Ovine Lentivirus Is Macrophagetropic and Does Not Replicate Productively in T Lymphocytes

MARK D. GORRELL,¹ MALCOLM R. BRANDON,² DARLENE SHEFFER,¹ ROBERT J. ADAMS,¹
AND OPENDRA NARAYAN^{1*}

*Division of Comparative Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205,¹
and Centre for Animal Biotechnology, The University of Melbourne, Melbourne, Australia²*

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The lentiviruses of sheep, goats, and horses cause chronic multiorgan disease in which macrophages are highly permissive for viral replication. Monocytes, which mature into macrophages, are thought to be latently infected with lentivirus, but the extent to which other leukocytes are infected is unknown. Dendritic cells have not been studied separately from monocytes and T-cell subsets have not been examined in previous attempts to identify infected cells in peripheral blood mononuclear cells (PBMC). We found no evidence of T-cell tropism using an animal-passaged, pathogenic ovine lentivirus. Phytohemagglutinin-stimulated infectious PBMC produced 20-fold less virus than differentiated macrophages, and cocultivation of infectious PBMC with fresh, uninfected phytohemagglutinin blasts did not facilitate virus replication. Furthermore, central lymph cells, the best in vivo source of purified lymphocytes, lacked virus and did not yield virus upon in vitro cultivation. In contrast, cultivated blood-derived macrophages were highly permissive for viral replication. To identify the latently infected PBMC, PBMC from infected sheep were selectively depleted of monocytes and B cells by passage over nylon wool and then of nonadherent cells bearing CD4, CD8, T19, $\gamma\delta$ T-cell receptor, CD45RA, or major histocompatibility complex class II antigens by panning. Removal of adherent monocytes and B cells or of adherent cells and the three major T-cell subsets ($CD4^+$, $CD8^+$, $T19^+$) did not decrease the infectivity of PBMC. The richest sources of infected cells in fresh PBMC were $CD45RA^+$ and major histocompatibility complex class II⁺ nonadherent cells, which are three characteristics of dendritic cells. Thus, the dendritic cell, and not the monocyte or the $CD4^+$ cell, is probably the predominant infected cell type in blood.

The lentiviruses of sheep are etiologic agents causing chronic wasting diseases involving one or more organ systems including the lungs (progressive pneumonia; maedi) the central nervous system (visna), the joints (arthritis), and the mammary glands (mastitis). These diseases have prolonged incubation periods and insidious onset and progress slowly, leading to cachexia (61). Lesions in infected tissues are characterized histologically by extensive infiltration and proliferation of mononuclear inflammatory cells. These consist mainly of macrophages, $CD8^+$ and B lymphocytes, and much lesser numbers of $CD4^+$ lymphocytes (86). Cachexic animals also have decreased numbers of circulating $CD4$ lymphocytes (33). Despite this, infected and even cachexic sheep do not develop opportunistic infections (AIDS). This is in clear contrast to human beings and macaques infected with their respective lentiviruses, human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV).

The major host cells that support replication of the primate lentiviruses are $CD4^+$ lymphocytes and macrophages. The profound immunosuppression that results from these lentiviral infections is associated with infection and elimination of the $CD4^+$ lymphocytes (71). In sheep, macrophages in pathologically affected tissues have been identified in previous studies as the major cell type that produces virus (16, 17, 61, 62, 74, 77, 86, 87). However, whether $CD4$ -bearing lymphocytes are also infected is not known. There is one report that in the central nervous system some cells expressing visna virus core protein have the morphology of lymphocytes (18). Earlier studies of goats infected with caprine arthritis-encephalitis virus (a lentivirus closely related to the

ovine lentivirus) and on sheep had shown that the infection is latent in the mononuclear cells in the blood (peripheral blood mononuclear cells [PBMC]) and that virus replication becomes productive when cultures of the PBMC give rise to adherent macrophages (16, 17, 62). Macrophages were shown to be the major producers of virus in these cultures. Since monocytes are precursors of macrophages and depletion of adherent and Fc receptor-positive cells from PBMC removed latently infected cells, it appeared that adherent monocytes are the major type of latently infected cells in the blood. However, it has since been shown that PBMC also contain Fc receptor-positive dendritic cells (34) which are permissive for HIV replication in culture (64, 65). Therefore, these previous experiments designed to study monocytes were actually studying both monocytes and dendritic cells.

The availability of monoclonal antibodies (MAbs) to various ovine leukocyte surface antigens and their use in separating subpopulations of mononuclear cells (41, 42) has facilitated a more thorough investigation of the identity of infected cells in the blood. The question whether $CD4^+$ cells are susceptible to infection and/or latently infected in blood can be addressed. In this study, we used PBMC and central lymph from infected sheep as sources of infected cells. This study confirmed that mature macrophages are highly permissive and showed that activated T lymphocytes are nonpermissive for replication of the virus. Central lymph, a source of purified lymphocytes rich in $CD4^+$ cells, contained very few infected cells and produced little virus in culture. Infected cells bearing latent virus in PBMC were neither monocytes nor $CD4^+$ cells but instead were nonadherent cells expressing major histocompatibility complex (MHC) class II antigens and CD45RA. The latter are characteristics of dendritic cells.

