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Slow, persistent replication of lentiviruses: Role of tissue macrophages and macrophage precursors in bone marrow

(visna virus/*in situ* hybridization/immunocytochemistry/macrophage infection/viral pathogenesis)

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ABSTRACT **Lentiviruses, as exemplified by visna virus of sheep, are nononcogenic retroviruses that cause slowly progressive diseases after prolonged periods of incubation. Earlier studies on visna have shown that the long incubation period of the disease is associated with constant production of minimal quantities of virus in tissues, whereas virus could be obtained by culturing monocytes and macrophages from explants of lymphatic tissues and inflamed organs. In this study the role of macrophages in lentivirus infection was explored using two sheep that were intrabronchially inoculated with virus. When sections of paraffin-embedded tissue, processed by a recently described technique which combines immunocytochemistry for the identification of macrophages and *in situ* hybridization for identification of viral nucleic acid, were examined, we found that virus replication is associated almost exclusively with infection in selected populations of macrophages in the interalveolar region of the alveoli, in inflammatory exudate cells in the lung, in lymph nodes, and in the spleen. Although large numbers of alveolar macrophages had viral RNA, few of these cells produced virus. While this minimally productive type of viral replication provides an explanation for the slow pace of the infection, restricted replication in terminally differentiated, short-lived macrophages does not explain persistent virus replication in the animal. With the discovery of clusters of infected macrophage precursors in the bone marrow, a mechanism for persistence was found. The macrophage precursor cells provide an important missing link in the virus–target-cell circuit and may be the reservoir of latently infected cells which perpetuate lentivirus infections in both animals and humans.**

Lentiviruses are nononcogenic retroviruses which cause slowly progressive diseases after unusually long periods of subclinical infection. Visna/maedi virus of sheep and caprine arthritis encephalitis virus of goats are the prototypes of this group of agents that cause paralysis, pneumonia, and arthritis months to years after initial infection (1). The nononcogenic retrovirus associated with acquired immune deficiency syndrome, AIDS, in humans shares genetic sequences with visna virus (2), has biologic properties similar to lentiviruses in cell culture (3), and shows parallels in virus–host interactions (4). These findings have stimulated interest in the mechanisms by which lentiviruses cause disease.

Lentivirus infection involves a virus–host interaction in which host defenses fail to eliminate the agent but do restrict virus replication to low levels such that small quantities of virus are produced. Studies in lentivirus-infected sheep have shown that the slow rate of virus replication is not determined by the age, the immunological status of the animal, or the immune responses to the virus (5–7). Host cell restriction of viral expression has been proposed as the mechanism for the slow rate of replication (8). Monocyte/macrophages were the major cell type infected in the animal (9, 10), and examination of monocytes in culture demonstrated a latent infection activated when the monocytes matured into macrophages (11). These *in vitro* studies suggested that viral gene expression requires maturation of infected cells.

We have investigated the mechanism of the lentivirus restriction in sheep by directly evaluating viral replication in cells and tissues from infected animals, using infectious center assays for isolated cells and a technique in which immunocytochemical identification of macrophages and *in situ* hybridization of viral nucleic acid are performed sequentially on the same tissue section (12, 13). Our experiments suggest that infection of cells in the macrophage lineage may be responsible for both viral persistence and "slow" replication in the animal.

MATERIALS AND METHODS

Virus. Lentivirus VMA5 was isolated in Idaho from a sheep with inflammatory lesions in the CNS, lungs, and joints, and is typical of viruses seen in cases of visna-, maedi-, and lentivirus-induced arthritis (14). The virus was cultivated in a cell line of sheep alveolar macrophages transformed by simian virus 40 (15). Supernatant fluids from these infected cultures had an ID_{50} (tissue culture) of 5×10^5/ml.

Infection of Sheep and Preparation of Infected Sheep Cells and Tissue. Two 3-month-old Corriedale lambs were anesthetized with halothane and 20 ml of viral fluid was deposited in the right anterior lobe of the lung by using fiber-optic endoscopy. At 2- to 3-week intervals endoscopy was repeated, and cells were lavaged from the lobe in Hanks' salt solution. Some lavaged cells were sedimented on pretreated glass slides using a cytocentrifuge, then fixed in periodate/lysine/paraformaldehyde/glutaraldehyde (16), processed for immunocytochemistry, and *in situ* hybridization. Other cells were processed for the infectious center assays.

