Ovine lentivirus-infected macrophages mediate productive infection in cell types that are not susceptible to infection with cell-free virus

Dinesh K. Singh,† Yahia Chebloune, Leila Mselli-Lakhal, Bradley M. Karr and Opendra Narayan

1 Department of Microbiology, Marion Merrell Dow Laboratory of Viral Pathogenesis, Kansas University Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160-7424, USA
2 Laboratoire des lentivirus des petits ruminants, Institut National de la Recherche Agronomique (INRA), Ecole Nationale Vétérinaire de Lyon 1, Av. Bourgelat, B. P. 83 69280 Marcy l’Etoile, France

Ovine lentiviruses and caprine arthritis–encephalitis virus (CAEV) are prototypic lentiviruses that replicate predominantly in macrophages of infected animals. In situ hybridization of pathologically affected tissues from diseased animals has shown that viral RNA exists in permissive macrophages as well as in non-macrophage cell types that do not support productive virus replication. These findings raise questions about the cellular tropism of these viruses in vivo and how this may relate to their pathogenesis and the establishment of persistent infections. In this study, the susceptibility of macrophages and fibro-epithelial cells derived from goat synovial membrane (GSM) to infection by 14 North American ovine lentivirus strains was examined. All 14 strains were macrophage-tropic, as indicated by expression of viral proteins and by fusion and development of syncytial cytopathic effects following co-culture of infected macrophages with GSM cells. In contrast, neither viral DNA nor viral proteins was detected in GSM cells inoculated with cell-free virus from nine of the 14 strains. Specific virus proteins were immunoprecipitated from restrictive GSM cells following culture with infected macrophages and serial passage of GSM cells to remove the macrophages. The lack of infection of GSM cells by cell-free virus from some ovine lentivirus field strains was circumvented by cell-associated virus infection from infected macrophages to GSM cells following cell-to-cell contact. This strategy could be one of the mechanisms involved in the escape from immune surveillance and establishment of persistent infection in infected animals.

Introduction

Lentiviruses of small ruminant animals, exemplified by maedi–visna virus (MVV), originally isolated from sheep in Iceland (Sigurdsson, 1954), ovine progressive pneumonia virus, from sheep in the United States (Lairmore et al., 1987), and caprine arthritis–encephalitis virus (CAEV), from North American goats (Crawford et al., 1980; Narayan et al., 1980), are closely related and are prototypic of this genus of retroviruses. Typically, these viruses cause persistent infections and slowly progressive diseases that appear insidiously after incubation periods of more than 2 years in adult animals (Narayan et al., 1977; Sigurdsson, 1954). The ovine/caprine lentiviruses are strongly monocyte/macrophage-tropic and, to date, is the main cell type shown to support productive virus replication in infected animals (Gendelman et al., 1985; Gorrell et al., 1992; Narayan et al., 1983). During subclinical infection, virus replication is minimal and is detected only in macrophages. In contrast, animals that develop clinical disease show dramatic increases in virus burden and frequency of infected cells in blood. Viral RNA and/or DNA is readily detected by in situ hybridization in macrophages of pathologically affected tissues. The virus genome can also be detected in cell types other than macrophages in these tissues (Brodie et al., 1995; Haase et al., 1990; Staskus et al., 1991; Zink et al., 1990). The significance of the infection of non-macrophage cell types during the disease process is not clear.
These findings raised the paradoxical question of the apparently wider cellular host range of the viruses in tissues than in cells. Does the virus enter non-macrophage cell types in which viral RNA has been detected by a different pathway that circumvents the use of a specific receptor on these cells? Further, can infected macrophages, which are fully permissive for virus replication, mediate infection of non-macrophage cell types?

In the present study, we approached these questions by examining the susceptibility of macrophages and a non-macrophage fibro-epithelial cell type to infection with 14 ovine lentivirus strains isolated from North American sheep. Fibro-epithelial cells derived from goat synovial membrane (GSM) were used in this study for two reasons: (i) viral RNA was detected in epithelial cells in various affected tissues from diseased animals (Brodie et al., 1995; Zink et al. 1990) and (ii) these cells are generally susceptible to infection with both ovine and caprine virus strains and are commonly used for isolation and propagation of these viruses (Lairmore et al., 1988; Narayan et al., 1980).