* Corresponding author.

MATERIALS AND METHODS

Virus. The most common ovine lentiviruses found in the United States are those causing progressive pneumonia with or without arthritis (33, 35, 63). Virus was obtained from synovial fluid of an arthritic pneumonic Border Leicester sheep (SH93) naturally infected with this virus (33, 60). The virulence of this agent was increased by seven serial passages of infectious tissue extracts from sheep SH93 into uninfected Border Leicester sheep (86). The virus used in this study was obtained from supernatant fluid of 14-day-old cultured macrophages derived from blood of a sheep (SH149) during *in vivo* passage 5 of the virus. This stock virus had a reverse transcriptase (RT) activity of 70,000 cpm/ml and an infectivity assay titer of 5×10^6 50% tissue culture infective doses per ml.

Sheep. Yearling (young adult) Border Leicester sheep were used in this study. Six sheep were inoculated intravenously with virus derived from SH149. All became persistently infected within 1 week as determined by culture of PBMC (see below), and three sheep developed pneumonia and cachexia within 1 year. Two other uninfected sheep maintained in a separate area were used as a source of uninfected PBMC.

Surgical technique. Infected sheep were placed under anesthesia by using halothane and prepared for surgery by standard procedures. The lymphatic duct central to the prescapular lymph node was located, close to the omocervical vein, and cannulated as described by Cahill et al. (7). Polyethylene tubing (PE60; Clay Adams, Lincoln Park, N.J.) coated with tridodecylmethyl ammonium coating heparinate (Polysciences, Warrington, Pa.) (11) was secured in the duct under surgically sterile conditions. Lymph drained into polypropylene bottles containing 100 μ g of gentamicin (GIBCO) per ml and 500 IU of heparin (Sigma) which were changed twice daily.

Leukocyte cultures. Leukocytes were purified from blood on Ficoll-Hypaque density gradients and cultured in two types of media to facilitate either proliferation of activated lymphocytes or differentiation of macrophages, respectively.

(i) **Activated lymphocytes.** PBMC were suspended at a concentration of 10^6 cells per ml of RPMI medium supplemented with 1 mM glutamine (GIBCO), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (GIBCO), 0.1 mM 2-mercaptoethanol (Sigma), 60 μ g of gentamicin (GIBCO) per ml, and 10% fetal bovine serum (GIBCO) (R10-FBS) and incubated at 37°C. Phytohemagglutinin (PHA) at a concentration of 2 μ g/ml was incorporated into the medium for the first 3 days and was replaced in a medium change with R10-FBS containing human recombinant interleukin-2 (IL-2) (100 U/ml) (Cetus Corp.). This culture medium produced proliferating lymphocytes (PHA blasts) and is referred to here as lymphocyte activation medium (LAM).

(ii) **Differentiated macrophages.** PBMC at a concentration of 10^6 cells per ml were suspended in RPMI medium with the additives listed above and supplemented with 20% heated (56°C for 30 min) lamb serum (GIBCO) instead of FBS. This medium is referred to here as macrophage differentiation medium (MDM). Cells were incubated at 37°C in Teflon bottles (Pierce), where they differentiated into macrophages during the following 10 days. However, the cells, including macrophages, remained in suspension (Fig. 1). Upon transfer of 10- to 14-day-old cells from Teflon bottles to tissue culture dishes, the cells became typically fully spread,

FIG. 1. Sheep PBMC cultured in MDM in a Teflon flask for 2 weeks. The largest cells are macrophages, and the smallest cells are lymphocytes. Giemsa stain. $\times 250$.

adherent macrophages within a few hours with incubation at 37°C.

Goat synovial membrane cells. Goat synovial membrane cells are derived annually in our laboratory from the carpal synovia of colostrum-deprived newborn goats (59). Synovia are explanted and cellular outgrowth is expanded by two cultivations, and the cells are frozen with dimethyl sulfoxide in liquid nitrogen. Thawed cells were subcultured 10 to 12 times and were cultivated in minimum essential medium and 10 and 1% FBS, respectively, for growth and maintenance. These cells fused rapidly (within 24 h) upon contact with infected macrophages and were used in this study only as an indicator of infectivity in macrophages by this fusion phenomenon.

Virus assays. (i) **ICA.** Macrophage cultures were the most sensitive indicators of infectivity. Ten-day-old normal ovine macrophages were transferred from Teflon bottles to polypropylene tubes in aliquots of 1 ml of MDM containing 10^5 cells. Doubling dilutions of a specific number of potentially infected cells were added to the macrophages, and the cocultures were incubated for 15 days. Cells in each tube were then transferred to individual wells in 24-well culture plates, and goat synovial membrane cells were added. The plates were incubated at 37°C for 3 to 5 days and then stained with Giemsa and examined for multinucleated cell formation on the principle that infected macrophages fuse rapidly with goat synovial membrane cells. Infectious center assay (ICA) results are expressed as number of infected cells per 10^6 cells.

