	>0.10
(d) Day of peak temp.	3.6 ± 0.3	2.6 ± 0.4	1.449	24	>0.10
2. Leucopenia (per cent)	100	100	—	—	—
(a) Day of onset	4.0 ± 0.4	3.7 ± 0.3	0.438	23	>0.10
(b) Duration (days)	4.3 ± 0.8	5.4 ± 0.4	1.362	23	>0.10
(c) Peak value (% reduction)	66.0 ± 9.0	69.0 ± 3.0	0.418	23	>0.10
3. Mortality (per cent)	90*	100	—	—	—
Day to death	9.5 ± 1.2	7.2 ± 0.9	1.010	24	>0.10
4. Intensity of anatomical changes	Severe	Severe	—	—	—
5. Lymphadenopathy (per cent)	100	100	—	—	—

* Two killed *in extremis*

oxytetracycline given in daily doses alone or in combinations from the first day of inoculation failed to prevent deaths in 21 banteng. All developed clinical JD after a mean incubation period of 3.45 ± 0.2 days. Mortality was nearly 100% (two were killed *in extremis*). The mean day to death was 9.5 ± 1.2 post inoculation (Table 1). Analysis of clinical parameters revealed no significant differences in responses between treated and untreated animals.

Banyuwangi Disease

Banyuwangi, in east Java was the scene of several outbreaks of a JD-like syndrome from November 1978. Fourteen of 18 kacamatans were affected. In the next six months, there were 497 deaths (31.2%) in a mixed population of banteng, Rambon cattle (cross between non-descript and banteng) and buffaloes. Tissue suspensions from one suspected case each in banteng and buffalo were inoculated into batches of two banteng at Denpasar. Only the buffalo suspensions caused a febrile response and equivocal clinical signs. Blood drawn during pyrexia from the reacting banteng was sub-inoculated into two other banteng, one being a known 'carrier' and the other susceptible. The latter developed pyrexia and leucopenia after an incubation period of four days. There was also regional adenopathy. This was the first isolation of JD agent from a buffalo. There was also histopathological evidence of JD infection in 10 of 17 'clinical' cases, two from banteng and four each from Rambon cattle and buffalo.

Research

Results of several laboratory studies conducted at the BPPH, Denpasar, during 1975–80 were as follows.

Experimental transmission

The causal agent of JD was readily transmitted to banteng, buffaloes, sheep and goats but clinical signs were manifest only in banteng. Pigs, guinea pigs, rabbits, mice, chicken and duck embryos were not infected. Although inoculated sheep and goats did not show clinical signs, they had viraemia which persisted for up to six weeks. Virus also persisted in the spleen for up to three months.

One of two experimentally-inoculated buffaloes developed fever and leucopenia on the fourth day. Fever lasted for five days. There were no other clinical abnormalities. When necropsied two months later, spleen was infective to banteng. Viraemia was demonstrable at earlier periods.

Cell cultures

The agent was not cultivable in primary banteng and pig kidney cells or chicken embryo fibroblasts. Cultured lymph node cells developed a CPE on the fifth day. There were foci of syncytia. Some individual cells showed eosinophilic cytoplasmic inclusions. The fifth day culture suspension induced an equivocal clinical response in a banteng. It had fever for two days but no leucopenia. Sub-inoculation of its blood into another banteng proved to be non-infective. Cultures of later ages were not infective.

Persistence and relapses

Recovery from clinical JD did not entail sterile immunity. Viraemia was demonstrable by inoculation into banteng, for several months. The longest period of virus recovery was 488 days from a clinically normal 'carrier'.

Relapses were common. In this regard, JD behaved like a rickettsial infection. Thirty-two of 35 carrier banteng (92%) relapsed. Nine relapsed twice, 4 thrice, 2 four times and 1 on six occasions. Sixteen of these cases died. During relapse infection, there was low-grade febrile response for two or three days and leucopenia of variable intensity. However, in many cases there was no leucopenia.

Tick transmission

In two separate trials in 1975–76, there was compelling circumstantial evidence of transovarian transmission of JD agent in *Boophilus microplus* ticks. Banteng used in these studies were obtained from Lombok, regarded as a JD-free island, and maintained in tick-proof premises at BPPH, Denpasar.

In the first study, female ticks that had engorged on the bodies of naturally-infected banteng were allowed to oviposit in test tubes. The larvae that emerged were placed on the bodies of three donor banteng and allowed to mature into adults in 21 days. Twelve, 21 and 25 days later, the three donors developed typical signs of JD and died at different intervals.

In the second trial, which was only a partial success, juvenile ticks were harvested on the 19th day from the bodies of three donor banteng infested with 'infected' larvae. On the 20th and 21st days, engorged adults were harvested. Suspensions from these two categories of ticks produced unmistakable clinical JD in inoculated banteng. However, the donor banteng did not show spontaneous clinical signs in an observation period of eight weeks. The animals were then subjected to stress in the form of repeated road transport. This precipitated JD in one,

proving that it had been infected by larvae. The other two banteng did not develop the disease.

Haematology

This was a fruitful area of research leading to interesting findings. Absolute lymphopenia and marked thrombocytopenia were consistently observed in spontaneous and experimentally-induced JD. In the febrile phase, mature and immature lymphocytes underwent a series of degenerative changes leading to lysis. In the recovery phase, blood contained a significant proportion of abnormal lymphocytes. The abnormalities were related to their size, morphology and tinctorial properties. Large lymphocytes developed alternative foci of protoplasmic condensation, the so-called blebs and thinning of plasma membrane. The cytosol was vacuolated and weakly basophilic or amphoteric. Karyomegaly, swelling of nucleoli, eccentricity of nuclei, occurrence of binucleate cells and fusion into small syncytia were other abnormalities.

In the post-febrile lymphocytosis phase, there was a mixed population of medium-sized lymhocytes often binucleate, lymphocytes with mitotic figures and plasma cells. Medium-sized to large lymphocytes and monocytes had particulate inclusions. In electron micrographs, these were recognised as platelets in different stages of degeneration.

Lymphocytes and monocytes had ultrastructural changes. Mitochondria were swollen, the endoplasmic reticulum was disrupted and there was an abnormal increase in polyribosomes. In light microscopy, it was often difficult to differentiate large lymphocytes from monocytes. In the EM, the latter had more granules in the cytosol, more endoplasmic reticulum and a higher cytoplasmic–nuclear ratio.

Immunity

Jembrana disease is a persistent viral infection resulting in a profound immunological disorder. There was no evidence of humoral immunity. Subsidence of clinical disease did not constitute true recovery as relapses were frequent. Formalinised or B-propiolactone-treated infective tissue suspensions given alone or suspended in Freund's adjuvant failed to protect banteng against challenge infection. Such animals did not develop neutralising antibodies.

Serology

Convalescent sera were anti-complementary. This interfered with the performance of a CF test. However, a precipitating antigen was demonstrable in the acute phase plasma. It gave a single line of precipitation in agar gels against concentrated convalescent sera. Sera required heat-inactivation (60 °C for 30 minutes) to dissociate antigen-antibody complexes and ensure denaturation of free antigen. These interfered with the precipitation reaction.

Several samples of convalescent sera examined at the Naval Aerospace Medical Research Laboratory, Florida (USA) were reported to be hypogammaglobulinaemic (R.J. Brown, 1976, pers. comm.). This was attributed to a significant depression in IgG levels.

Characterisation of JD virus

The agent was filterable, the estimated particle size being 100–200 nm. It was inactivated after exposure to 55 °C for 15 minutes and to extremes of pH (3.0 and 11.2). It was resistant to the action of sodium deoxycholate (1:1000), diethyl ether and a range of antibiotics. It was readily inactivated by formaldehyde and β-propiolactone. Infectivity in meat persisted up to 36 hours at 22–25 °C and for 72 hours at ±4 °C. The agent stored well for several months at −70 °C.

Epidemiologic shifts

In the period since its discovery, JD has undergone marked changes in attributes such as virulence, rate of spread and species susceptibility. The virgin epizootic of 1964–67 was characterised by fast spread. Mass exodus of banteng might have acted as a contributory factor. However, excessive virulence might also be an associated factor.

In the early studies by the FAO team in 1974–75, rates of spread in the three village herds at Sesetan, Nagara and Panjer were moderate. The numbers of banteng with clinical disease were 22, 28 and 33 respectively and the periods taken for development of the disease in these cases were 5, 7 and 8 weeks respectively.

Whereas the 1964–67 outbreaks had affected buffaloes, this species was spared in subsequent outbreaks in Bali. However, there was evidence of natural disease in buffaloes in East Java where they outnumber banteng and crossbred cattle populations. Reduced susceptibility to tick infestation may be an important factor.

In Bali, general incidence of JD has declined significantly since 1976. Reported cases in five years from 1974 were 4584, 4610, 1970, 1188 and 1739 with a mean score of 1632 for 1976–1978. Mortalities and case fatality rates (in parenthesis) were 336 (7.3%), 345 (7.5%), 203 (10.3%), 125 (10.5%) and 55 (3.2%) respectively. The disease was recognised throughout the year. There was no correlation between incidence and climatic factors such as mean rainfall, relative humidity or diurnal temperatures. In a limited survey, spraying with insecticides significantly reduced the incidence of the disease.

A critical study of clinical parameters of experimentally-induced disease revealed that the infection had become progressively milder since 1977. For instance, mean duration of fever and mean peak temperature were significantly lower in later periods than in 1975–76. Whereas most infected banteng died in less than 11 days post-infection in 1975–76, the mean day-to-death was prolonged in 1977–78 (54 ± 12) and in 1978–79 (28 ± 9). In 1976–77, seven of 31 fatalities occurred in a mean period of 11.9 ± 0.6 days, six in 24 ± 2 days and the rest in 50 ± 4 days.

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Clinical Changes in Bali Cattle and Other Ruminants Following Infection with Jembrana Disease Virus

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Abstract

Clinical signs of Jembrana disease in Bali cattle (*Bos javanicus*), induced by intravenous inoculation of Jembrana disease virus (JDV), occurred after an incubation period of between 4 and 12 days. There was a linear relationship between the dose of virus inoculated and the incubation period. Clinical signs persisted for 5–12 days, followed by recovery of most affected animals. The major clinical signs were an elevated rectal body temperature persisting for seven days (range 5–12 days), lethargy, anorexia, enlargement of the superficial lymph nodes, a mild ocular and nasal discharge, diarrhoea with blood in the faeces and pallor of the mucous membranes. Not all of these clinical changes occurred in all affected cattle. The major haematological changes included a leucopenia, lymphopenia, eosinopenia and a slight neutropenia, a mild thrombocytopenia, a normocytic normochromic anaemia, elevated blood urea concentrations and reduced total plasma protein. The mortality rate in the experimentally-infected cattle was 17%.

Other cattle types were also susceptible to JDV infection. Infection of Friesian (*Bos taurus*) and crossbred Bali cattle (*Bos javanicus* × *Bos indicus*) induced clinical changes and lesions consistent with those detected in Bali cattle, although they were milder and consequently would have been difficult to detect under field conditions. Ongole cattle (*Bos indicus*) and buffalo (*Bubalus bubalis*) also developed a mild febrile response after inoculation with JDV but no other overt clinical signs of the disease; no haematological investigations were conducted on these animals after they were inoculated.

Jembrana disease virus was present to high titres in the blood of Bali cattle during the febrile period. The titre of virus in blood then declined concurrent with the regression of the clinical changes. The virus persisted at low titres in the blood of recovered cattle for at least 25 months. Buffalo, Ongole cattle, Ongole × Bali cattle, and Friesian cattle also developed a persistent infection; although the virus was recovered from blood or spleen tissue of infected buffalo for at least nine months after infection, it persisted for only 3–6 months in the other species. Sheep and goats did not develop a febrile response after infection with JDV. Sheep, however, developed a viraemia that persisted for 3–6 months after infection. Some pigs inoculated with JDV developed a fluctuating febrile reaction after infection but JDV was never recovered from the inoculated animals. Seventeen of 18 Bali cattle in which Jembrana disease had been experimentally-induced up to 22 months previously did not develop clinical signs when re-challenged with JDV.

WITH the recognition that Jembrana disease was caused by a bovine lentivirus designated Jembrana disease virus (JDV) (Kertayadnya et al. 1993; Chadwick et al. 1995a,b), the disease has been recognised as an unusual lentivirus disease: an acute disease with a short incubation period (Chadwick et al. 1995b).

Under field conditions, the clinical signs of Jembrana disease are difficult to define and are frequently complicated by the occurrence of secondary infections such as bacterial pneumonia (Teuscher et al. 1981). The disease can be readily transmitted to susceptible Bali cattle by inoculation of blood or spleen tissue from infected cattle (Soeharsono et al.

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1990), and the pathological changes in experimental and naturally occurring disease were reported to be very similar (Teuscher et al. 1981). The authors examined the sequential clinical and haematological and virological changes occurring in Bali cattle experimentally infected with Jembrana disease virus (JDV) in order to define the changes that do occur during the disease.

There have been no confirmed reports of clinical disease, typical of Jembrana disease in Bali cattle, in other cattle types or other animal species. Experiments were undertaken to confirm this apparent host specificity, and are described in this paper. These experiments involved the experimental infection of other cattle types and ruminant species with JDV and a detailed examination of the sequential clinical and haematological changes that occurred in these animals.

Materials and Methods

Experimental animals

Bali cattle used for experimental infection studies were approximately 18 months of age. They were obtained from Nusa Penida, a small island adjacent to Bali, where Jembrana disease had not been reported. Experience has shown that these cattle are consistently susceptible to experimental challenge with the Jembrana disease agent. On arrival, the animals were kept in screened animal houses and given free access to *Penissetum purpureum* grass and water. They were kept under observation for a minimum of seven days prior to use.

Other ruminant species and pigs used in the experiments were purchased from various areas of Bali or Java: Friesian cattle, buffalo, Indonesian sheep, goats and pigs from Bali, Ongole cattle (*Bos indicus*) from East Java, Rambon cattle (developed by crossbreeding *Bos indicus* × *Bos javanicus* cattle) from East Java, and the Madura breed of cattle (also derived by crossbreeding *Bos indicus* and *Bos javanicus*) from Madura island. All animals were clinically normal at the time of purchase and were monitored for several days prior to use.

Jembrana disease virus

Three isolates of JDV were used: Klungkung/85, Singaraja/86 and Tabanan/87. Unless otherwise stated, the Tabanan/87 isolate was used. All isolates were obtained from naturally-infected animals exhibiting typical signs of Jembrana disease: 10 mL of heparinised blood from the affected animal was injected intravenously into susceptible Bali cattle, the recipient animals were killed two days after the development of fever, spleen tissue was aseptically collected and distributed into aliquots, stored frozen at −70 °C and used as a source of infectious agent. When required, the spleen tissue was thawed, a 10% homogenate was prepared with a mortar and pestle with medium 199 (Flow Laboratories) as a diluent, and then clarified by centrifugation at 650 g for 20 min.

Method of infection and examination of experimental animals

Bali cattle were inoculated with various dilutions of whole blood or plasma obtained from experimentally-infected Bali cattle two days after they developed a febrile reaction in excess of 39.5 °C, as previously described (Soeharsono et al. 1990). Prior to infection and for up to 28 days after infection, the animals were examined daily: clinical signs were recorded and heparinised blood samples were obtained for haematological examination.

Clinical signs were recorded on a subjective scale of from 1 to 3 (1 = mild, 2 = moderate, and 3 = marked). Any animals that became recumbent and unable to rise were killed. A complete pathological examination was conducted on any animals that died, on animals that became recumbent and unable to rise, and all animals at the conclusion of the experiment. The results of the pathological examinations will be reported separately.

Haematological examinations

Blood samples were collected with heparin as an anticoagulant. Thin blood smears were made immediately after collection and stained with Giemsa for differential leucocyte counts. Total leucocyte, erythrocyte and thrombocyte counts, packed cell volume (PCV), haemoglobin (Hb), erythrocyte sedimentation rate, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin count (MCHC), blood urea, creatinine concentrations, total plasma protein and fibrinogen were determined as described by Soesanto et al. (1990).

Titration of infectious virus in blood and plasma

To quantitate the infectious agent present in the blood of infected Bali cattle, animals were infected intravenously with 1 mL of a 10% homogenate of spleen from an animal infected with the Tabanan/87 isolate of the Jembrana disease agent. Two days after the rectal temperature of the recipient animal exceeded 39.5 °C, a heparinised blood sample was obtained and serial 10-fold dilutions of the blood were made in antibiotic-free medium 199 with 10% foetal bovine serum (FBS). One mL of each dilution was inoculated intravenously into two Bali cattle.

Control animals were inoculated with 1 mL of medium 199 with 10% FBS.

All infected animals were examined daily after infection for evidence of Jembrana disease infection. The principal signs used were an increase in rectal temperature in excess of 39.5 °C which persisted for more than two days and a concurrent leucopenia (less than 4000 leucocytes per uL). Confirmation of the clinical diagnosis was made in most cases by the detection of typical gross and microscopic pathological changes (Teuscher et al. 1981) after the animal was killed. An approximate 50% Bali cattle infectious dose (ID_{50}) was determined as the highest dilution of the inoculum producing Jembrana disease in at least one of the two inoculated animals.

Infection and detection of persistent infections in ruminants and pigs

Ongole and Friesian cattle, buffaloes, sheep, goats and pigs were infected with JDV obtained from Bali cattle with experimentally-induced Jembrana disease as described above. They were infected with either blood collected and inoculated immediately after collection, or with a 10% suspension in medium 199 of frozen and thawed spleen from Bali cattle which were killed during the febrile period. The blood used to inoculate the goats and pigs was concurrently titrated in Bali cattle as described above and the dose per inoculated animal determined as 10^8 ID_{50}. All inoculated animals were examined for clinical signs of Jembrana disease at daily intervals for 28 days post-inoculation. To detect persistent JDV infection in the inoculated animals, at intervals after infection, tissues were collected and sub-inoculated into Bali cattle which were then monitored for typical clinical signs of Jembrana disease. Tissues used were either heparinised blood samples or 10% suspensions of spleen prepared from animals which were killed.

Resistance of Bali cattle to re-infection

Eighteen Bali cattle which had previously been experimentally infected with the Klungkung/85 or Singaraja/86 isolates of the Jembrana disease agent were challenged at various intervals after infection (Table 1) with 1 mL of a 10% suspension of spleen from a Bali animal infected with the Tabanan/87 isolate. Eight control animals were also challenged with the same inoculum. The rectal temperature was determined daily for 21 days after challenge, and blood samples were obtained from any animals exhibiting a febrile reaction to confirm the presence of haematological changes typical of Jembrana disease (Soesanto et al. 1990).

Table 1. Susceptibility of recovered cattle to challenge with Jembrana disease virus. Cattle were challenged with 1 mL of a 10% suspension of spleen from an animal experimentally infected with the Tabanan/87 strain of Jembrana disease virus and killed two days after the development of fever.

Animal	Strain used in initial infection	Time since initial infection (months)	Susceptibility to challenge with Tabanan/87 strain
442	Klungkung/85	20	—
451	Klungkung/85	22	—
186	Klungkung/85	22	—
477	Singaraja/86	7	—
484	Singaraja/86	9	—
485	Singaraja/86	6	—
491	Singaraja/86	4	+
501	Singaraja/86	3	—
502	Singaraja/86	4	—
510	Singaraja/86	5	—
512	Singaraja/86	4	—
514	Singaraja/86	4	—
515	Singaraja/86	4	—
518	Singaraja/86	4	—
521	Singaraja/86	3	—
522	Singaraja/86	3	—
523	Singaraja/86	3	—
492	Singaraja/86	2	—

Analysis of data

As Bali cattle were inoculated with varying dilutions of blood and there was a linear relationship between the dilution of blood and the incubation period before the development of clinical signs, the clinical signs and haematological data recorded at daily intervals after infection were analysed on the basis of time after the development of a febrile response. The results are expressed as a mean of the individual results in animals that developed a febrile response within 14 days after infection, and include the data from animals that died or were killed *in extremis*.

Results

Experimentally-induced disease in Bali cattle

A febrile response was detected in 18 animals inoculated with various dilutions of whole blood from cattle which had been experimentally infected with JDV. As shown in Figure 1, the incubation period (time from inoculation to the onset of the febrile period) varied from 4 to 12 days, and there was a linear relationship between the duration of the incubation period and the dose (dilution of the blood or plasma) inoculated into each animal. The febrile response persisted for seven days (range 5–12 days) but there was no relationship between the duration of

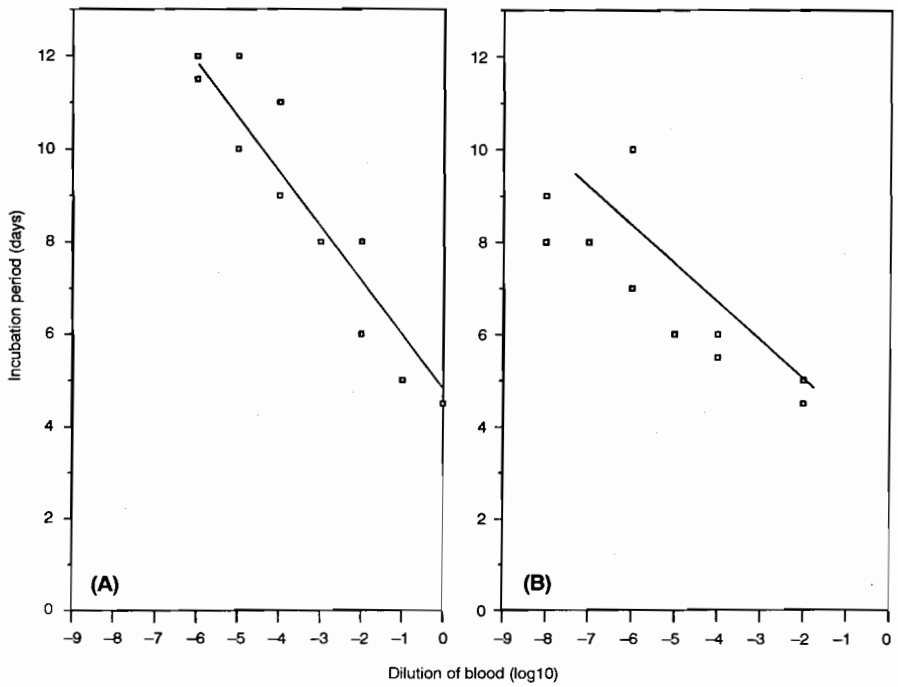

Figure 1. Relationship between the dilution of blood used to inoculate cattle and the incubation period before the onset of clinical signs. The blood was obtained from two experimentally-infected cattle (A and B) on the second day of the febrile reaction. In animal A, r=0.95 ($P<0.01$); in animal B, r=0.8.

the febrile period and the dose of virus inoculated. The severity and duration of other clinical signs are shown in Table 2.

The most common clinical signs, occurring in more than 16 of the 18 cattle, were anorexia, lethargy, erosions of the oral mucosa and enlargement of the superficial lymph nodes, particularly the prescapular, prefemoral and parotid lymph nodes. The erosions of the oral mucous membranes were located postero-laterally on the ventral surface of the tongue, were frequently bilateral, usually well defined and approximately 1 cm in diameter. Hypersalivation, nasal discharge, diarrhoea and blood in the faeces were detected in 10–13 of the 18 animals. Pallor of the mucous membranes was detected in 6 of the 18 animals between 4 and 13 days after the onset of the febrile response. Although no conjunctival or corneal lesions were detected, a slight serous lachrymal discharge was observed in 6 of the 18 animals; it persisted for periods of between 2 and 7 days and occurred between 3 and 15 days after the start of the febrile response. No neurological signs were detected in any of the cattle.

Of the 18 animals that developed Jembrana disease, two died and one was found recumbent and unable to rise and was considered to be *in extremis* and was killed. Including the recumbent animal, the mortality was 17%. The three animals died from 9 to 20 days after the development of a febrile response; one died during a febrile period which had persisted for eight days before death, and the other two died 4 and 13 days after recovery from a febrile period of 9 and 8 days, respectively.

All animals developed a leucopenia which coincided with the febrile period and was principally due to a lymphopenia, although there was also a moderate neutropenia towards the end of the febrile period persisting for approximately seven days (Fig. 2). There was almost a complete absence of eosinophils 2 to 12 days after the start of the febrile period, which then slightly increased but remained below normal until the observations were discontinued 21 days after the start of the febrile period. No change was detected in the number of monocytes or basophils.

Table 2. Severity and duration of clinical signs detected in Bali cattle after experimental infection with Jembrana disease virus.

Clinical sign	Per cent of 18 cattle affected	Severity of clinical signs* in relationship to onset of fever																	
		Days after onset of fever																	
		−2	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Anorexia	94			1	1	1	2	2	2	2	2	2	2	2	2	1	1	1	1	
Lethargy	89		1	1	1	1	2	2	2	2	2	2	1	1	1	1	1		1	1
Enlargement of prescapular LN	100		1	1	1	1	2	2	3	3	3	3	3	3	3	2	2	2	2	2
Enlargement of prefemoral LN	100			1	1	1	1 2	2	3	3	3	3	3	3	3	2	2	2	2	2
Enlargement of parotid LN	100			1	1	1	1	1	2	3	3	3	3	3	2	2	2	1	1	1
Erosions of oral mucosa			1	1	1	1	1	1	2	2	2	2	2	2	1	1	1			
Hypersalivation	72					1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Diarrhoea	13						1	1	1	1	1	1	1	1	1	1	1			
Blood in faeces	13					1	1		1	1	2	1	1	1	1					
Nasal discharge	56						1	1	1	1	1	1	1	1	1	1	1	1	1	
Pallor of mucous membranes	33							1	1	1	1	1	1	1	1	1	1			

* Results are a mean of the severity of the signs observed and are expressed on a scale of 1 to 3 (1 = mild, 2 = moderate and 3 = marked severity). Blanks indicate that lesions were not detected.

There was a reduction in the PCV, number of erythrocytes and Hb values (Fig. 2) which commenced at the start of the febrile period and persisted throughout the period of observation (up to 21 days after the onset of the febrile period). The MCV, MCH and MCHC remained within normal values indicating a normocytic normochromic anaemia. There was a reduction in the number of thrombocytes commencing 1–2 days prior to the onset of the febrile period and persisting until three days after the end of the febrile period (Fig. 2). No change was detected in the erythrocyte sedimentation rate.

A decrease in the amount of plasma protein occurred from the onset of the febrile period but it returned to normal approximately 15 days later. Blood urea concentrations increased markedly commencing three days after the start of the febrile period but in those animals that recovered they returned to normal 9–10 days later (Fig. 2); in 2 of the 3 animals that died or which were killed *in extremis*, blood urea concentrations in excess of 160 mg per dL were detected. Similar high blood urea concentrations were seen in only 2 of the other 15 animals that survived the infection. No significant change was detected in the concentrations of fibrinogen or creatinine.

Experimentally-induced disease in Ongole cattle

Six of 7 Ongole cattle inoculated with 1 mL of a 10% suspension of spleen tissue from an affected animal developed a febrile response (a rectal temperature in excess of 39.5 °C) of 2 to 5 days duration after an incubation period varying from 6 to 17 days. No other clinical signs were detected and no haematological examinations were conducted. All the Ongole cattle survived the infection.

Experimentally-induced disease in Friesian cattle

Two Friesian cattle infected with JDV developed similar clinical and haematological changes. A febrile period began four days after infection and persisted for five days (Fig. 3). There was a slight reduction in appetite during the febrile period, and slight enlargement of the prescapular lymph nodes was observed on the first day of fever and persisted until 10 days after infection (Fig. 3). Haematological changes included a transient leucopenia, due to lymphopenia and neutropenia, during the febrile period (Fig. 3). There was a reduction in the number of thrombocytes extending from the start of the febrile period to the termination of the experiment 11 days after inoculation (Fig. 3). No change was detected in the PCV (Fig. 3) or number of erythrocytes.

The two Friesian cattle were killed 11 days after inoculation. In one animal (F81) the only macroscopic pathological change detected was slight enlargement of the spleen and lymph nodes; histopathological changes were detected in the spleen (a slight parafollicular proliferation of lymphoreticular

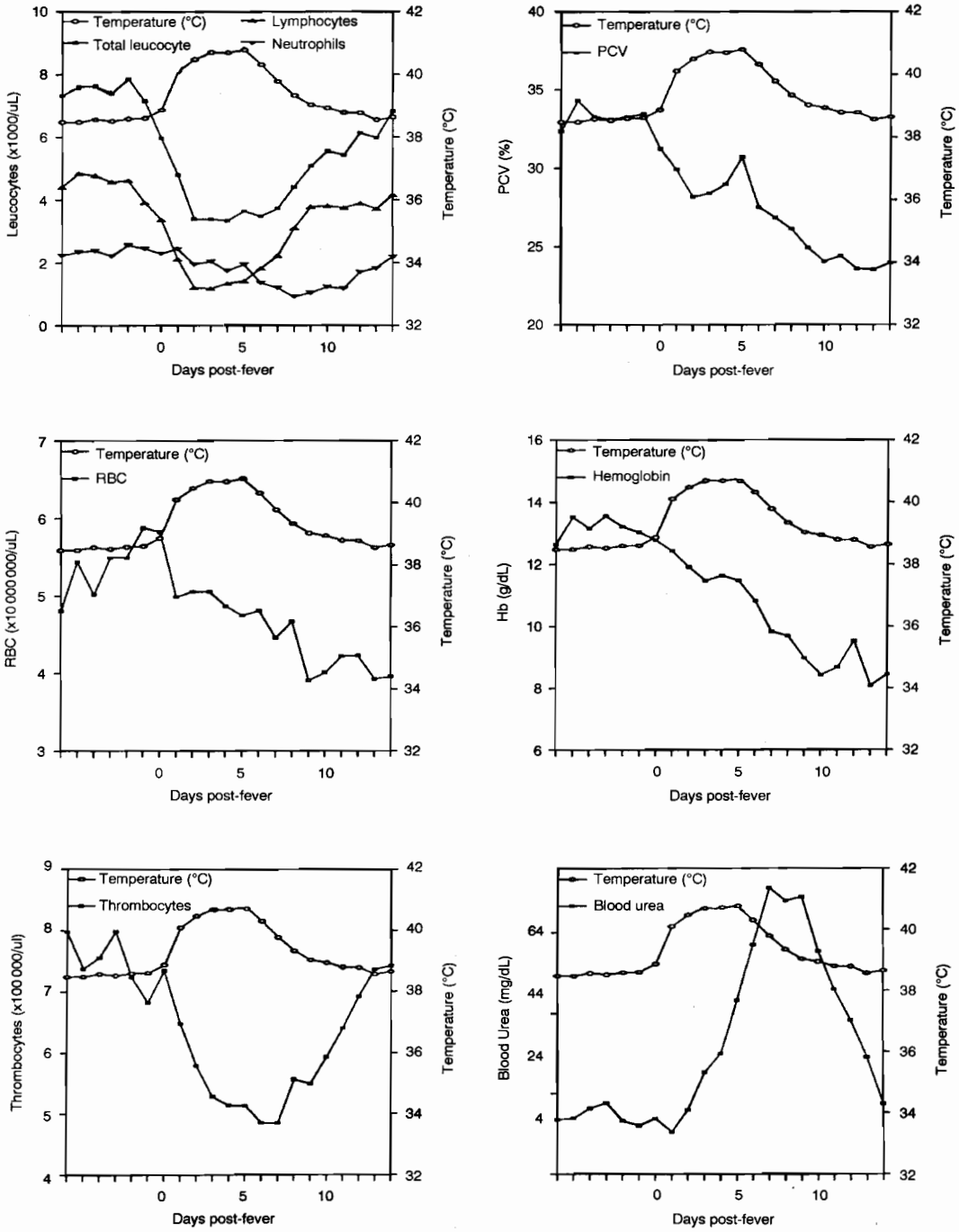

Figure 2. Changes in rectal temperature, peripheral blood leucocytes, RBC concentrations, PCV, haemoglobin concentrations, thrombocyte concentrations, and urea concentrations in Bali cattle during the course of experimentally-induced Jembrana disease. The results are the mean of results from 18 affected cattle.

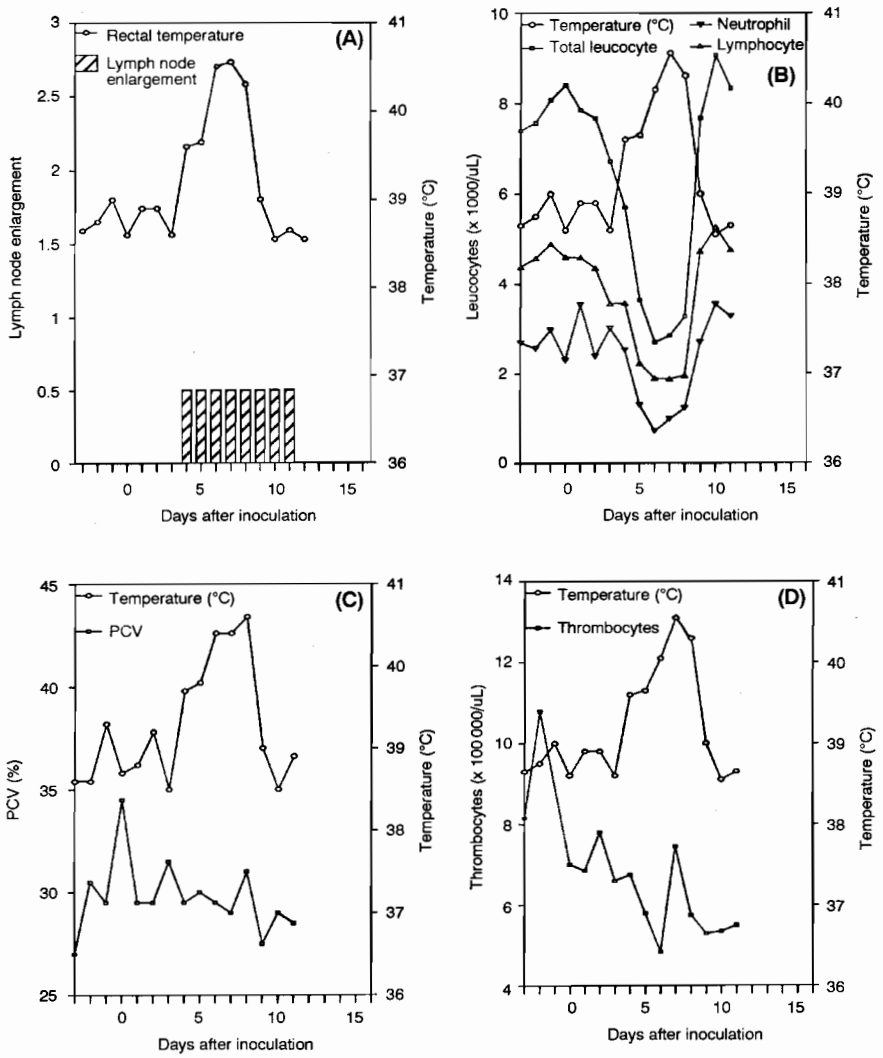

Figure 3. Clinical and haematological changes in Friesian cattle at intervals after infection with JDV. Shown are the changes in rectal temperature and degree of subcutaneous lymph node enlargement (A), peripheral blood leucocytes (B), PCV (C), and thrombocyte concentrations (D). The results are the mean of results from two inoculated Friesian cattle.

cells), liver (mild infiltration in the portal triads with lymphocytes), kidney (moderate infiltration of lymphoreticular cells into the intertubular areas), lungs (mild interstitial pneumonia with thickening and infiltration of the septa with lymphoreticular cells, and mild leucostasis in small blood vessels), and uterus (mucosa infiltrated with mononuclear cells). These changes in F81 were similar to those in mild cases of Jembrana disease in Bali cattle. In the other animal (F82) there were no macroscopic changes and histopathological changes were detected in only the lungs (alveolar septa slightly thickened and infiltrated with polymorphs), bronchi (erosions of the epithelial surface surrounded by polymorphs), and intestine (focal necrosis of Peyer's patches with polymorph infiltration). There was a good follicular response in the spleen and lymph nodes. These changes in F82 were not typical of lesions in Bali cattle with Jembrana disease but were those of a mild suppurative bronchitis and pneumonia. In neither

Table 3. Detection of virus in blood and/or spleen at various intervals after infection from Jembrana disease.*

Species/breed	Tissue	Time after infection (months)											
		0	1	2	3	4	6	7	8	9	11	19	25
Bali	Blood							+			+		+
Ongole	Blood	+	–		+		–		+			+	
	Spleen		+										
Madura	Blood	+	+		+		–						
Rambon	Blood	+	+		+		–						
Friesian	Blood	+	+			–							
Buffalo	Blood	+	+		+		+			+			
Sheep	Blood	–											
	Spleen		+	+		+							

* Detection of the virus involved subinoculation of the tissue into susceptible Bali cattle and subsequent observation of the inoculated animal for clinical signs of Jembrana disease.

animal were significant histopathological lesions detected in the lymph nodes, heart, adrenal gland, pancreas, gastrointestinal tract, bone marrow, tongue, thymus, urinary bladder, brain or choroid plexus.

Three other Friesian cattle were infected with JDV and were monitored at intervals after infection for evidence of viraemia. A febrile response of 1–3 days duration after an incubation period of 7–8 days developed in 2 of these 3 animals. Subinoculation of blood from these animals into susceptible Bali catttle indicated that the JDV survived in these Friesian cattle for at least one month but for less than four months after inoculcation (Table 3).

Experimentally-induced disease in Madura cattle

Eight of 10 Madura cattle inoculated with JDV developed a rectal body temperature more than 39 °C for two or more days after infection (Fig. 4). The mean febrile response in these eight animals began five days after inoculation and persisted for four days (range 2–5 days). In pyrexic animals moderate enlargement of the superficial prescapular, prefemoral and parotid lymph nodes was detected (Fig. 4) but there were no further clinical changes and all animals survived the infection.

In pyrexic cattle there was leucopenia (less than 4×10^9 leucocytes per μL), lymphopenia and neutropenia for 3 days during the febrile period (Fig. 4). The number of thrombocytes also decreased during the febrile period but was always greater than 500×10^9 per μL (Fig. 4). There was a reduction in the PCV during the febrile period (Fig. 4) but no significant change was detected in the number of erythrocytes, concentration of total plasma protein or blood urea. No haematological changes were detected in the cattle that did not show a febrile response after inoculation.

Two of the Madura cattle were killed at the end of the febrile period, 10 days after inoculation. The only macroscopic change detected was a slight enlargement of the visceral and subcutaneous lymph nodes, and spleen. Histopathological changes consistent with a mild form of Jembrana disease in Bali cattle were detected and included mononuclear cell proliferation in the non-follicular areas of the lymph nodes and spleen, alveolitis and leucostasis in the lungs, and mononuclear cell infiltration in the kidneys and adrenal medulla.

Reinfection of the remaining eight Madura cattle with JDV 48 weeks after the initial infection did not cause a febrile response.

Experimentally-induced disease in Rambon cattle

Six of 10 Rambon cattle inoculated with JDV showed a rectal body temperature exceeding 39 °C for two or more days (Fig. 5). The mean febrile response in these animals began 5 days after inoculation and persisted for four days (range 2–8 days). In the febrile animals there was enlargement of the subcutaneous prefemoral, prescapular and parotid lymph nodes from the start of the febrile period and persisting in some animals until 19 days after infection (Fig. 5); the persistence of the lymph node enlargement was greater but the degree of enlargement was less than in the Madura cattle. No other clinical changes were detected and all animals survived the infection.

In the six Rambon cattle that showed a febrile response, there was a mild reduction in the total number of leucocytes but the mean number did not decrease below 4×10^9 per μL (Fig. 5). The mild leucopenia was due to a transient lymphopenia

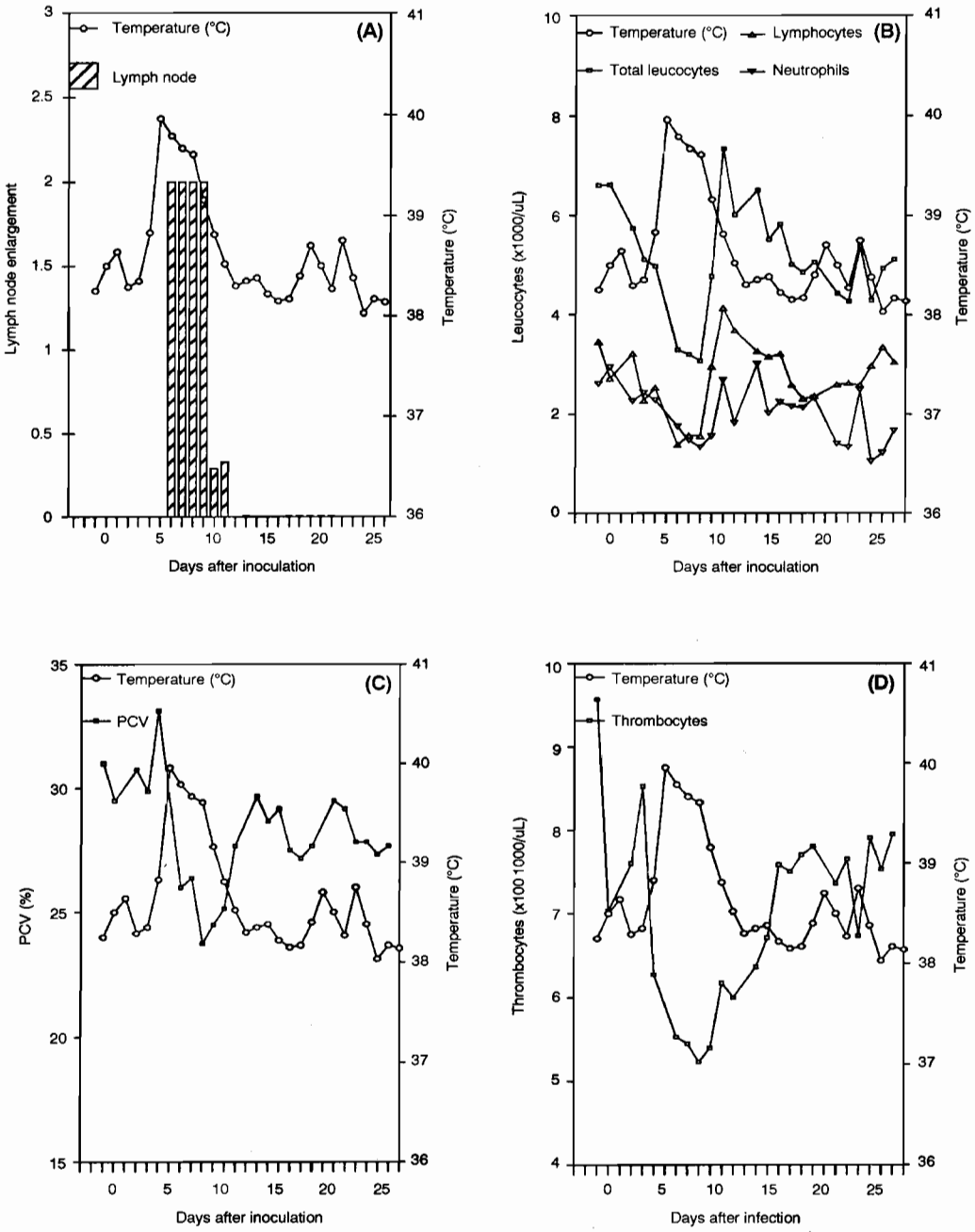

Figure 4. Clinical and haematological changes in Madura cattle at intervals after infection with JDV. Shown are the changes in rectal temperature and degree of subcutaneous lymph node enlargement (A), peripheral blood leucocytes (B), PCV (C), and thrombocyte concentrations (D). The results are the mean of results from 8 of the 10 inoculated Madura cattle that developed a febrile reaction.

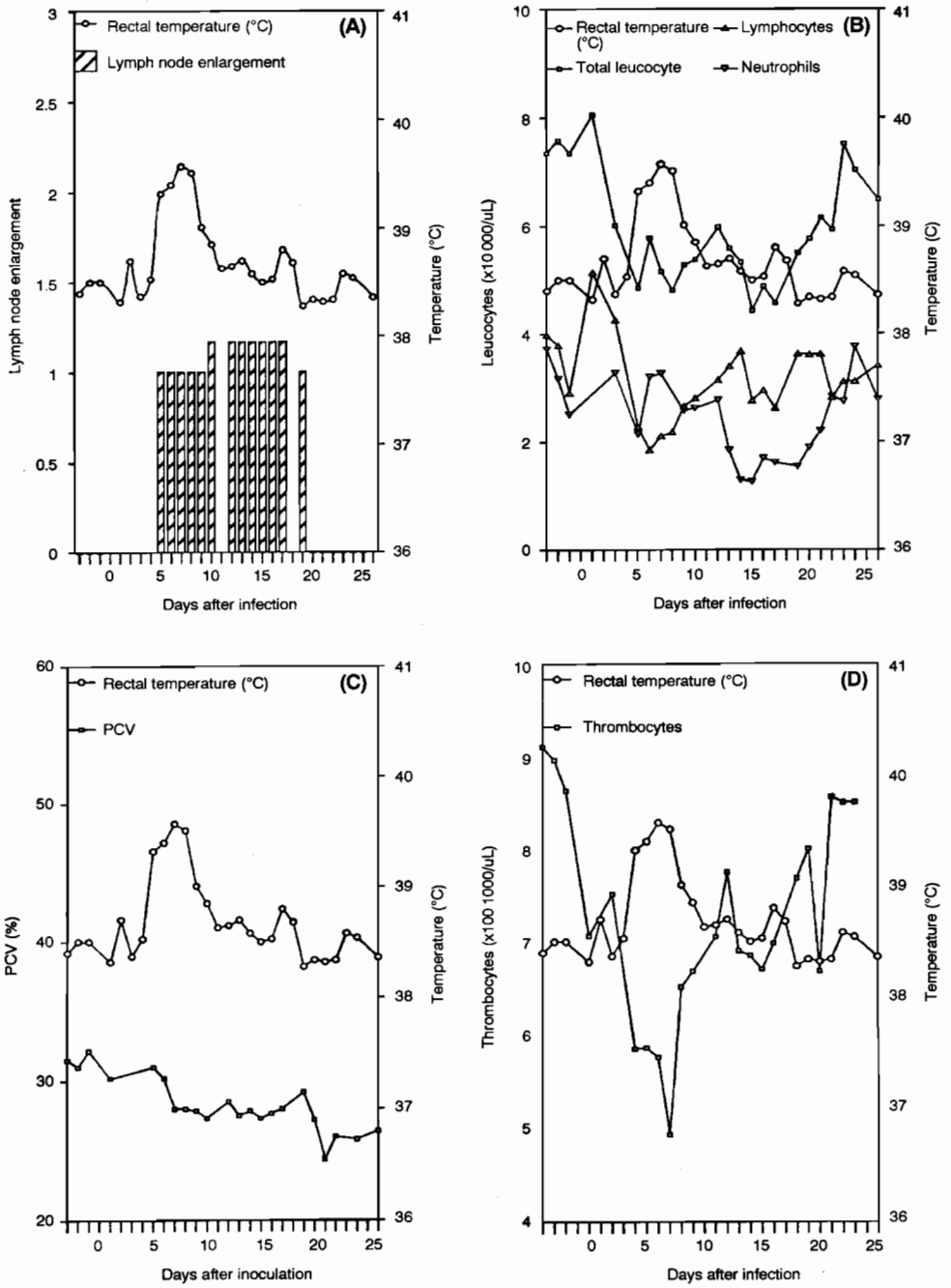

Figure 5. Clinical and haematological changes in Rambon cattle at intervals after infection with JDV. Shown are the changes in rectal temperature and degree of subcutaneous lymph node enlargement (A), peripheral blood leucocytes (B), PCV (C), and thrombocyte concentrations (D). The results are the mean of results from 6 of the 10 inoculated Rambon cattle that developed a febrile reaction.

during the febrile period and a transient neutropenia in the immediate post-febrile period (Fig. 5). Febrile animals showed a transient reduction in the number of thrombocytes, similar to that detected in Madura cattle (Fig. 5). There was a progressive decline in the PCV (Fig. 5) from the start of the febrile period and continuing until the end of the observation period (27 days after infection). No change was detected in the number of erythrocytes, concentration of total plasma protein or blood urea in any of the cattle that developed a febrile response, and no haematological changes were detected in the four cattle that did not become pyrexic.

One Rambon animal with a febrile response was killed 14 days after inoculation and one 19 days after inoculation. In both animals, consolidation and oedema of the lungs were detected macroscopically and histopathologically. Other changes included lymphocyte proliferation in the follicular and non-follicular areas of the lymph nodes and spleen, lymphocytic infiltration into other organs including the liver, kidneys and lungs, and a severe bronchopneumonia. The changes were consistent with mild lesions of Jembrana disease in Bali cattle, complicated by secondary bacterial pneumonia.

Reinfection of the remaining eight Rambon cattle 46 weeks after the initial infection did not produce a febrile response.

Experimentally-induced disease in buffalo

Five of eight buffalo developed a rectal body temperature of greater than 39.5 °C of 2–4 days duration after an incubation period varying 8–16 days. No other obvious clinical signs were detected but no haematological examinations were conducted. All buffaloes survived the infection.

Experimentally-induced disease in sheep, goats and pigs

No consistent febrile response or any other clinical signs of Jembrana disease were detected in inoculated sheep (n=9), goats (n=6) and pigs (n=6).

Persistence of the virus in infected animals

The persistence of the Jembrana disease virus in the blood or spleen of Bali cattle and the other inoculated species is shown in Table 3. Virus was recovered from the blood of Bali cattle for 25 months after recovery from clinical disease, from Ongole, Rambon and Madura cattle for 3 months but not after 6 months, from Friesian cattle for 1 month but not after 4 months, from buffalo for 9 months and from sheep for 4 months after inoculation. The agent was not detected in any of the tissue samples from inoculated goats or pigs, or from the buffalo before they were inoculated.

Inoculation of blood or spleen tissue from the persistently-infected Bali cattle produced a disease in subinoculated Bali cattle that was indistinguishable from that induced by subinoculation of tissues from animals with acute Jembrana disease.

In sheep the agent was detected by subinoculation of spleen tissue 1, 2 and 4 months after infection, but was not detected by subinoculation of whole blood obtained seven days after infection (Table 3). In Ongole cattle the agent was detected in spleen tissue one month after infection but not detected in whole blood obtained at the same time.

The approximate titre of JDV in blood of infected Bali cattle was determined at intervals after infection and the results are shown in Table 4. The titres (ID_{50} per mL) in blood were $<10^4$ until one day before the onset of fever when they increased to $>10^4$. Titres of 10^8 were detected on the second and third day of the febrile reaction, and 10^5 one day after the end of the febrile period. The titre decreased to 10^2 32 days after infection and a low titre of about 10^1 was detected 47 and 72 days after infection.

Resistance of recovered Bali cattle to re-infection

Only one of 18 Bali cattle previously affected with Jembrana disease that was induced experimentally with one of either two strains of JDV developed clinical signs of Jembrana disease when they were re-challenged with a third Tabanan/87 strain of JDV. This animal that reacted had been infected four months earlier with the Singaraja/86 strain, when it had developed a febrile reaction four days after infection which persisted for six days. After re-challenge, this animal developed a febrile reaction six days post-inoculation which persisted for six days. The animal was recumbent and unable to stand

Table 4. The titre of infectious virus in peripheral blood of Bali cattle at intervals after experimental infection with JDV.

Days after infection*	1	2	3	4	5	7	8	12	32	47	72
Titre (log ID_{50}/mL)	<4	<4	<4	>4	>4	8	8	5	2	1	1

* The febrile period in the sampled animals commenced six days after infection and persisted for six days.

two days after the rectal temperature had returned to normal, and was killed. Macroscopic and microscopic lesions typical of Jembrana disease were detected. All of the eight control animals challenged with the same inoculum developed typical clinical signs of Jembrana disease.

Discussion

The titre of infectious virus in blood and plasma on the second day of the febrile reaction was consistently about 10^8 per mL and it was estimated that all the animals other than Bali cattle were inoculated with at least 10^7 ID_{50} of JDV. Bali cattle were inoculated with doses of virus varying from 10^1 to about 10^8 ID_{50} and although there was a linear relationship between the dose and the incubation period there was no detectable relationship between the dose inoculated and the severity or duration of the disease induced.

The most common clinical changes encountered in the cattle experimentally infected with JDV were anorexia, lethargy, fever, erosions of the oral mucous membranes and enlargement of the superficial lymph nodes. Other signs less frequently observed were hypersalivation, nasal discharge, diarrhoea with blood in the faeces, pallor of the mucous membranes and a mild serous ocular discharge. These signs were accompanied by leucopenia principally due to a lymphopenia but also partly due to a neutropenia, an eosinopenia, a normocytic normochromic anaemia, thrombocytopenia, elevated blood urea concentrations and decreased plasma protein. Most of these haematological changes occurred during the febrile period, although the nadir of the neutropenia occurred after the febrile period had ended and the eosinopenia persisted until the observations were discontinued 21 days after the start of the febrile period. These clinical and haematological changes are similar to those reported in Jembrana disease by Teuscher et al. (1981); however, these authors reported that the thrombocytes practically disappeared during the febrile phase whereas the thrombocytopenia detected in the current study was only partial, and also that the erythrocyte sedimentation rate showed a considerable increase and this was not detected in the current study.

The high concentrations of blood urea in excess of 160 mg per dL in two of the three animals that died suggest this may have been a contributing factor to the death of these animals. These high values for blood urea are likely to be associated with the kidney lesions consistently detected in animals with Jembrana disease (Teuscher et al. 1981; Dharma et al. 1991).

The specific diagnosis of Jembrana disease in the field is difficult and is based upon clinical signs, principally anorexia, lethargy, fever, diarrhoea with blood in the faeces, enlargement of the superficial lymph nodes and 'blood sweating' (Teuscher et al. 1981). These changes are very similar to those detected in the experimentally-infected cattle except for the so called 'blood sweating'. 'Blood sweating', the appearance of pin-point drops of blood on the cutaneous surfaces, especially of the limbs, was not observed in any of the 18 experimental animals examined in this report and it does not appear to be directly related to the disease process; although the aetiology is unknown it may be related to arthropod bites (Teuscher et al. 1981) and is perhaps associated with the thrombocytopenia, which may contribute to delays in the clotting mechanism.

The differential diagnosis of Jembrana disease in Bali cattle includes the sheep-associated form of malignant catarrhal fever (MCF), an enzootic disease in Indonesia to which Bali cattle are susceptible (Sudarisman et al. 1986). The clinical signs of MCF can also include anorexia, fever, depression, lymph node enlargement, oral erosions, and diarrhoea or dysentery (Plowright 1986). Although the clinical disease associated with MCF in Bali cattle may often be peracute, the clinical signs of MCF may vary considerably (Hoffmann et al. 1984), and individual cases of MCF and Jembrana disease may be difficult to distinguish. However, the clinical signs of Jembrana disease such as the ocular and nasal lesions and diarrhoea are always mild and never progress to the severe forms sometimes observed in MCF (Selman et al. 1974); the erosive lesions in the oral cavity are also mild and generally confined to the postero-lateral surface of the tongue. Nervous signs observed in MCF (Selman et al. 1974; Plowright 1986) were not observed in the animals with experimentally-induced Jembrana disease. Other features distinguishing the two diseases are that with Jembrana disease the incubation period after experimental infection was consistently 12 days or less whereas in MCF the incubation period is highly variable, ranging from 18 days to over 100 days (Liggitt et al. 1978). The mortality rate observed in the present study of experimental Jembrana disease was 17%, whereas in MCF the disease is progressive and the mortality rate is virtually 100% (Plowright 1986).

The inability to induce clinical signs of Jembrana disease other than a transient febrile response in Ongole cattle, Ongole × Bali cattle, Friesian cattle, buffalo and pigs, and the lack of a febrile response in inoculated sheep, goats and pigs, is consistent with field observations that clinical signs of Jembrana disease occur only in Bali cattle and no other cattle

breeds or other animal species. However, while these other species did not develop an overt clinical disease they did develop mild clinical and haematological changes that were consistent with Jembrana disease in Bali cattle, and these same bovine species and sheep were shown to be infected with JDV for considerable periods after infection. However, the period of persistence of JDV in Friesian cattle, Ongole cattle and crossbred Bali × Ongole cattle was much less than in Bali cattle. In two of these animal species there may have been a longer period of persistence: in sheep, even though they did not develop any clinical response to JDV infection, virus was recovered from spleen tissue for at least four months after infection; the Indonesian buffalo, which are common draught animals in Indonesia, developed a persistent infection that persisted for at least nine months. The results suggest that species of cattle and particularly buffalo may be capable of maintaining the infectious agent in populations of animals in areas where there is only a low population of Bali cattle. It is also possible that if Bali cattle were not present, the Jembrana disease virus could persist in cattle populations without being recognised.

Sheep did develop a persistent infection, even though they showed no clinical reaction after they were inoculated with the infectious agent. However, the inability to recover the infectious agent from the blood of sheep seven days post-inoculation, and the need to inoculate spleen material to recover the agent, suggests that in sheep the number of infectious virus particles persisting after infection was very low. The apparently low titre of virus persisting in sheep, and the inability to detect the virus in goats and pigs after inoculation, suggests that sheep, goats and pigs are unlikely to act as significant vectors of the virus.

All Bali cattle developed clinical signs of Jembrana disease after infection but only some of the Ongole, Ongole × Bali cattle, and buffalo developed a febrile response. For example, only 80% and 60% of the Madura and Rambon cattle, respectively, developed clinical or haematological signs. However, although a febrile response may not have been detected, most of these animals were infected. As reported separately in these Proceedings by Hartaningsih et al. an antibody response to JDV was detected in all the Madura cattle (including two that did not show a febrile response) and 7 of the 8 Rambon cattle tested (including 3 of the 4 animals that did not show a febrile response). These serological results indicated the absence of clinical signs in some animals was not due to a failure to infect them with JDV.

Only limited studies were undertaken of the infection in Ongole cattle, buffalo, sheep, goats and pigs but more extensive studies were undertaken in the Friesian and crossbred Bali (Madura and Rambon) cattle. The clinical changes in Friesian and crossbred Bali cattle were a febrile response and concurrent lymphadenopathy. The extent and duration of the febrile response in these cattle types were less than in Bali cattle (Fig. 6) and the enlargement of the superficial lymph nodes during the febrile period, a striking characteristic of Jembrana disease in Bali cattle, was also less than that in Bali cattle (Fig. 6). Other clinical signs detected in Bali cattle with Jembrana disease were also of lesser severity or absent in Friesian and crossbred Bali cattle: lethargy and anorexia, common in Bali cattle, were detected only in the Friesian cattle and these changes were mild; oculo-nasal discharge, pallor of the mucous membranes and bloody diarrhoea, which occurred in some Bali cattle with Jembrana disease, were not detected in any crossbred Bali or Friesian cattle.

More extensive clinical and haematological studies were undertaken in the Friesian and crossbred Bali cattle than in Ongole cattle, buffaloes, sheep, goats and pigs. Changes in the crossbred Bali cattle occurred only in those animals that developed a febrile reaction after infection. The changes detected in Friesian and crossbred Bali cattle, including the leucopenia, lymphopenia, neutropenia, and thrombocytopenia, were also less severe than those reported in Bali cattle. Some haematological changes consistently detected in Bali cattle were not seen in the Friesian and crossbred animals: decreased plasma protein concentrations were not detected. Likewise, increased blood urea concentrations, consistently detected in Bali cattle and in severe cases associated with marked glomerular swelling and hypercellularity in the kidneys (Dharma et al. 1991), were not detected; decreased PCV and erythrocyte concentrations consistently detected in infected Bali cattle, indicating a normocytic normochromic anaemia, were not detected in the Friesian and crossbred Bali cattle. There were some changes indicating anaemia in the other animal species but these were mild: in the crossbred Bali cattle there was a small decrease in the PCV, which was transient in Madura cattle but persisted for the observation period in Rambon cattle. No marked change occurred in the PCV of infected Friesian cattle, and the erythrocyte count remained normal in all crossbred and Friesian cattle.

Although only two Madura, Rambon and Friesian cattle were examined post mortem, all had developed a febrile response and were killed during the immediate post-febrile period, when striking lesions were consistently detected in Bali cattle (Dharma et al. 1991). Histological lesions consistent with a mild form of Jembrana disease in Bali cattle were

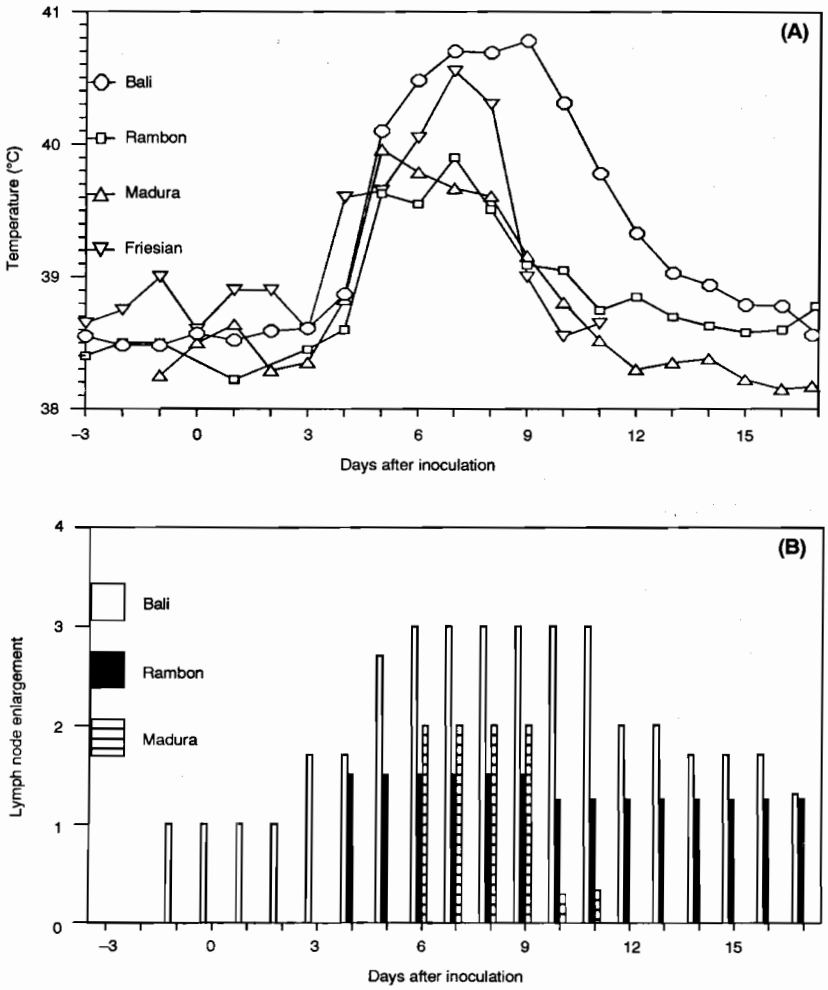

Figure 6. Comparative temperature (A) and lymph node changes (B) in Bali cattle, Friesian, Rambon and Madura cattle at intervals after infection with Jembrana disease virus. The changes were more severe and of longer duration in Bali cattle than the other cattle types.

detected in both the Madura cattle, but they were seen in only one of the two Friesian cattle, and in one of the two Rambon cattle.

The predominant histological lesions reported in Bali cattle with Jembrana disease (proliferation of lymphoreticular and lymphoblastoid cells in the parafollicular regions of the spleen and lymph nodes) occur in lymphoid organs (Dharma et al. 1991). This was also true of lesions detected in the crossbred Bali and Friesian cattle. However, whereas in the spleen and lymph nodes of Bali cattle there was follicular atrophy and a scarcity of plasma cells in the lymph nodes and spleen until five weeks after infection (Dharma et al. 1991), there was a marked follicular response in the spleen and lymph nodes of the crossbred Bali and Friesian cattle during the immediate post-febrile period. Infiltration of mononuclear cells into non-lymphatic organs such as the liver, kidneys, adrenal, heart and choroid plexus was common in Bali cattle (Dharma et al. 1991); however, when detected in the Madura, Rambon and Friesian cattle, it was less intense. Pulmonary histological lesions were consistently detected in Bali cattle with Jembrana disease (Dharma et al. 1991). In

the crossbred Bali and Friesian cattle, similar although mild pulmonary lesions consisted of infiltration of lymphoreticular cells into the septa and mild leucostasis in small blood vessels. As in Bali cattle, pulmonary lesions were sometimes complicated by histopathological lesions of bacterial pneumonia; Pasteurella pneumonia is common in Indonesian cattle (Dharma et al. 1991) and possibly the pneumonia pre-existed or was exacerbated by JDV infection.

Friesian and crossbred Bali cattle, unlike Bali cattle (Dharma et al. 1991), showed no parafollicular proliferation of mononuclear cells in intestinal lymphoid tissue. Haemorrhagic lesions, seen in infected Bali cattle (Dharma et al. 1991) were also not detected. Glomerular swelling and hypercellularity in the kidneys associated with increased blood urea concentrations, suggested as a possible cause of death in some cases of Jembrana disease in Bali cattle (Dharma et al. 1991), were not seen in the present study.

Although the lesions detected in the two types of crossbred Bali cattle were similar, they were of greater severity in the Madura than in the Rambon cattle. In Madura cattle, a higher percentage of animals became febrile, enlargement of the subcutaneous lymph nodes was greater, the leucopenia was of greater magnitude, and the antibody response to JDV was greater. The differences in magnitude of the response in the two types of crossbred cattle may be due to genotypic differences between them. Madura cattle belong to a stable breed derived by crossbreeding Bali and *Bos indicus* cattle. They are possibly more closely related genotypically to Bali cattle than are animals of the Rambon type (F1 generation Bali × Ongole (*Bos indicus*)). There is anecdotal evidence that F1 male progeny of Bali × Ongole cattle are infertile (Rouse 1972) and development of the Madura breed would have required further crossbreeding with male Bali cattle.

Although JDV has an antigenic (Kertayadnya et al. 1993) and genetic (Chadwick et al. 1995a,b) relationship with bovine immunodeficiency-like virus (BIV), there were major differences between the disease detected in the Friesian (Holstein) cattle infected with JDV and those that have been reported for BIV. In the Friesians leucopenia due to a lymphopenia and a neutropenia was detected. In contrast, the R29 strain of BIV inoculated into Holstein-Friesian calves was reported to cause a mild transient leucocytosis, lymphocytosis and lymphadenopathy (Van der Maaten et al. 1972), and mild follicular hyperplasia in lymphoid tissue five to six weeks after inoculation (Carpenter et al. 1992). Direct comparison of the histological changes in JDV and BIV-infected Friesian cattle is difficult as these have not been described for BIV-infected cattle until about five weeks after infection, at which time lesions in JDV-infected cattle are regressing and there is a marked increase in the lymphoid follicular reaction (Dharma et al. 1991). In the one Friesian animal infected with JDV that showed pathological changes 11 days after infection, there was a good follicular response in the lymph nodes and the degree of proliferation of lymphoreticular cells in the parafollicular areas of the spleen, a marked characteristic of the disease in Bali cattle (Dharma et al. 1991), was less than in Bali cattle. Further comparative studies of the earlier changes in *Bos taurus* cattle infected with BIV and later changes after infection with JDV are warranted.

Only one of 18 animals in which Jembrana disease had been experimentally induced up to 22 months previously developed Jembrana disease when re-challenged with the infectious agent. These results indicate that immunity does develop after primary infection with JDV.

In conclusion, it was demonstrated that JDV can replicate and produce lesions in a range of cattle types in addition to Bali cattle. However, while infection caused a severe disease in Bali cattle, it resulted only in a mild disease and sometimes a subclinical infection in the other cattle types. The disease in these other cattle types would be difficult to detect under field conditions. These experimental observations were consistent with serological evidence that in areas of Indonesia where the disease is endemic, cattle other than Bali cattle are sometimes infected (Hartaningsih et al. 1993).

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The Pathology of Jembrana Disease

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Abstract

The pathology of experimental and natural field cases of Jembrana disease is similar. The striking gross lesions during the acute phase, approximately 2–4 weeks post-infection, are generalised lymphadenopathy and splenomegaly.

Microscopically, in the first week post-infection (Phase 1), there is a general lymphoreticular reaction affecting both follicular and non-follicular compartments. The acute phase (Phase 2), 2–4 weeks post-infection is signified by follicular atrophy and an intense non-follicular lymphoreticular hyperplasia and a similar infiltrative and proliferative process in liver, kidneys, adrenal medulla and elsewhere. Leucostasis, mainly occuring in the smaller blood vessels of the lungs, has a very high diagnostic value. In the third phase, starting from the fifth week of infection, there is a marked lymphoid follicular reaction and plasma cell formation. Residual lesions occur up to 60 days post-infection.

Temporary immunosuppression appears to occur during the acute phase of the disease as indicated by follicular atrophy, a decrease in the immunoglobulin G-containing cells in the lymphoid organs and a decrease in the BoCD4:BoCD8 T-lymphocyte ratio in lymph node follicles.

AFTER a major epizootic on Bali island in 1964, Jembrana disease (JD), a disease of viral (retrovirus) aetiology and characterised by fever, lymphadenopathy, anorexia, rhinitis, hypersalivation and enteritis, is now endemic in Bali, Lampung, West Sumatra and South Kalimantan. The disease has also been reported in the Banyuwangi district in East Java. This paper summarises the pathological changes of field cases of JD and sequential pathological changes and immunohistochemical findings in Bali cattle experimentally infected with Jembrana disease virus (JDV).

Pathological Changes of Field Cases of Jembrana Disease

The major pathological changes of field cases of JD from Bali (1983–95), Lampung (1976), West Sumatra (1992), South Kalimantan (1994) and East Java (1978) are principally the same. The general post mortem picture is dominated by reactive changes in the lymphoid system namely lymph nodes and spleen, and haemorrhages in various tissues such as epicardium, endocardium, serous and mucous membranes. The striking histopathological changes consist of proliferation of lymphoreticular cells in the lympho-haemopoietic system and infiltrative changes in other organs such as lungs, adrenals, liver, kidneys and choroid plexus. Leucostasis is consistently detected in the lungs and other tissues. Using an electron microscope, the large mononuclear cells with abundant cytoplasm causing leucostasis were proven to be intravascular macrophages (Budiarso and Rikihisa 1992). There are no significant changes observed in the central nervous system. It is concluded that the general pathological changes observed in recent cases of JD are basically as same as those described by Adiwinata (1967), Pranoto and Pudjiastono (1967), Ishitani (1968), Budiarso and Hardjosworo (1976, 1977) and Teuscher et al. (1981).

Sequential Pathological Changes in Bali Cattle Experimentally Infected with Jembrana Disease Virus

This experiment was conducted to understand better the pathogenesis of JD. In this experiment, animals were killed at specified intervals after intravenous

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inoculation of the Jembrana disease virus (JDV). Twenty Bali cattle were allotted into 10 groups of two animals each and were all intravenously inoculated with 1 mL of a 10^{-5} dilution of 220 nm filtered plasma from a donor animal. Two animals were then killed and necropsied at various intervals after infection. In addition, four animals were intravenously inoculated with 1 mL of medium 199 containing 10% foetal bovine serum and kept in close contact with the experimental animals, then killed and necropsied at 2 and 10 days after inoculation. They served as in-contact control animals.

This experiment revealed that infection of Bali cattle with plasma from a JD-infected donor animal caused a well-defined pathological response. The general gross picture was dominated by signs of vascular damage in the form of mild exudates and haemorrhages, and reactive changes in the lymphoreticular system especially. The most striking gross changes were lymphadenopathy and splenomegaly. Enlargement of lymph nodes was first observed at day six post infection (PI), becoming severe from days 10 to 15, and still apparent up to day 30 PI. Splenomegaly was first observed two days PI and became most marked from days 8 to 15 PI. Mild splenomegaly was still seen at day 42 but not at day 60 PI. Petechial and ecchymotic haemorrhages and oedema were observed in various visceral organs including the gastrointestinal tract, heart and kidneys. Mild apical consolidation of the lungs and multifocal greyish foci of the heart and kidneys were also constantly present.

Microscopic examination revealed three distinct phases of JD. The initial phase, occuring in the first week of infection, was indicated by a general lymphoreticular reaction involving both follicular and non-follicular compartments of the lymphoid organs. The second phase, occuring from 8 to 21 days PI, was signified by an intense non-follicular proliferative response by reticular and lymphoblastic cells in lymphoid organs, with a similar infiltrative and proliferative process in the lungs, heart, liver, kidneys, adrenal medulla and choroid plexus. The third phase, which developed from the fifth week of infection, was signified by a marked lymphoid follicular reaction and plasma cell formation which was prominent in the medullary cords of the lymph nodes and the non-follicular areas of the spleen. The cytology and histological distribution of proliferative changes in the lymphoid system suggested that during the acute phase of the disease, a predominantly T–lymphocytic reaction took place, perhaps associated with transient humoral immunosuppression. Residual lesions occurred up to day 60 PI. Detailed results of this experiment have been published (Dharma et al. 1991).

Sequential Immunohistochemical Findings in the Lymphoid Organs of Bali Cattle after Inoculation with JDV

This study was carried out using the peroxidase-antiperoxidase test for immunoglobulin–containing cell (ICC) assessment and the indirect test for lymphocyte subset assessment.

Assessment of ICC

For ICC assessment, formalin–fixed lymphoid tissues (spleen and lymph node) from animals used for the sequential study of the pathology of experimental JD were used. Tissue preparation and staining procedures are described by Dharma (1992). Briefly, this study revealed that the prevalence of immunoglobulin–G–containing cells (IgG-CC) in the follicular and medullary cord compartments of the lymph nodes, and follicular and non-follicular compartments of the spleen, dropped from day 2 PI until day 21 PI, then increased sharply thereafter until day 60. This pattern was similar to the pattern of antibody detected in the blood (Hartaningsih et al. 1993). Immunoglobulin G-CC were most prevalent in the medullary cords of the lymph nodes and the non-follicular compartment of the spleen. ImmunoglobulinM- and IgA-CC were, however, scattered in all compartments of the lymphoid organs but the numbers were low and the standard deviations were high.

Assessment of lymphocyte subsets

To assess lymphocyte subsets in the lymph nodes of Bali cattle after infection with the JDV, animals were allotted into three groups of three animals each and were all intravenously inoculated with 1 mL of the 10^{-5} dilution of 220 nm filtered plasma from the donor animal. All animals were subsequently killed by exsanguination and necropsied on days 4 (incubation phase), 10 (acute phase) and 42 (recovery phase) PI. Three animals were intravenously inoculated with 1 mL medium 199 containing 10% FBS and killed and necropsied at day 14 PI to serve as control animals. Tissue preparation and staining procedures are described by Dharma (1992).

Briefly, this study revealed that BoCD4 and BoCD8 lymphocytes were detected in all lymph node compartments in all sections from every experimental animal. They were found in the largest numbers in the parafollicular and paracortical areas of the lymph nodes. In this study no significant differences in the ratio of BoCD4:BoCD8 lymphocytes in the paracortex were found between different clinical phases of the disease. The numbers of BoCD4 and BoCD8 lymphocytes in the follicles

varied remarkably between follicles within the same section. In this study, a significant decrease ($P < 0.05$) in the mean BoCD4:BoCD8 ratio in the follicle was noted during the acute phase of the disease. Scattered BoCD4 and BoCD8 lymphocytes were also seen in the sinuses and the medullary cords of the lymph nodes but no clear numerical difference was apparent. Detailed results of the experiment on the immunopathology of JD have been published (Dharma et al. 1994).

Conclusions and Recommendations

Jembrana disease appears to be unique in Bali cattle and seems to have no obvious parallel in any other species. The pathology of JD has been well documented and can be used as diagnostic tools. The pathology of experimental and natural field cases of JD is basically the same. It induces severe lymphoreticular hyperplasia in the non-follicular areas of the lymphoid organs in a neoplastic-like fashion but regression eventually follows. Prominent 'leucostasis', such as seen in the lungs of an infected animal has not been described in other bovine diseases. Without complication by secondary bacterial infection, the acute phase is usually followed by recovery.

Knowledge of the pathogenesis of JD at this stage is far from complete. Therefore, more detailed sequential lymphocyte subset studies in the lymphoreticular tissues and peripheral blood are recommended to understand better the immunosuppressive mechanism in JD. Identification of the precise target cells, for example, by a double-labelling immunoperoxidase test, is suggested to provide more information on the kinetics of JDV, particularly during the early immune response which is related to the antigen presentation mechanisms. The recently developed in situ hybridisation technique may later be applied to detect minute amounts of viral nucleic acid in the tissues, to explain the persistence of the virus.

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Bali Cattle: Origins in Indonesia

J.W. Copland[1]

THE origin of Bali cattle, *Bos javanicus* d'Alton, in Indonesia, is part of the complex evolution of all cattle over a long time. The evidence is clouded in antiquity. There is some information from fragments of fossils and limited evidence from anthropological and archaeological findings, from ancient and modern history. Although there is no formal basis on which to make a statement on the origin of Bali cattle, there are intriguing clues which suggest a possible process of evolution.

Origin of Cattle

The earliest known type of wild cattle, *Bos acutifrons* comes from a part of a fossil found in India (Payne 1970). The wild cattle (*Bos acutifrons*) then developed into two streams: the aurochs (*Bos primigenius*) that inhabited the forests of Asia, North Africa and Europe; and the urus (*Bos namadicus*) found in India and other parts of Asia. One view is that the centre of origin of the cattle family, the Bovidae, was the old world tropics or subtropics of Asia, North Africa and Europe (Pilgrim 1947). Most views expressed on the origin of cattle focus on the auroch as the foundation of most of the cattle species and breeds developed in the world. The auroch, although an ancient cattle type, has survived into historical memory as the last recorded animal was killed in a Polish forest in the seventeenth century (Payne 1970). They were large animals with enormous horns, the latter a characteristic found in modern cattle species.

