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Antigenic and genetic stability of bovine immunodeficiency virus during long-term persistence in cattle experimentally infected with the BIV_{R29} isolate

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Experimental infection of cattle with bovine immunodeficiency virus (BIV) is characterized by persistent, low levels of virus replication in the absence of clinical disease. A virus neutralization (VN) assay was developed to examine the role of VN antibodies in controlling virus replication in cattle experimentally infected with the BIV_{R29} isolate of BIV. All animals developed VN antibody, but there was no correlation between VN titres and restriction of virus replication *in vivo*. BIV infection did not induce high-titred, cross-neutralizing antibody and there was no evidence for antigenic variation through more than 4 years *in vivo*. Genetic comparisons among the BIV_{R29} inoculum virus and viruses isolated from infected animals identified only limited genetic variation during 4 years *in vivo*. Moreover, there was no evidence that the observed variation was due to selection. Analyses of genetic diversity in the virus stock used for inoculation indicated a fairly homogeneous population. In the absence of high levels of virus replication and overt clinical disease, there appeared to be little selection of virus variants, resulting in antigenic and genetic stability of BIV_{R29} during long-term, persistent infection.

Introduction

Bovine immunodeficiency virus (BIV), a member of the lentivirus subfamily of retroviruses, was originally isolated from a dairy cow with persistent lymphocytosis, lymphoid hyperplasia and perivascular cuffing in the brain (Van Der Maaten *et al.*, 1972). Serological studies indicate that BIV is present worldwide (Cockerell *et al.*, 1992; Forman *et al.*, 1992; Horner, 1991; Amborski *et al.*, 1989; McNab *et al.*, 1994), with high seroprevalence among some herds in southeastern regions of the United States (St Cyr Coats *et al.*, 1994; Snider *et al.*, 1996). BIV seropositivity has been correlated with decreased milk production in dairy cattle (McNab *et al.*, 1994), but has not been directly linked with clinical disease in naturally infected cattle. An increased incidence of encephalitis and secondary bacterial infections has been reported in herds with high BIV seroprevalence (Snider *et al.*, 1996), and there is a report of atypical lymphosarcoma in a calf experimentally infected with BIV (Rovid *et al.*, 1996). However, there has been no demonstration that BIV played a direct role in the aetiology of these syndromes. In many cases, such a demonstration is complicated by the presence of confounding factors, including co-infection with bovine leukaemia virus (BLV). Experimental infection of calves with BIV stocks derived from the original BIV_{R29} isolate resulted in a transient increase in lymphocytes and a lymphoid hyperplasia similar to that found early after infection with immunosuppressive lentiviruses (Carpenter *et al.*, 1992; Suarez *et al.*, 1993). Alterations in immune function and suppressed antibody response to other antigens have been observed (Zhang *et al.*, 1997*b*; Flaming *et al.*, 1993, 1997; Onuma *et al.*, 1992). To date, however, there have been no reports of clinical disease or

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signs of immune suppression in BIV_{R29}-infected cattle after more than 4 years post-infection (Flaming *et al.*, 1997).

Lentivirus infections are typically characterized by the establishment of a persistent, lifelong infection and a slow, progressive disease course in the naturally infected host. There is wide variation in clinical disease both among and within the different members of the lentivirus subfamily. Two factors which influence the clinical outcome of infection are the virulence of the inoculum virus and the effectiveness of the host immune response. Reports of clinical disease syndromes in herds with a high incidence of BIV seroprevalence (Snider *et al.*, 1996; St Cyr Coats *et al.*, 1994) suggest that field isolates of BIV may be more pathogenic than the cell culture-adapted BIV_{R29} isolate. In addition, calves experimentally infected with the field-derived BIV_{FL112} isolate exhibited a more pronounced leukocytosis (Suarez *et al.*, 1993) and higher levels of virus replication *in vivo* in comparison to BIV_{R29}-infected calves (S. Carpenter & Y. Wannemuehler, unpublished observations). Attenuation of the BIV_{R29} isolate following extensive passage in cell culture may contribute to the lack of clinical disease observed in cattle experimentally infected with this isolate. In addition, the persistent low level of virus replication during long-term experimental infection with BIV_{R29} may be attributed to an effective host immune response. In the present study, we examined the role of virus-neutralizing (VN) antibody in controlling BIV replication in cattle experimentally infected with BIV_{R29}.

Methods

■ **Animal inoculation, serum collection and clinical monitoring.** Eight male Holstein calves, 2–4 months of age, were experimentally inoculated with $1{\cdot}8 \times 10^4$ syncytia-forming units (SFU) of BIV propagated in foetal bovine lung cells (FBL) as previously described (Carpenter *et al.*, 1992; Isaacson *et al.*, 1995). Five of the eight animals, #344, 345, 347, 348 and 349, received 10^7 peripheral blood mononuclear cells (PBMC) from a lymphocytotic cow persistently infected with BLV as part of a separate study on the interactive effects of the two viruses. The other three calves, #340, 341 and 342, received 10^7 PBMC from a BLV-negative cow. The groups were housed in separate pens and monitored for signs of clinical disease. Blood was collected by jugular venipuncture on a weekly basis for the first 11 weeks post-inoculation (p.i.) and every 8–12 weeks thereafter through 4 years p.i. Haematological evaluation, including total and differential leukocyte analysis, clinical evaluation and evaluation of immune function have been reported previously (Flaming *et al.*, 1993, 1997; Rovid *et al.*, 1995). Results from these *in vivo* studies indicated that animals co-infected with BIV and BLV did not differ from singly infected animals in any parameters of immune function, virus replication or pathological sequelae that have been examined. Therefore, the two groups are not considered separately in the analyses described herein.