(ii) **RT assay.** Supernatant fluids from cultures were examined for RT activity as described previously (53). Clarified cell culture fluids were diluted 1/2 in RPMI medium, and then virus was pelleted and resuspended in 50 mM Tris-HCl (pH 7.8)–0.1% Triton X-100–20 mM KCl–10 mM dithiothreitol–5 mM $MgCl_2$ –0.1 mM dATP–0.1 mM dCTP–0.1 mM dGTP–0.05 optical density units of poly(rA)-oligo(dT) per ml–20 μ Ci of [^3H]TTP per ml. Labeled DNA was precip-

TABLE 1. MABs to sheep leukocyte antigens

Antigen(s)	Specificity	MAB(s)	Reference(s)
CD1, T6	B and cortical T cells, Langerhans cells, some macrophages	20.27	45
CD4	MHC class II-restricted T cells	44.38, 44.97	49
CD8	MHC class I-restricted T cells	38.65	49
T19	CD2 ⁻ CD4 ⁻ CD8 ⁻ CD5 ⁺ $\gamma\delta$ C ⁺ T cells	19.19	43, 46
CD45RA	B, naive T, dendritic' cells?, monocytes?, NK cells?	20.96	41, 47
$\gamma\delta$ T-cell receptor	CD2 ⁻ CD4 ⁻ CD8 ⁻ , T19 _H , some CD8 ^{lo}	86D	43
MHC class II	B cells, activated T cells, macrophage-dendritic cell-monocyte lineage	49.1, 42.20, 28.1	70
MHC class I	All leukocytes	41.19	20
CD11a, LFA-1	Broad	F10-150	41
Unknown	All leukocytes	72.87	23
Unknown	Macrophages, granulocytes	ILA22, ILA24	12

itated by adding 400 mg of salmon sperm DNA and 20% trichloroacetic acid in 0.1 mM sodium PP_i, and the radioactivity was counted by scintillation spectroscopy. The RT assay background from uninfected cultures was 400 to 1,300 cpm/ml.

MABs. The MABs used are listed in Table 1. MABs ILA22 and ILA24, which bind to a surface antigen of macrophages, monocytes, and granulocytes (12), were kindly provided by J. Ellis, Wyoming State Veterinary Laboratory, University of Wyoming, and MABs F10-150, to LFA-1 (41), and 86D, to the sheep $\gamma\delta$ T-cell receptor (43), were kind gifts from C. R. Mackay of The Basel Institute for Immunology. The other MABs were produced at the Centre for Animal Biotechnology, Melbourne, Australia.

MAB 20.96 recognizes the high-molecular-weight (220,000/205,000) isoform of leucocyte common antigen, CD45RA, which, as in humans, is on B cells and naive T cells and is not expressed by memory T cells, macrophages, or granulocytes (41, 47). It is unclear whether sheep monocytes, dendritic cells, or natural killer (NK) cells express CD45RA, but these cells are probably similar to their counterparts in humans (21). Human CD45RA is expressed on some NK cells and on both monocytes and dendritic cells in blood (85). All four subsets of sheep MHC class II antigens are recognized by MAB 49.1 (70). Also included were MABs specific for T-cell antigens CD4, CD8, and T19, which define the three major T-cell subsets of blood and lymph in ruminants (45, 46, 49), and MAB 41.19, to MHC class I (20). MAB 72-87 (immunoglobulin G2a [IgG2a]) reacts with a 180/95-kDa molecule on the surface of all lymphocytes, monocytes, and granulocytes

in thymus, lymph node, blood, and lymph of sheep and cattle (23). Expression of the 72-87 antigen was not increased on PHA-stimulated lymphocytes (Fig. 2).

Cell separations. Sheep PBMC were obtained from blood by using Ficoll-Hypaque. Adherent PBMC were removed by incubation in plastic flasks for 1 h at 37°C and then by passage over nylon wool (28, 62). In some experiments, the nylon wool was compressed to retrieve adherent cells. Specific subsets of nonadherent cells were subsequently removed by incubation with MABs and then by panning in petri dishes coated with anti-mouse antibody by the method of Mage (50). Bacteriological-grade 90-mm petri dishes (VWR) were incubated overnight at 4°C with 10 ml of 0.05 M Na₂HPO₄ containing sheep anti-mouse IgG Fc (Jackson ImmunoResearch, West Grove, Pa.) at 4 μ g/ml. This antibody was discarded, and then nonspecific binding sites were blocked by incubation with lamb serum at 1/500 in the phosphate buffer. Ascitic fluid diluted 1/800 (or 1/400 for anti-CD4) in Hanks buffer containing 4% lamb serum was incubated with 1×10^7 to 3×10^7 leukocytes for 30 min at 4°C, and then the cells were resuspended in fresh Hanks buffer containing 4% lamb serum and placed in anti-mouse Ig-coated petri dishes for 30 min at 4°C before unbound cells were harvested.

The effectiveness of these procedures was assessed by Giemsa and immunoperoxidase staining of cytopspins, by cultivation of PBMC in chamber slides (Nunc), and by flow microfluorimetry (Fig. 3; Table 2).

Leukocyte phenotyping. Cytocentrifuged preparations of cells were made with a Shandon cytospin 2 centrifuge. The

FIG. 2. Immunofluorescence profiles of nonadherent PBMC cultured for 24 h with PHA (+PHA), or after inoculation with virus (+virus), or without either virus or PHA (control). PHA treatment induced increased expression of MHC class I antigens, CD44, and MHC class II antigens, but there was no response to inoculation with virus.