Very little is known of the origin of *Bos* (*Bibos*) type cattle in Southeast Asia. They appear to have become separated from the *Bos* type in the Upper Pliocene period (Zeunner 1963). Cattle of this type superficially resembled Zebu, as they possessed a hump, although the bone structure of the head is quite different. It has been suggested that the modern *Bos* (*Bibos*), such as Bali cattle, is more similar to ancestral cattle than other modern types.

The widespread distribution of wild species of cattle in the temperate and subtropical climatic zones of the 'old world' and findings in the Upper Pleistocene period make it particularly difficult to discover the original centre(s) of domestication (Payne 1970). Domestication could have occurred at one point or several points at the same time. It is thought that there must have been at least two centres of domestication of cattle in Southeast Asia. The geographical distribution of *Bos* (*Bibos*) types of cattle suggests that the centre of domestication was Indo-China and Malaysia, later spreading to Bali. The other centre of domestication was probably Assam-Burma and led to the development of the gayal.

The centre of domestication is a geographical region where a type of cattle migrated and evolved into another type due to natural selection and the influence of man. The cattle may have migrated naturally or as a result of man's role as a hunter. In these centres, the results of natural selection over a long time transformed the surviving animals into a type which best suited the biophysical environment. In the first instance, natural selection was the force as it is most unlikely to have been influenced by man. However, as man became a sedentarised farmer, he started to utilise all the resources around him, including cattle. The process of domestication integrated cattle into the agricultural system and developed an interdependence between man and animals.

A tentative and general picture of the origins of Bali cattle is given in Figure 1 and the global location of the main cattle breeds of today in the world is given in Figure 2.

The evolution and origins of Bali cattle could be summarised as follows (adapted from Friend 1970).

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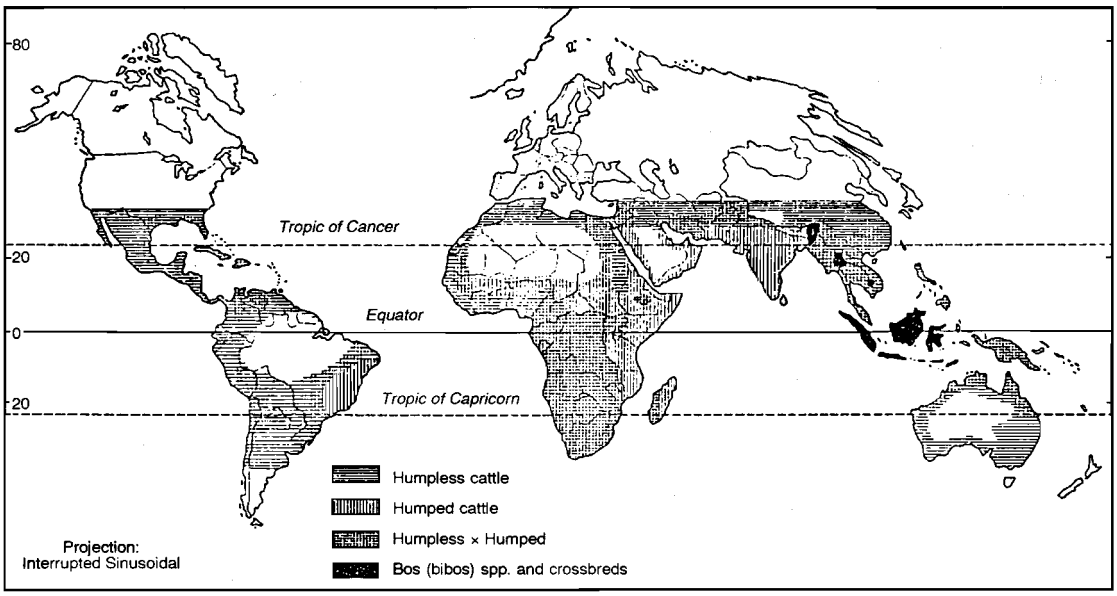

Figure 1. Distribution in the tropics and subtropics of the major types of cattle (Payne 1970).

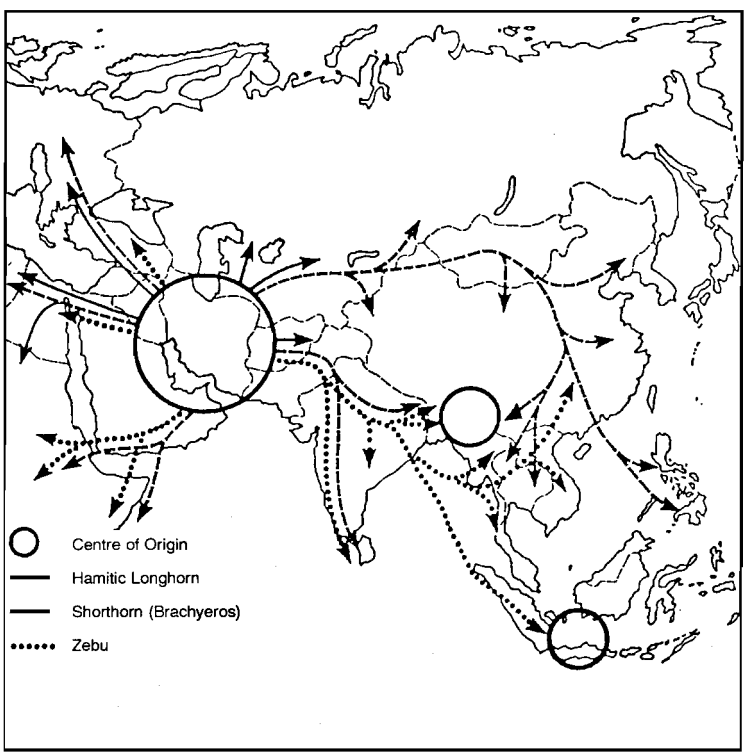

Figure 2. Possible migration routes of domestic cattle in Asia (Payne 1970).

Common Ancestors

Pliocene period: some 7 million years ago

Natural selection and migration leading to the following ancient breeds

Pleistocene period 2 million years ago leading to

Bos bonanus	European bison
Bos gaurus	Guar of India
Bos grunniens	Yak
Bos nomadicus	Wild cattle of India
Bos primigenius	Wild Aurochs
Bubalus bubalus	Indian Buffalo
Bos banteng	Banteng of Burma

Domestication and migration of man and *Bos banteng*

Domestication period of *Bos banteng* 10 000–5000 years ago in Indonesia

Bos javanicus	Indonesia, Thailand, Indo-China, Australia

Not all cattle types have been domesticated. For example, the American bison, in spite of many attempts by American Indians, have not been domesticated. Bali cattle in one sense are not fully domesticated, as they have the capacity to revert readily to the wild state, called the banteng, at any stage of life. Conversely, they can be domesticated relatively easily. The ability to move between the wild and domestic states is a possible reflection of recent domestication in Indonesia.

Indonesia probably became the centre of Bali cattle domestication some ten to five thousand years ago. The process of domestication probably started in prehistoric times on Bali and Java, according to Meijer (1962).

Bali cattle migrated in the 19th and 20th centuries from Indonesia into Malaysia (Devendra et al. 1973) and into Australia (Letts 1962; Calaby 1975). Often the number imported was small. The 20 animals imported into Australia in the early 1800s resulted in a wild feral population of more than 2000 cattle 150 years later (Letts 1964). Interestingly, the importation of Bali cattle from Timor did not introduce Jembrana disease, but it may have resulted in the introduction of cattle tick and buffalo fly into Australia.

Indonesia has two types of Bali cattle, a domestic type, called Bali cattle and a wild type named banteng. There are not many wild bantengs left in Indonesia. They are now mainly found in the national parks such as Ujung Kulon and Baluran (Wind 1978).

Taxonomy of Bali Cattle

The taxonomy of a species can reflect its origins. The scientific name of Bali cattle has been loosely used in the scientific literature during the past century, perhaps the result of wild and domesticated examples of the species existing at the same time. The taxonomy was resolved by Hooijer (1956) in a study of the authenticity of earlier reports and observations.

The scientific name of Bali cattle is *Bos javanicus* (d'Alton); d'Alton identified the genus and species and described the taxonomy of Bali cattle in 1823 which precedes all other descriptions and names.

The formal classification of Bali cattle is as follows:

Kingdom	Animal	
Order	Artiodactyla	Cloven hoofed animals
Class	Ruminantia	Animals with a rumen
Family	Bovidae	
Genus	Bos	
Species	javanicus (d'Alton)	

Synonyms of *Bos javanicus* are:

Bos leucoprymnus by Quoy and Gaimard (1830). This is rejected because it was based on hybrid and was after d'Alton's description.

Bos sondaicus by Müller (1840). Rejected as it was after d'Alton's description.

Bos banteng by Temminck (1840). Rejected.

Bos bantinger by Schlegel and Müller 1855. Rejected.

The holotype of the species is located in the Leiden Museum in the Netherlands. It is a male skeleton from Java and described by d'Alton and subsequently confirmed, although misnamed by other taxonomists (Schlegel and Müller 1845, and Rutimeyer 1867).

Without any doubt, Bali cattle (*Bos javanicus*) are an independent and distinct cattle species.

Indicators that banteng and Bali cattle are the same species of *Bos javanicus* d'Alton

Morphological evidence is given by Hayashi (1981) in a study of the multiseriate craniometric analysis of the relationships between wild banteng and five types of native Asian cattle in Indonesia, Philippines and Korea. In this study, Bali cattle and wild banteng were found to have similar cranial relationships.

The chromosomes of the two types are identical and consist of 29 accrocentric chromosome pairs and two submetacentric sex chromosomes, with a diploid of 2n = 60 (Fischer 1971).

In a global survey of the skin of cattle types and breeds, Jenkinson and Nay (1973) examined the sweat glands of Bali cattle and banteng and found

that they were similar. The shallow hair follicle in *Bos javanicus* is part of the reason for their superior heat tolerance.

The Australian experience described by Letts (1962, 1964) of importing domestic Bali cattle (*Bos javanicus*) from Bali and Timor and then letting them become feral has shown that the domestic Bali cattle can revert to the wild banteng quickly and become indistinguishable from the native banteng as seen in Indonesia.

The uniqueness of Bali cattle

Bali cattle are different from all other species of cattle as a result of their origin and evolution. They will crossbreed with European cattle, *Bos taurus*, but the male offspring as reported by Jellinek et al. (1980), is usually infertile. Bali cattle do have some distinct characteristics which have made them the cattle breed of choice in many parts of Indonesia. Differences between Bali cattle and *Bos taurus* and *Bos indicus* may influence their responses to physiological and pathological events such as Jembrana disease and may explain the variation seen in the susceptibility observed in the field.

Some of the unique differences of Bali cattle from other cattle which make them a valuable asset in Indonesia are:

Size	Smaller than Zebu and European cattle (Anon. 1983)
Colour	Strikingly uniform markings (Payne and Rollinson 1973)
Behaviour	Timid and can become wild readily
Reproduction	High conception rates
Lactation	Ability to stop lactating and survive a bad dry season
Feed utilisation	Better able to utilise low quality feed base
Heat tolerance	Better heat tolerance than buffalo and other cattle (Moran 1973)
Water turnover	Lower water turnover than *Bos taurus*/Shorthorns (Siebert and Macfarlane 1969, Jenkinson and Nay 1973)
Meat quality	Marked fat deposition sites and limited fat in muscle mass
Disease pattern	Stated to be resistant to external and internal parasites except liver fluke, *Fasciola gigantica*. Increased resistance to malignant catarrhal fever and Jembrana disease.

Due to the difference in origin, Bali cattle are genetically unique compared to other cattle, and are likely to have a subtle and different spectrum of characteristics of production and disease responses. They are well adapted to the Indonesian environment, particularly in Eastern Indonesia, and once Jembrana disease is controlled, they have the potential to be a major national asset for the livestock sector.

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Bali Cattle — Their Economic Importance in Indonesia

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Abstract

Indonesia covers an area of 9.8 million km^2 of which 1.9 million is land. The population was about 195 million in 1995, and Gross National Product (GNP) per capita was US$900 in 1994. The agricultural sector has made a major contribution to the Indonesian economy, growing more than 4% per year during the past decade, with the contribution of livestock to agricultural GNP increasing from 6% in 1969 to 11.5% in 1994. This paper examines the role of livestock in Indonesia from the point of view of its contribution to food production, and as a means of generating income and employment, of sustaining agriculture and the environment, and of reducing poverty. The economic importance of Bali cattle is discussed, as well as their role in livestock development in Indonesia.

INDONESIA is an archipelago of more than 17 000 islands extending 5100 km from west to east and 1888 km from north to south. Administratively, Indonesia consists of 27 provinces, 243 districts, 60 municipalities, 3839 subdistricts and 65 554 villages. Out of a population of about 195 million (1995), about 109 million (54%) live on the island of Java, which accounts for only 7% of the total land area. Gross National Product (GNP) per capita in 1994 was about US$900.

Thirty years ago, Indonesia was one of the poorest countries in the world, with a GNP per capita of US$50, half that of India and Bangladesh. In 1969, the Government of Indonesia (GOI) adopted a new strategy for development, the First Twenty-Five Year Long-Term Development Plan (PJP 1), and pursued it through successive Five-Year Development Plans (Pelita) for broad-based economic growth, particularly related to rural development. Agricultural development was given one of the highest priorities, with food self-sufficiency as a target.

The agricultural sector made a major contribution to the Indonesian economy, growing more than 4% per year during the past decade. A large part was due to a rapid growth in rice production, but also to steady growth in livestock, estate crops and fisheries. The result was that poverty among rural smallholders was reduced.

Although the contribution of agriculture to GNP decreased during the past 25 years from 42% in 1969 to 18% in 1993, the role of agriculture in the Indonesian economy is still important, given that 51.1% of householders are still engaged in agriculture. On the other hand, the contribution of livestock to agricultural GNP increased from 6% in 1969 to 11.5% in 1993.

Role of Livestock in the Indonesian Economy

Livestock for food

The contribution of livestock to food supplies in Indonesia is increasing at a high rate. Meat production rose by 375% between 1969 and 1994, from 309 300 tonnes to 1 469 200 t. During the same period, egg production increased by 905% from 57 700 tonnes to 580 300 t, and milk by 1244% from 28 900 t to 388 600 t.

As income determines the protein intake of people, particularly in urban areas, higher incomes result in higher demand for animal products. Of the different animal species, meat production from monogastric animals (poultry and pigs) increased faster than that of small ruminants (sheep and goats) or large ruminants (cattle and buffalo). Trends in meat production for different animal species in Indonesia are presented in Table 1.

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Table 1. Meat production by species 1969–1994 (Indonesia) ('000 tonnes).

Year	Cattle	Buffalo	Goats	Sheep	Pigs	Horses	Poultry	Total
1969	164.9	48.5	12.0	9.7	34.2	0.8	39.2	309.3
1970	167.3	49.2	12.2	9.8	34.6	0.8	38.7	312.6
1971	177.2	52.1	12.9	10.4	36.7	0.8	42.1	332.2
1972	196.1	57.1	13.9	11.0	38.6	0.7	48.8	366.2
1973	202.6	59.6	14.9	11.7	41.2	0.9	48.5	379.4
1974	212.8	62.3	15.4	12.0	44.5	0.9	85.2	433.1
1975	224.5	65.2	17.4	13.0	47.8	0.9	66.2	435.0
1976	225.5	64.6	19.4	8.4	51.2	1.5	78.3	448.9
1977	224.5	65.5	23.5	14.1	46.7	1.4	92.0	467.7
1978	225.4	64.2	26.3	14.3	47.7	1.4	65.3	444.6
1979	213.7	64.4	35.2	17.1	54.2	1.6	100.3	486.5
1980	220.8	65.4	36.3	17.6	57.3	1.6	172.3	571.3
1981	227.8	67.8	38.5	18.2	59.0	1.7	183.0	596.0
1982	235.5	70.2	40.2	18.8	60.8	1.7	201.3	628.5
1983	203.7	44.2	65.5	23.5	93.9	1.8	218.9	651.5
1984	216.4	48.2	48.3	28.8	119.1	1.7	279.7	742.2
1985	227.4	48.6	49.5	29.8	133.2	1.7	318.7	808.9
1986	227.8	49.4	61.8	31.7	163.7	1.3	343.3	879.0
1987	234.8	43.7	61.5	30.6	141.0	1.5	382.4	895.5
1988	238.1	41.7	66.2	31.0	154.3	1.6	404.1	937.0
1989	242.8	43.1	62.9	32.2	136.3	1.4	442.4	971.1
1990	259.2	44.3	58.3	31.7	123.8	1.7	508.7	1027.7
1991	262.2	47.5	57.0	37.4	110.0	1.5	583.5	1099.1
1992	297.0	45.0	68.8	30.2	149.9	1.8	646.6	1239.2
1993	346.3	51.2	71.2	40.1	169.3	1.6	698.6	1378.3
1994*	358.2	53.6	72.1	41.2	184.2	1.7	758.3	1469.2

*Preliminary figures.

Livestock for income and employment

At the farm level, livestock provide income and so increase the economic stability of households because small stock act as a cash buffer, and large stock as a capital reserve. They also act as a hedge against inflation and reduce the risk associated with crop production in mixed farming systems.

At the national level, livestock products represent 11.4% of total agricultural output. The livestock subsector has achieved the greatest growth in production during the past 25 years and it is expected to increase by 6.4% in Pelita VI, with food crops increasing by 2.5%, estate crops by 4.2% and fisheries by 5.2%.

Livestock are a source of general employment. Milk production can be very labour-intensive at the farm level, and sheep, goats and poultry provide an important source of part-time jobs for rural families. During Pelita V, livestock generated 328 000 jobs, and during Pelita VI, a target of 456 000 jobs has been set.

Livestock in sustainable agriculture and the environment

Mixed farming systems that include livestock offer many advantages by providing the basis for a more profitable and sustainable agricultural system by adding value to crop residues, and by providing manure. Large animals also provide draught power.

The use of livestock to graze vegetation under coconut, oil palm and rubber plantations increases production and reduces the costs of weed control. Such systems also safeguard the environment and avoid pollution while supplying additional organic matter to the soil.

Feeding crop residues to livestock and using their manure as fertilizer and as a soil conditioner benefits the environment directly. Soils treated with manure have better structure, water retention and draining capacity. As a result, crops grow faster, provide good ground cover and reduce erosion.

The number of cattle and buffalo used as draught animals, as well as for meat and milk production, has increased by 70% and 7% respectively during the past 25 years. Trends in livestock populations for the different animal species are shown in Table 2.

Contribution of livestock to poverty reduction

In general, livestock can best contribute to poverty reduction through improving efficiency in integrated farming systems other than through the addition of free standing intensive enterprises.

Experience under the recent GOI — Smallholders Cattle Development Project indicates that the income of farmers with cattle was higher than those without them, primarily because of the capacity to use cattle as draught animals, use of crop residues for fodder, grazing on land otherwise not used, and the contribution of manure to soil fertility.

The importance of livestock as a source of income for poor farmers is demonstrated by the fact that in Bangladesh, the Grameen Bank, which assists the "poorest of the poor", provides about 50% of its loans for the purchase of livestock. The same applies in Indonesia where 70% of funds for "desa terting gal" under "Inpres Desa Tertinggal" (IDT) were used for purchasing livestock.

Economic Importance of Bali Cattle

The role of Bali cattle in agriculture is almost the same as that of livestock in general agriculture. The species contributes to meat production, acts as a source of income, generates employment, provides

Table 2. Livestock population by species 1969–1994 (Indonesia) ('000 head).

Year	Dairy cattle	Beef cattle	Buffalo	Goats	Sheep	Pigs	Horses
1969	52	6447	2940	7544	2998	2878	642
1970	59	6137	2885	6336	3362	3169	692
1971	66	6245	2918	6943	3146	3382	665
1972	68	6286	2822	7189	2996	3350	693
1973	70	6389	2276	6665	3480	2662	636
1974	86	6380	2415	6517	3403	2906	600
1975	90	6242	2432	6315	3374	2707	627
1976	87	6237	2284	6904	3603	2947	631
1977	91	6217	2292	7232	3864	2979	659
1978	93	6330	2312	8051	3611	2902	615
1979	94	6362	2432	7659	4071	3183	596
1980	103	6440	2457	7691	4124	3155	616
1981	113	6516	2488	7790	4177	3364	637
1982	140	6594	2513	7891	4231	3587	658
1983	198	8894	2398	10970	4789	4248	527
1984	203	9236	2743	9025	4698	5112	659
1985	208	9318	3245	9629	4885	5560	668
1986	222	9432	3496	10783	5284	6216	715
1987	233	9510	3296	10392	5354	6339	658
1988	263	9776	3194	10606	5825	6484	675
1989	288	10094	3224	10996	5910	6936	683
1990	294	10410	3335	11298	6006	7136	683
1991	306	10667	3311	11484	6108	7612	695
1992	312	11211	3342	12062	6235	8135	678
1993	329	10829	3057	11502	6240	8704	582
1994*	330	11010	3109	11886	6485	9010	585

*Preliminary figures.

manure for improved soil fertility, converts roughage into high value products, and provides draught power.

In Indonesia, Bali cattle are considered an original species with several economic advantages, compared to other species.

Historical background

Bali cattle *(Bos sondaicus)* is a domesticated species of banteng *(Bibos banteng)* which was known from ancient times in Burma, Thailand, Indochina, the Malaysian peninsular, Sumatra, Java and Bali.

Wild banteng are still found in West Java (Ujung Kulon Wildlife Reservation) and East Java (Baluran Wildlife Reservation). Bali cattle are similar in type and appearance to wild banteng, but where and when their domestication took place is still a matter of scientific debate. However, a Special Veterinary Conference in Pamekasan Madura in 1934 concluded that domestication of wild banteng did take place in Indonesia, sustaining a certain species purity on Bali Island.

Distribution

Bali Island is recognised as the main source of pure Bali cattle. From Bali, they spread to other parts of Indonesia, and to Malaysia and Australia. Bali cattle were imported to Lombok island by the ancient monarch and Lombok subsequently became the second source of pure Bali cattle. They were introduced to South Sulawesi in the 1890s and to Java in 1907. They performed satisfactorily in South Sulawesi and Mojoagung, East Java, but unsuccessfully in West Java, due to malignant catarrhal fever, a virus infection carried by sheep. Bali cattle were then introduced to Timor, Sumbawa, the Moluccas, Irian Jaya and other parts of Sumatra and Kalimantan. Trends in Bali cattle numbers and distribution are shown in Table 3.

Table 3. Bali cattle and other breed populations and distribution in Indonesia (1988).

Provinces	Ongole	P.O	Bali	Madura	Other	Total
DI. Aceh	3863	3107	84	32881	339995	379930
Sumatera Utara	9106	31224	2455	5143	131096	179024
Sumatera Barat	11849	13370	6867	12910	307604	352600
Riau	0	0	26489	0	53848	80337
Jambi	0	0	20047	1523	49376	70946
Sumatera Selatan	0	0	14939	0	300300	315239
Bengkulu	126	50	21228	6026	61676	89106
Lampung	8787	1925	83730	10808	57774	163024
DKI Jakarta	0	0	0	0	0	0
Jawa Barat	5973	8599	620	9709	126179	151080
Jawa Tengah	29269	177549	14236	23196	872099	1116349
DI. Yogyakarta	2389	26949	185	797	155033	185353
Jawa Timur	104565	363502	42234	888658	1613718	3012677
Bali	0	0	432927	0	0	432927
NTB	289	629	305388	2745	33979	343030
NTT	34056	5905	438138	20517	113239	611855
Timor Timur	0	0	9326	0	47813	57139
Kalimantan Barat	110	1320	46236	54068	0	101734
Kalimantan Tengah	0	4342	11244	5584	0	21170
Kalimantan Selatan	25870	21667	60093	2326	0	109956
Kalimantan Timur	0	3761	9923	0	21619	35203
Sulawesi Utara	2319	19217	1833	233	207025	230627
Sulawesi Tengah	9541	31815	69848	46818	170985	329007
Sulawesi Selatan	11982	58234	987463	7429	133126	1198234
Sulawesi Tenggara	0	0	14367	0	160499	174866
Maluku	0	0	4571	0	62988	67559
Irian Jaya	0	0	7654	0	25422	33076
Total in Indonesia	260094	773165	2632125	1131371	5045393	9842148
(Percent of total cattle in Indonesia)	(2.64)	(7.85)	(26.75)	(11.5)	(51.26)	(100)

Source: Buku Statistik Peternakan, 1995.

Comparative advantages

Pioneer cattle

Because Bali cattle were seen to thrive in South Sulawesi, Kalimantan, Irian Jaya and other areas where cattle had not previously been developed, they were regarded as a pioneer species that could go wild or semi-wild if left in the jungle, but could be easily domesticated.

High fertility rate

The fertility rate is the percentage of normal calving produced by a group of mated female animals within a year. Some researchers found that the fertility rate of Bali cattle was very high.

Some observations by scientists in a number of locations were as follows:

- Aalfs (1934) on Bali island, 83%;
- Wardoyo (1950) in South Sulawesi, 82%;
- Davendra (1973) in Malaysia, 82%;
- Moran (1971) in northern Australia, 90%;
- Kirby (1972) in northern Australia, 100%.

Normal length of pregnancy and ease of calving

The average length of pregnancy of Bali cattle observed during the Bali Cattle Breeding Project at Pulukan, Bali, was 287.6 days, with male calves taking 2.7 days longer than female calves. In general, all cattle breeds have a similar length of pregnancy, between 275 and 298 days.

Observations of 1000 calvings of Bali cattle (in Bali) showed that more than 90% of foetuses were in the anterior position which enabled calving to take place easily; the rest were in the posterior position. Dystocia cases caused by malposition (parturition abnormalities) were rare.

Pregnancy rate at first mating

Pregnancy rate at first mating is a very important determinant of the fertility of a group or breed of cattle. As already noted, the pregnancy rate of Bali cattle is high.

Observations in Penebel, Marga and Sukasada for the three years 1978–1980 of 2000 pregnancies showed an average rate of 88%. Other researchers found rates between 80% and 86%. The pregnancy rate of *Bos taurus* is between 50% and 70% while that of *Bos indicus* is lower, ranging between 35% and 61% (Oloufa 1955).

Higher calving percentage

The calving percentage per year of a group of cattle is the number of calvings divided by the number of adult cows, multiplied by 100. Darmadja (1980) found the calving percentage of Bali cattle was 52.15%. Other research in Indonesia indicated the calving rate for Bali cattle on Timor was 64% ±1.6%, Flores 78% ±12.8%, Sumbawa 72% ±21.5%, Lombok 74.4% ±11.3%, with the average 72.1% ±14.3% higher than Ongole cattle 40.4% ±21.4%.

The Bali Cattle Breeding Project reported that by strict selection (culling of infertile and unproductive cows) the fertility rate of Bali cattle is increasing from 74.08% to 93% with the calving interval reduced from 538 days to 421 days. It is expected that the calving percentage per year will increase by about 29%, from 52.15% to about 80.6%.

High adaptability

As has been mentioned, Bali cattle have a high adaptability, being able to survive in any area, climate or condition, such as the wet, arid and semi-arid areas of Indonesia, from Sumatra to Irian Jaya.

High response to fattening

Research in Bali by Nitis (1979) on the effect of replacing 30% of forage with concentrated feed showed that the body weight of Bali cattle fed on concentrates was 2.7 times heavier than those given green roughage, or 1.7 times heavier than cattle raised by traditional farmers. On such findings, it could be concluded that Bali cattle have a high response to fattening.

High carcass percentage

Researchers have recorded the following carcass percentages for Bali cattle: Aalfs (1934) 52%; Moran (1978) 55%–56%. Compared with other original Indonesian cattle, the carcass percentage of Bali cattle is the highest, with Ongole cattle at 45%, and Madura cattle at 47.8% (Tajib 1956).

Comparative disadvantages

Slow growing cattle

Bali cattle are known to be slow growing, but males especially continue to grow until they reach a considerable slaughter weight. The live weights of male and female Bali cattle, from birth to slaughter age, are presented in Table 4.

Growth of the female is slower than that of the male, and the older the female, the slower the growth. At 5.5 years of age, female growth stops. Weight gain between 2.5 years and 3 years was only 8.1 kg and between 5.5 and 6 years, only 1 kg.

Males grow fairly fast until weaned, grow slower for a period, then grow faster from 1–1.5 years on. Good quality bulls grow well until 4.5 years old and can achieve a high body weight.

Bali cattle given high quality feed produced an average daily gain (ADG) of only 660 grams over a period of 154 days, lower than for Grati and Ongole cattle, and buffalo, which produced a AGD of 900 grams, 750 grams and 730 grams respectively (Moran 1971).

High calf mortality

Darmadja (1980) reported average calf mortality up to 6 months of age (182 days) was 7.33% and Sumbung et al. (1977) reported calf mortality of Bali cattle in South Sulawesi was 7%.

The Bali Cattle Breeding Project recorded mortality rates of young cattle (205–550 days) at 3.95%, while the percentage on traditional farms was 6%.

Several observations on adult cattle mortality have been made both on Bali and outside Bali. The Bali Cattle Breeding Project has recorded adult Bali cattle mortality at 4%, while Sumbung et al. (1977) recorded adult mortality at 2.7% in South Sulawesi. The high mortality of adult Bali cattle is caused by Jembrana disease which is threatening the cattle industry in Indonesia. Other causes are bacterial diseases and toxic pesticides. The causes of adult mortality recorded by the Bali Cattle Breeding Project are shown in Table 5.

Susceptibility to Jembrana and other specific diseases

Jembrana is the name given to a disease affecting Bali cattle which was first recognised in 1964 in the Jembrana district of Bali. The disease spread rapidly to surrounding districts and by August 1965 had occurred throughout Bali and an estimated 26 000 Bali cattle died out of a total population of 300 000. In the Jembrana district, the reported mortality was 19 000 out of a total of 31 000. Another outbreak of a similar disease occurred in Tabanan district of Bali in 1971 and 1972, with a mortality rate of 13%.

A more endemic condition of Bali cattle disease, currently known as Jembrana disease (JD) has been the topic of research since 1972.

Currently, JD or a Jembrana-like disease has been reported in three areas: in Bali, JD is now endemic; in Lampung Tengah, Rama Dewa disease is now endemic; and in Banyuwangi, East Java, the disease is known as Banyuwangi disease.

Malignant catarrhal fever (MCF) affects cattle and buffalo sporadically and with low incidence, but its case fatality rate is very high, up to 95%. MCF is caused by a virus. The first report of MCF in Indonesia was in 1894, affecting buffalo in Kediri (Partadiredga et al. 1988). Bali cattle are the most susceptible to MCF, followed by Madura cattle and buffalo, while Ongole and Friesian Holstein are relatively resistant (Peranginangin 1988).

Table 4. Production and reproduction performance of Bali cattle.

Items	Sulsel	NTT	NTB	Bali	P3 Bali*
Puberty age (month)	20	22	22	20	18
Av. weight at first mating (kg)	165	170	170	180	185
Calving percentage (%)	76	70	72	69	86
Calving interval (days)	475	520	510	530	430
Av. birth weight (kg)	12	12	13	16	18
Av. weaning weight (205 days) kg	70	75	72	86	94
Av. weight at 1 year (kg)	112.5	115	115.4	127.5	140
Av. weight at 2 years (kg)					
— male	210	220	222	235	260
— female	170	180	182	200	225
Av. weight at 5 years kg					
— male	350	355	300	395	484
— female	225	235	238.5	264	300

Source: Ismed Pane, 1980.
*P3 Bali = Bali Cattle Breeding Project.
NTT = Nusa Tengqara Timur province.
NTB = Nusa Tengqara Barat province.
Sulsel = Sulawesi Selatan

Table 5. The cause of adult mortality at Bali Cattle Breeding Project.

Mortality causes	Year									
	1978		1979		1980		1981		Average	
	ND	%	ND	%	ND	%	ND	%	ND	%
Accident	—	—	2	4.65	1	1.85	3	4.47	6	2.90
Feed poisoning	2	6.25	3	6.97	2	3.70	2	2.78	9	4.48
Pesticide poisoning	2	6.25	4	9.30	5	9.26	9	12.50	20	9.95
Jembrana	17	53.13	17	39.53	27	20.00	33	45.83	94	46.77
Coryza	2	6.25	2	4.65	2	3.70	1	1.39	7	3.48
Bacterial diseases	4	12.50	5	11.63	3	5.55	10	13.89	22	10.95
Internal parasites	—	—	2	4.65	8	14.81	7	9.72	17	8.46
	—	—	1	2.33	—	—	—	—	1	0.50
Other diseases	2	6.25	2	4.65	4	7.40	6	8.33	14	6.97
Unknown	3	9.37	5	11.63	2	3.70	4	5.56	14	6.97
	32	100	43	100	54	100	72	100	201	100

Source: Bali Cattle Breeding Project.
ND = Number of deaths.

Another specific disease affecting Bali cattle is Bali Ziekte (BZ), which attacks sporadically and with clinical signs similar to photosensitivity caused by liver function disorder. Ressang (1984) suspected that a virus or poisonous plant was the causal agent. Sobari (1980) stated that *Lantana camara* is a poisonous plant which causes BZ. He proved that the clinical signs of BZ occurred in Bali cattle fed on 1 kg of dried *Lantana camara* leaves. Dharma et al. (1982) also said that the clinical signs and necropsy was indistinguishable between BZ and the disease caused by poisoning with *Lantana camara*.

Role of Bali Cattle in Livestock Development

Bali cattle as draught animals

Bali cattle grow faster than any other breed. In 1967, the proportion of Bali cattle was 11% of total cattle numbers and this has now increased to 26.8% (Table 6). In line with this, the role of Bali cattle as draught animals has become more important.

Bali cattle and meat production

According to a sampling carried out by the Directorate of Livestock Programming in 1991, the percentages of different cattle species slaughtered for meat were Bali cattle 38%, Peranakan (crossbred) Ongole (*Bos indicus*) 25%, Ongole 14%, Madura cattle 5%, local cattle 16% and dairy cattle 2%. Based on this study, the contribution of Bali cattle to beef production in Indonesia was the highest.

Table 6. The cattle population of different breeds in Indonesia (1984–1994) in '000s.

Year	Ongole	PO	Bali	Madura	Others	Total
1984	231	741	2199	1042	5023	9236
1985	238	749	2300	1064	4967	9318
1986	245	757	2406	1086	4938	9432
1987	252	765	2516	1108	4869	9510
1988	260	773	2632	1131	4980	9776
1989	267	781	2753	1154	5139	10094
1990	276	790	2879	1178	5287	10410
1991	284	798	3011	1203	5371	10667
1992	293	806	3150	1228	5734	11211
1993	301	715	2895	1253	5665	10829
1994*	310	765	2954	1279	5702	11010
(%)	(2.8)	(6.9)	(26.8)	(11.6)	(51.8)	100.0

Source: Directorate of Livestock Programming.
* Preliminary figures.
PO = Peranakan Ongole (crossbred Ongole).

Distribution of Bali Cattle through GOI projects

The Government of Indonesia (GOI) has distributed Bali cattle throughout Indonesia through several projects.

(1) The Second Kalimantan Livestock Development Project (ADB Loan No. 706 — INO) has provided loans for the procurement of 58 000 female and 4900 male cattle, of which 21 000, or 33% were Bali cattle.

Table 7. Distribution of cattle under government projects in Indonesia (1992–1995).

Provinces	Different breeds of cattle (head)							
	Bali	BC	SC	SO	PO	Madura	Other	Total
DI. Aceh	1994	3969	3495	366	0	0	1172	10996
Sumatera Utara	557	891	0	0	684	0	0	2132
Sumatera Barat	8734	695	0	1083	3008	0	1441	14962
Riau	31812	0	0	0	497	1249	0	33558
Jambi	15839	2936	0	0	0	4294	201	23269
Sumatera Selatan	16073	211	0	0	798	1552	362	18996
Bengkulu	30251	2176	0	0	1231	4260	0	37918
Lampung	22391	1319	0	0	2971	3545	539	30765
DKI Jakarta	0	0	0	0	0	0	0	0
Jawa Barat	0	2082	518	0	0	0	0	2600
Jawa Tengah	11480	0	0	0	4515	0	1695	17690
DI. Yogyakarta	0	0	0	0	652	0	3409	4061
Jawa Timur	475	0	0	0	11072	1494	6305	19345
Bali	574	0	0	96	0	0	0	670
NTB	20189	0	0	0	0	0	45	20234
NTT	26291	0	0	2322	0	1006	0	29620
Timor Timur	12323	0	0	0	0	0	0	12323
Kalimantan Barat	9248	10021	0	0	7829	2227	0	29325
Kalimantan Tengah	10650	6619	0	0	5393	5457	0	28119
Kalimantan Selatan	15886	11104	1064	674	2689	0	0	31418
Kalimantan Timur	9319	11306	0	0	6375	3378	0	30377
Sulawesi Utara	1752	0	0	0	55	0	36	1843
Sulawesi Tengah	9656	0	0	0	1329	0	0	10985
Sulawesi Selatan	29553	0	0	1329	0	0	0	31191
Sulawesi Tenggara	6979	1738	1226	0	0	0	0	9943
Maluku	8783	0	0	811	0	2639	0	12232
Irian Jaya	11995	0	0	0	0	0	0	11995
Total 1995	312804	55066	6303	6991	49097	31102	15205	476567
1994	304113	57821	8888	5780	47758	30450	14894	469703
1993	295457	56858	8515	5628	41916	29458	11603	449435
1992	258042	54175	8198	4343	26857	26857	10875	402658

Legend: Bali = Bali Cattle, BC = Brahman Cross, SC = Sahival Cross, SO = Sumba Ongole, PO = Peranakan (crossbred) Ongole, Madura = Madura Cattle.

(2) The Smallholder Cattle Development Project IFAD Phase I (Loan 35 — ID) has procured and distributed 54 410 head of cattle, of which 51 405 or 98% were Bali cattle and the rest (2%) were Madura cattle.
(3) IFAD Phase II Project (IBRD — 2628 and IFAD ID — 171) distributed 76 000, mostly Bali cattle.
(4) Cattle distributed under the GOI distribution and redistribution program up to 1995 were reportedly numbered 476 567, of which 312 804 (64.5%) were Bali cattle; the rest were Brahman cross (11.6%), Sahiwal cross (1.3%), Sumba Ongole (1.5%), Peranakan Ongole (10.3%), Madura cattle (6.5%) and others (3.2%).

Distribution trends under GOI projects are presented in Table 7.

Bali cattle related projects

There are two main projects related to the development of Bali cattle:

(1) The Bali Cattle Breeding Project in Bali (Pulukan), Lampung, West Nusatenggara (Dompu) and South Sulawesi (Bone). The objective of this project, which began in 1978, was to improve the genetic potential of Bali cattle. It was conducted with New Zealand technical assistance (1978–1995).
(2) The Bali Cattle Disease Investigation Unit (BCDIU) is part of the Disease Investigation Centre (DIC) at Denpasar, Bali, which was established a part of the Smallholder Cattle Development Project. The objectives of the study

are to determine the basic characteristics of the disease and the aetiological agent involved, to develop a methodology for detection and diagnosis, and to determine the distribution and method of transmission.

Acknowledgment

The author thanks ACIAR for the opportunity to present this paper.

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The Physicochemical Characteristics of a Virus Associated with Jembrana Disease

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Abstract

The etiological agent of Jembrana disease in Bali cattle (*Bos javanicus*) is present to a high titre of about 10^8 infectious agents per mL in the plasma of cattle during the acute clinical disease. Plasma from such cattle was used to characterise the infectious agent, shown by exclusion membrane filtration to be 50 to 100 nm in size, indicating it was a virus and not a rickettsia as previously proposed. Electron microscopic evidence, including the size (75 to 130 nm), morphology and morphogenesis of the virions in plasma and tissue from affected animals, indicated the virus was a retrovirus. The virus banded at a density of 1.15 g per mL in sucrose gradients, and contained an intravirion reverse transcriptase. The morphology and apparent morphogenesis of the virions most closely resembled that of lentiviruses. Further evidence that the virus was a lentivirus was the antigenic relationship of an immunodominant 26K protein of the virus to the 26K capsid protein of bovine immunodeficiency virus.

JEMBRANA disease of Bali cattle (*Bos javanicus*) can be consistently reproduced in susceptible Bali cattle by intravenous or intraperitoneal inoculation of blood or spleen tissue from infected cattle (Soeharsono et al. 1990). The inoculated cattle develop clinical and pathological signs of Jembrana disease that are indistinguishable from those in natural cases (Teuscher et al. 1981; Dharma et al. 1991; Soeharsono et al. 1990).

Since the first outbreak of the disease in 1964, there have been several hypotheses regarding the cause of the disease. It was initially considered to be caused by rinderpest virus (Adiwinata 1968; Pranoto and Pudjiastono 1975), and subsequently to be caused by a rickettsia-like agent (Hardjosworo and Budiarso 1975) possibly an *Ehrlichia* (Ressang et al. 1985). A more recent hypothesis based on the ability of the infectious agent to pass through a 220 nm membrane filter, the resistance of the agent to antibiotics, and the nature of the histopathological changes, was that the disease was caused by a virus (Ramachandran 1981; Teuscher et al. 1981).

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Difficulty in characterising the infectious agent associated with Jembrana disease has been caused by the inability to culture the agent in any in vitro system. However, studies showed there was a high titre of the infectious agent in the plasma of infected Bali cattle during the acute stages of the disease (Soeharsono et al. 1990) and this suggested plasma from such cattle could be used as a source of the infectious agent, and enable its identification and characterisation. This report describes such studies of the physicochemical characteristics of the agent present in plasma and spleen of infected animals which indicated Jembrana disease was caused by a retrovirus, possibly a lentivirus.

Methods and Results

Source and maintenance of the infectious agent

Three 'isolates' of the infectious agent were used and designated Klungkung/85, Singaraja/86 and Tabanan/87. Unless otherwise stated, the Tabanan/87 isolate was used. All isolates were obtained from naturally-infected animals exhibiting typical signs of Jembrana disease. Ten mL of heparinised blood from the affected animal was injected intravenously into susceptible Bali cattle and the recipient animals were

killed two days after the development of fever. Spleen tissue was aseptically collected, distributed into aliquots, and stored frozen at −70°C. When required, the tissue was thawed, a 10% homogenate was prepared with a mortar and pestle with medium 199 (Flow Laboratories) as a diluent, and then clarified by centrifugation at 650 g for 20 minutes.

Bali cattle used for experimental infection studies were approximately 18 months of age and obtained from Nusa Penida, a small island adjacent to Bali, where Jembrana disease has not been reported. Experience has shown that these cattle are consistently susceptible to experimental challenge. The animals were kept in screened animal houses and kept under observation for a minimum of seven days prior to use.

Titration of the infectious agent

The Jembrana disease agent was titrated using Bali cattle as an indicator host. Serial 10-fold dilutions of tissue were prepared in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS). One mL of each dilution was inoculated intravenously into two susceptible Bali cattle and an approximate 50% infectious dose (ID_{50}) per mL was determined as the highest dilution of the inoculum producing typical clinical signs of Jembrana disease (Soesanto et al. 1990) in the inoculated cattle. Negative control animals were inoculated with DMEM with 10% FCS. Plasma from infected cattle collected at sequential intervals were also titrated as described above.

Titration of the infectious agent present in blood of Bali cattle at intervals after experimental infection showed that the titre of the agent in whole blood was less than 10^4 ID_{50} per mL until one day before the onset of the fever, then rapidly increased to approximately 10^8 ID_{50} per mL during the febrile period. A titre of 10^5 ID_{50} per mL was detected one day after the end of the febrile period, and very low titres of approximately 10 ID_{50} per mL were detected 47 and 72 days after infection. During the febrile period, there was no difference between the titre of the agent in whole blood and the plasma fraction.

Infection of laboratory animals

Attempts to maintain the infectious agent in suckling Swiss mice were unsuccessful. Litters of suckling Swiss mice were inoculated intracerebrally with 0.02 mL of plasma from infected and febrile Bali cattle containing 10^8 ID_{50} per mL. The mice were killed seven days after inoculation and the brains homogenised in DMEM and subinoculated into the brains of further newborn mice. After an additional 10 days, these mice were also killed and the brains and the spleens from them were suspended in DMEM. The suspensions were clarified by centrifugation and inoculated into Bali cattle. No clinical signs were detected in the inoculated mice. Subinoculation of brain suspensions from the inoculated mice into Bali cattle did not result in the development of Jembrana disease.

Intraperitoneal inoculation of Wistar rats, guinea pigs and rabbits with plasma containing an estimated 10^8 ID_{50} per mL of the infectious agent was also carried out. This did not result in the development of clinical signs of disease in any of the inoculated animals. The animals were killed 7 to 10 days after inoculation and spleen tissue from the animals subinoculated into Bali cattle. This did not result in the development of Jembrana disease in the inoculated animals.

Replication of the infectious agent in cell cultures

Attempts were made to determine if the infectious Jembrana disease agent would replicate in various continuous cell lines, namely Vero (African green monkey), CV1 (African green monkey), BHK-21 (hamster kidney), EBTr (embryonic bovine turbinate) and Madin-Darby bovine kidney (MDBK) cells. Attempts were also made to adapt the agent to replicate in primary foetal bovine kidney, lung, spleen, testis and muscle cells prepared from foetuses of Bali cattle by conventional techniques. All cell cultures were grown in 25 cm^2 plastic flasks using DMEM containing 10% FBS and antibiotics, and maintained at 37°C in a 5% CO_2-in-air-atmosphere. Confluent monolayers were maintained in the same medium but with only 2% FBS. To infect the cells, medium was removed from confluent cell culture monolayers and 0.5 mL of plasma from infected cattle was added for one hour at 37°C. A further 5 mL of maintenance medium was then added to the cultures which were incubated at 37°C. After 7 days, the cultures were rapidly frozen and thawed and inoculated (passaged) onto other uninfected cell cultures. To detect the infectious agent in the cultures, they were examined daily for any cytopathic effect, and the cells from the inoculated cultures were suspended in the culture medium by scraping the monolayers with a rubber spatula and the suspension inoculated intravenously into susceptible Bali cattle.

Attempts were also made to determine if the infectious agent would replicate in peripheral blood mononuclear cell (PBMC) cultures. These were prepared from heparinised blood collected from uninfected Bali cattle. The PBMC were separated using Nycoprep (Nycomed Pharma AS, Roeshov, Norway), washed two times in DMEM by centrifugation at 500 g for 10 minutes, counted and

resuspended in DMEM containing 20% FBS and antibiotics to a dilution of 2×10^6 cells per mL. Five mL of the diluted cells were dispensed into 25 cm^2 plastic tissue culture flasks and incubated at 37°C in a 5% CO_2-in-air atmosphere. The PBMC cultures in 25 cm^2 flasks were infected by the addition of 0.5 mL of plasma from infected cattle. The cultures were maintained at 37°C and additional normal PBMC were added to the cultures at seven day intervals. The infected cell cultures were inoculated into Bali cattle to detect the presence of the infectious agent. As a control, a 10% suspension of plasma in DMEM without mononuclear cells was also incubated at 37°C and after seven days 1 mL of this material was inoculated into susceptible Bali cattle.

There was no evidence of virus replication in any of the adherent continuous cell lines or primary cell cultures, but there was evidence of limited persistence of the infectious agent in the cultured PBMC. No cytopathic effect was detected in any of the inoculated continuous cell lines or primary bovine cell cultures during two blind passages of the agent, each of seven days duration. Sub-inoculation of these cultures that were maintained for 7 and 14 days into susceptible Bali cattle did not produce clinical signs of Jembrana disease. In the infected PBMC, there was also no cytopathic effect detected but subinoculation into susceptible Bali cattle of the cultures maintained for 7 days (6/6 cultures tested) and in some cultures maintained for periods between 7 and 28 days resulted in the development of Jembrana disease in the inoculated cattle. The agent was not detected in control uninfected PBMC cultures or in medium inoculated with plasma in the absence of mononuclear cells and incubated for seven days at 37°C (0/2 cultures tested).

Size of the infectious agent determined by membrane filtration

To determine the size of the infectious agent, exclusion membrane filtration was used. Plasma from infected Bali cattle (approximate titre of 10^8 ID_{50} per mL) was sequentially filtered through membrane filters (Sartorius GmbH, Germany) of 220, 100 and 50 nm. Each successive filtrate was titrated in susceptible Bali cattle as described above. As controls, the reovirus Nelson Bay virus (NBV) and Newcastle disease virus (NDV) were added to plasma samples before filtration; the filtrates were therefore also inoculated onto Vero cell monolayers to detect NBV and into the allantoic cavity of 10-day old embryonated eggs to detect NDV.

There was no reduction in titre of the infectious agent in plasma after it was filtered through a 220 nm filter. Filtration of the plasma sequentially through a 220 nm and then a 100 nm filter also did not cause any detectable reduction in titre of the infectious agent in two separate experiments. Filtration of plasma sequentially through 200 nm, 100 and 50 nm filters caused a reduction of titre to less than 10^2 ID_{50} per mL (the lowest dilution that was inoculated into cattle). NDV added to plasma samples before sequential filtrations was excluded by the 100 nm filters but not by the 220 nm filters, and NBV was excluded by the 50 nm filters but not by the 100 and 220 nm filters.

Susceptibility of the infectious agent to diethyl ether

To determine the ether-sensitivity of the infectious agent, plasma from infected Bali cattle (approximately 10^8 ID_{50} per mL) was filtered through 220 nm filters, and then divided into two aliquots. One aliquot was left untreated and to the other was added diethyl ether (20% final concentration). Both treated and untreated aliquots were then incubated at 4°C for one hour, then titrated in Bali cattle as described above.

After ether treatment, the titre of the agent was reduced from 10^8 ID_{50} to less than 10^3 ID_{50} per mL in one experiment and to less than 10^1 ID_{50} per mL in a repeat experiment. There was no reduction in the titre of the agent in the untreated plasma samples.

Stability of the infectious agent in plasma

The stability of the infectious agent present in plasma during storage at 4°C and to freezing at −70°C was determined. Plasma from infected Bali cattle (approximately 10^8 ID_{50} per mL) was titrated immediately after it was collected. Other aliquots of the same plasma were stored at either 4°C or rapidly frozen −70°C and then subsequently rapidly thawed and titrated in Bali cattle.

Storage of plasma at 4°C for 24 hours resulted in a decrease in titre of the agent from 10^8 ID_{50} to 10^2 ID_{50}. Rapid freezing and immediate thawing of plasma at −70°C also caused reduction in titre from 10^8 ID_{50} per mL to between 10^3 and 10^4 ID_{50} per mL; the titre remained at this level when the plasma was stored at −70°C for 1 and 2 months.

Sucrose gradient centrifugation of the infectious agent

Purification of the infectious agent by sucrose gradient centrifugation of plasma from infected cattle was attempted. Plasma from infected Bali cattle on the second day of the febrile period (approximate titre 10^8 ID_{50} per mL) was filtered through a 220 nm membrane filter and centrifuged (96 000 g for two

hours at 4 °C) in an SW28 rotor in a Beckman ultracentrifuge (Beckman Instruments, USA). The supernatant was discarded and the pellet resuspended in Tris-NaCl buffer (0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4) to 1% of the original volume. This suspension was layered on a 20–50% (w/v) continuous sucrose gradient and centrifuged (96 000 g for four hours at 4 °C) in a SW28 rotor. Visible bands were collected and examined for infectivity in cattle, by electron microscopy, for reverse transcriptase (RT) activity, for JDV-specific antigenicity against serum from convalescent animals by an enzyme-linked immunosorbent assay (ELISA) as described by Hartaningsih et al. (1993) and by Western immunoblotting as described by Kertayadnya et al. (1993).

After centrifugation, an easily visible light-scattering band was consistently detected in the gradients at a sucrose density of 1.15 g per mL. Inoculation of the visible band into susceptible Bali cattle reproduced Jembrana disease. The band reacted with convalescent serum of infected cattle by ELISA and Western immunoblotting procedures (see below), contained large numbers of virus particles when examined by electron microscopy (see below) and was associated with RT activity (see below).

Detection of reverse transcriptase (RT) activity associated with the infectious agent

The RT assays were performed by the DEAE-micromethod of Gregersen et al. (1988) on the light scattering band of density 1.15 g per mL in sucrose density gradients as previously described (Kertayadnya et al. 1993).

In virus purified from the blood, where the time of sample preparation was more than 24 hours, clear evidence for the presence of the RT activity was not obtained. When samples were processed and the RT assays conducted on samples processed within 12 to 14 hours of collection of the plasma, RT activity was demonstrated (Kertayadnya et al. 1993). Variation in the concentration of divalent cations was shown to have a marked effect on RT activity but no clear preference for either Mg^{2+} or Mn^{2+} was demonstrated (Kertayadnya et al. 1993).

Identification by Western immunoblotting of proteins associated with the infectious agent

Western immunoblots were conducted using the agent present in the light scattering band in sucrose gradients as an antigen, and sera from convalescent animals as probes. The light-scattering band detected in sucrose gradients was centrifuged (96 000 g for two hours at 4 °C) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Tris-glycine buffer system (Laemmli 1970) with a 12% separating gel and a 4.5% stacking gel. For Western immunoblotting, the proteins were electrophoretically transferred to nitrocellulose paper, probed with bovine serum collected from convalescent animals, and subsequently reacted with peroxidase-conjugated anti-bovine IgG as described by Kertayadnya et al. (1993).

The reactivity of the Jembrana disease agent proteins with bovine immunodeficiency virus (BIV) antisera and of BIV antigens with sera from Jembrana disease convalescent animals was also examined. Western blots of BIV-infected bovine embryonic lung cells were supplied by Dr C.A. Whetstone (National Animal Disease Center, Ames, Iowa) and were prepared as described by Whetstone et al. (1991). Anti-BIV serum was a gift of Dr J. Brownlie (Royal Veterinary College, University of London).

Western immunoblots of the samples prepared from plasma of cattle with Jembrana disease and probed with bovine serum collected at intervals after recovery from experimentally-induced Jembrana disease detected several proteins not present in plasma of uninfected cattle and not reactive with serum from uninfected animals. Proteins with an estimated molecular weight of 45k, 42k, 33k, 26k, and 16k were consistently detected by sera collected 12 months after infection with the Jembrana disease agent. Additional proteins including a 100K and 15K were detected in blots probed with some but not all sera collected 12 months after recovery from Jembrana disease.

Analysis by Western blots of the proteins showed an antigenic relationship between BIV and JDV. The 26k of JDV was detected by the anti-BIV serum and the 26k protein in extracts of BIV-infected cells was consistently detected by serum from Bali cattle that had recovered from Jembrana disease.

Morphology of the agent by electron microscopy

The morphology of the infectious agent in plasma and in lymphoid tissue was examined by electron microscopy. Plasma containing an estimated 10^8 ID_{50} per mL of the infectious agent was centrifuged (96 000 g for four hours at 4 °C) and the supernatant discarded. The pellets and 1 mm^3 blocks of lymphoid tissues collected from infected cattle were fixed with 5% phosphate buffer glutaraldehyde, washed in Sorenson's buffer, post-fixed in 1% Dalton's chrome osmic acid for one hour further, dehydrated through a graded series of ethanols and cleared in propylene oxide. The tissues were then passed through various mixtures of propylene oxide and Epon 812 before vacuum embedding in Epon 812. Ultra-thin sections were prepared and stained with uranyl acetate and

lead citrate before examination with a Philips 301 transmission electron microscope.

The light scattering band of density 1.15 g per mL in sucrose gradients were dialysed against 0.01 M Tris for 24 hours, negatively stained with 1% phosphotungstic acid, placed on formvar coated grids and examined as above.

Large numbers of uniform virus-particles were detected in ultra-thin sections of pelleted material prepared from plasma of infected cattle. The virus like particles were circular and varied in diameter from 96 to 124 nm (average 98 nm). Nucleoid-like structures, from 30 to 44 nm in diameter, were frequently eccentrically located and observed in more than 50% of the particles. The virus particles were surrounded by a bilaminar membrane and membrane projections were not observed.

Negatively stained preparations of the light scattering band of density 1.15 g per mL in the sucrose gradients showed numerous spherical particles approximately 85 to 95 nm in diameter. The particles were surrounded by an electron-luscent halo-like structure approximately 10 nm wide with no surface projections. Internally, most particles contained homogenous electron-dense material.

Virus-like particles were detected in ultra-thin sections of spleen and lymph node tissues. The particles were located extracellularly, and intracellularly within cytoplasmic vacuoles. The extracellular particles varied in diameter from approximately 100 to 130 nm (average 115 nm) and surrounded by a bilaminar membrane. Membrane projections were not observed. Nucleoids, centrally and eccentrically located and ranging from 40 to 56 nm in diameter were found in more than 50% of the particles. Some particles of a similar size had a relatively clear centre with electron-dense granules and contained no nucleoid. The intracellular particles were located within membrane-bound cytoplasmic vacuoles from 4.6 × 1.0 to 2.0 × 4.0 μM and contained mainly virus particles with an eccentric nucleoid, and some with a relatively clear centre, similar to the extracellular particles. C-type budding from the plasma membrane was also detected.

Discussion

The infectious agent of Jembrana disease present in the plasma of infected cattle was filterable through membrane filters of 220 nm and 100 nm, but was not excluded by 50 nm filters. These results indicated that the size of the agent was between 50 to 100 nm, was not cell-associated in the plasma, and was a virus and not a rickettsia as previously suggested by Hardjosworo and Budiarso (1975) and Ressang et al. (1985). The agent has been subsequently referred to as Jembrana disease virus (JDV).

The presence of JDV in the plasma and the tissues of infected animals was confirmed by electron microscopy, and its presence in these tissues also provided evidence of its association with the disease process. Additional evidence of the association of the virus with the disease process was the high titre of the infectious agent present during the disease process, and the consistency with which the disease could be induced in susceptible cattle with even high dilutions of plasma from animals with acute disease. Antibody to JDV was also only detected in areas where the disease was endemic (Hartaningsih et al. 1994).

There were several indications that JDV was a retrovirus, possibly a lentivirus, including the morphology of the virus, the presence of RT activity, and the antigenic relationship of the 26K protein of the virus with BIV.

The size of the particle determined by filtration (50 to 100 nm) was consistent with the size of the particles detected by electron microscopy 96 to 124 nm (average 98 nm), and the ether-sensitivity of the agent was also consistent with the presence of a lipid-containing envelope suggested by electron microscopy. The general morphological features of JDV in infected plasma and tissues were similar to those described in lentiviruses (Cheevers and McGuire 1988), although the bar-shaped nucleoids characteristics of lentiviruses including BIV (Gonda et al. 1987) were not found. The eccentric nucleoid detected in JDV has been detected in other lentiviruses (Palmer et al. 1985).

An interesting feature of JDV was the rapid decline in infectivity of the virus present in plasma stored at 4°C, and its instability when frozen at −70°C. The rapid decline of viral infectivity over 24 hours was also associated with almost complete loss of detectable RT activity over this period of time; intravirion RT activity was only detected in samples processed within 14 hours of collection from cattle.

There was a good concordance between viral antigen titre detected by ELISA and RT activity in the light scattering band of density 1.15 g per mL detected in sucrose gradients ((Kertayadnya et al. 1993), which provided evidence for the association of RT activity with virions. The RT enzyme was able to use both Mg^{2+} and Mn^{2+} and, unlike most retroviruses and including lentiviruses (Coffin 1990), no clear preference of the RT for either divalent cation was determined.

Antigenic cross-reactivity occurs between the capsid antigens of some lentiviruses (Cheevers and McGuire 1988) and the evidence indicated there was an antigenic relationship between the 26K capsid protein of BIV and the 26K of JDV. This and the

similarity of the protein structure of JDV detected by Western immunoblotting and the reported protein structure of BIV (Whetstone et al. 1991; Gonda, 1992) suggested a close relationship between these viruses.

There was no evidence that JDV was related to bovine leukemia virus (BLV). The apparent morphogenesis of JDV in lymphoid tissue of affected cattle did not resemble BLV, and antibody to BLV was not detected in the experimentally-infected cattle after they had recovered from Jembrana disease (Wilcox et al. 1992). The characteristics of JDV were distinct from bovine syncytial spumavirus: this virus is readily cultured in bovine cell cultures, the envelope contains very prominent and numerous radiating spikes 5-15 nm long, and it has Mn^{2+}-dependent RT activity (Hooks and Gibbs 1975).

Sequencing of the viral genome is required to confirm that JDV is a lentivirus and to fully understand the antigenic relationship detected between JDV and BIV. It is also suggested the further examination of the morphology and morphogenesis will require the adaptation of JDV to cell culture; most electron microscopic studies of retroviruses have been conducted with virus-infected cell cultures (Coffin 1990). Limited replication of JDV in PBMC was obtained but optimisation of the conditions for culture will be required.

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Nucleotide Sequence Analysis of Jembrana Disease Virus: A New Bovine Lentivirus

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Abstract

The complete nucleotide sequence of the RNA genome of Jembrana disease virus (JDV) was obtained. The genome was that of a typical lentivirus. In addition to the *gag*, *pol* and *env* genes and flanking long terminal repeats (LTRs) that characterise all retroviruses, a number of accessory genes were identified. These were represented by small, often multiply spliced open reading frames (ORFs) in the central and 3' terminal regions of the genome, including *vif*, *tat*, *rev*, and possibly *tmx*. Phylogenetic analysis of 598 bp of *pol* gene sequence data indicated that JDV was closely related to bovine immunodeficiency virus (BIV). The genome of JDV was 7732 bp in length, which was 750 bp shorter than that of the BIV127 strain (Garvey et al. 1990) and the smallest lentivirus genome yet reported. Alignment of the entire genomes of JDV and BIV127 revealed many deletions throughout JDV relative to BIV127, the largest of which were 471 bp from the *env* gene and 157 bp from the U3 region in the LTR. There were also several insertions of up to 33 bp in the JDV genome relative to BIV127, mostly found in the *env* gene and small ORFs that overlap *env*. Other significant genomic differences between JDV and BIV127 included changes to *cis*-acting sequences throughout the genome such as promoter and enhancer sequences in the LTR, the *trans*-activation response region, splice sites and frameshift sequences; alterations to the *gag* precursor protein cleavage sites and thus the processed products; loss of the *vpw* and *vpy* ORFs; and amino acid changes in all coding regions. These genomic differences were discussed in relation to the differences in pathogenicity between JDV and BIV. The nucleotide sequence reported here was deposited in GenBank (Accession number U21603).

JEMBRANA disease virus (JDV) has recently been characterised as a retrovirus on the basis of reverse transcriptase (RT) activity, virus morphology and C-type budding from cell membranes (Kertayadnya et al. 1993). Morphological characteristics of the nucleocapsid and strong serological cross-reactivity between the capsid proteins of JDV and bovine immunodeficiency virus (BIV), suggested that JDV may be a lentivirus related to BIV.

Lentiviruses are typically associated with chronic, progressive diseases involving long incubation periods (Narayan and Clements 1990). An acute lymphoproliferative disease with a short incubation period such as Jembrana disease (Dharma et al. 1991) is not typical of lentivirus infections, although a mild acute illness involving some lymphoproliferative changes has also been associated with several lentiviruses during the early stage of infection, including human immunodeficiency virus type 1 (HIV-1) (Sinicco et al. 1990), simian immunodeficiency virus (SIV) (Benveniste et al. 1988), feline immunodeficiency virus (FIV) (Yamamoto et al. 1988), ovine maedi/visna virus (OMVV) (Lairmore et al. 1988) and BIV (Carpenter et al.

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1992; Suarez et al. 1993). More severe acute diseases have been associated with equine infectious anaemia virus (EIAV) infection in horses (Narayan and Clements, 1990) and with infection of pig-tailed macaques by $SIV_{SMMPBj14}$, a lethal variant which emerged following in vivo laboratory passage of SIV of sooty mangabey monkeys (Fultz et al. 1989; Fultz 1994). The acute syndrome induced by $SIV_{SMMPBj14}$ was remarkably similar in many respects to that induced by JDV. Like JDV, $SIV_{SMMPBj14}$ induced a severe lymphopenia initially, followed by a rapid, intense lymphoproliferative disorder in which large numbers of blastic lymphocytes were seen in the parafollicular regions of lymph nodes, spleen and lymphoid tissues of other organs, and very high levels of infectious virus occurred in the plasma (Lewis et al. 1992).

In this paper, the authors report the nucleotide sequence of the complete genome of JDV. The genomic organisation and its relationship to BIV and other lentiviruses are described. Typical retroviral long terminal repeats (LTRs) were identified, as well as the structural genes *gag*, *pol* and *env*. A number of accessory genes which are typical of lentiviruses were also identified, represented by small open reading frames (ORFs) located in the central and 3' terminal regions of the genome (Narayan and Clements 1990).

Methods

Infectious virus was purified by sucrose gradient centrifugation from the plasma of Bali cattle experimentally infected with the Tabanan/87 isolate of JDV as previously described (Wilcox et al. 1992). Random cDNA fragments were produced from viral genomic RNA using Superscript (BRL) primed with both the random hexamers provided and oligo-dT, then ligated into pGEM (Promega) for cloning. Sequencing was performed using the 373A automated DNA sequencing system (Applied Biosystems) with primers complementary to the SP6 and T7 (or M13) elements adjacent to the pGEM multiple cloning site.

Sequence data were aligned with the BIV127 complete genome (GenBank #M32690) using the services offered by the National Institutes of Health (BLAST at NCBI). Phylogenetic analysis was performed using the PHYLIP suite of programs (version 3.5c; J. Felsenstein, 1993). Protein and LTR alignments were performed using ClustalV (D. Higgins). Other software used for genomic analysis as described in the text included ESEE (The Eyeball Sequence Editor; version 1.09e; E. Cabot), SeqAid II (D. Rhoads), MacVector™ (version 3.5) and PCFold (version 4.0; M. Zuker).

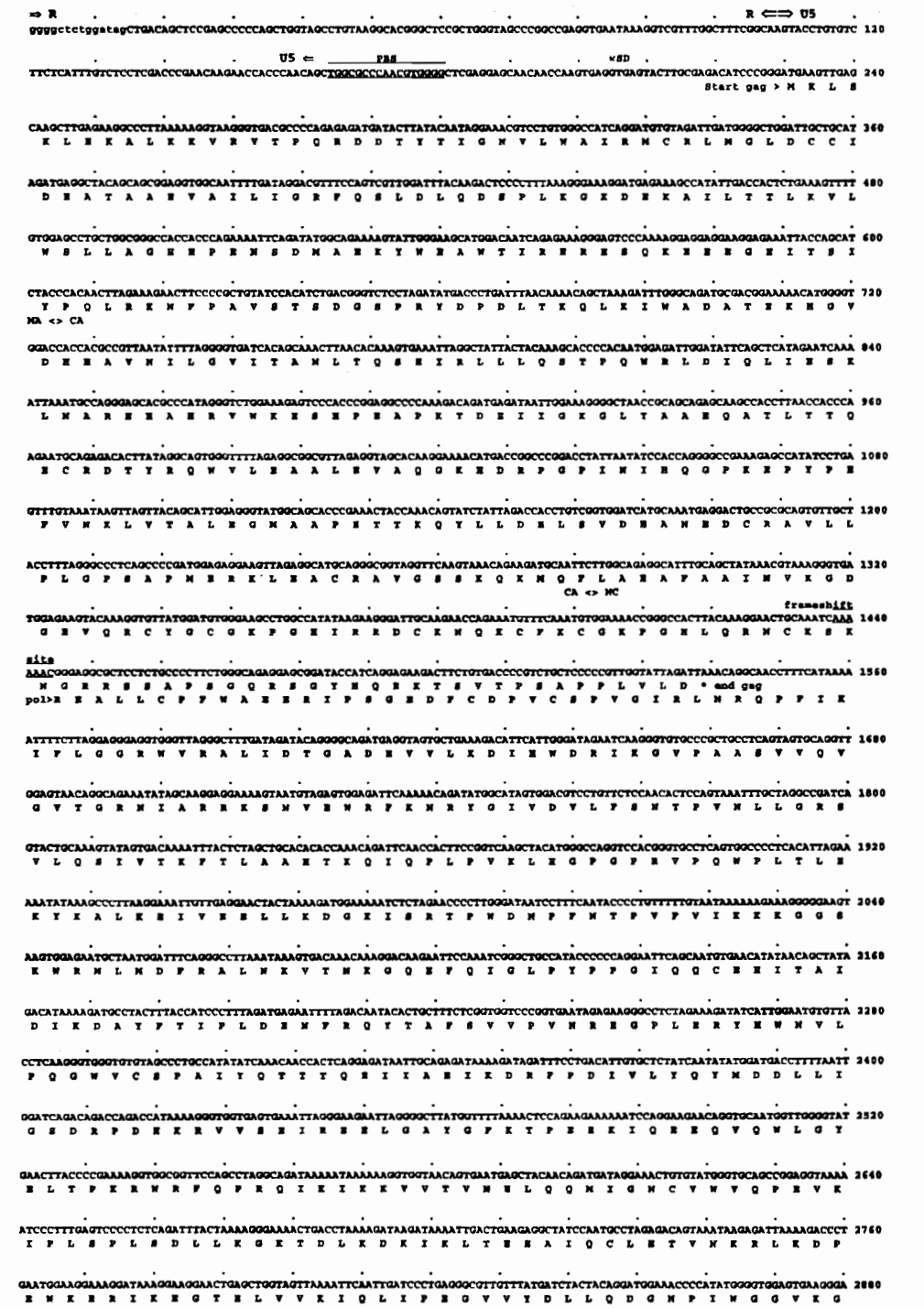
Results

Approximately 80 pGEM clones, selected by successive cycles of hybridisation and sequencing, were used to derive the entire JDV genome. Clones contained cDNA inserts ranging from 300 b to 4 kb. The nucleotide sequence of the complete RNA genome of JDV, 7732 bp in length, is shown in Figure 1, with translations of ORFs. Phylogenetic analysis based on 598 bp of sequence data, derived from a conserved RT-coding region of the *pol* gene, indicated that JDV was a lentivirus, with a close genetic relationship to BIV127.

Despite being 750 bp shorter than the BIV127 sequence reported by Garvey et al. (1990), there was a high degree of nucleotide and amino acid homology observed throughout the two viral genomes, which allowed them to be aligned if 27 deletions totalling 897 bp and 17 insertions totalling 147 bp were introduced into JDV. Most putative features of the JDV genome were then identified by means of this alignment. The genomic organisation of JDV was almost identical to BIV127, and was consistent with that of all lentiviruses in possessing a number of small ORFs in the central and 3' terminal regions.

The LTR identified for JDV, delineated by the inverted repeats adjacent to the polypurine tract and the primer binding site, was 397 bp long which was 192 bp shorter than that of BIV127. Alignment of the LTRs revealed a major deletion of 157 bp from the U3 region of JDV relative to BIV127 and a smaller deletion of 41 bp from the U5 region (Fig. 2). Some specific *cis*-acting sequences within the LTR were strongly conserved between the two genomes, including the TATA box of the basal promoter, polyadenylation and transcription termination signals, 7 bp inverted repeats, polypurine tract and the primer binding site. By contrast, enhancer elements within U3 were generally quite poorly conserved and although both JDV and BIV127 contained an NF-κβ-binding enhancer element, that of JDV was located 167 bp from the promoter while that of BIV127 was 310 bp from its promoter. The CAT enhancer sequence (consensus sequence CCAAT) found in most eukaryotic genes and in BIV127, was absent from JDV.

A region corresponding to the *trans*-activation response (TAR) region of BIV was identified for JDV. The first stem-loop structure (nt 1–31), which has been previously demonstrated by deletion analysis to be the most important for transactivation (Carpenter et al. 1993; Liu et al. 1992), was quite similar to that of BIV, although subsequent structures were quite dissimilar.


⇒ R
R ⟸ U5
U5 ⇐
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Start gag > M R L S
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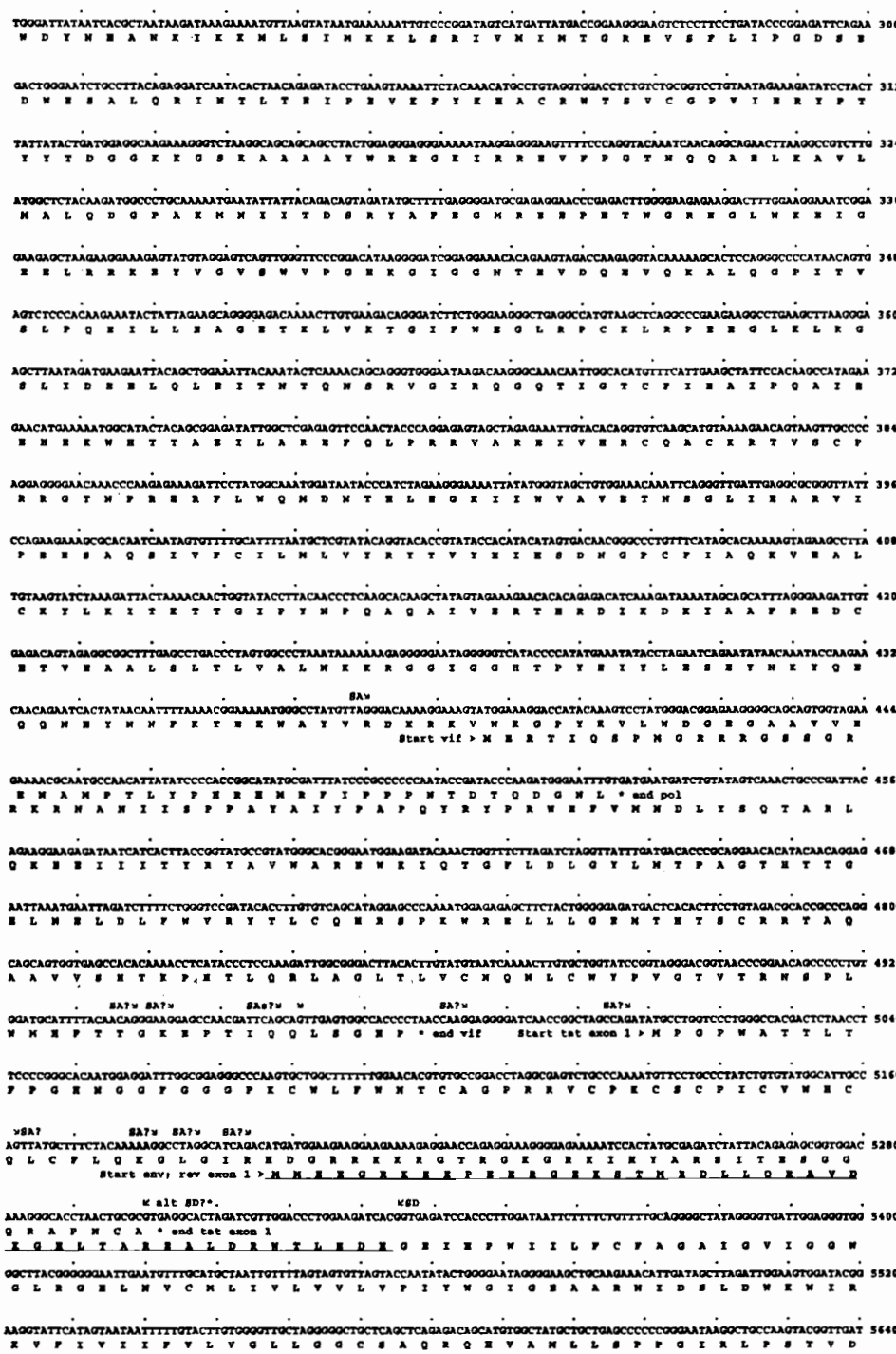
pol>R E A L L C F F W A E E R I F S G E D F C D F V C S F V G I R L M R Q P F I K

continued



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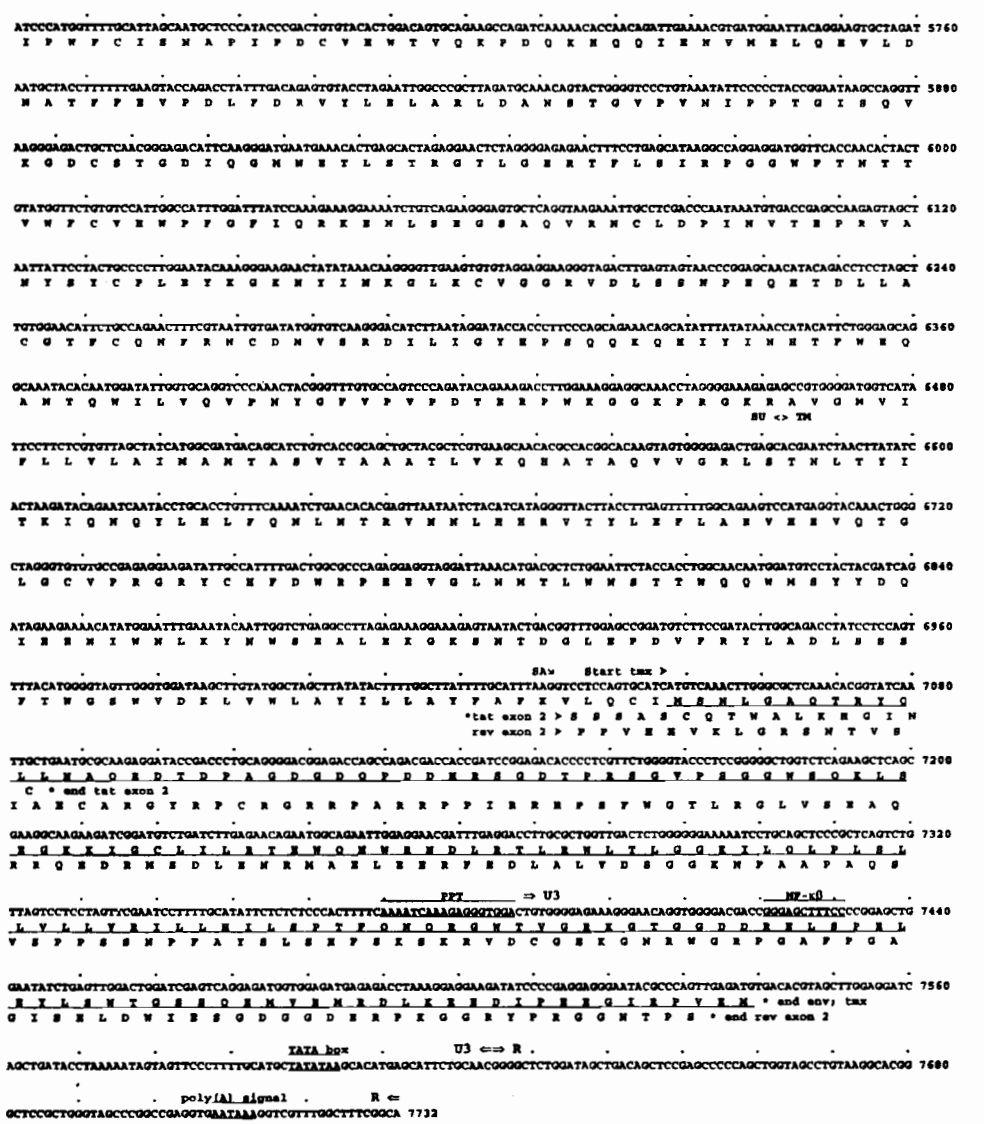

Figure 1. The complete nucleotide sequence of JDV genomic RNA. Nucleotides in lower case at the 5' end of the genome were inferred from the R region at the 3' end. Translations of all ORFs identified are provided (underlined amino acids indicate where the *rev* (exon 1) and *tmx* ORFs coincide with the *env* ORF). Annotations indicate important *cis*-acting sequences; R, U5 and U3 boundaries; potential splice donor site (↙SD) and splice acceptor sites (SA↘).