The animals were killed by anesthetic overdose at either 6 or 16 weeks after inoculation. Each was perfused through the heart with 10 liters of phosphate-buffered saline (P_i/NaCl) followed by 25 liters of periodate/lysine/paraformaldehyde/glutaraldehyde fixative (16). Small pieces of tissue were embedded in paraffin, and serial sections were cut and placed onto pretreated glass slides for routine histological staining, immunocytochemistry, *in situ* hybridization, and combined immunocytochemistry and *in situ* hybridization (13, 17).

Infectious Center Assays. The assay was performed on lavaged cells as described (11).

Immunocytochemistry. Antibodies to sheep alveolar macrophages were prepared in rabbits. These antibodies bound specifically to monocytes and selected populations of mac-

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rophages in sections of fixed tissues from normal sheep (15). Cells or tissue sections were incubated with the IgG fraction of the anti-macrophage serum followed by incubation with avidin–biotin-coupled peroxidase complex (Vector Laboratories, Burlingame, CA) and visualized with 3,3'-diaminobenzidine tetrahydrochloride (18).

***In Situ* Hybridization.** Cloned DNA of visna virus (19) and measles virus (20) were used in these studies. The cloned visna virus DNA was cleaved with the restriction endonuclease *Sst* I and the visna virus fragment (9.1-kilobase) representing more than 90% of the viral genome was separated from pBR322 by electrophoresis in a low-melting-point agarose gel and subsequently purified from the agarose (21). The measles virus DNA probe, clone N, is complementary to the mRNA coding for the nucleocapsid protein. The DNA probes were radiolabeled with deoxyadenosine 5'-(α-[^{35}S]thio)triphosphate and deoxycytidine 5'-(α-[^{35}S]thio)triphosphate (Amersham; specific activity $>5 \times 10^8$ cpm per μg DNA) by nick-translation (22) modified as described (17) to yield probes 50 to 80 base pairs long. Labeled DNA was added at a concentration of 0.2 μg/ml in 5 μl to cytocentrifuged preparations or tissue sections which had first been treated with acid, heat, and proteinase K as reported (13). This was followed by an acetylation step (23) to further reduce nonspecific binding of the probe. The slides were incubated for 50 hr at room temperature, washed extensively, and autoradiographed 1–10 days. Silver grains in cells indicated the presence of viral RNA. The controls for specificity of hybridization were absence of grain development when the visna probe was applied to uninfected cultures or tissue sections and when the measles probe was applied to sections of visna virus-infected tissue. The specificity of the measles probe was shown in *in situ* hybridization experiments on measles virus-infected cell cultures and sections of measles virus-infected mouse brain (17).

For detection of viral DNA, the sections were acetylated and then treated with 200 μl of RNase at 100 μg/ml. Probe was placed onto the specimens which were then heated in sealed plastic containers for 6 min at 90°C to denature the DNA. The slides were cooled to 4°C and then allowed to hybridize at 22°C for 50 hr. Control experiments were the application of the probe to RNase-treated specimens without the denaturation step. Therefore, grain development following RNase and heat treatments was interpreted as hybridization of the probe to visna viral DNA.

Combined Immunocytochemistry and *in Situ* Hybridization. Preparations were first processed for immunocytochemistry and then for *in situ* hybridization (13). Our previous studies showed that specimens processed for immunocytochemistry had reduced *in situ* hybridization. Consequently, this procedure was used to qualitatively identify macrophages containing viral nucleic acid. To accurately quantitate the number of viral genomes per cell, another set of specimens were processed at the same time for *in situ* hybridization only. The combined procedure was used because it provided unequivocal identification of the type of infected cells in tissues and determined whether viral DNA or RNA was present in these cells.