FIG. 3. Immunofluorescence profiles of sheep PBMC adherent to nylon wool, nonadherent PBMC, and nonadherent PBMC after 24 h of culture in 10% FBS without mitogen or IL-2. This indicates that many CD45RA^{hi} and MHC class II^{hi} cells adhered to the nylon wool. A phenotypic profile of the nonadherent cells is shown in Table 2.

immunocytochemical localization of MAb binding on ethanol-fixed cells by using the indirect immunoperoxidase technique was as described by Gorrell et al. (22), except that the peroxidase-conjugated rabbit anti-mouse IgG (Dako) was used at 1/25 diluted in 5% normal sheep serum in fresh phosphate-buffered saline, and cells reacted with MAb ILA24 were not fixed until after the anti-mouse IgG incubation.

Immunofluorescence staining of cell suspensions involved incubating 10^6 leukocytes with 0.1 ml of hybridoma supernatant or of mouse ascites diluted 1/400 in 10% FBS-RPMI and used dichlorotriazinyl amino fluorescein-conjugated anti-mouse IgG Fab (Jackson ImmunoResearch) as described previously (22, 54). Flow microfluorimetry analyses were performed on a FACSTAR (Becton Dickinson), FACSCAN (Becton Dickinson), or EPICS (Coulter Electronics) machine, gating lymphocytes, monocytes, or macrophages as appropriate. MAbs ILA22 and ILA24 were used to help delineate the latter two gates (12, 13).

RESULTS

LAM and MDM were used to cultivate PBMC from infected sheep. Preliminary experiments showed that when

TABLE 2. Phenotypic profiles of sheep PBMC before and after subset depletion

Surface antigen	% of total lymphocytes stained by MAbs to these surface antigens		
	Unseparated	Non-adherent	CD8-depleted nonadherent
CD4	20	44	58
CD8 ^{hi}	12	10	3
CD8 ^{lo}	5	6	1
T19	21	ND ^a	16
$\gamma\delta$ T-cell receptor	42	7	18
CD45RA	35	23	12
MHC class II	40	22	ND
Ig	32	ND	10

^a ND, not done.

TABLE 3. Virus replication in PBMC cultured in MDM and LAM

Expt no.	n ^a	Treatment	Macro-phages present ^b	ICA titer ^c	RT (cpm/ml)
1	22	MDM	+	ND ^d	97,050
2	3	MDM	+	>2,049	41,700
3	1	MDM	+	80	35,000
4	1	MDM	+	ND	39,800
5	3	MDM	+	338	4,000
6	3	MDM	+	183	6,210
No virus	8	MDM	+	<0.1	1,000
1	9	LAM	ND	ND	5,250
2	3	MDM + IL-2	+	>2,049	9,300
3	1	MDM + IL-2	+	<0.2	ND
4	1	MDM + IL-2	+	ND	8,400
5	3	MDM + PHA + IL-2	+	<2.9	1,050
6	3	MDM + PHA + IL-2	+	<2.9	1,100

^a n, number of replicate cultures.

^b Presence of macrophages determined by morphology, flow microfluorimetry, and/or adherence.

^c ICA results are expressed as the number of PBMC yielding virus per 10^6 PBMC.

^d ND, not done.

PBMC were cultivated in LAM, T lymphocytes were activated and proliferated predictably but macrophages failed to differentiate. Many PBMC became adherent to the plastic but did not spread or develop processes. In contrast, cultivation of the PBMC in MDM resulted in development of typical large adherent macrophages (Fig. 1) having phagocytic properties. After 2 weeks, MDM cultures contained about 40% CD4⁺ cells, 15% CD8⁺ cells, 20% B cells, few $\gamma\delta$ ⁺ and T19⁺ cells, and 20% macrophages. The phenotypes of these macrophages, defined as such by morphology or by 0- and 90-degree scatter, were examined by flow microfluorimetry of macrophages from suspension cultures and by immunoperoxidase staining of both adherent cultures and cytocentrifuged cells. Macrophages were MHC class I⁺ MHC class II⁺ CD45RA⁺ CD4⁺, many (30 to 80%) were CD1⁺, and most were ILA24⁺. Approximately 5×10^7 PBMC from infected sheep were cultivated in 20 ml of LAM and MDM for 14 days at 37°C, after which supernatant fluids from each culture were examined for RT activity. Table 3 shows that after 2 weeks, the MDM contained approximately 20-fold more RT counts than the LAM (Table 3). PBMC from uninfected sheep cultivated in either type of medium failed to produce RT activity (Table 3). To offset the possibility that different media interfered with the RT assay, spent media of either type obtained from cultures of uninfected cells were added 1:1 to the heterologous RT-containing medium. These mixtures were incubated for 1 h at 37°C and processed for RT activity. The type of medium had no effect on RT counts (data not shown). This experiment confirmed earlier reports that differentiating macrophages from infectious PBMC are the highest producers of virus particles (16, 62). PBMC cultured in LAM produced low but significant levels of RT activity, but whether these virus particles were produced by lymphocytes or incompletely differentiated macrophage precursors was not clear. Whether proliferating CD8⁺ cells among the PHA blasts caused reduced virus production by infected cells was examined by depleting infectious PBMC of CD8⁺ cells