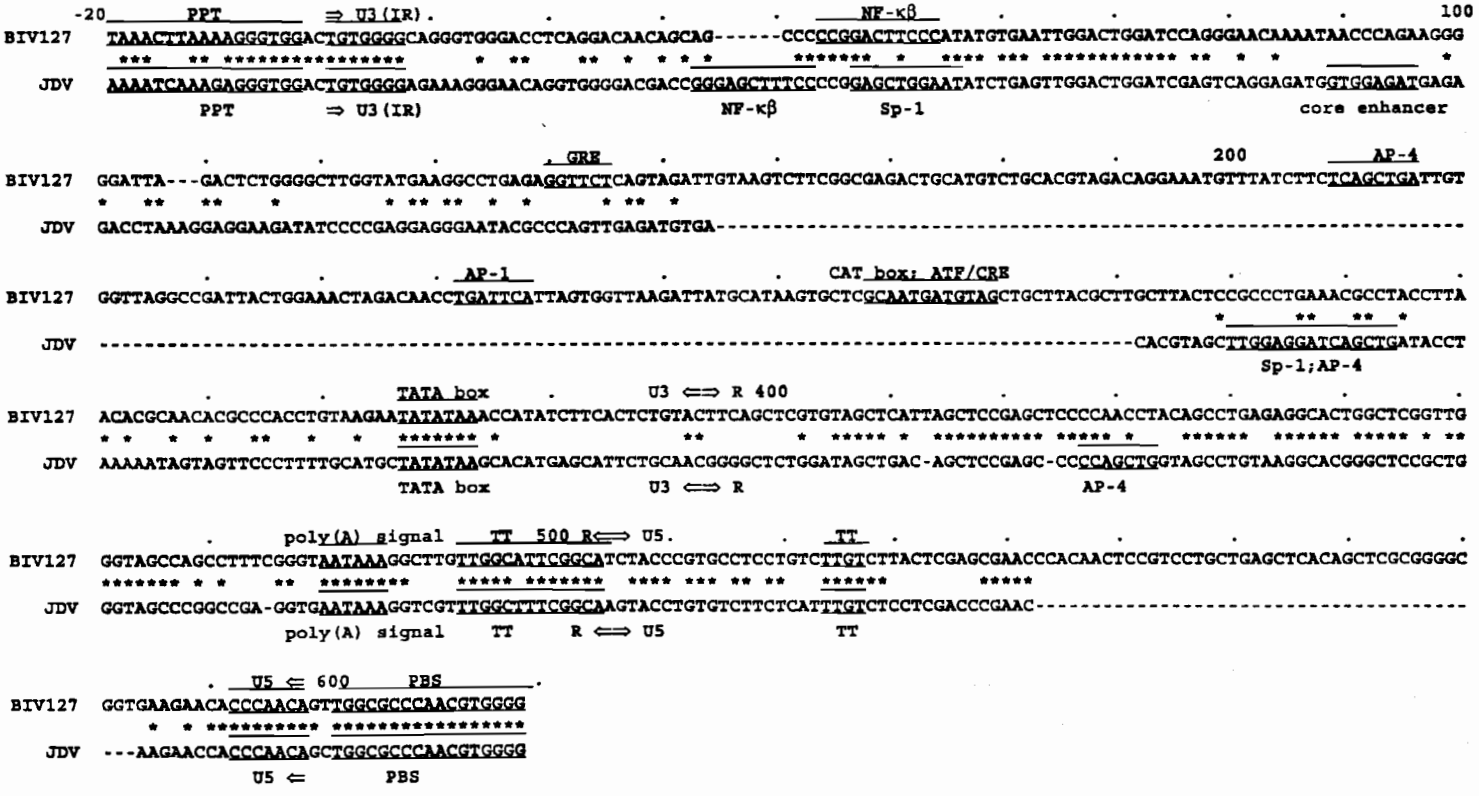

Figure 2. Comparison of the nucleotide sequences of the LTR of BIV127 and the putative LTR of JDV. Nucleotides are numbered from the start of U3. Annotations indicate U3, R and U5 boundaries; promoter and enhancer elements identified; the primer binding site (PBS); the polypurine tract (PPT); and transcription termination signals (TT). Asterisks indicate identifical nucleotides.

The *gag* gene of retroviruses is typically translated as a single precursor protein and cleaved by the viral protease into an N-terminal matrix protein (MA), a capsid protein (CA), and a C-terminal nucleocapsid protein (NC). The *gag* precursor of BIV, however, is unusual in containing five cleavage sites, giving rise to three additional short processed products designated p2L, p3 and p2 (Tobin et al. 1994). The *gag* gene products of JDV and BIV127 showed strong amino acid sequence identity overall, which was consistent with the serological cross-reactivity previously observed between the capsid proteins of BIV and JDV (Kertayadnya et al. 1993). Only two of the five cleavage sites were conserved in JDV, which suggested that the JDV *gag* precursor would be processed into MA, CA and NC proteins only (Table 1). The p2L short protein would be incorporated into CA and both the p3 and p2 short proteins would be incorporated into NC. The duplicated $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ motifs of the NC of BIV127, suggested to resemble the 'zinc-finger' regions seen in nucleic acid-binding proteins of retroviruses (Garvey et al. 1990), were conserved in JDV. The first two amino acids of the MA of JDV (M-K) were also conserved, which probably precludes its myristylation.

The *pol* ORF of most retroviruses and all lentiviruses is translated as a Gag-Pol precursor by a −1 ribosomal frameshift, and includes the protease (PR), RT and integrase (IN) proteins. Recent reports have also described a dUTPase product encoded by a short region between the RT and IN domains of the *pol* gene of all non-primate lentiviruses except BIV, in which it is only a non-functional remnant (Elder et al. 1992; Threadgill et al. 1993). The *pol* gene product of JDV showed 68% amino acid sequence identity to that of BIV127 (Table 1), with only two deletions of 6 bp and 3 bp. Like BIV, there was no identifiable region that could potentially encode a dUTPase protein.

Cis-acting sequences that may be involved in the frameshifting mechanism have been identified for BIV. These included a putative heptanucleotide frameshift site (AAAAAAC), a potential stem-loop structure 8 bp downstream from it, and a downstream pentanucleotide that may be involved in the formation of a pseudoknot structure (Battles et al. 1992; Chamorro et al. 1992). With regard to the corresponding sequences of JDV, the heptanucleotide frameshift site was perfectly conserved, the GC-rich inverted repeats of the stem were extended from 12 bp to 13 bp, and the complementarity and proximity of the downstream pentanucleotide were improved for the potential pseudoknot formation. The frameshifting mechanism of JDV may therefore be more efficient than that of BIV.

Table 1. Comparison of putative protein products of JDV and BIV.

	JDV		BIV		
Protein	Amino acids	Mass (kDa)	Amino acids	Mass (kDa)	% Identity
Gag precursor	436	43.8	476	53.4	62%
MA	125	14.3	126	14.6	60%
p2L	—	—	22	2.5	
CA	226	25.3	219	24.6	75%
p3	—	—	25	2.7	
NC	85	9.2	66	7.3	63%
p2	—	—	18	1.9	
Gag/Pol precursor	1432	163	1475	168	66%
Pol precursor	1027	118	1035	118	68%
Env precursor	781	88.8	904	102	31%
SU	422	47.8	555	62.1	24%
TM	359	41.1	349	40.2	39%
Vif	197	22.9	198	22.8	55%
Tat	97	10.7	103	11.7	54%
(alt. Tat)*	(114)	(12.5)	—	—	
Rev	213	23.8	186	20.7	35%
(alt. Rev)*	(201)	(22.4)	—	—	
Tmx	164	18.5	159	18.0	29%
Vpw	—	—	54	6.6	
Vpy?	—	—	80?	9.5?	

* Alternate forms of Tat and Rev potentially generated by utilisation of an alternative splice donor site at nucleotide 5299.

The *env* ORF of JDV encoded a precursor polyprotein of 781 amino acids, which was predicted to be processed into a surface protein (SU) and a transmembrane protein (TM) utilising a cleavage site at a conserved R-A motif (Fig. 3). Alignment of the JDV *env* precursor with that of BIV127 required the introduction into the JDV genome of 10 in-frame deletions totalling 173 amino acids and 17 in-frame insertions totalling 49 amino acids. The deletions included one of 471 bp in the hypervariable V2 region of SU, where variations of up to 312 bp have been reported to occur between various BIV isolates, and deletions of 87 bp relative to BIV127 appear to be the norm (Garvey et al. 1990; Suarez and Whetstone 1995). The SU of JDV is thus substantially smaller than that of BIV127 (Table 1), a difference that is exaggerated by the lower number of potential N-linked glycosylation sites identified in the JDV *env* precursor (Fig. 3). The TM protein of JDV, however, was very similar in size to that of BIV127.

Splice site sequences potentially utilised for the expression of *env* and all accessory genes were identified according to the consensus sequences (C/A)AG.**GT**RAGT for the splice donor site (SD) and Y(N)NY**AG**.G for the splice acceptor site (SA). Only the dinucleotides of the intron immediately adjacent to the cutting site (bold type) were described as invariant (Nevins 1983). One, two or even three errors were permitted in the remaining six positions of the SD consensus sequence on the basis of previous reports (Viglianti et al. 1990; Purcell and Martin 1993).

The sequence identified as the major splice donor site in the pre-*gag* leader region of BIV127 (Liu et al. 1992; Oberste et al. 1993), to which the coding exons of all *env* and accessory gene transcripts are spliced, was perfectly conserved in the leader region of JDV (nt 194). The SA usually utilised to generate the BIV127 *env* and *rev* mRNA transcripts (Oberste et al. 1993) was not conserved in JDV. Generation of *env* transcripts for JDV could be achieved by utilisation of any one of three potential SAs (Fig. 1).

There were at least three accessory genes identified in the JDV genome, including *vif*, *tat* and *rev* (Fig. 1). By comparison, the primate lentiviruses possess at least six accessory genes, including *vif, tat, rev, vpr, nef* and *vpu* or *vpx*, and BIV possesses *vif*, *tat, rev,* and three potential accessory genes *vpw, vpy* (formerly *W* and *Y*) and *tmx* (Garvey et al. 1990; Gonda 1992). JDV potentially contained a fourth gene analogous to the *tmx* gene of BIV127 and *ttm* of EIAV (Biesel et al. 1993), found in the 3' terminal region in the same reading frame as *env* and expressed from a singly spliced transcript utilising the same SA as the second exons of *tat* and *rev* (see below; Gonda 1992). The *vpw* ORF, described only for BIV127 (Gonda 1992) and a South African strain of visna virus (Querat et al. 1990), was not functional in JDV due to stop codons. The *vpy* ORF, for which there is no evidence of expression in BIV127, was also not functional in JDV.

The putative *tat* gene of JDV was predicted to be expressed from a multiply spliced transcript that included two coding exons derived from separate ORFs in the central and 3' terminal regions respectively (Fig. 1). In the first coding exon there was strong conservation of the cysteine-rich region and downstream basic domain found in the Tat proteins of most lentiviruses including BIV127 (Garvey et al. 1990), which has been suggested to be important to their nucleic acid-binding properties. The SD site utilised by BIV127 at the end of exon 1 (Liu et al. 1992) was perfectly conserved in JDV (nt 5335) and the SA of exon 2 was strongly conserved (nt 7032), suggesting that they would be utilised to splice the JDV *tat* transcript. Like the BIV127 Tat protein, the presence of a stop codon prior to the SD implied that utilisation of these splice sites would generate a Tat protein translated entirely from the first exon (Liu et al. 1992).

The authors also identified a potential alternative SD (nt 5299) immediately prior to the stop codon in the JDV *tat* gene that was not conserved in BIV127. Utilisation of this alternative SD would generate a longer Tat protein that would contain 17 amino acids translated from the second exon (Table 1). This potential SD contained three errors relative to the consensus SD sequence described above, in comparison to the two errors contained in the fully conserved SD, and was therefore considered likely to be utilised by the splicing machinery at a significantly lower efficiency, if at all. The simultaneous use of alternative cryptic SDs in the splicing events of lentiviral accessory genes is precedented (Viglianti et al. 1990; Purcell and Martin 1993), although the use of alternative cryptic SAs has been more commonly reported (Viglianti et al. 1990; Oberste et al. 1993; Purcell and Martin 1993).

The putative JDV *rev* gene also consisted of two coding exons derived from separate ORFs, of which exon 2 encoded the greater part of the protein, as it does for most lentiviruses. The first coding exon used the same reading frame, start codon and SA as the *env* gene described above, and the second coding exon was predicted to be spliced to the first utilising the same SD and SA as the *tat* gene. Amino acid sequence identity between the Rev proteins of JDV and BIV was low (Table 1). Utilisation of the alternative SD of JDV would generate a putative Rev protein containing 12 fewer amino acids translated from exon 1 (Table 1).

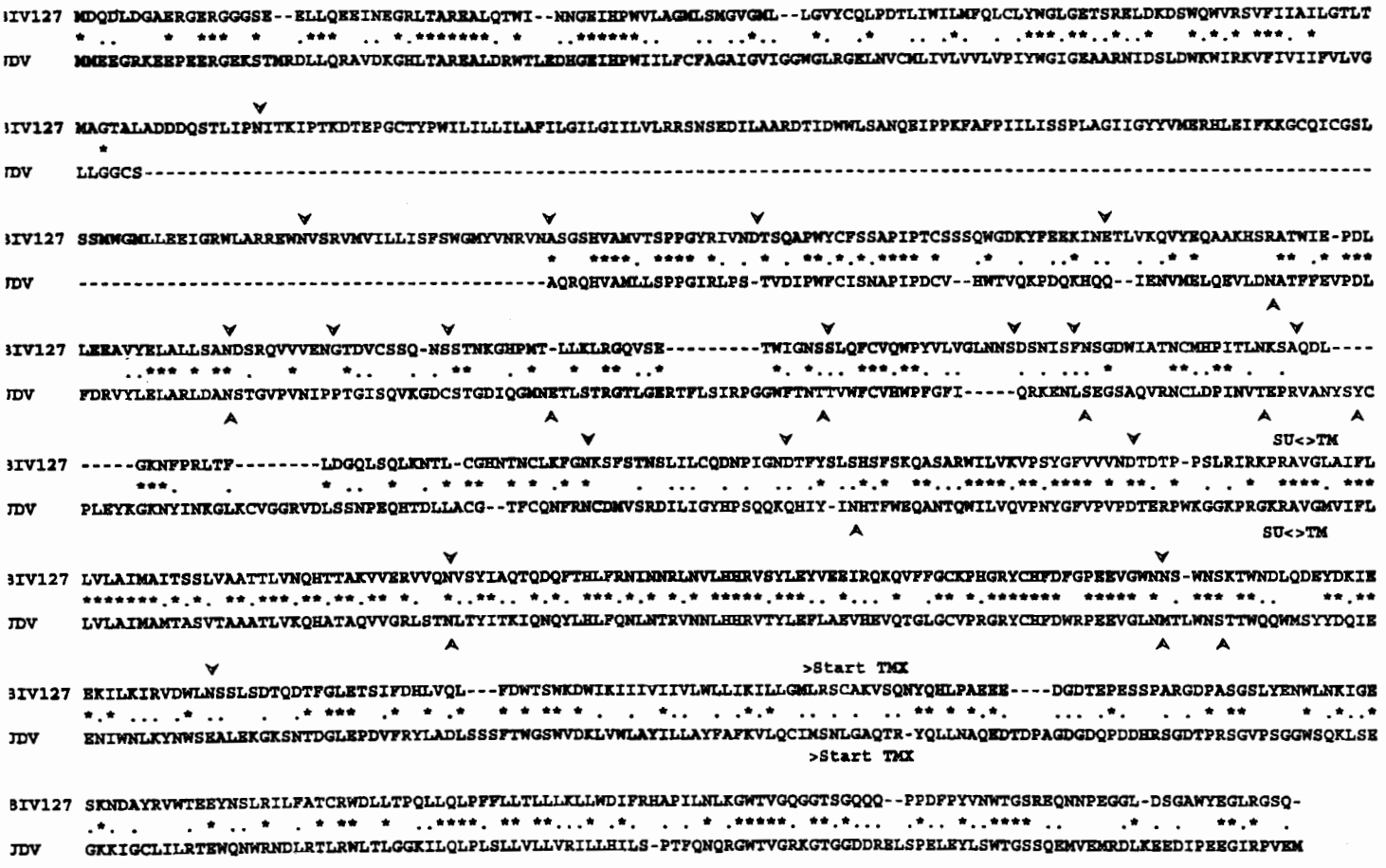

Figure 3. Alignment of the env precursor proteins of BIV127 and JDV. The alignment was produced using ClustalV (D. Higgins). Identical amino acids are indicated by an asterisk; conservation substitutions are indicated by a dot. Potential glycosylation sites are indicated by arrowheads.

Discussion

With an RNA genome of 7732 bp, JDV is the smallest lentivirus genome yet reported, being marginally smaller than EIAV (7984 bp) and 750 bp smaller than BIV127. The most striking feature of the JDV genome was the large number of deletions relative to BIV127. The largest of these were 471 bp from the *env* gene, 157 bp from U3 and 41 bp from U5 in the LTR, 42 bp from the untranslated leader region and 2 deletions of 57 bp and 45 bp from coding regions of the *gag* gene. There were also several insertions in the JDV genome, although these were generally smaller, ranging up to 33 bp, found in the *env* gene and the LTR. Despite the error-prone nature of lentiviral RTs and the extensive genomic variability observed in lentiviral systems in vivo, it is unprecedented for two such closely related lentiviruses to feature so many deletions and insertions relative to each other, rather than simple substitutions. The extraordinary degree of genomic variation attributable to deletions/insertions observed in the bovine lentivirus system including JDV strains of BIV may reflect a basic difference in the functional properties of the RTs of these viruses relative to those of other lentiviruses. It is appropriate to consider that the failure of a dUTPase-deficient laboratory strain of EIAV to replicate in macrophages in vitro, may be the result of dUTP misincorporations during reverse transcription, leading to high mutation rates and hyper-recombination (Threadgill et al. 1993). It is plausible that any unusual properties of the RT of the bovine lentivirus group may relate to the lack of functional dUTPase in this group, although it is unclear why the primate lentiviruses should have no need of such a dUTPase function.

In contrast to the mild nature of symptoms associated with BIV infection, JDV infection is associated with a severe, acute and often lethal disease with striking similarities to that associated with $SIV_{SMMPBj14}$ infection. It is remarkable that among a group of viruses generally known for their ability to cause chronic infections of a relentlessly progressive nature, that two viruses such as JDV and $SIV_{SMMPBj14}$ should emerge that induce an acute lymphoproliferative disease. Previous authors have noted the similarities between the symptoms of acute $SIV_{SMMPBj14}$ infection and acute infection with HIV or typical SIV, and the acute virulence of $SIV_{SMMPBj14}$ has been suggested to result from the production of extremely high levels of virus in vivo (up to 10^5 infectious units/mL) associated with the induction of inflammatory cytokines which correlated strongly with the ability of the virus to replicate in resting macaque lymphocytes and to activate and induce proliferation lymphocytes in vitro, (Fultz 1991, 1994; Novembre et al. 1993). The relationship between BIV and JDV may be similar to that between $SIV_{SMMPBj14}$ and typical SIV strains.

Despite the relatively small number of genomic differences between $SIV_{SMMPBj14}$ and the parental strain SIV_{SMM9}, it has proved difficult to specifically define the genetic determinants of the altered biological characteristics. The essential requirements are complex; they consist of sequences in both 5' and 3' parts of the genome, and probably involve a duplicated NF-κβ- enhancer element in the LTR (Novembre et al. 1993; Dollard et al. 1994).

The authors have considered the large number of significant genomic differences between BIV127 and JDV that may contribute to the acute virulence of JDV. The enhancer elements identified for JDV were markedly different to those of BIV127 and the NF-κβ binding site in particular, was much closer to the TATA box than that of BIV127. Both the SU and TM-coding regions of the *env* gene, which also showed extensive changes in JDV, have been commonly reported to affect viral tropism, cytopathic effects and ability to induce proliferation of resting lymphocytes in vitro, including both B-cells and T-cells (Nair et al. 1988; Johnston et al. 1993). These properties have in turn, been associated with altered pathogenicity in vivo (Fultz 1991). A specific association between the C-terminal portion of TM and induction of lymphocyte proliferation (Nair et al. 1988; Johnston et al. 1993) adds further significance to recent reports indicating that this TM fragment is also expressed independently as an accessory gene product in EIAV and BIV (Gonda 1992; Beisel et al. 1993), and possibly JDV.

Lymphoproliferative changes have been described in mice transgenic for the *tat* gene of OMVV (Vellutini et al. 1994). Other reports have linked the lentiviral Tat protein with cellular activation (Neuveut et al. 1993), and induction of IL-2 (Westendorp et al. 1994). The JDV Tat protein differed from that of BIV127 by a considerable number of amino acid substitutions and it is possible that the utilisation of an alternate SD site at the end of the first coding exon could lead to translation of an additional 17 amino acids from the second coding exon.

In addition, mechanisms of alternative splicing which affect the expression of lentiviral accessory genes from singly and multiply spliced transcripts allow for substantial and unpredictable modulations in the patterns of viral expression and even viral infectivity to result from relatively minor genomic mutations to constitutive splice site sequences (Viglianti et al. 1990; Purcell and Martin 1993; Martarano et al. 1994). There are numerous changes

to the splice site sequences of the accessory genes of JDV relative to BIV.

Other significant genomic differences between JDV and BIV127 included changes to the *gag* precursor protein cleavage sites and thus the processed products, alterations to the frameshift sequences in the region of overlap of *gag* and *pol*; loss of the *vpw* and *vpy* ORFs, and amino acid changes in all coding regions.

Finally, in view of their extensive genomic similarities, it is arguable as to whether JDV and BIV should be considered to represent different viruses, or simply different strains of the same virus, in which case an alternative naming strategy such as 'BIV-1' and 'BIV-2', or bovine lentivirus 'type 1' and 'type 2' may be more appropriate (Suarez et al. 1993). The authors believe it is appropriate to retain the name JDV, and to consider it and the American BIV isolates as distinct viruses for reasons of both pragmatism and technical taxonomic accuracy. Pragmatically, the diseases caused by the respective viruses are radically different, and JDV is associated with a specific and unique disease syndrome. Technically, the respective viral genomes are sufficiently different by virtue of deletions/insertions to warrant consideration of these viruses as separate entities, despite extensive regions showing a high degree of nucleotide and amino acid sequence identity that would normally only be associated with strains of the same virus.

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Anti-JDV Monoclonal Antibodies and Immunocytochemical Tests

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Abstract

Murine monoclonal antibodies (MAbs) were produced against the 26 kilodalton (K) and 16K virion proteins of Jembrana disease virus (JDV) using hybridoma technology. Western immunoblot analysis showed that the MAbs against the 26K proteins of JDV cross-reacted with the 26K (capsid) protein in BIV-infected cells, confirming previous results obtained with polyclonal antiserum. Immunoperoxidase staining of frozen sections of lymph node tissue from infected cattle, using MAbs against the 26K protein of JDV as a probe, detected JDV specific antigen in mononuclear cells. The potential future application of the MAbs that were produced is discussed.

JEMBRANA disease virus (JDV), is the cause of Jembrana disease virus in the lentivirus group. Characterisation of JDV showed the capsid protein (26K) of JDV was antigenically related to the cognate protein in bovine immunodeficiency virus (BIV) (Kertayadnya et al. 1993).

The development of serological assays such as ELISA (Hartaningsih et al. 1994) and Western immunoblotting (Kertayadnya et al. 1993) have enabled detection of antibodies to JDV. The studies reported in this paper were aimed at producing monoclonal antibodies (MAbs) against the proteins of JDV. MAbs may provide reference antibodies for the immunocytochemical assays for JDV antigen in tissue of infected cattle, and enable the development of a non-biological assay for detection of JDV in individual cattle.

Material and Methods

Preparation of JDV antigen

Plasma from JDV-infected cattle collected on the second day of fever was centrifuged (96 000 × g, 2 hours, 4 °C) and the pellet used as a 'crude antigen'. Some pellets were purified by sucrose density gradient centrifugation. Both crude and sucrose purified antigens were treated with Triton X-100 (final 1% concentrations) to disrupt the virus. The antigens were stored at −20 °C before use.

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Mouse immunisation

Female Balb/C 6 to 8 weeks of age were immunised with either a crude viral antigen or sucrose purified antigens. The mice were initially inoculated intraperitoneally with 0.2 mL of an emulsion of the antigen and Freund's complete adjuvant, then four times with the same antigen emulsified in Freund's incomplete adjuvant (Sigma Chemical Co. USA) at weekly intervals. Three days before use, the mice were injected with 0.2 mL of antigen without adjuvant.

Myeloma cell preparation, cell fusion and monoclonal antibody production

Myeloma (NS1) cell lines, obtained from the School of Veterinary Studies, Murdoch University, Perth, Australia, were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS), 0.22 mg/mL sodium pyruvate, 0.58 g/mL glutamine, 200 units mL and 200 ug/mL penicillin-streptomycin, and 0.0025 mg/mL fungizone. Cells were cultured in 25 cm^2 plastic tissue culture flasks at 37 °C in a 5% CO_2 atmosphere.

The fusion and monoclonal production techniques were introduced by Mr W. Coackley, of Murdoch University. The fusion of a mixture of 10^8 spleen cells from immunised mice and 20 × 10^6 NS1 cells was induced by warm polyethylene glycol (PEG) M.W. 1300 to 1600 (Sigma Chemical Co. USA), for about 2 minutes. The PEG was added in drops to the cell mixture for 1 minute while gently tapping the

side of the tube to keep the cells in suspension. The mixture was held with gentle shaking for a further 2 minutes in a 37 °C water bath and slowly resuspended with 10 mL DMEM without serum. The tube was gently agitated and centrifuged (800 r/min for 5 minutes). The supernatant was removed and the cells resuspended in 50 mL complete DMEM with HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine). Spleen cells from normal mice were added as feeders to the cell mixture (final concentration of 10^6 cells per mL). The cells were gently mixed and distributed in 0.1 mL amounts into wells of 96-well tissue culture microplates (Linbro). The plates were incubated at 37 °C in a 5% CO_2 atmosphere.

The plates were examined daily for the production of hybridomas. On the fourth day after the fusion, 2 drops (by Pasteur pipette) of HAT medium was added to each well. When the hybridomas were about 1 mm diameter, the medium was tested for a specific antibody against JDV by ELISA (techniques were similar to the procedures described by Hartaningsih et al. 1994). The hybridomas with ELISA positive reactions were aspirated and subcultured into other wells. An equal volume of HT medium (complete medium without aminopterin) containing 2×10^6 feeder cells per mL was added to each well.

Maintenance, cloning and storage of MAbs.

The ELISA positive hybridomas were maintained in HT medium and eventually stored in liquid nitrogen. In order to obtain colonies of cells derived from a single hybridoma cell, the hybridomas with a positive ELISA reaction were cloned by a limiting dilution method (Mc Kearn 1980). Serial dilution of hybridomas were made in 96-well plates. Following growth of hybridomas, colonies from a single cell were subcultured, tested for antibodies, resubcultured and some were stored in liquid nitrogen. After several passages in small wells, the cloned hybridomas were grown into larger wells of 24-well flat bottom plates (Linbro), then into 25 cm^2 flasks and finally into 75 cm^2 flasks. ELISAS were continuously performed to ensure that the hybridomas continued to secrete antibodies.

Actively-growing hybridoma cells were inoculated intraperitoneally (5×10^6 cells per mouse) into adult mice. Prior to the inoculation, the mice were injected with Ehrlich tumour ascites cells. When the mice developed very extended abdomens, ascites fluid was collected by hypodermic needle, tested for antibodies and stored at −20 °C.

Western immunoblotting and immunocytochemical assays

The Western immunoblotting technique used was described by Kertayadnya et al. (1993). The JDV antigens used were described above. Western immunoblots of BIV were supplied by Dr C.A. Whetstone of the National Animal Disease Center, Ames, IOWA, USA. The antigen for these blots were prepared from detergent (CHAPS)-solubilised BIV-infected cells as described by Whetstone et al. (1991).

The membranes were blocked with 0.5% gelatin in PBS-tween (0.1% tween 20 in PBS, pH 7.2) overnight at 4 °C. After washing three times with PBS-Tween, the membranes were then incubated at room temperature for 2 hours with MAbs, anti-JDV serum or anti-BIV serum. The membranes were then incubated at 37 °C for 1 hour. The immunoreactive proteins were visualised using 4-chloro-naphthol chromogen.

The immunocytochemical method (indirect immunoperoxidase test) for detection of JDV antigen in tissue as previously attempted by Dharma (1993) was used. Frozen sections of lymph node and spleen tissues from infected and uninfected animals were prepared and mounted on poly-L-lysine coated glass slides. The sections were probed with tissues culture fluid containing MAb (a 1:10 dilution of hybridoma culture medium or a 1:100 dilution of ascites fluid) for 60 minutes at room temperature. The slides were gently washed twice with phosphate buffered saline (PBS), pH 7.2, and twice with distilled water for two 5 minutes periods. The slides were again air dried, and covered with peroxidase-conjugated rabbit anti-mouse immunoglobulin diluted 1:60 in PBS with 5% normal bovine serum and 0.1% Triton X-100, for 60 minutes at room temperature. The sections were again washed with PBS and distilled water as above, and covered with 0.005% diaminobenzidine (DAB) tetrahydrochloride (Sigma Chemical Co., USA) in PBS containing 0.2% hudrogen peroxidase for 10 minutes. After washing with tap water, the sections were counterstained with Mayer's haematoxylin for 5 minutes, again washed in tap water, cleared in xylene, then mounted with DPX.

Results

Detection of hybridomas

Fusion was attempted with four mice immunised with a Triton X-100 treated crude viral antigen (pelleted plasma) and four mice immunised with a Triton X-100 treated sucrose gradient purified preparation of JDV.

A total of 1200 hybridomas were detected during the eight fusions (which were dispensed into a total of forty 96-well plates) as shown in Table 1. ELISA results showed that 235 (19.65%) of these hybridomas secreted antibody that reacted with the control antigen derived from plasma of uninfected cattle and with the sucrose gradient purified antigen from plasma of infected cattle (Table 1). Only 61 (5.08%) of the hybridomas secreted antibody that reacted with the sucrose gradient purified JDV antigen, not with the control antigen, and these were selected as possibly secreting JDV-specific MAb. Most hybridomas were detectable 7 to 14 days after the fusions were performed.

Table 1. Detection of hybridomas and results of ELISA screening for antibodies in wells containing hybridomas resulting from fusions with splenocytes from eight immunised mice.

Fusion	Antigen used for immunisation	Wells with visible hybridomas tested	ELISA reactivity	
			Plasma protein[a]	JDV[b]
1.	Crude antigen[c]	129	34	11
2.	Crude antigen	141	41	6
3.	Crude antigen	156	42	3
4.	Crude antigen	165	43	10
5.	Sucrose purified[d]	202	82	9
6.	Sucrose purified	128	54	4
7.	Sucrose purified	138	51	11
8.	Sucrose purified	141	48	6
TOTAL 1200		235 (19.58%)	61 (5.08%)	

[a] These hybridomas produced a positive reaction with the control plasma antigen from uninfected cattle.
[b]These hybridomas produced a positive ELISA reaction with the sucrose JDV antigen but not with control plasma antigen from uninfected cattle.
[c] Mice immunised with a Triton X-100 treated crude pelleted preparation of plasma from infected cattle.
[d] Mice immunised with Triton X-100 treated sucrose gradient purified JDV antigen.

After the potential JDV-specific MAb-secreting hybridomas were cloned, only 22 hybridomas continued to produce JDV-specific MAb. Analysis by Western immunoblotting showed that these MAbs reacted with two different JDV proteins; 14 MAbs reacted with a 26K protein, and 8 MAbs with a 16K protein. High antibody titre ascites fluid was produced against two of these hybridomas, designated BC10 (reacting with the 26K protein) and BC16 (reacting with 16K protein).

Cross-reactivity of MAb with BIV antigens

Western immunoblot analysis of the reactivity of the two MAbs with BIV-infected cell culture lysates showed that MAb BC10 (against the 26K protein of JDV) reacted with the 26K protein and 26K-precursor proteins of BIV. No reactivity of the monoclonal BC16 (against the 16K protein of JDV) with lysates of BIV-infected cells was detected.

Immunocytochemical test of tissue with MAb

Indirect immunoperoxidase staining using both MAbs (BC10 and BC16) produced intracytoplasmic brown staining in mononuclear type cells, particularly in the parafollicular regions of lymph node tissue collected from infected cattle. These cells were not identified but morphologically resembled macrophages, lymphoblasts and small lymphocytes. No staining was detected in the cells of control tissues from uninfected cattle.

Discussion

Hybridoma technology enables the production of highly specific MAbs directed against single antigenic determinants, even when impure antigens are used for immunisation. Purified antigens may increase the frequency of specific antibody-producing hybridomas but their use is not essential (Cote et al. 1982; Dilworth and Griffin 1982). In the attempts to produce MAbs that were reported in this paper, there were no marked differences in the rate of detection of hybridomas secreting JDV-specific MAbs when the immunisation of mice was conducted using a crude virus preparation and when it was conducted with sucrose gradient purified JDV.

Early attempts to produce JDV-specific MAbs in this laboratory, the results of which are not reported, were unsuccessful: not only were no JDV-specific MAbs produced but the number of hybridomas produced was very low. These early attempts used similar antigens and apparently identical fusion techniques to the successful attempts that were reported. The only difference in the techniques used was that the early attempts to produce JDV-specific MAbs utilised antigen preparations to immunise mice that were not treated with Triton X-100. Whether this was actually due to the effect of Triton X-100 on JDV, or to other variations in the technical procedures used, is unknown but it should be considered in future attempts at MAb production against JDV. It is of interest that the successful production of MAb against BIV reported by Horzinek et al. (1991) also utilised Triton X-100 treated virus for the immunisation of mice. In the current study, there was a high titre of infectious JDV in the plasma used as a source of virus to immunise mice against JDV, and detergent disruption of this intact virus may be required to expose internal virion protein antigens.

Twenty two MAbs reacted with two virion proteins of JDV. One panel of 14 MAbs reacted with the 26K protein of JDV, which is presumed to be the capsid (major core) protein of the virus (Kertayadnya et al. 1993). This protein was shown to be immunodominant during the immune response to JDV in Bali cattle. Another panel of 8 MAbs recognised a lower molecular weight (16K) protein. This 16K protein, by analogy with the putative protein structure of BIV, is probably either the matrix (MA) protein, of 16K in BIV (Gonda 1992), or the nucleocapsid (NC) protein, of 13K in BIV (Gonda 1992). Both the MA and CA proteins of BIV are coded for by the *gag* gene (Gonda 1992). The absence of antigenic cross-reactivity between this 16K protein of JDV with either the 16K or 13K protein of BIV by polyclonal antisera (Kertayadnya et al. 1993) or with MAbs suggests the 16K protein of JDV is not antigenically related to the cognate protein of BIV.

The production of MAbs against the 26K virion protein of JDV has enabled the confirmation of the antigenic relationship between the 26K protein of JDV and the CA protein of BIV. The MAb BC16 against the 16K protein of JDV did not react with any protein in lysates of BIV-infected cell cultures, confirming previous results with polyclonal bovine antiserum that this protein of JDV is not antigenically related to any protein in BIV (Kertayadnya et al. 1993).

The development of MAbs against virion proteins of JDV also enabled the development of an immunocytochemical assay for the detection of JDV-infected cells in tissue. The technique was succesful in identifying viral protein in cells when both MAbs to the 26K and to the 16K protein were used. The reactive, and presumably JDV-infected cells, morphologically resembled macrophages, lymphoblasts and small lymphocytes, and were distributed predominantly in the parafollicular regions of spleen and lymph nodes, corresponding in distribution to the major location of proliferative cells in the tissues (Dharma et al. 1993). The results indicated that the major site of virus replication occurs in these areas, and that the principal target cell for JDV replication in Bali cattle is either or both lymphocytes and macrophages. Further studies utilising double-labelling procedures with specific bovine cell markers will be required to comfirm the identity of these cells containing JDV antigens. Additional investigations with techniques such as in situ hybridisation to identify JDV nucleotide sequences in tissue cells would also be of value in confirming the result obtained with the immunocytochemical staining technique. These studies would be invaluable in furthering the understanding of the pathogenesis of JDV in Bali cattle, and perhaps in the selection of appropriate cells to use for further attempts to culture JDV in an in vitro culture system.

An additional potential use of the immunocytochemical assay utilising MAb would be the development of a specific method of confirming the presence of JDV in the tissue of cattle, and as an ancillary aid in the confirmation of a clinical and pathological diagnosis of the disease. Unfortunately, the assay has so far been unsuccessful in identifying JDV antigens in formaldehyde-fixed tissue (results not reported) and this will probably be necessary before immunocytochemical assay becomes a practical aid to the diagnosis of Jembrana disease in Indonesia.

Another potential use of the MAbs produced is in the development of methods for the immunological detection and quantitation of JDV in the blood of infected cattle. The only assay currently available is a biological assay requiring the inoculation of susceptible Bali cattle (Soeharsono et al. 1990), and this is time-consuming and very expensive. The high titre of JDV in plasma during the acute clinical phase of JDV, and the ability to quantitate JDV antigens in plasma by ELISA (Hartaningsih et al. 1994), suggest that this should be possible, perhaps by a capture ELISA. This could also provide a valuable ancillary aid for the confirmation of a clinical diagnosis of acute Jembrana disease.

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Detection of Jembrana Disease Virus in Paraffin-Embedded Tissue Sections by In Situ Hybridisation

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Abstract

An in situ hybridisation technique was developed that detected Jembrana disease virus (JDV) genomic RNA in formalin-fixed paraffin-embedded tissue sections, using a digoxigenin-labelled riboprobe. Large numbers of JDV-infected cells were demonstrated in many tissue sections from experimentally-infected animals early in the disease course, which was consistent with the extremely high circulating viraemia reported to occur during the febrile phase. Results suggested that virus replication occurs predominantly in the spleen initially, from where it spreads rapidly to other tissues including lymph nodes, lungs, bone marrow, liver, kidney, myocardium and elsewhere. The relatively high level of infection found in bone marrow progenitor cells suggested that bone marrow may be more important in the pathogenesis of JDV infection than has previously been recognised. Infected cells were also identified in the general circulation and within unusual intravascular lesions in lung sections.

ALTHOUGH the clinical and pathological changes associated with acute infection with Jembrana disease virus (JDV) have been comprehensively described, the pathogenesis is poorly understood. It has been widely assumed, but not yet demonstrated, that the high-levél viraemia observed early in the disease course results from viral replication in lymphoid tissues and cells of the lymphoproliferative infiltrate observed in other tissues. It remains somewhat anomalous, however, that the viraemia peaks by 12 days post-infection (p.i.) in most animals, but the pathological degree of lymphoproliferation continues to increase in severity until five weeks p.i., when regression of lesions commences (Dharma et al. 1991).

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Bone marrow is the most important haematopoietic tissue of mammals, where undifferentiated stem cells mature into erythrocytes, circulating granulocytes (neutrophils, eosinophils and basophils), megakaryocytes (platelet precursors), most B-lymphocytes, and all cells of the mononuclear phagocyte system (Jain 1986). A number of haematological changes associated with JDV infection, including lymphopenia, eosinopenia, and a mild thrombocytopenia, neutropenia and anaemia, suggest that viral infection of bone marrow progenitor cells may be important (Soesanto et al. 1990). However, pathological changes described in bone marrow are subtle and not present in all cases (Dharma et al. 1991). Bone marrow infection is important in the disease pathogenesis of some lentivirus infections, including equine infectious anaemia virus and human and simian immunodeficiency viruses (Kitagawa et al. 1991; Pise et al. 1992; Valli 1993), although it has not been reported for bovine immunodeficiency virus.

The technique of in situ hybridisation (ISH) has been widely applied for the detection of viral genomes in infected tissues, especially since the use of non-isotopic methods such as biotin or digoxigenin

(DIG)-labelled probes has largely replaced isotopic methods (McQuaid et al. 1990; Desport et al. 1994). The technique is particularly suited to the examination of formalin-fixed paraffin-embedded specimens. In this study, an ISH technique using a DIG-labelled riboprobe was developed for the detection of JDV-infected cells in paraffin-embedded tissue sections, and used to investigate aspects of the pathogenesis of Jembrana disease.

Methods

Riboprobes were synthesised using an in vitro transcription system based on T7/SP6 RNA polymerase (Promega) according to the manufacturer's instructions, except that a DIG-labelled nucleotide mixture (Boehringer) was substituted for the radio-labelled nucleotides. The positive riboprobe template consisted of a 683 bp fragment of cDNA derived from the *pol* gene of JDV genomic RNA (nt 1421–2103) cloned into pGEM (Promega), and was linearised with *Sal I* for antisense transcription. A negative control probe was synthesised from a similar clone transcribed in the positive sense (retrovirus-infected cells contain no antisense copies of the RNA genome). A second negative control probe was transcribed from non-linearised pGEM provided. Products of the 20 µL transcription reactions were ethanol precipitated, resuspended in 100 µL nuclease-free water with 100 units of RNAasin provided, and stored in aliquots at –80 °C. Positive and negative riboprobes were equilibrated for intensity by dot-blot hybridisation.

Tissue samples were obtained from two eighteen-month-old female Bali cattle that had been experimentally inoculated with JDV (Tabanan/87 strain). The first animal had been infected eight days prior to necropsy and was four days into the febrile period, while the second animal had been infected for five days and febrile for one day. The samples were fixed in 10% neutral buffered formalin for 24 hours and paraffin-embedded. Sections from these and archival paraffin-embedded specimens were cut at 6 µm onto silanated slides.

The protocol for in situ hybridisation was similar to one previously reported (Desport et al. 1994). Prior to hybridisation, sections were incubated at 75 °C for 15 minutes, deparaffinised in xylene and rehydrated through graded ethanols to distilled water. Nucleic acids were unmasked by digestion with protease VIII (0.25 mg/mL; Sigma) for 10–20 minutes at 37 °C, although archival specimens, whose formalin fixation time was unknown, usually required longer digestion for optimal unmasking. Sections were then washed in distilled water, dehydrated in 100% ethanol and air dried.

Riboprobe or DNA probe was then applied to sections at the rate of 0.25 µL/section (approx. 25 ng/section) in 50 µL of hybridisation solution consisting of 50% formamide, 5% dextran sulphate, 2 × SSC (final concentrations of 0.3 M NaCl, 0.3 M Na citrate), 2.5 mM Tris.HCl (pH 8.0), 0.25 mM EDTA, and 400 µg/mL denatured herring sperm DNA. Sections were then coverslipped (but not sealed), placed in small humidified chambers, and incubated firstly at 80 °C for 15 minutes to allow denaturation, then at 55 °C for 2 hours to allow hybridisation. All solutions used to this point were made with DEPC-treated water.

Following hybridisation the coverslips were washed off with 4 × SSC and the slides washed twice in 4 × SSC for 5 minutes; twice in 1 × SSC for 5 minutes; and once in 0.1 × SSC at 75 °C for 15 minutes. (Tissue sections were not allowed to dry out during washes, or at any stage of the post-hybridisation processing.) Sections were then blocked by incubation with 3% (w/v) bovine serum albumin and 0.05% (v/v) triton-X 100 in buffer 1 (100 mM Tris.HCl, pH 7.5; 150 mM NaCl) at room temperature for 30 minutes, followed by a further 30 minutes with the same blocking solution containing sheep anti-DIG antibody conjugated to alkaline phosphatase (Boehringer), diluted 1:500. Slides were then washed twice in buffer 1 for 5 minutes and equilibrated in buffer 3 (10 mM Tris.HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$) for 2 minutes. Colour was developed by overnight incubation with the NBT/X-phosphate system (Boehringer). The reaction was stopped by immersion in running tap water for 15 minutes. Nuclei were counterstained with 1% neutral red, and 0.8% light green (in 0.2% acetic acid) was applied for a light cytoplasmic counterstain. Slides were then immersed in acetone for 20 seconds to remove non-specific staining, flushed with 100% ethanol, then with xylene, and permanently mounted.

Results

Tissue sections obtained from JDV-infected Bali cattle consistently showed cells that gave a strong signal when stained with the positive (antisense) riboprobe, but no signal when stained with the negative (positive sense) riboprobe or the pGEM-derived negative riboprobe. Staining was consistently strongest in the nucleus, although usually present also in the cytoplasm. No signal was seen in tissues from uninfected animals stained with either positive or negative riboprobe.

As described in previous reports, optimal unmasking of nucleic acids by protease VIII digestion was found to be critical to the procedure,

particularly when using archival specimens (Desport et al. 1994; McQuaid et al. 1990).

A large number of JDV-infected cells were found in all tissue sections examined from the first animal, including lymph node, spleen, lung, liver, kidney and femoral bone marrow. Sections of spleen and lymph node consistently contained the most infected cells, which were distributed in a multifocal to diffuse pattern throughout parafollicular areas, and areas where follicular architecture had been largely obliterated by sheets of proliferating lymphoid cells (Fig. 1). It was estimated that as many as 5–10% of cells in non-follicular areas stained positive, while less than 1% were infected in follicular areas.

Lung sections contained infected cells predominantly within alveolar septae, particularly in areas of thickened septae where pathological changes were consistent with interstitial pneumonia (Fig. 2). Many intravascular lesions also contained a number of infected cells, which morphologically appeared to be immature monocytes rather than of lymphoid origin, which is consistent with previous reports (Dharma et al. 1991; Budiarso and Rikihisa 1992). Infected cells were also commonly found randomly distributed within the characteristic lymphoid infiltrate observed in sections of liver and kidney (Fig. 3). As a generalisation, wherever lymphoproliferative cells congregated in these tissues it was estimated that approximately 5% of such cells were demonstrably infected with JDV. Interestingly, no positive staining at all was seen in mitotic cells, although they were commonly observed in the infiltrate in most sections.

Bone marrow sections were found to contain many infected cells, despite showing no changes on routine histopathological examination. Most infected cells were large lymphocytes or haematopoietic stem cells, which could not be differentiated by morphological characteristics. A few infected cells were identifiable as myeloid or erythroid precursors, and a small number of megakaryocytes were weakly positive.

Finally, it was noted that all tissue sections from the first animal contained infected cells in blood vessels, indicating the presence of significant numbers of infected cells within the general circulation. The

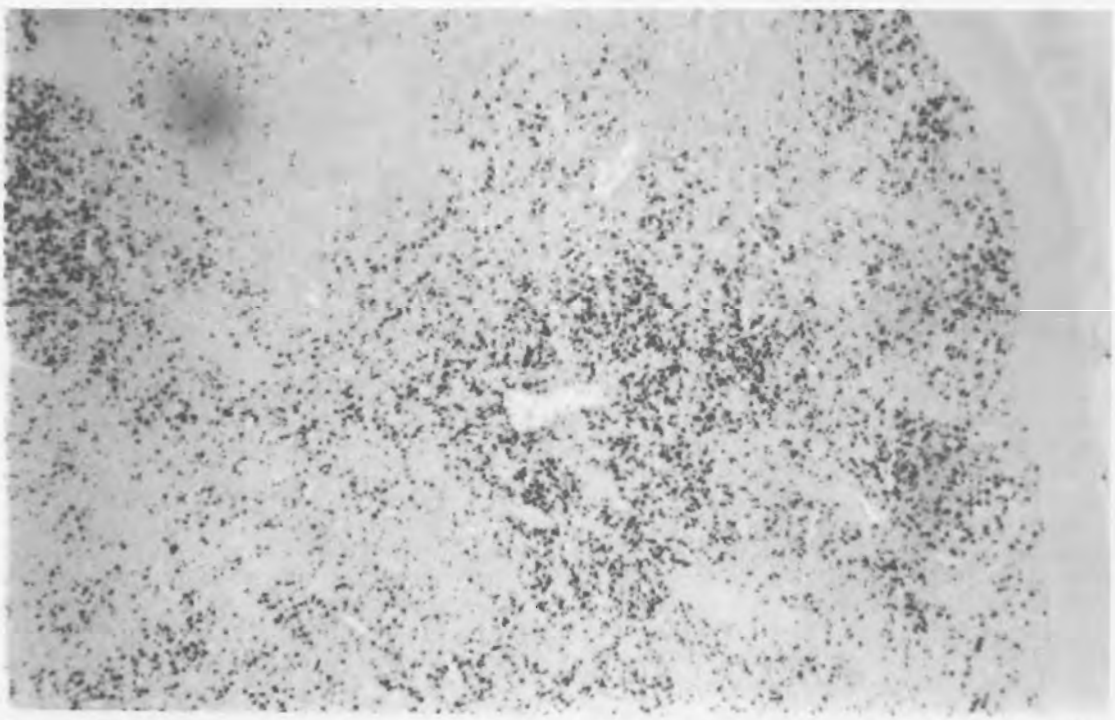

Figure 1. Section of spleen from the first animal, necropsied on the fourth day of the febrile period, stained with the JDV-specific riboprobe (× 180). Follicular architecture has been largely obliterated by sheets of proliferating lymphoid cells. It was estimated that as many as 5–10% of cells in non-follicular areas stained positive, while less than 1% were infected in follicular areas.

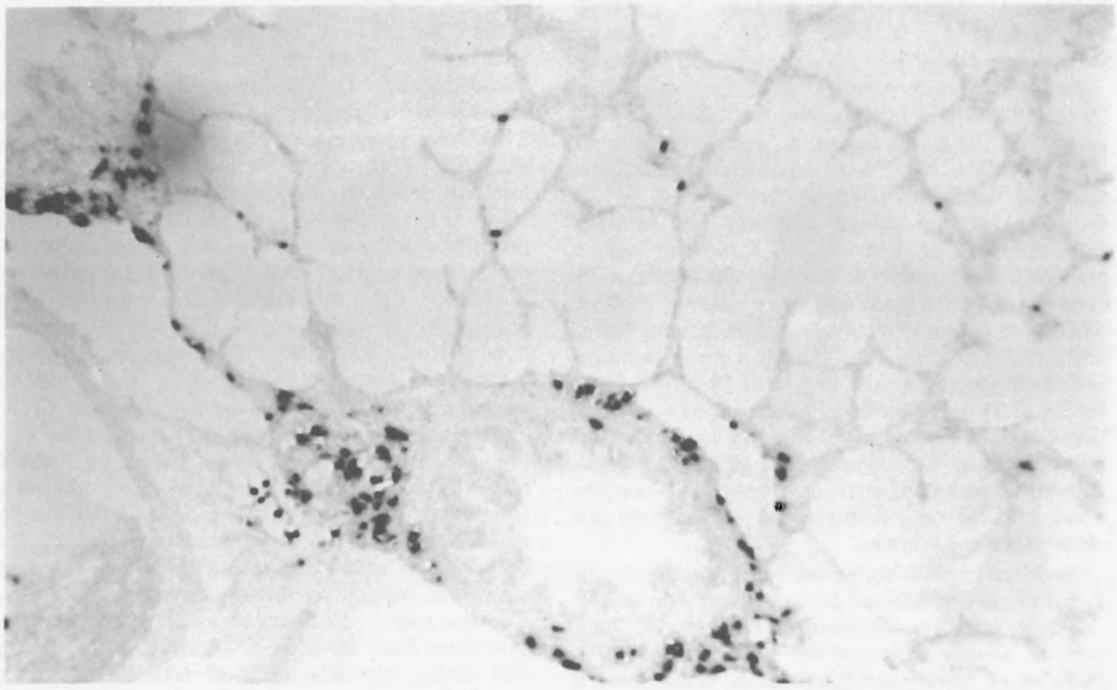

Figure 2. Lung section from the first animal showing infected cells predominantly within alveolar septae and in peribronchial areas of lymphoid infiltration (× 600).

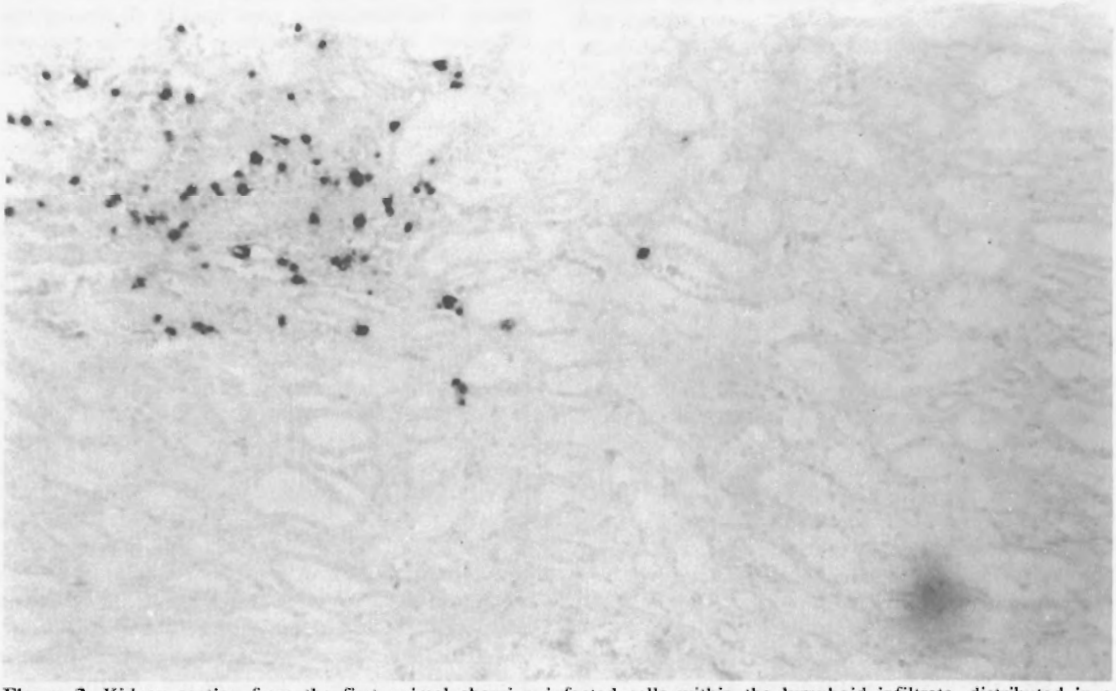

Figure 3. Kidney section from the first animal showing infected cells within the lymphoid infiltrate, distributed in a multifocal pattern throughout glomeruli and tubular areas (× 600).

morphology of these cells was consistent with that of monocytes or large lymphocytes.

Splenic sections from the second animal showed a considerably greater density of infected cells than those of the first animal, despite the follicular architecture of the tissue appearing relatively normal. Approximately 10–15% of cells throughout non-follicular areas appeared to be infected, while follicular areas contained relatively few infected cells. Unlike the first animal, however, only a relatively small number of infected cells were seen in sections of lymph node and bone marrow, both of which also appeared relatively normal histologically, and sections of lung, kidney, liver, and myocardium, which all showed a mild degree of lymphoid infiltration. Only very occasional infected cells were seen in sections of thyroid, adrenal gland, brain, choroid plexus, tongue and pancreas, all of which tissues showed no pathological changes on routine examination. Infected cells within blood vessels were not observed in any sections.

Archival paraffin-embedded specimens were also found to contain infected cells, although not usually in large numbers. Somewhat surprisingly, lung sections usually contained the most infected cells. Sections of spleen and lymph node often showed almost complete obliteration of normal follicular architecture by proliferating lymphoid cells with a high mitotic index, but contained very few infected cells. Similarly, sections of lung, liver, kidney and myocardium all contained a heavy cellular infiltrate, but only a small number of infected cells. Overall, less than 0.5% of infiltrating round cell populations were found to be infected. Staining in positive cells was considerably weaker than in tissues from the experimentally-infected animals, and was generally restricted to the nucleus. As for those animals, no mitotic cells were found to be infected.

Discussion

The technique of ISH using a DIG-labelled riboprobe to detect viral RNA was found to be a sensitive technique for the demonstration of JDV-infected cells in paraffin-embedded tissue sections. Specific staining in infected cells was both nuclear and cytoplasmic, although considerably more prominent in the nucleus. This was consistent with the biology of retroviruses, which are transcribed in the host cell nucleus and translated in the cytoplasm. The notable lack of productive JDV infection in mitotic cells was also consistent with the biology of lentiviruses, which generally replicate in activated cells, rather than the dividing cells that are the typical host cells for the oncogenic retroviruses (Bishop 1978; Narayan and Clements 1990).

ISH with riboprobes identified JDV-infected cells in many tissues including spleen, lymph node, lung, bone marrow, liver, kidney and myocardium. All tissues obtained from both experimentally infected animals were found to contain infected cells wherever a lymphoproliferative infiltrate was observed. With the exception of bone marrow, infected cells were not generally seen in tissues which did not contain such an infiltrate. The vast majority of infected cells appeared to be members of the infiltrating round cell population and either of lymphoid origin or of the monocyte/macrophage lineage, which was consistent with previous suggestions (Dharma et al. 1991, 1994), and generally consistent with the tendency of other lentiviruses to infect these cell types (Narayan and Clements 1990).

The greatest concentration of infected cells was found in the spleen of the second animal, necropsied on the first day of the febrile period. Splenic sections from the first animal, necropsied on the fourth febrile day, contained lower numbers of infected cells. In contrast, all other tissue sections from the first animal, including lymph node, bone marrow, lung, liver and kidney, contained many more infected cells than those from the second. These results suggested that during the early phase of JDV infection, the initial virus proliferation occurred predominantly in the spleen, from where it then spread to lymph nodes, bone marrow, lungs, kidneys and other tissues. The cumulative virus load in all tissues was consistent with the extremely high-level viraemia which occurs during the febrile phase (Soeharsono et al. 1990).

The abundance of JDV-infected cells in the bone marrow was of particular significance as pathological changes had not been detected. Only mild changes have been reported in bone marrow, which has therefore not previously been implicated in the pathogenesis of the disease. Infection of marrow progenitor cells by JDV would be consistent with a number of previously reported clinical findings associated with Jembrana disease, including anaemia, thrombocytopenia, eosinopenia and transient neutropenia (Soesanto et al. 1990; Teuscher et al. 1981). Bone marrow may have also been the source of infected mononuclear cells identified in the general circulation and in intravascular lesions in lung sections. Abnormal leukocytes previously described during the febrile phase may have also originated from bone marrow (Teuscher et al. 1981). Bone marrow infection may therefore be as important in the pathogenesis of Jembrana disease as it is for other lentivirus infections.

In archival paraffin-embedded specimens, the results were similar to those in the experimentally-infected cattle, except that infected cells generally

numbered far fewer, even in sections that showed a markedly greater degree of pathological lymphoproliferation. While approximately 5% of infiltrating round cells appeared to be infected in tissues from the first animal, less than 0.5% were demonstrably infected in most archival specimens. This confirmed that the genetic and immunological events which regulate viral replication are at least partly independent of the direct or indirect mechanisms which underlie the proliferative effects of JDV infection in vivo.

The ISH technique described here will be useful for more extensive studies of the pathogenesis of JDV infection than could be undertaken with the case material available for this study. It may also be useful, in conjunction with immunocytochemical labelling, for investigating the specific cell types infected by JDV, and for investigating the direct role, if any, that JDV may play in illnesses that occur in chronically-infected animals.

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Current Information on Jembrana Disease Distribution in Indonesia

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Abstract

The antibody to Jembrana disease virus (JDV), the aetiological agent of Jembrana disease in Bali cattle (*Bos javanicus*), was detected in cattle on four islands of Indonesia: Bali, Sumatra (Lampung, Sumatra Barat, Jambi and Riau provinces), Java (in the Banyuwangi district of Java Timur), and Kalimantan (Kalimantan Selatan and Kalimantan Tengah provinces). Diseases indistinguishable from Jembrana disease have been reported in all areas where the antibody was detected, except Jambi, Riau and Kalimantan Tengah. The antibody to JDV was not detected in cattle from Madura island (Java Timur province), Sulawesi Selatan, Timor island (Nusa Tenggara Timur province and East Timor), and Lombok island (Nusa Tenggara Barat province), areas where there have been no reports of clinical Jembrana disease in Bali cattle.

BALI cattle are widely distributed throughout Indonesia with a total population of slightly more than 3 million. A serological survey of cattle was undertaken, mainly during the period 1990–1991, but with additional sera being collected in 1992 and 1995. The objectives of the study were to determine the prevalence of cattle with antibodies to JDV; to provide a basis for a control program designed to prevent the movement of cattle and spread of the infection from serologically-positive areas to serologically-negative areas; and to provide an early warning system for the presence of JDV in areas where Jembrana disease has not been recognised.

Serological distribution

Serological examinations for antibodies to JDV were conducted by an enzyme-linked immunosorbent assay (ELISA) as described by Hartaningsih et al. (1994).

Antibodies to JDV in cattle or buffalo were detected by ELISA in four islands of Indonesia: Bali, Sumatra (Lampung, Sumatra Barat, Jambi, Riau, Bengkulu and Sumatra Selatan provinces), Java (in the Banyuwangi district of Java Timur), and Kalimantan (Kalimantan Selatan and Kalimantan Tengah provinces). Antibodies to JDV were not detected in cattle from Nusa Penida island (Bali province), Madura island (Java Timur province), Sulawesi island (Sulawesi Selatan province), Timor island (Nusa Tenggara Timur and Timor Timur province), and Lombok island (West Nusa Tenggara province).

Detection of antibodies during 1990–1991

In the period 1990–1991, antibodies were detected in serum samples from cattle in only three provinces in Indonesia: Bali, Java Timur and Lampung. No antibodies were detected in cattle in the districts which were sampled in Jambi province (Sumatra), in the Kupang district of Timor island (Nusa Tenggara Timur province), in Lombok (Nusa Tenggara Barat province), and in Sulawesi Selatan province.

In Bali province, antibodies were detected in all districts on the island of Bali but not on the adjacent island of Nusa Penida, a subdistrict of the Klungkung district. There was a higher prevalence of antibody-positive cattle in the western districts (Jembrana, Tabanan and Buleleng) than in other districts, which corresponded to the reported prevalence of clinical cases of Jembrana disease in cattle in Bali province. Antibodies were also detected in 13 of 51 buffalo sampled in the western district of Bali island.

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In Lampung, antibodies were detected only in Lampung Tengah district but not in the adjacent district of Lampung Selatan. In Lampung Tengah, there was a higher prevalence of antibody-positive cattle in the subdistricts of Seputih Raman, Seputih Banyak and Sekampung than in other subdistricts. In East Java province, antibodies were detected in cattle in the Banyuwangi district but not in cattle from the island of Madura.

Detection of antibodies to JDV during 1992

Sumatra Barat: Antibodies were detected in Timpeh in the Sawahlunto subdistrict in 1992.

Riau and Jambi provinces: Although antibodies were not detected in Jambi province in serum samples collected during 1990–1991, and serum samples were not obtained from Riau province, antibodies were detected in cattle in both Riau and Jambi provinces (Sumatra island) as shown in Table 1.

Kalimantan Selatan and Kalimantan Tengah provinces: Antibody positive cattle were detected in several districts in Kalimantan Selatan province, and were also detected in one district (Barito Selatan) in Kalimantan Tengah, a district which borders Kalimantan Selatan.

Detection of antibodies to JDV during 1995

Bengkulu and Sumatra Selatan province: Antibodies to JDV were detected in Bengkulu and Sumatra Selatan province, provinces adjacent to Lampung province of Sumatra island. The occurrence of Jembrana disease in these two provinces was reported by the Disease Investigation Centre Region III, Lampung (Prabowo, pers. comm.).

Discussion

The presence of Jembrana and Jembrana-like diseases in the three known endemic areas, and the

Table 1. Distribution of antibody to Jembrana disease agent in cattle in Indonesia.

Province	District	Subdistrict	ELISA RESULT (Positive/sera tested)			
			Bali cattle		Ongole cattle	Bali × Ongole cattle
Lampung	South Lampung	Natar	0/514	(0%)	—	—
		Palas	3/48	(6%)	—	—
		Sidomulyo	4/110	(4%)	—	—
	Central Lampung	Punggur	1/17	(6%)	0/2	1/3
		Seputih Raman	79/226	(35%)	3/25	0/25
		Seputih Mataram	14/76	(18%)	1/15	—
		Seputih Banyak	60/204	(29%)	1/3	0/1
		Raman Utara	10/84	(12%)	—	—
		Rumbia	15/157	(10%)	—	—
		Seputih Surabaya	33/180	(18%)	—	—
		Sehampung	16/49	(33%)	—	—
	North Lampung	Blambangan Umpu	0/92	(0%)	—	—
		Banjit	0/60	(0%)	—	—
West Sumatra	Sawahlunto/Sijunjung*		28/201	(14%)	—	—
Jambi	Unit IX, X, XIV		0/208	(0%)	—	—
East Java	Madura island		—			0/301**
	Banyuwangi		31/100	(31%)	—	—
South Kalimantan	Banjarbaru	Unknown	8/24	(33%)	—	—
	Tanah Laut	Ambungan	11/18	(61%)	—	—
		Panggung	46/71	(64%)	—	—
		Panggung Baru	10/13	(76%)	—	—
		Pabahanan	5/32	(15%)	—	—
	Barito Kuala	Barambai	5/29	(17%)	—	—
	Kota Baru	Unknown	13/20	(65%)	—	—
	Unknown	Unknown	8/24	(33%)	—	—
Central Kalimantan	Barito Selatan	Unknown	3/14	(21%)	—	—
South Sulawesi			0/210	(0%)	—	—
East Nusa Tenggara	Kupang		0/214	(0%)	—	—
West Nusa Tenggara	Lombok island		0/155	(0%)	—	—

* Outbreak of Jembrana disease in 1992
** Madura breed of cattle

concurrence of this with the presence of JDV virus antibodies in cattle in only these three areas in 1990, indicated that the detection of antibodies to JDV would provide an additional method of confirming the presence of suspected Jembrana disease in areas where the disease was previously unknown. Previously, Jembrana disease has been diagnosed, or suspected, only on the basis of pathological and histopathological changes in affected animals (Teuscher et al. 1981; Prabowo and Ishitani 1984; Sudana et al. 1979). While ideally, the development of a method of isolating JDV or detection of JDV antigen in tissues of affected cattle would provide a more definitive diagnosis in individual cattle, the detection of antibodies to JDV in a population of cattle, in association with typical clinical and pathological changes in individual cattle, would provide a valuable method of confirming a pathological diagnosis. The detection of antibodies to JDV in the absence of clinical signs of disease would also indicate that close examination of the cattle in an area might lead to the detection of the disease and an early warning system, especially in areas where there are only low densities of Bali cattle.

In Bali, the reported prevalence of Jembrana disease cases is higher in the western districts of the island (Jembrana, Tabanan and Buleleng) than in other districts. The reason for this is unknown. There was a correlation between the prevalence of cattle in Bali with antibodies to JDV and the number of reported cases of Jembrana disease. There have been no reported cases of Jembrana disease (Hassan, pers. comm.) and there was no serological evidence of Jembrana disease in the Nusa Penida district of Bali province.

In Lampung Tengah, there was also a correlation between the prevalence of serologically positive cattle and the prevalence of the disease. There was a higher prevalence of serologically positive animals in the Seputih Raman, Seputih Banyak and Sekampung subdistricts; the majority of clinical cases of 'Rama Dewa' disease were reported to occur in the Seputih Raman and Seputih Banyak subdistrict.

Distribution of antibodies to JDV after 1991

An outbreak of a disease clinically indistinguishable from Jembrana disease occurred in a small transmigration area in Timpeh village (Sawahlunto-Sijunjung subdistrict), Sumatra Barat, in April 1992, which was associated with a high mortality rate of cattle in the affected area (Tazril, pers. comm.). Typical pathological lesions of Jembrana disease were detected in tissues of affected cattle obtained by the Disease Investigation Centre in Bukittinggi in Sumatra Barat (D.M.W. Dharma, pers. comm.). As there was a close concurrence between clinical disease and the presence of antibodies in other areas, the detection of antibodies to JDV in the affected cattle provided confirmation of the clinical and pathological diagnosis of Jembrana disease. Serum samples were not obtained from this province during the 1990–1991 period, so it was not possible to verify that cattle in this area were antibody-negative before the outbreak.

In Riau and Jambi provinces, which are adjacent to Sumatra Barat, clinical and/or pathological evidence of Jembrana disease has not been detected, and antibodies to JDV were not detected in Jambi during the serological survey conducted in 1990–1991. The detection of antibodies during 1992, however, indicates that JDV has recently been introduced into these two provinces and further examination of cattle will probably result in the detection of clinical cases of the disease.

Tissues from a Bali animal that had died in Kalimantan Selatan in 1992 were submitted by the Disease Investigation Centre in Banjarmasin (Kalimantan) to the Disease Investigation Centre in Denpasar (Bali) and Jembrana disease was suspected, based on histological lesions (D.M.W. Dharma, pers. comm.). Although no other cases of Jembrana disease were identified, the detection of antibodies to JDV in affected cattle in Kalimantan Selatan provided evidence that the virus is present in the region, and the serological results further indicated that the virus was widespread in cattle in this province. Antibodies were also detected in Barito Selatan, a subdistrict of Kalimantan Tengah, which borders on South Kalimantan, suggesting that cattle in this province are also affected and that further examination of cattle in these two provinces might identify further clinical and pathological evidence of Jembrana disease.

The current population of Bali cattle in Kalimantan Selatan and Kalimantan Tengah is about 13 000 and 8000, respectively (Kalianda, pers. comm.). They are widely scattered and the density of Bali cattle throughout the two provinces is low. In addition to Bali cattle, there are also large numbers of crossbred Bali cattle, Ongole and Madura cattle, and buffalo. The outbreak of Jembrana disease in Kalimantan was apparently not associated with high morbidity and mortality rates, as in other initial outbreaks of Jembrana disease in Bali, Lampung and Sumatra Barat. Possible reasons why evidence of the disease has not been detected include:

(1) the presence of only a low density of Bali cattle in Kalimantan;
(2) the likelihood that affected animals were sold for slaughter by the owners and consequently not reported;

(3) that the provincial veterinary staff were unaware of the presence of JDV in Kalimantan resulting in misdiagnoses;

4) the relatively poor communication systems in Kalimantan that have made investigations of disease difficult.

Clinical observations and serological results indicated that there has been limited spread of Jembrana disease from endemic areas in Bali, East Java and Lampung (Sumatra island) into adjacent areas or to neighbouring islands. Although the disease has occurred in Bali since 1964, there has been no clinical evidence of Jembrana disease and there was no serological evidence of Jembrana disease in Bali cattle on the islands of Lombok and Nusa Penida which, at the closest points, are 10–20 kilometres from Bali. In Lampung province, the only animals positive for JDV antibodies in 1992 were in the Lampung Tengah district of Lampung province, the only district where Jembrana disease has been detected since 1976 and until 1992. The serological evidence obtained in 1992 suggests, however, that the virus has spread to cattle in Lampung Selatan even though there have been no reports of clinical cases of the disease.

The detection of JDV antibodies in Bengkulu and Sumatra Selatan suggested that the disease has spread into this area. As Bengkulu and Sumatra Selatan provinces are adjacent to Lampung province, movement of cattle from an infected area in Lampung to a previously uninfected area in Bengkulu is likely to have occurred.

Although the mechanism of transmission of Jembrana disease is unknown it has been suspected to be arthropod-transmitted. However, the limited distribution of antibody-positive cattle and the limited spread of the disease from the endemic areas over a prolonged period suggests that if Jembrana disease is transmitted by arthropod vectors that this mode of transmission is limited. There are no physical barriers to the spread of free-flying arthropods from the endemic areas in Lampung Tengah to the adjacent district of Lampung Utara where the disease does not occur, and there are only short distances separating Bali from the adjacent islands of Lombok and Nusa Penida where the disease has not occurred.

There have been no confirmed field reports of the occurrence of clinical Jembrana disease or other diseases indistinguishable from Jembrana disease in cattle other than Bali cattle. However, in the current study, there was serological evidence that Ongole (*Bos indicus*) and crossbred Bali cattle in Lampung Tengah, and buffalo in Bali, had been infected with JDV. The detection of antibodies in these cattle is consistent with detection of a mild or subclinical infection and viraemia in Ongole, Madura and Rambon cattle and buffalo when they were experimentally-infected with JDV (Soeharsono et al. 1990) and the detection of antibodies to JDV in experimentally-infected cattle (Hartaningsih et al. 1994). Their role in perpetuation of the virus infection in populations of cattle and transmission of the infection to Bali cattle require further investigation.

Conclusions

1. Jembrana disease is slowly speading from infected areas to uninfected areas in Indonesia. The most likely cause of spead is movement of carrier animals.
2. Provinces eastward of Bali are still free from JD.

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The Transmission and Persistence of Jembrana Disease Virus in Cattle

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Abstract

Two probable methods of transmission of Jembrana disease caused by Jembrana disease virus (JDV) and evidence for these modes of transmission are described. During the acute disease there is a high titre of JDV of about 10^8 50% cattle infectious units (ID_{50})/mL in the blood of affected Bali cattle. During this phase, two methods of transmission are possible: (1) direct transmission of virus in secretions between cattle in close contact with each other; (2) mechanical transmission of virus by haematophagous insects. Recovered cattle are also a potential source of infection. Recovered cattle are persistently viraemic but the titre of infectious virus in blood by 60 days after recovery from the acute disease is only about 10^1 ID_{50}/mL, virus cannot be detected in secretions during this phase, and mechanical transmission by haematophagous insects is unlikely.

THE cause of Jembrana disease in Bali cattle (*Bos javanicus*) is Jembrana disease virus (JDV), a recently-described lentivirus that is antigenically and genetically related to, but distinguishable from, bovine immunodeficiency-like virus (Chadwick et al. 1995a, b). The acute nature of Jembrana disease with recovery of most affected animals and no recurrence of disease in the recovered animals is unusual for a lentivirus.

Transmission of JDV

The mechanism of transmission of Jembrana disease is poorly understood. After it was first detected on Bali island in 1964, it apparently required about 12 months to spread throughout the entire cattle population on the island. Clinical disease has since occurred in three other areas of Indonesia: Lampung and West Sumatra provinces on Sumatra island, and in East Java. It is now endemic in all these areas and, although there has been remarkably limited spread of the disease from the endemic areas to adjacent areas, there is serological evidence that it is now present also in other provinces of Sumatra and Kalimantan (Soeharsono, these Proceedings).

In Bali, Jembrana disease is endemic and outbreaks of the disease with a low number of affected cattle in a small area are common (Putra, these Proceedings). Based on an early hypothesis that the disease had a rickettsial aetiology (Adiwinata 1967) and known role of arthropods in the transmission of many rickettsial diseases, arthropods including ticks (Ramachandran, these Proceedings) were suspected for many years to be involved in the transmission of the disease, and insecticides were used to assist in control of disease outbreaks (unpublished observations). Rickettsia are no longer considered to be involved in the disease and with the recognition that the disease is caused by a lentivirus (Kertayadnya et al. 1993; Chadwick et al. 1995b) there has been a re-examination of the probable methods that may be involved in transmission.

A feature of the disease that seems particularly relevant in the development of an hypotheses on the method of transmission of the disease is that there

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appear to be two distinct phases of virus replication during the disease process. During the acute clinical disease, there is a high titre of about 10^8 ID_{50}/mL of infectious virus in blood of affected animals. In recovered animals there is a persistent viraemia although the titre of virus is low, approximately 10 ID_{50}/mL (Soeharsono et al. 1990, 1995a).