Our results demonstrated that all 14 ovine field virus strains caused productive infection in inoculated sheep macrophage cultures or co-cultures of infected macrophages with GSM cells. In contrast, nine of these virus strains did not cause infection in GSM cells inoculated with cell-free viruses. We suggest that GSM cells lack functional specific receptors for some of the ovine lentivirus strains used in this study and that infected macrophages overcame this defect to cause infection in GSM cells by cell-associated virus following cell-to-cell contact. We propose that viral RNA detected in non-macrophage cell types of various affected tissues of diseased sheep and goats may be the result of macrophage-mediated infections of non-permissive cells by cell-associated virus. This mechanism, whereby cells lacking specific receptors for virus entry nevertheless become infected, may be important for the persistence of infection in the ecology of these viruses during the disease process and in transmission of these viruses to other animals within the species or to members of closely related species.

Methods

**Viruses.** Field strains of ovine lentivirus (FV1–FV14) were isolated from 14 Border Leicester sheep in a naturally infected flock in Maryland, USA. These strains were propagated in single co-cultures between peripheral blood mononuclear cell (PBMC)-derived macrophage cultures from these animals and PBMC-derived macrophages from a non-infected sheep. The Icelandic MVV strain 1514 (Petursson et al., 1976) was kindly provided by Neal Nathanson (John Hopkins University, Baltimore, USA) and was propagated in sheep choroid plexus cell cultures. CAEV strain CO (CAEvco; Narayan et al., 1980), originally obtained from an encephalitic goat, was passaged and propagated in GSM cells. These prototype strains of ovine/caprine lentivirus were used as controls for productive virus replication as indicated in the text.

**Cells.** Sheep PBMC were isolated from 50 ml blood collected in 2 mM EDTA from a non-infected sheep. Purification of PBMC by centrifugation through Ficoll–Hypaque gradients and cultivation in a macrophage differentiation medium has been described previously (Narayan et al., 1983).

Monocytes were differentiated into macrophages by cultivation at 37 °C in Teflon flasks for 1 week. Macrophages were then seeded into tissue culture dishes, where they became mature, adherent, esterase-positive phagocytic cells within 1 week. Non-adherent cells were washed away by rinsing the culture with serum-free medium prior to virus inoculation. Inoculated cells were examined routinely for degeneration and/or development of cytopathic effects (CPE). Portions of the inoculated macrophage cultures were co-cultivated with GSM cells and monitored for development of multinucleated giant cells. Parallel fractions of inoculated macrophages were used for the detection of virus-specific proteins by immunoprecipitation.

**GSM Cells.** GSM cells were obtained originally from an explant of carpal synovial membrane isolated from a colostrum-deprived newborn kid (Narayan et al., 1980). These cells were propagated by cultivation in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) and stored in liquid nitrogen. Typical monolayer cultures were propagated in MEM plus 10% FBS and confluent monolayers between passages 7 and 12 were used. Prior to inoculation with virus, cultures were rinsed with serum-free MEM and then inoculated with virus in medium containing 2% lamb serum. The inoculated cultures were routinely monitored for development of syncytial CPE 3 days post-inoculation (p.i.). Parallel cultures of inoculated cells were used for detection of the virus genome by PCR and virus-specific proteins by immunoprecipitation.

**CPE on macrophages and GSM cells.** To determine whether sheep macrophages and GSM cells were susceptible to infection and productive virus replication following inoculation with the 14 ovine field viruses, parallel cultures were inoculated at an m.o.i. of 0.1 or 1. Infected cells were maintained in culture for 1–3 weeks and the medium was changed every 3–4 days. One week after inoculation, macrophage cultures inoculated with each virus were co-cultured with indicator GSM cells and the co-cultures were observed for development of syncytial CPE and/or cytolysis as described previously (Chebloune et al., 1996a). GSM cultures inoculated with each virus were monitored for development of CPE for 1–2 weeks. Cultures not showing CPE were passaged after dissociation with trypsin, subcultured and observed for CPE development for an additional week.

