JOURNAL OF VIROLOGY, July 1986, p. 37–44
0022-538X/86/070037-08$02.00/0

Vol. 59, No. 1

Neutralizing Antibodies to Visna Lentivirus: Mechanism of Action and Possible Role in Virus Persistence

SUZANNE KENNEDY-STOSKOPF[1] AND OPENDRA NARAYAN[1,2]*

Division of Comparative Medicine[1] and Department of Neurology,[2] The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Received 5 December 1985/Accepted 7 April 1986

Lentiviruses are nononcogenic retroviruses that cause persistent infections and slowly progressive diseases. Visna virus, a lentivirus of sheep, persists in cells of the macrophage lineage despite the presence of neutralizing antibodies in the animal. These antibodies are measured by prevention of virus replication in sheep fibroblast cell cultures. In this study we have compared the antiviral properties of the antibodies in sheep fibroblast and macrophage cell cultures, the latter being more relevant to infection in the animal. Using infectivity assays, binding of radiolabeled virus to cell membranes, cellular processing of labeled virus into acid-precipitable and acid-soluble components, and in situ hybridization of viral nucleic acid, we show that the antibodies prevented virus replication in both fibroblasts and macrophages. However, the site of neutralization differed between the two cell types. In fibroblasts, the site of virus neutralization was at the cell membrane, when the antibodies prevented virus attachment. In macrophages, virus incubated with the antibodies was phagocytized rapidly, followed by uncoating of the virions. However, virus RNA was not transcribed. Despite this ability of the antibodies to abort virus replication in macrophages, the kinetics of binding of the antibodies to the virus was much slower than the binding of virus to the macrophages. Therefore, persistent virus replication in immune sheep may be the result of virus spreading from macrophage to macrophage before the agent can be neutralized by antibodies in the plasma.

Lentiviruses are nononcogenic, exogenous retroviruses which cause visna (wasting) and maedi (dyspnea) in sheep (19, 42, 43), arthritis-encephalitis in goats (6, 7), infectious anemia in horses (3, 4), and acquired immune deficiency syndrome in humans (12, 16). These diseases are characterized by long incubation periods, insidious onset, and slowly progressive clinical courses. Infection is persistent, but only a few hosts ever develop clinical disease (9, 10). Replication of the virus is restricted in the host (21), and pathologic responses are immune mediated (2, 8, 37) rather than caused by the direct effects of the virus.

Visna virus causes a productive, lytic infection in cultures of sheep choroid plexus (SCP) fibroblasts (44, 45). In previously reported studies, we used these cells as indicators for measuring infectivity and specific neutralizing antibodies in studies of infection in sheep (17). These studies showed that the animals produced small amounts of virus neutralizing antibodies 4 weeks or more after inoculation, but the virus could still be obtained from the animal by cocultivation of peripheral blood leukocytes with the fibroblasts. In some persistently infected sheep, virus in cells coexisted indefinitely with neutralizing antibodies in plasma, whereas in other sheep, nonneutralizable mutant viruses, in addition to the parental virus, were obtained from the peripheral blood leukocytes (30, 31). This development of antigenic mutants, selected by neutralizing antibodies, represents a unique mechanism for survival of the agent in an immune environment.

Recent studies have shown that the target cells of the virus in the persistently infected animal are monocyte macrophages (34, 36) and not fibroblastic cells. Since macrophages have important roles in immunologically specific defenses as well as the nonspecific defenses of the host, we have reexamined the neutralization of visna virus in sheep to determine whether the macrophage target cell may have a role in the ability of the virus to persist in the animal in the face of neutralizing antibodies. Using plaque-purified, cell-free visna virus, serum from a sheep hyperimmunized with visna virus, and fibroblast and macrophage cell cultures, we show here that antibodies to the virus were effective in neutralization, irrespective of the target cell. However, the site of neutralization differed in the two cell types. Neutralization occurred at the cell surface of fibroblasts and intracellularly in macrophages. Surprisingly, however, the virus had a greater affinity for macrophage target cells than did antibodies for the virus. This may thus provide a mechanism for the ability of these agents to replicate persistently in the immune host whether or not antigenic drift in the virus occurs.

MATERIALS AND METHODS

Cell cultures. SCP fibroblasts were derived from early subcultures of the choroid plexus of a newborn, colostrum-deprived lamb. The cells were grown in minimum essential medium (MEM) plus 10% fetal bovine serum and maintained in MEM plus 1 or 2% lamb serum heated for 30 min at 56°C as previously described (33).

Sheep alveolar macrophages immortalized with simian virus 40 were used in this study. The establishment and characterization of the cell line (transformed macrophages, TM) was reported previously (14). The macrophages were cultivated as confluent monolayers in Dulbecco MEM (DMEM) supplemented with 20% lamb serum and were maintained in DMEM plus 2% lamb serum. The cells were shown to be macrophages by phagocytic properties, pres-

* Corresponding author.

ence of Fc receptors, presence of nonspecific esterases, and ultrastructural morphology. Infection of these macrophages with different strains of lentiviruses resulted in productive virus replication for all strains (14).

Virus. Icelandic visna virus strains 1514 was used throughout the study (39). The virus was plaque purified in SCP cell cultures as described previously (32). In this study infectivity was measured by plaque formation or quantal assays in SCP cultures. The methods were interchangeable because 1 PFU was equivalent to one 50% tissue culture infectious dose ($TCID_{50}$).

Radiolabeled virus. SCP fibroblast cultures in tissue culture flasks (150 cm^2) were infected with virus 1514 at a multiplicity of 0.1. When 50% of the monolayer showed cytopathic effect (CPE), the cells were washed with Hanks balanced salt solution, and 0.25 mCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 5 ml of methionine-free MEM was added. The flasks were rocked overnight at 37°C in 5% CO_2. The medium was then removed and stored at 4°C, and 10 ml of methionine-free medium was added to the cultures. The medium was changed at 4- to 6-h intervals twice, and the supernatant fluids were stored at 4°C. The cultures were then relabeled with isotope overnight as described above, and the medium was removed and pooled with the other supernatant fluids at 4°C.

The pooled, labeled virus supernatants were clarified at 1,500 × *g* for 20 min at 4°C and centrifuged at 18,500 × g for 2 h at 4°C. The labeled virus pellets were suspended in NET buffer (50 mM NaCl, 5 mM EDTA, 10 mM Tris hydrochloride, pH 7.4) and added to a step gradient of 5 ml of 55% (wt/wt) sucrose and 7 ml of 20% (wt/wt) sucrose which was centrifuged at 40,000 × *g* for 2 h at 4°C in a vertical rotor. The virus was collected at the interface of the two sucrose solutions and dialyzed against NET plus 1% lamb serum in a microconcentrator (Centricon; Amicon, Danvers, Mass.). The labeled virus preparation was divided into 100-μl samples and stored at −70°C. The infectivity of the preparation was determined by assay in monolayer cultures of SCP cells as previously described (32). The amount of radioactivity in the labeled virus preparation was determined in a liquid scintillation counter to determine the ratio of counts per minute in infectious units (cpm/$TCID_{50}$).