■ **Cells and virus.** Primary FBL cells (Whetstone *et al.*, 1991) were maintained in DMEM supplemented with 10% foetal calf serum (FCS) and antibiotics. A number of BIV stocks and/or isolates were derived from BIV_{R29} following *in vivo* and/or *in vitro* passage of the original virus (Van Der Maaten *et al.*, 1972). The virus stock used for experimental inoculation of the eight animals was propagated in FBL cells (Carpenter *et al.*, 1992) and is designated as $BIV_{R29\text{-}4093}$. $BIV_{R29\text{-}346M}$ was derived by *in vitro* culture of monocyte-derived macrophages collected from a steer (#346) experimentally infected with $BIV_{R29\text{-}4093}$ (Rovid *et al.*, 1996). BIV_{FL112} (Suarez *et al.*, 1993) was provided by D. Suarez. All virus stocks were maintained by passage in FBL cells. Additional *in vivo* isolates were obtained from experimentally infected cattle at 4 years p.i. by co-cultivation of PBMC with FBL as described below.

■ **Virus isolation.** Whole blood was collected by jugular venipuncture and PBMC were isolated by centrifugation, collection of the buffy coat, and flash lysis of erythrocytes (Roth *et al.*, 1981). Approximately 10^7 PBMC were co-cultivated with FBL cells in the presence of 8 μg/ml polybrene (Carpenter *et al.*, 1992). Cultures were passaged twice-weekly and visually monitored for syncytium formation. At each passage, replicate cultures were assayed for the presence of BIV, BLV, bovine spumavirus and bovine viral diarrhoea virus using immunofluorescence or immunoperoxidase assays as previously described (Carpenter *et al.*, 1992; Wannemuehler *et al.*, 1993). In most cases, cells were sub-cultured at least six times before they were considered virus-negative. Viruses isolated from experimentally infected cattle at 4 years p.i. which were used for antigenic and genetic characterization are referred to as $BIV_{R29\text{-}341}$, $BIV_{R29\text{-}342}$, $BIV_{R29\text{-}345}$, $BIV_{R29\text{-}347}$ and $BIV_{R29\text{-}348}$.

■ **Virus neutralization assay.** Sera collected from BIV-infected cattle prior to or post-inoculation were heat-inactivated to destroy complement, serially diluted twofold in DMEM with 10% FCS, and used in a VN assay similar to that previously described (Carpenter *et al.*, 1987). Virus stocks were prepared from clarified BIV-infected culture supernatant and were diluted to 200 SFU in a 250 μl volume. Virus was added to an equal volume of serially diluted serum, incubated for 15 min at 4 °C and inoculated in duplicate onto FBL cells seeded the previous day at a density of 2×10^4 cells/cm^2 into 6-well tissue culture plates. Cultures were incubated at 37 °C with 5% CO_2 for 24 h, at which time the BIV/serum inoculum was removed and fresh DMEM with 10% FCS was added. After 3 days, cells were fixed in methanol, and BIV-induced syncytia were detected by immunocytochemistry (ICC) using monoclonal antibody to BIV Gag as previously described (Wannemuehler *et al.*, 1993). The number of BIV-specific SFU were counted and the serum dilution that resulted in at least 80% reduction in SFU as compared to negative control sera was considered to be the virus neutralization titre.

■ **Cloning and sequence analysis.** Total DNA was isolated from virus-infected FBL using SDS–proteinase K digestion. BIV *env* sequences were amplified by PCR using primers to conserved regions of the gene. The upstream primer sequence was 5′ CTATGGATCAGGACCTAGAC (5′ nt 5413) and the downstream primer sequence was 5′ CAGCAC-AAGCAGGAATATTGC (5′ nt 7092). The nucleotide sequence numbers of the primers are based on BIV_{127} (Garvey *et al.*, 1990). Approximately 100 ng of total DNA was amplifed by PCR using the following conditions: 94 °C for 2 min, 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, 2 min extension at 72 °C with a final 5 min extension at 72 °C. For direct sequencing of PCR products, amplified DNA was purified by column chromatography using a commercial kit (Promega) and sequenced using internal primers. For analysis of individual variants present in the $BIV_{R29\text{-}4093}$ inoculum, 1 μl of amplified DNA was ligated to pCR2.1 (Invitrogen) and individual clones were isolated and sequenced. For amplification of viral sequences in the absence of selection

for replication on FBL, DNA was isolated from PBMC collected from steer #348 at 4 years p.i. and a 656 nt fragment was amplified with the upstream primer 5′ GTTGTCCCATATGTAGTTGGC (5′ nt 5737), located in *tat* exon 1, and the downstream primer 5′ GCAAACTT-TGGAGGTATTTC (5′ nt 6393), located in *env*. Amplification conditions were similar to those described above. The amplicon was cloned, and individual clones were picked and sequenced as above.

