Variations in lentiviral gene expression in monocyte-derived macrophages from naturally infected sheep

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Seventy-nine 1-year-old lambs from three individual farms and a feedlot were examined for natural lentivirus infection. We used three different methods to detect infection and to identify the stage of the ovine lentivirus life cycle in blood-derived macrophages. Cytopathic infectious virus was obtained from 14/14 Border Leicester animals obtained from a naturally infected flock. Neither virus particles, virus proteins, virus specific antibodies nor viral DNA were detected in samples from 34 lambs from two South Kansas City farms. However, among 31 feedlot lambs, we identified 11 infected animals. Specific viral proteins were immunoprecipitated from macrophages of one animal, but no infectious cytopathic virus was isolated from these cells. Cells from ten of the other feedlot animals harboured viral DNA but neither viral particles nor proteins could be detected by our techniques. Thus, in these naturally infected animals, the virus life cycle either proceeded to completion, subject to differentiation of infected precursor cells in blood, or remained arrested at the DNA stage despite maturation of monocytes to macrophages. Sequence analysis of the env gene of viral genomes from two of the ten feedlot sheep showed sequences distinct from those of known ovine and caprine lentiviruses. Surprisingly, these sequences have a higher identity (of nucleotide and derived amino acid sequences) to caprine arthritis-encephalitis virus than to the ovine prototype, maedi-visna virus. These data suggest that the ovine and caprine lentiviruses found in North American sheep may have a common ancestral genotype that is closely related to the caprine virus.

Introduction

Infection in sheep and goats with ovine–caprine lentiviruses occurs worldwide (Cork & Narayan, 1980; Honger et al., 1990; Narayan & Cork, 1985; Sigurdsson et al., 1957), with the main mechanism of virus transmission being ingestion of infectious colostrum or milk by newborn animals (Adams et al., 1984; Cutlip et al., 1988; Houwers & van der Molen, 1987; Lerondelle & Ouzrout, 1990; Ouzrout & Lerondelle, 1990; Rowe et al., 1991). The prototype ovine virus is maedi–visna virus (MVV) obtained originally from Icelandic sheep during an epizootic of pneumonia (maedi) and progressive paralysis (visna) that developed among these animals following introduction of European rams into local flocks during the 1950s (Sigurdsson, 1954; Sigurdsson et al., 1957). The prototype caprine virus, caprine arthritis–encephalitis virus (CAEV) was isolated initially from the synovial fluid of an arthritic goat (Crawford et al., 1980) and from the brain of an encephalitic kid (Narayan et al., 1980), and subsequently from goats in many other countries (Agrimi et al., 1987; Dahlberg et al., 1981; Krieg & Peterhans, 1990; Dawson et al., 1983; Rowe et al., 1992a, b) including Australia, where MVV has never been identified in sheep (Ellis et al., 1983; Surman et al., 1987). The complete genomes of both prototype viruses have been sequenced (Braun et al., 1987; Sonigo et al., 1985; Staskus et al., 1991; Saltarelli et al., 1990; Querat et al., 1990). MVV and CAEV share approximately 74.8% and 77.5% identity in the amino acid sequences encoded by the gag and pol genes respectively, but only 60% identity in the env gene-encoded sequences. The env genes of two commonly used strains of CAEV, CAEV-co and CAEV-63 have 90% identity with each other (Knowles et al., 1991; Saltarelli et al., 1990).
Unlike the human lentivirus, human immunodeficiency virus (HIV), which replicates to high titres in lymphoid tissues during the early phase of infection, the lentiviruses of small ruminant animals infect mainly macrophage-lineage cells, replicate at a low rate for an indefinite period, and are rarely present in a cell-free state in tissues during subclinical infection (Narayan et al., 1982). This type of replication, with an apparent incomplete virus life cycle, continues for years. A few of these persistently infected animals eventually develop chronic progressive disease, affecting specific organ systems. In such animals, virus can usually be recovered from cell-free homogenates of the affected tissues (Narayan et al., 1982; Kleijer Anderson et al., 1984). However, the virus life cycle remains at the incomplete state in macrophages from other parts of the body (Narayan et al., 1983a).