Immune serum. A purified virus suspension containing approximately 10^8 $TCID_{50}$ of strain 1514 was treated with Nonidet P-40 detergent at a final concentration of 0.5%. A 2-ml volume of this material was emulsified in 2 ml of complete Freund adjuvant and injected intradermally into four sites of a sheep. Three similar injections of virus antigen emulsified in incomplete adjuvant were given sequentially 2 to 3 weeks apart and followed by two intravenous injections, 1 week apart, of 5 ml of pure antigen. The sheep was bled for serum 7 days after the last injection (30). Normal serum was a lot of commercial lamb serum (GIBCO Laboratories, Grand Island, N.Y.) which had been screened for the absence of neutralizing antibodies to strain 1514. All sera were heated for 30 min at 56°C before use to inactivate mycoplasmas and serum-bovine viruses.

Serum neutralization. Virus 1514 (1,000 $TCID_{50}$) was mixed with doubling dilutions of serum and incubated for 30 min at 37°C. Four replicates of each dilution were added to confluent layers of SCP cells in a 96-well microtiter plate. Plates were evaluated for CPE after 10 to 14 days.

Kinetics of neutralization. Serum diluted 1:50 in 10 ml of MEM and 10^6 $TCID_{50}$ of virus 1514 in 10 ml of MEM plus 1% lamb serum were warmed to 37°C and then mixed. At various intervals, 1-ml samples were taken and added to 100 ml of MEM at 8°C. Half-log_{10} dilutions were made from these samples, and residual infectivity was measured by quantal assay in a 96-well microtiter plate.

Determination of labeled virus binding to cells. Cells were cultivated in 35-mm tissue culture dishes, inoculated with 1 ml of labeled virus at a multiplicity of 0.2 to 0.4, and incubated either at 37°C in a CO_2 incubator or at 8°C in sealed chambers containing 95% air and 5% CO_2. Labeled virus inocula were removed at sequential intervals from individual dishes, and 200-μl samples were transferred to 5 ml of scintillation fluid (Liquiscent; National Diagnostics, Somerville, N.J.). The cell monolayers were washed three times with DMEM and lysed with 0.5 ml of 1% sodium dodecyl sulfate in deionized, distilled water. Cell lysates were transferred to liquid scintillation vials containing 5 ml of scintillation fluid. An additional 5 ml of scintillation fluid was added to each tissue culture dish and then transferred to a scintillation vial. Radioactivity in these samples and in the labeled virus inocula was determined as described above.

Determination of the effect of temperature on virus binding. Confluent monolayers of cells in tissue culture dishes were inoculated with labeled virus as described above and incubated at 8°C for 14 h. The inocula were then removed and saved. Cells were washed three times with DMEM, replenished with 1 ml of maintenance medium, and transferred to 37°C. At sequential intervals, the supernatant fluids and lysates of washed cells were saved to determine whether the inoculated virus was present in the supernatant fluid, on the cell surface, or intracellularly. To distinguish between virions adherent to the cell surface and endocytosed virions, inoculated cell cultures at 8 or 37°C were washed three times with DMEM and treated with 0.5 mg of trypsin and 0.2 mg of EDTA (GIBCO) at 37°C for 10 min (SCP fibroblasts) or 30 min (TM). Cell suspensions, dissociated by the trypsin, were washed twice in MEM plus 5% fetal bovine serum and then lysed and counted as described above. Labeled virions which could not be removed from the cells with trypsin-EDTA were considered either irreversibly bound to the cell surface or endocytosed virus.

Determination of acid-soluble radioactivity in cultures infected with labeled virus. Appearance of acid-soluble cpm in supernatant fluids was considered to be indicative of cellular uncoating of virions. This measurement was performed by adding 1 ml of 0.01 M sodium pyrophosphate and 1 ml of 3.5% perchloric acid in 0.01 M sodium pyrophosphate sequentially to 100-μl samples of labeled virus inocula or cell culture supernatants at 4°C. The samples were mixed on a Vortex mixer and incubated on ice for 30 to 50 min. The precipitates were collected by vacuum on glass-fiber filters and rinsed with 0.01 M sodium pyrophosphate and 0.1 M HCl, then with 95% ethanol to facilitate drying. Filters were dried and placed in liquid scintillation vials to which 5 ml of 4% PPO (2,5-diphenyloxazole) in toluene (Omnifluor; New England Nuclear Corp., Boston, Mass.) was added. Counts quantified in the liquid scintillation counter represented acid-precipitable radioactivity. Acid-soluble radioactivity was determined by subtracting acid-precipitable cpm from the total radioactivity of a 100-μl sample in liquid scintillation fluid.

Statistics. All experiments were performed in triplicate, and the statistical significance of the data was evaluated by a three-way analysis of variance.

In situ hybridization studies. In situ hybridization studies were performed at 37°C using cultured macrophages which had been inoculated with virus or virus preincubated with

neutralizing antibodies. At 48 h after inoculation, cells were trypsinized, cytocentrifuged onto pretreated slides (13), and processed for in situ hybridization.

Cloned DNA of virus 1514 (28a) was radiolabeled by nick translation using [^{35}S]dATP and [^{35}S]dCTP (Amersham Corp.), as described previously (Moench et al., in press). Probe with a specific activity of 4×10^8 to 6×10^8 cpm/μg was applied to cytocentrifuged preparations in hybridization protocols designed to detect either viral DNA or RNA after autoradiography (20, 28a).

RESULTS

Virus assay. The purified, [^{35}S]methionine-labeled virus 1514 preparation had an infectivity titer of 10^8 $TCID_{50}$/ml. Comparison of the initial radioactivity with the infectivity showed a ratio of 0.5 cpm/PFU.

Neutralization of virus 1514 by immune serum in SCP and TM cultures. The highest dilution of the immune serum which protected SCP cells against virus-induced CPE was 1:320. Since the virus did not cause clear-cut CPE in TM cultures, supernatant fluids from these cultures that had been inoculated with mixtures of virus and diluted serum 2 weeks previously were transferred, well for well, to confluent SCP cultures. Here, CPE developed only in the cultures receiving virus plus serum diluted more than 1:320. Thus, the neutralizing capacity of the serum for virus 1514 was similar when measured in SCP or TM cultures.