The program SITES, version 1.1 (Hey & Wakeley, 1997), was used to identify the number of synonymous (S) and non-synonymous (N) nucleotide changes, as well the number of synonymous sites and non-synonymous sites. These values were used to calculate the N/S and Kn/Ks ratios. SITES was also used to perform Tajima's D test, which tests whether observed mutations are due to positive Darwinian selection or to neutral mutations (Tajima, 1989).

Results

Development of VN antibodies in experimentally infected cattle

A VN assay was developed to examine the role of neutralizing antibody in controlling BIV replication in cattle experimentally infected with BIV_{R29}. In initial studies, serially diluted sera samples were assayed for antibodies capable of neutralizing $BIV_{R29\text{-}346M}$. This isolate, related to the $BIV_{R29\text{-}4093}$ inoculum, replicates to sufficient titres *in vitro* for use in the VN assay. In all cases, sera collected from animals prior to experimental infection were negative for BIV-neutralizing antibody (Table 1). In seven of the eight animals, VN antibody titres were detectable by 17 weeks p.i. The single animal negative for VN antibody at 17 weeks had low but detectable titres by 22 months p.i. There was wide variability in VN antibody titres among the eight animals at all time-points examined, with maximum titres ranging from 16 (#344 and #340) to 256 (#342). In some cases, VN antibody titres increased throughout the 4 year period, with maximum titres observed at 44 months p.i. In two animals, #347 and #345, maximum titres were reached at 17 weeks p.i. and remained unchanged. Antibody titres in #348 peaked at 17 weeks p.i., declined by 22 months p.i., and increased until 44 months p.i., although titres at that time remained lower than those observed at 17 weeks p.i.

To determine the relationship between VN antibody titre and virus replication, we compared the VN titre with recovery of infectious virus from PBMC (Table 1). Results of virus isolation are expressed as the number of *in vitro* passages required for detection of BIV-specific syncytia. The highest VN titre was observed in #342, an animal from which BIV was consistently and readily isolated. In contrast, animals with the lowest VN titres, #340 and #344, were those which were most often negative for virus recovery. Over the 4 year period, we observed no evidence that VN antibody was effective in the clearance of BIV *in vivo*.

Table 1. Neutralizing antibody titres and virus isolation from BIV-infected cattle

Animal		Pre-infection	Time post-infection				
			2 weeks	17 weeks	22 months	33 months	44 months
340	VN titre*	< 8	< 8	< 8	8	8	16
	BIV isolation†	—	p2	p2	p4	—	—
341	VN titre	< 8	< 8	16	32	32	64
	BIV isolation	—	p2	p2	p4	p3	p3
342	VN titre	< 8	< 8	128	128	128	256
	BIV isolation	—	p2	p2	p3	p2	p4
344	VN titre	< 8	< 8	8	16	16	16
	BIV isolation	—	p2	—	—	—	—
345	VN titre	< 8	< 8	32	32	32	32
	BIV isolation	—	p2	p2	p4	p3	p3
347	VN titre	< 8	< 8	64	64	64	64
	BIV isolation	—	p2	p2	p2	p2	p3
348	VN titre	< 8	< 8	128	32	32	64
	BIV isolation	—	p2	p2	p6	p2	p3
349	VN titre	< 8	8	64	32	64	64
	BIV isolation	—	p2	p2	p4	p3	—

* VN titres indicate serum dilution which gave at least 80% reduction in SFU of $BIV_{R29\text{-}346}$ as compared to virus in diluent alone. VN assays were repeated two or three times.

† Virus was isolated by co-cultivation of PBMC with FBL cells and tested for BIV by ICC. Results are expressed as the passage at which BIV-specific syncytia were first detected. Cells were passaged at least six times before being considered negative (—).

Table 2. Analysis of antigenic variation in BIV-infected cattle

Virus	Animal	VN titre	
		Year 3 sera	Year 5 sera
R29-4093	342	64	64
	345	16	16
	348	8	16
Homologous year 4 virus*	342	128	128
	345	32	16
	348	16	16

* Virus was isolated from PBMC collected from steers #342, 345 and 348 at 4 years p.i. and used in a VN assay with sera collected from the same animal at 3 and 5 years p.i.

Table 3. Assessment of VN antibody to heterologous virus in sera from BIV-infected cattle

Sera from BIV-infected steers were tested for VN antibody to $BIV_{R29\text{-}4093}$ and BIV_{FL112}.