Earlier studies have shown that peripheral blood mononuclear cells (PBMC) from subclinically CAEV-infected goats and ovine lentivirus-infected sheep harbour incompletely expressed viral genomes and that cultivation of these cells in medium which promotes maturation of monocytes into macrophages results in virus production by the macrophages (Narayan et al., 1983a; Gendelman et al., 1986). These mature macrophages, differentiating in vitro, not only produce virus but frequently develop viral cytopathic effects similar to those seen during cultivation of mature macrophages from diseased tissues.

In this study we examined PBMC from 14 animals from a naturally infected flock of Border Leicester (BL) sheep, 34 lambs from two single flocks, and 31 feedlot (FL) lambs that had been previously acquired from different farms, for evidence of lentivirus infection. Our results show three types of infection of monocytes/macrophages in sheep; 14 animals were found to be productively infected and infectious cytopathic viruses were isolated from their PBMC-derived macrophages. Among the FL lambs, cells from one animal expressed viral proteins and its serum contained antibodies specific to ovine lentivirus proteins but no infectious cytopathic virus was recovered from its macrophage cultures. Cells from ten other FL sheep had viral DNA but did not produce viral proteins. Among these samples in vitro differentiation of blood monocytes to macrophages failed to induce expression of the viral genomes, and examination of sera from these animals by immunoprecipitation failed to detect antibodies specific to viral proteins of ovine and caprine lentiviruses. To determine the genetic nature of these inexpressible viral genomes in the FL sheep, a 1.6-kb PCR fragment from the env gene was cloned and sequenced from two of the ten sheep. DNA sequences from the two sheep were unique and distinct from the molecularly cloned strains of MVV and CAEV used in our laboratory. Surprisingly, these DNA sequences have 86% identity with CAEV but only 72% with MVV. These data demonstrate not only different types of lentivirus infection in nature but also suggest that North American ovine lentiviruses may be of caprine origin, a phenomenon also observed in European sheep.

Methods

- **Plasmids.** Plasmid pLV1-ILKS1 was a gift from K. A. Staskus (Staskus et al., 1991) and contains the complete proviral genome of the MVV 1514 clone inserted into the SalI site of pBR322. Plasmid pCAEV (Saltarelli et al., 1990) contains a large part of the proviral sequence of CAEV introduced into the HindIII site of pUC18.

- **Sheep.** A total of 79 animals was examined in this study. All animals were approximately 1 year of age and healthy. Fourteen healthy animals were sampled from a single Border Leicester (BL) flock in Maryland. A few animals in this flock had clinical ovine progressive pneumonia and were excluded from this study. Twenty (DH1) and fourteen (DH2) Dorset Horn lambs were sampled from two single flocks near Kansas City. These flocks were kept close to introduction of any new animals for more than 10 years and no history of lentivirus infection had been observed during this period of time. Thirty-one feedlot (FL) lambs were sampled from a local Kansas feedlot. About 250 lambs in this feedlot (group of animals collected from different breeding farms and commercialized for meat) were a cross-bred collected from local farms in Kansas and Missouri. They had been housed together for 2 days before blood was taken. Serological and virus isolation experiments were performed on sera and cells from the blood samples from all of the animals studied.

- **Derivation of macrophages from PBMC and assays on cultivated macrophages.** Blood collected in EDTA from each animal was sedimented to remove plasma (saved for antibody determinations) and the cells were resuspended in Hanks' balanced salt solution. The mononuclear cells (PBMC) were prepared and macrophages were differentiated in culture as previously described (Narayan et al., 1983a). The PBMC were obtained by centrifugation of the cell suspension through Ficoll–Hypaque gradients. Approximately 10⁸ PBMC were resuspended in 30 ml of macrophages differentiation medium (MDM) containing of RPMI supplemented with 10 mM-HEPES buffer, pH 7:3, 50 μg/ml gentamicin, 5 × 10⁻⁶ M-2-mercaptopoethanol, 2 mM-glutamine, (sRPMI) + 20% heated lamb serum and cultivated in Teflon bottles at 37 °C for 2 weeks with a complete medium change at 1 week. This method yielded suspensions of macrophages which, when seeded into tissue culture dishes, became adherent and spread into typical esterase-positive cells fully capable of Fc receptor-mediated endocytosis of antibody-coated red blood cells within 4 h at 37 °C. Mature macrophages were transferred from Teflon bottles to tissue culture dishes where they were maintained for a further 2 weeks. Cells in different dishes were used for different experiments. Two dishes were maintained and examined either for degeneration (cytolysis) and/or for development of syncytial cytopathic effects (CPE) as previously described (Chebloune et al., 1996). Cells in two dishes were co-cultivated with GSM (indicator), cells which are highly susceptible to fusion CPE induced by ovine–caprine lentiviruses. (see below). The co-cultivation procedure accelerated development of CPE if any of the macrophages were producing virus. Portions of the macrophage cultures were also used for detection of viral proteins by immunoprecipitation and other portions for detection of viral DNA by PCR assay as described below.