TABLE 4. Infectivity and virus replication in central lymph cells

Treatment	No. of infected cells/10 ⁶ lymph cells (ICA) or cpm/ml (RT) on postcannulation day:			
	0 (n = 3)	1 (n = 4)	2 (n = 2)	3 (n = 2) ^a
ICA				
Fresh cells	ND ^b	ND	<1	0.25
2 weeks in MDM	3.3	0.7	ND	<0.13
RT after 2 weeks in:				
MDM	1,050	1,288	525	1,300
LAM	ND	1,300	500	1,375
IL-2-FBS	ND	ND	600	1,100

^a For day 3, RT results have n = 2 but ICA results have n = 1.^b ND, not done.

before cultivation in LAM. Removal of CD8⁺ cells had no significant effect on RT counts (data not shown).

To identify cells capable of amplifying virus from latently infected cells, we inoculated 4×10^6 infectious PBMC into two preparations of 1×10^7 uninfected PBMC, cultured in LAM and MDM, respectively. The cells in LAM received an equal number of fresh uninfected PHA blasts at weekly intervals for 4 weeks as is done for primary isolation of HIV (73). The MDM cultures were ended at 2 weeks. No significant RT activity developed in the LAM, whereas supernatant fluid from cells in the MDM had an RT count in excess of 50,000 cpm/ml. These results suggested that PHA-activated uninfected PBMC were incapable of rescuing and amplifying virus from latently infected cells in infectious PBMC.

Lymph. Infectivity of cells in PBMC was compared with that in central lymph. Cultivation of cells from three collections of lymph at 1×10^6 , 4×10^6 , and 8×10^6 cells per ml in MDM, LAM, or LAM without PHA for 14 days resulted in no significant production of virus as measured by RT (Table 4). Further, less than 1 cell per 10^6 freshly collected cells had infectivity when measured by infectious center assays in macrophage cultures (Table 4). These few infected cells were probably in the contaminating dendritic cell-monocyte subset. The central lymph contained 5×10^7 cells per ml, composed of 44% CD4⁺, 24% CD8⁺, 44% CD45RA⁺, 32% MHC class II⁺, 23% Ig⁺, 5% T19⁺, 7% $\gamma\delta$ ⁺, and <1% ILA24⁺ cells, 0.2 to 0.5% monocytes and dendritic cells, and 0.4 to 1% neutrophils. After 2 weeks in LAM, the lymph cells were 40% CD4⁺, 38% CD8⁺, 24% $\gamma\delta$ ⁺, and 10% CD45RA⁺. Equivalent lymph cells cultivated in MDM were 50% CD4⁺, 21% CD8⁺, and 20% CD45RA⁺.

TABLE 6. Phenotypic profiles of sheep PBMC

Leukocyte antigen	% of total lymphocytes stained by MAbs to these leukocyte surface antigens					
	Uninfected sheep			Infected sheep ^a		
	109	157	155	149	153	174
CD4	22	20	20	17	28	28
CD8 ^{hi}	15	12	15	11	10	18
CD8 ^{lo}	4	5	3	6	8	5
T19	18	21	16	17	23	10
CD5	70	67	59	66	82	ND ^c
Ig	31	32	32	40	23	ND
CD45RA	40	35	53	48	35	43
MHC class II	38	40	40	56	ND	47
MHC class II 49.1 ^b	71	60	77	56	ND	77

^a These blood samples were taken 4 months (sheep 149), 7 weeks (sheep 153), or 5 months (sheep 174) after infection.^b More cells are stained by MAb 49.1 than are stained by other anti-MHC class II MAbs (22).^c ND, not done.

These results showed clearly that lymph was a very poor source of this virus compared with blood (Tables 3 and 5).

Identification of infected PBMC. Our experiments designed to identify the latently infected cells in PBMC from infected sheep involved depleting specific subpopulations and performing ICAs with cultured macrophages as the indicator cells. Lentivirus infection of sheep PBMC is latent, and no virus was detected in the plasma of the sheep under study.

The PBMC phenotypes of these ewes (Table 6) were all within the normal range (6, 22, 33, 46). The blood leukocytes of these sheep were about 50 to 70% lymphocytes, 30 to 40% neutrophils, and 3 to 4% monocytes. Dendritic cells cannot be identified by Giemsa stain or flow microfluorimetry, so they could have been counted as lymphocytes or as monocytes. Infected PBMC were selectively depleted of monocytes and B cells by adherence to glass beads and nylon wool. The removal of most B cells, which express high levels of CD45RA and MHC class II (CD45RA^{hi} MHC class II^{hi}), was confirmed by immunoperoxidase staining of cytocentrifuged cells (data not shown) and by flow microfluorimetry (Fig. 3; Table 2). Most of the nonadherent, CD45RA^{hi} MHC class II^{hi} cells were Ig⁻ (data not shown). The preparations of nonadherent PBMC contained less than 0.5% "monocytes" as assessed by Giemsa staining. An unknown portion of these "monocytes" were probably dendritic cells, which do not adhere to nylon wool. Nonadherent cells were then depleted of cells bearing CD4, CD8, T19, $\gamma\delta$ T-cell receptor,