Animal	Virus inoculum	Serum collected (years p.i.)	VN titre	
			BIV_{FL112}	$BIV_{R29\text{-}4093}$
342	$BIV_{R29\text{-}4093}$	5	< 4	64
345	$BIV_{R29\text{-}4093}$	5	< 4	16
348	$BIV_{R29\text{-}4093}$	5	< 4	16
1268	BIV_{FL491}	3	< 8	< 4
1275	BIV_{FL112}	3	8	< 4

Limited antigenic variation during persistent BIV infection

Antigenic variation is a common feature of lentiviruses and the higher levels of virus replication observed in some animals (i.e. #342) may have been due to replication of virus antigenically distinct from the $BIV_{R29\text{-}346M}$ virus used in the neutralization assay. To explore this possibility, virus isolated from individual animals at 4 years p.i. was antigenically compared with the inoculum virus. $BIV_{R29\text{-}342}$, $BIV_{R29\text{-}345}$ and $BIV_{R29\text{-}348}$ were used in a VN assay with sera collected from steers #342, #345 and #348 at 3 and 5 years p.i. In all three animals tested, sera collected at 3 years p.i. had neutralizing antibody to the inoculum virus, $BIV_{R29\text{-}4093}$ (Table 2). Surprisingly, the same year three sera had equivalent or higher VN antibody titres to virus isolated at year 4 p.i. Moreover, VN titres to year 4 virus were essentially identical in sera collected at 3 and 5 years p.i. In all cases, VN titres to the inoculum virus and year 4 virus did not differ by more than twofold, indicating little or no antigenic variation between the inoculum virus and year 4 viruses.

Neutralizing antibody does not neutralize heterologous isolates of BIV

Studies with other lentiviruses have shown that later time-points after infection are characterized by a maturation of the immune response and development of broadly acting neutralizing antibody (Cole *et al.*, 1997; Hammond *et al.*, 1997). Therefore, the similarities in VN antibody titre to the inoculum virus and year 4 viruses may have been due to the presence of high-titred, cross-reacting neutralizing antibody. To test this possibility, sera collected from the experimentally infected animals were used in a VN assay against the heterologous BIV_{FL112} isolate (Suarez *et al.*, 1993). No VN antibody to BIV_{FL112} was detected in year 5 sera samples from any of the animals inoculated with $BIV_{R29\text{-}4093}$ (Table 3). To ensure that BIV_{FL112} was susceptible to *in vitro* neutralization in our assay system, serum from cattle experimentally infected with BIV_{FL112} or the related BIV_{FL491} were tested for type-specific and cross-neutralizing antibody. Sera from steer #1275, inoculated with BIV_{FL112}, had low levels of VN antibody to BIV_{FL112}, but no detectable VN antibody to $BIV_{R29\text{-}4093}$. Partial neutralization of BIV_{FL112}, but not BIV_{FL493}, was observed at higher dilutions of #1275 sera. Sera from #1268, inoculated with BIV_{FL491}, was unable to neutralize either the related BIV_{FL112} or the unrelated $BIV_{R29\text{-}4093}$. The results indicated that BIV infection did not elicit high titres of cross-neutralizing antibody in either $BIV_{R29\text{-}4093}$-inoculated steers or in calves inoculated with Florida isolates of BIV. Therefore, the comparable VN antibody titres to the $BIV_{R29\text{-}4093}$ and the year 4 viruses (Table 2) reflect the antigenic similarity of these viruses rather than the presence of cross-neutralizing antibody.

Limited genetic variation of BIV during long-term persistent infection

Our immunological analyses over the course of persistent infection suggested limited antigenic variation of BIV *in vivo*. To examine the extent of genetic variation over the same time-period, we compared the *env* sequence of $BIV_{R29\text{-}4093}$ with the *env* sequences of virus isolated at 4–5 years p.i. from five of the experimentally infected steers. Virus was recovered from infected animals by co-cultivation of PBMC with FBL cells, and total DNA was isolated from virus-infected cells. Amplification of *env* sequences was done using primers conserved among heterologous isolates of BIV. Direct sequencing of the PCR products indicated limited sequence heterogeneity between

the $BIV_{R29\text{-}4093}$ inoculum and virus recovered from infected animals at 4 years p.i. (Fig. 1 and Table 4). Nucleotide diversity between $BIV_{R29\text{-}4093}$ and *in vivo* virus ranged from 0·40% in steer #348 to 1·74% in #347. Over 75% of the nucleotide changes were non-synonymous, with the resulting amino acid variation between 1·19–5·23%. The ratio of non-synonymous to synonymous nucleotide changes (N/S) was greater than one, and the Kn/Ks ratio was greater than 0·33, suggesting that variation may have been due to positive Darwinian selection. Analysis of the five *in vivo* isolates using the Tajima test (Tajima, 1989), however, indicated that the observed variation was due to neutral mutations rather than positive selection.