- **Indicator cell lines.** Goat synovial membrane (GSM) cells were obtained originally from explanted carpal synovial membrane from a colostrum-deprived, newborn goat (Narayan et al., 1980). The lines of cells were expanded by cultivation in Minimum Essential Medium + 10% fetal bovine serum and stored in liquid nitrogen. Typical monolayer cultures were passaged at 1:3 split ratios and used for seven to ten passages. GSM and SCP are highly susceptible to fusiogenic infection with CAEV and lytic infection by MVV.

- **Viruses.** The Icelandic MVV strain 1514, kindly provided by Neal
Nathanson (Petursson et al., 1976), was cultivated in SCP cell cultures. The CAEV strain CAEV-co, isolated from the brain of an encephalitic goat, (Narayan et al., 1980) was cultured in GSM cells. Field virus BL.93 had been obtained from the synovium of a BL ram with synovitis and pneumonia. This virus was expanded in a single culture of inflammatory cells aspirated from the swollen joint together with PBMC-derived macrophages from an uninfected sheep. The titre of this virus was approximately 10^7 TCID50/ml when titrated in macrophages.

**Virus-specific protein immunoprecipitation.** To test for the presence of virus-specific antibodies in the serum and viral proteins in the macrophages of lambs used in this study we selected the immunoprecipitation technique. Detection of virus-specific antibodies capable of immunoprecipitating virus-specific proteins was performed on macrophages co-infected with CAEV and MVV. Detection of viral proteins in macrophages was performed on 2–3-week-old cultures of blood-derived macrophages from each animal. Macrophage monolayers were fixed twice with RPMI medium without serum and methionine and incubated for 2 h in the same medium. Proteins were radiolabelled by addition of 100 μCi/ml [35S]methionine/cysteine (ICN) to the medium, and incubation overnight (16–18 h). Culture medium was harvested and cell debris removed by centrifugation in a microfuge (5 min, 12000 r.p.m.). The clarified cell culture medium was made 1 x with respect to RIPA buffer, and nuclei and cell debris were removed by centrifugation in a microfuge (5 min, 12000 r.p.m.). Clarified cell culture medium and cell lysis were incubated and rotated overnight at 4 °C in the presence of serum or other virus. Culture medium was harvested and inoculated into normal ovine macrophage cultures. All of these cultures had cytopathic virus (data not shown). Thus, macrophage cultures derived from all 14 BL sheep were not productively infected with a cytopathic virus. Medium from the cultured macrophages of these animals was harvested and inoculated into normal ovine PBMC-derived macrophage cultures. All of these cultures degenerated; parallel cultures developed syncytia when co-cultivated with GSM cells and culture medium from all of the cultures had cytopathic virus (data not shown). Thus, macrophages from all 14 BL sheep produced infectious, cytopathic virus after 2 weeks of culture.

**Results**

**Assays on cultivated macrophages**

Macrophage cultures derived from all 14 BL sheep either degenerated or developed syncytial cytopathic effects when co-cultivated with the GSM cells. This result indicated that macrophages from these animals contained a replication-competent virus. Medium from the cultured macrophages of these animals was harvested and inoculated into normal ovine PBMC-derived macrophage cultures. All of these cultures degenerated; parallel cultures developed syncytia when co-cultivated with GSM cells and culture medium from all of the cultures had cytopathic virus (data not shown). Thus, macrophages from all 14 BL sheep produced infectious, cytopathic virus after 2 weeks of culture.

Macrophage cultures from the 20 DH1, 14 DH2 and 31 BL sheep obtained from the Kansas City area appeared normal (data not shown). These cells did not develop fusion for up to 2 weeks either by themselves or after co-cultivation with GSM cells. This indicated that the macrophage cultures from these sheep were not productively infected with a cytopathic virus.