One method of transmission is suggested by the high titre of virus in blood of approximately 10^8 ID_{50}/mL during the acute clinical phase of Jembrana disease. During this period, there would be a high probability that the disease could be transmitted by the mechanical transfer of blood or blood-contaminated products from affected to susceptible cattle. In contrast, during and after the recovery period when the titre of virus declines to low levels of approximately 10 ID_{50}/mL, the chance of mechanical transmission by blood or blood-contaminated products would be relatively low.

Two methods of mechanical transmission of the virus in blood from cattle during the acute disease are probable: by multi-use needles during vaccination programs, and by arthropods. The high titre of the virus in blood of 10^8 ID_{50}/mL during the acute disease suggests that vaccination procedures involving the multiple use of a single syringe and needle, sometimes practised in the areas where Jembrana disease is endemic, could mechanically transmit the virus. This mode of transmission has been reported for equine infectious anaemia virus (Issel et al. 1990). It is also possible that transmission of the virus on the mouth parts of haematophagous arthropods during interrupted feeding would be possible. Transmission by haematophagous arthropods such as *Tabanus* species of equine infectious anaemia virus occurs from acutely infected horses, in which virus titres of about 10^6 infectious virus particles/mL of blood have been detected (Issel and Foil 1984; Issel et al. 1990), less than is detected during Jembrana disease.

Mechanical transmission of JDV by arthropods, as seems likely, has not been responsible for extensive spread of Jembrana disease from endemic to adjacent areas: the disease has not spread from Bali island to two closely adjacent islands, Nusa Penida and Lombok, since the disease initially occurred in Bali in 1964, and there has been limited spread of the disease from the endemic area of Lampung province in Sumatra island to adjacent areas since the disease occurred there in 1976 (Hartaningsih et al. 1993; Soeharsono et al. 1995a).

A second method of transmission also seems likely: during the acute phase of the disease there is considerable evidence that it is contagious. Cross-infection from infected to susceptible cattle housed in direct contact with each other in screened animal rooms was reported by Soeharsono et al. (1990); the disease in the in-contact susceptible cattle occurred approximately 14 days after acute signs of the disease were detected in the inoculated animals.

Contact transmission is probably associated with JDV present in secretions, including saliva and possibly urine, and in lactating animals also, in milk, of animals during the acute febrile phase of the disease (Soeharsono et al. 1995a). Furthermore, while it is well known that transmission of infection is possible by intravenous or intraperitoneal inoculation of JDV into susceptible animals (Soeharsono et al. 1990), it is also possible to infect animals by administration of JDV via the oral and intranasal routes and by installation of JDV onto the conjunctival mucous membranes (Soeharsono et al. 1995a). However, as the virus was only detected in secretions during the acute stage of the disease, when there is a high titre viraemia in affected cattle, transmission via these routes would be likely only from cattle with the acute disease and where there was intimate contact between affected and susceptible cattle.

Further evidence of the importance of close contact between animals for the transmission of Jembrana disease is that cattle owned by individual farmers tend to be either all infected (antibody-positive) or all uninfected (antibody-negative), suggesting that cross-infection of animals owned by the same farmer had occurred to a greater extent than between animals owned by different farmers in the same village (Soeharsono et al. 1995a). Cattle owned by individual farmers are frequently tethered together while grazing, and housed in isolation from other farmers' cattle; there is frequent intimate contact between these cattle but minimal direct contact between these cattle and other cattle in the same village. Even if the transfer of JDV between cattle owned by the same farmer was not the result of contact with infected body fluids, and if an arthropod vector was involved, it would suggest that arthropod transmission occurs to a greater extent if there are only short distances involved.

Transmission of JDV in semen during natural mating or by artificial insemination is not a common means of dissemination of JDV, or if it does occur, it is an infrequent event, as the virus could not be detected in semen of experimentally infected cattle before and immediately after the febrile phase of the disease (Soeharsono et al. 1995a).

Transmission of JDV in milk, demonstrated to be an important mode of transmission in the caprine arthritis-encephalitis lentivirus (Issel et al. 1990) is also a possible method of transmission of JDV. The virus was detected in milk and the disease could be transmitted by oral instillation of virus into

susceptible cattle (Soeharsono et al. 1995a). However, the ability to detect JDV in milk only during the acute febrile phase of the disease suggests this route of infection is of limited importance.

Persistence of JDV

Bali cattle are viraemic for at least two years after recovery from clinical disease (Soeharsono et al. 1990) but the role of these persistently viraemic recovered animals in the transmission of the disease is unknown. The titre of virus in blood 72 days after infection is low (about 10 ID_{50}/mL), and virus could not be detected in secretions in recovered animals. Mechanical transmission by arthropods or contact transmission would therefore be unlikely from these animals. There is no evidence of recurrence of clinical disease in recovered animals, or of any variation in the level of viraemia, although this latter possibility has not been excluded. There is anecdotal evidence that the occurrence of Jembrana disease in Sumatra and Java (Hartaningsih et al. 1993) was associated with the illegal movement of cattle from Bali (unpublished observations), and persistently viraemic recovered cattle were possibly the source of the infection in these areas.

The role played by other animal species in the transmission and perpetuation of JDV is not understood but it is possible they could be involved (Soeharsono et al. 1995b). It is known that *Bos taurus*, *Bos indicus* and crossbred Bali (*Bos javanicus* × *Bos indicus*) cattle can be infected with JDV and remain viraemic for 3–6 months (Soeharsono et al. 1995b). Virus also persists in sheep for at least four months after infection, and viraemia has been detected in the Indonesian buffalo (*Bubalus bubalis*) for at least nine months after infection (Soeharsono et al. 1990). Whether JDV could persist in populations of any of these animals in the absence of *Bos javanicus* cattle is unknown.

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Serological Test for JDV Antibodies and Antibody Response of Infected Cattle

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Abstract

An enzyme-linked immunosorbent assay (ELISA) was developed that detected antibodies in Bali cattle (*Bos javanicus*) to Jembrana disease virus (JDV). In infected cattle, antibodies were not detected in most of the cattle until 11 weeks after infection, a maximum antibody response was detected 23 to 33 weeks after infection, and persisted until at least 59 weeks after infection. Western immunoblotting (WB) indicated the major immunodominant protein of JDV was a 26 kilodalton (K) protein. An antibody response was detected in most JDV-infected Madura and Rambon (crossbred *Bos javanicus*) cattle, although clinical signs of disease were detected in only some of these cattle and the clinical signs that occurred were considerably milder than those in Bali cattle.

DURING the acute febrile stage of Jembrana disease there was a high titre of about 10^8 50% Bali cattle infectious doses (ID_{50}) of JDV per mL in blood and plasma of affected cattle (Soeharsono et al. 1990). The infectious agent in blood and plasma was a virus with characteristics of a retrovirus (Wilcox et al. 1992; Kertayadnya et al. 1993). Only limited replication of the virus in cell cultures has been obtained (Wilcox et al. 1992) and plasma from infected cattle has been used as a source of infectious virus. This paper describes the developed ELISA which detected antibodies in infected cattle and the antibody response of infected cattle.

Serological test

An enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to JDV in cattle and buffalo serum was conducted as previously described by Hartaningsih et al. (1994). The antigen was prepared by sucrose gradient purification of virus (Kertayadnya et al. 1993) present in the plasma of cattle experimentally infected with the Tabanan/87 isolate of JDV (Soeharsono et al. 1990). All assays were standardised against a reference positive serum (PM90) obtained from a naturally-infected animal in the endemic Jembrana disease area of Tabanan, Bali.

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Cattle

Nineteen Bali cattle were obtained from Nusa Penida, an island adjacent to Bali where Jembrana disease does not occur. Eight Madura cattle (a breed in Indonesia developed by crossbreeding *Bos javanicus* and *Bos indicus* cattle) were obtained from Madura island, and eight Rambon cattle (a breed developed by crossbreeding *Bos javanicus* and *Bos indicus* cattle) from an area of East Java where Jembrana disease has not been reported. All cattle were infected with the Tabanan/87 isolate of JDV by intravenous inoculation of virus, and sera were collected 3 days prior to infection and at intervals of 2 weeks after infection. The sera were stored at –20°C until tested by ELISA for antibody against JDV.

Antibody Response to JDV in Experimentally-infected Cattle

Bali cattle

The ELISA results with sera from 19 Bali cattle at intervals after they were infected with JDV and had developed Jembrana disease are shown in Figure 1. Antibodies were not detected in any cattle at the time of infection. After infection, antibodies were detected in 18 of the 19 cattle but were not detected in one animal. A positive ELISA result was initially detected 2 weeks after infection in a low percentage of the cattle but it was not until 8 weeks after infection that the mean absorbance of the sera from

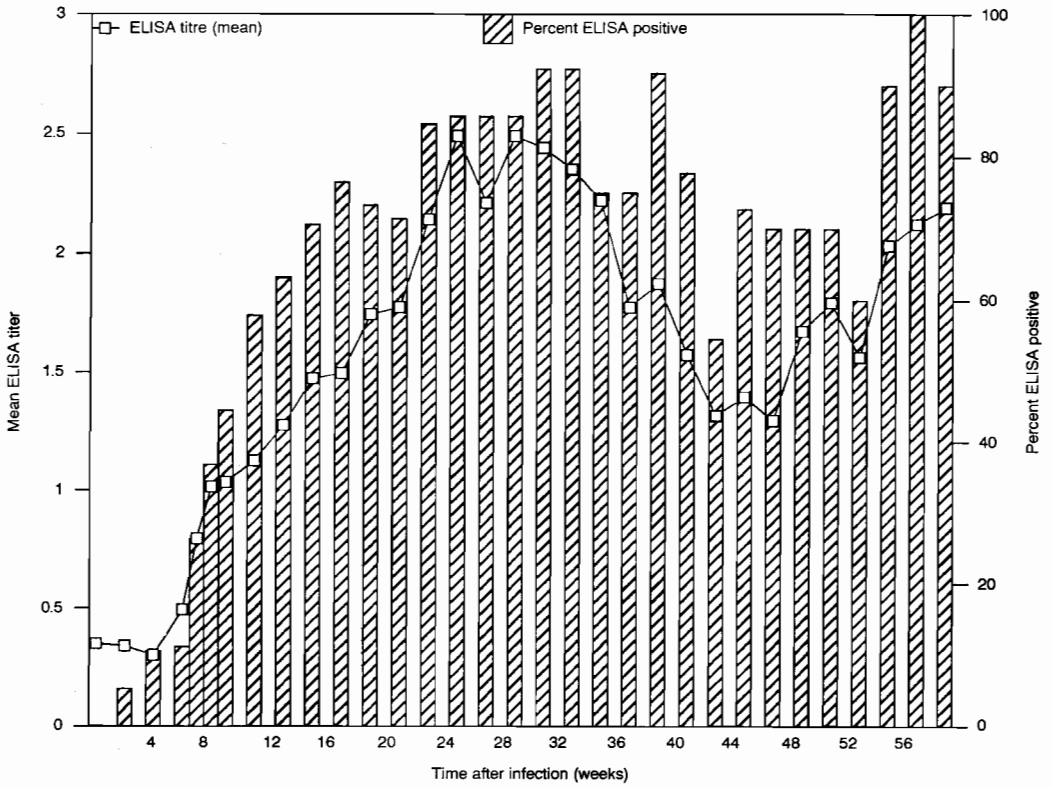

Figure 1. Sequential antibody response to JDV in Bali cattle. The ELISA titres are expressed as a ratio as detailed in the text. A value of 1.0 or greater in an individual animal was interpreted as a positive result.

all cattle exceeded that of the selected positive absorbance value, and 11 weeks after infection before sera from most of the cattle gave a positive result. The maximum number of positive cattle and the peak mean absorbance of the sera occurred between 23 and 33 weeks after infection. The antibodies persisted in most of the cattle until 59 weeks after infection, when the observations ceased. There was a transient decrease between 29 and 55 weeks post-infection in the number of antibody-positive cattle and the mean titre of antibody detected by the ELISA. Western blotting analysis (Kertayadnya et al. 1993) of sera from three Bali cattle (CB767, CB773 and CB783), which gave a positive ELISA result, detected antibodies reactive to individual proteins of JDV (data not shown). The reaction to the 26K (capsid) protein was immunodominant and there was a close association between the detection of antibodies to the 26K protein by Western immunoblotting and ELISA results in individual serum samples, although other proteins were also detected when sera collected at later stages after infection were used as probes.

Madura and Rambon cattle

The antibody response detected by the ELISA at intervals after infection in Madura and Rambon cattle is shown in Figures 2 and 3, respectively. In Madura cattle, antibodies were first detected 2 weeks after infection in three of the eight inoculated animals and all cattle were antibody-positive 13 weeks after infection. Most cattle remained antibody-positive until 48 weeks (when the experiment was terminated) although there was a cyclic pattern to the mean antibody titre with successive peaks at approximately 12, 24 and 43 weeks with a marked decline in the mean antibody titres after 43 weeks. In Rambon cattle, the antibody response detected at intervals after infection was similar to that observed in Madura cattle: antibodies were first detected in one animal 4 weeks after infection and seven of the eight cattle were antibody-positive 13 weeks after infection; antibodies were not detected in one animal. There appeared to be a cyclic pattern to the mean antibody response with peaks at 11, 25 and 38 weeks after infection.

Discussion

The antibody response in infected Bali cattle was delayed: antibodies were detected in a low percentage of cattle in the first 6 weeks after infection, but were not detected in most of the infected cattle until 11 weeks after infection and a maximal response was not detected until 23–33 weeks. The lag period after infection before an antibody response was detected in most of the cattle by the ELISA may be partly due to the fact that the ELISA detected only IgG. Although IgM may be produced in the period before IgG production, attempts to detect JDV-specific IgM in recovered cattle using a similar ELISA were unsuccessful (data not shown).

In Bali cattle, the levels of JDV-specific IgG induced after infection and detected by ELISA varied from animal to animal. One animal (CB777) did not produce detectable antibodies after infection. Some animals produced consistently high levels of antibody as the sera were consistently positive according to ELISA results. Others produced only low levels of antibodies inconsistently detected by ELISA. This variation was not due to variation in the ELISA procedure, as similar results were obtained by Western immunoblotting. The reason for the low levels of antibodies in some animals is unknown but it may be associated with apparent immunosuppression in infected animals. The inconsistent detection of antibodies in some animals indicated that while the ELISA would be of value in detecting evidence of JDV infection of Bali cattle on a herd basis, the procedure would be an unreliable method of confirming JDV infection in individual animals. This unreliability would be exacerbated by the delayed antibody response detected in most animals.

The delayed antibody response detected by ELISA also concurred with histological studies in infected cattle (Dharma et al. 1991). The inability to detect a significant antibody response until approximately 8 weeks after infection is probably associated with the absence of a significant follicular reaction

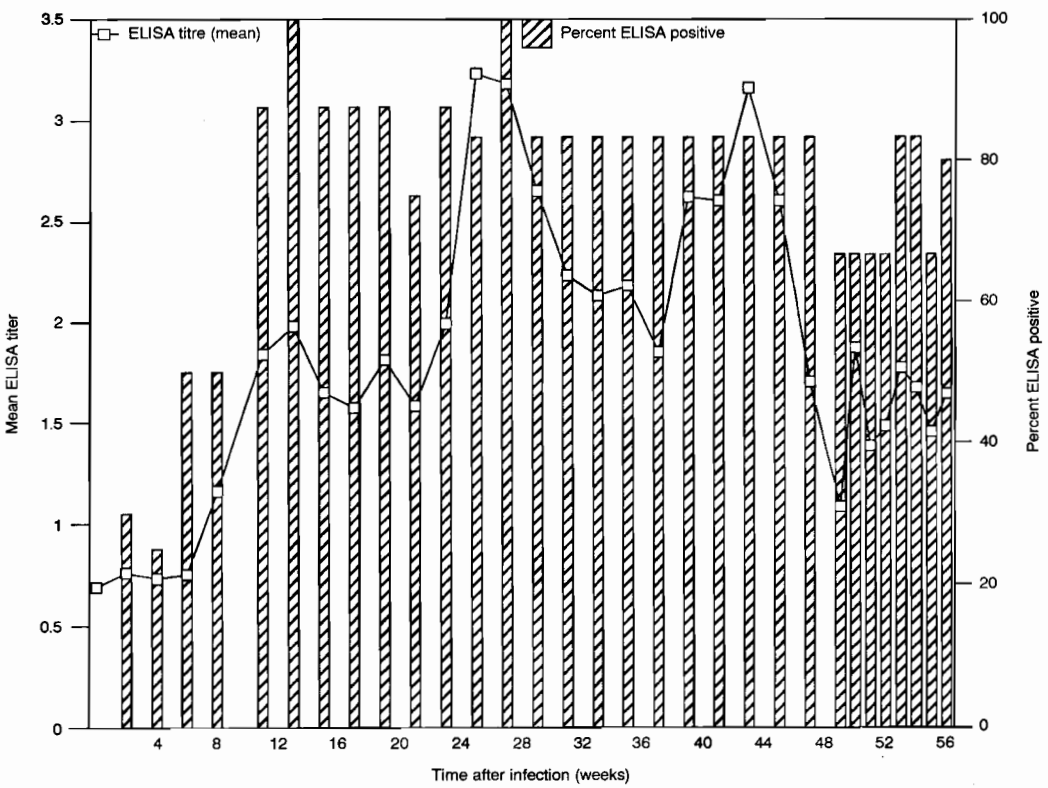

Figure 2. Sequential antibody response to JDV in Madura cattle. The ELISA titres are expressed as a ratio of the absorbance of the test sera and the mean absorbance of 1:640 dilutions of the reference positive (PM90) serum plus 3 standard deviations of the mean. A value of 1.0 or greater in an individual animal was interpreted as a positive result.

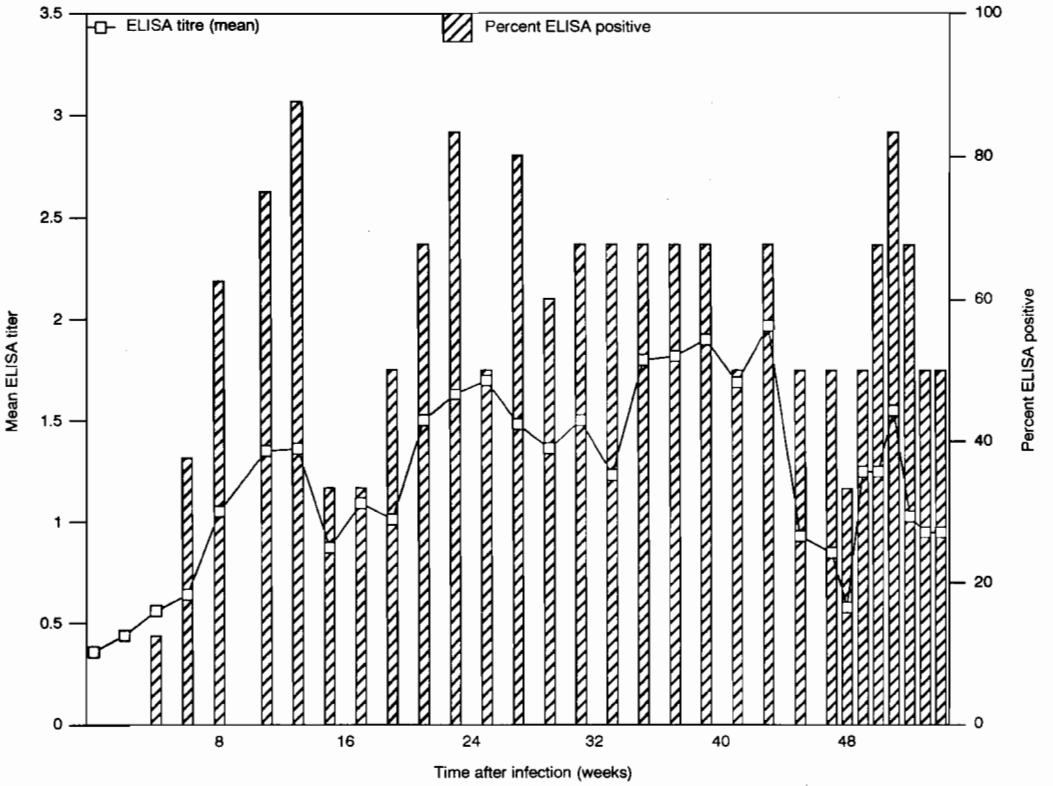

Figure 3. Sequential antibody response to Jembrana disease virus in Rambon cattle. A value of 1.0 or greater in an individual animal was interpreted as a positive result.

and the scarcity of plasma cells in lymph nodes or spleens during the clinical and early recovery stages of the disease.

The detection of antibodies by the ELISA in 15 of 16 crossbred Bali cattle (Madura and Rambon) cattle clearly indicated that Jembrana disease virus can infect crossbred Bali cattle, even though crossbred Bali cattle developed only some clinical signs and pathological changes normally seen in Jembrana disease in Bali cattle. In these cattle and in other large ruminants such as Friesian cattle, Ongole cattle, and buffaloes, which can also be infected (Soeharsono et al. 1990), the clinical signs that occurred would be difficult to detect under field conditions.

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Investigation of the Cell-Mediated Immune Response to Jembrana Disease Virus Proteins in Cattle

S. Wareing

Abstract

The cell-mediated immune response to Jembrana disease virus proteins in cattle could be investigated in Indonesia. Lymphocyte proliferation could be assessed using the MTT assay with the production of cytokines interleukin-2, interleukin-4 and interferon-gamma being assessed using the reverse-transcriptase polymerase chain reaction.

JEMBRANA disease virus (JDV) is a lentivirus that causes an acute, severe disease in Bali cattle (*Bos javanicus*) (Chadwick et al. 1995). Infection with JDV results in a case fatality rate of approximately 20% with the major clinical signs being fever, lethargy, anorexia and enlargement of the superficial lymph nodes (Soesanto et al. 1990).

Tests have shown that the antibody response to Jembrana disease is delayed. Antibodies are not detected in a majority of cattle until 11 weeks post infection, with the maximum antibody response occurring between 23 and 33 weeks post infection (Hartaningsih et al. 1994). It is hypothesised that the mechanism of recovery from Jembrana disease is cell mediated. In order to investigate the cell-mediated immune response to JDV, protocols are being developed to measure the cell-mediated immune response to JDV recombinant proteins in cattle. Once developed, these protocols will be directly applied to measuring the cell-mediated immune response to JDV.

Cell-mediated immunity (CMI) refers to an immune response that is primarily mediated by activated T lymphocytes and macrophages (Sites et al. 1994). CMI reactions typically result in lymphocyte proliferation and the release of numerous immunoregulatory cytokines. Numerous assays have been developed to assess CMI reactions. Many, however, are beset by difficulties such as biological variability, complexity, standardisation and expense (Sites et al. 1994). With these factors in mind, the author has chosen to assess the CMI response to JDV proteins by monitoring lymphocyte proliferation and cytokine production in conjunction with antibody production, a humoral response.

Lymphocyte proliferation assays assess the proliferation of previously in vivo sensitised lymphocytes that have been exposed to antigen in vitro. Peripheral blood mononuclear cells (PBMC) are separated from whole blood by density gradient centrifugation. The PBMC are exposed to antigen and cultured for 72 hours, at which time the proliferation is assessed.

Lymphocyte proliferation could be assessed using the MTT assay. The assay was developed by Mosmann (1983) as an alternative to the traditional radioactive nucleotide incorporation assays. Mitochondrial enzymes of viable cells convert the tetrazolium salt MTT to blue-coloured formazan (Denizot and Lang 1986). The amount of formazan produced is proportional to the number of cells. Hansen et al. (1989) modified the original method and yielded signals that were 100% higher than previously published results. The principal modification was changing the composition and pH of the extraction buffer to ensure all of the formazan produced was solubilised. The assay has several advantages in that it is rapid, does not require the use of radioisotopes (Mosmann 1983) or sophisticated equipment. To ensure the sensitivity of the assay, a standard titrated thymidine incorporation assay will be conducted in parallel during the study.

Cytokines regulate the way in which the immune system responds during an immune challenge (Ito and Kodama 1996). Identifying the cytokines that participate in an immune response enables examination of lymphocyte communication, leading to a better understanding of immunity and immunopathology (Covert and Splitter 1995). Traditionally, biological assays that use cytokine-dependent cell lines and

immunoassays have been used to measure cytokine production. Biological assays are both sensitive and quantitative, but lack specificity and only measure secreted cytokines and not those that are membrane bound (Hutchinson et al. 1994). Immunoassays are both specific and sensitive, but their availability for cytokine detection is limited. The reverse transcriptase polymerase chain reaction (RT-PCR) overcomes these problems in that it is highly specific, highly sensitive, can detect many cytokines simultaneously (Covert and Splitter 1995) and detects all transcribed cytokines (Hutchinson et al. 1994).

The RT-PCR procedure requires the isolation of PBMC and extraction of cellular RNA. Cytokine mRNA is reverse transcribed to cDNA and amplified using specifically designed primers and the polymerase chain reaction (PCR). Amplified products are electrophoresed in an agarose gel and analysed.

Three cytokines that could be chosen for analysis are interleukin-2 (IL-2), interleukin-4 (IL-4) and interferon-gamma (IFN-γ). IL-2 synthesis and secretion is primarily triggered by antigen-induced activation of mature T lymphocytes (Nicola 1994). The binding of IL-2 to its receptor mainly induces the proliferation of activated T lymphocytes, although it has also been shown to induce proliferation and cytokine expression in B lymphocytes and natural killer (NK) cells (Nicola 1994). IL-2 also suppresses cells of the Th2 subset (Mosmann and Sad 1996). IL-4 is mainly produced by activated T lymphocytes with basophils and mast cells being a minor source. IL-4 has been shown to have numerous properties in vitro including its ability to promote immunoglobulin class switching (IgE and IgG1) (Roitt 1994), induction of lymphocyte proliferation, induction of cytotoxic T cell differentiation (Nicola 1994) and the suppression of Th1 cells (Mosmann and Sad 1996). IFN-γ is produced by T lymphocytes and NK cells (Roitt 1994) and was originally identified by its anti-viral activity (Nicola 1994). IFN-γ activates macrophages to become tumorcidal and kill intracellular parasites, induces the expression of class 1 and class 2 MHC molecules and enhances NK cell activity.

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The Development and Use of a Vaccine for the Control of Jembrana Disease

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Abstract

Results obtained in a series of vaccination experiments indicate it is possible to induce a partial protective immune response by vaccination with whole virus vaccines prepared from tissues of affected cattle. Optimal procedures developed were the inactivation of the virus in tissues with Triton X-100, the use of a mineral oil adjuvant already used for the commercial production of a haemorrhagic septicaemia vaccine in Indonesia, and vaccination three times at monthly intervals. The use of spleen tissue rather than plasma as a source of viral antigens would reduce the cost of preparation of the vaccine to enable the production of about 3000 vaccine doses per donor animal. Three injections of the vaccine did not prevent the development of clinical signs in the vaccinated cattle when they were challenged with 100 ID_{50} of infectious JDV one month after the last vaccine doses, but it markedly reduced the severity of the clinical signs that did develop and it is likely that if the developed procedure was used to vaccinate cattle under field conditions it would result in the development of a reasonable level of herd immunity and prevent high mortality rates in cattle in naive areas.

THE evidence that cattle develop a protective immunity after recovery from Jembrana disease (Soeharsono et al. 1990; Hartaningsih et al. 1993) suggested that it may be possible to experimentally induce a protective immunity in Bali cattle by vaccination with the appropriate viral antigens. In this paper, a series of experiments is reported which indicate that it was possible to immunise Bali cattle with whole virus antigens and tissue preparations and to protect them from challenge with virulent JDV.

Methods and Results

A series of experiments has been conducted to determine if it is possible to immunise cattle and prevent the development of Jembrana disease.

Formaldehyde inactivated vaccine

In this experiment, an attempt was made to induce a protective immune response in three cattle by immunisation with a formaldehyde-inactivated virus preparation emulsified with Freund's incomplete adjuvant. Formaldehyde-inactivated plasma-derived vaccines, containing different concentrations of plasma were emulsified with incomplete Freund's adjuvant (IFA) and administered to cattle on 1, 2 or 3 occasions at monthly intervals. The animals were then challenged with an estimated 1000 or 100 ID_{50} of JDV as detailed in Table 1. This attempt at inducing a protective immunity was unsuccesful and all the cattle were still susceptible to Jembrana disease when inoculated with approximately 1000 or 100 ID_{50} of virus one month after the final immunisation.

Detergent (Triton X-100) inactivated virus vaccines

An experiment was conducted to determine if Triton X-100 inactivated plasma-derived virus antigen (the equivalent of 30 mL of plasma per dose) emulsified in IFA and administered three times would induce a protective immunity against challenge with 100 ID_{50} of JDV one month after the last vaccine dose. The

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results indicated that three inoculations of Triton X-100 inactivated plasma-derived vaccine emulsified in IFA resulted in a partial protective immunity in the four immunised cattle when challenged with 100 ID_{50} of virulent JDV (Table 1). Protective immunity was indicated by a marked reduction in the duration of the febrile period, and a reduction in the duration of leukopenia. There was also a reduction in the severity of the histological lesions after challenge (data not shown).

Comparison of IFA and Quil-A as adjuvants

The relative efficacy of IFA as an adjuvant was compared to Quil-A. The antigens were plasma-derived: the antigen when used with IFA was pretreated with Triton X-100 but not when used with Quil-A. The overall result indicated that there was a reduction in the severity of the disease following vaccination three times with a Triton X-100 inactivated JDV vaccine emulsified in IFA and that this method of vaccination was more efficacious than vaccination using the same antigen preparation mixed with Quil-A (Table 1).

Comparison of IFA and MOA as adjuvant

The relative efficacy of the two adjuvants IFA and MOA were compared, using a similar Triton X-100 treated plasma-derived antigen and similar challenge procedures to those used in the above experiment comparing the efficacy of IFA and Quil-A. The results indicated that the use of plasma-derived antigen inactivated with Triton X-100 and emulsified with MOA or with IFA provided a partial protective immunity (a non-significant reduction in the duration of the febrile period, a significant reduction in the duration of the leukopenia, an intermittent leukopenia, and a reduction in the severity of the histological lesions) following challenge of the vaccinated cattle with 100 ID_{50} of virulent virus. There was a significantly greater efficacy using MOA rather than IFA. The use of three rather than two injections of vaccine resulted in a slight (but not significant) reduction in the duration of the febrile period and duration of leukopenia.

Comparative efficacy of vaccination with plasma-derived antigen and a spleen-derived antigen

Experiment 1

The comparative efficacy of immunisation three times with three different doses of the plasma-derived antigen (the equivalent of 30, 5 and 1 mL of the original plasma), and 1 mL of a 10% spleen suspension was examined. All preparations were inactivated with Triton X-100 and emulsified in MOA, and inoculated three times at one month intervals. Vaccinated cattle and controls were challenged with 100 ID_{50} of infectious virus one month after the last vaccine dose. The results indicated that vaccination three times at monthly intervals with 1 mL of a 10% spleen suspension induced a similar degree of protective immunity to that produced by the equivalent of 30 mL of plasma when the vaccinated cattle were challenged one month after the last vaccine dose. The levels of JDV-specific antibody induced by the two procedures were similar. The protection was not complete but was indicated by a decrease in the duration of the acute febrile reaction associated with Jembrana disease, and a decrease in severity of the pathological lesions compared to those normally detected during Jembrana disease.

Experiment 2

To confirm the similar efficacy of the spleen-derived antigen demonstrated in Table 1, a similar experiment was conducted. Animals were vaccinated with either plasma-derived antigen or spleen-derived antigen emulsified in MOA and administered three times at monthly intervals. All animals, including non-vaccinated controls were challenged with an estimated 100 ID_{50} of JDV one month after the last vaccine dose. As shown in Table 1, all 5 control cattle developed typical clinical signs of Jembrana disease with a febrile period of 5 to 7 days duration and a concurrent leukopenia also of 5 to 7 days duration. In cattle vaccinated three times with a plasma-derived vaccine, all five cattle developed clinical signs of Jembrana disease but the mean duration of the febrile and leukopenic periods was reduced by about 1.5 days in comparison to the control cattle. In cattle vaccinated three times with the spleen-derived antigen, only 2 of 5 cattle developed clinical signs of Jembrana disease and the mean duration of the febrile and leukopenic periods in the five cattle was reduced by 4.8 days and 5.4 days, respectively.

Immune response to JDV antigens

No antibodies were detected in the sera of any cattle before they were immunised or infected with JDV. However, several vaccination procedures resulted in the development of JDV-specific antibody which was detected by ELISA. Triton X-100 inactivation provided a better antibody response than formaldehyde-inactivation when plasma-derived antigens were used as vaccines. IFA and MOA in combination with Triton X-100 inactivation of plasma-derived antigens induced a similar antibody

Table 1. The result of the sequential series of experiments that were conducted using tissue preparations from JDV-infected animals as immunogens and a variety of methods of inactivation of the virus, different adjuvants, different dose rate, and different dose of challenge.

Type of antigen	Equivalent plasma volume (mL) per dose	Method of inactivation of virus	Adjuvant	Vaccine doses	Challenge dose (ID_{50})	Degree of protection			Comment on efficacy
						Fever > 2 days	Leucopenia > 2 days	Mortality	
Plasma	10	Formaldehyde	IFA	1	1000	3/3	2/3	0/3	No protection
	10	Formaldehyde	IFA	2	1000	5/5	5/5	0/5	No protection
	100	Formaldehyde	IFA	3	100	5/5	3/5	2/5	No protection
		Control			100	2/3	2/3	1/3	
	30	Triton X-100	IFA	3	100	1/4	0/4	0/4	High efficacy
		Control			100	2/2	2/2	2/2	
	30	Triton X-100	IFA	3	100	0/6	0/6	0/6	High efficacy
	30	Quil A	Quil A	1	100	4/6	2/6	0/6	Poor efficacy
				2	100	2/5	2/5	0/5	Moderate efficacy
				3	100	3/5	3/5	0/5	Moderate efficacy
		Control			100	4/4	1/4	2/4	
	30	Triton X-100	IFA	2	100	5/5	4/5	0/5	No protection
				3	100	4/5	5/5	0/5	Poor efficacy
	30		MOA	2	100	5/5	5/5	0/5	No protection
				3	100	4/5	4/5	0/5	Poor efficacy
		Control			100	5/5	5/5	0/5	
	30	Triton X-100	MOA	3	100	4/5	3/5	0/5	Moderate efficacy
	5			3	100	4/5	3/5	0/5	Moderate efficacy
	1			3	100	3/4	3/4	0/4	Poor efficacy
Spleen	10% spleen	Triton X-100	MOA	3	100	3/5	2/5	0/5	High efficacy
		Control			100	5/5	5/5	0/5	
Plasma	30	Triton X-100	MOA	3	100	4/4	4/4	0/4	No protection
Spleen	10% spleen	Triton X-100	MOA	3	100	1/5	0/5	0/5	High efficacy
		Control			100	5/5	5/5	0/5	

response, which was greater than the response induced by the same antigen combined with Quil-A. Three doses of Triton X-100 inactivated plasma-derived antigen emulsified in MOA induced higher antibody titres than only two or one dose of the same vaccine. Vaccination with Triton X-100 inactivated plasma-derived antigen and spleen-derived antigens emulsified in MOA induced similar levels of JDV-specific antibody in the vaccinated cattle.

The major virion protein detected by Western immunoblotting using serum from vaccinated animals as probes was the 26K capsid protein, and this was detected soon after the first vaccination (data not shown).

Discussion

The results of the sequential series of experiments that were conducted using tissue preparations from JDV-infected animals as immunogens and a variety of methods of inactivation of the virus, different adjuvants, and different dose rates, indicated that no vaccination procedure completely prevented clinical signs of Jembrana disease in all cattle but they resulted, to a variable extent, in a reduction in the severity of the disease that was induced by challenge with an estimated 100 ID_{50} of JDV. This was indicated by a reduction in several parameters used to assess the severity of the disease: the duration of the febrile period, a reduction in the period of leukopenia, the sometimes intermittent nature of the fever and leukopenia during the acute disease period and a reduction of the severity of the histological lesions compared to those that occurred in control (non-vaccinated) cattle (data not shown). Using these parameters, the formaldehyde-inactivated plasma-derived whole virus vaccine with IFA failed to provide any protection against an intravenous challenge dose of 100 ID_{50} of JDV. Three doses of a plasma-derived antigen inactivated with Triton X-100 emulsified in IFA did induce a protective immune response; this immunisation regime resulted in a reduction in severity of clinical disease. A similar vaccine incorporated in Quil-A, which should have simultaneously provided inactivation of the infectious virus and adjuvant activity, appeared to provide a slightly lower protective immune response than the Triton X-100-inactivated vaccine in IFA; the number of cattle that reacted to challenge was higher in the groups of cattle immunised with the Quil-A vaccine than in groups of cattle immunised with Triton X-100-inactivated vaccine. Subsequent comparison of the plasma-derived antigen using Triton X-100 as an inactivating agent with two adjuvants, IFA and a mineral oil adjuvant (MOA) used for the production of haemorrhagic septicaemia vaccine in Indonesia, indicated a similar or slightly higher efficacy of the MOA than IFA. When a 10% suspension of spleen tissue was used as an alternative to plasma as a source of virus for incorporation in the vaccine, the results indicated that this provided a higher efficacy than the plasma-derived antigen when the equivalent of 30 mL of plasma was used for each vaccine dose.

Although it will require verification, it is likely that the degree of protective immunity that was induced by plasma-derived or spleen-derived antigens emulsified in MOA and administered three times at monthly intervals would provide a sufficient degree of herd immunity to reduce the severity of the disease in naive cattle populations and reduce the case fatality rate associated with Jembrana disease.

The degree of efficacy was, however, difficult to quantify. To adequately quantify the efficacy, several readily measurable factors need to be considered. In previous studies (Soesanto et al. 1990; Soeharsono et al. 1990), the parameters used to indicate Jembrana disease were a concurrent febrile response and leukopenia of >2 days duration. In the current study, these two factors alone were probably insufficient to assess efficacy as with the small number of animals used in each vaccination group there was little significant variation in the response in vaccinated and control groups and most vaccinated animals would have been deemed to have developed Jembrana disease. It was evident, however, that although most vaccinated animals developed some clinical signs of Jembrana disease, there was a reduction in the severity of the disease that occurred following some vaccination regimes.

Numerous but variable clinical and haematological changes occurred in Jembrana disease in Bali cattle and could be considered as measurements of the efficacy of vaccination: the major clinical signs in experimentally-infected cattle were an elevated rectal body temperature persisting for a mean of 7 days (ranging from 5 to 12 days), lethargy, anorexia, enlargement of the superficial lymph nodes, and mild ocular and nasal discharge and diarrhoea with blood in the faeces, but not all of these changes occurred in all affected cattle. Major haematological changes included a leucopenia characterised by a lymphopenia, eosinopenia and slight neutropenia. The pathological changes were also complex, although Dharma et al. (1991) considered the major changes during the acute and immediate clinical phase were the proliferative lesions in the parafollicular areas of lymphoid tissues, and the infiltrative lesions in visceral organs but especially the kidney and lung.

It is apparent then, that simple parameters such as fever, leucopenia, and mortality are insufficient indices of efficacy, and the indices used must consider the variation in the nature of the lesions that

occur. The ultimate criterion of efficacy could be a reduction in the case fatality rate, but in experimentally-infected unvaccinated cattle, this was 17%, too low to enable a reduction in the case fatality rate to be used as an index of efficacy; of the 25 control cattle used in the current experiments, only 6 of 25 died within 3 weeks of challenge, and in some control groups no animals died after challenge with JDV. It was noted, however, that most of the immunisation procedures prevented mortality in the challenged cattle.

A challenge dose of JDV of about 100 ID_{50} was used in most of the experiments that were conducted. This was achieved by preparing a 10^{-6} dilution of blood from donor cattle on the second day of the febrile reaction after they were experimentally infected with JDV. Estimation of the ID_{50} contained in the challenge dose was based on the assumption that the titre of JDV in blood at this stage of the infection was usually 10^8 ID_{50}/mL. The challenge dose rate could, however, have varied in each experiment and it would be preferable if in future experiments a standard suspension of infectious JDV could be prepared, titrated and stored frozen in liquid nitrogen. The rationale for using a small challenge dose of 100 ID_{50} was based on reports that protection against some retrovirus-induced diseases, for example, simian immunodeficiency virus (SIV) and feline leukemia, can be induced and demonstrated only if a low dose of virus is used to challenge the immunised animals (Gardner 1990; Fukuyama et al. 1993). The estimated 100 ID_{50} used in the JDV vaccination experiments may also simulate what happens under field conditions: if it is assumed that the disease could be transmitted by mechanical transfer of blood from affected animals during the acute stage of the disease by biting flies such as tabanids, and that tabanids (*Tabanus fuscicostatus*) may carry about 0.006 µL of blood on their mouth parts (Montelaro and Issel 1990), this would contain 600 infectious virus particles (assuming a virus titre of 10^8 ID_{50} of virus/mL of blood), only a part of which could be expected to be transferred into the susceptible cattle. Mosquitoes or other biting insects may be less efficient in transmitting JDV between animals.

The appearance of JDV-specific antibodies were detected by ELISA often following the first vaccine dose but the titre of antibody determined by the ELISA further increased with each vaccine dose, and three vaccine doses induced not only a better protective immunity but also higher antibody titres than only two vaccine doses. These findings are consistent with the results obtained with other experimental lentivirus vaccines (Desrosiers et al. 1989; Murphey-Corb et al. 1989) where monkeys required repeated high doses of inactivated SIV before high level of antibodies against SIV were produced. The titre of JDV-specific IgG measured by the ELISA was also usually significantly correlated with the degree of protective immunity. The ELISA therefore probably does not measure antibody to other virion proteins that may be of greater importance in the induction of a protective immunity. Assay of the neutralising antibody response would be potentially valuable in assessing the immune status of vaccinated cattle.

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Epidemiological Observations of Jembrana Disease In Bali

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Abstract

A field study to determine the profile of antibodies to Jembrana disease virus (JDV) in naturally-infected Bali cattle was conducted in the Jembrana district where Jembrana disease (JD) is known to be endemic. Sera samples were taken monthly for 12 months, in two different farming systems, called traditional and intensive.

During the 12-month survey, monthly herd immunity on both farms varied from 15.3% to 71.1%, and during those periods no clinical cases of JD occurred either on traditional or intensive farms. The mean monthly herd immunity was found to be higher on an intensive farm (39%) than on traditional farms (25%), indicating that the mode of transmission of JD in the two areas was different. Although there were no statistical differences ($P>0.05$), it seems that the prevalence of seropositive animals was influenced by farm size. As farm size increases, the prevalence of seropositive animals and the percentage of farms becoming infected are also likely to increase. It was concluded that under field conditions, for transmission of JD to occur, both healthy and carrier (infected) animals would have to be mixed close together.

With regard to season, it was found that the incidence of JD antibodies in previously seronegative animals occurred at the end of the dry season or at the beginning of the wet season. Three different features were found about the duration of JD antibodies in naturally-infected Bali cattle. One group of animals (21%) continuously showed JD antibodies over the 12 months of observations, another group (50%) was seropositive only once, and the last group (29%) showed fluctuating JD antibodies. It was also found that prevalence of JD antibodies was related to the age of the animals; when the animals became older, the number which were seropositive increased.

THIRTY years after the first epidemic, JD is regarded as endemic throughout the Island of Bali. Now, the disease occurs all year round, with sporadic outbreaks which seem to occur in some areas or villages at intervals of 4–5 years. This feature may be due to changes in the population of susceptible animals, either through a decline in naturally-acquired immunity or by an increase in the number of non-immune new-born calves.

It was demonstrated that recovered Bali cattle become immune, while at the same time remain carriers of JDV (Soeharsono et al. 1990). The role of this immune carrier animal in the occurrence of outbreaks in the field is not yet fully known. While relapse of recovered animals has been noted (Soeharsono 1988), one field veterinarian has indicated that animals with ELISA-detected antibodies were known to resist JDV (Dr Ismed Pane, pers. comm.). The data indicate that protective antibodies could develop in recovered animals under field conditions. Accordingly, the role of herd immunity in its relation to protecting the population from JD outbreak warrants further investigation. For haemorrhagic septicaemia, a proportion of herd immunity of about 60% could protect the cattle population from outbreak (Putra 1992).

The objective of the present study was to investigate the profile of JD antibodies in naturally-infected Bali cattle in two different farming systems (traditional and intensive) in the Jembrana district where JD is endemic.

Materials and Methods

Sampling sites

The survey was conducted in the Jembrana district of Bali. Two villages with different farming systems

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(called traditional and intensive) were selected. A traditional farm is defined as a smallholder farm with 1 to 5 Bali cattle, with a grazing area and/or feed through zero grazing, with the cattle generally used as draught animals for ploughing. For this criteria, Pangkungjajang village in the Melaya subdistrict was selected. In the intensive farm system, about 250 Bali cattle are reared in paddocks and have the same grazing land, and the animals are generally not used for ploughing. One such farm is located in Pulukan village, Pekutatan subdistrict, and is owned by the government as the centre for the Bali Cattle Development Project. Vaccination against haemorrhagic septicaemia in both farms was carried out every 6 months.

Serum samples

Serum samples were collected every month for 12 months, beginning in August 1992. The number of Bali cattle observed throughout the study was 132 on traditional farms and 164 on the intensive farm. The presence of JD antibodies was detected using an enzyme-linked immunosorbent assay (ELISA) as described by Hartaningsih et al. (1994).

Incidence rate

The incidence rate is defined as the number of new cases in the population at risk. New cases are the animals that previously were seronegative, then became seropositive. For accuracy of data analysis, only those animals that were bled more than 10 times were included in the analysis.

Results

Observations of clinical JD during the survey

During 12 months of observations, no clinical cases of JD were found in either study group.

Herd immunity and its relation to the seasons

As shown in Table 1, the period of prevalence of JD antibodies (herd immunity) was higher in the intensive farm system than in the traditional farm system, during either the wet or dry seasons. In both farm systems, higher mean monthly herd immunity was found during the wet months compared to the dry months.

The incidence rates of JD antibodies in both systems varied from 2% to 43%, and occurred during the dry season and the beginning of the wet season (June to January) (Table 2).

Table 1. Period of prevalence of Jembrana disease antibodies in naturally-infected Bali cattle in traditional and intensive farming systems.

Month	Farm system			
	Intensive		Traditional	
	Number of samples	Prevalence (%)	Number of samples	Prevalence (%)
Wet season				
November	114	71.1	111	38.7
December	137	40.1	99	23.2
January	131	50.4	97	38.1
February	126	36.5	85	15.3
March	133	34.6	96	20.8
Mean ± SD		46.5 ± 15.0		27.2 ± 10.6
Dry season				
April	99	31.3	83	19.3
May	90	30.0	73	19.2
June	88	21.6	77	15.6
July	129	35.7	76	21.1
August	153	50.3	127	27.6
September	143	28.7	99	32.3
October	130	32.3	105	35.2
Mean ± SD		32.8 ± 8.8		24.3 ± 7.4
Mean ± SD		38.6 ± 13.2		25.5 ± 8.5
Range		21.6 – 71.1		15.3 – 38.7

SD = Standard deviation.

Herd size and prevalance of JD antibodies

Although there was no statistically significant difference ($X^2 = 5.95$, df = 4, $P>0.05$), there was a tendency that when the farm (herd) size increased, the prevalence of JD antibodies also increased (Table 3). Similarly, the number of infected farms was found to increase as the herd size increased; however, the difference was not statistically significant ($X^2 = 9.36$, df = 4, $P > 0.05$).

The duration of JD antibodies in naturally-infected Bali cattle

Of 296 Bali cattle bled during the survey on both farms, 39 animals were bled continuously for 12 months. Of those 39 animals, 24 became seropositive on one occasion or more, while the other 15 were seronegative (Table 4). The duration of JD antibodies in 24 naturally-infected Bali cattle varied, with at least three different groups observed. The first group (5 out of the 24 or 20.8%) harboured JD antibodies continuously for the 12 months of

Table 2. The incidence rate of Jembrana disease antibodies during 12 months of observations in both traditional and intensive farming systems in the Jembrana district, Bali.

Month	Number of susceptible animals*	Number of new cases	Incidence rates (%)	Number of susceptible animals	Number of new cases	Incidence rates (%)
		Traditional farm			Intensive farm	
August	65	17	26.1	93	40	43.0
September	48	5	10.4	53	5	9.4
October	43	0	0	48	1	2.1
November	43	3	6.9	47	17	36.2
December	40	0	0	30	2	6.7
January	40	9	22.5	28	4	14.3
February	31	0	0	24	0	0
March	31	0	0	24	0	0
April	31	0	0	24	0	0
May	31	0	0	24	0	0
June	31	0	0	24	2	8.3
July	31	0	0	22	0	0
Total	65	34	52.3	93	69	74.2

*Susceptible animals are Bali cattle that carry no JD antibodies.

Table 3. The period of prevalence of Jembrana disease antibodies in naturally-infected Bali cattle in traditional and intensive farming systems using enzyme-linked immunosorbent assay. Data are described based on the herd size.

Farm size (head)	Number of Bali cattle observed	Number of seropositive (%)	Number of cattle infected					Number of farms observed	Number of farms infected	Infection rates per farm (%)
			1	2	3	4	5			
1	11	3 (27.3)	3	—	—	—	—	11	3	27.3
2	58	29 (50.0)	9	10	—	—	—	29	19	65.5
3	33	21 (63.6)	—	3	5	—	—	11	8	72.7
4	20	12 (60.0)	—	1	2	1	—	5	4	80.0
5	10	7 (70.0)	—	1	—	—	1	2	2	100.0

sampling. The second group (12 out of the 24 or 50%) and the third group (7 out of the 24 or 29.2%) showed fluctuating antibodies.

Jembrana disease antibodies for different age groups

On both farms, the prevalence of JD antibodies was found to increase significantly as the animals became older ($X^2 = 32.1$, df = 4, $P<0.05$, Table 5).

Maternal antibodies

Of 12 seropositive cows, 8 of their calves (66.7%) aged 2–7 months, also carried JD antibodies (Table 6). Eight of 11 calves (72.7%) aged 3 months or less also carried JD antibodies, the majority of them born of seropositive cows. Four calves were found to be without JD antibodies, even though they were born of seropositive cows. No calves born of seronegative cows carried JD antibodies.

Table 4. The duration of Jembrana disease antibodies in naturally-infected Bali cattle during 12 months of observations on two farms.

Animal code	JD antibody titre*											
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
						Traditional farm						
63	—	1.1	—	—	—	—	—	—	—	—	—	—
57	—	—	—	—	—	1.4	—	—	—	—	—	—
58	—	—	—	—	—	1.4	—	—	—	—	—	—
2	—	—	—	—	—	1.4	—	—	—	—	—	—
1	—	—	—	—	—	1.8	—	—	—	—	—	—
61	3.3	13.2	6.1	1.0	2.8	3.9	—	3.6	2.7	2.2	2.9	3.1
94	2.6	—	1.3	2.2	1.5	—	—	1.6	—	—	—	—
62	—	2.8	—	1.0	—	1.2	—	—	—	—	—	—
124	10.2	11.2	12.7	15.7	3.3	4.5	3.0	4.2	3.2	3.6	3.1	2.8
117	9.7	12.7	14.5	17.5	3.6	2.2	3.8	4.3	4.0	2.6	2.7	2.8
123	5.0	6.8	7.4	10.3	1.8	2.4	1.4	2.7	2.8	2.0	1.9	1.9
118	8.7	8.8	10.1	11.0	2.5	3.6	2.3	2.8	3.0	2.6	2.2	2.5
						Intensive farm						
826	—	—	—	1.3	—	—	—	—	—	—	—	—
823	—	—	—	2.3	—	—	—	—	—	—	—	—
817	—	—	—	—	—	1.5	—	—	—	—	—	—
763	2.3	—	—	—	—	—	—	—	—	—	—	—
731	—	—	—	—	—	—	—	—	—	—	1.1	—
811	—	—	—	2.6	—	—	—	—	—	—	—	—
813	—	—	—	2.0	—	—	—	—	—	—	—	—
732	2.4	—	—	2.0	—	—	—	—	—	—	—	—
805	—	1.3	—	1.1	—	—	—	—	—	—	—	—
746	—	—	—	2.4	—	1.8	—	—	—	—	1.2	—
814	5.1	—	1.2	9.5	1.7	2.3	1.4	1.6	2.3	2.1	2.8	2.4
785	5.9	1.6	1.1	10.9	3.6	3.1	1.5	2.7	2.9	2.5	2.3	3.1

*Antibody titre is expressed as the ratio of mean absorbent of tested sera to the mean of control positive sera that was diluted 640 times. The ratio of ≥1.0 was classified as positive, while <1.0 is negative (—).

Table 5. The prevalence of Jembrana disease antibodies in naturally-infected Bali cattle in both traditional and intensive farming systems using enzyme-linked immunosorbent assay. Data are described based on different age groups.

Age group (months)	Farming system						Total samples	Total prevalence (%)
	Intensive			Traditional				
	No. of samples	No. of seropositive animals	Prevalence (%)	No. of seropositive animals	No. of samples	Prevalence (%)		
1–6	12	11	91.6	9	30	30.0	42	47.6
7–9	3	2	66.6	5	9	55.0	12	58.3
12–18	71	43	57.7	15	32	47.0	103	56.3
24–36	51	44	86.3	36	52	69.0	103	77.7
>48	27	27	100.0	7	9	78.0	36	94.4
Total	164	127	76.2	72	132	54.5	296	66.5

Table 6. Relationship between the presence of JD antibodies in pregnant cows and JD antibodies in their calves.

Pregnant cows code	Antibody titre of the pregnant cow	Calf code	Calf age (months)	Antibody titre of the calf
Intensive farm				
78589	3.1	762.92	7	2.1
77289	5.2	763.92	6	2.3
85485	3.4	764.92	3	2.8
89389	4.2	765.92	3	—
85285	5.7	767.92	3	5.3
86186	4.0	771.92	3	3.3
72587	5.1	775.92	3	2.8
75188	2.2	776.92	3	4.7
81083	6.9	772.92	2	5.2
84085	dna	766.92	3	4.1
87685	dna	768.92	2	—
85785	dna	773.92	2	5.9
74288	—	761.92	4	—
Traditional farm				
85	—	85a	10 days	—
1	—	1a	15 days	—
79	—	79a	19 days	—
3	—	3a	25 days	—
5	—	5a	25 days	—
72	—	72a	1	—
123	2.7	123a	1	—
67	—	67a	1	—
69	—	69a	1	—
82	—	82a	4	—
74	2.6	74a	5	—
54	1.2	54a	6	—
55	—	55a	10	—

dna = data not available. — = negative. The antibody titre was the absorbance of the ELISA conducted as described by Hartaningsih et al. (1994).

Discussion

The interesting feature of JD is the occurrence of immune carrier animals. After experimental infection, antibodies to JDV were observed for up to 2 years, the animals resisted challenge, while the blood of the animal remained infectious (Soeharsono et al. 1990). Accordingly, the presence of JD antibodies in Bali cattle in this survey indicated that these animals had been infected by JDV under natural conditions, and were thus immune carrier cattle. As shown in these observations, with the proportion of herd immunity at about 30% throughout the year, no clinical cases of JD were observed. In contrast, the incidence rates of JD antibodies on both farms were very high, indicating that infection did occur, perhaps with mild clinical signs (subclinical). Therefore, the titre of JDV in the blood may influence the severity or pathogenesis of the disease. Putra (1993) has shown that when 10 mouth parts of *Tabanus* were contaminated with JDV, the suspension made by washing those contaminated mouth parts was not able to induce disease in susceptible Bali cattle, but could induce the development of antibodies. By increasing the number of *Tabanus* used to 15, clinical cases of JD were observed.

The antibody profile observed in the traditional and intensive farming systems showed strong evidence that there is the need for a close association between carrier (infected) and susceptible animals. Since the titres of JDV are very high in the blood of infected Bali cattle during fever (remaining infectious at 10^{-8} dilution) (Soeharsono et al. 1990), it can be speculated that blood sucking insects could transmit the disease mechanically in the field.

Recently, it was also found that a suspension, made by washing the mouth parts of 200 mosquitoes contaminated with JDV, was able to induce clinical cases of JD (Putra et al. 1995), and the mosquitoes were probably able to transmit the disease under field conditions (Putra and Sulistyana 1995). The fact that herd immunity was higher during the wet

season than the dry season coincides with the increasing population of insects, thus increasing mechanical transmission.

It can be concluded that the involvement of blood sucking insects in spreading JDV in the field cannot be disregarded. The limited spread of JD to adjacent areas, for example from Bali Island to Nusa Penida Island, and quick spread at the village level (Putra et al. 1983) would also support the notion that blood sucking insects are involved. When the disease was first reported, it took about 8 months to spread throughout Bali Island (Adiwinata 1967).

The role of mechanical transmission through blood sucking insects will be limited by: the times (distance) as JDV was found to be unstable outside the host (Wilcox et al. 1992; Kertayadnya et al. 1993); titre of JDV in the blood; and the flight range of the insects. Patterns of this type of disease transmission have been shown for equine infectious anaemia which is also due to a retrovirus (Hawkins et al. 1976).

Acknowledgments

The authors wish to thank: the Director of Animal Health (Jakarta) for providing funds for this study; the staff of Dinas Peternakan, BPPH Denpasar and P3 Bali who were involved during the survey; Mr Ketut Mayun and Mr Tjokorda Kresnaananda for their technical assistance, especially in examining the sera.

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Jembrana Disease in Lampung

H. Prabowo[1]

Abstract

A disease of Bali cattle broke out in Central Lampung of Lampung Province, Southern Sumatra for the first time in Rama Dewa village in May 1976. By field and laboratory examinations, the disease could not be differentiated from Jembrana disease in Bali. Based on clinical signs, macroscopic and histopathological changes in the affected animals and ELISA results using sera of Bali cattle collected from several subdistricts of Lampung until the end of 1995, the disease has spread out to eight subdistricts of Central Lampung and three subdistricts of South Lampung. In 1994, a similar disease occurred in Bengkulu and South Sumatra Provinces.

THE Province of Lampung lies between 103° 50′ and 103° 40′ east and between 3° 45′ and 6° 45′ south, with a total area of more than 35376 sq km. Lampung has a tropical climate with temperatures between 22 °C and 33 °C and an annual rainfall of 2000–3000 mm. For local government administration, the Province of Lampung is divided into five districts (Bandar Lampung, South Lampung, Central Lampung, North Lampung and West Lampung) and then further divided into subdistricts and villages.

The first outbreak of Jembrana disease (JD) was reported in the village of Rama Dewa, Seputih Raman subdistrict of Central Lampung in May 1976. At that time, the disease was called Seputih Raman disease (Soeharsono and Darmadi 1976).

Then an evaluation team from the Directorate-General of Livestock Services used the term Rama Dewa Disease, instead of an unknown enzootic disease which had initially been called Seputih Raman disease (Anon. 1977). However, results from field and laboratory diagnoses indicated that the disease could not be differentiated from JD which had appeared in Bali. During the first outbreak until the end of 1976, the number of reported cases was 1001 head of cattle of which 885 died in four subdistricts.

In 1987, a second outbreak occurred for the first time in Rama Gunawan village of Seputih Raman subdistrict, spreading rapidly to six subdistricts. In the period from January to December 1987, the number of reported cases was 618 head of cattle of which 319 died (Prabowo et al. 1987).

Only Bali cattle were affected with the disease. Ongole cattle and other animals including buffalo were mixed with affected animals, but did not show any symptoms.

The clinical and pathological symptoms of the disease were anorexia, fever, a mild ocular and nasal discharge, diarrhoea or diarrhoea with blood in the faeces, enlargement of the superficial lymph nodes, haemorrhages in various organs, and splenomegaly.

Field diagnoses of JD at the Disease Investigation Centre (DIC) Region III, Bandar, Lampung, are usually based on epidemiology, clinical signs and gross pathology. Laboratory confirmation is mainly based on histopathology. For serological examination, specimens were submitted to the Bali Cattle Disease Investigation Unit (BCDIU) Denpasar, Bali, and analysed by ELISA.

In 1994, a similar disease occurred in Bengkulu and South Sumatra Provinces.

Materials and Methods

This paper has been prepared based on data of JD outbreaks in 1976, 1987 and the situation of the disease until the end of 1995. The specimens used for histopathological examination were derived from fatal field cases. Tissues were fixed in buffered

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formalin and then embedded in paraffin and stained with haematoxylin-eosin.

Sera for serological tests were collected from several parts of Lampung Province and submitted to BCDIU, Denpasar.

Results

Distribution of Bali cattle in Lampung

Soeharsono and Darmadi (1976) said that a group of Balinese transmigrants took 29 head of Bali cattle from Bali to Lampung in 1959.

Mr Pan Rentog, a Balinese transmigrant, said that in 1962 he took 40 head of Bali cattle from Tabanan district, Bali, to Rama Gunawan village, Seputih Raman district of Central Lampung through Palembang harbour, South Sumatra. Another person took 12 head from the same area in 1963, and another took 40 head in 1964.

In 1974, a livestock trader was given an import licence by the Livestock Service of Lampung Province to import 300 head of Bali cattle from Nusa Penida Island, Bali, through Panjang Harbour of Lampung Province. Since then, there has been a rapid increase in the numbers of Bali cattle in most Lampung Province areas, distributed by the Small Holder Cattle Development Project (International Fund for Agricultural Development Project/World Bank Project), especially in transmigration areas.

Occurrence of the disease

The disease broke out for the first time in Rama Dewa village, subdistrict of Seputih Raman, Central Lampung in May 1976, and so has been called Rama Dewa disease. In the period from May 1976 to January 1977, the disease occurred in the four subdistricts of Seputih Raman, Seputih Banyak, Rumbia and Raman Utara and 885 out of 6098 head of Bali cattle died or were condemned. Only Bali cattle suffered from this disease. Morbidity rate, mortality rate and case fatality rate were 16.41%, 14.50% and 88.41%, respectively (Anon. 1977).

The second outbreak occurred in January 1987 for the first time in Rama Gunawan village, Seputih Raman subdistrict of Central Lampung. The disease spread rapidly among the cattle population in that village and then spread to neighbouring villages and other subdistricts. Until December 1987, six subdistricts were affected: Seputih Raman, Seputih Banyak, Rumbia, Raman Utara, Seputih Mataram and Seputih Surabaya.

Morbidity, mortality and case fatality rates were 3.25%, 1.67% and 51.61%, respectively (Prabowo et al. 1987). The population of Bali cattle in these subdistricts was about 19 000 head.

In October 1990, the same disease occurred in Sekampung subdistrict of Central Lampung. In the period from October to December 1990, 23 head of Bali cattle died or were condemned. In 1992 and 1993, the disease occurred also in Sidomulyo, Palas and Sukoharjo subdistricts of South Lampung.

Breed susceptibility

There have been no reports of clinical Jembrana disease in cattle other than Bali cattle. Both sexes of Bali cattle are susceptible to JD. The incidence of JD is highest in cattle aged 2 to 8 years.

Clinical findings

The disease is characterised by high fever (usually more than 41 °C), decrease of appetite or anorexia, depression, hypersalivation, ocular and nasal discharge, and erosion of the oral mucous membranes. Diarrhoea or bloody diarrhoea were observed frequently, as well as enlargement of the superficial lymph nodes, particularly the prescapular, prefemoral and parotid lymph nodes.

Macroscopic Pathology

Lymphoreticular system

Palpable lymph nodes particularly prescapular, prefemoral and paratid were consistently enlarged. The size varied from two to six times normal. Similar changes were observed also in the other lymph nodes. The cut surfaces usually were haemorrhaged, oedematous and the cortex and medulla could not be differentiated.

Spleens were swollen, enlarged to about six times normal, and congested or haemorrhagic. The cut surfaces were dark red and swollen. The pulp was friable.

Respiratory system

In upper respiratory organs, mucous membranes of nose, larynx and trachea were congested and petechial haemorrhages were scattered. The lungs usually showed patchy red or grey hepatisation, but in some specimens, the lungs were apparently normal.

Alimentary system

Erosions were observed in the tongue, pharynx and oesophagus. Erosions, petechial haemorrhages and oedema were observed in the mucosae of the abomasum, small intestine, large intestine and rectum, but sometimes severe haemorrhages showed in these organs. The liver was enlarged, yellowish brown in colour and foci of haemorrhages could be noted. The gall bladder was enlarged and contained

dark green bile fluid. Petechial haemorrhages were found in the mucosa of the gall bladder.

Circulatory system

The epicardium showed petechial or ecchymotic haemorrhages especially at the coronary grooves and over both ventricles. The endocardium showed petechial haemorrhages. Haemorrhages were observed also in the myocardium.

Urinary system

The kidney usually was slightly swollen, and a yellowish brown in colour. Foci of haemorrhages could be observed in the cortex. In some cases, infarctions were noted. The urinary bladder showed few petechial or ecchymotic haemorrhages in the mucosa.

Central nervous system

No macroscopic changes were detected in the brain, but in some cases the vessels in the meningi were congested.

Histopathology

The most common and prominent findings microscopically were proliferation of lymphoid cells throughout the body, although some variation in severity occurred according to the duration of infection.

Lymphoreticular system

The histological findings in lymph nodes and spleen were similar. Destruction and decrease of mature lymphocytes were noted in the follicular areas of the lymph nodes. Prominent findings were proliferation of lymphoid cells occupying paracortical areas. Lymphoblasts were the main components, and mitotic figures could be detected. Hyperplasia of recticular cells and increase of macrophages were constant findings. Plasma cells, neutrophils and eosinophils were also found although their numbers varied from case to case.

Characteristic changes of the spleen were nearly the same as those of the lymph nodes. Destruction and decreases in numbers of lymphocytes were observed in the follicles. Lymphoid cells proliferated principally in the spaces around the arteries of follicles and red pulp. Reticular cells showed hyperplasia.

Respiratory system

Upper respiratory organs showed degeneration and desquamation of epithelium, haemorrhage, oedema and diffuse proliferation of lymphoid cells. The lung showed interstitial pneumonia. Lymphoid cell proliferation was manifested diffusely in the alveolar septum and perivascular and peribronchial areas.

Alimentary system

Similar lesions were found in the oral mucosa and oesophagus. Microscopic examination indicated that coagulation necrosis had occurred, and diffuse lymphoid cell proliferation was found in lamina propria. Haemorrhage and oedema were often seen in erosive lesions.

Epithelial degeneration and erosion were observed in low alimentary organs such as the abomasum, and small and large intestines. Marked lymphoid cell proliferation, haemorrhage and oedema were found, and perivascular lymphoid cells were present.

Histological examination of the liver revealed characteristic changes in all cases. The lymphoid proliferation and increase of reticular cells were marked in Glisson's sheath, especially in periportal and pericholangeal tissues. In the lobules, infiltration of lymphoid cells, activation of endothelial cells, and some fatty changes of liver cells were observed.

In the epicardium of the heart, haemorrhage, infiltration and accumulation of lymphoid cells were common. In the myocardium, interstitial infiltration of lymphoid cells was observed.

In the kidney, proliferation or infiltration of lymphoid cells were present. The lymphoid cells were distributed in perivascular, periglomerular and interstitial tissues in the renal cortex.

Degeneration of transitional epithelium, haemorrhage, proliferation of lymphoid cells and lymphoid vasculitis of veins in the urinary bladder were observed.

In the brain, there was generalised hyperemia and perivascular haemorrhages, but in most cases only congestion was found.

Serological tests

Results of serological tests through ELISA examination indicated that antibodies were detected in serum samples of Bali cattle from several parts of Lampung Province. Antibody positive animals were detected in the Central Lampung district in Seputih Raman, Seputih Banyak, Rumbia, Raman Utara, Seputih Mataram and Seputih Surabaya subdistricts, and from South Lampung district in Sidomulyo, Palas and Kalirejo subdistricts.

A new outbreak of Jembrana disease, as diagnosed clinically, pathologically and serologically, occurred in July 1994 in Manna subdistrict, South Bengkulu district of Bengkulu Province, but antibody positive animals were also detected recently in

Seluma subdistrict (Anon. 1994). In June 1994, Jembrana disease occurred also in Buay Madang subdistricts of South Sumatra Province. Results of serological tests indicated that antibody positive animals were detected also in Belitang, Martapura and Mesuji subdistricts.

Discussion

The historical background of JD in Lampung is that it originally came with Bali cattle carriers sent from Bali to Lampung Province. Balinese transmigrants and the Livestock Service of Lampung Province introduced Bali cattle from Bali to Lampung (Soeharsono and Darmadi 1976; Prabowo and Soesilo 1987). These people brought the animals from Tabanan district in Bali. The clinical signs, macroscopic features, histopathological changes and results of serological examinations were similar in Lampung to those of JD that occurred in Bali Province.

The serological survey indicated that antibodies were detected in serum samples from cattle in five provinces of Indonesia: Bali, East Java, Lampung, West Sumatra and South Kalimantan. In Lampung, antibodies were detected only in Central Lampung and South Lampung (Soeharsono et al., these Proceedings). The detection of antibodies to Jembrana disease virus in areas where the disease occurs, and not in other areas of Indonesia, suggests that all of these diseases are closely related or the same (Hartaningsih et al. 1993).

From the field point of view it was recognised that three main factors are important in the transmission of Jembrana disease. Firstly, the farmer himself; secondly, the cattle trader, and thirdly, the vector. The farmers do not want to lose their animals to the disease, but if they own sick cattle, they try as soon as possible to sell the sick animals for cash, even if the price is low. The cattle trader tries help the farmers obtain the cash. The cattle trader sends the sick animals to other places, possibly infecting cattle in new areas.

As a result of field investigations of the Jembrana disease outbreak in the Manna subdistrict of Bengkulu Province in 1994, there is no doubt that Bali cattle from an infected area were introduced to that place.

A tropical country like Indonesia is characterised by high humidity, providing good conditions for a great number of ectoparasites. During the clinical stage of the disease, there is a high titred viraemia and contact infection between animals in intimate contact, and mechanical transmission by biting arthropods is likely during this phase. It was known that *Tabanus rubidus* is one potential vector for the disease (Putra 1993). The incidence of the disease at the outbreak was decreased after insecticide spraying was carried out, further suggesting that a vector played an important role in the transmission of the disease.

To inhibit the spread out of the disease, the Local Government closed the infected areas and the importation of Bali cattle from infected areas to other areas (free areas) was prohibited. In the district where the disease occurred, affected animals should be kept separate from the herd, housing should be clean and dry, and there should be adequate food. The use of broad spectrum antibiotics and control by insecticide sprays of the vector of the disease is recommended.

Since 1995, 1100 head of Bali cattle from four subdistricts of South Lampung have been vaccinated against Jembrana disease using the inactivated vaccine described by Hartaningsih et al. (these Proceedings).

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Jembrana Disease in West Sumatra

Tazril Tembok[1] and Erinaldi[1]

Abstract

Between 1985 and 1991, Bali cattle were imported to West Sumatra from East Nusa Tenggara, West Nusa Tenggara and South Sulawesi. Before 1985, the only cattle breeds in West Sumatra were Ongole, Friesian Holland, Simenthal crosses, Limousine crosses, and local cattle.

Jembrana disease appeared in West Sumatra in April 1992, attacking Bali cattle that had been imported from East Nusa Tenggara in March 1990. While Jembrana disease had not occurred previously in East Nusa Tenggara, it had been known in Sumatra island since 1976 in the Province of Lampung. There is no direct communication with or transportation between Lampung and the isolated Beringin Sakti and Muaro Takung villages (District of Sawahlunto Sijunjung) where Jembrana disease killed 133 (26.7%) Bali cattle and infected 353 (70.8%) of the other total Bali cattle population of 498.

Since 1993, under the guidance of the Bali Cattle Disease Investigation Unit (BCDIU), about 700 Bali cattle in West Sumatra have been vaccinated against Jembrana disease, using 3 mL doses three times a year. Serology tests carried out by BCDIU in 1995 on 324–379 Bali cattle showed antibody rates between 83.1% and 97.2%.

It is still difficult to determine the source of Jembrana disease in West Sumatra, but special attention must be given to the suspected carriers, ticks and blood-sucking flies, and to the re-use of unsterile needles. Although Jembrana disease is now under control in West Sumatra, further investigation and research is recommended for the whole of Indonesia, wherever Bali cattle have been distributed.

WEST Sumatra is only one of 27 provinces in the Republic of Indonesia. Situated in the western sector of Sumatra Island, it is divided into six municipalities, eight districts, 103 subdistricts and 2465 villages. About 4.2 million people live in a total area of 4.2 million hectares, of which about 50.2% is forest, about 30.1% is used for agriculture, and 19.3% for other uses. About 60%–70% of the people live in rural areas, earning their living from agriculture and livestock. They can be classified as subsistence and traditional farmers.

The livestock in West Sumatra includes beef cattle, buffalo, dairy cattle, horses, goats, sheep, pigs and poultry (chickens and ducks). Use of the livestock relates to the income, social welfare and social status of the people; livestock are a source of food, capital and easily converted savings, and of draught power. The total numbers of livestock in West Sumatra (1994–1995) are presented in Table 1.

Jembrana Disease

Supported by local and national funds, 500 head of Bali cattle were imported for the first time to West Sumatra from East Nusa Tenggara in December 1985, joining other breeds such as Ongole, Friesian, Simenthal crosses, Limousine crosses, and local cattle. Between 1987 and 1991, about 7700 Bali cattle were imported from East Nusa Tenggara, West Nusa Tenggara and South Sulawesi, supported by the International Fund for Agricultural Development (IFAD), and distributed to the Districts of Sawahlunto Sijunjung, Pesisir Selatan, Pasaman, Solok and District 50 Kota transmigration settlements.

The Jembrana disease outbreak occurred in West Sumatra for the first time in April 1992 at Beringin

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Table 1. Population of livestock in West Sumatra.

Livestock	1994	1995
Beef cattle	390094	410644
Dairy cattle	992	997
Buffalo	196178	204669
Goats	243194	260639
Sheep	1678	1733
Pigs	45223	46283
Horses	6072	6198
Native chicken	9521514	9921501
Broilers	807427	30863483
Layers	1620413	1642999
Ducks	1504403	1659552

Sakti village (Timpeh II) in the District of Sawahlunto Sijunjung. Five hundred and fifty head of Bali cattle had been distributed to this location in March 1990. When the outbreak occurred on April 7, 1992, there were 398 head of Bali cattle at Beringin Sakti. To September 16, 1992, Jembrana disease had killed 105 Bali cattle (26.38%) and infected 312 others (78.39%). On September 27, 1992, Jembrana disease appeared at Muaro Takung village (about 25 km from Beringin Sakti) and from a total population of 100 Bali cattle, 28 died (28%) and 41 were clinically affected (41%). Both areas are isolated and far from public activities or facilities, and about 200 km from the provincial centre (five hours by four-wheel-drive vehicle) or two hours from the district centre. Some data on the disease outbreak are presented in Tables 2 and 3.

The suspected areas of the outbreak are:

Area I: Timpeh I, Timpeh III, Timpeh Kampung and Air Amo, with a total population of 576 head of cattle;

Area II: The whole District of Sawahlunto Sijunjung, with a total population of 1658 head of cattle;

Area III: The Provinces of West Sumatra, Riau and Jambi, with a total population of 53 278 head of cattle.

Clinical Findings and Pathological Changes

Clinical findings included somnolence, loss of appetite, fever (39.5 °C to 41.5 °C), swelling of lymph glands, especially prescapulars and prefemorals, hypersalivation, exudant from noses and eyes, diarrhoea, haemorrhage, and sometimes, petechial haemorrhages.

Pathological changes included swelling of all lymph glands to three to five times normal size, splenomegaly (three to five times normal size), hepatomegaly with yellowish colour, enlargement and fullness of gall bladder, haemorrhage of the small intestine and colon, oedema of lungs, spotted haemorrhages of epicardium and endocardium.

Table 2. Morbidity, mortality and case fatality rates at Beringin Sakti.

Month	Population	Sick	Deaths	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
April	398	87	29	21.86	7.29	21.5
May	369	81	28	21.95	7.59	23.6
June	341	72	26	21.11	7.62	27.1
July	315	41	11	13.02	3.49	21.2
August	304	30	10	9.87	3.29	25.0
September	294	1	1	0.34	0.34	50.0
October	293	0	0	0.00	0.00	~
November	293	0	0	0.00	0.00	~
December	293	0	0	0.00	0.00	~

Table 3. Morbidity, mortality and case fatality rates at Muaro Takung.

Month	Population	Sick	Deaths	Morbidity rate (%)	Mortality rate (%)	Case fatality rate (%)
September	100	1	1	1.00	1.00	50.0
October	99	17	12	17.17	12.12	41.4
November	87	8	5	9.20	5.75	38.5
December	82	15	10	18.29	12.20	40.0

Epidemiology

It is difficult to determine the source of Jembrana disease in West Sumatra. While Jembrana disease had been unknown in Nusa Tenggara, the original source of Bali cattle, it had been known since 1976 in the Lampung Province of Sumatra Island. There is no communication or transportation between Lampung and the location of the outbreak, since Beringin Sakti and Muaro Takung are in an isolated area, practically surrounded by forests. However, sometimes people from neighbouring villages brought Ongole cattle or buffalo to use as draught animals at the two villages. Ticks and blood-sucking flies (Tabanidae, Haimatobia and Stomxys) are suspected of being vectors for the spread of the disease. Another possibility is the re-use of unsterile needles.

Controlling Jembrana Disease

Steps taken to control the disease included immediate field observation and monitoring of cases of infection as soon as the report of the outbreak was received on April 21, 1992. The Disease Investigation Centre (DIC) was notified and reports made to the Governor of West Sumatra, the Director General of the Livestock Service, and the head of the regional office of the Department of Agriculture in West Sumatra. Samples were sent to the Bali Cattle Disease Investigation Unit in Denpasar, Bali.