**Immunoprecipitation.** Immunoprecipitation analysis of ovine/caprine lentivirus proteins using the hyperimmune serum of a goat inoculated with both CAEV and MVV (Ge2) has been described previously (Chebloune et al., 1996a). Briefly, macrophage and GSM cultures inoculated with each of the 14 viruses were incubated at 37 °C for 1–2 weeks. Cell monolayers were washed twice with serum-free MEM and incubated for 2 h in methionine/cysteine-free MEM. Proteins were radiolabelled by addition of 100 μCi/ml [35S]methionine/ [35S]cysteine (ICN) for 16–18 h. Virus-specific proteins released into the culture medium or present in the cell lyysate fraction were then immunoprecipitated with Ge2 serum. The immunoprecipitated proteins were separated by SDS–PAGE and then autoradiographed by standard methods.

**PCR amplification of proviral DNA.** PCR was used to determine whether the provirus genome was present in GSM cells inoculated with the nine field virus strains that failed to cause CPE or production of viral proteins. Cell monolayers were dissociated with trypsin and resuspended in MEM at a concentration of 2 × 10⁶ cells/ml. Approximately 2 × 10⁶
cells were lysed for PCR as described previously (Chebloune et al., 1996b). Two rounds of PCR amplification with oligonucleotide primers specific to the gag gene of CAEV were used to detect the virus genome. Primers GEX5 (5' GAAAGTGTGCTGCGAGACTTGGTTC 3') and GEX3 (5' TGGCTGTACCATGTTAAGCTTGC 3') correspond to bases 393–416 and the complement of 1268–1291, respectively, of CAEVco (Saltarelli et al., 1990). The first round of PCR was performed in a Perkin-Elmer thermocycler by initial DNA denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 92 °C for 1 min, annealing at 56 °C for 1.5 min and primer extension at 70 °C for 3 min. To increase the sensitivity of the DNA amplification, 5 µl PCR product was used as the template for a second round of amplification with primers GINS (5' GATAGAGACATGGCGAGGCAAGT 3') and GIN3 (5' GAGGGCA-TGTCGATTTGCTACTGT 3'), which correspond to bases 524–546 and the complement of 1013–1036, respectively, of CAEV gag. As an internal control for the integrity of the DNA lysates, oligonucleotide primers specific to the human β-actin gene were used (Joag et al., 1994). Following PCR amplification, 10 µl aliquots from each sample were separated by electrophoresis on a 1% agarose gel. DNA products were subsequently visualized by staining with ethidium bromide.

### Co-culture of virus-infected macrophages with GSM cells.

Primary cultures of sheep macrophages, grown in 7-96 cm² (3 cm diameter) culture dishes, were inoculated with field viruses at an m.o.i. of 0.1. At 1 week p.i., approximately 1 × 10⁵ fresh GSM cells in MEM plus 10% FBS were added to the infected macrophages. The macrophage–GSM co-cultures were maintained at 37 °C and monitored for development of CPE. Fusion of GSM cells occurred within 24 h of coculture. To separate co-cultured GSM cells from macrophages, cocultures were dissociated by trypsin action, which selectively dissociates the GSM cells, and then GSM cells were transferred to a new culture flask. Trypsin dissociation and passage of these GSM cells derived from the macrophage–GSM co-culture was repeated three times to ensure the purity of the GSM culture. After the third passage, expression of viral proteins in these cells was assayed by immunoprecipitation, as described above. In addition, culture medium from this passage of GSM cells was harvested and used to inoculate fresh macrophage and GSM cultures.