Neutralization kinetics. Neutralization kinetics was determined to learn the rate at which neutralizing antibodies inactivated virus 1514 infectivity. The immune serum was diluted 1:50 in DMEM, mixed with equal volumes of virus suspension containing 10^6 PFU, and incubated at 37°C. Samples (1 ml) were removed at regular intervals, diluted 1:100 in DMEM at 4°C, and assayed for residual infectivity in SCP cells. No reduction of viral infectivity occurred during the first 15 min of incubation (Fig. 1). Ninety-nine percent of viral infectivity was lost after 60 min, and no infectivity was detected after 90 min.

Virus 1514 binding to cells at 37°C. Confluent monolayers of SCP fibroblasts and TM (7×10^5 cells per tissue culture dish) were inoculated with labeled virus in DMEM plus 2% normal lamb serum (multiplicity of infection = 1) at 37°C to determine the rate of binding of virus to the cells. Counts representing nonspecific binding (number of cpm in cultures after addition and immediate removal of inocula followed by three washings) were subtracted from sequential determinations of cell-associated radioactivity. Counts were converted to PFU based on the relation of infectivity to radioactivity, which was adjusted to allow for the decay of the [^{35}S]methionine. Virus began to attach to the cells during the first 5 min of incubation, and by 15 min 4,500 and 8,200 PFU had bound to SCP fibroblasts and TM, respectively (Fig. 1). Thus the rate of virus binding to cells was faster than the rate of neutralization.

Effect of the immune serum on binding of labeled virus to SCP fibroblasts. Using information from the kinetics of neutralization study, we diluted the immune serum 1:50 and incubated this with labeled virus for 90 min at 37°C, then cooled the mixtures to 8°C before adding them to SCP cells at 8°C. In control experiments, the labeled virus was incubated with normal lamb serum diluted 1:50 and treated similarly. Increasing numbers of cpm adsorbed to the cells with time. Of the 45,000 cpm added to each tissue culture dish, an average of 11,133 ± 1,553 cpm bound to the SCP

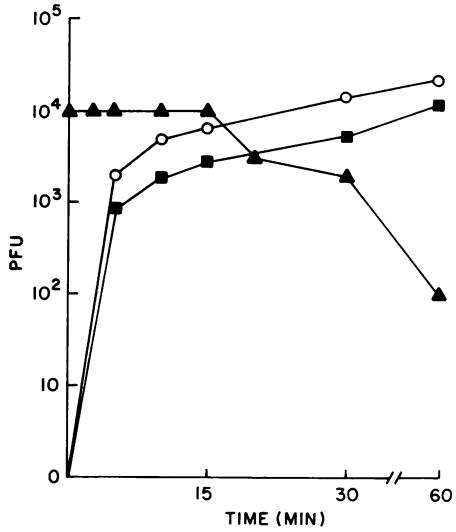

FIG. 1. Comparison of kinetics of neutralization of virus 1514 with serum from a hyperimmunized sheep with the rate of adsorption of the virus to SCP fibroblasts and TM at 37°C. Immune serum was mixed 1:1 with 10^6 PFU of virus 1514 at 37°C. At intervals, samples were removed, diluted 100-fold, and assayed for residual infectivity (▲). In virus-cell interaction studies, SCP fibroblasts (■) and TM (○) in 35-mm dishes were inoculated with 7×10^5 PFU of [^{35}S]methionine-labeled virus 1514 and incubated at 37°C. Radioactive counts that bound to the cells were determined sequentially. Counts were converted to PFU using the ratio of infectivity to radioactivity, adjusted for the decay of [^{35}S]methionine.

cells in the presence of control serum (Fig. 2A), but only 6,718 ± 569 cpm bound in the presence of immune serum (Fig. 2B) after 24 h at 8°C. Further, approximately 40% of the adsorbed cpm from the virus-control serum mixture was irreversibly bound to the SCP cells, in contrast to 11% of the cpm from the immune serum mixture. Only minimal amounts of acid-soluble radioactivity were detected in the inocula of these cultures, indicating that no virus had penetrated and uncoated at 8°C.

Although preincubation of labeled virus 1514 with neutralizing antibodies effectively inhibited irreversible binding of the virus to SCP cells by 87%, a small number of cpm had become irreversibly bound to the cells. To determine whether the virus would penetrate the cells, the cultures were shifted to 37°C after washing with DMEM. Before the temperature shift in this experiment, an average of 4,038 ± 1,853 cpm had adsorbed to the SCP cells, but during the first 15 min of incubation at 37°C, more than 50% of this amount had eluted into the culture medium. Less than 9% of this amount was acid soluble (Fig. 3B). The number of acid-soluble cpm did not increase significantly in the culture medium during further incubation. In contrast, 50% of the cpm that bound initially to control cultures at 8°C (9,343 ± 3,815 cpm) was present in the culture medium 6 h after the temperature shift, and 44% of this was acid soluble (Fig. 3A). Thus, the small amount of virus that bound to the SCP cells in the presence of neutralizing antibodies did not penetrate the cells.

To determine whether the immune serum would prevent virus replication of adsorbed virus, we added labeled virus at a multiplicity of infection of 0.4 to the SCP fibroblasts and incubated the culture at 8°C for 14 h. The cultures were then washed three times with DMEM at 8°C, and 1 ml of medium containing either 2% control serum or 2% immune serum