Coordination with the local and central government was increased, with the infected areas closed. Extension and guidance efforts were increased on matters such as sanitation, keeping stock areas clean to try to keep livestock healthy, stopping grazing to prevent cross-infection between animals, insecticide spraying of stables, disposal of dead animals, and a prohibition on the slaughter of sick animals.

Vaccination of about 700 head of Bali cattle began in Indonesia for the first time in the village of Beringin Sakti in February, 1993, under the guidance of the BCDIU, and continued three times a year at one month intervals, using 3 mL doses of Jembrana vaccine for intra-muscular injection as described by Hartaningsih et al. (these Proceedings). The vaccination program has continued up to 1995 and will be continued further.

Serology tests conducted by BCDIU produced good results, as shown in Table 4.

Table 4. Results of serology tests conducted by BCDIU.

Date of testing	Total number of sera	Result of serology test	(%)
First test (April 1995)	379	315/379	83.1
Second test (June 1995)	324	315/324	97.2
Third test (July 1995)	366	337/366	92.1
Fourth test (September 1995)	354	328/354	92.7

Conclusion

Although Jembrana disease is presently under control in West Sumatra, further investigation and research is recommended for the whole of Indonesia, wherever Bali cattle have been distributed.

Biology of the Bovine Immunodeficiency Virus

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Abstract

The bovine immunodeficiency virus (BIV) was the first bovine lentivirus (lenti meaning 'slow') to be isolated and characterised. Several genetically distinct strains of BIV have been isolated in the USA, and serologic analyses indicate that bovine lentivirus infections in cattle are worldwide. BIV molecular biology has been extensively studied. The BIV genome is 9 kb and contains the structural genes *gag*, *pol*, and *env*, flanked on the 5' and 3' termini by long terminal repeats. There are six putative nonstructural regulatory genes termed *rev*, *tat*, *vif*, *vpw*, *vpy*, and *tmx*. Rev and Tat are transactivators that function to upregulate viral gene expression; both are indispensable for virus replication. The remaining nonstructural regulatory gene functions are under investigation. The mature Gag products, p16, p26, and p7, are derived from proteolytic cleavage of the Gag precursor, Pr53. The mature Env proteins, gp100 and gp45, are derived from the post-translational processing of the Env glycoprotein precursor, gp145. In vitro, BIV replicates in a variety of primary and established canine, lapine, and bovine cells, and in vivo, the target cell appears to be monocytes/macrophages. BIV has a broader host range than most lentiviruses as it can infect sheep and rabbits, as well as cattle. Natural and experimental infections with BIV are nonacute, persistent, and induce an immunologic response. There is variable outcome in the clinical manifestations in BIV-infected cattle. Experimentally-infected, well-cared-for animals have a mild disease, including lymphocytosis, lymphadenopathy, and encephalitis. Signs of disease in natural infections are more prominent and extensive and include lymphoid tissue depletion and secondary diseases, which appear to be intensified by co-factors.

THE first bovine lentivirus, bovine immunodeficiency virus (BIV), was discovered in the late 1960s during the search for bovine leukemia virus (BLV), the etiological agent of enzootic leukemia/lymphosarcoma in cattle (Van Der Maaten et al. 1972). Little scientific interest was shown in this lentivirus until 1983, when the human immunodeficiency virus (HIV), the etiologic agent of the acquired immunodeficiency syndrome (AIDS), was discovered (reviewed in Gonda 1992; Gonda and Oberste 1992; Gonda 1994a,b; Gonda et al. 1994) and later shown to be a lentivirus (Gonda et al. 1985). It was demonstrated that BIV is a member of the lentivirus genus of retroviruses and is morphologically, genetically, and serologically related to HIV (Gonda et al. 1987); similarities in their biology became more evident as research on the two lentiviruses unfolded. Several names have been used to identify the first bovine lentivirus. Due to similarities in the biology of BIV and that of the primate immunodeficiency lentiviruses, it was determined by the International Committee for the Taxonomy of Viruses that the prototypic bovine lentivirus should be named bovine immunodeficiency virus or BIV (Murphy et al. 1995). Today, the lentivirus genus of retroviruses includes members from cattle, cats, goats, sheep, horses, nonhuman primates, and humans (Fig. 1). The purpose of this paper is to provide an overview of the biology of BIV.

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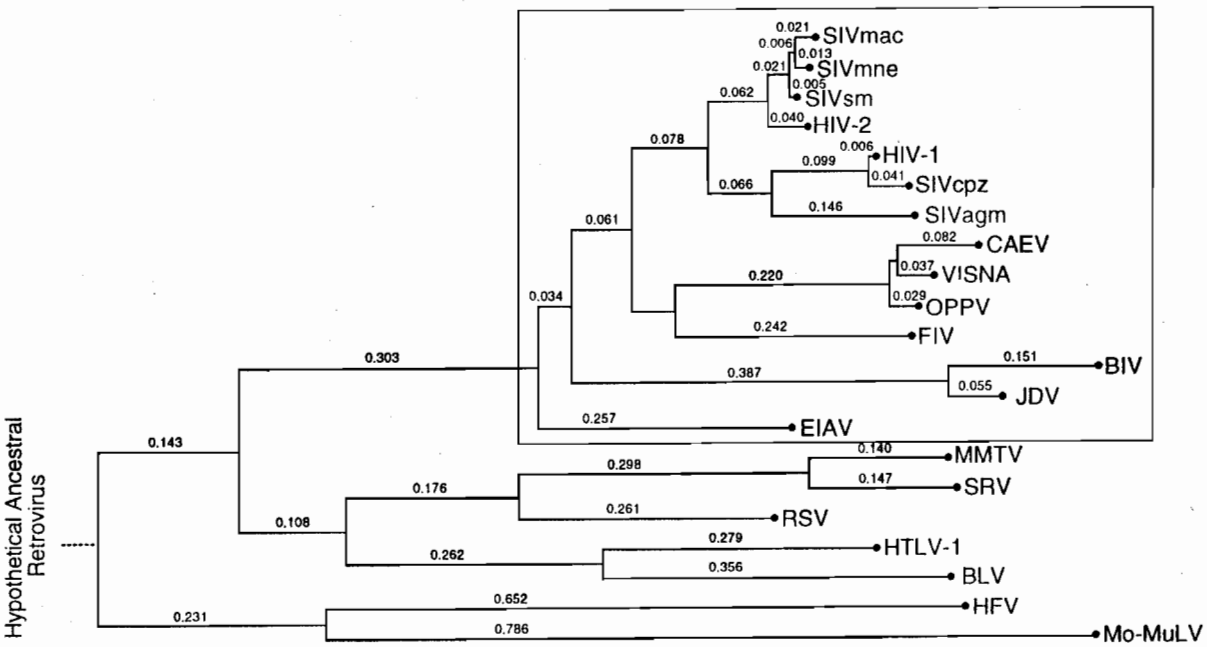


Historical Perspective

The prototypic bovine lentivirus strain was isolated from cow R-29 in Louisiana, USA (Van Der Maaten et al. 1972). To date, most of the reported research on BIV has been performed using BIV strain R-29, or derivatives thereof. More recently, the isolation and characterisation of several genetically distinct strains of bovine lentiviruses from USA and Bali cattle have been reported (Kertayadnya et al. 1993; Suarez et al. 1993). Of these, Jembrana disease virus (JDV) is the most genetically divergent bovine lentivirus described to date (74% nucleotide homology between BIV and JDV in a conserved region of *pol*) and has been shown to induce an acute disease in the domesticated banteng cattle species (*Bos javanicus*) indigenous to Bali (Soesanto et al. 1990; Chadwick et al. 1995). There are now numerous serologic reports from Europe, Central and South America, Canada, China, New Zealand, and others, using BIV R-29 reagents, indicating that infections of cattle with BIV and/or bovine lentivirus strains related to the prototypic BIV are worldwide. The strongest antibody reactions to BIV R-29 proteins come from animals located in or originating from the southernmost portions of the USA. Cattle sera from Maryland and other northern states had been analysed by enzyme-linked immunosorbent assays (ELISA) and demonstrated by radioimmunoprecipitation that positive ELISA results correlated only with cross-reactivity in the major immunodominant capsid protein, p26 (Gonda et al. 1994; unpublished data), suggesting that distinct bovine lentivirus serotypes exist, even within the USA, that are geographically isolated. Similar observations by Western blotting have been made for cross-reactivity between BIV and JDV (Kertayadnya et al. 1993). Thus, broad-based serological screening of cattle from geographically isolated locales, using ELISAs or Western blot assays based on prototypic BIV antigen, will at best yield a minimal estimate of the true penetration of bovine lentivirus infection and will not be discriminatory. This is due to the limited cross-reactivity between the immunodominant BIV p26 and these sera. It will be important to obtain isolates of these novel serotypes that will provide more relevant regional information on their serology to truly assess the extent of bovine lentivirus infection in herds and correlate disease patterns.

Molecular Biology

An understanding of the molecular biology of BIV was made possible by the molecular cloning of several complete, fully functional, integrated BIV proviral genomes from BIV-infected cells (Gonda et

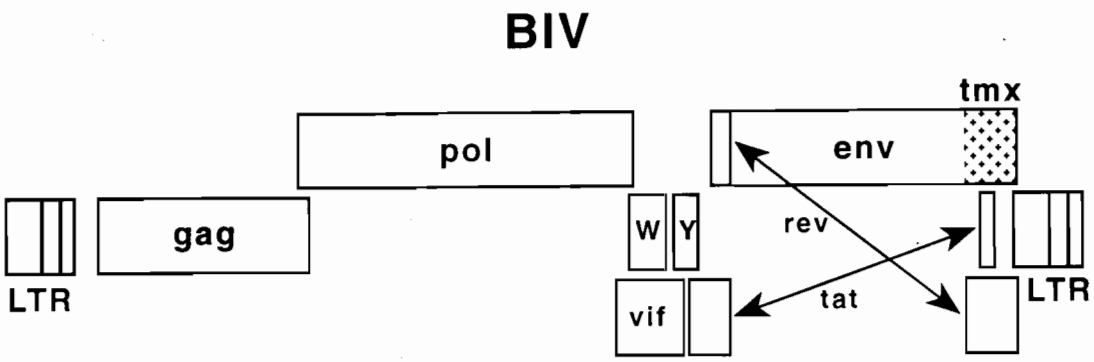
al. 1987; Braun et al. 1988). These proviral molecular clones (BIV106 and BIV127) have provided a vehicle for a number of important studies of the structure and function of BIV genes and served as a resource for isolating genes for the expression of recombinant proteins useful in antibody production and assay development to probe BIV infections. The BIV genome organisation (Fig. 2) was derived by DNA sequencing of these molecular clones (Garvey et al. 1990). BIV has the obligate retroviral structural genes *gag*, *pol*, and *env*, flanked on the 5' and 3' termini by the long terminal repeats (LTRs). The LTRs contain all of the necessary information for the initiation, enhancement, and termination of viral transcription (Pallansch et al. 1992; Carpenter et al. 1993; Fong et al. 1995). In addition, there are six putative nonstructural regulatory genes, *tat*, *rev*, *vif*, *vpw*, *vpy*, and *tmx*. Functional and immunological studies using the BIV proviral molecular clones and cDNAs have provided information on several of the nonstructural regulatory genes, as discussed below.

Tat and Rev nonstructural regulatory proteins

BIV Tat and Rev ($p14^{Tat}$ and $p23^{Rev}$, respectively) are transactivators of viral gene expression and are very similar in function to those of HIV. Both *tat* and *rev* mRNAs are derived by multiple splicing of the viral genomic RNA and consist of three exons. Differential splicing results in two forms each of *rev* and *tat* mRNAs. For *rev*, the significance of differentially spliced variants appears inconsequential, as similar Rev proteins are derived from their translation. However, with *tat*, differential splicing results in the translation of two distinct Tat proteins, which differ in the number and composition of their carboxyl-terminal amino acid residues and their transactivating activities. The products of *rev* and *tat* provide their essential functions by interacting with sequences found in the viral RNA. The RNA target for Tat is called the transactivation responsive (TAR) element, which has been localised to a 31-nucleotide RNA stem-loop structure in the 5' termini of the LTR R region sequences, and is present in all viral transcripts. Rev also works through an RNA target called the Rev responsive element (RRE), which has not been molecularly identified for BIV, but is believed to reside in the sequences encoded by the extracellular domain of the transmembrane protein. These sequences are found in all unspliced and singly spliced viral RNA transcripts, with the exception of BIV *tmx*, as in other lentiviruses. Both Tat and Rev are phosphorylated and have a nuclear localisation; however, while Rev is found both in the nucleus and nucleolus, Tat is found almost exclusively in the nucleolus in the infected cell. Tat and Rev are indispensable for replication and are key elements in upregulating virus expression (Liu et al. 1992; Oberste et al. 1991, 1993; Oberste and Gonda 1992; Carpenter et al. 1993; Chen and Frankel 1994; Fong et al. 1995; unpublished data).

Other nonstructural regulatory proteins

Vif ($p26^{Vif}$) has been identified immunologically, but cDNAs of its putative transcript have not been reported. BIV Vif shares common amino acid sequence motifs with Vifs of other lentiviruses. Vif-coding sequences for all lentiviruses also reside in a similar location in the genome. A transcript predicted to encode *vif* has been identified by Northern blotting. Vif is not phosphorylated, has a cytoplasmic localisation, and is also found in the virion. Preliminary studies using BIV proviruses with a truncated *vif* have suggested that BIV Vif, while not essential for replication, appears to enhance virus infectivity of the virus. The *tmx* mRNA is singly spliced, and its product, Tmx ($p19^{Tmx}$), has been identified immunologically. Tmx does not appear to



be phosphorylated, has a cytoplasmic localisation, and is also found in the virion. The function of Tmx and the remaining nonstructural regulatory proteins of BIV in the virus life cycle are currently under investigation (Oberste et al. 1991; Oberste and Gonda 1992; Gonda 1992; 1994a,b; Tobin et al. unpublished data).

Gag, Pol, and Env structural proteins

The BIV *gag* gene is translated from the viral RNA as a large precursor, Pr53. Gag proteins are also translated with those of Pol by a -1 frameshifting mechanism, which permits Gag and Pol proteins to be simultaneously expressed from the same message as a 170-kDa Gag-Pol polyprotein. The majority of the mature matrix (MA), capsid (CA), and nucleocapsid (NC) Gag proteins found in the virion are from cleavage of Pr53 by the viral protease and a minority from Pr170. A number of immunologic and physical methods have been used to precisely identify and map the gene order and major cleavage products of the BIV Gag precursor. The identity and order of proteins specified by the BIV *gag* are: NH-p16^{MA}-p2L-p26^{CA}-p3-p7^{NC}-p2-COOH. The functions of the smaller peptides (p2L, p3, and p2) are unknown. The Pol products of BIV have not been identified. The *env* open reading frame of BIV is very complex. Beginning at the 5' ATG, it specifies the first coding exon of Rev and is followed by a signal peptide and the Env precursor, which also includes a form of Tmx. The mature surface and transmembrane Env proteins, gp100 and gp45, respectively, are derived from the post-translational cleavage of the Env glycoprotein. During post-translational processing by a cellular protease the signal peptide is removed from the Env precursor, which simultaneously removes the preceding Rev amino acids. At the amino acid level, the BIV *env* gene shows the greatest variability with as much as 50% sequence divergence between U.S. isolates for the surface glycoprotein (Garvey et al. 1990; Rasmussen et al. 1990; Battles et al. 1992; Rasmussen et al. 1992; Tobin et al. 1994; Suarez and Whetstone 1995; unpublished observations).

Recombinant BIV proteins

A number of studies have utilised the information derived from the BIV DNA sequence (Garvey et al. 1990) to express segments of *gag* and *env* as recombinant antigens in various prokaryotic and eukaryotic systems. These recombinant proteins are useful in serological diagnosis of BIV infections (Rasmussen et al. 1990; 1992; Atkinson et al. 1992; Chen et al. 1994). Information obtained from the DNA and amino acid sequencing studies of BIV (Garvey et al. 1990; Tobin et al. 1994) has been used to express precisely the MA, CA, and NC and Pr53 proteins. These recombinant proteins were used to generate well defined polyclonal and monoclonal antibodies for immunohistochemical analyses and p26^{CA} antigen capture ELISAs to study BIV infections (Rasmussen et al. 1990; Battles et al. 1992; unpublished data). Like HIV in infected humans, an immunologic response to Gag proteins is only made to MA and CA in BIV-infected animals.

BIV Infections and Pathogenesis

Lentiviruses can be categorised as causing acute and nonacute infections. The acute infections are rare and are only known for simian immunodeficiency virus (SIV) strain $_{SMMPBj}$, the Wyoming strain of equine infectious anaemia virus (EIAV$_{wyo}$), and JDV (Martin 1990; Soesanto et al. 1990; Israel et al. 1993). The remainder of the known lentiviruses are more characteristic of this unique retrovirus genus and are nonacute and slow acting, taking months to years from infection to onset of disease; however, there are wide variations in the clinical manifestations and debilitory effects induced by lentiviruses of different species (Gonda 1994a; Gonda et al. 1994). BIV, however, is more characteristic of most lentiviruses and, in cattle, causes a nonacute disease associated with lymphadenopathy, transient lymphocytosis, encephalitis, secondary infections, and wasting (Van Der Maaten et al. 1972; Gonda et al. 1994). It also has recently been reported that there is a depletion of immune tissues associated with secondary infections in BIV-infected dairy herds indicative of an immunocompromised state (Snider et al. 1996; in press). There is an unusually high turnover in dairy cattle due to culling for various health and productivity problems in BIV endemic herds. It has been reported that there is also a decrease in milk production in BIV-infected animals (Jacobs et al. 1995). The full economic impact of BIV infections on dairy and beef cattle remains to be determined.

BIV pathogenesis in naturally and experimentally-infected cattle has been difficult to characterise. This is primarily due to the nonacute slow acting nature of the virus, the long duration from initial infection to the onset of signs, nutritional status of the herd, presence of other secondary infections which may be contributory to the overall disease, but not a primary cause, and the limited number of pathological and virological investigations into this aspect of BIV biology. In experimental infections, BIV induces a mild lymphocytosis, lymphadenopathy, and encephalitis (Van Der Maaten et al. 1972; Carpenter et al.

1992; Gonda et al. 1994). A variety of other haematologic and immunologic anomalies have been observed in experimental animals that have not been confirmed. The lack of a more severe disease in experimentally-infected cattle may relate to the absence of important natural stimuli provided under practical conditions, which are needed for virus activation and/or provide physiologic stress on the host immune system (i.e., concurrent infections, stress of dairy farming, crowding, transport, climate, etc.).

Rabbits and sheep can be experimentally infected with BIV (Pifat et al. 1992; Van Der Maaten et al. 1992; Jacobs et al. 1994). In rabbits, there is a persistent infection (> two years) with a rapid and strong immunological response to Gag and Env. The infection appears to be targeted to cells of the immune system and brain (Pifat et al. 1992). PBMCs from BIV-infected rabbits also appear to have a decreased proliferative response to mitogens and there is a marked impairment of follicle development in response to antigen accompanied by follicular and paracortical lymphoid depletion in lymph nodes (Hirai et al. 1994; Kalvatchev et al. 1995). In infected sheep DNA can be found in PBMC DNA by PCR; however, virus cannot be rescued from these animals, the immunologic response is sporadic, and significant signs of disease have not been detected. Interestingly, transgenic mice made with BIV proviruses have provided some clues to the pathogenicity of BIV. BIV transgenic animals develop a persistent humoral immune response to virus expression. Encephalitis, chronic skin lesions, lymphoid tissue depletion, runting, and early death, presumably due to brain lesions, are found. The level of virus expression is low and correlates with pathologic lesions. Molecular investigations into virus gene expression and the host immune response may help explain the pathology seen in these mice and cattle and lead to a better understanding of cells involved in the disease process.

Areas for Future Study

Recent findings with SIV and HIV suggest that chronicity and the maintenance of high virus loads is central to the development of pathogenesis. In SIV_{SMMPBj}, enhanced virus replication capacity appears to correlate with elements in the viral LTR and the structure of the *nef* gene. Certainly, for 'fast-acting slow viruses' such as the JDV, $EIAV_{wyo}$, and SIV_{SMMPBj} lentivirus strains, increased virulence appears to correlate with rapid production of high virus titres in the plasma. In the case of SIV_{SMMPBj}, the severe disease in infected monkeys, which is reminiscent of the disease caused by JDV in banteng cattle, appears to correlate with physiological imbalances resulting from a strong inflammatory response to the rapid rate of virus replication. This can be overcome with proper medical intervention in the case of SIV_{SMMPBj}-infected primates. Host factors may also play a role in disease outcome (Soeharsono et al. 1995). It will be important to determine and compare the key viral factors that play a role in pathogenesis in BIV and JDV. The BIV nonstructural regulatory proteins and LTR are potential targets. Preliminary studies, in which the components of enhancer elements in the BIV LTR were altered, have yielded interesting results. Both basal level and Tat-activated LTR-directed gene expression can be enhanced. These derivatives replicate faster in tissue culture and are highly cytopathic. Rabbits infected with a BIV LTR derivative developed a severe lymphadenopathy and interstitial pneumonia within six months of infection (Fong et al. unpublished observations). Future studies into the role of virus load, the target cell for infection in vivo, immunosuppressive effects, and molecular pathology in animals naturally and experimentally infected with BIV will lead to a better understanding of bovine lentivirus disease. It may also be possible to vaccinate naive animals, for example, with attenuated strains of BIV or noninfectious pseudovirions prior to their exposure to herds in which BIV is endemic in order to reduce pathogenicity, if not infection (Tobin et al. in press). Such a strategy may have economic importance and implications for JDV as well as BIV.

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Regulation of Gene Expression in Bovine Lentiviruses

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Abstract

Lentivirus pathogenesis is dependent, in part, on sites and levels of virus gene expression within an infected individual. To better understand the pathogenesis of bovine immunodeficiency virus (BIV), the authors examined a number of viral, cellular, and host factors important in the regulation of BIV gene expression in vitro and in vivo. In vitro studies included analyses of Tat-mediated transactivation and in vitro host cell tropism. In vivo studies examined virus persistence and the development of a virus-specific host immune response in cattle experimentally infected with the R29 isolate of BIV. In each case, BIV gene expression was found to occur at relatively low levels, as compared to primate lentiviruses and equine infectious anaemia virus. The molecular mechanisms which contribute to the low level of BIV gene expression, and their potential role in BIV pathogenesis, are discussed.

BOVINE immunodeficiency virus (BIV) is a naturally-occurring lentivirus which is found in cattle populations throughout the world. Lentiviruses are associated with a lifelong, persistent infection and a variable disease course. The clinical outcome of infection may depend, in part, on levels of virus gene expression and replication within cells of the immune system. Therefore, understanding the mechanisms which regulate lentivirus gene expression is of interest in attempts to identify the underlying factors which contribute to clinical disease and viral pathogenesis. In the broad sense, lentivirus gene expression is regulated by viral regulatory proteins, by the host cell environment, and by the host immune response. In each case, complex interactions among virus and host proteins modulate overall levels of virus replication at the transcriptional and post-transcriptional level of gene expression. In an effort to define the molecular basis of lentivirus pathogenesis, laboratory staff have examined a number of viral, cellular, and immune factors which regulate BIV replication in vitro and in vivo.

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Transcriptional Regulation of BIV Gene Expression

Lentiviruses have evolved a complex mechanism of differential splicing to temporally regulate viral gene expression (Cullen 1992). The early, regulatory phase of lentivirus replication is characterised by the appearance of small, fully spliced mRNAs encoding the viral regulatory proteins Tat and Rev. Tat acts to increase overall levels of viral transcription, and Rev acts post-transcriptionally to activate the expression of incompletely-spliced viral RNAs encoding structural proteins and genomic RNA. All lentiviruses thus far characterised have been shown to encode a viral transactivating protein, Tat, which increases the rate of transcription of genes linked to the viral LTR (Pallansch et al. 1992; Liu et al. 1992; Hess et al. 1985; Sherman et al. 1989; Arya et al. 1985; Hungnes et al. 1992). Interestingly, the mechanisms of transactivation differ among various members of the lentivirus subfamily. In HIV, EIAV, and simian immunodeficiency virus (SIV), transactivation requires the presence of a highly structured RNA sequence, termed transactivation response region (TAR), located downstream of the transcription initiation site (Berkhout et al. 1989; Hauber et al. 1988; Dorn and Derse 1988; Carvalho and Derse 1991; Sherman et al. 1988; Fenrick et al. 1989; Huang and Jeang 1993; Feng and Holland 1988; Polacino et al. 1993). In most cases, the LTRs exhibit relatively low

levels of basal activity, yet are highly activated in the presence of homologous Tat (Sakuragi et al. 1991; Cullen 1992; Derse et al. 1987). In contrast, LTR sequences required for transactivation by visna virus and feline immunodeficiency virus (FIV) are located in the U3 region of the LTR, and include AP-1 and AP-4 enhancer sequences (Sparger et al. 1992; Hess et al. 1989). Both visna virus and FIV LTRs exhibit a high level of basal activity in most cell types, but are weakly transactivated (Hess et al. 1985; Sparger et al. 1992; Davis and Clements 1989). At the protein level, HIV and SIV Tat contain highly conserved functional domains which include a cysteine-rich domain, a highly basic domain, and a conserved–core region (Ruben et al. 1989; Green and Loewenstein 1988; Hauber et al. 1989; Berkowitz et al. 1993). EIAV Tat lacks the cysteine-rich domain, but retains a conserved–core region as well as a highly basic domain (Stephens et al. 1990; Dorn et al. 1990). Similar, functionally analogous domains are absent in the transactivating protein of visna (Davis and Clements 1989; Gourdou et al. 1989), as well as the putative FIV Tat (Olmsted et al. 1989; Sparger et al. 1992).

The regulatory mechanisms of BIV are less well characterised. BIV has been found to encode a transacting protein derived from an open reading frame located in the *pol/env* intragenic region (Pallansch et al. 1992; Liu et al. 1992). Computer analysis of the BIV RNA for the presence of TAR-like secondary structure in the 5' end identified two potential stem-loop structures between nucleotides +4 and +70. The structural similarities of BIV Tat and the presence of a TAR-like element in the extreme 5' end of the BIV RNA are highly suggestive that viral transactivation is mediated through an RNA enhancer similar to EIAV and primate lentiviruses. To test this hypothesis, the complete BIV LTR was inserted at the HindIII site into the expression plasmid pCAT-BASIC (Promega Biotec, Madison, WI) upstream of the gene for bacterial chloramphenicol acetyl transferase (CAT). A series of deletion and substitution mutations in the BIV LTR were generated and the resultant plasmids used in transient expression assays to examine the effect of these mutations on viral transactivation (Carpenter et al. 1992).

Results indicated that BIV transactivation required the presence of sequences downstream of the transcription initiation site, including the presence of the potential RNA stem-loop structure located at +4 to +31. Although the data did not establish whether the BIV TAR structure acts as a RNA or DNA element, taken together with the structural similarities among BIV Tat and primate lentivirus Tat proteins, these findings indicate that the mechanism of BIV transactivation is similar to primate lentiviruses.

Several structural and spatial features are required for optimal transactivation of HIV-1 TAR, including the presence of a base-paired stem structure (Berkhout et al. 1989; Hauber and Cullen 1988; Roy et al. 1990) as well as specific nucleotides within both the bulge and loop (Roy et al. 1990; Feng and Holland 1988; Berkhout and Jeang 1989). The latter includes a CUG sequence which is conserved in primate lentiviruses, as well as EIAV (Berkhout and Jeang 1989; Feng and Holland 1988; Carvalho and Derse 1991). Examination of specific nucleotide sequences within the bulge and loop of BIV identified several differences between BIV and the primate lentiviruses which might affect overall levels of transactivation. These included potential base pairing within the BIV bulge, as well as the absence of a CUG sequence in loop 1. A limited number of BIV TAR mutations were analysed in order to determine if levels of BIV transactivation could be increased over that observed in the wild-type LTR.

To determine if elimination of the base pairing would increase BIV transactivation, the G at +11 was mutated to a C. Rather than increasing transactivation, this single nucleotide change reduced transactivation by 60%. Mutations in the loop region of TAR resulted in only a slight increase in levels of transactivation. This result was surprising, as transactivation by HIV-1 Tat can be highly sensitive to point mutations in the loop region (Berkhout and Jeang 1989; Feng and Holland 1988). However, more recent studies confirm that BIV Tat does not require loop sequences for activity (Chen and Frankel 1994), presumably because Tat-TAR interactions are sufficiently stable in the absence of additional cellular factors. It is interesting to speculate that the absence of a cellular loop-binding protein may contribute to the relatively low level of transactivation observed with BIV Tat, as compared to primate lentiviruses and EIAV. The secondary structure of BIV TAR differs from the predicted structure of Jembrana disease virus (Chadwick et al. 1995) at several points required for Tat binding, including the bulge and the distal stem. The biological significance of these differences are not known. A thorough understanding of both the similarities and differences among bovine lentiviruses in the mechanisms controlling viral replication will be needed to identify key events contributing to the alternate disease course.

In Vitro Host Range of BIV

The U3 region of the LTR upstream of TAR includes the enhancer region which contains a number of transcription factor binding motifs that contribute to basal levels of viral transcription. The presence or

absence of transcription factors may vary among cell types, and thus may contribute to overall levels of gene expression within a given cell type. To better characterise factors which regulate BIV expression in vivo, the authors examined a number of bovine cell types for their ability to support BIV replication in vitro. Cell types analysed included primary foetal bovine lung cells (FBL), transformed bovine macrophages (BOMAC) (Stabel and Stabel 1995), Concanavalin A-stimulated lymphoblasts, peripheral blood derived endothelial cells (PBEC), and primary astrocytes. Cells were infected with a variety of BIV-R29 isolates as well as a field isolate from Florida, FL112 (Suarez et al. 1993). Following inoculation, cells were blind-passaged for one or two months and tested weekly for BIV replication. Supernatant samples were collected biweekly and tested for virus production using assays for reverse transcriptase activity. At each passage, duplicate cultures were fixed in methanol, and assayed for BIV protein production by immunocytochemistry using BIV Gag-specific monoclonal antibody (Wannemuehler et al. 1993). After one month, total DNA or RNA were isolated from replicate cultures for detection of BIV by the polymerase chain reaction (PCR) or Northern blot analyses, respectively. All isolates of BIV were found to replicate in FBL, BOMAC, PDEC, and astrocytes. In contrast, no detectable virus production was detectable in lymphoblast cultures by RT assay or infectivity assays. BIV proviral DNA remained detectable in lymphoblast cultures after 30 days, suggesting a low level of virus spread in these cells.

A cell-free infectivity assay was developed in order to quantify virus replication in vitro. This assay allowed comparison of the kinetics and overall level of replication among different isolates of BIV in a variety of cell types. No cell type was identified which supported high levels of BIV replication in vitro; the highest titres obtained were approximately 10^4 focus-forming units/mL supernatant. Differences among isolates were observed in both the rate and the overall level of virus replication. Most isolates replicated faster, and to higher levels, in FBL cells as compared to other cell types and there was some variability among the isolates in how quickly they adapted to each cell type. All of BIV isolates used in these studies were originally selected for replication on FBL cells and the results of the in vitro analyses may reflect the selective pressure applied during original isolation. To examine the host range of BIV in vivo, peripheral blood mononuclear cells (PBMC) were isolated from eight steers persistently infected with BIV-R29. Cells were co-cultured with FBL cells and with BOMAC, and virus replication was assayed as above. In seven of eight samples, BIV was recovered from FBL co-cultures. In contrast, only two samples co-cultured with BOMAC replicated BIV. These results suggest that FBL cells are highly permissive for replication of BIV R29 related isolates, even years after the original inoculation.

In summary, bovine cells were found to differ in permissiveness for replication of BIV, with primary lymphoblasts among the least permissive for productive replication of cell-free virus. BIV isolates differed in the kinetics and overall level of replication among the cell types examined; however, the biological significance of this variability is not known. All isolates replicated most efficiently in FBL cells, with maximum titres of approximately 10^4 focus-forming units/mL. No cell type was identified which supported high levels of BIV replication.

Immune Control of BIV Replication In Vivo

The persistent low level of BIV replication observed during long-term experimental infection with BIV R29 suggests that the immune response may be effective in restricting virus replication in vivo. Studies were undertaken to characterise the development and persistence of virus-specific antibody in cattle experimentally infected with BIV-R29 or co-infected with BIV-29 and bovine leukemia virus (BLV) (Isaacson et al. 1995). All eight experimentally-infected animals produced antibodies reactive to BIV *gag*- and *env*-encoded proteins by four weeks post inoculation (p.i.). By 40 weeks p.i., seven of eight cattle had dramatically decreased Gag-specific antibodies, and anti-Gag reactivity remained very low or undetectable through 190 weeks p.i. In contrast, antibodies to a recombinant BIV Env protein were readily detectable in all eight animals. During the period of declining Gag antibody, infectious virus was recoverable from PBMC of each animal; however, all animals remained clinically normal. In a single animal, antibodies to both recombinant Gag and Env proteins persisted through four years p.i. Interestingly, this animal was repeatedly negative for virus recovery beyond 17 weeks p.i. The mechanism(s) of Gag-specific antibody loss are not clear, and may include restricted expression of Gag proteins and/or immunosuppression of Gag-specific immune response. These findings provide evidence that a decline in Gag-specific antibody reactivity in a lentiviral infection can occur without increasing viral replication and progression to immunosuppressive disease.

Lentivirus persistence is associated with the periodic appearance of antigenic variant virus which is able to escape circulating neutralising antibody. A virus neutralisation assay was developed in order to characterise the development of BIV neutralising antibodies in experimentally-infected cattle and to

examine the role of antigenic variation in BIV persistence. During a four year period, 8/8 experimentally-infected cattle developed neutralising antibodies to homologous virus; however, differences were observed in neutralising titre and rate at which neutralising antibodies appeared. In general, there was a direct correlation between virus load and neutralising antibody.

To examine the antigenic and genetic variation of BIV over the course of experimental infection, sera collected from animals at three and five years p.i. were reacted with virus recovered from the homologous animal at four years p.i. *Env* sequences encoding gp110 were amplified from each virus by PCR, and the predicted amino acid sequence of year four virus isolates were compared with the inoculum sequence. Surprisingly, there was little antigenic and/or genetic variation found among the isolates. The low level of BIV replication in vivo, and the perhaps limited heterogeneity in the original inoculum, may contribute to persistence of relatively homogeneous, low–virulent viral genotypes.

Conclusion

Experimental infection of cattle with BIV-R29 results in transient, clinicopathological changes without overt clinical signs of disease. Analysis of BIV gene expression at the transcriptional and post-transcriptional level consistently depicts low levels of virus replication in vitro and in vivo. Together, these findings support the hypothesis that the clinical outcome of lentivirus infections correlates with the virus load. Therefore, the absence of overt disease in cattle experimentally infected with BIV-R29 is likely due to the low virulence of the virus inoculum. In the absence of high levels of virus replication in vivo, there appears to be little immune selection of antigenic variant virus, and little genetic variation in persisting virus. R29 appears to be the least virulent of BIV isolates characterised to date, and it is possible that more virulent isolates of BIV exist in the field. Continued analysis of field isolates of BIV, together with comparative studies of Jembrana disease virus, are needed to understand better the virus-host interactions which regulate virus gene expression and contribute to clinical disease.

Acknowledgments

The authors express appreciation to, and acknowledgement of, their collaborators in these studies. Collaborators include James A. Roth, Jeffrey A. Isaacson, Cecilia Whetstone, Susan Nadin-Davis, and Charles Wood. This work was supported in part by PHS grants CA50159, CA 59125, and T32 AI07378.

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The Comparative Pathology of the Lentiviruses

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Abstract

Eight lentiviral infections are known, of which only one, human immunodeficiency virus (HIV), occurs in man. Two have been identified in large ruminants, so-called bovine immunodeficiency virus (BIV) and Jembrana disease (JD) of Indonesian cattle.

While the term lentivirus implies a slow (chronic) disease, recent work has identified fast (acute) forms including JD, and subclinical infections. Latency is an important feature of the group.

The pathology of lentiviruses has some common features such as immunological responses, infiltrations of major organs and a tendency to secondary infections of many types promoted by immunodeficiency. But they are very heterogeneous in the way the pathology is expressed. Four general patterns are known: (i) acute transient infections in which a majority of animals recover (JD); (ii) chronic lymphoproliferative diseases which often end in failure of the immune system and death (HIV, FIV); (iii) chronic lymphoproliferative diseases without immunodeficiency leading to organ failure (maedi, visna (MVV) of sheep); and (iv) acute episodic disease associated with an immunological haemolytic anaemia (equine infectious anaemia).

Secondary infection is a common hazard of all lentiviral diseases and presents special problems of diagnosis.

IN the fields of veterinary and medical pathology, the lentiviruses show a remarkable global and species distribution. Their pathology demands special attention if researchers are to understand the clinical features of the individual diseases.

The term lentivirus stemmed from the concept of slow (i.e. chronic) virus infection proposed by Sigurdsson 1954a,b in a series of lectures at the University of London. His concept was based on his classical studies of an ovine syndrome in Iceland expressed clinically as a pneumonia (maedi) or as a neurological disorder (visna). The associated virus is now termed maedi-visna virus (MVV). Both lesions are usually expressed to some extent in affected animals.

For a proper understanding of any lentiviral infection the pathology should be analysed in detail at all stages of development since it may change greatly with the passage of time. The data for several major forms are still incomplete.

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Although the term lentivirus implies slow development of disease (It. lento = slow), some members of the group cause a relatively acute, and in one form at least (African SIVsmmPBj14 in Asian macaques), a peracute illness. Initial acute infections may be followed by one of several sequels including recovery (e.g. Jembrana disease), progressive systemic disorders (e.g. maedi) or sustained immunodeficiency with secondary complications and death (HIV, FIV). For example, AIDS is a chronic lentiviral infection complicated by secondary infection with any of a myriad of other potential pathogens including viruses, bacteria, protozoa and others.

The term lentivirus is therefore not entirely satisfactory for the whole group and may well have to go back to the taxonomic drawing board. The genetic relationship of the known members is undeniable but from the point of view of the pathologist and the clinician they are a very heterogeneous set of diseases.

A further characteristic of all lentiviruses is their capacity to exist subclinically (latent infections) sometimes as incomplete or restricted virus (Narayan and Clements 1989). Little is known of the associated

pathology which will be of critical importance to an understanding of the group especially in relation to the immune system. About 15% of HIV positive patients may not develop AIDS. The key to this phenomenon probably lies at the T lymphocyte CD4 or fusin receptor level (Berger et al. 1996).

General Biology

Recent work by Chadwick et al. 1995 based on pol gene analysis of the Jembrana disease (JD) virus has added to knowledge of the genomic relationships between the lentiviruses (Fig. 1). This picture is remarkable not only for the subdivisions of the viruses but also for the sharp clustering of the host species. It therefore may reflect host genetics as well.

Two other features may be derived from this tree. Within the species or even breeds e.g. bovine, simian, ovine, differences in susceptibility exist. For example Jembrana disease virus is more pathogenic in Bali cattle than in *Bos indicus* or *Bos taurus* (Soeharsono et al. 1995). Secondly, the species are sharply separated by the pathology sustained (Fig. 2). Thus the group can be subdivided into acute, transient infections (large ruminants), chronic or slow immunodeficiency diseases (primates, cats), slow, progressive systemic conditions (small ruminants) and the unique episodic syndrome of Equidae which suffer haemolytic anaemia.

Clearly much productive work lies ahead for the molecular biologists, immunologists and geneticists to elucidate more clearly the fundamental reasons for these demarcations. The task of the present writer is to concentrate on the diverse pathology.

General Pathology

Even with the present incomplete data base, it seems that the early pathology of all lentiviral infections has much in common but within the group there is later great diversity in their duration, dynamics, immunological and pathological responses. All infections cause marked changes in the antigen-processing and immunological systems. Indeed lentiviral diseases are primarily disorders of the immune system.

Involvement of parenchymatous organs is mainly by lymphoproliferative processes and infiltrations rather than by direct injury. In this way the respiratory or nervous systems may be severely damaged with corresponding clinical signs and fatal results.

The primary acute phase tends to be clinically non-specific even in the more chronic and devastating conditions such as AIDS. In Jembrana disease, an acute response can be seen in the lymphoreticular system, pulmonary alveoli, glomerular mesangial cells and in cellular infiltrates body-wide. A majority of animals recover.

Little is in fact known about the primary phase of the immunodeficiency diseases because the patient usually survives until the later development of AIDS. For this reason, feline immunodeficiency virus (FIV) infection may be a valuable model of the human disease. Preliminary observations on early FIV infection show many similarities to JD. The later immunological complications of HIV and FIV do not apply to JD and are uncertain in bovine immunodeficiency virus (BIV) infection which are short-lived clinical conditions although the virus may persist.

It should not be overlooked however that the more acute JD and BIV (Snider et al. 1996) may also be complicated by secondary infection under both field and experimental conditions. Bacterial pneumonia and helminthiasis have been noted. Earlier workers using cruder inocula observed rickettsia-like structures in their material (Budiarso 1980).

The general pathology of lentiviral infections can therefore comprise two distinct but inter-related components (Figs 3 and 4). The first reflects the interaction between the virus and the immune system of the host which may range from transient responses leading to a successful immune reaction (JD) to a prolonged struggle ending in failure of the immune system and complicated by secondary infection promoted by immunodeficiency (HIV, FIV).

The second aspect of the pathology arises from involvement of other systems with eventual organ failure, e.g., glomerulonephritis and uraemia (JD), terminal pneumonia (maedi) or encephalitis (HIV, visna).

The interpretation of lentiviral pathology therefore often involves more than a simple virus-cell relationship. The pathogenesis is complex.

Comparative Pathology

Because of a certain correlation between host species and type of pathology, the lentiviruses will be discussed briefly in groups, with emphasis on the species affected.

Bovine

(a) Jembrana disease

The pathology and immunology of this condition are discussed elsewhere in these Proceedings (Dharma et al. 1991) and therefore are not given expanded treatment here. The pathology, however, falls into the main categories of a response by the immune and other systems and may be complicated by the effects

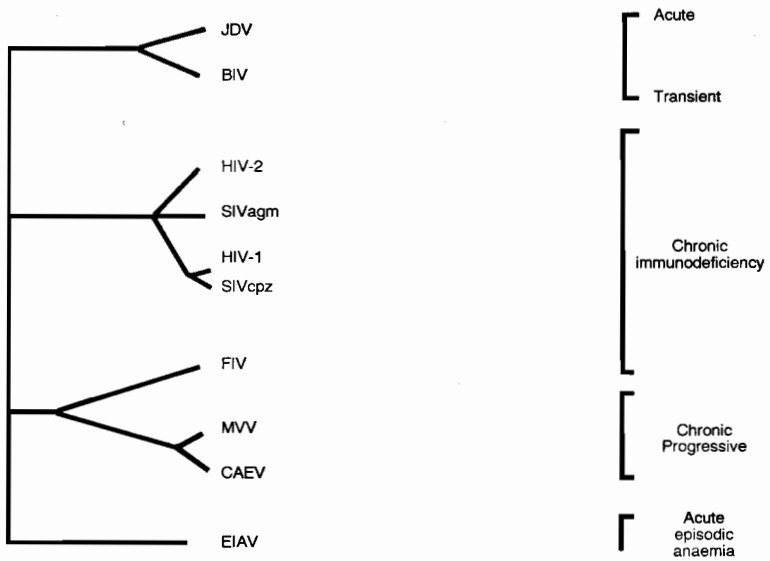

Figure 1. Relationships of the lentiviruses and their diseases. JD = Jembrana disease; BIV = Bovine immunodeficiency virus; HIV-2 = Human immunodeficiency virus Type 2; SIVagm = Simian immunodeficiency virus of African green monkeys; HIV-1 = Human immunodeficiency virus Type 1; SIVcpz = Simian immunodeficiency virus of chimpanzees; FIV = Feline immunodeficiency virus; MVV = Maedi-visna virus; CAEV = Caprine arthritis-encephalitis virus; EIAV = Equine infectious anaemia virus.

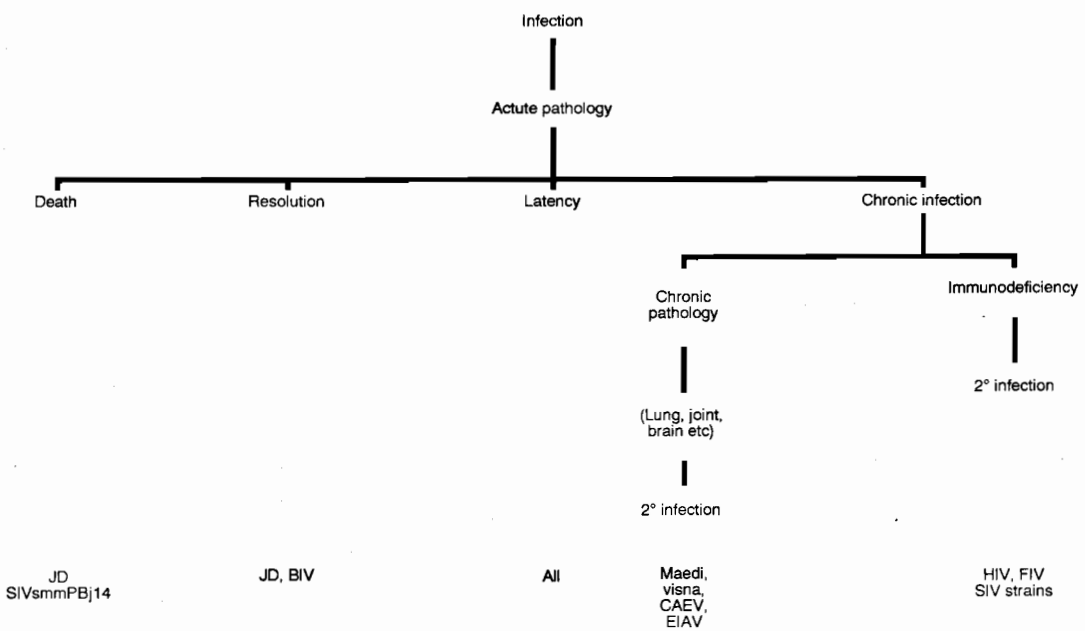

Figure 2. Lentiviral dynamics.

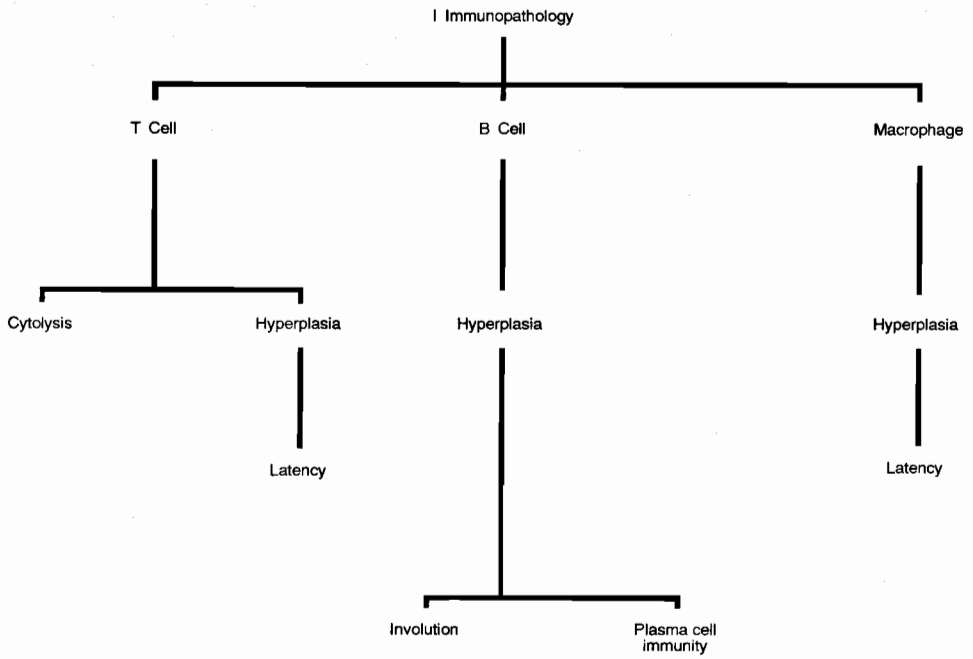

Figure 3. Pathology of lentiviruses.

II Systemic pathology

Pneumonia

Glomerulonephritis

Interstitial
nephritis
hepatitis
etc

Encephalomyelitis

Arthritis etc

All are expressions of antigen processing
or immune response
or immune failure

Susidiary functional effects include
autoimmunity
renal failure
neural failure etc

Secondary infection is a constant risk in progressive infections

Figure 4. Pathology of lentiviruses.

of superimposed infection. A notable difference from most if not all other lentiviral infections is the lack of significant changes in the central nervous system. Previous studies at field and laboratory levels suggests that up to 25% of animals may have concurrent infections. Unlike most lentiviral infections, however, 80% or more of affected animals can recover.

It is also imperative to distinguish the pathology at different stages of an acute cycle that lasts for about eight weeks compared with the immunodeficiency diseases with a span of months or years and consequently greater diversity of tissue change.

If secondary infection occurs, e.g., bacterial pneumonia, it may overlay the lentiviral effects and make interpretation of the primary condition difficult. This is more likely to occur in field outbreaks as in South Sumatra.

A further feature to note is that although *Bos indicus*, *Bos taurus* and the buffalo *Bubalus bubalis* generally do not react clinically, lesions may be seen histologically.

(b) Bovine 'immunodeficiency' virus (BIV)

The majority of field and experimental reports of this infection indicate a mild or subclinical condition. A recent report associates BIV infection with a range of

other diseases (BLV, mastitis, secondary infections, hepatic diseases and infections of the feet) in dairy cattle (Snider et al. 1996).

Unlike the agent of Jembrana disease to which it is closely related (Chadwick et al. 1995), the virus has been propagated in tissue culture. The infection, usually without clinical disease, has been diagnosed world-wide by serological surveys yielding prevalence rates varying from 5–95%. This aspect will be discussed separately.

The pathologist should note that BIV may co-exist with other agents including the virus of bovine leukaemia and bovine syncitial virus. Pathological data are so far fragmentary and difficult to interpret. Among the experimental changes described are lymphadenopathy with follicular hyperplasia and some paracortical reaction (Carpenter et al. 1992; O'Neill 1995). Low-grade encephalitis, usually slight, with perivascular infiltrates of lymphocytes and other mononuclears have been seen. The disease process seems to be transient and on current evidence does not clearly lead to immunodeficiency despite the current nomenclature.

Present evidence suggests some similarities in the pathology of JD and BIV infections but it is too early to draw sharp conclusions. The viruses are, however, closely related (Chadwick et al. 1995; Fig. 1).

Small ruminants

Although the lentiviral infection, equine infectious anaemia, was described as early as 1904 (Carre and Vallee 1904) the first detailed study of a virus (MVV) from the group was carried out by Sigurdsson and his colleagues in Icelandic sheep (Sigurdsson 1954a,b; Sigurdsson et al. 1952, 1957). Subsequently a related lentiviral infection of goats, caprine arthritis-encephalitis (CAEV) was recognised. CAEV is transmissible to sheep without clinical effects (Banks et al. 1983).

(a) Maedi-visna virus (MVV)

Pathologically this group of viral strains cause chronic progressive involvement of major organs. In maedi there is severe pneumonia; in visna encephalitis is prominent. Immunodeficiency is not a feature.

Comparative studies in the United States and elsewhere show that the host species may influence the outcome. American breeds are comparatively resistant.

An unanswered question is the prevalence of MVV, if any, in tropical areas where sheep and goat populations are very large. Reports to date originate mainly from the northern hemisphere and Australasia.

Clinical descriptions of maedi have emphasised the respiratory effects but other systemic effects are seen in the kidneys, mammary glands and joints as well as the nervous system. There is both genetic and pathological overlap with visna and CAEV.

Uncomplicated early maedi is based at the alveolar level of the lungs (Jubb et al. 1993) not unlike Jembrana disease, but is followed by intense lymphoproliferative changes in the interstitial components of the lungs and in the lymph nodes. The lungs become consolidated and rubbery in consistency. Further disruption is commonly caused by secondary infections by pyogenic bacteria or Pasteurella spp. with consequent bronchopneumonia, abscess formation or hepatisation. Helminth infections may also be present.

Synergistic interaction with the agent of pulmonary adenomatosis (jaagziekte) caused by another retrovirus, has been described in several countries as complicating the pathology further. The intensity of the basic MVV lesion may depend on viral strain differences as well as on the breed of sheep (Lairmore et al. 1988).

Visna, the neurological form of MVV infection, like maedi, is slowly expressed clinically from the age of two years when, e.g., mild ataxia and trembling of facial muscle appear. There is a prolonged encephalomyelitis with demyelination (Jubb et al. 1993). The process originates from around the ependyma suggesting intra-ventricular spread of virus from the choroid plexus which itself is infiltrated by lymphoid and other cells. In Jembrana disease, the choroid lesions may be severe but the process does not extend into the nervous system.

Other systemic lesions in visna as in maedi include interstitial pneumonia and focal proliferative glomerulonephritis (cf. Jembrana disease). Immunodeficiency is not a feature.

MVV infection has been used as a model for the study of restricted viral expression which is of fundamental importance in some of the lentiviral infections (Narayan et al. 1983; Haase 1986; Reyburn and McConnell 1993). Restriction ensures that the basic viral genome is maintained within infected cells. For example proviral DNA can be identified in explanted choroid plexus (Haase et al. 1977).

(b) Caprine arthritis-encephalitis virus (CAEV)

This agent is closely related to MVV (Fig. 1) and shows many of its pathological features in the respiratory and nervous systems. It is, however, unique in its affinity for joints where villous hypertrophy, vascular proliferation and mononuclear infiltration may proceed to necrosis (Wilkerson et al. 1995).

As in MVV, neonates may be infected with colostral milk but contact infection also occurs. In some young goats, the disease is expressed as an acute leucoencephalomyelitis while remaining latent in others. In adults the condition is more chronic in the form of an arthritis especially in the carpal joints. Chronic mastitis, pneumonia and encephalomyelitis are often present.

As in other lentiviral infections, the macrophage is thought to be the most potent carrier of virus. Specific populations of macrophages may be involved (Gendelman and Morahan 1992).

Equidae

Equine infectious anaemia virus (EIAV) is widely distributed throughout the world though its prevalence and distribution in many countries with large populations of equidae is still unknown. It is the archetypal lentiviral infection, having been described by Carre and Valle in 1904. The virus stands alone on its phylogenetic branch (Fig. 1) and has a number of unique pathological features.

Transmission of virus is usually caused by insect vectors including Tabanids and mosquitoes especially in wet swampy areas and tends to have a seasonal incidence. Although horses may die in the initial acute phase, the disease is usually periodic with tissue virus declining in time.

The basic pathology of EIAV resembles other lentiviral infections with reactive changes in the lymphatic organs and lymphoproliferative infiltrates in organs, notably liver including Kupffer cells, and kidneys where the glomerular mesangial cells are infected. Blood monocytes and macrophages are highly susceptible to virus. Recurrence is due to renewed replication in macrophages of viral variants. The virus is unstable and undergoes antigenic drift.

Resulting immune complexes are deposited in tissues including erythrocytes resulting in a severe haemolytic anaemia. This differentiates EIAV from all other lentiviruses. Its pathology is consequently much more complex when the primary changes are overlaid by widespread focal haemorrhages in major organs and tissues. Liver, kidneys, spleen and lymph nodes are further enlarged by the erythrophagocytosis and haemosiderin deposition of phagocytic cells.

Anaemia also causes degeneration and even necrosis of major viscera. Within blood vessels endothelial swelling and proliferation occur. Functional organ and vascular failure follow. For this reason oedema of subcutaneous and other interstitial tissues are often seen.

Death from EIAV is therefore likely to be due to severe and extensive disorders comprising both immunological and haematological changes expressed in both tissue and biochemical pathology. The anaemia is at first macrocytic but later becomes normocytic and normochromic. Leucocytosis includes the appearance of siderocytes (Granzien and Newton 1968). While serum albumin declines, gamma-globulin rises to depress the albumin: gamma-globulin ratio. In acute cases, the serum iron is raised (Jubb et al. 1993).

EIAV therefore most clearly expresses the heterogeneous nature of lentiviral pathology. It can be acute, chronic or subclinical (latent) with persistence of virus sometimes lasting for life.

Feline immunodeficiency virus (FIV)

This is the first of the chronic (slow) immunodeficiency-inducing lentiviruses to be discussed. In contrast, a transient inhibited immune reaction may occur in other lentiviral infections. For example, Jembrana disease shows a brief deficiency in the acute stage but this is quickly followed by a conventional and often effective immune response (Dharma et al. 1991; Wilcox et al. 1995).

FIV has a world-wide distribution as shown by both clinical and seroepidemiological studies (Robinson and Menrath 1995). The disease appears to be a valuable model for HIV infection, a feature noted by several very active international research groups. The availability of an easily accessible species like the cat means that the earlier changes can be studied in a way that is seldom possble in HIV infection where patients usually survive the acute stage. Because the focus to date has understandably been on the immune reaction, other systemic responses are still poorly understood.

The clinical pathology of FIV infection and probably other members of the immunodeficiency group can be summarised as follows in terms of dynamics and pathology (Robinson and Menrath 1995).

(i) Acute viraemia associated with generalised lymphadenopathy, malaise, diarrhoea and fever. Lymphoid changes are marked in neonates but may be absent in aged cats.

(ii) Latency. An effective early immune response may suppress viraemia for months or years. After 25–40 weeks, lymphocyte and neutrophil numbers in the blood may stabilise.

(iii) FIV-AIDS related complex (FARC). Although similar clinical signs may occur in non-FIV infected cats (e.g., stomatitis, cystitis, dermatitis, and abscessation), those with FIV show decreased CD4+ T lymphocyte numbers and a depressed CD4+:CD8+ count.

(iv) Feline AIDS (FAIDS). When a decline in B cells is added to low CD4+ levels, the animal is passing into an AIDS-like syndrome. Lymphoid infiltration of the lung, kidneys and brain cause additional effects, e.g., renal failure. Neoplastic infiltration may emerge. Experimental studies indicate possible complications with such agents as feline calicivirus (FCV), feline syncitial-forming virus (FSV), feline leukaemia virus (FLV), herpesvirus Type 1, Toxoplasma and other pathogens.

The process differs from that of JD and MVV in the chronic immunodeficiency, and is sharply divided from JD in its long duration and usual failure to recover. Restriction of viral expression may occur in FIV infection as in the HIV and MVV syndromes.

In terms of pathology, the early stages show follicular lymphoid hyperplasia in primary and secondary lymphoid organs, bone marrow and elsewhere. During this period, antigen-antibody complexes are formed which may be responsible for plasma cell reactions (Callanan et al. 1993). Mononuclear infiltrates affect the intestine, liver, kidneys, lungs and brain where gliosis occurs (Brown et al. 1991). Astrocytes and microglia are probably targetted by virus. Neuronal loss is a critical side-effect.

Secondary infections will add their own pathology to the basic picture. Further variation in the disease picture may be due to strain differences and cell susceptibility, both of which may depend on antigenic drift of the FIV virus.

The primates

This group, which includes the apes and man, are usually treated separately although their respective viruses share close nucleotide homology and, significantly, a tropism for CD4+ lymphocytes in the immune system. The closeness of their genetic relationship is also shown in one branch of the dendrogram in Figure 1. They have similarities in pathology as well.

(a) Simian immunodeficiency virus (SIV)

There is always something new out of Africa, said the Roman historian Pliny, and he could have been anticipating SIV infection from which HIV virus may have been derived.

While some African SIV strains may circulate subclinically in monkeys, e.g., chimpanzees, it has been shown that SIV in predominantly Asian macaques causes a syndrome like human AIDS (Martin 1990). A more unusual and so far unique peracute disease is caused by a strain of SIV (SIVsmmPBj14) from sooty mangabey monkeys in macaques. Some animals died with peracute dysentery in eight days. This highlights the acute nature of some lentiviral infections.

There is considerable variation in pathogenicity (or host-susceptibility) among the SIV group. But where the virus induces immunodeficiency, e.g., in rhesus monkeys, the pathology closely resmbles HIV infection (Baskerville et al. 1990). Thus initial lymphoid hyperplasia is followed by involution and depletion of cells. Mononuclear infiltration of major organs such as intestine, lungs, heart and kidneys was seen. In the kidneys also there was glomerular mesangial hypercellularity. Encephalitis was characterised by gliosis and both perivascular and meningeal lymphocytic infiltration. Lymphoid depletion may develop from day 52 p.i. and as the disease progresses secondary pathogens may come into play. Hirsch et al. 1991 noted cytomegalovirus and disseminated *Mycobacterium avium*, both potential pathogens in man. Congenital infection can take place as in the human subject.

SIV infection may therefore, in some species, provide a useful model for HIV infection in man, though at some cost. The use of cats with FIV seems to be a more convenient system. Though the full pathology in the cat remains to be worked out, available evidence shows a close resemblance to its human counterpart.

(b) Human immunodeficiency virus (HIV)

Few diseases have been studied with the intensity or at the depth of HIV infection with its common termination in the acquired immunodeficiency syndrome (AIDS). Yet information is lacking on the histopathology of some of the earlier changes that occur in the short acute and longer latent phases as patients survive to succumb only after months or years from the complex AIDS syndrome with various complicating infections. Much data however can be gleaned from the clinical pathology and immunology of the blood throughout the disease.

Early signs of HIV in adults are influenza-like and last for about 2–3 weeks. A prolonged lymphadenopathy continues for weeks, months or years, while AIDS develops in 5–7 years (Narayan and Clements 1989; Levy 1993). The final phase is introduced by failure of CD4+ T helper lymphocytes, immunodeficiency and the onset of opportunistic infections. Congenital infection can occur in children with severe respiratory and neurological symptoms.

HIV may infect many other cell types including follicular dendritic cells, B cells, blood monocytes, tissue macrophages, microglia and others not all of

which are CD4 positive. The initial lymphadenopathy is characterised by follicular and parafollicular hyperplasia in lymphoid organs. Seroconversion occurs initially with hypergammaglobulinaemia but in most patients is followed by follicular involution and marked depletion of lymphoid and related cells.

Systemic changes are seen in many other organs such as the lungs, intestine, kidneys and brain with corresponding clinical signs.

A wide variety of secondary pathogens may complicate AIDS which impose their own pathology on an already devastated body.

Discussion

While the lentiviral infections are a varied group (Fig. 1) a common pattern of pathology runs through all of the diseases described. Three pathological strands can be identified that may occur in all of them but are subject to variations due to genetic, immunological and other factors little understood.

The three lentiviral pathologies are:

(i) Lymphoproliferative (immunological) response:
Acute follicular and parafollicular hyperplasia followed by a conventional immune response (JD) or a prolonged deterioration leading to immunodeficiency and death (HIV, FIV);
Lymphoproliferative infiltrations of major organs such as lungs (MVV) or brain (MVV, HIV, FIV);
EIA is differentiated by the intensity of its immunologically-based haemolytic anaemia.

(ii) Structural changes in other organs associated with antigen processing cells (mesangial glomerulonephritis) or infiltrates (nephritis in JD, pneumonia in MVV, encephalitis in most except JD);
Associated functional pathology such as uraemia (JD), respiratory failure (MVV), neurological failure (MVV, HIV) including dementia in man.

(iii) The pathology of secondary infection which depends on the type of pathogen. Thus viruses (bovine leucosis virus, cytomegalovirus etc), bacteria (pasteurellae, mycobacteria), protozoa (*Toxoplasma*), fungi, helminths and others create their own pathology that becomes mixed with the basic lentiviral process.

Changes are further complicated by the duration of the infection. The task of the pathologist, in cooperation with microbiologists and others, is to interpret the overall picture.

Little is known of the basic reasons for the differences in the pathology of members of the lentiviral genus. The affinity of gp120 of HIV for the CD4+ receptor is the key to the eventual immune failure. The same process may operate in SIV and FIV infections. Other lentiviral infections are known to have an affinity for cells of the macrophage lineage yet sharp differences are present within them. JD and BIV are relatively mild and transient whereas MVV is chronic, progressive and fatal in some breeds. The answers will be found at the molecular level among the many receptors and epitopes and through them at a deeper genetic level. That is beyond the scope of this paper.

For a proper understanding of lentiviral infections, it is, however, essential that the virologist, pathologist, epidemiolologist and clinician work together to obtain a clear picture of the individual disease (Campbell 1995).

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Pathogenesis of Bovine Immunodeficiency Virus

S. Carpenter[1] and L.D. Miller[2]

Abstract

The clinical, immunological, and pathological effects of long-term infection with bovine immunodeficiency virus (BIV) were studied in cattle experimentally infected with the R29 isolate of BIV. Experimental infection of calves with BIV-R29 induced mild lymphoproliferative changes early after infection, including transient lymphocytosis and follicular hyperplasia. Over a four-year period, BIV-infected animals exhibited transient suppression of neutrophil and macrophage function, with little evidence of clinical immunosuppression. A single BIV-infected calf developed an atypical T-cell lymphosarcoma concurrent with progressive monocytosis at six months post-inoculation. All other BIV-infected animals remained clinically normal for more than five years following infection. Four BIV R29-infected animals were necropsied at five years post-inoculation. Histopathological changes included follicular hyperplasia and, in some cases, follicular involution. In these animals, proviral DNA was detected in a variety of tissue samples, including the brain, but there was no correlation between the presence of lesions and the sites of proviral DNA. These results indicate that BIV R29 is only mildly pathogenic, even after long-term infection. Identification and characterisation of additional field isolates of BIV are needed to better assess the range of clinical and pathological consequences of BIV infection.

CLINICAL and pathological manifestations of lentiviral infections vary widely, both among the different members of the lentivirus family as well as among individuals inoculated with the same virus. The differences in clinical disease among the various members of the lentivirus family are due primarily to the target organ affected and, in some cases, to the tempo of virus replication and induction of disease. Bovine immunodeficiency-like virus (BIV) is a member of the lentivirus family which has received only limited in vivo study (Van Der Maaten et al. 1972; Gonda et al. 1987). In 1972, Van Der Maaten et al. reported the isolation of a virus from cattle with persistent lymphocytosis. At necropsy, affected cattle were found to exhibit lymphoid hyperplasia and mild lymphocytic perivascular cuffing in the brain. Experimental infection of colostrum-deprived calves resulted in mild transient leukocytosis and moderate lymphadenopathy. Electron microscopy examination demonstrated that the causative virus was morphologically similar to visna virus (Van Der Maaten et al. 1972; Boothe and Van Der Maaten 1974). More recent studies have shown that the bovine lentivirus is antigenically and genetically related to other members of the lentivirus subfamily of retroviruses, including HIV, and in 1987 the virus was named bovine immunodeficiency-like virus (BIV) (Gonda et al. 1987). The structural similarities between BIV and HIV, as well as the occurrence of lymphadenopathy in experimentally-infected calves, suggests that the pathogenesis of BIV may be similar to other immunosuppressive lentiviruses. Therefore, a long-term study was undertaken to examine the in vivo effects of BIV following experimental infection of calves.

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Early Pathogenic Effects of BIV in Experimentally-infected Calves

A series of experiments was undertaken to characterise the in vivo virulence of BIV R29 during the first six weeks after infection and to determine if viral virulence could be increased following rapid in

vivo passage (Carpenter et al. 1992). Following inoculation, calves were examined at least three times per week for changes in temperature and haematological profiles. The normal range of lymphocyte and neutrophil counts were determined from a total of 123 samplings from age-matched calves. For the purposes of this study, the normal range for the lymphocyte and neutrophil counts was defined as the range that contained 95% of the values from these control animals. Increased numbers of lymphocytes were observed in several BIV R29 passage calves, notably p2 and p5 calves. However, the lymphocyte values were all within the normal range. Isolated days of fever occurred in p2 and p3 calves, while a more extended febrile response, most likely associated with pneumonia, was observed in the p5 calf. No consistent, progressive haematological changes were identified in the BIV R29 passage calves which could be correlated with successive passage of BIV.

Whole blood collected from each calf was tested at least twice during the experimental period for the presence of infectious BIV and antibody to BIV. All animals inoculated with BIV R29 infected cells seroconverted by six weeks post inoculation (p.i.) and BIV was recoverable from each animal at two weeks p.i. However, levels of BIV replication in vivo appeared to be low. In situ hybridisation studies indicated that during peak periods of viral replication in vivo, less than 0.03% of peripheral blood mononuclear cells (PBMC) were expressing detectable levels of viral RNA. Moreover, the levels of viral RNA in PBMC in vivo were less than one-tenth the levels observed in persistently infected cells in vitro. These results suggested that in vivo passage of BIV R29 did not dramatically alter levels of virus replication in vivo.

None of the calves in these experiments were infected with bovine viral diarrhoea virus (BVDV) prior to BIV inoculation; however, antibody to BVDV was detected in post-inoculation sera collected from p1, p2 and p3 calves. These results indicated that the BIV R29 stock contained infectious BVDV. Interestingly, BIV R29 p4 and p5 calves did not seroconvert to BVDV by 5–6 weeks p.i., although both had seroconverted to BIV in a similar time frame. This serological evidence indicates that p4 and p5 calves are BVDV-free and, therefore, suggests that the cycles of neutropenia observed in the p4 calf are a consequence of BIV infection.

At 5–6 weeks p.i., BIV R29 passage calves were euthanased and examined for gross and histopathological changes. Grossly, lymph nodes often had slight-to-moderate swelling seen as bulging of the parenchyma when the capsule was incised. Infrequently, entire lymph nodes or portions of them were hyperemic. Hemal nodes were pink to bright red and ranged from 2 mm to 1.5 cm in diameter. Consistent histologic changes characterised as lymphoid follicular hyperplasia were found in lymph nodes, hemal nodes, spleen, tonsil, Peyer's patches and other gut-associated lymphoid tissue in infected calves. Sections of brain, spinal cord, salivary gland, esophagus, trachea, lung, thymus, heart, liver, pancreas, adrenal, kidney, abomasum, small intestine, large intestine and bone marrow were normal.

The histopathological changes observed in BIV-infected calves were similar to changes found early after infection with the immunosuppressive lentiviruses, including HIV-1 (Carpenter et al. 1992). The most frequent histopathological finding was lymphoid hyperplasia, primarily due to expansion of the number and size of follicles. Secondary follicles occupied most of the subcapsular cortical space and were frequently observed in the deep cortex, especially in areas adjacent to fibrous trabeculae. Cells in the center of hyperplastic follicles were larger and generally less identifiable as histiocytic or lymphocytic types with the exception of macrophages containing cytoplasmic tingible bodies. In the paracortex, scattered large pale histiocytic cells and occasional mitotic figures were present among the predominating large and small lymphocytes. In hemal nodes, moderate to marked follicular hyperplasia was commonly observed and was similar to that observed in the lymph nodes. The degree of lymphofollicular hyperplasia was variable among the lymph nodes and hemal nodes from different anatomic locations and between nodes and other lymphoid organs within an infected animal. The degree of hyperplasia also varied among the infected animals; however, pathological changes did not increase in severity with progressive passage of BIV nor could differences be attributed to the presence or absence of concurrent BVDV infection.

Longitudinal Studies of BIV in Experimentally-infected Cattle

To study the long-term effects of BIV in experimentally-infected cattle, 18 calves were randomly assigned to one of four groups and experimentally infected with either BIV-R29, bovine leukemia virus (BLV), BIV-R29 and BLV, or sham-infected (Flaming et al. 1996). Blood was collected at regular intervals over a four-year period for analysis of immune cell subset and function, virus persistence, and virus-specific antibody (Flaming et al. 1996; Isaacson et al. 1995; Rovid et al. 1995). At five months post-inoculation, a single calf inoculated with BIV only developed T-cell lymphosarcoma concurrent with progressive monocytosis (Rovid et

al. 1996). Tumor cells were $CD2^+$, $CD4^-$, $CD8^-$, and were found in multiple lymph nodes, the thymus, and brain. Infectious BIV was recovered from splenic tissue and peripheral blood, and BIV proviral sequences were detectable by PCR in most tumor samples. All samples were negative for BLV using similar assays. This case of lymphosarcoma differed from each of the four classical forms of bovine lymphosarcoma, and raises the possibility that BIV infection may predispose animals to the development of atypical lymphosarcomas, similar to HIV-1 and FIV infection (Rovid et al. 1996).

In studies of immune function over a four-year period following experimental infection, BIV-infected animals exhibited transient suppression of neutrophil and macrophage function, as measured by selected in vitro assays (Flaming et al. 1996; Rovid et al. 1995). Animals co-infected with BIV and BLV did not have a consistently different response from that observed in animals infected with each virus alone. With the exception of the single animal that died of lymphosarcoma, all animals remained clinically normal. At five years post-inoculation, four animals were killed and examined for gross and histopathological changes.

The majority of lymphoid tissues were in a state of hyperplasia comparable to that described for cattle examined during the earlier stages of BIV infection (Descoteaux and Matlashewski 1990). However, some nodes or portions of them had changes characteristic of involution, with the most prominent changes including a marked reduction or absence of small lymphocytes in the follicular mantle zone and looseness or separation between the remaining follicular and interfollicular cells. Remarkable changes were not found in other organs. BIV was recoverable from PBMC throughout the five years post-infection. Therefore, it appears that long-term persistent infection of cattle with BIV-R29 is associated with sub-clinical pathological changes which include follicular hyperplasia and follicular involution. Similar changes have been described in persons infected with HIV-1 (Biberfeld et al. 1987).

To determine if histopathological changes were associated with the presence of BIV, total DNA was isolated from a variety of tissue samples and BIV *pol* sequences were amplified by the polymerase chain reaction. Proviral sequences were detectable in a number of tissue samples; however, there was variability among animals as to which tissues were BIV-positive. The most consistently positive tissue was the brain. Interestingly, no lesions were observed in the brains of any BIV R29-infected animals; however, microscopic brain lesions were found in two animals inoculated with the Florida isolates of BIV (Suarez et al. 1993) and necropsied at six months post-inoculation. These lesions were characterised by mild scattered meningeal infiltrates composed chiefly of lymphocytes and histiocytes. Encephalitic lesions occurred as occasional foci of lymphocytic perivascular cuffing and cell nodules in the cerebrum, cerebellum, and brain stem. Sites of virus replication in animals inoculated with the Florida isolates were examined by immunohistochemistry using rabbit antisera specific for BIV Gag (Wannemuehler et al. 1993). Virus-specific staining was observed in a number of lymph nodes and in the tonsil. However, it was difficult to determine if the staining reflected replication in lymphoid cells, or was due to virus trapping within the reticular network of the lymph node. Therefore, the correlation between virus replication and pathological changes was not conclusive.

Conclusion

Experimental infection of calves with the R29 isolate of BIV induced mild lymphoproliferative changes early after infection, including transient lymphocytosis and follicular hyperplasia. Little evidence of clinical immunosuppression was observed over a four-year period following experimental infection of calves with BIV R29. However, long-term infection with BIV R29 was associated with histopathological changes that included follicular hyperplasia and, in some cases, follicular involution. Additional pathological changes were observed in calves inoculated with the more recently derived field isolates of BIV (Suarez et al. 1993). Together these results suggest that pathogenesis of BIV in experimentally-infected animals is determined, in part, by the virus inoculum. It is clear that BIV R29 is only mildly pathogenic; identification and characterisation of additional field isolates of BIV are needed to define better the range of clinical and pathological consequences of BIV infection.

Acknowledgments

The authors express appreciation to, and acknowledgement of, their collaborators in these studies. Collaborators at Iowa State University include James A. Roth, Anna Rovid, Kevan Flaming, Yvonne Wannemuehler and Eric M. Vaughn. Collaborators at the National Animal Disease Center, Ames, Iowa include Cecilia Whetstone, David Suarez, Janice Miller, and Martin Van Der Maaten. This work was supported in part by PHS grants CA50159, CA 59125, T32 AI07378 and USDA fellowship grant 88-38420-3832.

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Bovine Lentivirus Infection in Bali Cattle (*Bos javanicus*) Following Inoculation with BIV

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Abstract

Bovine immunodeficiency virus (BIV) was isolated in North America. Inoculation of BIV into domestic North American cattle (*Bos taurus*) produces only mild clinical signs, i.e., transient lymphocytosis. Jembrana disease is an acute, infectious, enzootic disease of Bali cattle (*Bos javanicus*) in Indonesia. Clinical signs include anorexia, leucopenia, fever, enlargement of peripheral lymph nodes, and a 17% mortality rate. When Jembrana disease virus (JDV) is inoculated into *Bos taurus*, infections are mild, similar to BIV. Recently, it was shown that JDV is a bovine lentivirus (Chadwick et al. 1995; Kertayadnya et al. 1993).

In the study reported here, two young bantengs (Bali cattle) and a Holstein calf were inoculated with BIV-FL112, a wild-type isolate that causes lymphocytosis. A sham-inoculated Holstein calf was used as a negative control. Necropsies were done at 8 weeks postinoculation (PI).

All calves remained clinically normal. All principals became BIV positive by PCR and virus isolation. The Holstein principal developed antibodies to BIV and showed a transient, B cell lymphocytosis. The bantengs did not develop detectable antibodies or a lymphocytosis. FACS analyses of preinoculation blood showed that bantengs had a higher percentage of CD3+, IL2R+, and γ/δ-TCR (N2) T cells compared to the Holsteins, and a lower percentage of monocytes. Postinoculation, the Holstein showed an increase in B cell, monocyte, and MHCII+ cells, consistent with a B cell lymphocytosis. The bantengs, however, showed a decrease in the percentages of γ/δ-TCR+, N2+, and N12+ cells with no change in the B cell population. Culture of lymphocytes with concanavalin A and pokeweed mitogen showed no significant differences in the response of cells from principal compared with control calves at the individual time points tested. Lymph node sections from the inoculated Holstein calf showed prominent follicular germinal centres. No significant pathologic change was noted in tissues from bantengs. In situ hybridisation and immunohistochemical studies did not detect the presence of BIV.