### Results

#### Sheep macrophages are permissive for replication of the 14 ovine lentivirus strains

The 14 field ovine lentivirus strains used in this study were isolated in the culture medium of monocyte-derived macrophage cultures from naturally infected sheep and were not passaged in *vitro*. Primary macrophage cultures from non-infected sheep were inoculated with the 14 ovine lentivirus strains. These monolayer cultures failed to develop CPE at 1 week p.i. but rapid fusion between the macrophages and GSM cells ensued after co-culture with indicator GSM cells. Inoculation of fresh macrophage cultures with cell-free medium, harvested from infected macrophage–GSM co-cultures, again resulted in fusion and CPE only after co-culture with GSM cells (data not shown). Immunoprecipitation of virus-specific proteins from inoculated macrophages revealed major viral proteins Gag p25 and Env gp135 in the culture media as well as Pr gp170 Env in the cell lysates (Fig. 1). The protein corresponding to Pr 55 Gag was not immunoprecipitated in this experiment. In fact, this precursor is rarely present in the cell lysates of infected macrophages, whereas it is commonly immunoprecipitated from cell lysates of infected GSM cells. Protein profiles from the 14 field virus strains were similar to those of the prototype strains of MVV and CAEV. These data demonstrated that all 14 virus strains replicated productively in macrophage cultures, thus confirming the macrophage-tropism of these viruses.

#### GSM cells support productive replication of some, but not all, field viruses

We next examined whether the 14 field virus strains were capable of infecting GSM cells and causing typical CPE. Interestingly, following virus inoculation at an m.o.i. of 0.1, GSM cells inoculated with nine of these viruses did not develop CPE or cell lysis, even after successive subculturing (data not shown). Immunoprecipitation analysis of the inoculated GSM cells resulted in the detection of virus-specific proteins exclusively from cells inoculated with five virus strains that induced CPE and not from cell cultures inoculated with the other nine strains (Fig. 2). To determine whether the early stages of the virus life-cycle were initiated in GSM cells inoculated with the viruses that did not induce CPE, we examined the inoculated cells by PCR for the presence of the virus genome at 48 h p.i. Since the genomic sequences of the field viruses are not known, we used oligonucleotide primers specific to the gag region of CAEV to detect viral DNA. We chose the gag region because it is highly conserved among the known ovine/caprine strains (Querat et al., 1990; Saltarelli et al., 1990). The use of these oligonucleotide primers on DNA samples isolated from macrophages infected by the 14 virus strains resulted in a specific 512 bp PCR product from all the samples (data not shown). The 512 bp gag-specific PCR product was amplified from GSM cells inoculated with lab-adapted CAEVco and FV3 used as positive controls but not from cells inoculated with the nine field viruses that failed to produce either CPE or viral proteins (Fig. 3). Thus, the absence of productive replication of the field virus strains in GSM cells correlated with the absence of both viral DNA and expression of virus-specific proteins in these cells. These results indicated that the resistance of GSM cells to infection with these viruses was most likely due to a defect at the level of specific entry into GSM cells.

#### Resistance of GSM cells to field virus infection can be circumvented by contact with infected macrophages

To determine whether the inability of these field viruses to infect GSM cells could be overcome by cell-to-cell contact with productively infected cells, macrophages inoculated with two of the nine viruses (FV12 and FV14) were co-cultured with non-infected GSM cells. GSM cells were separated from these co-cultures by dissociation with trypsin and subcultured three
Fig. 1. Immunoprecipitation of viral proteins from infected macrophage cultures. Monolayer cultures of sheep macrophages were inoculated at an m.o.i. of 0–1 with various ovine virus strains and the laboratory strains of CAEV and MVV. At 1 week p.i., proteins produced by infected macrophages were labelled with $[^{35}S]$methionine/$[^{35}S]$cysteine and virus-specific proteins were immunoprecipitated from culture medium and cell lysates by using a goat hyperimmune serum (G62) (Chebloune et al., 1996a). M, culture medium; C, cell lysate; NI, non-infected macrophages. Names of the various virus strains are indicated above the respective lanes. The field viruses FV6–FV11 and FV13 are not included in this figure. Positions and sizes (in kDa) of molecular mass markers are shown on the left and positions of the major viral Gag protein p25 and Env gp135 and the precursor Pr gp170 Env are indicated on the right.