TABLE 5. Infectivity in sheep PBMC depleted of various subsets

PBMC	No. of infected cells/10 ⁶ fresh PBMC in expt (ICA):							
	1	2	3	4	5	6	7	8
Total PBMC	ND ^a	ND	43	2.1	1.25	14	500	43
Nonadherent PBMC	143	111	ND	2.1	3.2	6	1,000	800
Nonadherent PBMC depleted of the subsets:								
CD4 ⁺	50	8.8	0.5	1.5	1.9	2.3	125	500
CD8 ⁺ and T19 ⁺	125	333	ND	ND	ND	ND	77	200
CD8 ⁺ and $\gamma\delta$ T-cell receptor	ND	8.5	0.5	0.9	7	ND	ND	ND
MHC Class II ⁺	0.4	<0.4	<0.6	0.13	<0.6	34	<2	1
CD45RA ⁺	ND	ND	ND	0.13	<0.3	ND	ND	ND
MHC IIC ⁺ and CD45RA ⁺	ND	<0.1	<0.47	ND	ND	ND	ND	ND

^a ND, not done.

TABLE 7. In vitro infection of sheep PBMC

Cell type depleted	LAM culture fluids (TCID ₅₀ /ml) ^a		Cells (no. of virus-bearing cells/10 ⁶)	
	0 d.p.i. ^b	12 d.p.i.	0 d.p.i.	12 d.p.i.
None	1 × 10 ¹	1.6 × 10 ¹	>286	32
Adherent	<1 × 10 ¹	1 × 10 ⁰	>250	5
Adherent, CD8 _H , T19 _H	1.6 × 10 ¹	<1 × 10 ¹	>200	1
Adherent, MHC class II _H	<1 × 10 ¹	<1 × 10 ¹	ND ^c	111

^a TCID₅₀, 50% tissue culture infective dose.^b d.p.i., days postinfection.^c ND, not done.

CD45RA, or MHC class II antigens by panning. Assessment by immunoperoxidase staining and flow microfluorimetry showed that panning removed greater than 80% of cells bearing each target antigen (Table 2; data not shown).

The ICA revealed that up to 0.05% of PBMC were infected (Table 5). ICA titers varied between experiments, perhaps because of variability of the level of infection in the sheep, but internal replicates indicated that the intraexperimental reproducibility was within a fourfold range. Removal of MHC class II⁺ cells or CD45RA⁺ cells by using the MAbs SBU.MHC.II.49.1 and SBU.20.96 consistently reduced the infectivity of nylon wool nonadherent PBMC by 100-fold or more (Table 5). The removal of adherent cells or CD4⁺, CD8⁺, or $\gamma\delta$ ⁺ cells had little or no effect on the level of infectivity.

Susceptibility of cultured PBMC to infection. PHA-stimulated PBMC were incubated with virus at 37°C for 3 to 6 h at a multiplicity of infection of 5, washed three times, and then cultured in IL-2 for 12 days. Virus bound to approximately 0.01% of both unseparated PBMC and nonadherent PBMC as determined by ICA, but the cells did not become productively infected. The number of virus-bearing cells was far less after 12 days, and culture fluids were negative in RT assays and contained very small amounts of virus by infectivity assays (Table 7). The depletion of CD8⁺ cells from nonadherent PBMC before inoculation with virus had no influence on the number of infected cells nor did it enable the PBMC to produce virus (Table 7).

This experiment suggested that the stock virus was exclusively macrophagetropic. To determine whether the stock virus contained some lymphocytotropic virus, we inoculated PBMC, cultured them in LAM, and passaged this culture into fresh PHA blasts in an attempt to amplify any lymphocytotropic virus that may have been present. Normal PBMC (4 × 10⁷) cultured for 5 days in either LAM or MDM were inoculated with 1 ml of stock SH149-derived virus (multiplicity of infection of 0.1). After 14 days, inoculated macrophages produced 30,000 cpm of RT, but PHA blasts developed no RT activity. Two milliliters of cells and supernatant of these inoculated PHA blasts were transferred weekly, for 6 weeks, into fresh PHA blasts. Supernatant fluids from the final passage were examined for infectivity and RT activity. There was no evidence of virus replication. The virus yield fell from 10⁴ to 10¹ at passage 2 and then to undetectable levels thereafter. Further transfer of passage 6 PHA blasts to normal macrophage culture in MDM failed to cause infection in the latter. This experiment showed that T lymphocytes failed to become infected after in vitro inoculation with stock virus.