Determination of Sensitivity of the *in Situ* Hybridization Procedure for Detecting Viral RNA. Cell cultures were infected with virus at a multiplicity of 1. At intervals of 12, 24, 48, 72, 96, and 120 hr after inoculation, cells were harvested for *in situ* hybridization and preparation of cellular RNA. For the latter procedure, the cells were solubilized in guanidine hydrochloride, and the RNA was purified as described (24) and then quantitated by dot blot hybridization (25, 26). Purified viral genome RNA was used as a standard. Nick-translated, cloned viral [^{32}P]DNA (specific activity 1×10^8 cpm/μg DNA) was the probe. The numbers of copies of viral RNA per cell were based on a genome of 1×10^4 bases for the viral RNA and previously published values for the amount of RNA per cell (27). Comparison of the two hybridization procedures in samples showed a linear relationship between the number of copies of viral RNA per cell and the number of grains obtained by *in situ* hybridization (Fig. 1). Using the 24-hr sample (see above), we determined that the latter technique was capable of detecting approximately 10 copies of viral RNA per grain above background after 10 days of autoradiographic exposure.

RESULTS

Previous studies on replication of lentiviruses in sheep and goats have shown that cell free homogenates of tissues rarely contained virus yet virus could always be recovered from explants of tissue (5) and from monocytes in blood (11). Brahic *et al.* (28) extended these observations showing that in sheep inoculated intracerebrally, replication of visna virus in cells of the choroid plexus was restricted at the level of transcription. This abnormal route of inoculation, however, precluded evaluation of the natural course of infection. Consequently, in this study sheep were inoculated via a natural route of infection and the course of the infection as spread from the lung to cause persistent systemic infection was followed.

Role of Alveolar Macrophages in Infection. Using fiber optic endoscopy, we lavaged the same lobe of the lung that had been inoculated. This procedure routinely yielded about 1×10^6 cells of which 70–90% stained with the anti-macrophage serum (Table 1). These cells represented macrophages lining the alveolar spaces. The combined labeling technique showed that virus-specific RNA was found exclusively in these alveolar macrophages and was not found in the epithelial cells also obtained in the lavage fluids. The concentration of viral RNA ranged from 50 to >1000 copies per cell 2–3 weeks after inoculation, but virus could not be obtained from these cells by 6 weeks after inoculation. Since the presence of viral RNA in the macrophages could have resulted either from phagocytosis of virions or from viral replication, we performed hybridization experiments to determine whether viral DNA, an indicator of viral replication, was present in the macrophages. Hybridization after RNase and heat treatment showed grains indicating the presence of viral DNA. Thus, alveolar macrophages were hosts for viral replication.

Comparison of *in situ* hybridization data with corresponding infectious center assays for each batch of lavaged cells showed that ≈1% of the cells that had viral RNA produced virus (Table 1). Furthermore, cultivation of the cells con-

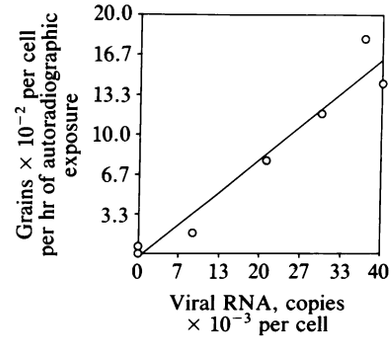

FIG. 1. Quantitation of viral RNA by *in situ* hybridization. Linear plot of the relationship between RNA copy numbers and grain counts determined by dot blot hybridization and *in situ* hybridization respectively (R = 0.97). The correlations were obtained from different batches of cell cultures which were harvested at 12, 24, 48, 72, 96, and 120 hr after infection (left to right in figure). All autoradiographs were exposed for 1–10 days.