The *env* sequences represent the predominant genotype of virus selected for *in vitro* replication in FBL cells and may not represent the predominant genotype *in vivo*. A more direct analysis of virus genotypes present in plasma or PBMC is difficult due to the low levels of BIV replication during persistent infection (Carpenter *et al*., 1992). However, it was important to determine if the *env* sequences obtained from virus propagated in FBL were reflective of virus sequences present *in vivo*. DNA was isolated from PBMC obtained from steers at 4 years p.i. and used to directly amplify *env* sequences present *in vivo*. In one of the animals, #348, we were able to amplify a 322 nt fragment from the 5′ *env* gene. Although this region did not contain the size-variable V2 region of *env*, it did allow us to determine if sequences obtained directly from PBMC were similar to those amplified from the infected cell culture. The amplicon was cloned, and six individual clones were sequenced for comparison with the $BIV_{R29\text{-}4093}$ and $BIV_{R29\text{-}348}$ consensus sequences obtained by direct sequencing of the PCR-amplified product. The six sequences obtained directly from PBMC were similar to the consensus sequences of virus propagated in cell culture (Fig. 1 and Table 4). At the amino acid level, the percent divergence between the individual clones and $BIV_{R29\text{-}4093}$ ranged from 0 to 3·4%, with the average divergence closely matching that observed for the entire $BIV_{R29\text{-}348}$ consensus sequence. The individual clones differed from the $BIV_{R29\text{-}348}$ consensus sequence by only one or two amino acids. The overall similarity among the consensus sequences and the individual clones indicates that the consensus sequences obtained following *in vitro* culture are reflective of sequences present *in vivo*. Moreover, the similarity between the *in vivo*-derived clones and the $BIV_{R29\text{-}4093}$ inoculum supports our finding of genetic conservation of BIV during long-term persistent infection.

Analysis of genetic diversity in the R29-4093 inoculum

The extent of genetic diversity of *env* in the $BIV_{R29\text{-}4093}$ stock was determined by PCR amplification, cloning and sequencing individual clones. Differences between individual clones and the $BIV_{R29\text{-}4093}$ consensus sequence ranged from 0·26 to 0·72% divergence at the nucleotide level and 0·78 to 1·76% divergence at the amino acid level (Fig. 1 and Table 4). The total number of nucleotide substitutions/deletions found in $BIV_{R29\text{-}4093}$ was only slightly less than that observed in the year 4 virus isolates (Table 4). It is possible that the observed nucleotide substitutions occurred during PCR amplification; however, the error rate of *Taq* polymerase using our standard laboratory conditions is 0·025%, about 20-fold less than the variation observed among the analysed sequences. Therefore, these findings suggest that BIV is genetically conserved during long-term persistent infection.

Previous studies of variation in the BIV surface (SU) protein have reported sequence divergence of up to 50% among unrelated BIV isolates (Suarez & Whetstone, 1995). In addition to nucleotide substitutions, insertions/duplications within the hypervariable V2 domain can result in a large amount of SU protein size variation (Garvey *et al*., 1990; Suarez & Whetstone, 1995, 1997). Comparison of BIV_{R29}-related sequences and sequences obtained from heterologous field isolates BIV_{FL112} and BIV_{OK40} of BIV demonstrate that BIV_{R29}-related isolates are genetically quite diverse from the more recently described BIV genotypes (Fig. 2). In addition, we did not detect amino acid insertions in the V2 domain in any of the $BIV_{R29\text{-}4093}$ variants or *in vivo* isolates (Figs 1 and 2). In fact, more than half of the observed amino acid differences among the BIV_{R29} variants were found to lie outside the previously described variable regions (Suarez & Whetstone, 1995). Therefore, both quantitative and qualitative differences in variation were observed in $BIV_{R29\text{-}4093}$-infected cattle as compared to cattle infected with more recently described field isolates of BIV.

Discussion

Longitudinal studies of cattle experimentally infected with the BIV_{R29}-related isolates have demonstrated virus persistence through 4 years post-infection with no progression to overt clinical disease (Flaming *et al*., 1997; Isaacson *et al*., 1995; Zhang *et al*., 1997*a*). One possible reason why BIV_{R29}-infected animals remain non-progressors is that BIV_{R29} is inherently apathogenic, with low levels of replication resulting in poor spread of the virus *in vivo*. In addition, an effective host immune response may control BIV replication and delay or prevent the onset of clinical disease. In the present study, a VN assay was developed in order to assess the role of BIV-neutralizing antibodies in controlling virus replication *in vivo*. During a 4 year period, eight of eight cattle experimentally infected with $BIV_{R29\text{-}4093}$ developed neutralizing antibodies; however, differences were observed in neutralizing titre and rate at which neutralizing antibodies appeared. Higher-titred ($\geqslant 128$) neutralizing antibody appeared faster in the animals from which virus was most easily and consistently recovered. The appearance of neutralizing antibody was