Infection and cellular activation. Lentivirus infection leads

to increased expression of MHC class II antigens, an activation marker, on macrophages in infected sheep (32) and also in macaque cells (14, 31) following infection. This prompted our investigation of the effect of virus inoculation on expression of activation antigens on PBMC. Expression of the MHC class I, MHC class II, and CD44 antigens was increased 24 h after exposure to PHA but not after exposure to virus at a multiplicity of infection of 5 (Fig. 2). Expression of the 72-87 antigen on lymphocytes was not increased by PHA stimulation, showing that flow microfluorimetry profiles were not influenced by the greater average cell size of the activated T lymphocytes. This experiment showed that the PHA-treated PBMC were indeed activated and that inoculation of the virus with unstimulated PBMC did not activate a detectable number of PBMC. The proportion of cells bearing virus after this inoculation was greater than 0.02% (Table 7) and perhaps greater than 0.2% (Table 3), but the limit of detection of increased expression of a molecule on PBMC is 1%. Therefore, this experiment probably would not have detected increased expression of a molecule if the increase was restricted to those cells that bound detectable infectious virus.

DISCUSSION

This study, using an animal-passaged, pathogenic ovine lentivirus, showed that the macrophage is the most permissive cell type for supporting replication of the virus and for amplifying latent virus in PBMC and that activated T cells do not support productive virus replication. Furthermore, very little virus was detected in samples of central lymph, the best source of purified lymphocytes and rich in CD4⁺ cells. Depletion of PBMC subsets provided indirect evidence that the predominant infected cell in PBMC is most probably the dendritic cell and not the monocyte or the activated CD4⁺ lymphocyte.

Cultivation of infectious PBMC in MDM, which favors the development of macrophages, resulted in massive virus production (Table 3). However, cultivation of infectious PBMC or inoculated, naive PBMC in LAM did not result in virus production. In contrast, human and macaque PBMC infected with HIV and SIV, respectively, are highly permissive for viral replication when cultured in LAM (53, 61, 71, 73). The PHA and IL-2 in LAM cause ruminant T cells to become activated and proliferate but preferentially induce the proliferation of activated CD8⁺ cells (52, 76). Further, primate CD8⁺ cells are known to suppress HIV and SIV in cultures of infected lymphocytes (26, 30, 36, 57, 68, 82). However, in our study, depletion of CD8⁺ cells from infectious ovine PBMC prior to cultivation in LAM or addition of infectious PBMC to fresh PHA-induced lymphoid blast cells derived from normal sheep did not result in virus production. This showed that the failure of activated CD4⁺ cells to produce virus was not due to inhibitory CD8⁺ cells. These two procedures enhance recovery of HIV from infectious human PBMC with cultures of activated CD4 lymphocytes (68, 73). Central lymph provided the best source of purified lymphocytes because it lacks monocytes, macrophages, dendritic cells (11, 44, 69), and NK cells (79). The extremely low level of infection among lymph cells provided additional evidence that this virus does not infect lymphocytes. This concurs with previous work showing that sheep lentivirus is not lymphocytotropic (16, 17, 74, 77) and contrasts the lentiviruses of sheep and goats with the primate lentiviruses. However, there is one report that in tissue sections of the central nervous system of Icelandic sheep, some cells ex-

pressing visna virus core protein have the morphology of lymphocytes (18). In contrast, others have searched and failed to find evidence of visna virus protein, RNA, or DNA in lymphocytes (16, 17, 74, 77). Perhaps there is a difference between Icelandic and North American breeds of sheep, or perhaps some members of the monocyte-dendritic cell-macrophage lineage can appear to have the morphology of a lymphocyte in tissue sections.

Addition of PHA and IL-2 or IL-2 to cultures of infectious sheep PBMC reduced viral replication (Table 3). This indicates that PHA and IL-2 reduce the ability of macrophages and/or their precursors to support viral replication by acting on these cells either directly or indirectly via a product of activated T cells or by inducing cytotoxic cells, such as CD8⁺ T cells or lymphokine-activated killer cells, to kill infected cells. The lack of evidence of CD8⁺ inhibitory cells is discussed above. However, a direct inhibitory effect of PHA-IL-2 on the development of adherent, fibrocytic macrophages was observed. IL-2 is known to redirect the maturation of human macrophage precursors such that they become NK-like cells rather than macrophages (39, 48). Therefore, the reduced virus production may have resulted from inhibition of the development of macrophage precursors into macrophages. Previous studies have shown that lentivirus production in PBMC correlated with development of macrophages in the cultures (16, 62), and virus production in these cells is contingent upon transcriptional activation factors that are produced by macrophages but not their precursors (15, 55). Both monocytes and dendritic cells in PBMC give rise to phagocytic, adherent macrophages in culture (3, 34, 64).