Table 1. Viral infection in alveolar cells lavaged from infected sheep

	2 weeks	4 weeks	16 weeks
Percent macrophages among lavaged cells*	90	75	92
Percent macrophages with visna viral RNA in lavaged cells*	15	0.1	0
Percent infectious centers in lavaged cells†	0.16	0.003	—
Percent RNA containing cells identified as macrophages*	100	100	—

Approximately 1×10^6 cells were lavaged from the inoculated lobe of lung of two sheep at indicated periods after inoculation except at 16 weeks when one sheep was done.
*Examined by combined immunocytochemistry and *in situ* hybridization.
†Examined by infectious center assay.

taining viral RNA for 3–5 days did not result in production of virus. These experiments indicated that either little virus was produced by cells containing viral RNA or the virus isolation technique was relatively insensitive. To determine the sensitivity of the infectious center assay procedure, we inoculated cultures of simian virus 40-transformed sheep alveolar macrophages with lentivirus at a multiplicity of 5 and harvested the cells for assay 16 hr later, when viral RNA was abundant but virions had not yet been assembled. More than 90% of the cells were infected (data not shown). It is clear, therefore, that the technique was sufficiently sensitive to detect permissively infected macrophages in the early stages of virus replication. Thus, the inability of the lavaged alveolar macrophages to produce virus despite the presence of large amounts of viral RNA must have been due to some posttranscriptional block in virion synthesis.

Infection in the alveolar macrophages disappeared with time, but by then the infection had spread to pulmonary macrophage populations in between the alveoli. These cells were not amenable to evaluation by the infectious center assay. However, since tissues from infected sheep usually contain minimal amounts of cell-free virus (5, 9, 11), we assumed that viral replication in tissue might be similar to that in alveolar macrophages. Other tissue sections were, therefore, examined to determine whether those macrophages were infected and whether the infection was confined to macrophages.

Virus Replication in Visceral Organs. Examination of tissue from the two sheep showed cells with viral RNA and DNA in the lung, lymph nodes, and spleen, but not in liver, kidneys, connective tissue, or muscle. Application of the combined labeling technique showed that viral RNA was confined almost exclusively to macrophage populations. In the lung, only small numbers of alveolar macrophages, and no epithelial cells, had RNA (Fig. 2 *A* and *B*). This confirmed results of the hybridization experiments on lavaged cells described above. Approximately 60% of the viral RNA in the lung was located in morphologically identifiable pulmonary macrophages in the interstitial spaces between alveoli. Small accumulations of mononuclear inflammatory cells had formed around bronchi and pulmonary blood vessels by 16 weeks after inoculation. Application of the combined labeling technique to these areas showed that a small number of the macrophages (≈1%) had viral RNA. The mononuclear cells in the inflammatory cuffs, which did not stain with the anti-macrophage antibodies, did not have viral RNA. This lack of infection in lymphocytes has been confirmed in ovine-peripheral-blood leukocyte cultures inoculated with visna virus (unpublished observations).

Widespread infection in macrophages (antibody-labeled cells containing viral RNA and DNA) was also found in the mediastinal lymph nodes draining the lung (Fig. 2*C*) and the spleen (Fig. 2*D*) and to a much lesser extent in the distant mesenteric lymph nodes. Despite this, ≈90% of the cells which had grains also had the macrophage marker. The mediastinal lymph node had undergone massive hyperplasia (≈15-fold normal size) by 16 weeks and large numbers of lymphocytic germinal centers were evident. In both this lymph node and the spleen, the infected macrophages were distributed either singly or in small groups in the sinusoidal areas and were distributed prominently in the marginal zones around the uninfected germinal centers (Fig. 2*D*).

Virus Replication in Bone Marrow. The inefficiency of lavaged alveolar macrophages, and possibly tissue macrophages, to package viral RNA into virions is compatible with both "slow" replication and the lack of infectivity in cell-free tissue suspensions. However, the relatively short life span of tissue macrophages and the apparently abortive infection in most alveolar macrophages suggests that infection in this cell type alone was not enough to maintain persistent infection in the animal. Further, since previous studies had shown infection in monocytes, we examined bone marrow for infection of the monocyte/macrophage precursors. Examination of sections dissected from the femur showed infection in cells in the bone marrow. *In situ* hybridization showed clusters of infected cells distributed at intervals (1–5 mm). In fields with infection, as many as 2% of the total cells had viral RNA, 100–500 copies per cell (Fig. 2 *D* and *F*). Application of the combined labeling technique showed that >90% of the cells containing viral RNA also had the macrophage antigen, indicating an almost exclusive infection of monocyte/macrophage precursors.