A BOVINE lentivirus known as bovine immunodeficiency virus (BIV) was isolated from a cow in Louisiana in 1969 during the search for an etiologic agent of bovine leukemia. BIV was initially described as a Visna-like virus because of similarities to the ovine lentivirus and was designated the R29 isolate (Van Der Maaten 1972). In early experiments, cattle inoculated with BIV showed only minor pathological changes with no disease or clinical immunodeficiency observed (Van Der Maaten et al. 1972). Molecular characterisation placed BIV in the lentivirus family (Garvey et al. 1990; Gonda et al. 1987).

BIV causes a persistent infection in affected cattle and serological data indicate it has a world-wide prevalence (Amborski et al. 1989; Black 1989; Horner 1991; Horzinek et al. 1991; Whetstone et al. 1990; Whetstone and Van Der Maaten 1996, unpublished data). BIV shares a similar genomic organisation with other lentiviruses including the *pol*, *gag*, *env* and *tat* genes (Garvey et al. 1990; Oberste et al. 1991). Antigenically, antisera to BIV p26 cross reacts with p24 of HIV-1, and antisera to equine

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infectious anaemia virus (EIAV) cross reacts with p26 and p24 of BIV (Gonda et al. 1987; Whetstone et al. 1991). Two BIV R29 derived molecular clones, BIV127 and BIV106, have been sequenced and show the virus to be distinct from but related to the other lentiviruses (Braun et al. 1988; Garvey et al. 1990).

The original experimental inoculations of BIV R29 in colostrum deprived calves induced a mild lymphocytosis and enlargement of peripheral subcutaneous lymph nodes, with no overt clinical signs of disease (Van Der Maaten et al. 1972). Studies examining immune function of BIV in vivo have demonstrated either mild or no immunosuppression based on lymphocyte blastogenesis tests and histopathological changes (Carpenter et al. 1992; Flaming et al. 1993; Marin et al. 1991). Available research results have not shown that BIV is a disease causing agent in cattle after experimental inoculations. These reports were all based on short term studies (Carpenter et al. 1992; Flaming et al. 1993; Marin et al. 1991; Van Der Maaten et al. 1972; Whetstone et al. 1990).

Jembrana disease is an acute, sometimes fatal disease of Bali cattle (*Bos javanicus*) that was recently identified as a bovine lentivirus (Kertayadnya et al. 1993), and shares sequence homology with, but is different from, BIV (Chadwick et al. 1995). When inoculated into *Bos indicus*, Jembrana disease virus (JDV) is subclinical (Wilcox et al. 1992; Soeharsono et al. 1995). The question arises whether JDV and BIV, both bovine lentiviruses, cause similar or different pathologies in genetically different cattle, *Bos taurus* and *Bos javanicus*. Although it is known that JDV is subclinical in *Bos indicus*, it is unknown whether BIV causes Jembrana disease in *Bos javanicus*.

Methods

Virus and animal inoculations

Two female banteng (*Bos javanicus*) calves, 8 and 10-months old, obtained from the San Diego Wild Animal Park, and two male Holstein (*Bos taurus*) calves, 3 to 4 months of age, were used. All animals were tested and found free of antibodies to bovine leukemia virus (BLV), bovine syncytial virus (BSV), bovine immunodeficiency virus (BIV), and bovine viral diarrhoea virus (BVDV). All animals were also negative for BIV by PCR to *gag* and *pol*. All animals were housed in containment facilities for a month prior to inoculation. Both bantengs and one Holstein calf were used as principals and the other Holstein calf was held as a mock-infected control.

The BIV FL112 isolate was grown in primary foetal bovine lung (FBL) cell cultures in roller bottles and tested for BVDV (performed by Dr Steve Bolin, National Animal Disease Center, Ames, IA). Inoculum from all of the roller bottles was combined and each principal received an equal quantity, approximately 30 mL of cells and cell culture media, in a combination of subcutaneous and IV inoculation in and around the jugular vein on the neck. The control calf was inoculated in the same way using cells and cell culture medium from the same passage of FBL cell cultures that were not infected with BIV.

Calves were bled 10 and 3 days preinoculation, the day of inoculation, and multiple times after inoculation. The WBC counts were determined with an automated cell counter. Blood smears were stained with a Hemal Stain (Hemal Stain Inc, Danbury, CT) and then counted to quantitate the number of polymorphonuclear cells and mononuclear cells. The absolute number of mononuclear cells was used for determination of absolute numbers of cell subtypes by multiplying total numbers by the percentage of each subtype as determined by flow cytometry.

Preparation and analysis of cells by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from blood as previously described (Whitmire and Harp 1991). For flow cytometric analysis, cells were incubated with primary monoclonal antibody to bovine lymphocyte surface antigens (Table 1), followed by incubation with secondary antibody (Table 1) conjugated to FITC or PE. Cells were analysed with a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) using FACSII software. Five thousand events were analysed from each sample. Data are presented as the mean $\pm$ SEM percentage of the total number of cells in the lymphocyte gate expressing a specific antigen. The lymphocyte gate was set according to standard light scatter properties of bovine lymphocytes. Cells used for sorting were FITC-labelled then sorted using an EPICS 752 cell sorter (Coulter Corporation, Hialeah, FL) (performed by Dr Kristi Harkin, Department of Zoology and Genetics, Cell and Hybridoma Facility, Iowa State University, Ames, IA). Between 30 000 and 300 000 cells were collected for each population examined with purity of cell populations estimated at > 95%.

Proliferation assays

PBMC were isolated from bovine blood and lymphocytes were assayed for proliferative response to mitogens as previously described (Whitmire and Harp 1991), with minor modifications. Proliferation

Table 1. Primary monoclonal antibodies and secondary anti-species antibodies used for flow cytometric analyses of bovine lymphocytes.

Receptor	Clone Number	Source
CD3	MM1A	VMRD[1]
CD4	GC50A1	VMRD
CD8	BAQ111A	VMRD
N2	BAQ4A	VMRD
N12 (TCR1)	CACT61A	VMRD
WC1	IL-A29	ILRAD[2]
Act 2	CACT26A, CACT77A	VMRD
IL-2R	CACT108A	VMRD
B cell (B4)	BAQ155A	VMRD
MHCII	TH14B	VMRD
Monocyte (M7)	BAQ151A	VMRD
anti-murine IgG-FITC	polyclonal	Caltag[3]
anti-murine IgM-PE	polyclonal	SBA[4]
anti-murine IgG1-FITC	polyclonal	SBA
anti-murine IgG2a-PE	polyclonal	SBA

1 VMRD, Pullman, WA, USA.
2 ILRAD, Nairobi, Kenya.
3 Caltag, South San Francisco, CA, USA.
4 SBA (Southern Biotechnology Associates), Birmingham, AL, USA.

data were measured as the mean counts/minute of incorporated radiation/well ± SEM.

Serological assays

Serum samples were tested for antibodies against BIV, BLV, and BSV by Western blots as previously described (Whetstone et al. 1990).

Virus isolations

Virus isolation was attempted at 1, 3, 5, 7, 9, 11, 15, 18, 21, 24, 29, 32, 35, and 41 days PI from all calves. Blood samples were collected in EDTA blood tubes and centrifuged. The buffy coat layer was removed and residual red blood cells were lysed by washing in distilled H_2O and 2X Hanks solution. Washed WBCs were cocultured with FBL cell cultures, and blind-passaged weekly until CPE was observed. Negative cultures were examined for 10 blind passages, with fresh low passage FBLs being added once during this period of time.

PCR methods

A nested PCR test targeted to the surface envelope, *env*, and the reverse transcriptase, *pol*, genes of the BIV genome was used as previously described (Suarez et al. 1995)

Statistical methods

WBC counts from all principals and control were examined to determine the peak WBC count and the largest difference between groups. Peak days were examined using a Student T-test analysis by comparison of the principals with preinoculation blood samples from the same animals and by comparison with a control animal that was sampled from the same day. Differences in mononuclear cell subtypes were examined by comparing absolute numbers of each subtype for control and principals on each day of sampling using the student T-test.

Results

All principals were infected with the FL112 isolate of BIV. Virus was isolated in first passage cell cultures by 7 days PI from all BIV principals, and confirmed by PCR. The sham-inoculated control remained negative for virus isolation throughout the experiment. PCR analyses of peripheral WBCs detected BIV proviral DNA by 3 to 5 days PI in all principals. The Holstein principal developed detectable antibodies to BIV p26 by day 14 PI and to other BIV polypeptides by 8 weeks PI; the two bantengs did not develop detectable antibodies specific to BIV by necropsy at 8 weeks PI; and the sham-inoculated control Holstein remained negative for antibodies to BIV.

In the Holstein principal, a transient increase in total number of WBC was observed that peaked at 14 days PI. Differential WBC counts showed that the primary cause of the increase was an increase of mononuclear cells and that polymorphonuclear cell numbers remained generally unchanged. Flow cytometry analyses showed that the increase in mononuclear cells was accompanied by an increase in the numbers of B cells in peripheral blood of infected compared with control calf, beginning day 10 PI, peaking at day 14 PI, and returning to control levels by the end of the study. This difference was statistically significant ($P<0.05$) at day 14 PI when comparing either the average of the presamples to the principal or a separate analysis of the principal with the control. A concomitant increase in numbers of cells expressing major histocompatability class II (MHC II) antigen was also seen, probably due in part to the increased numbers of B cells. Changes in other mononuclear cell subtypes, including CD3+ (T cells), CD4+ ($T_{helper\ cells}$), and CD8+ ($T_{cytotoxic/suppresser}$ cells) were not significant.

In the banteng principals, there was no increase in total WBC numbers following infection with BIV. Flow cytometry analyses (Table 2) showed that, pre-inoculation, bantengs had a higher percentage of null

cell populations (N2, γ/δ TCR, and IL2R) and a lower percentage of monocytes and MHCII positive cells than the Holstein. After infection, bantengs showed a decrease in the null cell populations (N12, N2 and γ/δ TCR), and no increase in MHCII or B cell populations.

Table 2. Flow cytometry analyses.

	Holstein (n = 1)		Banteng (n = 2)	
Cell subject	PRE	PI	PRE	PI
CD3	50–70%*	NC	60–70%	NC
CD4	15–25%	NC	14–20%	NC
CD8	12–22%	NC	9–12%	NC
N12	25–30%	NC	25–40%	15–25%
N2	25–30%	NC	40–70%	25–40%
γ/δ-TCR	25–30%	NC	40–70%	25–40%
IL2R	20–25%	NC	40–55%	NC
MHCII	20–35%	40–80%	10–20%	NC
B CELL	10–20%	15–60%	8–15%	NC
MONOCYTE	8–20%	30–35%	1– 4%	NC

* Percentages are the range of samples taken days −10, −3, and 0 preinoculation, and days 9, 11 and 15 postinoculation.

Lymphocyte proliferative responses to the mitogens concanavalin A and pokeweed mitogen showed no significant differences in the response of cells from principal compared with control calves at the individual time points tested.

Discussion

In this study, banteng were infected with BIV, as shown by virus isolation and PCR, but did not develop either clinical signs of Jembrana disease or a lymphocytosis usually associated with BIV infection in *Bos taurus*. Interestingly, banteng did not develop detectable antibodies to BIV by 8 weeks PI, similar to what has been described for JDV infection in Bali cattle (Hartaningsih et al. 1994).

Although BIV and JDV are both bovine lentiviruses, they are not identical genetically (Chadwick et al. 1995). Experimental studies have shown that JDV causes milder clinical disease in the F1 offspring, *Bos javanicus* × *Bos indicus*, and is subclinical in *Bos indicus* (Soeharsono et al. 1995). These data indicate that not only are the two bovine lentiviruses different, but also the pathology of JDV is dependent on the genetic background of the infected host. BIV is subclinical in *Bos taurus* (Carpenter et al. 1992; Suarez et al. 1994), causing lymphoproliferative changes including a transient lymphocytosis and follicular hyperplasia of regional lymph nodes. The study presented here suggests that BIV may have even less effect in Bali cattle, again suggesting that the genetic background of the infected host may be significant in determining the outcome of the bovine lentivirus infection.

This study further shows that *Bos javanicus* differ from *Bos taurus* in the normal makeup of their lymphocyte subsets in that the percentages of monocytes are much lower in bantengs than Holstein and the percentages of null cells are much higher. This may have implications in the basic immune response to pathogens.

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Studies of Bovine Lentivirus Infection in Sheep

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Abstract

Six yearling sheep were inoculated intraperitoneally with peripheral blood from two sheep infected with the bovine immunodeficiency-like virus (BIV) strain R29. An additional five sheep served as sham-inoculated controls. Of the six sheep given BIV, five seroconverted and one remained seropositive for the duration of the study. The polymerase chain reaction demonstrated BIV provirus in three of five serologically positive animals. BIV exposure was associated with significant but transient increases in neutrophils, eosinophils, B cells, $CD2^+$ cells, $CD4^+$ cells, and $CD4^+$:$CD8^+$ ratios. There were no significant associations between BIV exposure and deficits in antibody production to chicken ovalbumin and canine red blood cells, nonspecific lymphoproliferative responses to concanavalin-A, lipopolysaccharide, and pokeweed mitogen; specific lymphoproliferative responses to ovalbumin and tuberculin purified protein derivative; or cutaneous delayed type hypersensitivity to tuberculin purified protein derivative. Significant lymphoid hyperplasia was found micoscopically one year post-inoculation.

BOVINE immunodeficiency-like virus (BIV) belongs to the family of lentiviruses and shares antigenic, genetic, and structural similarities with the human immunodeficiency virus (HIV) type I (Gonda et al. 1987; Braun et al. 1988; Jacobs et al. 1992). The first isolation of BIV, designated BIV R29, was from an emaciated cow with lymphadenopathy and lymphocytosis. In initial transmission experiments, BIV R29 caused emaciation, weakness, non-persistent lymphocytosis, mild lymphoid pneumonia, lymphadenopathy, and mild perivascular lymphoid cuffing in the brain (Van Der Maaten et al. 1972). BIV R29 has now been propagated in tissue culture over many generations. In one study (Carpenter et al. 1992), calves inoculated with the virus propagated in vitro showed transiently increased lymphocyte counts and follicular hyperplasia at 5–6 weeks after inoculation. In other circumstances, the virus propagated in vitro did not cause lymphocytosis or lymphadenopathy (Suarez et al. 1993c). Differences between studies may have resulted from contamination of early viral inocula with bovine virus diarrhoea virus or possible attenuation caused by prolonged culture (Garvey et al. 1990; Carpenter et al. 1992; Flaming et al. 1993; Suarez et al. 1993c). No association between BIV and disease has been proved, but BIV seropositivity has been associated with a significant decrease in milk production (McNab et al. 1994).

It was thought that sheep would be a convenient model for the study of a ruminant lentivirus and might be more susceptible than cattle to the effects of BIV, since lentiviruses may display greater pathogenicity when entering a new species. Sheep are susceptible to experimental infection with BIV; however, sheep showed a delayed anti-BIV antibody response and small volumes of blood were incapable of transmitting infection between sheep, unlike cattle, suggesting that sheep may be relatively resistant to BIV infection (Whetstone et al. 1991). Sheep pastured with BIV-infected cattle may become infected (Bouillant and Archambault 1990) and an apparent natural infection has been reported in sheep (Smith and Jacobs 1993). The author reviews the findings of studies in sheep as previously reported (Smith et al. 1994; Jacobs et al. 1994; Jacobs et al. 1996).

Materials and Methods

Two yearling mixed-breed sheep were housed together in an isolation unit. The sheep were negative for antibodies to bovine leukemia virus, maedi

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visna virus, bovine syncytial virus, and bovine viral diarrhoea virus using routine methodologies and bovine immunodeficiency-like virus (BIV) using Western blot analysis (WBA) as described previously (Jacobs et al. 1992). The sheep had been dewormed and were repeatedly parasite-free, based on faecal analysis by flotation and Baerman protocols. Peritoneal macrophage formation was stimulated by inoculation with 180 mL of sterile 4% aqueous sodium thioglycolate solution intraperitoneally (i.p). A similar protocol was used to establish chronic HIV-1 infection in rabbits (Felice et al. 1988). After three days the sheep were inoculated i.p. with 180 mL of BIV (R29)-infected Madin-Darby bovine kidney (MDBK) cells and supernatant (kindly donated by Dr J. Black, American BioResearch Inc., Milton, Tenn.). Four months after the inoculation of these two sheep, 11 sheep (virus negative, parasite-free) were inoculated i.p. with 180 mL of sterile 4% aqueous sodium thioglycolate. Three days later, 1200 mL of blood was drawn from each of the two BIV-inoculated sheep in 200 mL aliquots. This blood was inoculated i.p. into six sheep such that each sheep received a total of 400 mL of pooled whole blood. The five remaining sheep served as controls such that 400 mL of anticoagulated whole blood was drawn from each sheep with each sheep receiving i.p. 400 mL of whole blood from another member of the control group.

Serology and hematology

Serological reactivity to BIV was determined by WBA (Jacobs et al. 1992). Short-term cultures of peripheral blood mononuclear cells or co-cultures of mononuclear cells with foetal bovine lung (FBL) cells were used to attempt virus isolation and to demonstrate reverse transcriptase activity. A nested polymerase chain reaction was used to demonstrate provirus as described previously (Suarez et al. 1993a,b). Complete blood counts were carried out and lymphocyte subpopulations were quantified using a battery of monoclonal antibodies as described elsewhere (Jacobs et al. 1994).

Humoral immune responses

At 10 months post-inoculation (p.i) with BIV, sheep were vaccinated with chicken ovalbumin (cOVA) and canine red blood cells (cRBC) as previously described for cattle and pigs (Glass et al. 1990; Mallard et al. 1990). Immune responses were measured in blood on days 0, 14, 21, and 30 using an ELISA and direct hemagglutination procedures as described elsewhere (Smith et al.1994).

Cell mediated immune responses

At 10 months p.i., non-specific lymphocyte stimulation to lipopolysaccharide (LPS), pokeweed mitogen (PWM), and concanavalin-A (con-A) was tested (Stirtzinger et al. 1986; Larsen 1979). The sheep were sensitised to tuberculin by the intradermal injection of BCG and delayed type hypersensitivity (DTH) was tested in vitro by the cutaneous reaction to PPD. Specific lymphocyte stimulation to cOVA and PPD was measured in vitro on days 0, 14, and 30 of the immunisation schedule (Glass et al. 1990; Paul et al. 1977).

Histopathological examination

One year after inoculation, the sheep were killed by a barbiturate overdose and complete necropsies were performed. Subjective and semiquantitative analyses were performed on the spleen and various lymph nodes (Jacobs et al. 1996).

Results

The culture supernatant derived from BIV-infected MDBK cells had RT activity between two and five times the activity found in control cultures. The first two sheep inoculated seroconverted by four weeks p.i. and remained seropositive. Five of the six sheep given whole blood from the two BIV-inoculated sheep seroconverted by four weeks p.i. However, four of these five sheep became seronegative at times varying from four weeks to six months p.i. One sheep did not seroconvert until six weeks p.i., but thereafter its reactivity was persistent. The remaining sheep had consistently high background staining that precluded the detection of specific anti-BIV responses. None of the sheep given whole blood developed anti-BVDV antibodies.

Virus isolation was unsuccessful and no blood cell cultures had significantly increased reverse transcriptase activity. BIV provirus was demonstrated by nested PCR in the two sheep initially inoculated with BIV-infected MDBK cells and at six months p.i. in three of the five sheep that seroconverted.

There were no significant changes in weights, rectal temperatures, or respiratory rates associated with BIV exposure. BIV-exposed sheep had transient significant increases in neutrophils and eosinophils and tended to have increased numbers of lymphocytes and monocytes at five to six months p.i. At two months p.i., BIV-exposed sheep had significant increases in B, $CD2^+$, and $CD4^+$ cells and $CD4^+$:$CD8^+$ ratios.

All the sheep responded to cOVA and cRBC. Both groups of sheep responded similarly to the

cRBC while the BIV-exposed showed a decreased anti-cOVA antibody response at 30 days after initial immunisation but the difference was not significant. Non-specific lymphocyte responses to Con-A, PWM, and LPS and the specific reactivity to cOVA did not differ between the two groups of sheep. Sheep exposed to BIV tended to have lower in vitro lymphocyte responses to PPD at 30 days p.i.; however, the difference was not significant. There was no difference in DTH responses to intradermal injection of PPD.

Mild lymphadenopathy was found in the BIV-exposed sheep at necropsy. There were no other gross findings in either group of sheep and histopathological changes were only found in the lymphoid organs of BIV-exposed sheep. Numbers of splenic periarteriolar lymphoid sheaths and secondary follicles in hilar and prescapular or popliteal lymph nodes were all significantly increased in those sheep exposed to BIV, as also was medullary sinus cellularity in prescapular or popliteal lymph nodes. The increased cellularity in lymph node medullary sinuses of the BIV-exposed sheep was due variably to lymphocytes, macrophages, and plasma cells. Lymph node post-capillary venules and transluminal post-capillary cellular traffic did not appear to differ in quantity or appearance between the two groups.

Discussion

The two sheep inoculated with BIV-infected MDBK cells became persistently infected since they seroconverted, remained seropositive, and had detectable proviral DNA in their peripheral blood leukocytes. Five of the six sheep given whole blood intraperitoneally from these two sheep seroconverted and three of them had detectable provirus, indicating that the blood transmission was successful.

The persistent anti-BIV antibodies in one sheep and the transient anti-BIV antibodies in the remaining four sheep indicated at least transient viral replication. Sheep appear to be relatively resistant to BIV infection compared to cattle, but persistent infection of some sheep has been achieved previously, as judged from the continuous presence of anti-BIV antibody (Whetstone et al. 1991). The biological behaviour of retroviruses suggests that the transiently seropositive sheep were infected but provirus was only temporarily expressed. The detection of BIV provirus by PCR in three of five sheep that seroconverted supports this reasoning. The phenomenon of transient seropositivity has been previously observed in experimentally and naturally-infected cattle (Whetstone et al. 1990; Issacson et al. 1993; Suarez et al. 1993b). Within months of infection, the BIV genome may become quiescent or cryptic, so that even the minute quantities of viral proteins needed to sustain an immune response are not produced. In the present study, BIV could not be re-isolated and in previous studies its isolation was exceedingly difficult (Whetstone et al. 1991). Even in persistently infected cattle, the frequency of BIV-infected cells in peripheral blood is exceedingly low (Carpenter et al. 1992) likely explaining the poor success rate of viral isolation from this tissue compartment. In some other lentiviral systems, persistently infected individuals have no antibody and no demonstrable provirus in peripheral blood mononuclear cells even using sensitive DNA amplification procedures; the lentiviral infection appears compartmentalised to more central lymphoid organs such as lymph nodes and spleen (Weiss 1993). Humans with acquired immune deficiency syndrome may become HIV-1 seronegative, possibly due to crippling of the immune system in the late stages of the disease. However, BIV-infected ruminants do not show signs of immune deficiency, suggesting that a paucity of BIV-infected cells in circulation, compartmentalisation of BIV-infected to central lymphoid organs and other uncharacterised host factors are responsible for the latency.

There was no evidence of weight loss, fever, or opportunistic infections due to BIV exposure and no signs of overt disease. The only significant haematological changes observed in the BIV-exposed sheep were mild transient increases in neutrophil and eosinophil counts. The possibility of subclinical infections was unlikely since there were no supportive clinical changes or lesions at necropsy. Although eosinophilia may result from parasitic infestation, the negative results from repeated faecal analyses do not support this etiology; there were no gross or microscopic lesions attributable to parasites.

The significantly increased numbers of B cells and $CD4^+$ cells and $CD4^+$:$CD8^+$ ratios in the BIV-exposed group of sheep may simply reflect an anti-BIV immune response. The results of immune function tests were similar to those previously reported for sheep and other ruminants (Glass et al. 1990; Stirtzinger et al. 1986; Larsen 1979; Paul et al. 1977; Burrells and Wells 1977; Burton et al. 1989). Although BIV-exposed sheep tended to have decreased anti-cOVA antibody responses and lower lymphoproliferative responses to PPD, none of the changes was significant. Due to the inherent variability in immune function tests, particularly those aimed at quantifying cell mediated immune responses, larger numbers of animals may be needed to prove a mild alteration in activity.

At one year p.i., BIV-exposed sheep showed mild but significant lymphoid hyperplasia. Both T- and B-dependent areas of lymphoid organs were affected; in the spleen, the numbers of periarteriolar lymphoid sheathes were increased and lymph nodes had significantly greater numbers of secondary follicles. Viral characteristics or differences in antigen-handling between central and peripheral lymphoid organs may be important determinants in the location of certain immune responses. Researchers may have been less able to determine lymph node paracortical cellularity than follicle frequency, thus decreasing the chances of detecting a mild change in paracortical cellularity. Other studies of BIV-inoculated calves have not revealed hyperplasia of T-dependent areas (Van Der Maaten et al. 1972, 1990; Carpenter et al. 1992); however, these studies characterised only the early events after infection. In the present study, degenerative lymphoid changes, characteristic of the transiton from latent infection to immunodeficiency in other lentiviral systems, were not found. It appears that if BIV is capable of causing such changes it will require more than a year to do so.

The sheep in this study were housed in isolation and were not exposed to many of the pathogens or stresses that sheep raised under natural conditions might encounter. Considering the prolonged period from exposure to the development of disease seen with most lentiviral infections, it was not unexpected that there were only mild, transient haematological changes in BIV-exposed sheep in the first year after exposure. Because of the difficulties in isolating BIV, most groups involved in BIV research have used the original R29 isolate. This strain has been passaged in vitro over many generations and has probably undergone mutations, possibly some affecting pathogenicity. Finally, BIV may have existed in cattle for a long period and become adapted to this host. If so, it might be expected to be more pathogenic in a different host, such as sheep, as has occurred when other lentiviruses have crossed species boundaries. However, the experiments presented here did not demonstrate this phenomenon.

Acknowledgments

This research was supported by a Strategic Grant from the Natural Sciences and Engineering Research Council of Canada and grants awarded through the Ontario Ministry of Agriculture, Food, and Rural Affairs and the Dairy Farmers of Ontario. The following researchers participated in the projects reviewed here: B. Jefferson, B. Mallard, L.J. McCutcheon, H.E. Smith, D.L. Suarez, V.E.O. Valli, and C.A. Whetstone.

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Immunological Responses in Cattle (*Bos taurus*) After Inoculation with BIV

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Abstract

Bovine immunodeficiency-like virus (BIV) was first isolated in 1972 (Van Der Maaten et al. 1972). Much work has been done on the molecular characterisation of BIV using the original BIV R29 isolate; however, R29 is believed to be cell culture attenuated since it no longer causes either mononuclear cell number increases or detectable enlargement of lymphatic nodules in experimentally-infected cattle. The host cell tropism and changes in host peripheral blood lymphocyte populations following infection with BIV are unknown.

Recently, the authors isolated and characterised a field isolate of BIV, FL112 (Suarez et al. 1993), that causes a transient, mononuclear cell lymphocytosis in experimentally-infected cattle. Cattle were inoculated with BIV FL112 and, using flow cytometry, it was shown that BIV causes a B cell lymphocytosis with no consistent, significant changes in other mononuclear cell populations including CD3+, CD4+, and CD8+ cells. Lymphocyte proliferative responses to the mitogens concanavalin A and pokeweed mitogen showed no significant differences in cells from principal compared with control calves. Cell sorting and PCR amplification showed that BIV is pantropic. Proviral DNA was present in CD3+, CD4+, CD8+, monocyte, B-cell, and WC-1 (γ/δ T cells, null cells) cell populations by 3–5 days postinoculation (PI) and also at 2.5 years post-inoculation.

THE bovine lentivirus, also known as bovine immunodeficiency-like virus (BIV), causes a persistent viral infection of cattle and appears to be widespread in the world cattle population (Suarez et al. 1994). The prevalence of viral infection is unknown, but serological evidence of infection varies widely when examining different herds, and it has been hypothesised, but not proven, that BIV is more prevalent in dairy than beef cattle (Amborski et al. 1989). The pathological effect on cattle has not been determined, although anecdotal reports have linked BIV to encephalitis, clinical immunodeficiency, and lymphoproliferation (Gonda et al. 1994). The only evidence for a deleterious effect of BIV comes from an epidemiological study that links BIV to lower milk yields in seropositive cattle (McNab et al. 1994). Difficulty in diagnosing BIV with currently described diagnostic tests makes the determination of the true role of BIV in cattle disease difficult (Suarez et al. 1994).

BIV was first isolated from an 8-year old dairy cow in Louisiana. The first isolate, R29, when used for experimental inoculation of cattle, caused a transient increase in mononuclear cell numbers approximately two weeks after inoculation and a smaller mononuclear cell increase several months later (Van Der Maaten et al. 1972). Enlargement of subcutaneous lymphatic nodules also was reported. The R29 isolate was the only BIV isolate available for study for more than 20 years and during that time it was cultured in multiple cell types with varying numbers of passages (Suarez et al. 1994). Evidence

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of attenuation, including absence of either mononuclear cell increases or detectable lymphatic nodules in cattle, was reported in recent studies of the R29 isolate (Carpenter et al. 1992). In contrast, low passage BIV isolates from Florida, FL112 and FL491, caused an early mononuclear cell increase when used in experimental inoculations (Suarez et al. 1993). The FL112 isolate caused a larger mononuclear cell increase than the closely related FL491, and neither isolate affected polymorphonuclear cells. However, neither isolate appeared to cause the late mononuclear cell increase or produce palpable enlargement of subcutaneous lymphatic nodules.

Several studies done in cattle attempted to assess changes in immune function with varying results including: decreased (Martin et al. 1991) and increased (Flaming et al. 1993) lymphocyte blastogenesis, and depressed (Onuma 1992) or unaffected (Rovid et al. 1995) monocyte function, and decreased (Flaming et al. 1993) neutrophil function. In all of those studies, cattle were inoculated with the attenuated R29 strain of BIV, often contaminated with a noncytopathic strain of bovine viral diarrhoea virus (BVDV) (Carpenter et al. 1992; Suarez et al. 1993). Moreover, most of the studies were done with small numbers of principals, sampling was not conducted in a temporal fashion, groups of cattle from different experimental studies were compared, and controls were not always used. These are possible explanations for conflicting reports in the literature on the effects of BIV on the bovine immune system.

Examination of changes of cell populations in peripheral blood has enhanced the general understanding of several immunodeficiency viruses, particularly the human immunodeficiency virus (HIV) and the feline immunodeficiency virus (FIV). Infection with either of these viruses results in a decline of the relative and absolute number of CD4+ cells in the peripheral blood accompanied by a decrease of the T_{helper} cells immune response. Loss of T_{helper} cells is thought to play an important role in the clinical immunodeficiency that is observed in HIV and FIV (Bendinelli et al. 1995; Hoffman-Fezer et al. 1992; Levy et al. 1993). Cell tropism also plays a role in HIV infection. Use of the CD4 molecule as a receptor for HIV attachment is important in its targeting of T_{helper} cells (Levy 1993). The receptor for FIV has not been determined, but it is not the CD4 molecule. FIV has a broader cell tropism than HIV, and can infect CD4+, CD8+ and Ig+ cells (Brown et al. 1991; English et al. 1993).

The cell tropism and effect of BIV on different white blood cell populations are unknown. A single experimental study of cattle infected with an R29-derived isolate of BIV did not show any significant changes in mononuclear cell subtype numbers when compared with control cattle (Flaming et al. 1993). Since the FL112 BIV isolate produces a mononuclear cell increase in cattle after experimental inoculation, it was selected for the following studies designed to determine possible changes in host peripheral blood lymphocyte populations following infection and to investigate BIV host cell tropism.

Methods

Virus and animal inoculations

Ten male Holstein calves, seronegative to the known bovine retroviruses in the USA, including BIV, bovine leukemia virus (BLV) and bovine syncytial virus (BSV), were housed in containment facilities in pairs by random selection, and inoculated between 8 and 12 weeks of age. Eight calves were used as principals and two calves were used as mock-infected controls. All calves were tested and found negative for BIV by PCR (Suarez et al. 1995) before being inoculated with the FL112 isolate.

The BIV FL112 isolate was grown in primary foetal bovine lung (FBL) cell cultures and tested for BVDV (performed by Dr Steve Bolin, National Animal Disease Center, Ames, IA). Each principal received approximately 30 mL of cells and cell culture media, subcutaneous and intravenous, and control calves were inoculated using cells and cell culture medium from the same passage of FBL cell cultures not infected with BIV.

Calves were bled 10 days preinoculation, the day of inoculation, and multiple times after inoculation. WBC counts were determined with an automated cell counter, blood smears were stained and counted for differentials, and the absolute number of mononuclear cells was used to determine absolute numbers of cell subtypes by multiplying total numbers by the percentage of each subtype as determined by flow cytometry.

In order to identify the specific cell subtypes infected by BIV both immediately after infection and after long term infection, two additional experiments were carried out. In one experiment, two calves were age matched, tested, housed as described above, and inoculated using the BIV FL112 isolate. Blood samples were taken on days -3 and 0 preinoculation and on days 3, 6, 9, 11, and 28 postinoculation (PI) with BIV. Cells were labelled for CD3, CD4, CD8, WC1 (null cell), monocyte, and B cell populations, analysed and sorted by flow cytometry, and used as template in a nested PCR procedure. In a second experiment, two cattle, one inoculated with FL112 and the other with FL491, were examined 2.5 years PI.

Preparation and analysis of cells by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from blood as previously described (Whitmire et al. 1991). For flow cytometric analysis, cells were incubated with primary monoclonal antibody to bovine lymphocyte surface antigens (Table 1), followed by incubation with secondary antibody conjugated to FITC or PE, then analysed with a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA) using HPLYSISII software. Cells used for sorting were FITC-labelled then sorted using an EPICS 752 cell sorter (Coulter Corporation, Hialeah, FL) (performed by Dr Kristi Harkin, Department of Zoology and Genetics, Cell and Hybridoma Facility, Iowa State University, Ames, IA). Between 30 000 and 300 000 cells were collected for each population examined with purity of cell populations estimated at ≥ 95%.

Table 1. Monoclonal antibodies used for flow cytometric analyses of bovine lymphocytes.

Receptor	Clone Number	Source
CD3	MM1A	VMRD[1]
CD4	GC50A1	VMRD
CD8	BAQ111A	VMRD
N2	BAQ4A	VMRD
N12 (TCR1)	CACT61A	VMRD
WC1	IL-A29	ILRAD[2]
Act 2	CACT26A, CACT77A	VMRD
IL-2R	CACT108A	VMRD
B cell (B4)	BAQ155A	VMRD
MHCII	TH14B	VMRD
Monoctye (M7)	BAQ151A	VMRD

[1] VMRD, Pullman, WA.
[2] ILRAD, Nairobi, Kenya.

Proliferation assays

PBMC were isolated from bovine blood and lymphocytes were assayed for proliferative response to mitogens as previously described (Whitmire and Harp 1991).

Serological assays

Serum samples were tested for antibodies against BIV, BLV, and BSV by Western blots as previously described (Whetstone et al. 1990).

Virus isolations

Virus isolation was attempted at 7 days PI from all calves. Blood samples were collected in EDTA blood tubes, centrifuged, the buffy coat layer removed, and residual red blood cells were lysed by washing in distilled H_2O and 2X Hanks solution. Washed WBCs were co-cultured with FBL cell cultures and blind-passaged weekly until CPE was observed. Negative cultures were examined for 10 blind passages, with fresh low passage FBLs being added once during this period of time.

PCR methods

A nested PCR test targeted to the surface envelope, *env*, and the reverse transcriptase, *pol*, genes of the BIV genome was used as previously described (Suarez et al. 1995).

Statistical methods

The WBC counts from all principals and controls were examined to determine the peak WBC count and the largest difference between groups. Principals were compared with preinoculation blood samples from the same animals and with control animals that were sampled from the same day. Differences in mononuclear cell subtypes were examined by comparing absolute numbers of each subtype for controls and principals on each day of sampling using the student T-test analysis.

Results

Eight calves were infected with the FL112 isolate of BIV. Virus was isolated in cell culture at 7 days PI from all BIV principals. Sham-inoculated controls remained negative for virus isolation throughout the experiment. PCR analyses of peripheral WBCs detected BIV proviral DNA as early as day 3 PI in four principals and in all principals by day 7 PI. Six of the eight principals developed detectable antibody to p26 by 14 to 21 days PI. No detectable antibody specific to BIV was detected in two calves that were necropsied at day 10 PI nor in the control calves at any time.

A transient WBC increase that peaked at 14 days PI was observed in the principals. Differential WBC counts showed that the primary cause of the increase was an increase of mononuclear cells and that polymorphonuclear cell numbers remained generally unchanged. Analyses of mononuclear cell subtypes showed that the increase in mononuclear cells was primarily a result of an increase in the numbers of B cells in peripheral blood of infected compared with control calves, and was statistically significant ($P>0.05$) at day 14 PI when comparing either the average of the presamples to the principals or a separate analysis of the principals with the controls.

There was a concomitant increase in numbers of cells expressing major histocompatability class II (MHC II) antigen, probably due in part to the increased numbers of B cells. No statistically significant changes were seen in other mononuclear cell subtypes, including CD3+ (T cells), CD4+ (T_{helper} cells), and CD8+ ($T_{cytotoxic/suppresser}$ cells) on day 14 PI.

There were no significant differences in the response of cells from principal compared with control calves at the individual time points tested in lymphocyte proliferative responses to the mitogens concanavalin A and pokeweed mitogen.

In the four additional calves that were inoculated with BIV in separate experiments, PCR detected proviral DNA in all cell types that were examined by day 7 PI and also at 2.5 years PI. Flow cytometry showed that the two calves that received BIV FL112 had increases in B cell and major histocompatibility class II cell populations after inoculation, similar to what was described for the other eight principals. Previous data from the two cattle infected for 2.5 years showed these animals had a mononuclear cell increase day 10 to three weeks PI.

Discussion

The validity of whether BIV causes a transient lymphocytosis was in doubt because it could not be reproduced in experimental inoculations with the R29 virus. However, a likely cause of the discrepancy may be attenuation of the R29 isolate because recent experiments with the new Florida field isolates confirmed the early transient lymphocytosis event (Suarez et al. 1994). Prior to the study reported here, the lymphocytosis was described as a mononuclear cell increase (Suarez et al. 1993; Van Der Maaten et al. 1972), but the identity of the affected cell population(s) remained unknown. The present study establishes that the mononuclear cell increase is predominantly B cells. Other lymphocyte subtypes were relatively unaffected during the time period studied.

Although the B cell increase in BIV inoculated calves accounts for most of the mononuclear cell increase, it does not explain the total increase in cells that express MHC Class II proteins. Other cell types must have also had an increased MHC II expression. Cats experimentally infected with FIV express MHC II proteins not only on B cells, but also on CD8+ and CD4+ cells (Rideout et al. 1992; Willet et al. 1993). A similar situation may occur in BIV infections but that possibility was not fully examined.

The B cell proliferation observed with BIV is different from what has been described with other agents. Although BIV is present in B cells, the virus does not appear to have a predilection for infecting only B cells. Therefore, B cell proliferation could be a direct result of the BIV infection or an indirect effect produced by infection of other cell types. Possible direct mechanisms include BIV acting in a superantigen role (Scott-Algra et al. 1994), or through a B cell proliferation signalling motif (YXXL/I)2 (Beaufils et al. 1993). The motif, present in bovine leukemia virus and Epstein Barr virus infections is thought to play a role in the B cell proliferation (Beaufils et al. 1993), sometimes leading to a B cell lymphoma. On the other hand, B cell proliferation might be a secondary response to a BIV-induced cytokine production in other infected cell types. The cause of BIV-induced B cell expansion was not determined in this experiment. It is also uncertain if the expansion is of a monoclonal or polyclonal nature. Although polyclonal B cell proliferation also has been reported in HIV-infected patients, the mechanism of this stimulation has not been determined (Levy 1993).

In this study, lymphocyte proliferative responses to mitogens concanavalin A and pokeweed mitogen were not significantly different between principals and controls. Previous studies have reported both increased (Flaming et al. 1993) and decreased (Martin et al. 1991) lymphocyte blastogenesis as well as reports of depressed neutrophil function (Flaming et al. 1993), depressed monocyte function (Onuma et al. 1992), and no change in monocyte function (Rovid et al. 1995). All of those studies were conducted using the attenuated R29 strain of BIV, often contaminated with a noncytopathic strain of BVDV (Carpenter et al. 1992; Suarez et al. 1993). In the study reported here, a low passage, field isolate of BIV, FL112, that has been shown to be free of contaminating BVDV (Suarez et al. 1993), was used and all principals and controls were sampled in a consistent, temporal fashion.

An important issue concerning BIV is the determination of what cell types are infected. Nested PCR analyses of six different cell types [CD3, CD4, CD8, B-cell, monocyte, and WC1 (null cell)], sorted by flow cytometry, showed that BIV infects a wide variety of cell types. Proviral DNA was observed in WBCs in calves by 5 days PI in all six cell populations, and as early as 3 days PI in most cell types. The same cell populations were also positive for proviral BIV DNA in samples obtained from cattle infected for 2.5 years. Although the FACS does not sort with 100% efficiency, cells were sorted for both leukocyte antigen markers and for size, and the results observed were consistent both in calves studied early PI, when PCR is at the limit of detection, and after long term infection. It is unlikely that

these cell surface markers are the receptors for BIV binding in cattle because FBL cells, which do not phenotypically express any of these cell surface markers when examined by flow cytometry analysis, readily support replication of the R29 and Florida isolates (Flaming and Suarez pers. comm.).

The data reported in this study clearly show that BIV induces a B cell proliferation early after infection and, unlike immunodeficiency viruses such as HIV and FIV, does not cause remarkable depletion of CD4+ cells. These data also show that infection with BIV is not limited to a select lymphocyte subset population, but rather can be detected in a wide range of lymphocytes both immediately and long-term after infection. While BIV, a lentivirus, shares genetic similarities with several immune deficiency causing lentiviruses, current immunological data from cattle experimentally infected with BIV do not support the hypothesis that BIV is an immune deficiency causing virus.

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Molecular Studies on BIV Infection and its Interaction with Other Bovine Viruses

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Abstract

Bovine immunodeficiency virus (BIV) is a bovine lentivirus that infects animals world-wide. The authors have cloned the BIV env and gag proteins and several sub-unit peptides spanning the entire coding regions of the two proteins as fusions to the bacterial *trp*E protein and expressed them in *Escherichia coli* (Spindler et al. 1984). These recombinant proteins can readily be expressed but the expression levels of these peptides varied. When these proteins were tested against a panel of bovine sera, only sera from BIV-infected animals reacted specifically. One major antigenic determinant was identified in the cartoxyl terminus of the p26 gag protein and another was identified in the transmembrane (TM) domain of the envelope glycoprotein.

The pathogenesis of the BIV infection has not been well characterised. Experimentally-infected animals did not develop immunodeficiency. It is possible that co-factors may play a role in enhancing the pathogenesis of BIV infection; and one of these co-factors could be bovine herpesviruses (BHV). BHV-1 co-infection of BIV-infected cells enhanced BIV replication and an immediate early gene of BHV-1 is involved. This herpes-viral gene product acts on the BIV promotor element to stimulate BIV expression. In vivo co-infection of animals with BIV and BIV-1 demonstrated that these viruses can interact in vivo, with BHV-1 reactivated BIV expression in infected animals.

BOVINE immunodeficiency virus (BIV) is a lentivirus, originally isolated from cattle with lymphocytosis, lymphadenopathy, neuropathy and progressive emaciation (Gonda 1992; Van Der Maaten et al. 1972). However, overt clinical disease in seropositive cattle is rare and the infection is difficult to reproduce experimentally (Carpenter et al. 1992; Flaming et al. 1993; Suarez et al. 1993; Whetstone et al. 1972). BIV antibodies have been detected in beef and dairy cattle in the USA, some European countries, Australia and New Zealand (Amborski et al. 1989, Horner 1991; Horzinek et al. 1991; St. Cyr Coats et al. 1994; Whetsone et al. 1991). However, serological screening of randomly-selected cattle sera for BIV antibody has shown a non-uniform distribution. Sera from eastern or northern parts of the USA are rarely positive for BIV, while approximately 4% of the sera from southern and southwestern regions are BIV positive (Black 1990) and average frequencies with individual herds in the south are considerably higher. For example, 40% of beef and 60% of dairy herds were found to be positive in Louisiana (Gonda 1994), and the seroprevalence of BIV among cattle herds in Mississippi was greater than 50% (St. Cyr Coats et al. 1994).

To screen for naturally-infected cattle, different serological approaches have been employed, including immunofluorescence assays (IFA), Western blot assay (Whetstone et al. 1972, 1991) and enzyme linked immunosorbant assay (ELISA) (St. Cyr Coats et al. 1994). Although these tests were able to detect BIV antibodies using BIV antigen or BIV-infected cells, they lacked sensitivities and required a large mount of native viral antigens. Since BIV can only be propagated well in primary bovine cell cultures and there is a lack of a continuous cell

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line expressing high levels of BIV, the use of native viral proteins for large scale serological testing of BIV is quite difficult. In addition, the IFA and ELISA tests can yield false positive results as compared with Western blot assay (Atkinson et al. 1992). Therefore, there is a need to use recombinant proteins as a source of antigens. Recombinant BIV proteins have been expressed both in bacterial, insect cells and eukaryotic cells (Rasmussen et al. 1990), and the bacterial recombinant gag and env proteins have been used to detect anti-BIV antibodies by Western blot analyses (Atkinson et al. 1992; Chen et al. 1993). These recombinant proteins should be an important source of antigens for large scale seroprevalence screening.

Studies on HIV have demonstrated that several human herpesviruses, such as herpes simplex type 1 (Mosca et al. 1987) and HHV-6 (Horvat et al. 1989), can be potential co-factors in HIV infection and the interactions between HIV and these viruses have been documented. BLV, BVDV, and BHV-1 are common infections in cattle and cause great economic losses to the cattle industry (Cockerell et al. 1992; Gibbs and Rweyemamu 1992; Xue et al. 1990). Studies have demonstrated that BIV and BHV-1 can interact in vitro, similar to what occurred between HIV and human herpesvirus (Geng et al. 1992). An investigation of BIV prevalence and its relationship to other viral infections will provide valuable information in understanding the pathogenesis of BIV infection.

Cloning and Expression of BIV *Gag* and *Env* Genes

BIV-infected animals develop strong antibody responses to various structural proteins like the capsid and the envelope glycoprotein. Antibodies against p26 capsid protein developed earliest (22 days post-infection) and strongest (up to 38 months post-infection) in calves that were experimentally infected by BIV (Whetstone et al. 1990). Therefore, recombinant capsid and envelope proteins can be used as an antigen source to detect animals infected by BIV. The authors used the pATH (protein amendable for making tryptophan hybrid) expression vector which uses the *trp* promoter to express the BIV *gag* and *env* genes. The cloned genes can be expressed as fusions to the trpE protein. The strategy centres on cloning and expression in bacteria of defined regions covering the entire *gag* and *env* coding regions using restriction enzyme deletions of the viral genome. The expressed proteins will then be tested for their reactivities with bovine sera obtained from animals that were infected by BIV. For the *gag* gene, six different clones spanning various regions of the *gag* open reading frame were generated (Fig. 1). These recombinant proteins, especially gag-2, gag-3, gag-4, and gag-5, can be expressed in high quantity and the average yields of the fusion proteins were about 1 mg of 70–80% pure protein harvested from about 200 mL of bacteria. Analysis of the various express gag proteins with a panel of immune bovine sera

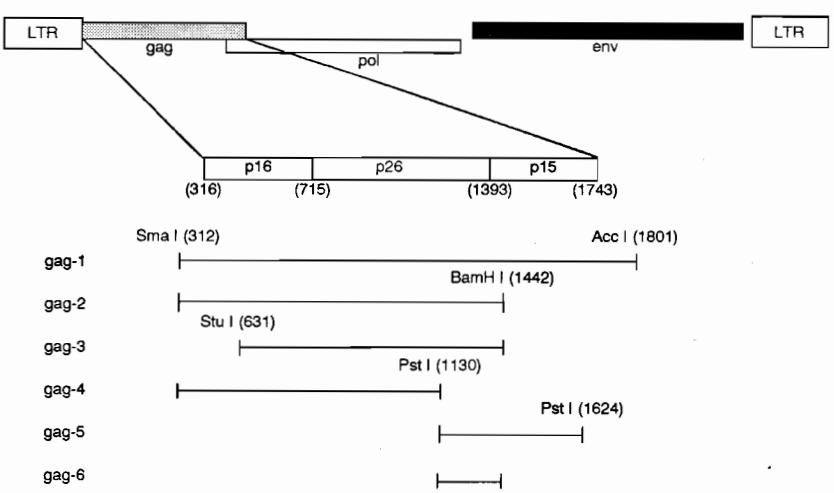

Figure 1. Summary of BIV *gag* constructs. Thick horizontal bars represent the relative genomic organisation of the *gag*, *pol* and *env* coding regions of BIV. Large open box represents the unprocessed $p53^{gag}$ precursor protein with nucleotide positions given below in parentheses. Thin horizontal bars represent coding regions contained within each *gag* construct. Numbers above in parentheses represent the nucleotide positions where specific restriction enzymes cut to generate the specific construct.

enabled identification of at least one major antigenic determinant which can be recognised by all sera tested. This determinant was encoded within the 47 kDa gag protein expressed by the gag-6 construct.

Similar strategy was used to generate nine different recombinant clones that span the various regions of the BIV envelope open reading frame (Fig. 2). These proteins were again expressed as fusions to the trpE protein in *E. coli*. The levels of these expressed proteins varied, some expressed at large quantity that can be detected easily by SDS polyacrylamide gel electrophoresis while others cannot. The recombinant env protein that had the strongest and most consistent reactiion with BIV-infected bovine sera was the protein expressed by env-8. This clone encodes a 134 amino acid peptide that represents the amino terminal region of the transmembrane domain of the envelope glycoprotein. It is interesting that a major antigenic determinant of HIV has also been mapped to a similar location on the HIV-1 gp41 transmembrane glycoprotein (Windheuser and Wood 1988).

Use of Recombinant Proteins to Screen for BIV Seroprevalence

Accumulated serological screening data on BIV seroprevalence in the USA showed a non-uniform distribution (Gonda et al. 1994). Very high seroprevalence was found in the southern United States, e.g., Louisiana and Mississippi (Gonda 1992; St. Cyr Coats et al. 1994), while cattle in the eastern or northern part of the USA are rarely positive. In central USA states, such as Colorado, the seroprevalence rate was about 21%. These differences

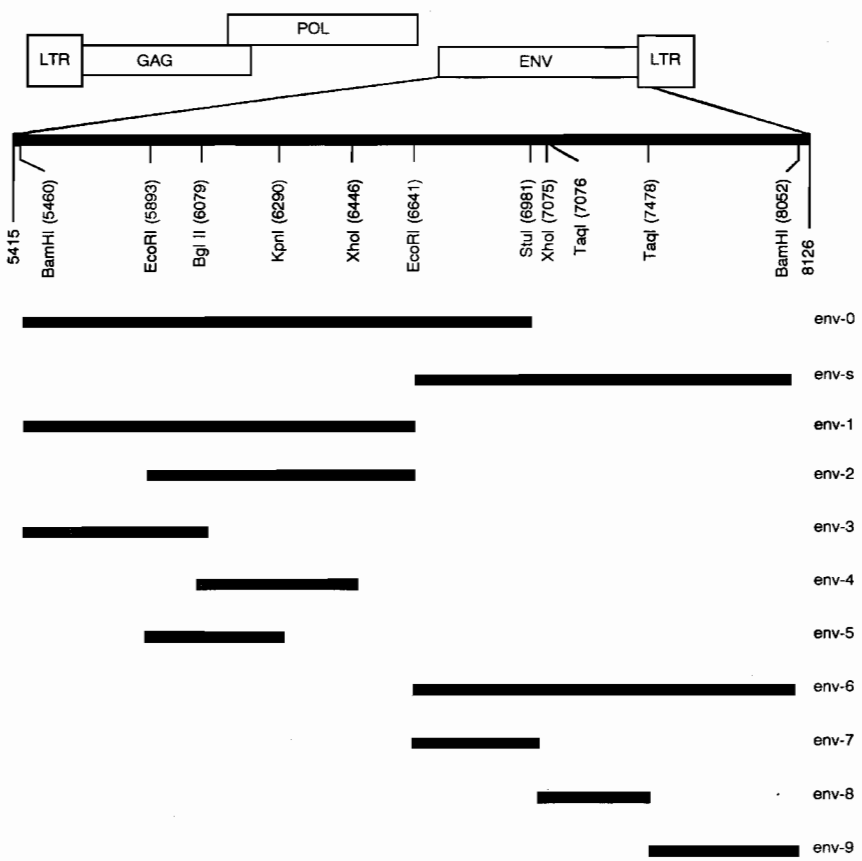

Figure 2. Schematic representation of various inserts of the pATH expression clones which cover most of BIV *env* ORF. The solid line represents the insert of BIV *env* sequence cloned in frame with the *trpE* gene using the indicated restriction enzyme sites. Their nucleotide positions are indicated according to the sequence published by Garvey et al. (1990).

could be due to differences in infection rate or due to the different assays or the source of antigens used in these studies. Because the recombinant BIV proteins can detect BIV antibodies specifically, reproducibly and are extremely sensitive, they were used to survey for the seroprevalence of BIV in cattle from other states in the central USA, such as Kansas. A collection of 155 sera from different parts of Kansas was tested with recombinant BIV gag protein in Western blot assays. Out of the sera tested, 23 were BIV positive (14.84%) (Table 1). The results are consistent with what was observed previously with the Colorado dairy herd based on an ELISA assay (Cockerell et al. 1992).

Table 1. Seroprevalence of BIV in Kansas cattle.

Town or cities	Number tested	Number positive (%)
El Dorado	11	1 (9.08%)
Kansas City A	24	5 (20.8%)
Kansas City B	19	4 (21.05%)
Kansas City C	30	4 (13.33%)
Kinsbury	6	0 (0.00%)
Manhattan	10	1 (10.00%)
Salina	4	1 (25.00%)
Williamsburg	25	5 (20.00%)
Other cities	26	2 (7.69%)
Total	155	23 (14.84%)

Although BIV infections have been reported in the USA, in European countries, Australia and New Zealand, the seroprevalence of BIV in some parts of Asia and of China is not known. In recent years, China has been importing both beef and dairy herds from various European countries and the USA, so it is possible that some of these imported cattle and their progeny may be infected by BIV. It is also possible that domestic herds in China may also be infected by BIV. In order to determine the seroprevalence of BIV, recombinant gag and env proteins were used in Western blot analyses to screen several imported herds and their progeny in the Tianjin area. In addition, domestic herds from several northern provinces of China were also screened. Positive samples were confirmed by PCR analyses using primers that specifically amplify the reverse transcriptase gene of BIV. The screening results are shown in Tables 2 and 3. Of the 689 imported cattle screened from two separate areas, 16 were found to be positive by Western blot and PCR. This represents a 2.32% seropositive rate. To further study the seroprevalence of BIV in other domestic herds in the northern part of China, 754 serum samples were collected from different counties of Zhangjiahou area (Table 3). Twenty-nine samples were found to be positive by Western blot analysis, and confirmed by PCR analyses. The 3–4% seropositive rate is similar to that reported in the south and southwestern regions of the US. However, more samples from various parts of China need to be tested to determine if the seropositive rate varies, as is the case in different geographical locations in the US.

Table 2. Western blot analysis of BIV infection in Tianjin area of China.

Origin of samples	Total number tested	Positive number	Positive rate %
Progeny in Area A	180	4	2.22
Progeny in Area B	509	12	2.36
Total	689	16	2.32

Table 3. Serological survey of BIV in different parts of Zhangjiahou area of China.

Area	Testing number	Positive number	Positive rate
Kangbao County	309	14	4.5%
Chabei Farm	21	2	9.5%
Chongli County	52	2	3.8%
Wei County	132	2	1.5%
Guyuan Farm	76	2	2.6%
Chicheng County	100	4	4.0%
Guyuan County	64	3	4.7%
Total	754	29	3.8%

Interaction between BIV and Bovine Herpesvirus-1 (BHV-1)

Numerous studies (Horvat et al. 1989) have demonstrated that exogenous viral infection, such as human herpesvirus, could be an important co-factor in HIV activation and pathogenesis. It is also possible that animal lentiviruses, like BIV, can also interact with animal DNA viruses to play a role in pathogenesis. These exogenous infections may also be responsible for activation of BIV replication in infected animals. In vitro studies have been conducted to determine the possible interaction between BIV and BHV-1. BIV-infected embryonic bovine lung (EBL) cells were

superinfected with varying doses of BHV-1. Reverse transcriptase (RT) in the culture supernatant was measured to quantitate the levels of BIV expression. Results showed that when BIV-EBL cells were infected with BHV-1, RT activity was detected earlier and at a higher level. The increase is proportional to the amount of herpesviruses added, indicating that there is an increase of BIV expression when herpesviruses were present.

Similar activation of the HIV LTR has been observed with HSV-1, and the IE gene products ICPO and ICP4 have been demonstrated to mediate such activations (Margolis et al. 1989). Because BHV-1 IE gene products BICPO and BICP4 share homology with the HSV-1 IE gene products, tests were conducted to determine if the BHV-1 IE gene can transactivate BIV LTR-directed gene expression.

A p601 fragment was tested that encodes the entire BHV-1 IE gene locus for activation of the BIV-LTR (Fig. 3). When the BHV-1 IE gene fragment p601 was transfected into EBL in the presence of BIV-CAT, only a very moderate (1.7 fold) activation of the BIV LTR was observed. Since p601 fragment contains both BICPO and BICP4 open reading frames, it is possible that they may affect the transactivation function of each other. BICPO and BICP4 were found to share a single promoter, and the mRNAs are generated by differential splicing (Wirth et al. 1991). In order to distinguish their effects on the BIV LTR, partial deletions were made to generate several deletion clones of p601. The p601–D1 has truncated the BICPO gene and p601–D2 has truncation in the BICPO gene (Fig. 3). The results indicated that only p601–D2, which contains an intact BICPO gene, could significantly transactivate BIV LTR. It has thus been shown that the IE gene product BICPO is a potent transactivator of co-infecting viruses like BIV. Since very little is known about the functions of BICPO, its transactivation of the BIV promoter will provide a well-characterised system to study the molecular mechanism involved in BICPO transactivation, whether BICPO acts by binding directly to a viral promoter, or indirectly via other cellular factors.

Due to the in vitro interaction between BHV-1 and BIV, it will be important to determine the seroprevalence of BIV and BHV-1 to determine whether BIV infection could be associated with BHV-1 infection. This study was conducted with 155 serum samples collected from the Kansas herds. In the cases of BIV and BHV-1 antibody detections, 13 of 155 (8.93%) serum samples were both BHV-1 and BIV seropositive. Seventy-five of 155 (48.38%) were BHV-1 seropositive while BIV negative. Statistical results indicated that BHV-1 seroactivities occurred independently of BIV infection (Table 4). There was no association between BIV and BHV-1 ($P>0.05$ in both cases). The results indicate that BIV infection did not clearly affect BHV-1 prevalences and BHV-1 infections did not significantly change the incidence of BIV infection. However, a larger panel of serum samples from different geographical locations should also be tested to determine the relationship between BIV and BHV-1 infection.

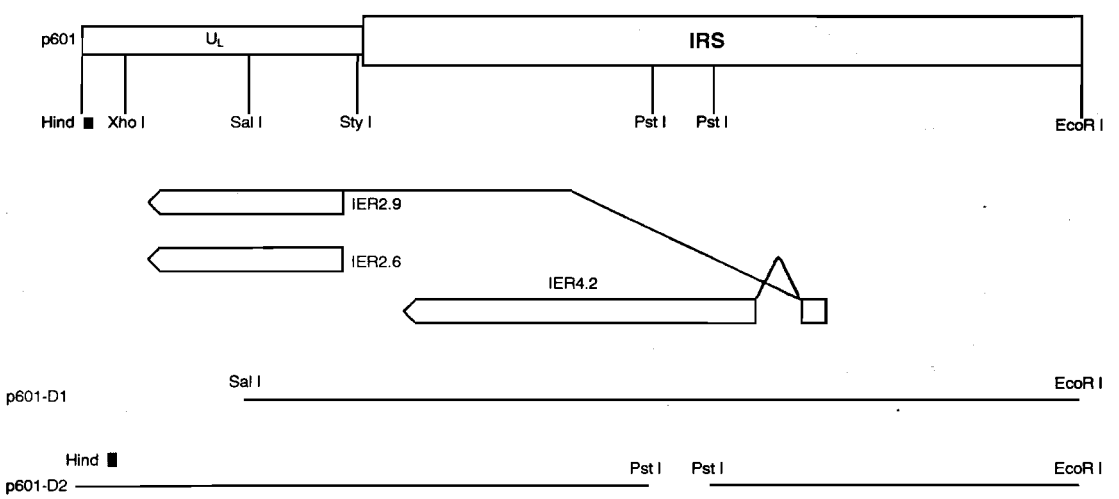

Figure 3. The gene map and partial deletions of p601. Sequences retained in the truncated plasmids are represented by solid lines. p601-D1 contains the complete ORF of IER4.2 (BICP4) and p601-D2 contains the complete ORF of IER2.9 (BICPO).

Table 4. Serological prevalance of BIV and BHV-1.

	BIV positive	BIV negative	Total
BHV-1 positive	13 (8.39%)	75 (48.38%)	88 (56.77%)
BHV-1 negative	10 (6.45%)	57 (36.77%)	667 (43.42%)
Total	23 (14.84%)	132 (85.16%)	155

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Expression of Recombinant Jembrana Disease Virus Env Protein in *Escherichia coli*

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Abstract

Jembrana disease virus (JDV) is a lentivirus belonging to the *Retroviridae* virus family, and contains envelope structural proteins essential for virus entry and replication. The Env protein consists of two components and the region cloned in this study contains major immunodominant and neutralisation domains of particular interest. A polymerase chain reaction (PCR) procedure was used to amplify the relevant regions from cDNA clones and form chimeric plasmids with a commercially available TA-cloning vector for PCR products. Recombinant plasmids for protein expression were constructed with the bacterial expression system, pGEX (Pharmacia). The glutathione-*S*-transferase fusion protein product was expressed for the ectodomain of the transmembrane (TM) component of Env in *Escherichia coli*. This was demonstrated by Western immunoblot using serum from a naturally-infected Bali cow (*Bos javanicus*). The product was purified by affinity chromatography for further use in immunological studies. The *E. coli*-expressed JDV-TM may be useful for trial as a vaccine as previous studies of other lentivirus recombinant TM protein have shown a protective neutralisation quality.

THE aetiology of Jembrana disease, Jembrana disease virus, is a lentivirus belonging to the *Retroviridae* family of viruses. Retrovirus genomes consist of three open reading frames, gag, pol and env among others. The envelope glycoprotein is located on the surface of the mature infectious virion and is involved in virus attachment and entry to the host cell. Env consists two functional units that are associated by non-covalent bonds. The surface unit (SU) is responsible for virus attachment and tropism via functional domains within its loop ultrastructure. The transmembrane unit (TM) anchors the env complex to the virion lipid membrane and facilitates membrane fusion allowing virus entry to the host cell. The aim of this study was to use recombinant techniques to produce recombinant JDV Env proteins for use in further immunological studies

The JDV *env* open reading frame was analysed by computational analysis, firstly by looking at the 'antigenic index' indicated by the MacVector™ program. Regions of high predicted antigenicity were compared with those reported for other lentiviruses. The region of JDV-TM chosen for expression utilised the naturally occurring *EcoR I* restriction enzyme site and resulted in a ≈300 bp product.

This region was determined to be best for study as it contained two important factors. The first is the fusogenic domain that is involved in membrane fusion, syncytium formation and entry to the host cell (Montelaro and Bolognesi 1995). Antibodies raised against this domain of HIV-1 have been shown to inhibit its function and thus stop infection (Wild et al. 1992, 1993, 1994; Earl et al. 1994). The second region, the principal immunodominant domain (PID), has in many lentiviruses been demonstrated to be one of the most immunogenic regions of Env (Pancino et al. 1994). It is predicted that the region of JDV-TM that was expressed in this study will also exhibit both of these useful properties.

The TM of JDV contains only three potential glycosylation sites, and these do not occur in the principal immunodominant or fusogenic domains (Chadwick et al. 1995). It was therefore considered an ideal candidate for bacterial expression, where there is no post-translational modification of proteins. Studies by Hartaningsih (1993) of JDV-infected cattle sera have shown the presence of antibodies against a ≈40 kDa protein, corresponding to the TM of the virus. A weak response was recorded against the SU region by Western immunoblot (Hartaningsih

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1993), suggesting the immunodominance of the JDV-TM region relative to that of SU.

There are many bacterial expression systems currently available, a vast majority using the bacteria, *Escherichia coli*. Most of these systems offer a unique purification procedure to yield high amounts of relatively pure recombinant protein. Commonly, expression plasmids contain a strong, inducible promoter to allow induction of high levels of protein synthesis. While selection with common antibiotics allows only the recombinant to be present in cultures, the fusion portion that is translated with the recombinant is fused to one terminus which consequently allows unique purification procedures. The GST-fusion expression system (Pharmacia) is one example of such a system where it involves cloning the protein coding region of interest in-frame with the glutathione-*s*-transferase enzyme. Expression of protein from the chimeric plasmid results in a polyprotein with the protein of interest fused to the GST-tag at the C-terminus. The GST partner allows detection and purification of recombinant proteins by immunological or colourimetric assays.

Expression of a portion of the external domain of TM, of apparent molecular weight 36 kDa, was achieved including the GST-tag. Recombinant JDV-TM was then purified by affinity chromatography utilising the GST-tag binding to a glutathione sepharose 4B matrix. Analogous regions of other lentivirus species have, however, been expressed in a variety of expression systems (Thorn et al. 1987; Kwang and Cutlip 1992; Chen et al. 1994; Calzolari et al. 1995; Cook et al. 1995; Pancino et al. 1995; Rosati et al. 1995). Western immunoblot studies showed the recombinant antigen was reactive with both GST-tag and JDV polyclonal antisera. Reactivity with JDV antisera was specific for the portion of JDV-TM expressed as no reactivity with the GST partner was observed.

Expression of the TM product of JDV will provide a valuable reagent for analysis as (1) a potential antigen for serological assays, particularly ELISA as described for JDV by Hartaningsih et al. (1993) and (2) as a potential vaccine for use in Indonesia.

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Cloning and Expression of Jembrana Disease Virus (JDV) in Yeast *Pichia pastoris*

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Abstract

The Jembrana disease virus (JDV) genes encoded for group antigen specific (*gag*) was cloned and expressed in the methylotropic yeast *Pichia pastoris* by placing the *gag* genes under control of the methanol inducible alcohol oxidase promoter. The JDV gag protein expressed intracellularly and recovered by breaking the cells with glass beads. The result of SDS-PAGE followed by Western blot analysis of the recombinant gag protein showed the reaction between gag protein and bovine anti-JDV sera.

JEMBRANA disease is an acute infectious disease of Bali cattle (*Bos javanicus*, syn. *Bos sondaicus*) in Indonesia. The disease can be readily transmitted to susceptible Bali cattle by the inoculation of tissue from infected cattle (Soeharsono et al. 1990). Jembrana disease virus (JDV) has recently been characterised as a retrovirus (Kertayadnya et al. 1993), and more recently as a lentivirus related to bovine immunodeficiency-like virus (BIV) on the basis of 598 bp of sequence data from a conserved region of the *pol* gene encoding the reverse transciptase (RT) protein (Chadwick et al. 1995).

The genome of JDV is 7732 in length, contains three major genetic elements characteristic of all retroviruses in the order of 5'-*gag-pol-env*-3' structural genes and flanking long terminal repeats (LTRs) as well as a number of accessory genes encoded for *vif*, *tat*, *ref*, and *tmx* (Chadwick et al. 1995; Tobin et al. 1994).

The *gag* genes are relatively conserved between JDV and BIV with 62% amino acid identity and primarily translated into 48.8 kD precursor polyprotein, which is subsequently processed by viral protease into mature gag protein of 14.3 kD matrix (MA) protein, 25.3 kD capside (CA) protein, 9.2 kD nucleocapsid (NC) protein (Chadwick et al. 1995).

The identification of JDV proteins is an important step toward obtaining a better understanding of the immune response of virus-infected animals and determining the function of individual proteins. Only one of the mature JDV proteins, the p26 CA protein was detected by bovine anti-BIV serum. The degree of amino acid conservation explains the serological cross-reactivity previously observed between capsid proteins of BIV and JDV (Kertayadnya et al. 1993). Antigenically, antiserum to BIV p26 cross reacts with p24 of HIV-1 and antiserum to equine infectious anaemia virus cross-reacts with p26 and p24 BIV (Suarez et al. 1993).

The env glycoprotein of JDV gp 90 is synthesised as a precursor polyprotein that is cleaved intracellularly into 50 kD surface unit (SU) and 42 kD transmembrane (TM) proteins (Chadwick et al 1995).

The methylotropic yeast *P. pastoris* has been developed as an efficient system for high level production of foreign proteins. A major advantage of this organism is the ease with which it can be grown in high density in a simple, defined medium. A number of proteins have been produced in *P. pastoris* in exceptionally high level secretion. The product may be in an almost pure form in the culture medium. HIV-1 env protein secreted from *P. pastoris* is hyperglycosylated and shows substantial proteolytic degradation (Scorer et al. 1993).

A clone of 2.9 kB JDV covering *gag* and part of the *pol* gene cloned in pGem-3zf was digested by *Eco*RI to obtain 2 kB which covered the whole *gag* and part of the *pol* and cloned into the *Eco*RI site of the *P. pastoris* expression vector, pHIL-D2 (*Pichia* Expression Kit, Invitrogen). Inserts were oriented by *Sal*I. The resulting plasmid was designated pHIL-D2/gag.

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The histidine requiring *P. pastoris* strain GS 115 (*his4*) was used in these studies. Media components (Bacto yeast extract, yeast nitrogen base, peptone, agar) were purchased from Difco. Dextrose, amino acids, PEG, sorbital were from Sigma. Yeast were grown in YPD medium at 30 °C prior to transformation, using electroporation in Gene-Pulser with Pulser Controller (BioRad). DNA for transformation was digested by *Not*I. His+ transformants were selected by plating on a defined minimal medium (RDB) lacking histidine. His+ transformants were checked for growth on media containing methanol as the sole carbon source (MM) compared to growth on media containing dextrose as sole carbon source (MD). Slower growth on MM compared to MD indicates disruption of the alcohol oxidase gene.

For protein analysis, 10 mL cultures were grown in MGY (minimal media containing yeast nitrogen base, biotin, and glycerol) for 2 days at 30 °C. Gag expression was then induced by changing the medium to MM (Minimal media containing yeast nitrogen base, biotin and 0.5% (v/v) methanol). Incubation was continued for 5 days with fresh methanol (10 × M) added on days 2, 4, 5, and 6. To replenish 10 mL cultures, 0.5 mL 10 × M (5 mL 100% ethanol in 95 mL water) solution was added, and the medium totally renewed on day 3 after induction. The cells were collected by centrifugation at 2000 × *g* and resuspended in 1 mL ice-cold breaking buffer containing 50 mM sodium phosphate (pH 7.4), 1 mM PMSF, 1 mM EDTA, and 5% glycerol (v/v). An equal volume of glass beads (0.5 mm diameter) was added and the mixture vortexed vigorously (8 × 30 s mixes) in a cold room. Cell breakage was monitored by light microscopy. The supernatant was centrifuged at 10 000 × *g* to remove debris and stored at −20 °C (Cregg et al. 1985, Cregg and Higgins 1995).

Protein samples were analysed by 12.5% SDS-PAGE followed either by Coomassie brilliant blue staining, or by immunoblotting using either monoclonal antibodies against JDV (mixture of Mab against p26 and Mab against p16 obtained from positively ELISA tested hybridoma culture supernatant against sucrose gradient-purified virus) or bovine anti-JDV positively ELISA tested sera produced from Bali cattle experimentally infected with JDV (Hartaningsih et al. 1994). Bound antibodies were visualised with horseradish peroxidase-conjugated anti-bovine IgG (Silenus) and 4-chloro-1-naphtanol horseradish peroxidase (BioRad) was used as a colorimetric substrate.

A recombinant plasmid pHIL-D2/gag was constructed, in which *gag* gene fragment recombined to pHIL-D2 cloning site in the right orientation after confirming with *Sal*I digestion. This recombination placed the cDNA under control of the alcohol oxidase 5' and 3' regulatory regions. Then *Not*I was used to separate vector sequences from yeast transcriptional units in order to promote homologous recombination into *AOX*1 gene. His+ transformants were obtained and screened for the production of gag on methanol induction. Gag protein was expressed intracellularly and recovered by breaking the cells with glass beads. The result of SDS-PAGE followed by Western blot analysis showed that crude preparation of the recombinant gag protein expressed intracellularly reacts with bovine anti-JDV sera. The p26 was detected together with larger uncleaved precursor proteins. The reaction could not be detected from gag protein against monoclonal antibodies. This could be due to incorrectly folded protein which does not react with monoclonal antibodies.

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Studies on bovine lentivirus infections in the U.K.

J. Brownlie[1]

BOVINE immunodeficiency-like virus (BIV) is one of eight characterised members of the sub-family *Lentiviridae* (Table 1) which share several common features. All viruses within the larger family of *Retroviridae*, to which the lentiviruses belong, are distinguished by the expression of the unique enzyme, reverse transcriptase, which facilitates transcription of infectious RNA into a complementary DNA copy. This viral DNA becomes incorporated into the cell nucleus as a provirus where it remains non-infectious and latent. Rescue of infectious virus from the provirus appears to depend on cell activation and the stimuli for this activation include an apparent legion of factors. The relevance of stress from crowding, long-distance transport and concurrent infection may be particularly important for the re-emergence of BIV from cattle latently infected with the virus.

The lentiviruses, apart from BIV, produce a clinical syndrome that is characterised by a gradual and progressive debilitation, which frequently leads to death. The incubation period for the development of disease varies but in most cases takes several years. By analogy, it would be exceptional if BIV were not to have a similar clinical syndrome but it may be unrealistic to find BIV overt disease with an incubation period of less than 3–5 years. It is the search for BIV-associated disease or rather the concern that such an associated disease remains unrecognised which has been considered important by some veterinary workers in Europe.

Central to the dilemma of bovine lentivirus research and investigation is that only one viral isolate has been available for study for the past 25 years. This isolate (R29), which was isolated from a cow with a wasting syndrome in the USA in 1969 (Van Der Maaten et al. 1972), has, over the years, been adapted to a range of laboratory cell lines and passaged many times. In the initial studies, Van der Maaten and others conducted acute experimental studies, thereby showing lymphoproliferative reactions with significant lymphadenopathy and persistent viraemia. Little clinical disease was observed in these studies, or any subsequent acute experimental study, with R29 virus. Longer term studies with R29 virus have only recently been concluded and preliminary reports do not reveal clinical lesions over a 5-year period although there is persistence of BIV in a variety of tissues (Johnstone, Heaton and Brownlie, in preparation).

A review of the potential of BIV to cause disease (Brownlie et al. 1994) has concluded that reliance solely on experimental studies with the R29 isolate as an indication of BIV pathogenesis could be misleading. It is clear that continual passage of any virus through cell culture can diminish virulence even to the point of establishing avirulent isolates suitable for live vaccine candidates. It is highly probable that present cultures of the R29 isolate have an apparent low in vivo virulence due to its multi-passaged history. Fortunately, two further wild-type BIV isolates have now been reported (Suarez et al. 1993). These were recovered from a group of cattle in Florida that were assessed clinically to be in poor health, whose carcasses were later rejected because of enlarged lymph nodes and in which their seropositivity to BIV was considerably elevated (C.A. Whetstone, J.M. Miller pers. comm.). Acute experimental infections with these isolates gave enhanced clinical lesions compared to the R29 virus studies (Suarez et al. 1993). From the European perspective, there is real interest in BIV and its potential to cause disease. Some limited studies from Europe are mentioned below.

The nature of the progressive pathology of all other lentiviruses has already been mentioned but, of greater relevance, are the recent reports of Jembrana disease, a fatal disease of Bali cattle due to the lentivirus designated Jembrana disease virus (Kertayadnya et al. 1993) (Table1). This is a virus closely related to BIV (Chadwick et al. 1995) that reveals major pathology in the lymphoid organs

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(Dharma et al. 1991). This work gives credance for bovine lentiviruses to act as pathogens in cattle although, in the case of JDV, the host range for the development of disease presently appears restricted to Bali cattle *(Bos javanicus)*.

Uncertainty still remains about the nature of BIV pathogenesis and whether there is an underlying immunodeficiency lesion. The present report illustrates some preliminary findings that analyse the cell tropism of BIV in an 8-week acute infection study in cattle *(Bos indicus)*.

Table 1. Designations of the eight characterised lentiviruses.