**Analysis of virus-specific proteins**

Culture media, as well as cell lysates of cultured macro-
Fig. 1. Immunoprecipitation of viral proteins from blood monocyte-derived macrophages. (a) Immunoprecipitation of viral proteins from culture medium (M) and cell lysate (C) of PBMC-derived macrophages from four of 65 sheep (FL, DH1 and DH2), negative for virus isolation, with a goat hyperimmune serum (G62). No viral proteins were found in either the culture medium or the cell lysate of macrophage cultures from the four animals (1–4). Similar results, with no viral proteins, were obtained from macrophage cultures of 60 other animals (not shown).

Immunoprecipitation of viral proteins from PBMC-derived macrophages from four of 14 BL sheep (5–8) from a naturally lentivirus-infected BL flock with a goat hyperimmune serum (G62). The major p25 gag and gp135 env viral proteins were identified in the culture medium of the four samples. The precursor gp170 env but not p55 gag was identified in cell lysates of the four samples. Samples from the other nine animals examined in this flock gave the same results (not shown). The absence of p55 could result from active viral replication and processing of viral proteins in macrophages. (b) Culture medium and cell lysate of cultured macrophages from a negative control sheep (1) and similar samples from macrophages infected in vitro with BL93 virus (2) were used as controls. A sample from one of the 65 animals negative for virus isolation from macrophage culture containing viral proteins in both the culture medium and cell lysate (3), but these proteins were not assembled into infectious virus particles. Positions of the major protein p25 gag, cleaved SU gp135 env and uncleaved precursor gp170 env are indicated. Positions of marker proteins are indicated.

Viral proteins were immunoprecipitated from cell lysates of all of the 79 (BL, DH1, DH2 and FL) sheep were studied by immunoprecipitation of virus-specific proteins using serum from a goat, G62, that had been inoculated numerous times with CAEV and MVV. Typical protein bands of ovine lentivirus were observed in both culture media and cell lysates of the macrophage cultures derived from the 14 BL animals (Fig. 1a, 5–8). However, minimal amounts of p55 Pr-gag precursors and p45 Pr-gag intermediate precursor were immunoprecipitated from the cell lysates (Fig. 1a). The high activity of virus replication in macrophages resulting in rapid maturation of viral proteins could explain the absence of accumulation of these viral proteins in the cytoplasm of infected macrophages. However, in a few samples biosynthesis of gp135-SU was minimal in both the culture and the cell lysate, as shown in Fig. 1a, lanes 7C and 7M. This could result from a lower level of replication of viruses which correlated with a lower amount of gp170 env and p25 gag, or inefficient processing of the precursor gp170 env. The difference in electrophoretic migration of gp135 observed between samples from different animals could result from a different level of glycosylation of the Env glycoprotein.
Fig. 2. PCR analysis on cellular DNA from sheep-farm animals. (a) Position of oligonucleotide primers used in PCR reactions. The structure of an ovine/caprine lentivirus genome, bordered by the two LTRs, is shown at the top. Coding sequences for structural gag, pol and env genes are represented as long open boxes. Positions of the beginning and the end of the env gene are reported. Positions of oligonucleotide primers are represented by arrows and the numbers are nucleotide positions in the sequence according to Saltarelli et al. (1990) for CAEV and Staskus et al. (1991) for MVV. Black bars indicate the PCR product, and numbers the size of this PCR product in base pairs. (b) PCR on DNA of cells infected in vitro and plasmid DNA using MVV-specific primers. Cellular DNA was isolated from macrophages infected in vitro with 10³ TCID₅₀ of MVV 1514 or 10³ TCID₅₀ of CAEV virus stock for each 35 mm dish; 0.5 μg of DNA isolated from these cultures was used for PCR and 5 μl (1/20) of PCR product was used for the nested-PCR. Reactions were performed as described in Methods. Ten μg of plasmid DNA was used for PCR and 5 μl of the PCR reaction was used for nested-PCR; 10 μl of nested-PCR reactions was loaded on to a 1.2% agarose gel. Lane 1, cells infected with MVV 1514; lane 2, cells infected with CAEV. Lane 3, pLV1-1LKS1 DNA; lane 4, pCAEV DNA. A 2.0 kb band (lane 1) and a 2.0-2.3 kb doublet (lane 3) resulting from bands amplified by PCR (2.3 kb) and nested-PCR (2.0 kb) are visible. Lanes M1 and M2, λ DNA digested with HindIII and a 1 kb ladder used as molecular mass markers. (c) PCR analysis of DNA from sheep-farm animals. Cellular DNA (0.5 μg) from each one of the 30 FL sheep (negative for virus isolation and virus proteins) was used to perform PCR; 5 μl of the resulting product was then used for the nested-PCR with the CAEV-specific primers (see a). Ten μl of nested PCR products was separated on a 1.2% agarose gel, transferred into nitrocellulose membrane then hybridized with a [³²P]dCTP labelled CAEV env probe. Numbers on the top from 1 to 30 identify different animals. Macrophages from a control animal negative for lentivirus infection and used as blood donor were either infected in vitro with CAEV, and DNA used as a positive control (lane 33), or DNA was isolated from non-infected cells (lane 32). A negative control for PCR without DNA is in lane 31.