DISCUSSION

We used a double labeling method to identify virus target cells in lentivirus-infected sheep and showed that cells of the macrophage lineage were the main cell type supporting viral replication in the animal. Therefore, the phenomena of persistent infection *in vivo* and the unusual type of "slow" virus replication in sheep are associated with infection of the macrophage lineage. Slow or restricted viral replication is explained best as a posttranscriptional block of replication in lavaged alveolar macrophages. Similar findings have been reported recently by Geballe *et al.* (10). Although alveolar macrophages are not important for maintaining infection,

FIG. 2 (*on opposite page*). Visna virus–macrophage interactions. Tissues, from an animal 16 weeks after infection fixed in periodate/lysine/paraformaldehyde/glutaraldehyde, were embedded in paraffin and processed for immunocytochemistry with antibody against sheep macrophages. (*A–E*) Tissue sections were hybridized with the visna viral DNA probe and in (*F*) with the measles virus DNA probe. All were counter stained with hematoxylin. (*A*) Section of lung showing labeled cells with viral RNA (curved arrow); labeled cells without viral RNA (point) and unlabeled pulmonary macrophages with viral RNA (straight arrows). (×150.) (*B*) Section of the inoculated lung showing viral RNA in alveolar macrophages (×300.) (*C*) Immunologically labeled macrophages showing viral RNA in a peri-germinal center in the mediastinal lymph node. (×375.) (*D*) Section of spleen showing cells containing viral RNA distributed around but not in the germinal center (straight arrows). (×50.) (*E*) A cluster of macrophage precursor cells in the bone marrow with visna viral RNA. (×375.) (*F*) A section of bone marrow immunocytochemically labeled with the anti-macrophage serum and hybridized with the labeled measles virus DNA probe showing specifically labeled promonocytes without grains.