The richest sources of infected cells in fresh PBMC were MHC class II⁺ and CD45RA⁺ nonadherent cells (Table 5). Removal of sheep monocytes and B cells by adherence to glass beads and nylon wool did not remove infectious PBMC. T cells, NK cells, and dendritic cells are nonadherent (3, 28, 34, 64, 66, 79), and the nonadherent cells contained most of the infectious PBMC. In sheep PBMC, naive T cells are CD45RA⁺ and memory and activated T cells are CD45RA⁻ (41), as is also true of humans (1, 51, 58, 78, 83). Conversely, MHC class II is expressed by memory and activated T cells (21, 41). Thus, MHC class II and CD45RA are mutually exclusive markers on sheep T cells. Furthermore, SIV-infected monkey T cells in PBMC are activated and memory cells and do not express CD45RA (84), so it is very unlikely that the CD45RA⁺ infected PBMC in sheep are T cells. Our finding that the removal of individual CD4⁺, CD8⁺, T19⁺, and $\gamma\delta$ T-cell-receptor-positive T-cell subsets did not decrease infectivity further indicates that T cells were not the infected CD45RA⁺ or MHC class II⁺ cells. Similarly, examination of NK-cell phenotypes indicates that NK cells were not the infected cell type identified in this experiment. Some CD45RA⁺ sheep cells are NK cells (37, 38, 41), but by analogy with human cells these are naive (4, 27) MHC class II⁻ (79) cells. Thus, the remaining PBMC subset, the dendritic cell, appears to be the infected cell type, and examination of its phenotype strongly supports this conclusion. Sheep dendritic cells are MHC class II⁺ (3, 5, 12, 13, 25, 44) and are probably CD45RA⁺ by analogy with human PBMC-derived dendritic cells (85), which compose about 3% of PBMC (34, 85). Preliminary flow microfluorimetry analyses indicated that at least some ovine dendritic cells and/or monocytes from blood are CD45RA⁺ (21). Thus, the dendritic cell is very likely the infected cell type detected in these experiments. Further confirmation of this finding awaits the development of the

means to directly identify sheep PBMC-derived dendritic cells.

Previous work from this laboratory (16, 62) showed that sheep lentivirus and the closely related lentivirus of goats, caprine arthritis-encephalitis virus, lack lymphocytotropism and suggested that the monocyte carries the bulk of blood-borne latent infection. One of these prior experiments (62) depleted PBMC simultaneously of both Ig⁺ and nylon wool-adherent cells. However, it is now known that dendritic cells carry Ig via their Fc receptors (3, 12, 25, 34) and are present in blood (34). Therefore, the technique used in this previous experiment would have removed dendritic cells in addition to monocytes and B cells. Since dendritic cells cultured in MDM mature into cells that are indistinguishable from macrophages derived from monocytes and since the macrophages derived from either cell type are highly permissive for virus replication, it is possible that these macrophages amplify virus from latently infected dendritic cells in the blood. This explains why the removal of Fc receptor-bearing and adherent cells or of phagocytic cells from cultures of infectious PBMC after 5 days *in vitro* removes the ability of those cultures to produce virus (62). Similarly, our present work builds on previous work which used the VMA-5 isolate of sheep lentivirus and found that infection in blood is restricted to "monocytes" (16). Dendritic cells are probably a subset of the cells previously identified morphologically as monocytes.

Our indirect evidence that dendritic cells rather than monocytes carry virus in the blood is concordant with the widespread observation that the macrophage is the principal cell type permissive for viral replication in tissues (86, 87) and also concurs with reports that human PBMC-derived dendritic cells are permissive for HIV replication (64, 65). Monocytes and dendritic cells from PBMC are bone marrow derived (75), and both give rise to cells of the mature macrophage lineage (3, 34, 64). This lineage includes alveolar macrophages, microglia (40), Kupffer cells, Langerhans cells, and interdigitating dendritic cells (56, 75). Numerous reports have shown that mature cells of this lineage are targets for virus replication in ungulates (16, 17, 87) and primates (8, 29, 67, 72, 80, 81). The link between each of the differentiated cell types and the two bone marrow-derived precursors has not been clearly established (19, 66). However, it is known that more than 56% of monocytes become Kupffer cells (9), whereas most dendritic cells are thought to become antigen-presenting cells in lymphoid tissue (2, 75). This work and the observation that ovine lentivirus infection is greater in lymphoid organs than in liver (60) are consistent with our new finding that the virus has a greater tropism for dendritic cells than monocytes.

Given the high affinity of the primate lentiviruses HIV and SIV and the feline immunodeficiency virus for CD4 and the reduced numbers of CD4⁺ cells in sheep with chronic disease (33), it is surprising that the ovine virus did not infect cells bearing this molecule. This finding and the lack of CD4 expression by sheep macrophages seen here and previously (22, 24) indicate that CD4 is unlikely to be the cellular receptor for sheep lentivirus. The observation that infected PBMC are within the MHC class II⁺ and CD45RA⁺ subsets supports, in part, the recent report that MHC class II acts as a cellular receptor for ovine lentivirus (10). Clearly, ovine lentivirus does not infect all MHC class II⁺ T and B cells, suggesting that receptor expression is insufficient for infection. The loss of CD4⁺ cells with chronic disease remains an enigma. However, one possibility is that, in view of our evidence that lymphocytes bind virus particles (Table 7),

such virus-bearing cells are eliminated by cytotoxic T cells or nonspecifically or in an antibody-dependent manner by lymphokine-activated killer cells, NK cells, or macrophages. The loss of CD4⁺ cells may be a property of all chronic lentiviral disease. Whereas in HIV infection this loss is believed to be due in part to infection of these cells with the virus (71), it is clearly not true of sheep lentivirus infection. It is tempting to speculate that CD4⁺ cell loss may be related to their interaction, via MHC class II and other cell surface molecules, with the virus-bearing antigen-presenting cells, macrophages, and dendritic cells.

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