and lysates from culture from only one FL animal (Fig. 1b, 3). The migration pattern of these proteins indicated cleaved forms of envelope gp135 env, capsid p25 gag and precursor gp170 env viral proteins. The doublet at 43 kDa probably derived from a cellular protein (observed in the non-infected samples also) and a second protein corresponding to the intermediate Gag precursor. Culture medium harvested from the other 64 macrophage cultures from 30 FL, 20 DH1 and 14
DH2 animals lacked virus-specific proteins in the culture medium and macrophage lysates (Fig. 1a, 1–4). No virus-specific bands were present in either the culture medium or cell lysate of a non-infected macrophage culture from a previously sampled lentivirus-free blood donor animal (Fig. 1b, 1). Viral proteins immunoprecipitated from macrophage cultures infected with the field virus isolate BL93 are shown in Fig. 1(b), 2 as controls for the virus-specific band patterns.

**DNA analysis by PCR**

To determine whether the PBMC-derived macrophages from the 64 sheep which did not produce infectious virus or viral proteins were completely free of viral infection, DNA from lysates of these cells was examined by PCR using both CAEV- and MVV-specific oligonucleotides (Fig. 2a). No positive signal was observed in any of the 64 samples when primers specific for MVV were used (data not shown). Control DNA from cells infected in vitro by MVV as well as pLV1 plasmid DNA (Fig. 2b, lanes 1 and 3) showed positive signals. DNA of CAEV-infected cells as well as pCAEV plasmid DNA (Fig. 2b, lanes 2 and 4) showed no positive signal when reacted with MVV-specific primers. Surprisingly, CAEV-specific primers detected a 1.3 kb specific band in samples from 10 of the 30 FL lambs (Fig. 2c). Hybridization with a 32P-labelled 3 kb env fragment derived from the CAEV genome confirmed that the 1.3 kb fragment detected in PCR products from 10 FL sheep was indeed viral DNA (Fig. 2c).

**Lack of virus-specific antibodies in sera from sheep positive for viral DNA**

The PCR results suggested that 10 out of 30 FL lambs were infected with a lentivirus, but no virus-specific proteins were detected in macrophages from these animals. We examined the serology of FL, DH1 and DH2 animals by immunoprecipitation. Sera from the 10 FL lambs that were positive for viral genome by PCR failed to detect any virus-specific protein (Fig. 3, 1–10). Sera from the 20 DH1, 14 DH2 and 20 remaining FL sheep that were negative for viral protein also failed to detect viral proteins by immunoprecipitation (data not shown). Contrasting with these results, the G62 serum, used as a positive control, detected the capsid p25 gag and envelope glycoproteins (Fig. 3, 11). These results suggested that sera from PCR-positive and PCR-negative animals do not contain virus-specific antibodies detectable by immunoprecipitation.

**Sensitivity of methods used for virus detection**

One possible explanation for the presence of viral DNA in cultures that lacked both virus and viral proteins is that very few infected macrophages were present in the dishes and that
Fig. 4. Immunoprecipitation of viral proteins from cells infected with various dilutions of BL93 virus stock. Cells were infected with 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} dilutions of BL93 virus stock as reported in the text. Infected cells were labelled with [^{35}S]methionine and viral proteins immunoprecipitated from culture media (M) and cell lysates (C). C-: control non-infected cells. Positions of specific viral proteins (gp170_{env}, gp135_{env} and p25_{gag}) are indicated. Positions of high molecular mass markers are indicated.