Fig. 2. Immunoprecipitation of viral proteins from GSM cells. GSM cultures were inoculated at an m.o.i. of 0–1 with the various virus strains and at 1 week p.i., cell monolayers were starved of methionine and cysteine and radiolabelled. After labelling of proteins with $[^{35}S]$methionine/$[^{35}S]$cysteine, virus-specific proteins were immunoprecipitated with the G62 serum from culture medium (M) and cell lysate (C) of GSM cells. NI, Non-infected GSM cells. Shown in this figure are five (FV2, FV5, FV12, FV14 and FV1) of the nine field viruses that did not induce CPE and three (FV8, FV4 and FV7) of the viruses that replicated in GSM cells. CAEVco and the Icelandic MVV strain K1514 were included as references. Sizes (in kDa) and positions of the molecular mass markers are shown on the left and viral proteins Gag p25, Env gp135 and Pr gp170 Env are indicated on the right.
times to ensure that the resulting cultures contained mainly GSM cells. Differentiated macrophages were selectively removed due to their resistance to trypsin dissociation. Interestingly, GSM cells separated from these co-cultures continued to develop typical syncytial CPE (data not shown). Immunoprecipitation analysis of proteins from these cells demonstrated the expression of viral proteins in both culture media and cell lysates (Fig. 4). This result suggested that the virus particles were introduced into GSM cells following cell-to-cell contact with the infected macrophages and the virus life-cycle then continued, leading to expression of viral proteins in GSM cells.

**Progeny virus from restrictive GSM cells is macrophage- but not GSM-tropic**

To determine whether infectious virus was produced by GSM cells obtained from the infected macrophage–GSM co-cultures, we inoculated culture medium from third passage GSM cells onto fresh cultures of macrophages and GSM cells. Inoculation of macrophage cultures with the culture medium from FV12- and FV14-infected macrophage–GSM-passaged cells resulted in productive virus replication, as indicated by expression of viral proteins (Fig. 5) and development of typical CPE after co-culture of the infected macrophages with GSM cells (data not shown). In contrast, GSM cells inoculated with culture medium from FV12- and FV14-infected macrophage–GSM-passaged cells did not develop any CPE or expression of viral proteins and the virus genome was not detected (data not shown). Titration of progeny virus stocks was performed by inoculating macrophage monolayers with various dilutions of these viruses, co-culturing with GSM cells at day 7 p.i. and observing CPE development 24 h later. Titres of $10^4$–10^5 TCID_{50}/ml were obtained for both viruses in the supernatants harvested from day 3 to day 7 p.i. These results indicated that virus particles produced by GSM cells after introduction of virus genomes by macrophages were capable of infection and...
productive replication in macrophages but not in GSM cells. Virus titres produced from the restrictive GSM cells following co-culture with infected macrophages were similar to those obtained from macrophages \((5 \times 10^3–10^6 \text{ TCID}_{50}/\text{ml})\). Titration of these viruses was done on macrophages, which were co-cultured with the indicator GSM cells at 6–7 days p.i.

**Discussion**

This report illustrates that lentivirus-infected macrophages, the main host cell of the virus in infected animals, can extend the host range of the virus by mediating infection of cells that are capable of supporting virus replication but which lack specific receptors for the viruses. This phenomenon may be relevant to the pathogenesis and epizootiology of these viruses. The use of synovial cells of goats to extend the host range of these field viruses from sheep infers that the phenomenon may even be important not only in transmission of the viruses from infected mothers to their offspring but also in cross-species transmission of the agents from goats to sheep and vice versa.

The data show clearly that all 14 North American ovine lentivirus strains tested in this report replicated efficiently in primary macrophage cultures. Earlier studies had shown that latent virus genomes in PBMC and cells in lymphoid tissues could be activated by induction of maturation of monocytes to macrophages (Gendelman et al., 1986; Narayan et al., 1983). Whether the virus-producing macrophages were indeed derived from latently infected monocytes or from some other type of latently infected cells such as dendritic cells is not known (Gorrell et al., 1992). By using experimental allergic encephalitis induction in sheep, we recently demonstrated that macrophages are the main cell type involved in virus invasion and viral protein expression in the nervous system (Chebloune et al., 1998). However, the results of the experiments described here demonstrate that macrophages were capable not only of transmitting virus to other cell types that were resistant to cell-free virus infection, but also of rescuing and amplifying virus from such cells.