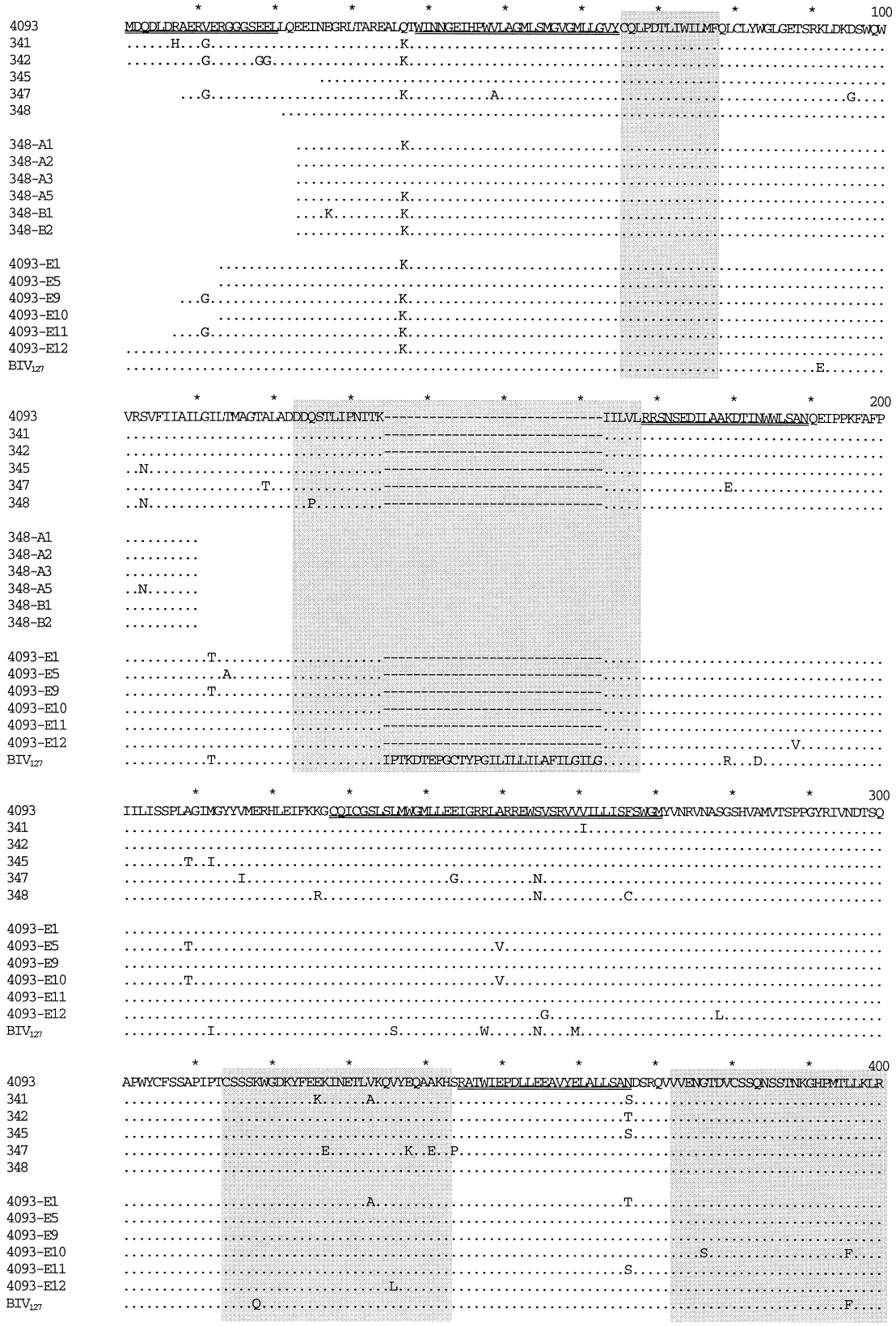

Fig. 1. For legend see facing page.