Fig. 5. PCR on DNA of cells infected with limiting dilution of BL93 virus stock. DNA (0.5 μg) from cells infected with a limiting dilution of BL93 virus stock (detail in the text and Fig. 4 legend) was used for PCR with CAEV-specific primers; 5 μl of the resulting PCR products was used for nested-PCR; 10 μl of the resulting products of nested-PCR was separated on a 1-2% agarose gel. M: DNA digested with HindIII used as molecular mass marker DNA. Lanes, 1, 2, 3 and 4 correspond to samples from cells infected with 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} dilutions of BL93 virus respectively. Lanes 5 and 6 correspond to non-infected cell DNA and DNA of cells infected with a 10^{-1} dilution of BL93 virus respectively. These two samples are used as negative and positive control respectively. The position and size of the specific viral band are indicated.
Fig. 6. For legend see p. 2048.
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Table 1. Percentage nucleotide identity in the \textit{env} gene of ovine and caprine lentivirus isolates as determined using PCGENE

<table>
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<tr>
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<th>CAEV-co</th>
<th>CAEV-63</th>
<th>Sh111</th>
<th>Sh8A</th>
<th>MVV</th>
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<tr>
<td>CAEV-co</td>
<td>100</td>
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<tr>
<td>CAEV-63</td>
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<td>84.9</td>
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<tr>
<td>Sh8A</td>
<td>86.2</td>
<td>84.9</td>
<td>99</td>
<td>100</td>
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<tr>
<td>MVV</td>
<td>70.8</td>
<td>70.2</td>
<td>72.2</td>
<td>74</td>
<td>100</td>
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From the highest dilution of the virus stock containing 1 to 10 TCID\textsubscript{50} when inoculated into 2 x 10\textsuperscript{8} cells, caused a productive infection in which viral DNA, proteins and CPE were all observed. Higher dilutions of the virus caused none of these effects. Therefore, conditions could not be created in cultured macrophages inoculated with a field virus to show the presence of viral DNA with exclusion of virus or viral protein production.

DNA sequence analysis

To determine whether the nucleotide sequences of PCR products amplified from DNA of the FL sheep were unique (distinct from CAEV and MVV), we cloned the 1.6 kb PCR fragment from two of the DNA-positive FL sheep into the pGEM-T vector. Inserts from two clones from each animal were sequenced and DNA sequences compared with both CAEVco and MVV \textit{env} sequences. The \textit{env} regions from both clones from the two sheep were highly identical (99\% nucleotide sequence identity) but were distinct from both CAEV and MVV genomes (Fig. 6). Interestingly, sequences from \textit{env} DNA of both FL sheep were found to share approximately 86\% identity with the CAEV \textit{env} gene but only 72.2\% identity with the MVV \textit{env} gene (Table 1, Fig. 6). The phylogenetic tree derived from these results shows clearly that \textit{env} DNA from clones Sh111 and Sh8A is closer to CAEV than VPP (Fig. 7). These data confirmed that the lentiviral DNA amplified by PCR from the naturally infected FL sheep is not only unique but is more closely related to CAEV than to MVV.

Discussion

This study on natural lentivirus infection in North American sheep has illustrated novel types of infection. Samples from 10 animals indicated the presence of viral DNA in macrophages whose cultivation failed to induce production of viral proteins. In another sample, viral proteins were identified but the naturally infected macrophages failed to produce infectious particles. This type of infection differs from earlier findings showing that virus production in 'latently' infected monocytes could be induced by maturation-differentiation of the cells \textit{in vitro} (Narayan \textit{et al.}, 1983b; Gogolewski \textit{et al.}, 1985; Kennedy \textit{et al.}, 1985). We show here that this virus-induction process was predictable in cells from 15 infected sheep but not in cells from 10 others. It is thus clear that healthy young sheep could be infected with a fully infectious lentivirus that is capable of productive virus infection, such as the agents obtained from the (as yet) clinically healthy animals in the disease-ridden BL flock, or with viruses that show no evidence of productive replication potential \textit{in vivo} or \textit{in vitro}. Whether the viral DNA in these cells was defective, latent or inducible by factors other than differentiation of monocytes to macrophages is not known. Sequences from the \textit{env} gene from macrophage cultures from two of these animals revealed
unique viral DNA with open reading frames encoding the SU and TM glycoproteins. Thus, the absence of virus replication could not be explained by structural mutations in the 1.6 kb of the env gene. It is of interest that whereas all of the BL sheep had antibodies to the virus, none of the FL animals that had unexpressed viral DNA in their differentiated macrophages had produced antiviral antibodies. Thus, other than the possibility that the animals had only recently become infected and did not yet have time to produce antiviral antibodies, we speculate that the viral DNA was not expressed in vivo.