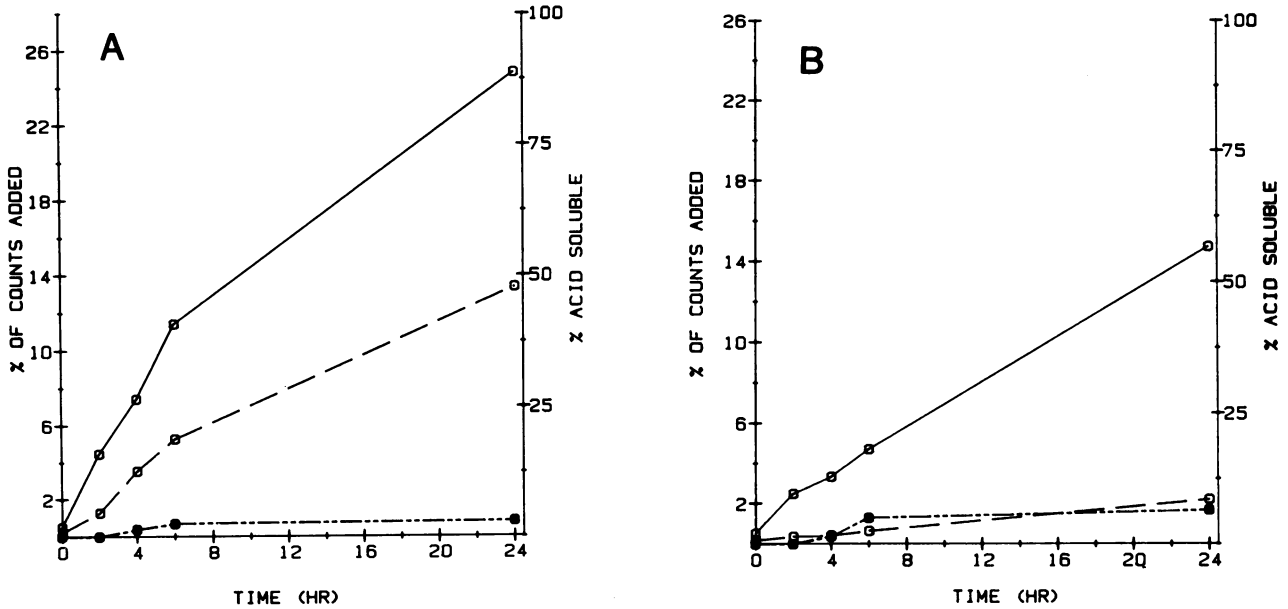

FIG. 2. Rate of adsorption of [^{35}S]methionine-labeled virus 1514 preincubated with normal lamb serum (A) and immune serum (B) to SCP fibroblasts at 8°C. (●) Percent of acid-soluble cpm (right axis) in the supernatant fluid. (O——O) Percent of radioactive cpm bound to the cells (left axis). (O---O) Percent of cpm irreversibly bound to the cells (left axis). Labeled virus was incubated with serum for 90 min at 37°C, cooled to 8°C, and then added to the dishes of SCP fibroblasts at 8°C. At indicated intervals, supernatant fluids were removed. Total and perchloric acid-precipitable cpm were determined for each sample and the percent of acid-soluble cpm was determined by subtracting acid-precipitable cpm from total cpm.

was added to each dish. Half of each of the two sets of cultures were shifted to 37°C immediately, and the other half were incubated for 4 h at 8°C to allow time for neutralizing antibodies to bind to virus. These cultures were then shifted to 37°C. Approximately 4,000 cpm (4,386 ± 396 cpm) of labeled virus 1514 bound to the SCP fibroblasts after 14 h at 8°C. The adsorbed virus penetrated and uncoated at similar rates at 37°C whether the cultures were treated with control or immune serum. This process was not altered by the further incubation of neutralizing antibodies with adsorbed virus at 8°C. Within 6 h after the temperature shift to 37°C, approximately 52% of the cpm that had bound to the cells at

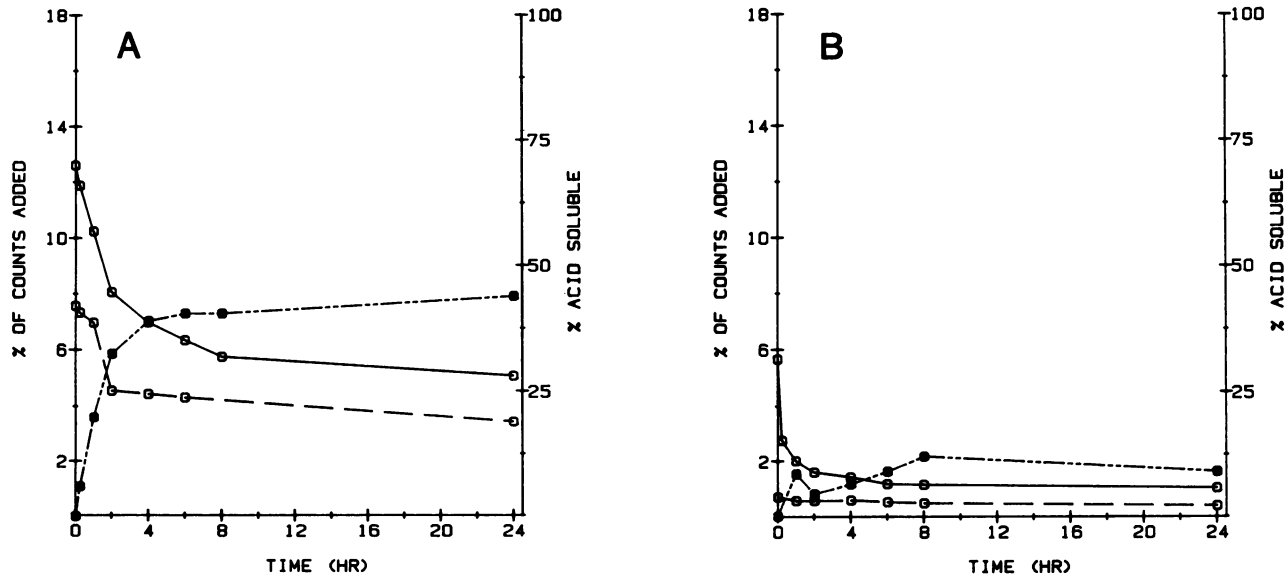

FIG. 3. Rate of penetration and uncoating of [^{35}S]methionine-labeled virus 1514 preincubated with normal lamb serum (A) and immune serum (B) in SCP fibroblasts at 37°C. (●) Percent of acid-soluble cpm (right axis) in the supernatant fluid. (O——O) Percent of radioactive cpm bound to the cells (left axis). (O---O) Percent of cpm irreversibly bound to the cells (left axis). Labeled virus was incubated with serum for 90 min at 37°C, and the mixtures were cooled to 8°C and added to dishes of SCP fibroblasts at 8°C. These were incubated for 14 h at 8°C. Inocula were then removed in three consecutive washes with cold medium. Cultures were replenished with maintenance medium and shifted to 37°C. As indicated in the figures, the cultures were examined sequentially for radioactivity that bound and irreversibly bound to the cells and for acid-soluble cpm in the supernatant fluids as indicated in the legend of Fig. 2.