These results demonstrated that GSM cells were permissive for expression of the genome of all virus strains tested but that cell-free virus inoculation with some strains did not result in infection of GSM cells. This restriction of virus replication could result from genetic modifications of the Env gp135 glycoprotein of these virus strains, leading to incomplete interaction with the GSM surface receptors and thus preventing internalization of virus particles. The fact that co-culture of GSM cells with infected macrophages, but not inoculation with cell-free viruses, resulted in productive infection of these cells emphasizes the fusogenic potential of the infected macrophages, which probably expressed the viral envelope glycoprotein gp41 on the cell surface. The restriction of infection of GSM cells by cell-free virus of some of the field virus strains was apparently circumvented following fusion between infected macrophages and GSM cells. This function of infected macrophages, using non-permissive cells as a reservoir for the viral genome and possibly rescuing the genome later for active virus replication, may be one mechanism of virus persistence in the host.

The ability of infected macrophages to transmit virus to heterologous cells may help to explain the presence of the virus genome in non-macrophage cell types in infected animals. Viral DNA and/or viral RNA have been identified in ependymal cells in brain (Staskus et al., 1991), epithelial cells in lungs with interstitial pneumonia (Brodie et al., 1995), synoviocytes in arthritic joints and glandular epithelium in infected mammary glands (Zink et al., 1990). The common link between these various pathologic manifestations of infection is the presence of virus-producing macrophages within the tissues. Whether the phenomenon is relevant to transmission of virus is open to speculation. However, it is known that infected macrophages in milk are the source of virus for nursing animals (Kennedy-Stoskopf et al., 1985; Lerondelle et al., 1989).

More recently, we demonstrated that ex vivo culture of milk epithelial cells from a naturally infected goat generated a persistently infected epithelial cell culture, while cultures from one infected and two non-infected goats were not infected. In vitro inoculation of these cell cultures with two CAEV strains resulted in a very high level of virus replication (Mselli-Lakhal et al., 1999). One can suppose that ingestion of infected macrophages by newborns could transmit the virus to intestinal epithelial or dendritic cells, leading to infection of macrophage populations in the new host. Such cell-to-cell transmissions could explain both maternal transmission of virus within the species (sheep to sheep and goat to goat) and also the transmission of virus from goats to sheep. This monotypism (macrophage-tropic virus) and dual-tropism (macrophage- and GSM-tropic virus) mimics the well-known human immunodeficiency virus type 1 (HIV-1) system. Indeed, most of the HIV-1 strains isolated from newly infected patients or during the long asymptomatic phase are macrophage-tropic (M-tropic) (Schuitemaker et al., 1991; Zhu et al., 1993). In contrast, viruses isolated from patients during the disease phase are mainly T lymphocyte-tropic (T-tropic) and dual-tropic viruses (Koot et al., 1993; Tersmette et al., 1989). While the T-tropic strains frequently induce syncytium formation in infected lymphocytes (SI strains), M-tropic strains do not (NSI strains). The recent identification of chemokine receptors (CCR5, fusin and CXCR4) as co-receptors with the primary CD4 molecule for HIV-1 (Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996) and their selective expression in T cells and macrophages has helped to demonstrate that HIV-1 tropism is related to the co-receptor usage of the various strains. Whether ovine and caprine lentivirus infections also require both a primary receptor and a co-receptor and whether a strategy similar to the HIV-1 system is used to establish macrophage and GSM tropism is still unknown.
This work was supported by Public Health Service Grant NS-12127 from the National Institutes of Health. We thank the Institut National de la Recherche Agronomique (INRA) and the Direction Generale de l’Enseignement et de la Recherche (DGER) for grant support. We also thank NARP II, DARE and the Indian Veterinary Research Institute (IVRI) for partial financial support of D.K.S.

References


Host-cell tropism of ovine lentivirus


Received 21 December 1998; Accepted 26 February 1999