Lentivirus	Abbreviation
Equine infectious anaemia virus	EIAV
Maedi-visna virus of sheep	MVV
Caprine arthritis-encephalitis virus	CAEV
Bovine immunodeficiency-like virus	BIV
Jembrana disease virus of Bali cattle	JDV
Feline immunodeficiency virus	FIV
Simian immunodeficiency virus	SIV
Human immunodeficiency virus	HIV

European Studies

There is currently no European isolate of BIV. This has some relevance as emerging data suggest that variants of BIV, which are antigenically distinct from BIV R-29 and demonstrate immunological cross-reactivity only with the major capsid protein (CA) (p26) are present in Europe (Horzinek et al. 1991); this is also the case in the Eastern United States (Gonda et al. 1994). Limited surveys in Holland (Horzinek et al. 1991), Germany (Muluneh 1994), France (Polack et al. 1996) and United Kingdom have all demonstrated serological evidence that BIV exists, albeit at a low level in clinically normal herds. The serological testing used for these studies was, in the most part, dependant on a crude enzyme-linked immunosorbent assay (ELISA), an immunofluorescent assay (IFA) and Western blotting (WB). Western blotting provides a more accurate assay of BIV antibodies but again relies on antigen derived from R29 virus; it is a real concern that viral variants may fail to be detected. The absence of a sensitive and reliable assay for BIV has been a constraint in both epidemiological and research studies in Europe.

Although it has been stated that BIV poses no apparent risk for human health, concern that BIV contamination should be present in milk for human consumption has been raised. In a series of inactivation experiments of BIV-infected milk by normal commercial procedures of *High Temperature Short Time* (HTST), Venables and colleagues from the Central Veterinary Laboratories, UK have shown complete inactivation of viable virus (Veterinary Record 1996, in press).

Tropism of BIV

Although considerable advances have been made in describing the genome of BIV, several important biological questions remain unanswered. The in vivo tropism of BIV is not defined but the expectation is that an immunodeficiency virus would have the ability to infect and perturb the function of lymphoid cells. To this end, experiments have been initiated to examine BIV tropism in peripheral blood mononuclear cells (PBMC) of cattle. The experimental approach is worthy of mention as similar experiments could be undertaken with Jembrana disease virus.

Animals

Calves require housing separately inside secure isolation facilities. All calves need evaluating for normal health and screened to be free of BIV, bovine leukaemia virus, bovine syncytial virus, bovine herpes virus -1 and -4, bovine respiratory syncytial virus and bovine virus diarrhoea virus.

Virus inoculum

Virus stocks used throughout the study have been obtained from a cloned inoculum of R-29/1023 of the original R-29 isolate, kindly supplied by C.A. Whetstone (NADC, Ames, USA). Virus was grown in foetal bovine lung cells to obtain virus inocula of $5 \times 10^{3.75}$ $TCID_{50}$ / animal dose; administration is by intravenous injection and intranasal droplet instillation.

Virus Isolation and Characterisation

Virus isolation is performed by overlaying PBMC or MACS-sorted leucocytes onto foetal bovine cells in 24-well plates (Nunclon, Denmark) and incubating for 3 days at 37 °C in 5% CO_2. If no cytopathic effect (CPE) is observed, cultures are passaged for a further two times. With no cytopathic effect, syncytia or specific fluorescence after three passages, cultures are deemed negative.

Leucocyte Preparation

Blood samples are taken by veno-puncture and collected into universals containing heparin. Leucocytes are separated from the blood by density

gradient centrifugation above Histopaque (Sigma; density 1.083) by centrifugation for 45 minutes at room temperature at 1200 g. Peripheral blood mononuclear cells (PBMC) at the interface are aspirated and washed three times in phoshate buffered saline or RPMI medium.

Monoclonal Antibodies

Four murine anti-bovine leucocyte antibodies used in this study will select and identify the different leucocyte sub-populations. All were of the IgG isotype.

CC21 — (BoCD21) recognised the majority of B-cells in PBMC and tissues and also cells with dendritic morphology in lymphoid follicles (Naessens et al. 1990).

CC39 — (BoWCI) recognises γδ T-cells which are present in high numbers in young ruminants but decrease with age. Distinctive distribution particularly in lymphoid, alimentary and skin tissues (Morrison and Davis 1991).

CC42 — (BoCD2) recognises αβ T-cells thereby accounting for the majority of $CD4^+$ and $CD8^+$ in both tissues and in PBMC (Davis and Splitter 1991).

CCG33 — (CCG33) present on the majority of monocytes and macrophages. May be expressed on some B-cells but not yet fully characterised (Howard and Sopp, pers. commun.) .

Magnetic Activated Cell Sorting (MACS)

PBMC are incubated on ice for 30 min with 2 mL of monoclonal antibody directed at either B-cell, $WC1^+$, $CD2^+$, or $CD14^+$ epitopes. Cells are washed once with PBSA plus 0.5% bovine serum albumin (BSA-Sigma) and then incubated for 15 minutes at 4 °C in the same medium containing 20 μL rat anti-mouse IgG_1 coated superparamagnetic beads (Miltenyi Biotech GmbH, Germany) per 1×10^7 PMBC. Two further washes and the labelled cells are isolated by passage over a MiniMACS™ column following manufacturer's instructions. The magnetic-labelled cell suspension is loaded on to the MiniMACS™ column and the eluate collected as a 'negative' cell population. The column was washed three times with medium before the column was removed from the magnetic field and the 'positive' cell population collected. This cell population was counted and an aliquot taken for evaluation by one colour immunofluorescence flow cytometry using a FACScan (Becton Dickinson, USA). Purity of the separated leucocyte sub-populations should be greater than 95% and most cases 98–99% (Heaton 1996).

Reverse Transcriptase, Non-radioactive Assay

Supernatents from cell culture of individual leucocyte subpopulations were assayed for enzymatic activity using the Reverse Transcriptase Assay, non-radioactive (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. The assay relies on the ability of the RT enzyme to incorporate both digoxigenin (DIG) and biotin-labelled dNTPs into the same freshly synthesised DNA. Measurement of the incorporation is by an ELISA, quantitation of DIG by peroxidase-labelled anti-DIG antibody and a subsequent assay of peroxidase activity. The optimal conditions for the BIV RT are a preference for Mg^{2+} (Gonda et al. 1987) at a concentration of 8 mM (+/– 2 mM), pH 7.8, KCL concentration of 120 mM (+/– 40 mM) and 0.2% Triton X-100.

PCR assay

DNA from cell fractions was extracted by standard techniques using the Nucleon 1 genomic DNA extraction kit (Scotlab Bioscience, Coatbridge, UK). Primer design was based on the complete genome sequences of molecular clones from the R-29 isolate (Garvey et al. 1990). Two primers were designed to amplify a fragment at the 3' region of the *gag* open reading. Amplification by PCR was performed using a Hybaid Omnigene Programmable thermocycler (Hybaid Ltd, UK) with the manufacturer's instructions.

Southern Blotting

Detection of the specific *gag* sequence in extracted DNA fragments was carried out as described by Southern (1975). Transfer of separated DNAs from agarose gels to nylon membranes was left to proceed overnight and, following transfer, the membranes were baked at 120 °C for 40 min to permanently bind the DNA. Digoxigenin-labelled BIV-*gag* probes were synthesised (Heaton 1996) for use in hybridisation studies to target specific *gag* sequences on the transfer nylon membrane. Detection of bound BIV DIG-labelled probe was performed by chemiluminescence and recorded on RX X-ray film.

Experimental Design

Selected calves should be held in isolation and bled on three consecutive weeks prior to BIV inoculation to establish haematological and virological baseline parameters.

Calves are inoculated with BIV while control animals are inoculated with uninfected foetal bovine

lung cells. All animals were examined and bled weekly over an 8-week period following inoculation. The in vivo tropism of the virus for the different leucocyte sub-populations was examined at weekly intervals.

Results

Preliminary results of the in vivo tropism of BIV for different leucocyte sub-populations are by both RT activity and by PCR/Southern blotting of the DNA extracted directly from separated leucocyte sub-populations. BIV-associated activity should not be detected in the control calf at any time during the course of the experiment or in the pre-infection samples from inoculated calves. Viral quantitation by the detection methods used was not possible.

Discussion

Earlier studies have shown that BIV can infect a range of bovine cell types (Van der Maaten et al. 1972; Gonda et al. 1987; Pifat et al. 1992) canine and laprine origin in vitro (Bouillant et al. 1989; Gonda et al. 1990; Pifat et al. 1992). It can infect bovine monocytes (Onuma et al. 1992), in which it can cause functional disturbances (Rovid et al. 1995). Infection of other leucocytes subpopulations has not been reported.

The MACS-sorting system for obtaining purified leucocyte populations is a versatile and valuable technique that can be used in most laboratories around the world that have access to defined monoclonal antibodies and fresh leucocytes. Some caution may be considered expedient from concluding proof of cell tropism on the basis of MACS-sorting of purified leucocytes. When the purified leucocyte populations cannot be assured to be 100% purified, if only 99% pure, then the possibility will always exist that low level contamination of infected cells may account for the positive signal in both RT assays and in the PCR/Southern blotting. The strength of viral signal in both assays, particularly when obtained directly from separated populations, would indicate that the observation is a real finding. However, further studies on the separated populations, e.g., in situ hybridisation or limited dilution studies, would need to confirm these results.

Providing evidence for in vivo tropism of BIV for leucocyte populations may be original but there are examples of such tropism with other lentiviruses. FIV has a tropism for immunoglobulin-positive B-cells, $CD4^+$, $CD8^+$ and monocytes (English et al. 1993). HIV has been shown to infect $CD4^+$, macrophage and also B-cells (Montagnier et al. 1984). However, the unusual feature of a pan-leucocyte tropism for BIV is that other ruminant lentiviruses (MVV and CAEV) are reported to have a tropism restricted to the monocyte/macrophage series (Narayan et al. 1982; Hasse 1986). Confirmation of the BIV tropism may help in the definition of this virus within the immunodeficiency viruses. It may also be an indication of the tropism of the closely related Jembrana disease virus and may direct research in the derivation of suitable cell culture systems for its isolation and growth.

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Jembrana disease and Jembrana disease virus
Refereed papers

Adiwinata, A.T. 1968. Some informative notes on a rinderpest-like disease on the Island of Bali. Bull. Off. Int. Epiz., 68, 5–13.

Towards the end of 1964, an acute contagious disease broke out among the cattle and buffaloes in the district of Jembrana on the island of Bali. It was January 1965 before the disease was brought to the attention of the Veterinary Services of the island, when thousands of cattle already succumbed to the disease. The first samples of material for laboratory diagnosis of the disease were only received in April by the Veterinary Institute in Bogor. A tentative diagnosis of rinderpest was made and vaccination was commenced but the clinical and pathological lesions encountered differed from classical rinderpest. Brief descriptions of the clinical disease and the pathological lesions observed are provided. Attempts to reproduce the disease were successful in Bali cattle (obtained from Lombok) and the course of the clinical lesions induced seemed unaffected by the administration of tetracyclines (which was thought by field veterinarians to have some curative effect on Jembrana disease). Zebu crossbred cattle and descendants of Bali cattle reared in East Java appeared to be clinically refractive. Pigs of Bali origin as well as European crossbred pigs were not susceptible. Rabbits, guinea pigs, adult mice and unweaned mice showed no ill effects after inoculation with infective materials. The inoculum did not effect embryonated eggs but the formation of multinucleated giant cells in calf kidney tissue cultures was observed 9 days post-inoculation.

Budiarso, I.T. and Hardjosworo, S. 1976. Jembrana disease in Bali cattle [letter]. Australian Veterinary Journal, 52, 97.

Provides a brief history of the initial outbreak of Jembrana disease in Bali cattle between December 1964 and September 1967 during which an estimated 60 000 cattle died from the disease, and the subsequent attempt to control the disease by vaccination with lapinised rinderpest vaccine. Describes a second outbreak of the disease that occurred in the Tabanan district of Bali in April 1972. After examining 39 clinical cases and conducting seven post-mortem examinations, the authors concluded the clinical signs and pathological changes in the 1972 outbreak were typical of those that were detected in the initial 1964–1967 outbreak of the disease. The authors reported the detection of intracellular rickettsial-like particles in smears from lymph nodes and spleens of the affected cattle, and that they were able to inject tissue suspensions from the infected cattle intraperitoneally into male guinea pigs and induce a haemorrhagic and/or necrotic orchitis with a fibrino-haemorrhagic peritonitis. They reported that smears from exudate of tunica vaginalis of the guinea pigs stained with Macchiavello or Giemsa showed numerous intracellular organisms resembling rickettsia. The authors concluded that the disease was not rinderpest but was possibly a rickettsial disease.

Budiarso, I.T. and Hardjosworo, S. 1977. Some notes on Jembrana disease of Bali cattle. Hemera Zoa, 69, 94–102.

Clinical and pathological features of the disease in groups of cattle were described from outbreaks of unknown origin in Tabanan county, on the Island of Bali in 1972. These animals had an illness that represented a hitherto unknown clinical pathological entity to Indonesian veterinarians. Clinical signs consisted of anorexia, fever (40–42 °C), generalised lymphadenopathy, nasal discharge, hypersalivation and anaemia but retention of good body condition. At the height of the fever animals usually showed signs of constipation followed by diarrhoea or dysentery. Mucosal erosions were rare but 'blood sweating' occurred in most cases. Haemorrhages were most often observed in the vaginal mucosa, base of the tongue and occasionally in the anterior chamber of the eye. Central nervous system disturbances were rare. Gross pathological findings included generalised lymphadenopathy, particularly affecting the prescapular and prefemoral lymph nodes, which were enlarged up to 20 times normal size, and vascular damage as shown by haemorrhages throughout the body. The spleen was enlarged and three to four times normal size. Single or clusters of intracellular rickettsia-like organisms were readily seen in smears from hepatic lymph nodes or spleen and occasionally from superficial lymph nodes and blood smears of animals which had body temperatures of 40.5 °C or higher. The organisms was best demonstrated by Macchiavello or Giemsa stain. Histological examination found vasculitis and perivasculitis; the predominant inflammatory cells were mononuclear types including histiocytes. Giant cells were occasionally observed. Small granulomatous nodules were consistently seen in the liver, kidney and occasionally in the lungs. Lymphoid tissue changes were both hypertrophy and hyperplasia.

Budiarso, I.T. 1980. The pathology of Jembrana disease. Media Vet., 2, 60–71.

The pathological changes observed in Jembrana disease occurring in the Tabanan district of Bali in 1972 [the second recorded outbreak of the disease] are described. Gross lesions were observed in the skin (congestion of blood vessels in the subcutaneous tissue and foci of haemorrhages), lymphatic system (enlargement of lymph nodes and spleen), digestive tract (inflammation and loss of surface epithelium, and mucosal haemorrhages). Haemorrhages were detected in several organs. The histological changes involved primarily the vascular system and the most prominent lesions detected in several organs were haemorrhage, vasculitis, perivasculitis, thrombotic endotheliosis, and lymphoid hyperplasia. In lymph nodes and spleen, an absence of follicles and proliferation of lymphoblasts admixed with reticuloendothelial cells and plasma cells is described. The brain was relatively normal. Clusters of rickettsia-like organisms were readily observed in smears and biopsies of lymph node and spleen. These changes had close resemblance to those of bovine petechial fever (Ondiri disease). Inoculation of guinea pigs intraperitoneally with suspensions of spleen and lymph node caused scrotal reaction and peritonitis. Rickettsia-like organisms were detected in the inflammatory cells of the scrotal fluids.

Budiarso, I.T., and Rikihisa, Y. 1992. Vascular lesions in lungs of Bali cattle with Jembrana disease. Veterinary Pathology, 29, 210–215.

Morphologic examination of 75 female Bali cattle between 19 months and 4 years old affected with Jembrana disease consistently revealed pulmonary granulomatous vascular lesions. The lesion were diffusely distributed throughout the lung. The principal lesion was the presence of a large number of intravascular macrophages that filled the lumen of pulmonary veins and pulmonary arteries of a vascular diameter of 20–200 μm, excluding the rest of the blood cellular components. Concentric layers of perithelial cells also with plasma cells and macrophages were occasionally present around both veins and arteries. Infiltration of polymorphonuclear leukocytes or small lymphocytes was not seen. Destruction or necrosis of tissues or blood vessels was rarely seen. Because this vascular lesion was found in the lungs of all affected cattle examined, this change was useful for post-mortem diagnosis of Jembrana disease. Moreover, its presence could be used to distinguish Jembrana disease from malignant catarrhal fever and other lymphoreticular proliferative conditions that are frequently found among cattle in Indonesia.

Chadwick, B.J., Coelen, R.J., Sammels, L.M., Kertayadnya, G. and Wilcox, G.E. 1995. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. Journal of General Virology, 76, 189–192.

A description of sequence data representing 598 bp of the *pol* gene, amplified by PCR from viral cDNA using broadly reactive universal primers for lentiviruses. When the sequence data were compared with that of known lentiviruses and other bovine retroviruses, the closest alignment was with bovine immunodeficiency-like lentivirus (BIV), showing 74% nucleotide sequence identity. This confirmed that JDV is a lentivirus and that it is indistinguishable from BIV. The pathogenesis of Jembrana disease is most unusual for a lentivirus infection and differs markedly from that reported for BIV infection.

Chadwick, B.J., Coelen, R.J., Wilcox, G.E., Sammels, L.M. and Kertayadnya, G. 1995. Nucleotide sequence analysis of Jembrana disease virus: a new bovine lentivirus associated with an acute disease syndrome. Journal of General Virology, 76, 1637–1650.

The complete nucleotide sequence of the RNA genome of Jembrana disease virus (JDV) was reported. In addition to the *gag*, *pol* and *env* genes and flanking long terminal repeats (LTRs) that characterise all retroviruses, a number of accessory genes represented by small multiply spliced ORFs in the central and 3' terminal regions of the genome, including *tat* and *rev* that are typical of lentiviruses, were identified. The genome of JDV was 7732 bp in length, 750 bp smaller than the genome of bovine immunodeficiency virus (BIV) strain BIV127. A striking feature of the genome was the many deletions relative to BIV127, the largest of which were 471 bp from the *env* gene and 157 bp from the U3 (promoter) region in the LTR. There were also several insertions of up to 33 bp in the JDV genome relative to BIV127 found in the *env* gene and small ORFs that overlap *env*. Other significant genomic differences between JDV and BIV127 included changes to cis-acting sequences throughout the genome such as promoter and enhancer sequences in the LTR, the transactivation response region, splice sites and frameshift sequences; alterations to the gag precursor protein cleavage sites and thus the processed products; loss of the *vpw* and *vpy* ORFs; and amino acid changes in all coding regions. The significance of these changes was discussed in relation to the differences in pathogenicity between JDV and BIV.

Dharma, D.M.N., Budiantono, A., Campbell, R. S. F. and Ladds, P. W. 1991). Studies on experimental Jembrana disease in Bali cattle. III. Pathology. Journal of Comparative Pathology, 105, 397–414.

Experimental infection of Bali cattle with Jembrana disease caused a well defined pathological response. The disease was generalised except for the central nervous system which did not show any significant cellular reactive changes. Following a generalised lymphoreticular reaction in the first week (Phase 1), the predominant effect was an intense non-follicular proliferative response by reticulum (dendritic) cells and lymphoblastoid cells in lymphoid tissue (Phase 2) and a similar infiltrative and proliferative process in liver, kidneys, adrenal medulla and elsewhere. In the kidneys, glomerular hypercellular swelling occurred which was associated with uraemia. Pulmonary alveolar cells reacted strongly to infection by swelling and proliferation especially in the anterior lobes. Mononuclear cell infiltration accompanied this response. In the lymph nodes and spleen, a marked lymphoid follicular reaction and plasma cell formation developed from the fifth week of infection (Phase 3). The cytology and histological distribution of proliferative changes in the lymphoid system suggests that, during the acute phase of the disease, a predominantly T lymphocytic reaction takes place which may be associated with transient immunosuppression. Residual lesions occurred up to 60 days post-infection. In all affected tissues, pleomorphic basophilic intracytoplasmic inclusions occurred, especially from the second week until about the fifth week of infection, but persisted in small numbers for more than 2 months. By light microscopy, both minute basophilic granular forms (Type 1) and large intravacuolar (Type 2) inclusions were consistently found in reticular cells, lymphoblasts, macrophages including Kupffer cells, pulmonary alveolar cells and occasionally in vascular endothelium. These structures appear to be of diagnostic value.

Dharma, D.M.N., Ladds, P.W., Wilcox, G.E. and Campbell, R.S.F. 1994. Immunopathology of experimental Jembrana disease in Bali cattle. Veterinary Immunology and Immunopathology, 44, 31–44.

Sequential immunohistochemical studies of the lymphoreticular responses of Bali cattle after inoculation with Jembrana disease virus were carried out using the peroxidase-anti-peroxidase test for immunoglobulin-containing-cell assessment and the indirect immunoperoxidase test for lymphocyte subset assessment. The prevalence of immunoglobulin G-containing cells declined during the acute phase of the disease but became significantly elevated during convalescence. This trend was consistent with serological responses previously observed using an enzyme linked immunosorbent assay. Temporary immunosuppression appeared to occur during the acute phase of the disease as indicated by a decrease in the immunoglobulin G-containing cells in the lymphoid organs and an observed decrease in the BoCD4:BoCD8 lymphocyte ratio in lymph node follicles.

Hartaningsih, N., Wilcox, G.E., Dharma, D.M.N. and Soetrisno, M. 1993. Distribution of Jembrana disease in cattle in Indonesia. Veterinary Microbiology, 38, 23–29.

An enzyme-linked immunosorbent assay (ELISA) was used to detect antibodies to Jembrana disease virus in sera of cattle in Indonesia. Antibodies were detected in cattle from Bali island and some districts of Lampung (Sumatra), West Sumatra and East Java provinces but not in other areas of Indonesia. Clinical Jembrana disease or Jembrana-like diseases have been reported to occur in Bali cattle (*Bos javanicus*) in all four areas where antibody was detected and not in the other areas where antibody was not detected. The results indicate there has been only limited spread of Jembrana disease from endemic areas to adjacent areas.

Hartaningsih, N., Wilcox, G.E., Kertayadnya, G. and Astawa, M. 1994. Antibody response to Jembrana disease in Bali cattle. Veterinary Microbiology, 39, 15–23.

An enzyme-linked immunosorbent assay (ELISA) and an agar gel immunodiffusion (AGID) test are described which detected antibody against Jembrana disease virus in infected Bali cattle. Both tests were specific and did not detect antibody in cattle from areas where clinical Jembrana disease has not been detected. The ELISA detected antibody in all infected cattle and had greater sensitivity than the AGID which detected antibody in less than 50% of infected cattle at any single time after infection. The antibody response to the virus was delayed; antibody was not detected by ELISA in a majority of infected cattle until 11 weeks after infection and a maximum antibody response was detected 23 to 33 weeks after infection. Antibody was still detectable 59 weeks after infection.

Kertayadnya, G., Wilcox, G.E., Soeharsono, S., Hartaningsih, N., Coelen, R.J., Cook, R.D., Collins, M.E. and Brownlie, J. 1993. Characteristics of a retrovirus associated with Jembrana disease in Bali cattle. Journal of General Virology, 74, 1765–1773.

A virus causing Jembrana disease in Bali cattle was demonstrated to have characteristics of a retrovirus. Reverse transcriptase activity was detected in virus purified by sucrose gradient centrifugation. Electron

microscopic examination of tissue from affected cattle indicated the virus matured by C-type budding through the plasma membrane and into intracytoplasmic vacuoles of cells in lymphoid tissue, with the formation of circular enveloped virus particles ranging in diameter from 96 to 124 nm with an eccentric nucleoid. Western immunoblotting using sera from recovered animals demonstrated virus proteins of 100, 45, 42, 33, 26, 16 and 14 kDa. The 26 kDa protein of JDV cross-reacted in Western blots with the 26 kDa capsid protein of bovine immunodeficiency virus (BIV). The apparent morphogenesis, protein structure and antigenic relationship with BIV suggested the virus was a lentivirus.

Soeharsono, S., Hartaningsih, N., Soetrisno, M., Kertayadnya, G. and Wilcox, G.E. 1990. Studies on experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. Journal of Comparative Pathology, 103, 49–59.

The agent causing Jembrana disease, an enzootic disease of *Bos javanicus* (Bali cattle) occurring in Bali, Indonesia, was shown to occur to high titres in the blood of animals during the febrile period of the disease and to persist in cattle for 25 months after clinical recovery. During the febrile period of the disease, most of the infectious agent appeared to be associated with the plasma fraction of whole blood. There was a linear relationship between the number of organisms inoculated into susceptible Bali cattle and the incubation period, which varied from 4.5 to 12 days. Seventeen of 18 animals in which Jembrana disease had been experimentally-induced up to 22 months previously did not develop clinical signs when re-challenged with the infectious agent. Ongole cattle (*Bos indicus*), Friesian cattle (*Bos taurus*), buffalo (*Bubalus bubalis*) and pigs but not sheep or goats developed a mild febrile response after inoculation with the Jembrana disease agent but no other overt clinical signs of the disease. Ongole and Friesian cattle, buffalo and sheep developed a persistent infection after inoculation; the infectious agent persisted in blood or spleen for at least 9 months in buffalo and for shorter periods in the other species.

Soeharsono, S., Wilcox, G.E., Putra, A.A., Hartaningsih, N., Sulistyana, K. and Tenaya, M. 1995. The transmission of Jembrana disease, a lentivirus disease of *Bos javanicus* cattle. Epidemiology and Infection, 115, 367–374.

Methods of transmission of Jembrana disease, an acute and severe disease of Bali (*Bos javanicus*) cattle caused by a recently identified bovine lentivirus known as Jembrana disease virus, are described. During the acute disease virus can be detected in saliva and milk. There is evidence of direct transmission from acutely affected animals in close contact with susceptible cattle, possibly by virus in these secretions infecting cattle by the conjunctival, intranasal, or oral routes, by which it was possible to experimentally infect cattle. During the acute disease the titre of infectious virus in blood is high, about 10^8 50% cattle infectious units (ID_{50}) per mL, and it is probable that the virus is also transmitted mechanically by haematophagous arthropods. Recovered cattle are also a potential but probably infrequent source of infection: recovered cattle are persistently viraemic but the titre of infectious virus in blood decreases to about 10^1 ID_{50} per mL by 60 days after recovery from the acute disease, and virus cannot be detected in secretions.

Soeharsono, S., Wilcox, G.E., Dharma, D.M.N., Hartaningsih, N., Kertayadnya, G. and Budiantono, A. 1995. Species differences in the reaction of cattle to Jembrana disease virus infection. Journal of Comparative Pathology, 112, 391–402.

Jembrana disease virus (JDV), a recently identified bovine lentivirus, causes an acute and severe disease in Bali cattle (*Bos javanicus*). Clinical Jembrana disease has not been reported in other types of cattle and this has led to the belief that the disease is unique to Bali cattle. This study showed, however, that other types were also susceptible. Infection of Friesian (*Bos taurus*) and crossbred Bali (*Bos javanicus* x *Bos indicus*) cattle induced clinical changes and lesions consistent with those detected in Bali cattle, although they were milder and would consequently have been difficult to detect under field conditions. The inoculated crossbred cattle were viraemic for 3 months and developed an antibody response to the virus that persisted for at least 46 weeks after infection. The duration of viraemia in Friesian cattle was not determined.

Soesanto, M., Soeharsono, S., Budiantono, A., Sulistyana, K., Tenaya, M. and Wilcox, G.E. 1990. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. Journal of Comparative Pathology, 103, 61–69.

The clinical and haematological changes in 18 Bali cattle (*Bos javanicus*) experimentally infected with Jembrana disease were described. The major clinical signs were an elevated rectal body temperature persisting

for 7 days (range 5 to 12 days), lethargy, anorexia, enlargement of the superficial lymph nodes, a mild ocular and nasal discharge, diarrhoea with blood in the faeces and pallor of the mucous membranes. Not all of these changes occurred in all affected cattle. The major haematological changes included a leucopenia, lymphopenia, eosinopenia and a slight neutropenia, a mild thrombocytopenia, a normocytic normochromic anaemia, elevated blood urea concentrations and reduced total plasma protein. The mortality rate in the experimentally-infected cattle was 17%. The similarity of Jembrana disease to malignant catarrhal fever and to diseases of cattle associated with Ehrlichia was discussed.

Sweatman, G.K. 1984. Potential arachnid vectors — Jembrana disease: an epidemiological study. In: Nutting, W.B., ed., Mammalian Disease aand Arachnids. Volume 1. Pathogen Biology and Clinical Manangement, CRC Press, Boca Raton, Florida. 247–251.

Arthropods associated with banteng cattle (*Bos javanicus*) were collected by hand, sweep net, and by placing CDC light traps overnight under the roof of shelters for the cattle, less than a metre from the backs of the housed cattle. No fewer than 39 arthropod species were collected. *Culicoides* spp., feeding on banteng, occurred in vast numbers. Mosquitoes were common feeders. Consideration of the whole ecosystem together with observations of types of arthropods recovered from banteng cattle leave no doubt that *Culicoides* spp. should be considered first in a program of control of the vectors of Jembrana disease. Mosquitoes would rank second. The biology of ticks, especially a one-host tick, cannot be matched to the characteristics of the spread of the disease. Ticks might, however, be part of a more complex vector system, and even muscid flies or droplet infection should not be overlooked during epidemics.

Teuscher, E., Ramachandran, S. and Harding, H.P. 1981. Observations on the pathology of 'Jembrana disease' in Bali cattle. Zentralblatt fur Veterinarmedizin Reihe A, 28, 608–622.

Jembrana disease is a transmissible infectious disease of Bali cattle, probably of viral origin. This study includes 13 field cases and 53 experimental animals. The gross changes consisted of a more or less generalised lymphadenopathy, splenomegaly, haemorrhages, oedema, ulceration of the mucous membranes, probable disturbances of coagulation and sometimes thrombosis. Haematological examinations showed leucopenia and anaemia, and abnormal lymphocytes were found in the blood. Microscopically, besides oedema and haemorrhages, the changes were characterised in lymph nodes, spleen, thymus and bone marrow by proliferation of abnormal lymphocytes. Multifocal infiltrations by lymphoid cells were seen in most organs, especially lung, heart, liver, and kidney. The brain showed oedema, vascular changes, thrombosis and haemorrhages. Many bovine disease have been considered in the differential diagnosis, but apparently it would appear that Jembrana disease has never been observed outside Indonesia.

Teuscher, E., Ramachandran, S. and Harding, H.P. 1982. Is 'Bali disease' in cattle a late complication of Jembrana disease. Zentralblatt fur Veterinarmedizin Reihe A, 29, 547–556.

'Bali disease' is a disease of cattle occurring in Indonesia and characterised by necrotic changes at the tips of the ears and in several parts of the skin. Generally a febrile episode is recorded a few weeks or months before the appearance of the typical changes. Plant poisoning and/or photosensitisation have been suggested as possible causes. From their observations on experimental animals, the authors suggest that 'Bali disease' may be a late complication of Jembrana disease. Gross microscopical lesions of two cases were described.

Wilcox, G.E., Kertayadnya, G., Hartaningsih, N., Dharma, D.M.N., Soeharsono, S. and Robertson, T. 1992. Evidence for a viral aetiology of Jembrana disease in Bali cattle. Veterinary Microbiology, 33, 367–374.

High titres of the infectious agent were detected in plasma during the febrile phase of the disease. Using this plasma as a source of the infectious agent, its size determined by membrane filtration was between 50 and 100 nm, indicating it was a virus and not a rickettsia as previously proposed. Spherical virus-like particles of 75 to 130 nm diameter with a smooth membrane and frequently with an eccentric nucleoid were detected by electron microscopy in plasma from infected animals. The virus replicated in mononuclear cell cultures of peripheral blood origin but not in other cell cultures. The virus and the associated disease had characteristics consistent with viruses in the family *Retroviridae*.

Wilcox, G.E., Chadwick, B.J. and Kertayadnya, G. 1995. Recent advances in the understanding of Jembrana disease. Veterinary Microbiology, 46, 249–255.

Review updating recent research on Jembrana disease. The clinical nature of the disease is described: it is a severe and acute clinical disease in Bali cattle with a case fatality rate of about 20%, and a mild sometimes

subclinical disease in other cattle types and buffalo. Progress in identification of the aetiology of the disease is also described: the aetiological virus has been identified as a lentivirus, designated as Jembrana disease virus (JDV). Preliminary sequence analysis has confirmed the identity of JDV as a lentivirus and has shown that it is distinguishable from BIV. There is antigenic cross-reactivity between the capsid protein of JDV and the previously identified bovine lentivirus designated bovine immunodeficiency virus (BIV). Serological tests that detect antibody to the capsid protein of JDV or BIV would not differentiate between antibody due to infection by either virus. The diseases induced by BIV and JDV in cattle are very different, and the pathogenesis of JDV infection in Bali cattle is unusual for a lentivirus infection.

Wilcox, G.E., Chadwick, B.J. and Kertayadnya, G. 1995. Jembrana disease virus — a bovine lentivirus producing an acute severe clinical disease in *Bos javanicus* cattle. In: Schwyzer, M., Ackerman, M., Bertoni, G. et al., ed., Immunobiology of Viral Infections. Proc. 3rd Congress European Society Vet. Virology. Fondation Marcel Merieux, Lyon, 349–352.

A review. Recent partial sequence analysis has identified Jembrana disease virus (JDV) as a new bovine lentivirus distinguishable from bovine immunodeficiency-like virus (BIV). Jembrana disease is, however, atypical of most lentivirus diseases. Infection of Bali (*Bos javanicus*) cattle with JDV produces an acute and severe disease syndrome after a short incubation period of 4 to12 days. Pathological changes during the acute disease primarily involve the lymphoid organs, with an intense parafollicular lymphoproliferative reaction and follicular atrophy. The clinical and pathological changes are very different to those reported in BIV-infected cattle, but are remarkably similar to those reported in the acute, severe disease syndrome recently described in pig-tailed macaques infected with a lethal variant of simian immunodeficiency virus (SIV), $SIV_{SMMPBj14}$.

Reports

Anonymous 1985. Jembrana disease. Foreign Animal Disease Report, United Stated Department of Agriculture, Washington, DC.

Laporan 1967. Kejadian penyakit hewan menular Rama Dewa pada sapi Bali di kecamatan Suputih Raman kabupaten Lampung Tengah. Pemerintah Propinsi Daerah Tingkat I Lampung, Dinas Peternakan, Kedaton, Lampung. [Language: Indonesian].

Prabowo, H. and Ishitani, R. 1984. Studies on Rama Dewa, the enzootic disease of cattle occurring in Lampung Province, Sumatra, Indonesia — its histopathology and critical views on name of the disease. Report to the Japan International Cooperation Agency.

Putra, A.A.G., Dharma, D.M.N., Soeharsono, S., Sudana, G. and Syafriati, T. 1983. Studi epidemiologi penyakit Jembrana di Kabupaten Karangasem. I. Tingkat morbiditas, tingkat mortalitas dan attack rate. Annual report on animal disease investigation in Indonesia during period 1981–1983. Disease Investigation Centre, Denpasar, Indonesia. [Language: Indonesian]

The morbidity and the mortality rates of the disease recorded were 63% and 20%, respectively. There were no significant differences in morbidity and mortality rates among different age groups or sexes in affected Bali cattle. In 91% of the mortalities, the death occurred in the first week following the first appearance of the clinical signs. Recovery was recorded in 61% of sick cattle without any treatment. The bimonthly attack rate curve showed that the arthropod vector seemed to play an important role in the disease ecology.

Putra, A.A.G., Dharma, D.M.N., Soeharsono, Sudana, G. and Syafriati, T. 1983. Studi epidemiologi penyakit Jembrana di Kabupaten Karangasem. II. Pengaruh pada Kebuntingan. Annual report on animal disease investigation in Indonesia during the period 1981–1982. Disease Investigation Centre, Denpasar, Indonesia. [Language: Indonesian]

Epidemiological studies were carried out during the outbreak of Jembrana disease in Karangasem district (East Bali) in 1981. Nine banjars (subvillages) within three infected villages were investigated. Abortion was recorded in 49% of pregnant cows affected with Jembrana disease. Abortion occurred at all stages of pregnancy. The abortions occurred in 70% of cases within 4 days of onset of the disease, and in 95.5% of cases within 7 days of onset of clinical signs.

Sudana, G. 1983. Beberapa aspek program penelitian dan pemberantasan penyakit Jembrana. Report: Balai Penyidikan Penyakit Wilayah IV. Denpasar, Bali, Indonesia. [Language: Indonesian]

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Adiwinata, R.T. 1967. Some informative notes on a rinderpest-like disease on the island of Bali. O.I.E.-F.A.O. Conference on Epizootics in Asia and the Far East. Tokyo, 2–9 October, 1967.

Adiwinata, A.T. 1975. Some informative notes on a rinderpest-like disease on the island of Bali. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Budiarso, I.T. and Hardjosworo, S. 1975. Jembrana disease of Bali breed cattle. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Chadwick, B., Sammels, L., Coelen, R.C. and Wilcox, G.E. 1994. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. Abstract in Veterinary Virology in Australia. University of Sydney, July 1994.

Dennig, H.K. and Ramachandran, S. 1975. Investigation on the transmission of Jembrana disease by ticks (*Boophilus microplus*). Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, September 22–24, 1975.

Harjosworo, S. and Budiarso, I.T. 1975. Jembrana disease of 1972–1975. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Hartaningsih, N., Soetrisno, Sulistyana, Kertayadnya, G. and Wilcox, G.E. 1988. Experimental transmission and immunological studies of Jembrana disease. Abstract in Proceedings of 6th Congress of Federation of Asian Veterinary Associations. Denpasar, Indonesia.

Pranato, R.A. and Pudjiastono 1975. An outbreak of a highly infectious disease in cattle and buffaloes on the island of Bali. I. Diagnosis based upon clinical and post-mortem findings. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Pranato, R.A. 1975. An outbreak of a highly infectious disease in cattle and buffaloes on the island of Bali. II. A report on necropsies and histopathological examination. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Ramachandran, S. and Madlangbayan, F.M. 1975. Therapeutic, serological and immunological aspects of Jembrana disease. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Ramachandran, S. 1975. Haematological findings in natural Jembrana disease of Bali cattle. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Ramachandran, S., Teuscher, E.E. and Darmadi, P. 1975. Experimental transmission studies: transmission to other species. Abstract in Seminar on Jembrana Disease. Disease Investigation Centre, Denpasar, Bali, Indonesia, 22–24 September, 1975.

Ressang, A.A. Budiarso, I.T. and Soeharsono, S. 1985. Jembrana disease: its similarity to bovine ehrlichiosis. Abstract in Workshop on Diseases Caused by Leucocytic Rickettsiae of Man and Animals. University of Illinois, Urbana-Champaign, Illinois, USA.

Soesanto, M., Budiantono, Tenaya, M. and Wilcox, G.E. 1988. Clinical and haematological characteristics of experimental Jembrana disease. Abstract in Proceedings of 6th Congress of Federation of Asian Veterinary Associations. Denpasar, Indonesia.

Wilcox, G.E., Kertayadnya, G., Hartaningsih, N., Dharma, D.M.N., Soeharsono, S. and Robertson, T. 1991. Evidence for a viral aetiology of Jembrana disease in Bali cattle. Abstract in Second European Veterinary Virology Congress. Uppsala, Sweden.

Wilcox, G.E. 1992. Jembrana disease: a retroviral disease of Bali cattle. Abstract in Australian Microbiologist, 13, A99.

Wilcox, G.E. 1993. Jembrana disease virus. Abstract in Australian Microbiologist, 14, A140.

Wilcox, G.E. 1994. An overview of current Jembrana disease research. Abstract in Veterinary Virology in Australia. University of Sydney, July 1994.

Wilcox, G.E., Chadwick, B. and Kertayadnya, G. 1994. Jembrana disease virus — a bovine lentivirus producing a severe and acute clinical disease in *Bos javanicus* cattle. Abstract in Third International Congress of Veterinary Virology. Interlaken, Switzerland, 4–7 September, 1994.

Bovine Immunodeficiency Virus publications

Amborski, G.F., Lo, J.L. and Seger, C.I. 1989. Serological detection of multiple retroviral infection in cattle: bovine leukemia virus, bovine syncytial virus and bovine visna virus. Veterinary Microbiology, 20, 247–253.

Animals used in studies on bovine leukemia virus (BLV) were screened for antibody to the three bovine lymphotropic retroviruses: bovine immunodeficiency-like virus (BIV), bovine syncytial virus (BSV), and BLV. Serum samples from 235 animals in four dairy and four beef herds were examined. Detection methods included indirect fluorescent antibody of virus-infected cell cultures (BLV, BIV and BSV), and agar gel immunodiffusion (BLV). Sera from BLV-infected animals in the dairy herds showed the highest single (50%) and multiple (30%) infections compared with 5% and <1%, respectively, in the beef herds. Single BIV infections were not detected in the dairy herds, but 11% of the sera contained antibodies to BIV plus BLV or BSV. Five sera from beef cattle had antibodies only to BIV and four were obtained from one herd. Only one beef serum of the 138 tested demonstrated multiple antibodies (BLV and BIV).

Archambault, D., Nadin-Davis, S., Lutze-Wallace, C. and Bouillant, AM. 1993. Le virus de l' immunodeficience bovine: mise au point 1990–1992. [The bovine immunodeficiency virus: 1990–1992 update]. Vet. Res., 24, 179–187.

Substantial progress has been shown on the bovine immunodeficiency-like virus (BIV) in 1990–1991 and up to mid-1992. The genomic sequences of BIV were analysed in detail and several subgenomic RNAs were identified. Nucleic acid molecular probes, PCR (polymerase chain reaction) amplification and a novel Western blotting procedure have been of great assistance for the experimental diagnosis of BIV. Antibody response after BIV infection has shown that antibodies to p26 antigen were always present in naturally and experimentally-infected animals. The experimental infection of sheep, goats and rabbits was confirmed. BIV causes an infection with no pathognomonic clinical signs in cattle and sheep for at least 3 and 4 years, respectively. Finally, there is not yet any evidence of BIV immune response disturbances similar to that of human AIDS.

Atkinson, B., Liu, Z.Q. and Wood, C. 1992. Use of bacterial trpE fusion vectors to express and characterise the bovine immunodeficiency-like virus core protein. Journal of Virological Methods, 36, 35–49.

The gag coding region from bovine immunodeficiency-like virus (BIV) was cloned into *E. coli* and expressed as a bacterial fusion protein. Six different clones spanning various regions of the gag open reading frame were generated. The resulting fusion proteins were expressed at high concentrations and readily purified. A panel of bovine immune sera specifically recognised the recombinant Gag proteins, as did immune sera from animals infected or immunised with lentiviruses related to BIV, such as equine infectious anemia virus (EIAV) and human immunodeficiency virus (HIV). Analysis of the deletion clones, using the bovine immune sera panel, enabled us to identify at least one major epitope which was specifically recognised by all bovine sera examined. The ease of expression, purification, and specificity of these fusion proteins should enable a thorough study of the epidemiology of BIV infection.

Baron, T., Mallet, F., Polack, B., Betemps, D. and Belli, P. 1995. The bovine immunodeficiency-like virus (BIV) is transcriptionally active in experimentally infected calves. Archives of Virology, 140, 1461–1467.

Studied infection by the bovine immunodeficiency-like virus (BIV) in three experimentally-infected calves, by polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR), from the peripheral blood mononuclear cells (PBMC). Two primer pairs located in the *gag* and *pol* regions of the viral genome allowed detection of the viral genomic DNA by PCR, as well as the unspliced genomic viral RNA transcript, by RT-PCR. We also present the evidence of the presence in peripheral blood mononuclear cells (PBMCs) of a mRNA transcript of the regulatory transactivator tat gene, according to the splicing pattern of the viral genome, by use of reverse transcription followed by nested PCR. The active expression of the virus in these animals was further assessed by the sequential rescue of the virus from unstimulated PBMCs in cell culture, from 4 weeks until 15 months following the infection.

Battles, J.K., Hu, M.Y., Rasmussen, L., Tobin, G.J. and Gonda, M.A. 1992. Immunological characterisation of the *gag* gene products of bovine immunodeficiency virus. Journal of Virology, 66, 6868–6877.

The bovine immunodeficiency virus (BIV) gag gene encodes a 53-kDa precursor (Pr53gag) that is involved in virus particle assembly and is further processed into the putative matrix (MA), capsid (CA), and nucleocapsid (NC) functional domains in the mature virus. Gag determinants are also found in the Gag-Pol polyprotein precursor. To immunologically identify the major precursors and processed products of the BIV gag gene, monospecific rabbit sera to recombinant BIV MA protein and Pr53gag and peptides predicted to correspond to the CA and NC proteins and the MA-CA cleavage site were developed and used in immunoprecipitations and immunoblots of BIV antigens. Monospecific antisera to native and recombinant human immunodeficiency virus type 1 proteins were also used to identify analogous BIV Gag proteins and to determine whether cross-reactive epitopes were present in the BIV Gag precursors or processed products. The BIV MA, CA, and NC Gag proteins were identified as p16, p26, and p13, respectively. In addition to BIV Pr53gag, the major Gag precursor, two other Gag-related precursors of 170 and 49 kDa were identified that have been designated pPr170gag-pol and Pr49gag, respectively; pPr170gag-pol is the Gag-Pol polyprotein precursor, and Pr49gag is the transframe Gag precursor present in pPr170gag-pol. Several alternative Gag cleavage products were also observed, including p23, which contains CA and NC determinants, and p10, which contains a peptide sequence conserved in the CA proteins of most lentiviruses. The monospecific antisera to human immunodeficiency virus type 1 CA (p24) and NC (p7) proteins showed cross-reactivity to and aided in the identification of analogous BIV proteins. Based on the present data, a scheme for the processing of BIV Gag precursors is proposed.

Belloc, C., Polack, B., Schwartzcornil, I., Brownlie, J. and Levy, D. 1996. Bovine immunodeficiency virus — facts and questions [review]. Veterinary Research, 27, 395–402.

Bovine immunodeficiency virus (BIV) is a lentivirus whose serologic prevalence is worldwide. Little is known about its impact on animal health status, pathogenesis and mode of transmission. Understanding BIV biology implies isolation of new viral strains and long-term studies on experimentally-infected cows and surrogate hosts such as rabbits.

Bouillant, A.M. and Archambault, D. 1990. Le virus de l' immunodeficience bovine: breve revue. [Bovine immunodeficiency virus: short review]. Ann. Rech. Vet., 21, 239–250.

A bovine visna-like virus was isolated by Van Der Maaten et al. 1972. but it did not draw attention since, at that time, most efforts were directed towards research on bovine leukemia virus. However, new interest was shown on the bovine visna-like virus after the isolation of the human immunodeficiency virus (HIV), because of the urgent need for developing animal models for the acquired immunodeficiency syndrome (AIDS). The purpose of this paper is to describe the different stages of the identification of the bovine virus and to up-date knowledge about it. The bovine visna-like virus has recently been named the bovine immunodeficiency-like virus (BIV) and is the sole bovine lentivirus known to-date. BIV shares morphologic, antigenic and genomic characteristics with other lentiviruses. It grows and induces large syncytia in vitro and generates virus-productive and latent infections in cell culture. It causes persistent infection and slow progressive disease in cattle and probably in sheep. As target cells of the virus are leukocytes, the type of which is unknown, perturbations of the immune system are expected. Consequently, BIV may potentiate the occurrence of secondary infections and play a role in retroviral, multiple infections. It is not oncogenic. Transmission appears to occur in cattle by contact, but evidence of transmission in human beings has not been shown. Finally, BIV may be a potential model in vitro and in vivo for HIV and AIDS.

Bouillant, A.M. and Becker, S.A. 1984. Ultrastructural comparison of Oncovirinae (type C), Spumavirinae, and Lentivirinae: three subfamilies of Retroviridae found in farm animals. J. National Cancer Inst., 72, 1075–1084.

The successive steps of maturation of seven retroviruses from five species of farm animals and one retrovirus from a mouse were compared in cell cultures. The viruses included three type C oncoviruses, one spumavirus, and three lentiviruses. Although members of the three subfamilies shared some gross morphologic features such as budding on plasma membranes, core, and surface projections, differences were noted in the ultrastructural detail of these features. Type C oncoviruses did not show any structural differentiation in identifiable form in the cytoplasm as opposed to characteristic features observed in the spumavirus and lentivirus subfamilies, respectively. Budding viruses were distinct among the 3 subfamilies. The type C bovine leukemia virus budding on vacuole membranes differed from the two other type C viruses by lacking an electron-lucent intermediate layer as did the lentiviruses. Differentiation between type C oncoviruses and lentiviruses could be confusing because of the similarity of the fully mature virions appearing in the intercellular space. However, each subfamily of retroviruses can be readily differentiated from one another when each morphologic stage of virus replication is examined by electron microscopy.

Bouillant, A.M., Ruckerbauer, G.M. and Nielsen, K.H. 1989. Replication of the bovine immunodeficiency-like virus in diploid and aneuploid cells: permanent, latent and virus-productive infections in vitro. Res. Virol., 140, 511–529.

Bovine immunodeficiency-like virus (BIV) is an infectious and leukotropic retrovirus, the sole lentivirus candidate which has been isolated from cattle. Although BIV has recently been shown to be related to the human immunodeficiency virus, there is very limited information on the replication and the pathogenesis of BIV. It is reported here that BIV can permanently infect diploid and aneuploid cells from four different species: bovine, canine, ferret and ovine. With the exceptions of a bovine diploid and a canine aneuploid cell line, all lines were virus non-productive. However, BIV was rescued by co-cultivation of virus non-productive cells with homologous BIV-free or canine cells (Cf2Th). A permanent and BIV-productive infection was established for 90-serial subcultures in a canine cell line. A BIV titre of 1 × 10(6)/0.1 mL was observed in stationary culture and 1 × 10(10)/0.1 mL in suspension culture. The canine cell line above was used for production of BIV antigens, whereas BIV-free canine cells were routinely used to isolate BIV from BIV non-productive cells infected in vitro and from blood from experimentally BIV-infected cattle. The different steps of virus maturation were similar by electron microscopy to those of lentiviruses. BIV results are compared to those of lentiviruses.

Braun, M.J., Lahn, S., Boyd, A.L., Kost, T.A., Nagashima, K. and Gonda, M.A. 1988. Molecular cloning of biologically active proviruses of bovine immunodeficiency-like virus. Virology, 167, 515–523.

A series of independent proviral molecular clones of bovine immunodeficiency-like virus (BIV) obtained from a genomic library of BIV-infected bovine cell DNA were physically and biologically characterized. Heteroduplex mapping shows that two of these BIV clones (106 and 127) contain uninterrupted proviral sequences approximately 9.0 kb in length, flanked by nonhomologous bovine cellular sequences. Microinjection of purified DNA from BIV clone 106 or 127 into susceptible bovine cells produces virus-specific cytopathic effects, including syncytium induction, supernatant reverse transcriptase activity, and infectious virus particle formation, similar to the effects produced by parental virus stock. Using restriction enzyme mapping, it was determined that the two infectious clones share 13 of 14 sites mapped within the provirus; thus, based on this criterion, the two clones are nearly identical, with the exception of a single polymorphic site recognised in the 3' half of the genome. BIV appears to be an exogenous pathogenic virus, because Southern hybridisation analyses detected no endogenous sequences related to BIV in DNA from a variety of uninfected bovine cells and tissues. Most of the BIV-related DNA found in cells 96 hr after infection is present as linear unintegrated viral DNA, although the presence of host flanking sequences in our proviral clones indicates that integration takes place. These biologically active clones of BIV will be of use in defining further the mechanisms of BIV pathogenesis and in engineering specific diagnostic reagents to determine the prevalence of BIV in cattle populations.

Brownlie, J., Collins, M.E. and Heaton, P. 1994. Bovine immunodeficiency-like virus — a potential cause of disease in cattle? Veterinary Record, 134, 289–291.

A short review of BIV concentrating on the role of BIV as a potential pathogen. Reference is made to the association that has been made but not proven between BIV and a recent wasting disease seen in many cattle in a Cheshire herd (see Report, 1994).

Carpenter, S. Nadin-Davis, S.A. Wannemuehler, Y. and Roth, J.A. 1993. Identification of transactivation-response sequences in the long terminal repeat of bovine immunodeficiency-like virus. Journal of Virology, 67, 4399–4403.

Transient expression assays using the reporter gene that encodes chloramphenicol acetyltransferase were used to identify cis-acting sequences necessary for bovine immunodeficiency-like virus (BIV) transactivation. Computer analyses identified two RNA stem-loop structures located immediately downstream of the transcription start site in the long terminal repeat. Deletion analysis of the long terminal repeat indicated that sequences containing the proximal stem-loop structure located between +4 and +31 are required for virus-specific transactivation. Therefore, BIV likely utilises a mechanism of transactivation similar to that of the human and simian lentiviruses.

Carpenter, S., Miller, L.D., Alexandersen, S., Whetstone, C.A., Van Der Maaten, M.J., Viuff, B., Wannemuehler, Y., Miller, J.M. and Roth, J.A. 1992. Characterisation of early pathogenic effects after experimental infection of calves with bovine immunodeficiency-like virus. Journal of Virology, 66, 1074–1083.

The early pathogenic effects of bovine immunodeficiency-like virus (BIV) were studied in calves experimentally inoculated with BIV. All animals inoculated with BIV R29-infected cells seroconverted by 6 weeks postinoculation, and BIV was recoverable from each animal at 2 weeks postinoculation. However, levels of BIV replication in vivo appeared to be low. In situ hybridisation studies indicated that during peak periods of viral replication in vivo, less than 0.03% of peripheral blood mononuclear cells were expressing detectable levels of viral RNA. Moreover, the levels of viral RNA in these cells in vivo were less than 1/10 the levels observed in persistently infected cells in vitro. BIV-inoculated calves had significantly higher numbers of circulating lymphocytes, and follicular hyperplasia was observed in lymph nodes, hemal nodes, and spleen. The histopathological changes observed in BIV-infected calves were similar to changes found early after infection with the immunosuppressive lentiviruses, including human immunodeficiency virus type 1.

Chen, L. and Frankel, A.D. 1995. A peptide interaction in the major groove of RNA resembles protein interactions in the minor groove of DNA. Proceedings of the National Academy of Sciences of the United States of America, 92, 5077–5081.

A 17-amino acid arginine-rich peptide from the bovine immunodeficiency virus Tat protein has been shown to bind with high affinity and specificity to bovine immunodeficiency virus transactivation response element (TAR) RNA, making contacts in the RNA major groove near a bulge. We show that, as in other peptide-RNA complexes, arginine and threonine side chains make important contributions to binding but, unexpectedly, that one isoleucine and three glycine residues also are critical. The isoleucine side chain may intercalate into a hydrophobic pocket in the RNA, Glycine residues may allow the peptide to bind deeply within the RNA major groove and may help determine the conformation of the peptide. Similar features have been observed in protein-DNA and drug-DNA complexes in the DNA minor groove, including hydrophobic interactions and binding deep within the groove, suggesting that the major groove of RNA and minor groove of DNA may share some common recognition features.

Chen, L. and Frankel, A.D. 1994. An RNA-binding peptide from bovine immunodeficiency virus Tat protein recognises an unusual RNA structure. Biochemistry, 33, 2708–2715.

The human immunodeficiency virus (HIV) Tat protein binds specifically to an RNA hairpin, TAR, located at the 5' end of its mRNA. Tat uses a single arginine residue within a short region of basic amino acids to recognise a bulge region in TAR. Here we show that a 17 amino acid arginine-rich peptide from the bovine immunodeficiency virus (BIV) Tat protein also binds to an RNA hairpin at the 5' end of its mRNA (BIV TAR), but recognises different structural features of the RNA, Mutagenesis, RNase mapping, and chemical interference experiments indicate that bulge and stem regions of BIV TAR are recognised simultaneously by the BIV peptide and that the RNA adopts an unusual structure. BIV Tat binds to its TAR site with high affinity and specificity and, unlike HIV Tat, does not appear to use cellular proteins to stabilise RNA binding in vivo. Thus, two related viral activators have evolved rather distinct ways to recognise their RNA targets.

Chen, P., Liu, Z.Q. and Wood, C. 1994. Use of TrpE fusion protein to identify antigenic domains within the BIV envelope protein. Journal of Virological Methods, 47, 331–343.

Nine different recombinant clones spanning various regions of the bovine immunodeficiency-like virus (BIV) envelope gene open reading frame were generated. These clones span the entire external glycoprotein as well as the transmembrane glycoprotein region. These proteins were expressed as fusions to the TrpE protein in *E. coli*. The levels of recombinant protein expressed varied, some clones expressed enough protein that can be detected in a Coomassie blue-stained gel, whereas other proteins could only be detected by Western blot analyses. A recombinant env protein representing the extracellular domain of the env protein was detected by BIV-infected bovine sera. In addition, a 134 amino acid peptide which may represent a major immunoreactive epitope was identified. This peptide is located at the amino terminus of the transmembrane glycoprotein and was specifically recognised by all BIV-infected calf sera tested. The identification of this epitope and the use of recombinant envelope protein will enable us to develop a more effective screening test to study the epidemiology of BIV infection.

Celum, C.L., Coombs, R.W., Jones, M., Murphy, V., Fisher, L., Grant, C., Corey, L., Inui, T., Wener, M.H. and Holmes, K.K. 1994. Risk factors for repeatedly reactive HIV-1 EIA and indeterminate Western blots — a population-based case-control study. Archives of Internal Medicine, 154, 1129–1137.

Objective: Causes of indeterminate results of Western blot testing (IWB) for human immunodeficiency virus (HIV) type 1 include seroconversion, HIV-2 cross-reactivity, and autoimmune disease, but most IWB results remain unexplained. This case-control study assessed risk factors for IWB results, including early HIV infection, other retroviral infection, autoantibodies, and other medical conditions. Design: Prospective study to determine HIV seroconversion rate, with a case-control design to assess other risk factors for IWB. Cases (persons with one or more repeatedly reactive HIV-1 enzyme immunoassay with IWB), their current sexual partners, and controls (persons with negative enzyme immunoassay and Western blot results) were recruited from blood banks, health department and prenatal clinics, and private providers in Washington and Oregon. Results: Of 244 cases enrolled, 206 were followed up for 6 months or longer, and six (3.0%; 95% confidence interval [CI], 0.7% to 5.3%) with recent HIV risk behaviors seroconverted. The Western blot banding patterns differed among groups; cases usually had p17 or p24 bands, while controls and cases' sexual partners usually had polymerase bands. Conditional logistic regression indicated that independent risk factors for IWB among male cases and controls were a tetanus booster in the past 2 years (odds ratio, 3.2; 95% CI, 1.2 to 8.6) and sexual contact with a prostitute (odds ratio, 3.0; 95% CI, 1.0 to 9.5). Independent risk factors for women were parity (odds ratio, 1.2; 95% CI, 1.02 to 1.4) and autoantibodies, either rheumatoid factor or antinuclear antibodies (odds ratio, 2.3; 95% CI, 1.03 to 5.6). No cross-reactivity was detected with HIV-2, human T-lymphotrophic virus type 1, feline immunodeficiency or feline leukemia, or bovine immunodeficiency viruses. Conclusions: Evaluation of persons with reactive HIV-1 enzyme immunoassays and IWB should include an assessment of HIV risk and other possible risk factors, such as alloimmunisation tie, parity or recent immunisation) or autoantibodies (i.e., antinuclear antibodies and rheumatoid factor). The relationship of IWB among men who reported sex with prostitutes is intriguing and warrants further study.

Chen, L. and Frankel, A.D. 1994. Tar Tat recognition in bovine immunodeficiency virus — an interesting system to study transcriptional regulation [Abstract]. Journal of Cellular Biochemistry Suppl. 18C, 57.

Studies on the RNA recognition-domain (amino-acids 49–57) of the HIV transcriptional activator TAT have been completed. The arginine rich RNA-binding domain is unusually flexible both in amino-acid sequence and in structure, with a single arginine providing the only sequence-specific RNA contact. A possible candidate RNA binding domain rich in arginines is found in the BIV Tat protein. The transactivation domains of BIV Tat and HIV Tat have similar sequences and may use similar mechanisms to transactivate their viral long terminal repeats. A 17 amino-acid arginine-rich peptide from the BIV Tat protein (amino acids 65–81) has been identified that binds specifically to an RNA hairpin and the 5' end of the mRNAs and recognises unusual structural features of the RNA. Binding of the BIV peptide to BIV TAR is considerably tighter and more specific than binding of HIV Tat to HIV TAR. Mutagenesis, RNAse mapping, and chemical interference experiments indicate that bulge and stem regions of BIV TAR are recognised simultaneously by the BIV peptide and that the RNA adopts an unexpected structure. In particular, two GC base pairs in the upper stem, near a single nucleotide bulge, are highly accessible to RNAse digestion. In vivo experiments confirmed the high specificity of the interaction and the identity of the RNA determinants. Transactivation experiments using HIV-BIV hybrid proteins in human and mouse cells have shown that, unlike HIV Tat, BIV Tat does not require additional cellular RNA loop-binding proteins for transactivation. Peptide mutagenesis, RNA binding

experiments and immunoprecipitation experiments have identified amino-acids in BIV Tat important for TAR binding. Unlike HIV TAR-Tat and RRE-Rev interactions, no conformational changes in BIV TAR have been detected by the circular dichroism upon peptide binding. The BIV system provides an interesting system to characterise the TAR-Tat interaction and the compare the mechanisms of BIV and HIV transactivation.

Clayton, J. 1994. Spectre of AIDS haunts reports of sick cows [news]. Nature, 367, 585.

Refers to a 'mystery illness' that 'swept through' a Cheshire dairy farm and has been linked to a possible infection with bovine immunodeficieny virus (BIV). It draws attention to the plight of a farmer whose 50-strong dairy herd appeared to be suffering from a wide-ranging clinical spectrum, including nerve degeneration, weight loss, mouth ulcers and respiratory infections. So far, two of the eight cows have tested positive for BIV by three methods — Western immunoblotting, immunofluorescence and ELISA — and others have shown positive results on one or two tests. Local companies are refusing to handle meat from the herd or to allow milk to be used for pig-feed.

Coats, K.S.C. 1995. Dual infection with bovine immunodeficiency virus and bovine leukaemia virus in Mississippi dairy cattle. Veterinary Record, 136, 269–270.

Following a previous report (Coats 1994) of a seroprevalence of antibody to BIV in two herds of 58% in a Mississippi State University (MSU) herd and a Coastal Plains (CP) herd of 38%, the authors also detemined the prevalence of antibody to bovine leukemia virus (BLV) in the two herds. The results demonstrated that the incidence of BLV infection in the two herds was between 20% and 25%, and was the same in the BIV-seropositive and BIV-seronegative animals. The results suggested that BIV and BLV infections were unrelated.

Coats, K.S., Pruett, S.B., Nash, J.W. and Cooper, CR. 1994. Bovine immunodeficiency virus — incidence of infection in Mississippi dairy cattle. Veterinary Microbiology, 42, 181–189.

Bovine immunodeficiency virus (BIV), a lentivirus, was originally derived from a Holstein cow with persistent lymphocytosis and severe wasting. The virus is known to occur sporadically throughout the United States and perhaps across the globe, but epidemiological data concerning the incidence of BN are meagre and the virus was previously unreported in Mississippi animals. This study examined the seroepidemiology of BIV infection from two Mississippi dairy herds (Coastal Plains and MSU). Serology revealed a 38% incidence of BIV infection in Coastal Plains animals and a 58% incidence in MSU animals. A cumulative BIV seroprevalence of 50% was found in the Mississippi animals, and BIV seroprevalence increased with increasing age of the animals. Peripheral blood leukocytes of age-matched BIV seropositive and seronegative animals were enumerated to assess any effect of BTV infection on leukocyte populations. No significant differences were found in total leukocyte populations or leukocyte subpopulations between BIV seropositive or seronegative animals. These data indicate that BIV infection is prevalent in Mississippi animals, but the role of BIV in bovine disease remains unclear.

Coats, K.S.C. 1995. Questions possibility of seminal transmission of bovine immunodeficiency virus — response [Letter]. American Journal of Veterinary Research, 56, 1401.

Correspondence relating to publication by Nash et al. 1995) reporting detection of bovine immunodeficiency virus (BIV) genomic material in bull semen by PCR.

Cockerell, G.L., Jensen, W.A., Rovnak, J., Ennis, W.H. and Gonda, M.A. 1992. Seroprevalence of bovine immunodeficiency-like virus and bovine leukemia virus in a dairy cattle herd. Veterinary Microbiology, 31, 109–116.

To determine the prevalence of single vs. dual infection with bovine immunodeficiency virus (BIV) and bovine leukemia virus (BLV), sera (n = 95) from a dairy cattle herd were analysed for anti-BIV and anti-BLV antibodies by an enzyme linked immunosorbent assay. Twenty-one percent (20/95) of samples were BIV-seropositive, while 52% (49/95) of the same samples were BLV-seropositive. A significantly greater percentage of BIV-seronegative samples were BLV-seropositive, 57% (43/75), than were BIV-seropositive samples, 30% (6/20). There was no significant correlation between data ranked from least to greatest amount of anti-viral antibody. Five cattle had persistent lymphocytosis (PL); all five were BLV-seropositive and two were BIV-positive. The mean anti-BLV titre was significantly greater in PL cattle, as compared at non-PL cattle, whereas there was no significant difference between the mean anti-BIV titre in PL cattle, as compared with non-PL cattle. These results provide additional information on the seroprevalence of naturally occurring BIV infection, and indicate that BIV can exist independent of other common infectious agents, such as BLV. Further, the results suggest that infection with BIV is not associated with an increased rate of infection with other infectious agents such as BLV.

Detilleux, J.C., Kehrli, M.E., Freeman, A.E., Whetstone, C.A. and Kelley, D.H. 1995. Two retroviral infections of periparturient Holstein cattle — a phenotypic and genetic study. Journal of Dairy Science, 78, 2294–2298.

Environmental and genetic factors affecting prevalences of antibodies to bovine leukosis virus and bovine immunodeficiency-like virus were studied on 137 periparturient Holstein cows selected for milk production. Environmental effects were obtained by logistic regression, and genetic parameters were determined using threshold animal models. Cows selected for high predicted transmitting ability for kilograms of milk fat plus protein had the highest prevalence of antibodies to bovine immunodeficiency-like virus and the lowest prevalence of antibodies to bovine leukosis virus. Heritability estimates for susceptibility to retroviral infections were close to zero.

Egberink, H. and Horzinek, M.C. 1992. Animal immunodeficiency viruses. Veterinary Microbiology, 33, 311–331.

Feline immunodeficiency virus (FIV) has morphological, physical and biochemical characteristics similar to human immunodeficiency virus (HIV), the cause of AIDS in man. However, it is antigenically and genetically distinct from HIV; an antigenic relatedness with equine infectious anaemia virus has been demonstrated. FIV has been molecularly cloned and sequenced. Diagnostic tests are commercially available and attempts at preparing inactivated, subunit and molecularly engineered vaccines are being made in different laboratories. During FIV infection a transient primary illness can be recognised, with fever, neutropenia and lymphadenopathy. After a long period of clinical normalcy a secondary stage is distinguished with signs of an immunodeficiency-like syndrome. The incubation period for this stage can be as long as 5 years, during which gradual impairment of immune function develops. Many FIV-infected cats are presented for the first time showing vague signs of illness: recurrent fevers, emaciation, lack of appetite, lymphadenopathy, anaemia, leucopenia and behavioural changes. Later, the predominant clinical signs observed are chronic stomatitis/gingivitis, enteritis, upper respiratory tract infections, and infections of the skin. Neoplasias, neurological, immunological and haematological disorder are seen in a smaller proportion. The immunodeficiency-like syndrome is progressive over a period of months to years. Concomitant infection with feline leukemia virus has been shown to accelerate the progression of disease. In vitro, phenotypic mixing between FIV and an endogenous feline oncovirus (RD114) has been demonstrated which leads to a broadening of the cell spectrum of the lentivirus. Bovine immunodeficiency virus (BIV) has been isolated only once, and all attempts to obtain additional isolates have failed; it has been recovered from the leukocytes of cattle with persistent lymphocytosis, lymphadenopathy, lesions in the central nervous system, progressive weakness and emaciation. As with the feline representative, BIV also was found to possess a lentivirus morphology and to encode a reverse transcriptase with Mg^{++} preference; it replicates and induces syncytia in a variety of embryonic bovine tissues in vitro. Antigenic analyses have demonstrated a conservation of epitopes between the major core protein of BIV and HIV. The original isolate has been molecularly cloned and sequenced. Besides the three large open reading frames (ORFs) comprising the *gag*, *pol*, and *env* genes common to all replication-competent retroviruses, five additional small ORFs were found. Numerous point mutations and deletions were found, mostly in the env-encoding ORF. These data suggest that, within a single virus isolate, BIV displays extensive genomic variation

Flaming, K., Van Der Maaten, M., Whetstone, C., Carpenter, S., Frank, D. and Roth, J. 1993. Effect of bovine immunodeficiency-like virus infection on immune function in experimentally infected cattle. Veterinary Immunology and Immunopathology, 36, 91–105.

Bovine immunodeficiency-like virus (BIV) is a bovine lentivirus which has antigenic and genetic homology with the human immunodeficiency virus. Little work has been reported on the effect of BIV infection on bovine immune function. This study was designed to evaluate lymphocyte blastogenesis, mononuclear cell subset numbers, function, haematology, and clinical signs in three groups of cattle. These groups were evaluated at 0–2 months post inoculation (PI, Group 1), 4–5 months PI (Group 2), or 19–27 months PI (Group 3). BIV infected animals were inoculated with the R-29 isolate of BIV in tissue culture cells, peripheral blood mononuclear cells from a R-29 infected calf, or a molecular clone of the R-29 isolate. Most inoculated animals seroconverted to BIV by Western immunoblot. BIV was reisolated from most of the animals inoculated. BIV infection was associated with an increase in the lymphocyte blastogenic response to the mitogen phytohaemagglutinin in Groups 2 and 3. Neutrophil antibody dependent cell mediated cytotoxicity and neutrophil iodination were decreased ($P<0.05$) in BIV-infected cattle (Groups 2 and 3 and Group 3, respectively). All animals were clinically normal during the evaluation periods. Notable differences were not observed in the other assessments performed. Work with additional BIV isolates and over longer time frames is warranted.

Fong, S.E., Pallansch, L.A., Mikovits, J.A., Lackmansmith, C.S., Ruscetti, F.W. and Gonda, M.A. 1995. Cis-acting regulatory elements in the bovine immunodeficiency virus long terminal repeat. Virology, 209, 604–614.

Functional cis-acting regulatory elements in the bovine immunodeficiency virus (BIV) long terminal repeat (LTR) were identified by deletion mapping and nuclear protein gel shift analysis using three BIV-infectible cell lines, Cf2Th, BLAC-20, and EREp. Deletion mapping studies indicated that putative NF-kappa B, GRE, AP-4, AP-1, CAAT, and ATF/CRE transcription factor elements positively contribute to LTR-directed gene expression in each cell line both in the presence and absence of the viral transactivator Tat Spl and overlapping AP-3 and retroviral core enhancer elements had variable effects on LTR-directed gene expression depending on cell type and presence or absence of Tat. In addition, a sequence spanning the LTR U5 region and the untranslated viral leader was strongly repressive in all cell lines. Tat transactivated the LTR 25-fold over basal levels in a TAR-dependent manner in Cf2Th cells. In contrast, Tat transactivated the LTR only 2.5-fold over basal levels in EREp and BLAC-20 cells in a TAR-independent manner. Probes for putative NF-kappa B, GRE, Spl, AP-4, AP-1, overlapping AP-3 and retroviral core enhancer, and juxtaposed CAAT and ATF-CRE elements specifically bound nuclear proteins from these three cell lines and HeLa cells, with the stoichiometry of binding being cell-type dependent. Probes for AP-4, AP-1, and juxtaposed CAAT and ATF/CRE elements exhibited greater protein binding with extracts from virus infected cells than with extracts from uninfected cells, suggesting that viral infection can modulate nuclear factor binding. The present studies indicate that several transcription factor elements in the BIV LTR have functional roles and that cell type can strongly determine the role they play in gene expression.

Forman, A.J., Gibson, C.A. and Rodwell, B.J. 1992. Serological evidence for the presence of bovine lentivirus infection in cattle in Australia [letter]. Australian Veterinary Journal, 69, 337.

A brief report of a small survey of 60 serum samples from two dairy herds in Victoria and 60 sera from four dairy herds in Queensland for antibody to the bovine immunodeficiency-like virus (BIV) by ELISA and Western blot. The herds were selected on the basis of a high prevalence of antibody to bovine leukosis virus. A positive result by one or both of the two tests indicated a prevalence rate of antibody to BIV of between 7% and 17%, in all of the six herds.

Garvey, K.J., Oberste, M.S., Elser, J.E., Braun, M.J., Gonda, M.A. 1990. Nucleotide sequence and genome organisation of biologically active proviruses of the bovine immunodeficiency-like virus. Virology, 175, 391–409.

The complete nucleotide sequences and translations of major open reading frames (ORF) of two distinct, infectious, proviral molecular clones (106 and 127) of the bovine immunodeficiency-like virus (BIV), obtained from a single virus isolation, were determined and compared. The genomes of BIV 127 and 106 are 8482 and 8391 nucleotides (nt), respectively, in the form predicted for the viral RNA. The structural organisation of the genomes of BIV 127 and 106 are identical to one another and most similar to that of the lentivirus subfamily of retroviruses. In addition to gag, pol, and env genes, the BIV genome contains five short ORFs between and overlapping pol and env in the 'central region,' a hallmark of the lentiviruses which is believed to play an important role in their pathogenesis. Three of the short ORFs in the central region of BIV have been identified by location and structural similarity to the nonstructural/regulatory genes (vif, tat, and rev) of other lentiviruses; we also discovered two unique ORFs, termed W and Y, which may serve as exons for novel genes. BIV does not have the nef gene found in primate lentivirus genomes. The proviral LTR of BIV 127 is 589 nt, contains regulatory signals for initiation, enhancement, and termination of viral transcription, and has sequences related to the Sp1 and NF-kappa B binding sites. A major deletion (87 nt) in the env gene and 2 minor deletions (2 nt each) in the R regions of the LTRs account for the smaller size of clone 106. Numerous point mutations were also present; some caused coding substitutions that were most prevalent in the env encoding ORF. These data suggest that, within a single virus isolate, BIV displays extensive genomic variation. These infectious clones of BIV represent well-defined tools with which to analyse the function of the various ORFs and to dissect the molecular mechanisms of replication and pathogenesis.

Geng, Y., Kashanchi, F. and Wood, C. 1992. Activation of bovine immunodeficiency-like virus expression by bovine herpesvirus type 1. Virology, 187, 832–836.

Bovine immunodeficiency-like virus (BIV) is a recently identified lentivirus that infects cattle. The virus has structural and genetic similarities to human HIV. The present study demonstrates that BIV can be activated by bovine herpesvirus type 1 (BHV-1), a pathogen frequently associated with cattle diseases. Activation of BIV

expression can be detected as increased BIV reverse transcriptase activity, increased in the number of syncytia induced by BIV, and increased in the steady state level of BIV-specific RNA upon BHV-1 super-infection. Additional transactivation studies using the BIV-LTR (long terminal repeat) were conducted. The BIV-LTR was linked to the chloramphenicol acetyl transferase gene (CAT) and transfected into bovine cell cultures in order to quantitate the levels of BIV-LTR expression. When the transfected cells were infected by BHV-1, there was an increase in CAT expression, indicating transactivation of the BIV-LTR by BHV-1. Most of the transactivation activities were abolished with an LTR construct that has deleted the NF-kappa B-like sequence located in the U3 region of the LTR. In order to further demonstrate that activation of the BIV-LTR involves factors that may bind to the LTR sequences, gel retardation assays were carried out using the BIV-LTR U3 region as probe. Our results showed that BHV-1 infection resulted in an induction of factor(s) that binds to the NF-kappa B-like sequence on the BIV-LTR. This suggests that transactivation of BIV by BHV-1 may be mediated by a bovine NF-kappa B-like protein that binds to the target sequence in the BIV promoter region.

Georgiades, J.A., Billiau, A. and Vanderschueren, B. 1978. Infection of human cell cultures with bovine visna virus. Journal of General Virology, 38, 375–

Fibroblastoid cell cultures derived from leukemic bone marrow were successfully infected with BIV (isolated and reported by Van Der Maaten et al. 1972). After 2 months of subcultivation the cultures showed the appearance of foci of altered cells, suggestive of malignant transformation. Such foci were absent in non-inoculated cell cultures. Both control and inoculated cultures had a limited life span, i.e., neither of them could be developed into continuous transformed cell lines. The presence of at least some BIV genome functions in the inoculated cells was demonstrated by (1) immunofluorescence using a reference BIV antiserum, (2) by detection in the supernatant culture fluid of sedimentable particles bearing RNA-dependent DNA polymerase activity with preference for Mg^{2+} ions, and (3) by electron microscopic detection of scarce cell-associated virus particles in one of the infected cultures. Infectious BIV could not be rescued. In contrast to leukemic bone marrow cultures, diploid human fibroblasts of various origin could not be infected with BIV.

Gonda, M.A. 1992. Bovine immunodeficiency virus [editorial review]. AIDS, 6, 759–776.

Gonda M.A., Braun, M.J., Carter, S.G., Kost, T.A., Bess, J.W., Arthur, L.O. and Van Der Maaten, M.J. 1987. Characterisation and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. Nature, 330, 388–391.