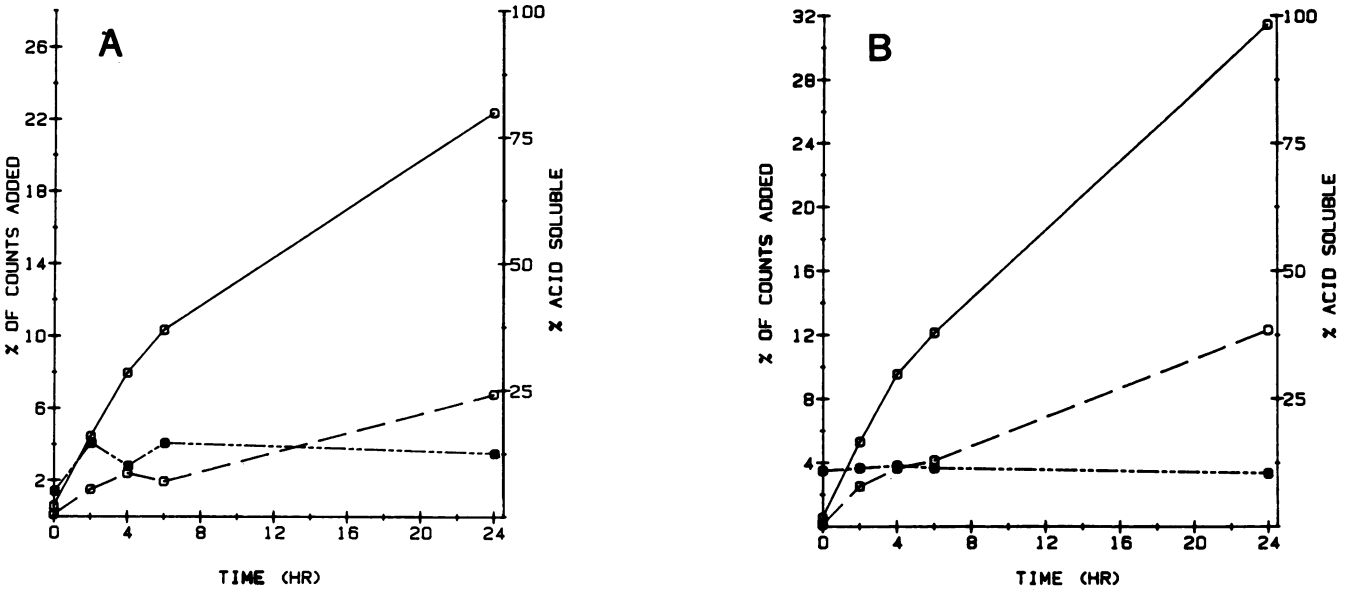

FIG. 4. Rate of adsorption of [^{35}S]methionine-labeled virus 1514 preincubated with normal lamb serum (A) and immune serum (B) to TM at 8°C. (●) Percent of acid-soluble cpm (right axis) in the supernatant fluid. (O——O) Percent of radioactive cpm bound to the cells (left axis). (O---O) Percent of cpm irreversibly bound to the cells (left axis). The protocol was similar to that described in the legend of Fig. 2.

8°C in both groups was found in the culture medium, and 43.5 ± 2.2% of this was acid soluble. Continuous incubation of replicates of these cultures resulted in development of CPE within 48 h. These results indicated that neutralizing antibodies had no effect on virus replication if the agent had already bound to the cell surface.

Effect of the immune serum on binding of labeled virus to TM. To determine whether the mechanism of neutralization of virus 1514 was the same in TM as in SCP fibroblasts, the binding experiments described above were repeated in macrophage cultures. Labeled 1514 virus was preincubated with normal or immune serum and added to TM at 8°C as described above. At 6 h, an equivalent amount of virus (average of 5,360 ± 2,251 cpm) adsorbed to the cells in the presence of either normal or immune serum (Fig. 4). However, the percent of irreversibly bound cpm was higher with the immune serum mixture (39.8%) than with the control mixture (28.2%). To determine whether the enhanced number of radiolabeled irreversibly bound virus in the virus-immune serum mixture penetrated and uncoated, the above

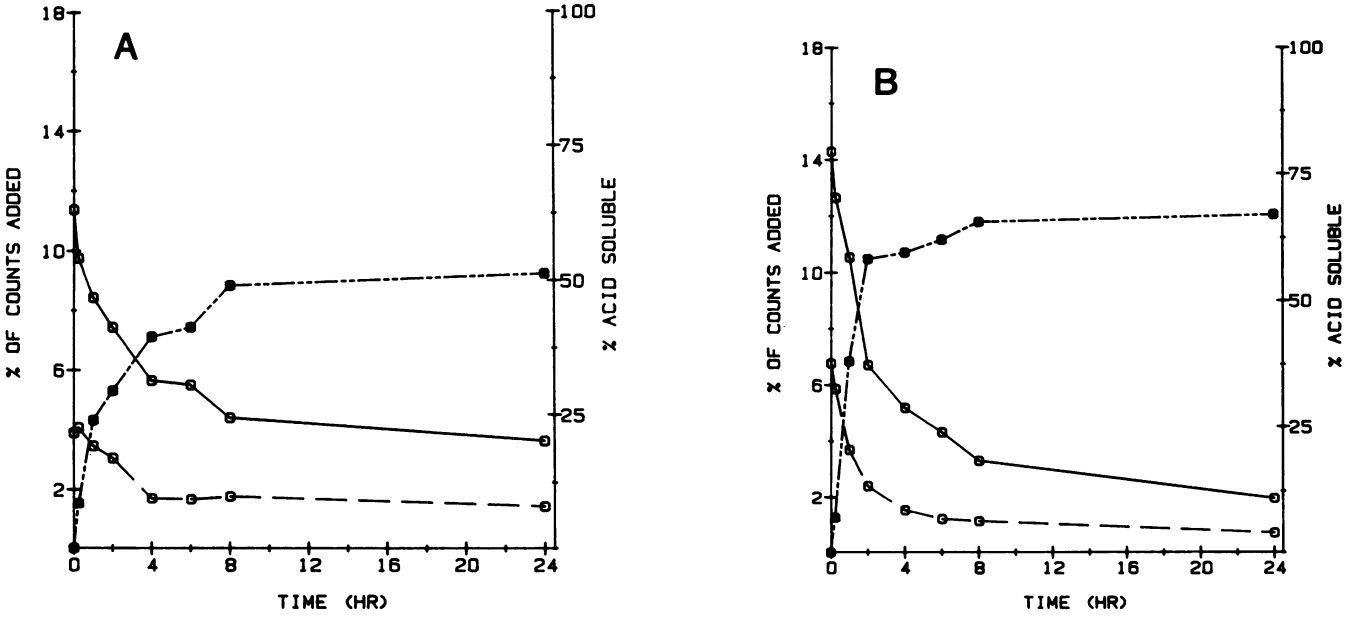

FIG. 5. Rate of penetration and uncoating of [^{35}S]methionine-labeled virus 1514 preincubated with normal lamb serum (A) and immune serum (B) in TM at 37°C. (●) Percent of acid-soluble cpm (right axis) in the supernatant fluid. (O——O) Percent of radioactive cpm bound to the cells (left axis). (O---O) Percent of cpm irreversibly bound to the cells (left axis). The protocol was similar to that described in the legend of Fig. 3.