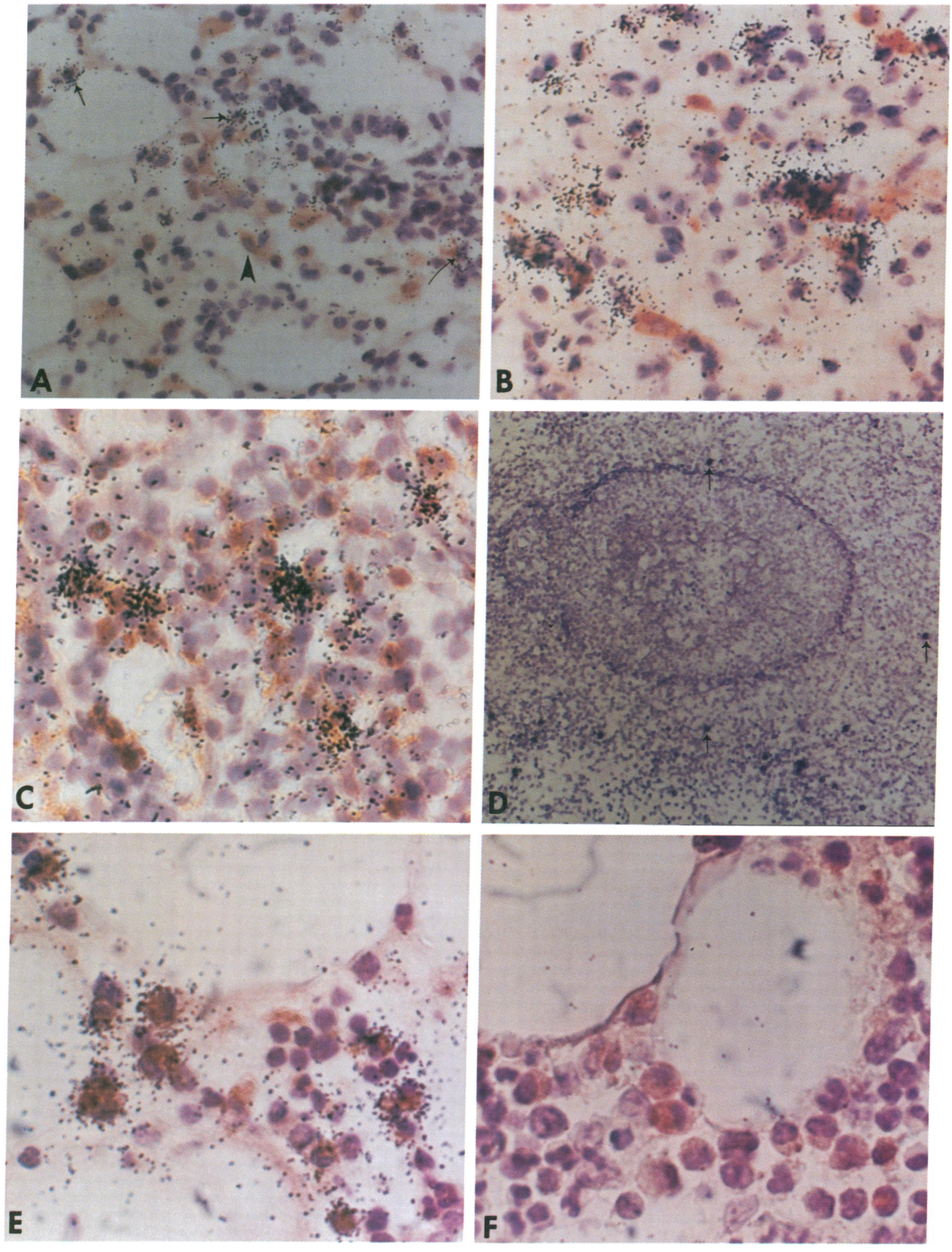

FIG. 2. *(Legend appears at the bottom of the opposite page.)*

similar restricted replication may occur in tissue macrophages because they contain many copies of viral RNA that are not processed into infectious particles. The mechanism of this type of replication remains to be determined. When cultured simian virus 40-transformed alveolar macrophages were inoculated with lentivirus, viral replication was more permissive. Whether transformation of these cells with simian virus 40 made them more permissive for the replication of lentiviruses is not known.

The lack of a permissive system for virus replication in tissue cells and the fact that terminally differentiated macrophages were the only infected cells in tissue strongly suggested the existence of a viral source not previously described. The infected macrophage precursors in the bone marrow satisfied this requirement. Infection in these cells may have been initiated by blood-borne, infected monocyte/macrophages, but the development of clusters of infected cells could have resulted only from mitosis of the infected precursor cells or from a burst of replication in a few cells with spread of the virus to contiguous cells. Since homogenates of bone marrow from infected sheep have little virus, it seems that the clusters might have originated from mitosis of latently infected cells (11).

Bone marrow was not considered to be important in the pathogenesis of these infections because, in contrast to the lymphadenopathy and severe inflammatory lesions in target organs (CNS, lungs, joints, and mammary glands) of animals with the disease no histologic abnormalities develop in bone marrow (1). It is thus intriguing that the bone marrow could be a reservoir of infected cells that do not become involved in pathologic processes until they leave the bone marrow and differentiate into tissue macrophages. Since infected macrophages were found in spleen, lymph nodes, and lung but not in connective tissues and liver, the infected bone marrow cells might have been committed to become macrophages in these affected tissues. Alternatively, macrophages in uninfected tissues may have shed the viral genome during maturation. It is also possible that expression of the viral genome may have been restricted by tissue-specific factors during the macrophage differentiation process, since tissue-dependent expression of immunoglobulin genes (29) and certain viral genes has been observed (30).

Despite the identity of viral target cells in these sheep we do not yet have a clear understanding of the mechanism underlying the relationship between lentivirus infection in macrophages and the lymphadenopathy and inflammatory lesions associated with lentivirus infections. While the lesions in the neuropil are thought to be "spill over" of virus from monocytes to neural cells (31), interstitial pneumonia, synovitis, and lymphocytic hyperplasia may be due to macrophage infection and concurrent disturbances in regulation of immune cells. Moreover, since the human retrovirus infection, AIDS, is also characterized by infection of the immune cells, lymphadenopathy, and involvement of the CNS (4), virus–host cell interaction similar to that seen in the animal infection may be at play, with mature cells of the immune system being involved in pathologic processes and a reservoir of latently infected cells being maintained in the bone marrow.

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