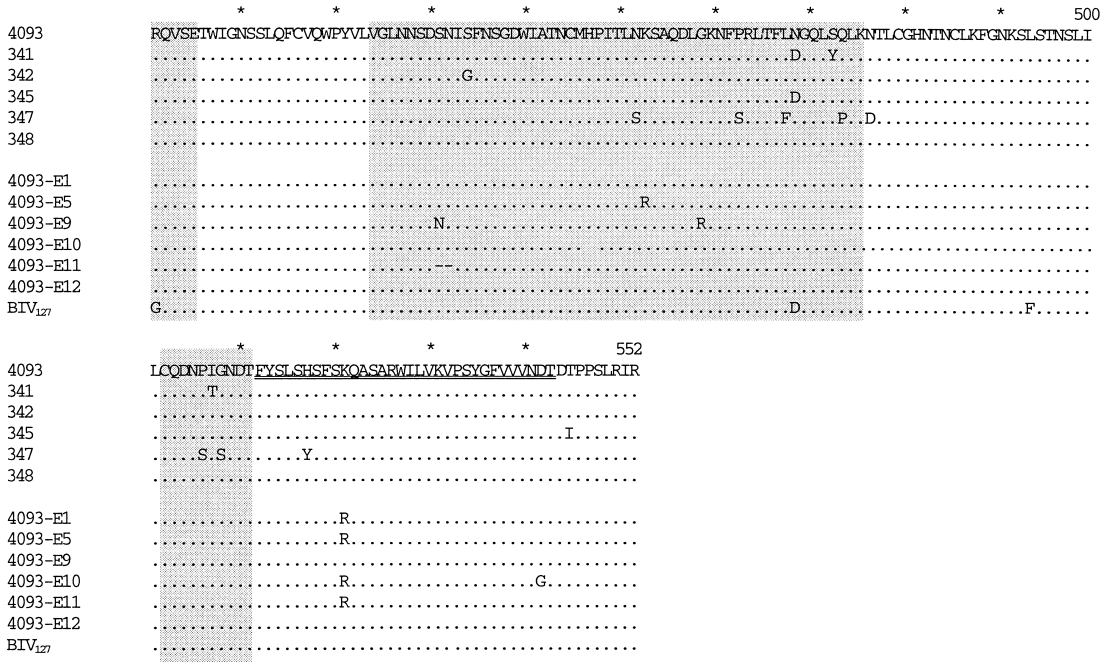

Fig. 1. Genetic heterogeneity among *in vivo* and *in vitro* isolates of BIV. The *env* sequences of $BIV_{R29\text{-}4093}$, $BIV_{R29\text{-}341}$, $BIV_{R29\text{-}342}$, $BIV_{R29\text{-}345}$, $BIV_{R29\text{-}347}$ and $BIV_{R29\text{-}348}$ were obtained from cell culture-propagated virus by direct sequencing of the PCR-amplified proviral DNA. The 348-A1, 348-A2, 348-A3, 348-A5, 348-B1 and 348-B2 *env* sequences are individual clones obtained from PCR amplification of proviral DNA isolated directly from PBMC collected from steer #348 at 4 years p.i. The 4093-E1, 4093-E5, 4093-E9, 4093-E10, 4093-E11 and 4093-E12 sequences are individual clones obtained following PCR amplification of proviral DNA isolated from $BIV_{R29\text{-}4093}$-infected FBL cells. The sequence of BIV_{127} (Garvey *et al.*, 1990), including the V2 insertion, was obtained from GenBank. Amino acid substitutions are indicated by the single letter designation. Dots represent amino acid identity and dashes indicate deletions. Previously described conserved and variable regions (Suarez & Whetstone, 1995) are indicated by double underlines and shaded boxes, respectively.

delayed, and peak titres were lower, in animals from which virus was not as readily recovered. Virus recovered from infected animals at 4 years p.i. was antigenically and genetically similar to the inoculum virus, and there was little evidence of immune selection by neutralizing antibody. Together, these results suggest that VN antibody does not play the major role in restriction of BIV_{R29} replication *in vivo*. It remains possible that an effective cell-mediated immune response is an important mechanism of immune control of BIV_{R29} replication *in vivo*.

Broadly reacting neutralizing antibodies are found in human immunodeficiency virus type 1-infected long-term non-progressors and may contribute to the lack of disease progression in these individuals (Cao *et al.*, 1997). In the present study, the neutralizing antibody response in BIV-infected cattle exhibited a fairly narrow specificity and only low levels of cross-reacting neutralizing antibody were detected. Despite this narrow specificity, VN antibody did not appear to contribute to immune selection of antigenically variant virus. The twofold differences in VN titre to the inoculum virus and the year 4 viruses are within the range of experimental error and cannot be considered as evidence of antigenic variation. Antigenic variation in the absence of immune pressure by neutralizing antibody has been reported in other lentivirus infections (Carpenter *et al.*, 1987; Cheevers *et al.*, 1993, 1999; Leroux *et al.*, 1997). It is possible that other immune mechanisms exert selective pressure *in vivo* and that antigenic differences between $BIV_{R29\text{-}4093}$ and year 4 viruses may be detectable by cytotoxic T lymphocytes and/or monoclonal antibodies. However, given the limited antigenic diversity observed, it was surprising that the host immune response failed to clear the virus after more than 4 years post-infection.

Previous studies of BIV variation in cattle experimentally infected with the Florida isolates of BIV identified nucleotide substitutions as well as insertions/duplications in the V2 region of the *env* gene (Suarez *et al.*, 1995; Suarez & Whetstone, 1997). The $BIV_{R29\text{-}4093}$ inoculum was derived from the same BIV_{R29} stock previously shown to contain at least two size variants, BIV_{106} and BIV_{127} (Garvey *et al.*, 1990). BIV_{127} contains 29 additional amino acids in the V2 region of *env* as compared to BIV_{106} (Garvey *et al.*, 1990). If the larger genotype has a selective advantage *in vivo* it might be expected to become the predominant genotype after 4 years *in vivo*. The only genotype detected in our studies was the smaller, BIV_{106}-