experiments were repeated and the cultures were incubated at 8°C for 14 h. An average of 8,000 cpm (8,441 ± 3,821 cpm) bound during this period. Cultures were then washed and shifted to 37°C. At 2 h after the shift to 37°C, 50% of the cpm that had adsorbed at 8°C appeared in the culture medium. Sixty percent of this was acid soluble (Fig. 5B). In contrast, only 40% of the cpm in the supernatant fluids from cultures with normal serum was acid soluble after as long as 4 h of incubation at 37°C (Fig. 5A). Thus, the immune serum not only enhanced the binding of labeled virus to TM, but also expedited penetration and uncoating of the virus in these cells.

Since the immune serum had prevented replication of infectious virus but nevertheless potentiated binding, penetration, and uncoating of the agent in macrophages, we asked whether the antibody-induced block in replication occurred at the stage of reverse transcription of viral RNA to DNA or at a later stage in the virus life cycle. When cultures of TM inoculated with virus-immune serum mixtures were examined for viral DNA by in situ hybridization 48 h after infection at 37°C, less than one grain per cell was detected. In contrast, cultures inoculated with virus and control serum had an average count of 40 grains per cell. Thus virus neutralization in the macrophage occurred at a stage between uncoating of the virion and transcription of the viral RNA.

Statistics. A three-way analysis of variance was performed to test for significance in the interactions among the three variables: (i) the four different treatments of cells (labeled virus + normal serum; labeled virus + normal serum + trypsin-EDTA; labeled virus + immune serum; labeled virus + immune serum + trypsin-EDTA); (ii) changes in cpm of [^{35}S]methionine-labeled virus 1514 associated with cells at different time intervals; and (iii) replicate experiments on different days. The interactions examined were treatments versus time (i × ii), treatments versus replicates (i × iii), and time versus replicates (ii × iii). The P values were <0.001 for each of the three interactions when determined for experiments involving either cell type. This meant that the three interactions were highly significant and permitted data from replicate experiments to be averaged. Data were graphed as the percent of cpm added which was cell associated.

DISCUSSION

Sheep infected with visna virus develop serum neutralizing antibodies gradually during 2 to 4 weeks after virus inoculation (33). The antibodies are found almost exclusively among the immunoglobulin G1 molecules, a class of antibodies that binds complement (but not *Staphylococcus aureus* protein A) (38). Sheep do not produce detectable amounts of neutralizing immunoglobulin M antibodies to visna virus (38), although such claims have been reported (28). Demonstration of specificity of low concentrations of neutralizing antibodies, as found in early immune sera, often requires a degree of purification of the immunoglobulin from serum proteins because undiluted serum from uninfected sheep (and from most other species) causes nonspecific inhibition of replication of the virus in cell cultures. Whereas addition of antiglobulins to virus-antibody mixtures enhances virus neutralization (35), addition of complement does not affect neutralization, and complement does not confer virus-neutralizing properties to dilutions of postinfectious sera in the absence of specific neutralizing immunoglobulin in the serum (24, 33, 35). Therefore, a role for complement in the neutralization of visna virus may not be critical.

Neutralization of visna virus with postinfectious serum requires prolonged incubation of virus-antibody mixtures (39). To examine the best possible effects of such antibodies, we used a hyperimmune serum with kinetics of neutralization much faster than those of postinfectious sera to show in this study that the antibodies block replication of the agent in both fibroblasts and macrophages by preventing virus adsorption to the former and by interfering with viral RNA transcription in the latter. Despite the effectiveness of these antibodies in neutralization, binding of antibodies to the virus in the neutralization process was slower than binding of virus to the target cells. Therefore, in the three-way interaction among virus, neutralizing antibodies, and target cells, it is theoretically possible for virus to infect cells in the presence of neutralizing antibodies.

To identify the stage of the virus life cycle interrupted by neutralizing antibodies in fibroblasts and macrophages, it was necessary to establish criteria for measuring adsorption, penetration, and uncoating of the virus and the transcription of viral genome. Labeled virus bound poorly to SCP fibroblasts and TM at 8°C; after 24 h, more than 75% of the added cpm was still present in the inoculum. However, low temperatures are known to inhibit adsorption of other retroviruses to target cells (1). Nevertheless, this procedure was necessary to prevent endocytosis and processing of the virus.

Shift of the inoculated SCP and TM from 8 to 37°C resulted in a sharp rise in acid-soluble cpm in the medium. This showed that the virions had been endocytosed and proteolytic digestion of the viral proteins had begun. Within 6 h after the shift, approximately 50% of the cpm bound to both cell types was present in the medium. Lack of further production of acid-soluble radioactivity after 6 h at 37°C suggested that virus 1514 which had adsorbed to the cells at 8°C had been endocytosed and uncoated within this period.

Proteolytic enzymes are the agents of choice for removal of viruses adsorbed to cell surfaces (15, 23), and trypsin has been used in previous studies to assess the binding of retroviruses (1). In this manner, labeled virus attached to the cell surface could be distinguished from virus which had been endocytosed and thereby protected from the protease. In this study, trypsin failed to remove approximately 50 and 30% of the cpm bound to SCP fibroblasts and TM, respectively, after the inoculated cultures had been incubated at 8°C for 14 h. These counts were considered irreversibly bound and probably represented virus which had fused to the cell surfaces and was no longer susceptible to the enzymatic activity of trypsin. Visna-maedi virus is known to cause cell fusion "from without" when inoculated at high multiplicities (22), and this fusion process may be an integral part of the replication cycle (5).

The irreversible binding of labeled virus to SCP fibroblasts was inhibited by more than 80% when the virus was pretreated with immune serum. Neutralizing antibodies therefore inhibited replication of the virus in these cells by preventing binding of the virus to putative cellular receptors. Inhibition of virus attachment is one of the classic explanations of virus neutralization, although this is usually seen under conditions in which excess antibodies are used (27, 41). It is unlikely that the antiserum used in this study had antibodies in great excess since the serum had a neutralizing titer of only 1:320 and was used at 1:50. The failure of the antibodies to prevent infection after virus had adsorbed to the cell emphasized the transient period of potential effec-

tiveness of the antibodies in curtailing replication and showed that after fusion of the virus to the cell surface had occurred, neutralizing antibodies were no longer effective.