An infectious virus which causes persistent lymphocytosis, lymphadenopathy, lesions in the central nervous system (CNS), progressive weakness and emaciation was previously isolated from the leukocytes of cattle. Our present studies show that this virus encodes a reverse transcriptase (RT) with Mg2+ cation preference, replicates and induces syncytia in a variety of embryonic bovine tissues in vitro, and has a morphology most similar to the human immunodeficiency virus (HIV). Moreover, serologic analyses have demonstrated a conservation of epitopes between the major core protein of this bovine retrovirus and HIV. Shared antigenic determinants were also observed with other pathogenic retroviruses of the lentivirus subfamily. To resolve the phylogenetic relationship of this virus, proviral molecular clones were derived and used to determine the nucleotide sequence of the highly conserved RT domain. The sequence data and serologic analyses together show that this bovine retrovirus is a novel lentivirus related to HIV and other lentiviruses. We propose that this virus be tentatively named bovine immunodeficiency-like virus (BIV) to reflect its genetic relationship and biological similarity to HIV.

Gonda, M.A., Fong, S.E. and Tobin, G.J. 1994. Bovine immunodeficiency virus — emerging biology of a nonacute pathogenic lentivirus of cattle [Review]. Food Microbiology, 11, 149–160.

BIV is a nonacute pathogenic member of the lentivirus genus of retroviruses that is related to HIV. The normal host range of BIV is cattle where it is associated with a transient lymphocytosis, persistent lymphadenopathy, chronic dermatitis, encephalitis, and possibly other immune-mediated lesions. Experimental studies indicate BIV infects cells of the immune system, primarily monocytes and macrophages, where it causes immune cell dysfunction. Existing seroepidemiological data on BIV prevalence suggest that virus infections are worldwide. In the US, the distribution appears to be nonuniform with the highest prevalence occurring in southern cattle populations. The molecular biology of BIV has been studied extensively and shown to have many parallels with HIV, including the presence of several non-structural accessory genes important in regulating virus gene expression and pathogenesis. Rabbit and transgenic mice animal models have been devised to study the mechanisms influencing BIV infection, replication, gene expression and pathobiology. Our increased knowledge of BIV-host interactions, as studied in natural and surrogate hosts, will lead to a better understanding of the immunopathogenic aspects of BIV infection and its effect on animal health.

Gonda, M.A., Luther, D.G., Fong SE. and Tobin GJ. 1994. Bovine immunodeficiency virus — molecular biology and virus-host interactions [review]. Virus Research, 32, 155–181.

Gonda, M.A., Oberste, M.S., Garvey, K.J., Pallansch, L.A., Battles, J.K., Pifat, D.Y., Bess, J.W. and Nagashima, K. 1990. Development of the bovine immunodeficiency-like virus as a model of lentivirus disease. Developments in Biological Standardisation (Karger, Basel), 72, 97–110.

The bovine immunodeficiency-like virus (BIV) is morphologically, serologically, and genetically related to the lentivirus subfamily of retroviruses which includes human and simian immunodeficiency viruses and other lentiviruses causally associated with debilitating diseases of domestic animals. There are many parallels in the biology and pathologic characteristics of BIV infections with those of HIV that make its development as a model of HIV-like infection and disease potentially attractive. In order to obtain a better understanding of the molecular basis of BIV-induced disease, two biologically active proviruses of BIV were molecularly cloned and sequenced. The BIV genome is 9.0 kilobases in the form of the proviral DNA. It contains the obligate retroviral structural genes, gag, pol, and env. In addition, in the BIV central region, between and overlapping pol and env, there are five potential coding regions for non-structural/regulatory genes; three are analogous to vif, tat, and rev in HIV and two, called W and Y, are unique to BIV. There is no coding region analogous to nef in BIV. Sequence comparisons of two functional proviruses obtained from the DNA of cells carrying an infection from a single virus isolation indicate that the genome of BIV is highly variable within a single biological isolate. Moreover, the greatest number of substitutions occur in the env gene. The results suggest the presence of multiple genotypes which may be of significance in defining the disease potential of a BIV isolate. These clones will be useful in dissecting the replicative cycle and mechanisms of pathogenesis of BIV in various animal models.

Gonda, M.A., Oberste, M.S., Garvery, K.J. et al. 1990. Contemporary developments in the biology of bovine immunodeficiency-like virus [review]. In: Schellekens, H. and Horzinek, M.C., ed., Animal Models in Aids. Elsevier, Amsterdam, 233–255.

Gonda, M.A., Pallansch, L A., Oberste, M S., Centanni, J M., Greenwood, J D. and Williamson, J.C. 1994. Pathogenesis of viral gene expression in bovine immunodeficiency virus (biv) transgenic mice [Abstract]. AIDS Research and human retroviruses, 10 (Suppl), S87–S87.

We have characterised functional proviral molecular clones and transgenic mice expressing these clones to understand the role of viral gene expression in pathogenesis. The biological effects of virus gene expression in vivo were studied in transgenic mice carrying complete proviral genomes. Two transgenic mouse lines with distinct integration sites developed antibodies to BIV proteins within six weeks of birth and significant clinical disease. Pathogenesis was observed in several organs including brain, thymus, spleen, lymph nodes, and skin, and one of the lines exhibits early mortality (50%) attributable to a virally induced meningoencephalitis. Virus gene expression was low, and could only be demonstrated by RT-PCR assay, and correlated with pathology observed in the affected organs.

Gonda, M.A., Wong-Staal, F., Gallo, R.C., Clements, J.E., Gilden, R.V. 1985. Heteroduplex mapping in the molecular analysis of the human T-cell leukemia (lymphotropic) viruses. Cancer Research, 45, 4553s–4558s.

The human T-cell lymphotropic virus (HTLV) family includes members associated with T-cell cancers (HTLV-I and HTLV-II) as well as the aetiological agent of the acquired immunodeficiency syndrome (HTLV-III). Molecular clones of these viruses were used in heteroduplex mapping experiments to study their structural and evolutionary relationships. The HTLV-I subgroup, despite some restriction enzyme site polymorphism, demonstrated a high degree of sequence conservation. Heteroduplexes of HTLV-I and HTLV-II demonstrated a significant amount of sequence homology, with the strongest region of conservation occurring in the 3' -most coding sequences, designated pX, and to a lesser, although substantial extent in the rest of the genome. Thus, the genomic organisation of HTLV-II appears to be very similar to that of HTLV-I. All HTLV-III molecular clones appeared to be identical, with a single exception, which showed heterogeneity in the env gene region. In heteroduplexes between HTLV-I and HTLV-III, very little homology was observed, being confined to the gap/pol region. In contrast to the latter result, a striking amount of homology was detected between HTLV-III and a morphologically similar, pathogenic, nononcogenic lentivirus, visna virus. These data provide strong evidence for a close taxonomic and thus evolutionary relationship between HTLV-III and the lentivirus subfamily of retroviruses. A taxonomic tree, based on the genetic relatedness and biological properties of the HTLV family, is proposed.

Grote, J. 1988. Bovine visna virus and the origin of HIV [letter]. J. R. Soc. Med., 81, 620.

Comments on report by Georgiades et al. 1978) of the presence of "bovine visna virus" (bovine immunodeficiency-like virus) in a high proportion of batches of fetal calf serum for used for cell culture. Due to the apparent similarity betweeen this virus and HIV, suggests that BIV be further investigated with regard to its possible causal role in AIDS.

Hidalgo, G. Flores, M. and Bonilla, J.A. 1995. Detection and isolation of bovine immunodeficiency-like virus (BIV) in dairy herds of Costa Rica. Zentralblatt fur Veterinarmedizin — Reihe B, 42, 155–161.

Serological (Western blot) detection of bovine immunodeficiency-like virus (BIV) in Holstein dairy herds is reported in Costa Rica for the first time, as well as the isolation of the virus, from a seropositive bovine, by cocultivation of peripheral blood mononuclear cells (PBMC) with embryonic rabbit epithelial (EREp) cells. The isolated strain, BIVCR1, reacted similarly in Western blot as the reference strain BIV R29 and is clearly distinguishable from bovine leukaemia virus (BLV). The data suggest an association between BIV infections and BLV infections, as it has been reported elsewhere. From these results it can be concluded that BIV is present in Costa Rica and it is suggested that these viral infections will probably follow the epidemiological parameters of BLV infections in Costa Rica, reaching high infection rates in dairy herds.

Hidalgo, G. and Bonilla, J.A. 1996. Lymphoproliferation assays in cattle naturally infected with bovine leukemia virus (blv) and bovine immunodeficiency-like virus (BIV). Zentralblatt Fur Veterinarmedizin — Reihe B, 43, 325–332.

Experiments were designed to evaluate the effect of BLV on mitogen-stimulated peripheral blood mononuclear cells (PBMC) from naturally infected cattle. BIV was also taken into consideration due to a recent report showing that in Costa Rica, most of the BLV-infected animals are also seropositive for BIV. The methodology was based on a non-radioactive technique to determine lymphoproliferation. A colourimetric assay using XTT (formazan salt) to measure cell multiplication was adapted for bovine PBMC. ELISA and Western blotting were used to determine the serologic status of the cattle. PCR was only available for BIV detection. Our results show clearly that, dually-infected cattle (BIV-BLV) have reduced lymphoproliferative responses to the mitogen Con A. Haematological abnormalities associated with viral infections were also observed, specially leukocytosis and lymphocytosis. Cows with lymphosarcomas are severely affected. The specific antibody response to different viral proteins could not be associated with the suppressive status of the animals. Due to the high rate of dual infections observed in Costa Rica, these results are not sufficient to clarify which virus is responsible for the suppressive activity, if one or both viruses are necessary, or if they act synergistically.

Hirai, N., Furuyama, H., Awaya, A. and Onuma, M. 1995. Effect of administration of serum thymic factor (FTS) in calves and rabbits infected with bovine immunodeficiency-like virus. Journal of Veterinary Medical Science, 57, 307–310.

The effect of serum thymic factor (FTS) administration in bovine immunodeficiency-like virus (BIV)-infected calves and rabbits was examined. We previously found that some of the macrophage functions were depressed and humoral immune responses against foreign proteins were delayed in BIV-infected calves compared to uninfected calves. After FTS administration, however, no delay of antibody responses against foreign proteins was observed in BIV-infected calves. Though the chemiluminescence (CL) responses of macrophages in BIV-infected calves were significantly depressed ($p<0.05$), FTS administration resulted in the recovery of the CL responses in the BIV-infected calves comparable to those in the control calves. Antibody responses against foreign proteins in BIV-infected rabbits were significantly depressed ($p<0.025$) as compared with those in uninfected rabbits, though the depression became no significant after FTS administration.

Hirai, N., Kabeya, H., Ohashi, K. Sugimoto, C. and Onuma, M. 1996. Detection of antibodies against bovine immunodeficiency-like virus in daily cattle in Hokkaido. Journal of Veterinary Medical Science, 58, 455–457.

Serological survey of bovine immunodeficiency-like virus (BIV) infection was performed in cattle of three different farms in Hokkaido, where a relatively high seroprevalence was recorded for bovine leukemia virus (BLV). About a half of 120 cattle tested were seropositive for BLV, while 7.5% of the cattle were seropositive for BIV. Though increased numbers of leukocytes were frequently observed in BLV-seropositive cows, no such changes were observed in BIV-positive but BLV-negative cows. No correlation was demonstrated between BIV- and BLV-seroprevalence of the cattle.

Hirai, N., Xuan, S.W., Ochiai K. and Onuma, M. 1994. Alteration of immune responses of rabbits infected with bovine immunodeficiency-like virus. Microbiology and Immunology, 38, 943–950.

Nine 3-month-old rabbits were inoculated with bovine immunodeficiency-like virus (BIV) to study the pathogenesis of BIV and alteration of the immune responses in experimentally infected rabbits. BIV proviral DNA and anti-BIV antibodies were detected from all rabbits inoculated with BIV-infected bovine embryo spleen (BESP) cells. Rabbits inoculated with spleen cells of the BIV-infected rabbit also converted to proviral DNA-positive and BIV-antibody-positive. The blastogenic responses to concanavalin A of peripheral blood mononuclear cells prepared from BIV-infected rabbits were not significantly different from those from uninfected controls at 2 and 4 months postinoculation (PI). The humoral immune responses against bovine serum albumin (BSA) were depressed in two of four BIV-infected rabbits at 1 to 3 months PI. The antibody responses against sheep red blood cells (SRBCs) were significantly depressed in all BIV-infected rabbits at 2 to 4 months PI. BIV was rescued by cocultivation of spleen cells of infected rabbits with BESP cells. Distinct development of lymphoid follicle was observed in lymph nodes and spleens of uninfected rabbits which received BSA and SRBCs. In contrast, moderate lymphoid cell depletion was observed in BIV-infected rabbits which received the same immunogens.

Horzinek, M., Keldermans, L., Stuurman, T., Black, J., Herrewegh, A., Sillekens, P. and Koolen, M. 1991. Bovine immunodeficiency virus: immunochemical characterisation and serological survey. Journal of General Virology, 72, 2923–2928.

Bovine immunodeficiency virus (BIV) was purified by isodensity centrifugation; viral activities were monitored in gradient fractions using the reverse transcriptase assay and a p26-specific monoclonal antibody ELISA. In the coincident peak fractions (density about 1.17 g/mL) proteins with Mr values of 26K, 17K, 53K, 14K and 100K (with decreasing intensity) were detected by Western blotting using serum of a calf after experimental BIV infection. When 957 randomly collected cattle sera from The Netherlands were tested by indirect immunofluorescence and confirmed using Western blot and/or radioimmunoprecipitation, 1.4% appeared seropositive. Thus BIV infection is not uncommon in one European cattle population.

Isaacson, J.A. Roth, J.A. Wood, C. and Carpenter, S. 1995. Loss of gag-specific antibody reactivity in cattle experimentally infected with bovine immunodeficiency-like virus. Viral Immunology, 8, 27–36.

The development and persistence of virus-specific antibodies were investigated in eight cattle experimentally infected with the R29 isolate of bovine immunodeficiency-like virus (BIV). By 4 weeks postinoculation (p.i.), antibodies reactive to BIV gag- and env-encoded recombinant fusion proteins were detectable by immunoblotting in all animals. By 40 weeks p.i., seven of eight cattle had dramatically decreased Gag-specific antibodies, and anti-Gag reactivity remained very low or undetectable through 190 weeks p.i. Immunoprecipitation experiments revealed a similar loss of reactivity to nondenatured BIV Gag in these animals. In contrast, antibodies to a recombinant BIV Env protein were readily detectable throughout the study in all eight cattle. During the period of declining Gag antibody, infectious virus was recoverable from peripheral blood mononuclear cells of each animal. However, there was no evidence for sufficient amounts of BIV p26-containing immune complexes to explain the loss of anti-Gag reactivity. Interestingly, the single animal that maintained detectable anti-Gag reactivity throughout the study was repeatedly negative for virus recovery beyond 17 weeks p.i. All animals have remained clinically normal for over 4 years p.i., with no evidence of consistent changes in mononuclear cell subsets. These findings provide evidence that in BIV infection an early decline in Gag-specific antibody reactivity can occur without evidence of increasing viral replication or progression to overt clinical disease.

Jacobs, R.M., Mccutcheon, L.J., Valli, V.E.O. and Smith, H.E. 1996. Histopathological changes in the lymphoid tissues of sheep exposed to the bovine immunodeficiency-like virus. Journal of Comparative Pathology, 114, 23–30.

Six yearling sheep were inoculated intraperitoneally with peripheral blood from two sheep infected with the bovine immunodeficiency-like virus (BIV) strain R29. An additional five sheep served as sham-inoculated controls. Of the six sheep given BIV, five seroconverted, one of them remaining seropositive for the duration of the study. The polymerase chain reaction demonstrated BTV provirus in three of the five serologically positive animals. At necropsy, 1 year after inoculation, histological changes were found only in the lymphoid tissues. In sheep exposed to BTV, mild though significant increases were seen in the (1) number of splenic periarteriolar lymphoid sheaths, (2) number of secondary follicles in hilar and prescapular or popliteal lymph nodes, and (3) medullary sinus cellularity in prescapular and popliteal lymph nodes.

Jacobs, R.M., Smith, H.E., Gregory, B., Valli, V.E. and Whetstone, C.A. 1992. Detection of multiple retroviral infections in cattle and cross-reactivity of bovine immunodeficiency-like virus and human immunodeficiency virus type 1 proteins using bovine and human sera in a Western blot assay. Canadian Journal of Veterinary Research, 56, 353–359.

Bovine antibovine immunodeficiency-like virus (BIV) antibodies were detected by Western blot analysis (WBA) using a chemiluminescence protocol. Bovine sera with anti-BIV activity, obtained from cows in two dairy herds, had antibodies directed against a variety of BIV-specific antigens indicating chronic infections. These sera were also tested for serological reactivity against bovine leukemia virus (BLV) and bovine syncytial virus (BSV). Cows most commonly had anti-BSV antibodies (12 of 39). Evidence for infection with BSV and BIV or BSV and BLV occurred with almost equal frequency (5 of 39 and 4 of 39, respectively) while only one instance of BIV and BLV coseropositivity was detected. The high prevalence of BSV seropositivity is consistent with a relatively infectious virus, which, as is known, may be transferred congenitally. Similar rates of coseropositivity of BIV or BLV with BSV in this population suggest that BIV is no more infectious than BLV and probably requires prolonged close contact for transmission. Seven of nine cows with anti-BIV antibodies detected primarily human immunodeficiency virus type 1 (HIV-1) p51 and p63 antigens by WBA using an alkaline phosphatase detection system, suggesting that HIV-1 proteins have potential usefulness in screening cattle for BIV seropositivity. Six human sera that showed strong reactivity against multiple HIV-1 proteins and the serum from one of three patients considered to be an 'indeterminate' HIV-1 reactor, cross-reacted primarily with BIV p26. This is the first report of human sera with antibody to BIV-specific proteins.

Jacobs, R.M., Smith, H.E., Whetstone, C.A., Suarez, D.L., Jefferson, B. and Valli, V.E. 1994. Haematological and lymphocyte subset analyses in sheep inoculated with bovine immunodeficiency-like virus. Veterinary Research Communications, 18, 471–482.

Bovine immunodeficiency-like virus (BIV) was passaged in vivo by intraperitoneal transfusion of ovine whole blood. Prior to transfusion, the recipient sheep were given sodium thioglycolate intraperitoneally to induce mild non-suppurative inflammation. The anti-BIV antibody response, haematology, and peripheral blood lymphocyte subsets (B, gammadelta, CD2+, CD4+ and CD8+) of recipient sheep were assessed for one year following transfusion. Passaging was successful since serum anti-BIV antibody responses were detected in 5 of the 6 recipient sheep; 1 of the 5 remained seropositive throughout the study. Lentivirus was not isolated from the recipient sheep, but provirus was detected by the polymerase chain reaction in DNA from peripheral blood leukocytes in 3 of the 5 sheep that seroconverted. In the BIV-inoculated sheep, neutrophils and eosinophils were significantly increased (p less-than-or-equal-to 0.05) at 3 months and between 6 and 8 months postinoculation, respectively. B, CD2+ and CD4+ cells and the CD4+:CD8+ ratios were significantly increased (p less-than-or-equal-to 0.05) 2 months postinoculation. Mild, transient haematological changes occurred in BIV-exposed sheep, but illness was not detected in the year.

Kakidani, H., Watarai, S., Onuma, M., Tomochika, K. and Yasuda, T. 1993. Suppressive effect of liposomes containing DNA coding for diphtheria toxin-a-chain on cells transformed with bovine leukemia virus. Microbiology and Immunology, 37, 713–720.

A recombinant plasmid which contained a gene for diphtheria toxin A-chain (DT-A) under the control of the long terminal repeat (LTR) of bovine leukemia virus (BLV) (BLV-LTR) was constructed to test a novel application of liposomes as antiviral agents. The promoter activity of BLV-LTR was estimated by the chloramphenicol acetyltransferase (CAT) assay using a plasmid which contains the coding sequence of CAT under the control of BLV-LTR (pBLVCAT). When BLV-infected cells were transfected with pBLVCAT, CAT activity was detected. BLV-uninfected cell lines, however, showed no detectable CAT activity. The plasmid DNA entrapped in liposomes was added to BLV-infected cells in culture. Syncytium formation induced by BLV-infected cells was effectively suppressed by the liposomes containing the gene for DT-A under the control of BLV-LTR. Conversely, liposomes containing the gene for DT-A without a promoter showed no such effect. DT-A gene-containing liposomes with BLV-LTR did not affect formation of syncytium induced by bovine immunodeficiency virus. These observations indicate that BLV-infected cells were readily targeted on the level of gene expression. This strategy could be applied to the treatment of BLV-induced B-cell proliferation of cattle, and further to other viral/neoplastic diseases where specific gene expression is exerted.

Kalvatchev, Z., Walder, R., Barrios, M. and Garzaro, D. 1995. Acquired immune dysfunction in rabbits experimentally infected with an infectious molecular clone of the bovine immunodeficiency virus (BIV127). Viral Immunology, 8, 159–1645.

To investigate the effect of bovine immunodeficiency virus (BIV) infection on the rabbit immune system, we studied the proliferative responses of peripheral blood lymphocytes (PBLs) of rabbits experimentally inoculated with BIV. All BIV127-inoculated rabbits seroconverted after 6 weeks and remained seropositive over a prolonged period of time. Assays for specific lymphocyte reactivity to concanavalin A (Con A) were performed monthly for over 1 year. One-hundred percent of infected rabbits developed abnormally low T cell responses, as measured by Con A stimulation. By 3 months postinoculation, the PBL response to Con A was diminished and remained depressed for 6 months. All animals were clinically asymptomatic within 14 months of BIV inoculation, By 15 and 16 months postinoculation, two of three infected rabbits exhibited recurrent lowering of the T cell responsiveness including a decrease in absolute PBL counts. One of these animals died unexpectedly. Our results further confirmed that a functional impairment of lymphocytes was induced early in the course of BIV infection, prior to clinical disease. These findings suggested that BIV infection may mimic asymptomatic infection of human immunodeficiency virus (HIV) and provided further evidence of the importance of BIV-induced disease in rabbits as a relevant model for the study of AIDS.

Kashanchi, F., Liu, Z.Q., Atkinson, B. and Wood, C. 1991. Comparative evaluation of bovine immunodeficiency-like virus infection by reverse transcriptase and polymerase chain reaction. Journal of Virological Methods, 31, 197–209.

Infection of embryonic bovine lung (EBL) cells by bovine immunodeficiency-like virus (BIV) were monitored by reverse transcriptase (RT), syncytia formation and polymerase chain reaction (PCR). Infection can be detected by PCR at 24 h while the presence of syncytia and RT were not detected until much later. The detection of BIV RT can be optimised by changing the pH and salt conditions. The enzyme is very sensitive to changes in pH but can tolerate a wider range of salt and $MgCl_2$ concentrations. Infection of primary human cell cultures by BIV was monitored by both PCR and RT. No active infection of human cells were detectable.

Liu, Z.Q., Sheridan, D. and Wood, C. 1992. Identification and characterisation of the bovine immunodeficiency-like virus *tat* gene. Journal of Virology, 66, 5137–5140.

A cDNA clone of the bovine immunodeficiency-like virus (BIV) transactivator gene (tat) was identified and characterised. The tat cDNA clone was generated by splicing, and on the basis of sequence analysis, the Tat protein was found to be encoded entirely by the first exon. It is 103 amino acids in size and shares sequence homology with the human immunodeficiency virus (HIV) Tat. The BIV tat clone can transactivate the BIV promoter effectively, as measured by the expression of the bacterial chloramphenicol acetyltransferase gene, when transfected into bovine cells. Besides activating the BIV promoter, the BIV Tat can also transactivate the HIV promoter effectively. It is possible that BIV Tat and HIV Tat employ similar mechanisms in transactivation of the viral long terminal repeat-directed gene expression. Molecular Sequence Databank No.: GENBANK/M90681.

Luther, D.G. Todd, W.J. Snider, R. Gonda, M.A. 1993. Nervous tissue lesions in cattle infected with the bovine immunodeficiency virus [abstract]. Journal of Cellular Biochemistry Suppl., 17E:33.

Holstein cattle infected with the bovine immunodeficiency virus (BIV) exhibit neurological signs of depression, ataxia, anorexia, and decreased milk production. These clinical signs progress to coma and death in the infected animals. The animals studied were naturally infected and had been sero-positive for BIV for a period of at least three years prior to development of clinical signs. Some of the affected animals had chronic mastitis and chronic foot-rot problems that failed to respond to traditional treatments. Although it was difficult to isolate the BIV from peripheral blood, the infection was easily transmitted when small quantities of blood were inoculated into BIV sero-negative calves. Necropsy of the cattle that died with the above clinical signs showed neurological lesions and inflammation of the brain and spinal cord. The microscopic examination revealed wide spread damage of neurons in the brain and spinal cord. The neuron pathology was thought to be caused by vascular lesions. The vascular lesions include proliferation of vascular endothelium and infiltration of the vessel walls with mononuclear and occasionally polymorphonuclear cells. These neurological lesions in animals infected with BIV are similar to the neurological lesions reportedly associated with HIV.

Martin, S.J., O' Neill T.P., Bilello, J.A. and Eiseman, J.L. 1991. Lymphocyte transformation abnormalities in bovine immunodeficiency-like virus infected calves. Immunology Letters, 27, 81–84.

Bovine immunodeficiency-like virus (BIV) is a lentiviral pathogen of cattle which is genetically and antigenically related to the human immunodeficiency virus (HIV-1). To determine the impact of BIV infection on the bovine immune system we studied the lymphocyte transformation responses of male Holstein calves inoculated with BIV strain R-29 to three mitogens: pokeweed mitogen (PWM), concanavalin A (Con A), and phytohaemagglutinin (PHA) at two and six months post-infection. By six months post-inoculation the response to all three mitogens was diminished compared to control animals and remained depressed 10 months post-inoculation. These results demonstrate that a functional impairment of lymphocytes can be observed early in the course of BIV infection, and prior to the onset of overt clinical disease.

McNab, W.B., Jacobs, R.M. and Smith H.E. 1994. A serological survey for bovine immunodeficiency-like virus in Ontario dairy cattle and associations between test results, production records and management practices. Canadian Journal of Veterinary Research, 58, 36–41.

A chemiluminescence Western blot analysis (WBA) for detecting antibovine immunodeficiency-like virus (BIV) antibodies, had good repeatability. The test was subsequently applied to a bank of serum samples from 928 adult cows from 265 herds in Ontario; the number of cows sampled within each herd ranged from 1 to 13. The overall prevalence of anti-BIV antibodies among cows was 5.5% with a 95% confidence interval of 4.2% to 7.2%. In contrast, 18.1% of herds had at least one reactor among cows tested, resulting in a herd-prevalence confidence interval of 13.8% to 23.4%. These estimates of prevalence were in the same range as previous reports from the US and Europe. Bovine immunodeficiency-like virus may have a worldwide distribution. Unfortunately, BIV test sensitivity and specificity are difficult to estimate because virus isolation is inefficient. Therefore, the apparent prevalences could not be adjusted for test sensitivity and specificity, to estimate the true prevalence of infection. The serum samples had previously been tested for antibodies to bovine leukemia virus (BLV). There were no significant associations between BIV and BLV test results. Least squares regression was used to investigate potential associations between BIV test results and selected production indices.

Monke, D.R. and Phillips, P. 1996. Questions possibility of seminal transmission of bovine immunodeficiency virus [letter]. American Journal of Veterinary Research, 56, 1401.

A response to paper by Nash et al. 1995. Detection of bovine immunodeficiency virus in blood and milk-derived leukocytes by use of polymerase chain reaction. American Journal of Veterinary Research, 56, 445–449. Authors argue that the detection of BIV in semen does not necesarily indicate that semen is responsible for the transmission of BIV.

Moore, E.C., Keil, D. and Coats, K.S. 1996. Thermal inactivation of bovine immunodeficiency virus. Applied & Environmental Microbiology, 62, 4280-4283.

Cell-associated bovine immunodeficiency virus (BIV) and cell-free BIV were subjected to increasing temperatures, including pasteurisation conditions. To determine the effect of heat treatment on BIV viability, reverse transcriptase activity and infectivity of the heat-treated virus were assessed. BIV was inactivated by heating to 47°C for 30 minutes and by low- and high-temperature pasteurisation conditions.

Moras, D. and Poterszman, A. 1996. Protein-RNA interactions — getting into the major groove. Current Biology, 6, 530–532.

The solution structure of the complex between a peptide derived from the bovine immunodeficiency virus tat protein and the TAR RNA to which it binds reveals a new motif in protein-RNA interactions.

Muluneh, A. 1994. Seroprevalence of bovine immunodeficiency-virus (BIV) antibodies in the cattle population in Germany. Zentralblatt fur Veterinarmedizin Reihe B-Infectious Diseases Immunobiology Food Hygiene Public Health, 41, 679–684.

Serum samples from 380 cattle were analysed for the presence of bovine immunodeficiency-virus (BIV) antibodies by focus immunoassay (cell-ELISA) and immunofluorescence assay (IFA). All specimens originated from dairy farms in the eastern part of Germany, which had been randomly collected during the period 1989–1991. The cattle were clinically healthy and free of bovine leukaemia virus (BLV) and bovine-virus diarrhoea-virus (BVDV) antibodies. Infection of cell lines with BIV was monitored by syncytia

formation, cell-ELISA, and immunofluorescence. The seroprevalence of BIV antibodies was 6.6 %, as determined by cell-ELISA. Comparison of IFA and cell-ELISA showed that all IFA positive sera were also positive in cell-ELISA. However, additional sera were reactive only in cell-ELISA. This first report suggests that BIV infection may cause minor problems in German cattle, while BIV is present in a similar prevalence to that reported from other countries.

Nadin-Davis, S.A. 1995. Detection of bovine immunodeficiency-like virus by the polymerase chain reaction. In: Becker, Y. and Darai, G. eds., PCR: protocols for diagnosis of human and animals virus diseases. Springer-Verlag (Berlin), 491–499.

Bovine immunodeficiency-like virus (BIV) is structurally, genetically and immunologically a typical lentivirus, but the long-term clinical consequences of BIV infection of cattle remain unclear. A previous report described the use of polymerase chain reaction (PCR) technology to detect two discrete portions of the BIV proviral genome in peripheral blood mononuclear cells (PBMCs) of naturally and experimentally infected animals. While this procedure is a useful experimental tool, it requires adaptation prior to use in a diagnostic setting for investigation of BIV prevalence. This report describes a modification of the previous PCR assays in which a 'drop-in/drop-out nested' (DIDON) format is used and the application of this technique to evaluate four bulls experimentally inoculated with BIV is demonstrated.

Nadin-Davis, S.A., Chang, S.C., Smith, H. and Jacobs, R.M. 1993. Detection of bovine immunodeficiency-like virus by the polymerase chain reaction. Journal of Virological Methods, 42, 323–336.

This report describes a test for the bovine lentivirus, bovine immunodeficiency-like virus, in which polymerase chain reaction (PCR) technology is employed. Two pairs of oligonucleotide primers directed to sequences within the coding regions of the gag and pol genes generated the expected PCR products from molecular BIV clones and from DNA of BIV-infected cell cultures but not from DNA of uninfected cultures. Data indicating the specificity and sensitivity of these PCRs for BIV detection and the potential utility of this technology for diagnostic applications are presented. Molecular Sequence Databank No.: GENBANK/X00376.

Nash, J.W., Hanson, L.A. and Coats, K.S. 1995. Detection of bovine immunodeficiency virus in blood and milk-derived leukocytes by use of polymerase chain reaction. American Journal of Veterinary Research, 56, 445–449.

Bovine immunodeficiency virus (BIV) is prevalent in beef and dairy cattle, yet the mode(s) of BIV transmission are undefined. Using polymerase chain reaction, which specifically targeted a 235-bp, highly conserved region of the BIV pol gene, BIV-infected leukocytes were detected in the blood and milk of BIV-seropositive cows. These data confirm the presence of BIV in milk and identify the potential for lactogenic transmission of this virus.

Nash, J.W., Hanson, L.A. and Coats, K.S. 1995. Bovine immunodeficiency virus in stud bull semen. American Journal of Veterinary Research, 56, 760–763.

Bovine immunodeficiency virus (BIV), a lentivirus, is prevalent in dairy and beef cattle in southeastern United States and may be associated with a lymphoproliferative disease. The mode(s) of BN transmission are undefined. Because artificial insemination is a common practice in dairy production, contaminated stud semen could serve as an important source of infection if the virus is harboured in seminal fluids. To evaluate this possibility, we procured 12 cryopreserved semen specimens from a stud semen repository. Leukocytes were purified from the specimens, and the leukocyte DNA was used as template in a polymerase chain reaction procedure that targeted a 235-base pair, highly conserved domain of the BIV pol gene. The target sequence was amplified from the seminal leukocyte DNA of 9 of the specimens (82%), and nucleotide sequencing confirmed the BIV specificity of the fragment. This finding provides evidence that stud bull semen may serve as an important reservoir of BIV suggesting the possibility that artificial insemination of dairy cows may have a major role in transmission and wide-spread dissemination of this bovine lentivirus.

Oberste, M.S. and Gonda, M.A. 1992. Conservation of amino-acid sequence motifs in lentivirus Vif proteins. Virus Genes, 6, 95–102.

The nonstructural/regulatory genes of human immunodeficiency virus type 1 (HIV-1) and other lentiviruses are believed to play an important role in the replication and pathogenesis of these viruses. In HIV-1 and other lentiviruses, the vif (viral infectivity factor) open reading frame (ORF) (also termed sor or Q in some lentivirus genomes) is located in the central region, overlapping the 3' end of the pol ORF, but in a different reading

frame. Among the lentiviruses, only equine infectious anemia virus lacks a vif ORF. The predicted Vif protein sequences from 38 lentiviruses were analysed for the presence of global and local sequence similarity. The Vif proteins of closely related lentiviruses are highly conserved (HIV-1HXB2:HIV-1mn = 91% identity), while those of more distantly related lentiviruses have diverged significantly (HIV-1HXB2:simian immunodeficiency virus max = 30% identity). A search for local sequence similarity revealed that a unifying feature of predicted lentivirus Vif proteins is the presence of at least one of two short, highly conserved sequence motifs, SL(I/V)X4YX9Y and SLQXLA. SLQXLA was present in 34 of 38 lentiviruses examined, while the remaining four lentiviruses had one (three viruses) or two (one virus) substitutions in this motif (of five total substitutions, three were conservative changes). The SL(I/V)X4YX9Y motif was found only in primate lentiviruses and in bovine immunodeficiency-like virus. Based on these findings, we suggest that the locus designation vif be used to denote all lentivirus ORFs previously called vif, Q, or sor.

Oberste, M.S., Greenwood, J.D. and Gonda, M.A. 1991. Analysis of the transcription pattern and mapping of the putative rev and env splice junctions of bovine immunodeficiency-like virus. Journal of Virology, 65, 3932–3937.

The bovine immunodeficiency-like virus (BIV) genome contains the obligatory structural genes of all retroviruses and, in addition, the complex central region of lentiviruses; this novel region may code for at least five nonstructural/regulatory genes in BIV (K.J. Garvey, M.S. Oberste, J.E. Elser, M.J. Braun, and M.A. Gonda, Virology, 175, 391–409, 1990). As a prelude to determining the function of these novel open reading frames, the transcriptional pattern of BIV was studied by Northern analysis of RNA from BIV-infected cells. Five size classes of BIV-specific RNAs of 8.5, 4.1, 3.8, 1.7, and 1.4 kb were detected. The 8.5-kb RNA contains sequences from all regions of the genome; it is the virion RNA and probably serves as the gag-pol transcript as well. By using gene-specific probes, subgenomic viral RNAs of 3.8, 1.7, and 1.4 kb were tentatively identified as the env, tat, and rev spliced messages, respectively. The 4.1-kb RNA could not be unambiguously identified but may encode vif. The hybridisation patterns of the putative tat and rev mRNAs suggest that they are the products of multiple splicing events. Discrete transcripts for the BIV W and Y central region open reading frames were not defined. The characterisation of partial cDNA clones has permitted the mapping of the env and putative rev splice junctions. Molecular Sequence Databank No.: GENBANK/M74711; GENBANK/M74712; GENBANK/M74756; GENBANK/M74757; GENBANK/M74758; GENBANK/M74759; GENBANK/M74760; GENBANK/M74761; GENBANK/M74762; GENBANK/M74763.

Oberste, M.S., Williamson, J.C., Greenwood, J.D., Nagashima, K., Copeland, T.D. and Gonda, M.A. 1993. Characterisation of bovine immunodeficiency virus *rev* cDNAs and identification and subcellular localisation of the rev protein. Journal of Virology, 67, 6395–6405.

One of the six putative accessory genes of bovine immunodeficiency virus (BIV) is similar to those identified as rev in the human immunodeficiency virus and visna virus genomes. To further analyse the BIV rev gene locus, protein, and function, rev cDNAs were cloned and characterised. BIV rev mRNA is derived from the full-length transcript by multiple splicing events and consists of three exons, including the untranslated leader sequence and two coding exons. BIV *rev* cDNA was expressed in bacteria and in a mammalian in vitro translation expression system. A 23-kDa *Rev* protein (p23rev) was immunologically detected in lysates from both systems by using an antiserum made to a synthetic Rev peptide. Recombinant p23rev made in bacteria was purified and used to make a polyvalent antiserum. Antisera to Rev peptide and recombinant p23rev immunoprecipitated p23rev from BIV-infected mammalian cells but not from virions. A mammalian expression vector using the BIV rev cDNA was constructed; p23rev was immunoprecipitated with anti-Rev serum from 32P-labeled lysates of monkey cells transfected with this plasmid, demonstrating that BIV Rev is phosphorylated. Immunofluorescence and immunoelectron microscopy with anti-BIV Rev antisera localised Rev in the nucleus and, particularly, in the nucleoli of BIV-infected cells. In functional studies, the expression of BIV Rev was shown to positively regulate the appearance both of Gag protein, which is translated from the unspliced primary viral transcript, and of singly spliced env mRNA but not that of the multiply spliced tat mRNA. These results demonstrate that BIV Rev activity correlates with the known function of lentivirus Rev proteins.

Onuma, M., Ogawa, Y. and Kawakami, Y. 1990. An evaluation of the syncytial assay for detection of bovine immunodeficiency-like virus. Nippon Juigaku Zasshi, 52, 1131–1133.

The syncytial assay was performed with cocultivation of R-29 BIV-infected bovine embryonic spleen cells (BESP) and cf2Th canine thymus cells. Strong positive syncytium formation was obtained 3 days after

cocultivation. Antigen detected by immunofluorescence was mainly associated with syncytia. The number of syncytia increased after 2 days and reached a peak 3–4 days after cocultivation. Polybrene at concentrations 2–5 μg/mL stimulated the production of syncytia. Syncytia were also produced in F81 cells (a cat cell line containing the murine sarcoma virus genome) when they were cocultivated with BIV-infected BESP cells. Polyclonal bovine antiserum against BIV (obtained from Van Der Maaten) at a final dilution of 10% inhibited syncytium formation.

Onuma, M., Koomoto, E., Furuyama, H., Yasutomi, Y., Taniyama, H., Iwai, H. and Kawakami, Y. 1992. Infection and dysfunction of monocytes induced by experimental inoculation of calves with bovine immunodeficiency-like virus. Journal of the Acquired Immune Deficiency Syndrome, 5, 1009–1015.

Three calves were experimentally inoculated with bovine immunodeficiency-like virus (BIV) to examine BIV pathogenesis. Inoculated calves produced specific antibody that could be detected from 3 to 5 weeks up to 1 year postinoculation (pi). Virus was isolated from peripheral blood mononuclear cells (PBMC) 3–4 weeks pi by syncytia assay. Thereafter, the virus could be continually isolated. BIV could be isolated from monocytes but not from T cells. Likewise, monocytes could be infected with BIV in vitro. Various monocyte functions of these BIV-infected calves and age-matched uninfected calves were tested; superoxide anion release, phagocytic activity, and chemotactic responsiveness of monocytes were depressed in BIV-infected calves compared with control calves. A slight delay in the humoral immune response against mouse serum protein was also evident. During the observation period of approximately 1 year, no significant clinical symptoms could be observed. One calf, however, was killed at 15 months pi. At the time of necropsy, BIV could be isolated from PBMC as well as from cells of the spleen, liver, and lymph nodes.

Pallansch, L.A., Lackman-Smith, C.S. and Gonda, M.A. 1992. Bovine immunodeficiency-like virus encodes factors which transactivate the long terminal repeat. Journal of Virology, 66, 2647–2652.

Lentiviruses are known to encode factors which transactivate expression from the viral long terminal repeat (LTR); the primary trans activator is the tat gene product. One of the putative accessory genes (tat) of the bovine immunodeficiency-like virus (BIV) bears sequence similarity to other lentivirus tat genes. This finding suggests that BIV may encode a transactivating protein capable of stimulating LTR-directed gene expression. To test this hypothesis in vitro, BIV LTR-chloramphenicol acetyltransferase (CAT) reporter gene plasmids were constructed and transfected into three cell lines established from canine, bovine, or lapine tissues that are susceptible to BIV infection. The level of BIV LTR-directed CAT gene expression was significantly elevated in BIV-infected cells compared with uninfected cells. The relatively high basal-level expression of BIV LTR-CAT in uninfected canine and bovine cell lines suggests that cellular factors play a role in regulating BIV LTR-directed gene expression. Additionally, by using a clonal canine cell line in which the BIV LTR-CAT plasmid is stably expressed, BIV LTR-directed CAT expression is elevated 15- to 80-fold by cocultivation with BIV-infected cells, supporting the notion that BIV encodes a transactivator. The relative specificity of this viral activation was assessed by coculturing the clonal BIV LTR-CAT cell line with bovine leukemia virus- or bovine syncytial virus-infected cells; these bovine retroviruses increased expression from the BIV LTR only two to threefold. Thus, BIV LTR regulatory elements in infected cells, like those of human immunodeficiency virus type 1 and other lentiviruses, are transactivated, presumably through the action of a Tat-like protein and cellular factors.

Pifat, D.Y., Ennis, W.H., Ward, J.M., Oberste, M.S. and Gonda, M.A. 1992. Persistent infection of rabbits with bovine immunodeficiency-like virus. Journal of Virology, 66, 4518–4524.

Chronic infection of rabbits was induced by a single intraperitoneal injection of bovine immunodeficiency-like virus (BIV)-infected cells. Ten BIV-infected animals were monitored serologically for up to 2 years. Results of serologic and virus rescue assays indicated that all animals became infected and demonstrated a rapid and sustained BIV-specific humoral response. BIV was rescued by cocultivation from spleen, lymph nodes, and peripheral blood leukocytes of infected animals. Viral DNA in immune tissues was confirmed by polymerase chain reaction amplification of BIV sequences. These data and specific immunohistochemical staining of mononuclear cells of the spleen for BIV antigen suggest that the infection is targeted to immune system cells.

Polack, B., Schwartz, I., Berthelemy, M., Belloc, C., Manet, G., Vuillaume, A., Baron, T., Gonda, M.A. and Levy, D. 1996. Serologic evidence for bovine immunodeficiency virus infection in France. Veterinary Microbiology, 48, 165–173.

We report herein on the first serologic detection of antibodies to bovine immunodeficiency virus (BIV) in France. Serum samples from dairy and beef cattle from southwestern and western France (Landes and Vendee)

were tested using a Western blot assay with a recombinant 53 kDa gag precursor derived from the Louisiana BIV R29 isolate. We performed our study on the oldest animals from 37 different herds that were under serologic follow up for previous bovine leukemia virus infection. Overall, 398 selected bovine sera were assayed and 15 serum samples from eight herds reacted with the recombinant 53 kDa BIV R29 gag. Interestingly, reactions obtained with French sera were weaker than with positive Louisiana sera, a finding that may indicate the occurrence of distinct French and Louisiana BIV variants.

Puglisi, J.D., Chen, L., Blanchard, S. and Frankel, A.D. 1995. Solution structure of a bovine immunodeficiency virus tat-tar peptide-RNA complex. Science, 270, 1200–1203.

The Tat protein of bovine immunodeficiency virus (BIV) binds to its target RNA, TAR, and activates transcription. A 14-amino acid arginine-rich peptide corresponding to the RNA-binding domain of BIV Tat binds specifically to BIV TAR, and biochemical and in vivo experiments have identified the amino acids and nucleotides required for binding, The solution structure of the RNA-peptide complex has now been determined by nuclear magnetic resonance spectroscopy. TAR forms a virtually continuous A-form helix with two unstacked bulged nucleotides. The peptide adopts a beta-turn conformation and sits in the major groove of the RNA. Specific contacts are apparent between critical amino acids in the peptide and bases and phosphates in the RNA. The structure is consistent with ail biochemical data and demonstrates ways in which proteins can recognise the major groove of RNA.

Rasmussen, L., Battles, J.K., Ennis, W.H., Nagashima, K. and Gonda, M.A. 1990. Characterisation of virus-like particles produced by a recombinant baculovirus containing the gag gene of the bovine immunodeficiency-like virus. Virology, 178, 435–451.

The entire gag gene of the bovine immunodeficiency-like virus (BIV) was inserted behind the strong polyhedron promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV). The resultant recombinant baculovirus (AcNPV-BIVgag) was used to infect insect cells in order to overexpress and characterise BIV gag gene products. The infection resulted in the high-level expression of a protein similar in size to the predicted BIV gag precursor (Pr53gag). BIV Pr53gag was detected in AcNPV-BIVgag-infected insect cells and in culture supernatants. Electron microscopy of these cells revealed an abundance of virus-like particles (VLPs) in the cytoplasm, budding from the cell membrane, and free in the culture medium. The size and morphology of the VLPs were similar to those of the immature forms of BIV observed in infected mammalian cells. The VLPs sedimented at a density of 1.16 g of sucrose per millilitre in linear gradients and were shown to contain the majority of the supernatant Pr53gag. Antigenic determinants on Pr53gag from VLPs were recognised by BIV and HIV-1 antiserum, and serum from rats immunised with VLPs reacted with recombinant and viral BIV Pr53gag and processed products. The protease (PR) activity in BIV virions was capable of processing recombinant Pr53gag; this activity was blocked by pepstatin A, a potent aspartyl PR inhibitor. Baculovirus-expressed BIV Pr53gag appears to be an excellent source of gag precursor; it may prove useful for structural studies and enable the development of assays to detect retroviral PR inhibitors. The data further suggest that unprocessed BIV Pr53gag plays a major role in the assembly of BIV particles. The expression of other BIV structural genes in insect cells may prove instructive in the study of molecular events involved in the assembly and processing of these BIV proteins.

Rasmussen, L., Greenwood, J.D. and Gonda, M.A. 1992. Expression of bovine immunodeficiency-like virus envelope glycoproteins by a recombinant baculovirus in insect cells [published erratum appears in Virology188, 959]. Virology, 186, 551–561.

The bovine immunodeficiency-like virus (BIV) env open reading frame (ORF) contains both sequences encoding env and sequences for exon 1 of the putative rev gene. Recombinant baculoviruses incorporating BIV env ORF sequences were constructed to characterise the expression, processing, and immunogenicity of products of the BIV env ORF in insect cells and to develop reagents to study native BIV Env glycoproteins. A recombinant baculovirus containing the entire env ORF synthesised a nonglycosylated, 20-kDa, BIV-specific protein, apparently unrelated to native BIV Env proteins. In contrast, a recombinant baculovirus containing a truncated env ORF in which the coding sequences for rev exon 1 were deleted synthesised three size classes of glycosylated proteins in insect cells related to the BIV Env precursor (gp145), surface (gp100), and transmembrane (gp45) glycoproteins observed in BIV-infected mammalian cells. Oligomers of recombinant BIV Env proteins also formed in these baculovirus-infected insect cells. Immunofluorescence staining of intact insect cells infected by the baculovirus expressing BIV Env with BIV-specific serum demonstrated that the recombinant Env glycoproteins were expressed on the cell surface. Antisera raised to recombinant Env

glycoproteins immunoprecipitated native gp145, gp100, and gp45 in BIV-infected bovine cells similar to sera from animals naturally or experimentally infected with BIV.

Report 1994. MAFF investigate dairy herd for BIV. Veterinary Record, 134, 154.

MAFF veterinarians in the U.K. are investigating a dairy herd in Cheshire in which chronic ill health in some animals may be linked to the presence of antibodies to bovine immunodeficiency virus (BIV). The farm has experienced a succession of problems including abortion, ill thrift and respiratory disease since importing consignments of heifers from Germany and the Netherlands last year. Veterinary investigation officers found two animals weakly positive to BIV among the 50 head herd and are currently carrying out further tests. The farm involved has been experiencing difficulties since an outbreak of ringworm affected the bought-in cattle and prevented them being sold on. The Milk Marketing Board initially decided to stop collections from the farm after a local dairy refused to accept the milk. It has since resumed collections but the milk is being collected separately and is not being used for human consumption. Antibodies to BIV have been found before in the U.K. herd during a small scale survey carried out in 1993.

Rovid, A.H., Carpenter, S. and Roth, J.A. 1993. Monocyte function and the recovery of monocyte tropic isolates of bovine immunodeficiency like virus in experimentally infected cattle [Abstract]. Journal of Immunology, 150, A229.

Bovine immunodeficiency-like virus (BIV) is a lentivirus of cattle which causes a mild transient lymphocytosis and lymphoid hyperplasia early after experimental infection of calves. We have examined whether monocytes are infected in BIV-infected cattle, and what effect BIV might have on monocyte function. A group of four Holstein calves were infected with the R29 strain of BIV and monitored for evidence of virus production in monocytes for 2 years. In addition, in vitro monocyte function assays were performed over an 18 month period starting 4 months after infection. Assays included phagocytosis of *Staphylococcus aureus*, antibody-dependent cell-mediated cytotoxicity (ADCC), and monocyte migration. Initially, purified monocytes did not produce detectable virus, although BIV could be isolated from unfractionated mononuclear cells. With increasing time after infection, virus could be isolated from monocytes in all four cattle. Virus isolated from monocytes of one R29-BIV infected animal was inoculated into another calf. This isolate, unlike the original R29 isolate, could be consistently isolated from monocytes from 2 weeks until at least 1 year post-inoculation (PI). Monocyte function did not, however, appear to be affected in BIV-infected cattle. Random and chemotactic migration and ADCC were not significantly different between BIV-infected and control animals. Phagocytosis tended ($P=0.06$) lower in the BIV infected animals than in the controls 4 to 8 months PI, but then returned to normal. We have therefore found evidence for development of a monocyte tropic isolate in cattle over time, with no effect of BIV on monocyte function.

Rovid, A.H., Carpenter, S. and Roth, J.A. 1995. Monocyte function in cattle experimentally infected with bovine immunodeficiency-like virus. Veterinary Immunology and Immunopathology, 45, 31–43.

The effects of bovine immunodeficiency-like virus (BIV) on monocyte function were examined in experimentally infected cattle and in monocytes infected in vitro. Infection with the R29 isolate of BIV appeared to have relatively little effect on monocyte function in cattle during the first 2 years postinfection (PI). For the first 4 to 8 months post infection, monocyte phagocytosis of Staphylococcus aureus tended to be lower ($P=0.06$) in BIV infected calves than in control animals. After 8 months PI, however, phagocytosis became equal between the two groups. Random and chemotactic migration and antibody-dependent cell-mediated cytotoxicity (ADCC) did not appear to be affected by BIV infection. Monocytes from BIV infected cattle were able to respond to in vitro treatment with interferon gamma similarly to monocytes from control cattle. Although experimental infection with BIV R29 resulted in minimal effects on monocyte function, this result could have been due either to a low virus burden in vivo or because BIV is intrinsically unable to affect monocyte function. To distinguish between these possibilities, monocytes from control, uninfected cattle were treated with BIV virus in vitro. Treatment of normal monocytes with cell-free virus significantly ($P<0.05$) increased phagocytosis and random and chemotactic migration and decreased ADCC, in a dose-dependent manner. It appears, therefore, that the normal function of peripheral blood monocytes in the BIV R29 infected animals may be due to a low virus burden rather than to the inability of BIV to affect monocyte function. The in vitro infection results also raise the possibility that the function of monocyte derived cells at local sites of BIV replication may be altered.

Sherman, M.P., Dock, N.L., Ehrlich, G.D., Sninsky, J.J., Brothers, C., Gillsdorf, J., Bryzgornia, V. and Poiesz, B.J. 1995. Evaluation of HIV type 1 Western blot-indeterminate blood donors for the presence of human or bovine retroviruses. AIDS Research and Human Retroviruses, 11, 409–414.

From 1985 through 1990, 1100 of 500 000 human blood donations in Syracuse, New York were repeatedly reactive by ELISA for antibodies to the human immunodeficiency virus type 1 (HIV-1). Nine hundred of the ELISA-reactive samples were confirmed as negative by Western blot (WB), 40 were confirmed as positive, and the remaining 160 sera were indeterminate, reacting mainly with HIV-l gag gene products. Twenty donors with the most reactive indeterminate WE were selected for follow-up studies. Four of these 20 donors admitted to retroviral risk factors and, interestingly, 12 (60%) had exposure to dairy cattle and drank unpasteurised milk. These 20 donors were analysed over a 3-year period for the presence of the pathogenic human retroviruses HIV-1, HIV-2, human T cell lymphoma/leukemia virus types I and II (HTLV-I and HTLV-II), as well as bovine immunodeficiency virus (BIV) and leukemia virus (BLV). Retroviral analyses included serology, plasma antigen capture, virus culture, and the polymerase chain reaction. Only one donor seroconverted and was clearly infected with HIV-1. None of the other 19 donor serological reactivities to HIV-1 changed, nor were they positive for any of the above-mentioned retroviruses. Although we cannot ascertain whether these latter 19 HIV-1 WE-indeterminate donors were exposed to human or bovine retroviral proteins, it is unlikely that their HIV-1 seroreactivity was caused by infection with HIV-1, HIV-2, HTLV-I, HTLV-II, BLV, or BIV.

Smith, H.E. and Jacobs, R.M. 1993. Serological evidence of bovine immunodeficiency-like virus infection in a sheep. Canadian Journal of Veterinary Research, 57, 305–306.

A six month-old sheep was entered into a control group in an experiment designed to study the effects of exposure to the bovine immunodeficiency-like virus (BIV). Anti-BIV antibodies were detected in the serum of this sheep prior to the start of this study; these antibodies persisted for 12 months at which time the animal was destroyed. The sheep was normal clinically and was grossly normal at postmortem examination. Blood from this sheep was inoculated into a recipient sheep which subsequently showed a transient anti-BIV antibody response beginning two months postinoculation. Sheep have been previously shown to produce anti-BIV antibodies after experimental inoculation with infected cell culture material or bovine blood and BIV infection was found in a sheep pastured with BIV-infected cattle. In the present case there was no contact with cattle; the source of the infection was not identified.

Smith, H.E., Jacobs, R.M. and Mallard, B. 1994. Cell-mediated and humoral immunity in sheep exposed to bovine immunodeficiency-like virus. Comparative Immunology, Microbiology and Infectious Diseases, 17, 29–39.

Six sheep were transfused intraperitoneally with whole blood from two sheep chronically infected with the bovine immunodeficiency-like virus (BIV). Five sheep were transfused intraperitoneally (i.p.) with normal ovine whole blood and served as controls. Five of six BIV-inoculated sheep seroconverted; four were transiently seropositive while one remained seropositive for the duration of the experiment. Tests for nonspecific lymphocyte reactivity to mitogens were performed monthly for one year. At approximately 10 months postinoculation, all sheep were immunised with chicken ovalbumin, canine red blood cells, and tuberculin. There were no significant associations between BIV exposure and deficits in antibody production to chicken ovalbumin and canine red blood cells; nonspecific lymphoproliferative responses to concanavalin-A, lipopolysaccharide, and pokeweed mitogen; specific lymphoproliferative responses to ovalbumin and tuberculin purified protein derivative; or cutaneous delayed type hypersensitivity to tuberculin purified protein derivative. Exposure to BIV did not alter the humoral or cell mediated immune responses of sheep in the first year of exposure.

Snider, T.G., Luther, D.G., Jenny, B.F., Hoyt, P.G., Battles, J.K., Ennis, W.H., Balady, J., Blasmachado, U., Lemarchand, T.X. and Gonda M.A. 1996. Encephalitis, lymphoid tissue depletion and secondary diseases associated with bovine immunodeficiency virus in a dairy herd. Comparative Immunology, Microbiology and Infectious Diseases, 19, 117–131.

Encephalitis, lymphoid tissue depletion and secondary infections occurred over a 5-yr-period in Holstein cows infected with bovine immunodeficiency virus (BIV). There were 59 cattle studied, the majority during 1991, when a severe environmental stress occurred, each with one or more primary causes of death, natural or by euthanasia, and most with several secondary diseases. The encephalitis was characterised by meningeal, perivascular and parenchymal infiltration with lymphocytes, occasional plasma cells and macrophages with perivascular oedema in some cows. Affected areas included the cerebrum, cerebellum, and spinal cord with no

particular distribution pattern recognised. The lymphoid depletion was primarily an absence of follicular development in nodes draining regions with secondary infections such as chronic mastitis and chronic suppurative pododermatitis. Paucity of lymphocytes in thymic-dependent regions of lymph nodes and the spleen suggested a primary depletion of T cells. Secondary infections were often multiple with each cow having several minor conditions, usually considered short-term and treatable. These included mastitis and pododermatitis, with many cows having non-responding abscesses, cellulitis and myositis attributed to injection site infections. A large number of the cattle had parturition difficulties such as dystocia, obturator paralysis, and metritis. Pulmonary, cardiovascular, and intestinal disease were recognised as both primary and secondary disease conditions. There was a high level of infection with bovine leukemia virus with 4 of the 59 cattle having lymphosarcoma. Under practical conditions, the infection with BIV has a different effect on the host than has been observed under experimental conditions. The presence of BIV combined with the stresses associated with parturition and a modern dairy production system were considered causal for the development of untreatable secondary diseases in immunocompromised cattle. The peak incidence in 1991 was attributed to increased environmental stress during renovation of the barn facility. During this time the cattle were kept on open pasture, exposed to an extremely wet winter, and spring weather conditions. The effect of co-infection with bovine leukemia virus, the influence of immunocompromise on the chronicity of mastitis, the relationship with laminitis and pododermatitis, and several questions related to viral transmission, complementarism with bovine leukemia virus, viral reactivation and immunoprophylaxis all remain as viable avenues for future investigations.

Suarez, D.L., Van Der Maaten, M.J., Wood, C. and Whetstone, C.A. 1993. Isolation and characterisation of new wild-type isolates of bovine lentivirus. Journal of Virology, 67, 5051–5055.

Two new isolates of bovine lentivirus, also known as bovine immunodeficiency-like virus (BIV), were obtained from a seropositive cattle herd in Florida. This is the first report of new isolates of BIV since the original BIV strain, R29, was isolated in 1969. The two new BIV isolates were derived from blood buffy coat cells cocultivated in vitro with fetal bovine lung cell cultures. The new isolates differed in vitro from the original R29 isolate in replication and syncytium formation in fetal bovine lung cells. Both new isolates were confirmed as BIV by immunofluorescence assay, Western blotting (immunoblotting), and polymerase chain reaction. Sequence analyses of the polymerase chain reaction pol gene product showed 92.6 and 93.6% homology to the published nucleotide sequence of BIV R29-127, a molecular clone derived from BIV R29. Each of the new BIV isolates was inoculated into two calves, and virus was recovered between 5 and 10 days postinoculation (p.i.), with BIV seroconversion between 10 and 21 days p.i. Virus was recoverable and antibody was detectable for at least 4 months p.i. Two calves developed a transiently elevated mononuclear cell count, similar to what was reported for BIV R29 in the original experimental calf inoculations. No other clinical abnormalities were observed. Molecular Sequence Databank No.: GENBANK/L06524; GENBANK/L06525; GENBANK/L06526.

Suarez, D.L., Van Der Maaten, M,J. and Whetstone, C.A. 1995. Improved early and long-term detection of bovine lentivirus by a nested polymerase chain reaction test in experimentally infected calves. American Journal of Veterinary Research, 56, 579–586.

A nested polymerase chain reaction (pen) test was developed to examine infection with the bovine lentivirus, bovine immunodeficiency-like virus (BIV), in cattle. Primers were designed to amplify two separate regions of the pol and env segments of the BIV genome. Two calves were experimentally infected with an isolate derived from the original strain of BIV, R29, or with a recent field isolate, FL491. Serial blood samples were collected and examined by virus isolation, protein immunoblot, and nested PCR. The nested PCR test detected BIV infection by 3 days after inoculation, earlier than the other two methods, and continued to identify infected cattle 9 to 15.5 months after inoculation, even when results from virus isolation and serology became negative. Nested PCR also detected multiple-size env products in samples obtained later in the infection from the calf that received FL491, giving evidence that viral quasi-species were selected during in vivo replication of the virus. Results indicated that the nested PCR test is more sensitive than virus isolation or serology for the detection of BIV infection in cattle.

Suarez, D.L. and Whetstone, C.A. 1995. Identification of hypervariable and conserved regions in the surface envelope gene in the bovine lentivirus. Virology, 212, 728–733.

The surface envelope (SU) gene of nine different isolates of the bovine lentivirus (BIV) were compared for nucleotide and deduced amino acid (aa) sequence diversity. Analyses were done both on isolates derived from the original reference strain, R29, and on field isolates of BIV. Six conserved and six hypervariable regions

were identified. Many of the hypervariable regions were located in areas predicted to be on the surface of the SU protein. The SU gene comparison among all isolates showed up to a 50% aa sequence divergence. When a conserved region of the reverse transcriptase gene was compared among eight of the isolates, there was less than 11% aa sequence divergence. When comparing all isolates, the greatest size differences in the SU gene are observed in the 2nd hypervariable region (V2) with up to a 104-aa difference between the largest and smallest variant. R29-106, an infectious molecular clone of the original isolate of BIV, has an 87-bp deletion in V2 as compared to prototype isolate R29-127. All R29-derived isolates sequenced for this study had a SU gene size similar to R29-106. The four field isolates sequenced for this study had SU genes larger than R29-127. R29-derived isolates may not be representative of BIV currently present in United States cattle.

Sundquist, W.I. 1996. Tattle tales. Nature Structural Biology, 3, 8–11.

Recent NMR structures of bovine immunodeficiency viral TAR RNA-Tat peptide complexes have revealed a new beta-hairpin RNA recognition motif. These complexes exhibit intriguing new variations on the recurring themes in nucleic acid recognition.

Tobin, G.J. and Gonda, M.A. 1993. Inhibition of bovine immunodeficiency like virus (BIV) in cell culture by anti-HIV 1 compounds [Abstract]. Journal of Acquired Immune Deficiency Syndrome, 6, 708–708.

To determine whether drugs that inhibit HIV-1 also inhibit BIV, we assayed three anti-HIV compounds for virus inhibition in BIV-infected cells. EREp cells were seeded into 96 well dishes, infected with cell-free BIV in the presence of inhibitors, and passaged every three days. The reverse transcriptase (RT) activity in the media was compared to culture wells lacking antiviral compounds to determine percent virus inhibition. The first two compounds, AZT and ddI, are nucleotide analogs known to inhibit HIV-1 RT. The concentrations of AZT and ddI that inhibited 50% of the RT values (IC_{50}) were both 1×10^{-6} M and were approximately 1000-fold lower than their LD_{50}s of 3×10^{-3} M and 2×10^{-3} M, respectively. The third compound was a rationally designed HIV-1 proteinase inhibitor that demonstrated and IC_{50} of 1×10^{-7} M with an LD_{50} greater than 1×10^{-5} M. These results indicate that BIV infection can be inhibited by anti-HIV-1 compounds that target two distinct catalytic processes in virus replication.

Tobin, G.J., Sowder, R.C., Fabris, D., Hu, M.Y., Battles, J.K., Fenselau, C., Henderson, L.E. and Gonda, M.A. 1994. Amino acid sequence analysis of the proteolytic cleavage products of the bovine immunodeficiency virus Gag precursor polypeptide. Journal of Virology, 68, 7620–7627.

Bovine immunodeficiency virus Gag proteins were purified from virions, and their amino acid sequences and molecular masses were determined. The matrix, capsid, and nucleocapsid (MA, CA, and NC, respectively) and three smaller proteins (p2L, p3, and p2) were found to have molecular masses of 14.6, 24.6, and 7.3 and 2.5, 2.7, and 1.9 kDa, respectively. The order of these six proteins in the Gag precursor, Pr53gag, is NH2-MA-p2L-CA-p3-NC-p2-COOH. In contrast to other retroviral MA proteins, the bovine immunodeficiency virus MA retains its N-terminal methionine and is not modified by fatty acids. In addition, the bovine immunodeficiency virus NC migrates as a 13-kDa protein in denaturing gel electrophoresis; however, its molecular mass was determined to be 7.3 kDa.

Toedter, G., Pearlman, S., Hofheinz, D., Blakeslee, J., Cockerell, G., Dezzutti, C., Yee, J., Lal, R.B. and Lairmore, M. 1992. Development of a monoclonal antibody-based p24 capsid antigen detection assay for HTLV-I, HTLV-II, and STLV-I infection. AIDS Research and Human Retroviruses, 8, 527–532.

A monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay (ELISA) was developed and employed to detect p24 capsid antigen from human T-cell lymphotropic viruses type I and II (HTLV-I, HTLV-II), simian T-cell lymphotropic virus type I (STLV-I)-infected cell lines, and from mononuclear cell cocultures of HTLV-infected humans and STLV-I infected monkeys. A monoclonal antibody specific for HTLV p24 and p53 capsid antigens was coated onto 96-well microtitre plates to capture HTLV/STLV antigen. Captured antigen was then detected by the addition of a polyclonal, biotinylated human anti-HTLV-I antibody, and colour developed with tetramethyl benzidine/H2O2 substrate. As little as 15 pg/mL of HTLV-I p24 antigen could be detected in this assay. Culture supernatants from HTLV-I-infected cell lines (HUT-102, MT-2, C5/MJ, HTLV-II-infected cell lines (Mo-T, Mo-B, PanG 12.1, NRA) and STLV-I-infected cell lines (Matsu, NEPC M39) were all positive in the assay. In addition, p24 was detected from peripheral blood mononuclear cell (PBMC) cocultures of 8 of 8 (100%) HTLV-I diseased patients, 14 of 20 (70%) HTLV-I and HTLV-II-infected, asymptomatic persons, and 8 of 8 (100%) STLV-I-infected, asymptomatic monkeys. Culture supernatants of cells infected with human immunodeficiency virus type (HIV-1), simian immunodeficiency virus

(SIV), Chlamydia trachomatis, cytomegalovirus (CMV), herpes simplex I and II (HSV), feline leukemia virus (FeLV), bovine leukemia virus (BLV), and bovine immunodeficiency virus (BIV) were all negative. Similarly, normal human peripheral blood mononuclear cells and uninfected, transformed human T cells, were also negative in the assay.

Van Der Maaten, M.J., Boothe, A.D. and Seger, C.L. 1972. Isolation of a virus from cattle with persistent lymphocytosis. Journal of National Cancer Institute, 49, 1649–1657.

Bovine embryonic spleen cells inoculated with leukocytes and tissues from a cow (R-29) with persistent lymphocytosis developed syncytia in the first and second passage. Electron microscopic examination of the cell cultures revealed virus-like particles similar in structure in maedi, visna, and progressive pneumonia viruses of sheep [and the virus was subsequently identified as a bovine lentivirus]. Colostrum-deprived, isolation-reared calves inoculated with the virus developed a mild lymphocytosis and a moderate lymphoproliferative reaction in the small subcutaneous lymphatic nodules.

Van Der Maaten, M.J. and Whetstone, C.A. 1992. Infection of rabbits with bovine immunodeficiency-like virus. Veterinary Microbiology, 30, 125–135.

New Zealand white rabbits, which had been prepared for inoculation by intraperitoneal treatment with thioglycollate, were inoculated intraperitoneally with bovine immunodeficiency-like virus (BIV). Infected materials from various sources were used including cultured cells and culture fluids, peripheral blood leukocytes from infected cattle and spleen tissue from previously infected rabbits. Virus isolations and serological responses detected by Western blotting provided clear evidence that infections had been established in inoculated rabbits and that the spleen was an important site of BIV infectivity. These results indicate that rabbits may be a useful species when testing for BIV infectivity in materials too toxic or highly contaminated to be inoculated directly into cell cultures. Furthermore, rabbits may also be useful in testing effects of coinfections with other bovine viruses on progression of BIV infection and for the initial evaluation of therapeutic regimens designed to suppress or eliminate BIV infections.

Van Der Maaten, M.J., Whetstone, C.A., Khramtsov, V.V. and Miller, J.M. 1990. Experimentally-induced infections with bovine immunodeficiency-like virus, a bovine lentivirus. Dev. Biol. Stand. (S. Karger, Basel) 72, 91–95.

A series of animal experiments were conducted. Experiment 1: five 4–6-month-old calves were inoculated with BIV grown in cell culture and thre seroconverted but none showed any clinical effects within two years of inoculation, although subinoculation of blood from these animals to other cattle indicated . Experiment 2: four calves inoculated with cell culture virus of higher titre than in the first experiment resulted in an active infection but no clinical signs of disease. Experiment 3: inoculation of cell culture virus into the cerebrospinal fluid of four calves resulted in an active infection but no clinical or haematological signs of disease. Experiment 4: cell culture virus was inoculated into two calves <1day of age and blood from a BIV-infected calf with persistent lymphocytosis into two cows more than 5 years of age. The aged cows developed an active infection but no haematological or clinical signs of disease. The two young calves developed an active infection and one of these calves developed a low level persistent lymphocytosis but no other signs of disease. Sheep-goat experiment: sheep and goats infected with either cell culture virus or blood from a recently infected calf seroconverted to the virus. Rabbit experiment: virus was recovered from the spleen of rabbits inoculated intraperitoneally with virus, and developed an antibody response to core proteins.

Walder, R., Kalvatchev, Z., Tobin, G.J., Barrios, M.N., Garzaro, D.J. and Gonda, M.A. 1995. Possible role of bovine immunodeficiency virus in bovine paraplegic syndrome: evidence from immunochemical, virological and seroprevalence studies. Research in Virology, 146, 313–323.

Bovine paraplegic syndrome (BPS) is a debilitating cattle disease of unknown origin that is characterised by leukocytosis, lymphocytopenia tend monocytopenia. The major clinical signs are difficulties in locomotion affecting hind limbs, hypoalgesia in the hind quarters, posterior paralysis and death within 72 to 96 hours after recumbency. To investigate the aetiological basis of BPS, we examined a possible association of the syndrome with infection by bovine immunodeficiency virus (BIV), a lentivirus implicated in immune system dysfunction and central nervous system lesions in cattle. Serum samples (n = 1, 278) were collected from both healthy and BPS-prevalent cattle herds in Venezuela, and organ extracts were prepared from euthanised animals (n = 11) suspected of having BPS. Sera were analysed for reactivity to recombinant BIV and bovine leukaemia virus gag precursor proteins by immunoblot procedures. Serum reactivity to BIV ranged from 12 to 66 % between

groups of BPS prevalent herds. The percentage of samples reactive to BLV antigen was much lower (2 to 17 %). Rabbits inoculated with extracts from BPS-afflicted animals exhibited an anamnestic immune response to BIV antigens as well as the presence of BIV gag antigens in their tissues. We present evidence for a possible association between BPS disease and a viral agent related to BIV. The role of BIV, in combination with malnutrition, in BPS is discussed.

Wannemuehler, Y., Isaacson, J., Wannemuehler, M., Wood, C., Roth, J.A., and Carpenter, S. 1993. In vitro detection of bovine immunodeficiency-like virus using monoclonal antibodies generated to a recombinant gag fusion protein. Journal of Virological Methods, 44, 117–127.

An *Escherichia coli* recombinant fusion protein containing the major core protein of bovine immunodeficiency-like virus (BIV) was used to immunise mice for generation of monoclonal antibodies to BIV p26. Eight hybridomas specific for BIV p26 were identified and two antibodies, designated 104 and 142, were further characterized. Both 104 and 142 antibodies were isotyped as IgG1; they reacted specifically with both BIV p26 and the recombinant fusion protein in Western immunoblot analyses. However, the epitope specificity of the antibodies was different. Immunoperoxidase assays were used to determine if antibodies 104 and/or 142 could detect BIV replication in cell culture. Both antibodies were found to react with BIV-induced syncytia and individual BIV-infected cells. The antibodies were also used successfully in a focal immunoassay for quantitation of BIV-infected cells. These antibodies will provide valuable reagents for detection and quantitation of BIV replication in studies of viral pathogenesis and immunity.

Whetstone, C.A., Sayre, K.R., Dock, N.L., Van Der Maaten, M.J., Miller, J.M., Lillehoj, E., Alexander, S.S. 1992. Examination of whether persistently indeterminate human immunodeficiency virus type 1 Western immunoblot reactions are due to serological reactivity with bovine immunodeficiency-like virus. Journal of Clinical Microbiology, 30, 764–770.

The bovine lentivirus, known as bovine immunodeficiency-like virus (BIV), is genetically, structurally, and antigenically related to human immunodeficiency virus type 1 (HIV-1). It is not known whether sera from persons exposed to BIV proteins would show either positive or indeterminate reactivity on HIV-1 antibody tests. We used a BIV Western blot (immunoblot) analysis to examine human sera characterised as HIV-1 antibody positive, HIV-1 antibody negative, HIV-1 persistently indeterminate, HIV-1 p17 antibody positive only, HIV-1 p24 antibody positive only, human T-cell leukemia virus type 1 (HTLV-1) p19 antibody positive only, or HTLV-1 p24 antibody positive only. None of these sera were positive by Western blot to BIV-specific proteins. Many of these sera, however, displayed strong reactivities to bovine cell culture antigens on blots prepared from both mock-infected and BIV-infected cell cultures. The HIV-1 p17 and p24 antibody-positive and the HTLV-1 p19 and p24 antibody-positive sera were further examined by Western blot to bovine leukemia virus (BLV) and were found to be negative. We examined sera from laboratory personnel at risk for BIV exposure, including two laboratory workers who were exposed to BIV by accidental injection with BIV-infected cell culture material, and found no evidence of seroconversion to BIV-specific proteins. We tested 371 samples of fetal bovine sera, each sample representing serum pooled from one to three fetuses. All samples were negative by BIV Western blot. To date, we have not detected any human sera with antibody to BIV-specific proteins. Our data indicate that persistently indeterminate results on HIV-1 Western blot are not caused by a human antibody response to BIV proteins.

Whetstone, C.A., Van Der Maaten, M.J. and Black, J,W. 1990. Humoral immune response to the bovine immunodeficiency-like virus in experimentally and naturally infected cattle. Journal of Virology, 64, 557–561.

Calves inoculated with bovine immunodeficiency-like virus (BIV) produced virus-specific antibodies that could be detected from 2 weeks to 2.5 years postinoculation by using both indirect fluorescent-antibody and Western immunoblot assays. Antibodies were primarily to p26. Virus and BIV-specific antibodies were isolated from calves given BIV-infected blood. Antibodies to BIV proteins were found in sera from naturally infected cattle.

Whetstone, C.A., Van Der Maaten, M.J. and Miller, J.M. 1991. A Western blot assay for the detection of antibodies to bovine immunodeficiency-like virus in experimentally inoculated cattle, sheep, and goats. Archives of Virology, 116: 119–131.

A cocultivation method was used to establish a cytocidal bovine immunodeficiency-like virus (BIV) infection in primary fetal bovine lung (FBL) cell cultures. Cultures were monitored for virus production using radial immunodiffusion and agar gel immunodiffusion. Pelleted virus and detergent (CHAPS)-solubilised

infected cell lysates from BIV-infected cell cultures were compared as sources of antigen for Western blots. Pelleted virus preparations from FBL-BIV cell cultures produced the best antigen for Western blot. Sheep and goats were inoculated with BIV and serum antibody responses were monitored up to 1 year post inoculation (PI). Sera from experimentally infected cattle, sheep, and goats reacted in Western blot assay with BIV viral induced polypeptides gp 110, p 72, p 55, p 50, gp 42, p 38, p 26, p 24, p 18, p 15, and p 13. Antibodies to p 26 were detected as early as 2 weeks PI in cattle, sheep, and goats. Antibodies to gp 110 were detected by 4 to 6 weeks PI in cattle, and by 9 months PI in sheep and goats. Antibodies to BIV proteins were still evident in cattle sera 2 1/2 years PI, and in sheep and goat sera 1 year I.

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Background: In lentiviruses such as human immunodeficiency virus (HIV) and bovine immunodeficiency virus (BIV), the Tat (transactivating) protein enhances transcription of the viral RNA by complexing to the 5' -end of the transcribed mRNA, at a region known as TAR (the transactivation response element). Identification of the determinants that account for specific molecular recognition requires a high resolution structure of the Tat peptide-TAR RNA complex. Results: We report here on the structural characterisation of a complex of the recognition domains of BIV Tat and TAR in aqueous solution using a combination of NMR and molecular dynamics. The 17-mer Tat peptide recognition domain folds into a beta-hairpin and penetrates in an edge-on orientation deep into a widened major groove of the 28-mer TAR RNA recognition domain in the complex. The RNA fold is defined, in part, by two uracil bulged bases; U12 has a looped-out conformation that widens the major groove and U10 forms a U . AU base triple that buttresses the RNA helix. Together, these bulged bases induce a similar to 40 degrees bend between the two helical stems of the TAR RNA in the complex. A set of specific intermolecular hydrogen bonds between arginine side chains and the major-groove edge of guanine residues contributes to sequence specificity. These peptide-RNA contacts are complemented by other intermolecular hydrogen bonds and intermolecular hydrophobic packing contacts involving glycine and isoleucine side chains. Conclusions: We have identified a new structural motif for protein-RNA recognition, a beta-hairpin peptide that interacts with the RNA major groove. Specificity is associated with formation of a novel RNA structural motif, a U . AU base triple, which facilitates hydrogen bonding of an arginine residue to a guanine and to a backbone phosphate. These results should facilitate the design of inhibitors that can disrupt HIV Tar-TAR association.