The existence of non-productive lentivirus infection has been reported previously in studies on goats naturally infected with CAEV in California (Rimstad et al., 1993). Using longitudinal analyses, Rimstad et al. showed that six animals that were seronegative but had viral DNA in PBMC remained precisely at this stage of infection when examined 8 months later. A similar type of phenomenon has also been observed in a few HIV-infected people who failed to develop evidence of virus replication several months after viral DNA had been detected in PBMC by PCR (Horsburgh et al., 1989). The suggestion of unexpressed viral genomes in these studies closely parallels our finding of unexpressed viral genome in maturing cells. One possible technical explanation for our findings is that only a few cells among the PBMC were infected, and that the more sensitive PCR detected viral DNA, but that the less sensitive immunoprecipitation procedure failed to detect viral proteins. This was unlikely because supernatant fluids lacked infectivity. Further, a limiting dilution assay that was designed to examine the effect of inoculation with a single infectious unit into at least 2 x 10^6 macrophages failed to duplicate the type of infection observed in vivo: 10^6 cells inoculated with less than 10 TCID50 of field virus BL93 developed viral DNA, viral proteins and infectious virus 1 week after inoculation. In contrast, cultures inoculated with higher dilutions of the stock virus failed to develop any of these three parameters of infection. Thus, even at the lowest m.o.i. with a replication-competent field virus, we could not duplicate the natural phenomenon. Therefore, the question of defective virus replication in vivo as suggested in this study, as well as in the longitudinal studies described by Rimstad et al. remains moot. One possible explanation of this phenomenon is that at the neonatal stage of life the animals had ingested virus (in colostrum) and this caused a non-productive infection in a number of stem cells of the monocyte-macrophage lineage. Terminally differentiated progeny from such cells could conceivably contain unexpressed and inexpressible viral DNA.

A surprising result in our studies was that the viral DNA in sheep macrophages, whether of the expressible type as seen in the BL animals or the non-expressible type as seen in the FL animals, was more easily detected with primers specific to CAEV than to MVV genomes. DNA from these field viruses was shown to have higher sequence identity to that of CAEV (86%) than to MVV (72%). These data strongly suggest that CAEV or a CAEV-like virus may have been the source of infection in these animals. Two of the ten samples of unexpressed DNA were sequenced to ensure that they were not contaminants by CAEV and MVV clones that were present in our laboratory. The sequencing data clearly distinguished between these viruses, showing that the field virus genomes were unique. However, the data showed that the env gene sequences of the two DNA samples were highly identical. Possibly, the animals could have been infected with the same virus. Whether these two animals, or indeed all ten of the animals that had the non-expressed viral DNA had originated from the same flock before introduction into the feedlot could not be ascertained. This finding of CAEV-like DNA in PBMC from sheep is compatible with husbandry data, since goat milk is used frequently to supplement milk in poorly lactating ewes and/or is used to feed orphan lambs. Such milk could be a vector for infecting sheep with CAEV. A recent study on naturally ovine lentivirus-infected sheep in France also showed that sequences from PCR products in the pol gene had a higher identity to CAEV than MVV pol gene sequences (Leroux et al., 1995). These data and the data in this report suggest that sheep in Europe and in North America may be infected naturally with viruses that are genetically closer to CAEV than to the Icelandic strain of MVV. However, whether this phenomenon is more common in different parts in the world remains to be determined.

In summary, lentiviral DNA that was inexpressible in maturing macrophages was found in 10 of 30 FL sheep. While these data cannot claim to be reflective of the extent of lentivirus burden in the sheep population in this country, they clearly indicate a new concept of lentivirus infection in nature. Our findings agree with other reports on longitudinally studied goats which failed to seroconvert after several months (indicating no production of viral proteins) despite prolonged maintenance of viral DNA in blood. The high frequency of this phenomenon suggests that this type of infection may be common in the natural history of lentiviruses in animals of the affected species, and may even occur in cases where closely related viruses jump the species barrier, as suggested by the presence of CAEV-like viruses in our sheep. Presumably, such animals remain ‘infected’ for life without ever developing signs of disease.

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