In contrast to the effect of neutralizing antibodies on infection of SCP fibroblasts, the antibodies enhanced binding, endocytosis, and uncoating of the virus in macrophages. Since virus replication did not occur in these cells and since virus DNA was not found in the cells by in situ hybridization, the block in the virus life cycle probably occurred at a stage between uncoating of the virion and transcription of the viral RNA. It is possible that these events, mimicking those of the early stages of virus replication, may have been mediated by Fc receptors on the surface of the macrophages. Phagocytosis of immune complexes via these receptors may thus have circumvented antibody inhibition of binding of virus to cellular receptors as seen in the SCP fibroblasts. This will be tested in future experiments using $F(ab')_2$ fragments from the immune serum.

The requirement for prolonged incubation (15 min at 37°C) of virus and antibodies before neutralization occurred suggests that more than one molecule of antibody is necessary to neutralize a virion (25) and that the interaction between neutralizing antibodies and virus particles may be initially reversible (11). This implies that the affinity of the antibodies for the infectious virion may be low. The unusually slow development of neutralizing antibodies in sheep infected with visna virus (17, 18, 39) and the small amounts (low titers) of these antibodies in the serum after prolonged infection support the concept of low affinity. Such antibodies may be a common response in lentiviral infections, and this may provide an explanation for lack of detectable neutralizing antibodies in goats infected with caprine arthritis-encephalitis virus (24, 35) as well as the low amounts of neutralizing antibodies in many humans infected with the acquired immune deficiency syndrome retrovirus (40, 47).

In view of the strong tropism of the ruminant lentiviruses for cells of the monocyte-macrophage lineage, it is possible that infection in these cells may be the major determinant for persistence of the viruses in the animal. This is supported by the fact that virus persists irrespective of whether the animal fails to develop neutralizing antibodies, as seen in goats infected with caprine arthritis encephalitis virus or in visna virus-infected sheep, which do develop neutralizing antibodies. Despite the susceptibility of visna virus to neutralization in macrophages, the greater affinity of the virus for the cell than of the antibody for the virus would favor spread of the agent in the presence of neutralizing antibodies. This provides a mechanism for the known ability of the virus to persist for years in the presence of neutralizing antibodies (32, 46). It also offers an explanation for the failure of nonneutralizable mutants of visna virus to replace the neutralizable parental virus in immune sheep in which antigenic drift of the virus had occurred (26). Thus, the ability of these lentiviruses to induce neutralizing antibodies in natural hosts offers no guarantee of cure of the infection. Similarly, the presence of neutralizing antibodies in the immunized host may not necessarily protect against invading virus because the kinetics of neutralization may be too slow to protect target cells.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants RR00130, NS12127, and NS15721 from the National Institutes of Health. S.K.S. is supported by a Special Emphasis Research Career Award (RR00017) from the Research Resources Division of the National Institutes of Health.

We thank Howard E. Gendelman for performing the in situ hybridization, Allyn Kimball for his advice on statistical evaluation of the data, and Linda Kelly for preparation of the manuscript. We also thank Janice E. Clements and Diane E. Griffin for their technical advice and suggestions.

LITERATURE CITED

1. **Anderson, K. B., and B. A. Nexo.** 1983. Entry of murine retrovirus into mouse fibroblasts. Virology **125**:85–98.
2. **Bowen, D. L., H. C. Lane, and A. S. Fauci.** 1985. Immunopathogenesis of the acquired immunodeficiency syndrome. Ann. Intern. Med. **103**:704–709.
3. **Cheevers, W. P., B. G. Archer, and T. B. Crawford.** 1977. Characterization of RNA from equine infectious anemia virus. J. Virol. **24**:489–497.
4. **Cheevers, W. P. and T. C. McGuire.** 1985. Equine infectious anemia virus: immunopathogenesis and persistence. Rev. Infect. Dis. **7**:83–88.
5. **Chippaux-Hyppolite, C., C. Taranger, J. Tamalet, G. Pautrat, and M. Brahic.** 1972. Aspects ultrastructuraux du virus visna en cultures cellulaires. Ann. Inst. Pasteur **123**:409–420.
6. **Clements, J. E., O. Narayan, and L. C. Cork.** 1980. Biochemical characterization of the virus causing leukoencephalitis and arthritis in goats. J. Gen. Virol. **50**:423–427.
7. **Cork, L. C., and O. Narayan.** 1980. The pathogenesis of viral leuko-encephalomyelitis-arthritis of goats. I. Persistent viral infection with progressive pathologic changes. Lab. Invest. **42**:596–602.
8. **Cork, L. C., and O. Narayan.** 1980. Pathogenesis of goat viral leukoencephalomyelitis. *In* Mechanisms of viral disease. Proceedings of the 31st Annual Meeting of American College of Veterinary Pathologists. American College of Veterinary Pathologists, **City, State.**
9. **Crawford, J. B., and D. S. Adams.** 1981. Caprine arthritis-encephalitis: clinical features and presence of antibody in selected goat populations. J. Am. Vet. Med. Assoc. **178**:713–719.
10. **Curran, J. W.** 1985. The epidemiology and prevention of the acquired immunodeficiency syndrome. Ann. Intern. Med. **103**:657–662.
11. **Daniels, C. A.** 1975. Mechanisms of viral neutralization, p. 79–97. *In* A. L. Notkins (ed.), Viral immunology and immunopathology. Academic Press, Inc., New York.
12. **Gallo, R. C., and F. Wong-Staal.** 1985. A human T-lymphotropic retrovirus (HTLV-III) as the cause of the acquired immunodeficiency syndrome. Ann. Intern. Med. **103**:679–689.
13. **Gendelman, H. E., T. R. Moench, O. Narayan, and D. E. Griffin.** 1983. Selection of a fixative for identifying T cell subsets, B cells, and macrophages in paraffin-embedded mouse spleen. J. Immunol. Methods **65**:137–145.
14. **Gendelman, H. E., O. Narayan, S. Kennedy-Stoskopf, J. E. Clements, and G. H. Pezeshkpour.** 1984. Slow virus-macrophage interactions. Characterization of a transformed cell line of sheep alveolar macrophages that express a marker for susceptibility to ovine-caprine lentivirus infections. Lab. Invest. **51**:547–555.
15. **Gollins, S. W., and J. S. Porterfield.** 1984. Flavivirus infection enhancements in macrophages: radioactive and biological studies on the effect of antibody on viral fate. J. Gen. Virol. **65**:1261–1272.
16. **Gonda, M. A., F. Wong-Staal, R. Gallo, J. E. Clements, O. Narayan, and R. V. Gilden.** 1985. Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. Science **227**:173–174.
17. **Griffin, D. E., O. Narayan, and R. J. Adams.** 1978. Early immune responses in visna, a slow viral disease of sheep. J. Infect. Dis. **138**:340–350.
18. **Gudnadottir, M., and P. A. Palsson.** 1966. Host-virus interaction in visna infected sheep. J. Immunol. **95**:1116–1120.
19. **Haase, A. T.** 1975. The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. **72**:101–156.
20. **Haase, A. T., L. Stowring, J. D. Harris, B. Traynor, P. Ventura, R. Peluso, and M. Brahic.** 1982. Visna DNA synthesis and the tempo of infection in vitro. Virology **119**:399–410.
21. **Haase, A. T., L. Stowring, O. Narayan, D. Griffin, and D. Price.**