Table 4. *In vivo* and *in vitro* variation in the BIV SU protein

		Nucleotide substitutions/ deletions			% Divergence from R29-4093	
		Total	S*	NS†	% nt	% aa
In vivo isolates	341	13	3	10	0·82	2·49
	342	7	1	6	0·45	1·34
	345	9	3	6	0·60	1·80
	347	27	6	21	1·74	5·23
	348	6	1	5	0·40	1·19
	Average	12·4	2·8	9·6	0·80	2·41
348 *in vivo* quasispecies‡	A1	1	0	1	0·38	1·13
	A2	0	0	0	0	0
	A3	0	0	0	0	0
	A5	3	1	2	1·13	3·41
	B1	3	1	2	1·13	3·41
	B2	1	0	1	0·38	1·13
	Average	1·17	0·33	0·83	0·44	1·32
R29-4093 quasispecies§	E1	8	3	5	0·52	1·56
	E5	9	4	5	0·59	1·75
	E6	4‖	0	4	0·26	0·78
	E7	11¶	4	7	0·72	2·15
	E8	11¶	4	7	0·72	2·15
	E9	5	0	5	0·32	0·97
	E10	9	2	7	0·59	1·76
	E11	8	4	4	0·52	1·55
	E12	8	3	5	0·51	1·53
	Average	8·11	2·67	5·44	0·53	1·58

* Number of synonymous substitutions.
† Number of non-synonymous substitutions.
‡ Sequences of individual clones derived by amplification and cloning 322 nt of *env* directly from year 4 PBMC.
§ Sequences of individual clones derived from amplification and cloning *env* from the $BIV_{R29\text{-}4093}$ virus stock.
‖ A 2 nt deletion resulted in a frameshift and premature truncation.
¶ A single nucleotide deletion resulted in a frameshift and premature truncation.

like, genotype. It is possible that the larger genotype was present *in vivo*, but was selected against during *in vitro* isolation in FBL cells. However, other studies have demonstrated selection of larger genotypes during *in vitro* replication of isolates containing mixed populations of larger and smaller genotypes (Suarez & Whetstone, 1997). Further studies are needed to clarify the role of the V2 region in BIV replication *in vivo* and *in vitro*.

Lentivirus infections are characterized by a high rate of genetic variation *in vivo*, and the genetic conservation in BIV *env* over a 4 year period *in vivo* was not expected. The percentage of non-synonymous nucleotide substitutions over 4 years *in vivo* was only slightly higher than that observed in the *in vitro* population. Although the ratios of N/S and Kn/Ks were higher than expected for random substitutions, the Tajima test indicated that the changes were due to neutral mutations. Moreover, the changes which did occur *in vivo* were not clustered in previously described hypervariable regions (Suarez & Whetstone, 1995), as might have been expected if the changes arose as a result of selective pressure. Together, these results strongly suggest that there was little selective pressure for virus variation *in vivo*. It is possible that the attenuation of BIV_{R29} following long-term culture *in vitro*, together with limited genetic diversity in the inoculum, contributed to the persistence of non-pathogenic virus genotypes *in vivo*. In the absence of high levels of virus replication and overt clinical disease, limited generation and selection of virus variants could result in antigenic and genetic stability of BIV_{R29} during long-term persistent infection. *In vivo* studies with BIV_{FL112} and BIV_{FL491} suggest that these isolates are somewhat more pathogenic than BIV_{R29}-related isolates (Suarez *et al.*, 1993), and it might be expected that other isolates of BIV are characterized by a higher rate of genetic and antigenic variation than observed in the present study. Delineating the factors which contribute to the genetic stability and persistence of BIV *in vivo* may provide insight into strategies that restrict lentivirus-associated disease in humans and animals.

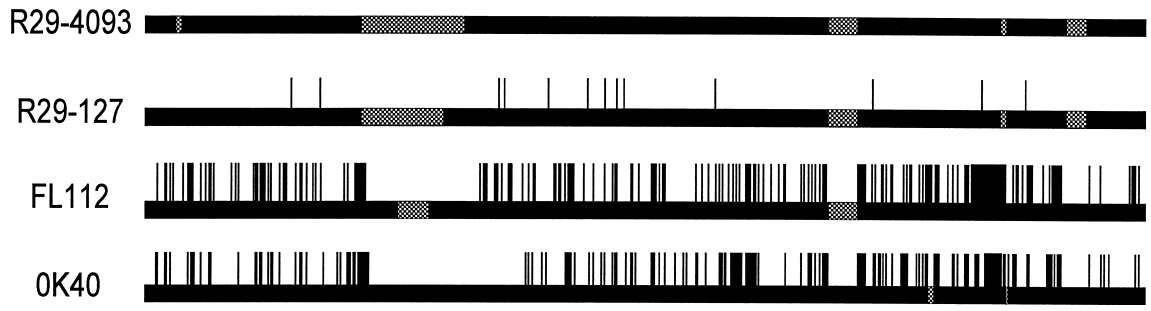

Fig. 2. Genetic heterogeneity in BIV SU among characterized isolates of BIV. The SU sequences of $BIV_{R29\text{-}4093}$, BIV_{127}, BIV_{FL112} and BIV_{OK40} are represented by a horizontal bar. Each vertical line represents a single amino acid substitution from the $BIV_{R29\text{-}4093}$ sequence. Stippled areas indicate deletions as compared to the larger genotype. BIV_{127}, BIV_{FL112} and BIV_{OK40} sequences were obtained from GenBank.

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