1977. Slow persistent infection caused by visna virus: role of host restriction. Science **195**:175–177.
22. **Harter, D. H., and P. W. Choppin.** 1967. Cell-fusing activity of visna virus particles. Virology **31**:279–288.
23. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell. Biol. **84**:404–420.
24. **Klevjer-Anderson, P., and T. C. McGuire.** 1982. Neutralizing antibody response of rabbits and goats to caprine arthritis-encephalitis virus. Infec. Immun. **38**:455–461.
25. **Lafferty, K. J.** 1963. The interaction between virus and antibody. I. Kinetic studies. Virology **21**:61–75.
26. **Lutley, R., G. Petursson, P. A. Palsson, G. Georgsson, J. Klein, and N. Nathanson.** 1983. Antigenic drift in visna: virus variation during long-term infection of Icelandic sheep. J. Gen. Virol. **64**:1433–1440.
27. **Mandel, B.** 1967. The interaction of neutralized poliovirus with HeLa cells. I. Adsorption. Virology **31**:238–247.
28. **Metha, P. D., and H. Thormar.** 1974. Neutralizing activity in isolated serum antibody fractions from visna-infected sheep. Infect. Immun. **10**:678–680.
28a. **Moench, T. R., H. E. Gendelman, J. E. Clements, O. Narayan, and D. E. Griffin.** 1985. Efficiency of *in situ* hybridization as a function of probe size and fixation technique. J. Virol. Methods **11**:119–130.
29. **Molineaux, S., and J. E. Clements.** 1983. Molecular cloning of unintegrated visna viral DNA and characterization of frequent deletions in the 3′ terminus. Gene **23**:137–148.
30. **Narayan, O., J. E. Clements, D. E. Griffin, and J. S. Wolinsky.** 1981. Neutralizing antibody spectrum determines the antigenic profiles of emerging mutants of visna virus. Infect. Immun. **32**:1045–1050.
31. **Narayan, O., D. E. Griffin, and J. Chase.** 1977. Antigenic shift of visna virus in persistently infected sheep. Science **197**:376–378.
32. **Narayan, O., D. E. Griffin, and J. E. Clements.** 1978. Virus mutation during 'slow infection': temporal development and characterization of mutants of visna virus recovered from sheep. J. Gen. Virol. **41**:343–352.
33. **Narayan, O., D. E. Griffin, and A. M. Silverstein.** 1977. Slow virus infection: replication and mechanisms of persistence of visna virus in sheep. J. Infect. Dis. **135**:800–806.
34. **Narayan, O., S. Kennedy-Stoskopf, D. Sheffer, D. E. Griffin, and J. E. Clements.** 1983. Activation of caprine arthritis-encephalitis virus expression during maturation of monocytes to macrophages. Infect. Immun. **41**:67–73.
35. **Narayan, O., D. Sheffer, D. E. Griffin, J. Clements, and J. Hess.** 1984. Lack of neutralizing antibodies to caprine arthritis-encephalitis lentivirus in persistently infected goats can be overcome by immunization with inactivated *Mycobacterium tuberculosis*. J. Virol. **49**:349–355.
36. **Narayan, O., J. S. Wolinsky, J. E. Clements, J. D. Strandberg, D. E. Griffin, and L. C. Cork.** 1982. Slow virus replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. J. Gen. Virol. **59**:345–356.
37. **Nathanson, N., H. Panitch, P. A. Palsson, G. Petursson, and G. Georgsson.** 1976. Pathogenesis of visna. II. Effect of immunosuppression upon early central nervous system lesions. Lab. Invest. **35**:444–451.
38. **Petursson, G., B. M. Douglas, and R. Lutley.** 1985. Immunoglobulin subclass distribution and restriction of antibody response in visna, p. 211–216. *In* J. M. Sharp and R. Hoff-Jorgesen (ed.), Slow viruses in sheep, goats, and cattle. Commission of the European Communities, Luxembourg.
39. **Petursson, G., N. Nathanson, G. Georgsson, H. Panitch, and P. A. Palsson.** 1976. Pathogenesis of visna. I. Sequential virologic, serologic, and pathologic studies. Lab. Invest. **35**:402–412.
40. **Robert-Guroff, M., M. Brown, and R. C. Gallo.** 1985. HTLV-III neutralizing antibodies in patients with AIDS and AIDS-related complex. Nature (London) **316**:72–74.
41. **Rubin, H., and R. M. Franklin.** 1957. On the mechanism of Newcastle disease virus neutralization by immune serum. Virology **3**:84–95.
42. **Sigurdsson, B.** 1954. Maedi, a slow progressive pneumonia of sheep: an epizoological and a pathological study. Br. Vet. J. **110**:255–270.
43. **Sigurdsson, B., P. A. Palsson, and H. Grissom.** 1957. Visna, a demyelinating transmissable disease of sheep. J. Neuropathol. Exp. Neurol. **16**:389–403.
44. **Sigurdsson, B., H. Thormar, and P. A. Palsson.** 1960. Cultivation of visna virus in tissue culture. Arch. Gesamte Virusforsch. **10**:368–381.
45. **Thormar, H.** 1963. The growth cycle of visna virus in monolayer cultures of sheep cells. Virology **19**:273–278.
46. **Thormar, H., M. R. Barshatzky, K. Arnesen, and P. B. Kozlowski.** 1983. The emergence of antigenic variants is a rare event in long-term visna virus infection *in vivo*. J. Gen. Virol. **64**:1427–1432.
47. **Weiss, R. A., P. R. Clapham, R. Cheingsong-Popov, A. G. Dalgleish, C. A. Carne, I. V. D. Weller, and R. S. Tedder.** 1985. Neutralization of human T-lymphotropic virus type III by sera of AIDS and AIDS-risk patients. Nature (London) **316**:69–72.