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Primary cultures of endothelial cells from the human liver sinusoid are permissive for human immunodeficiency virus type 1

(CD4 receptors/von Willebrand factor/immunocytochemistry)

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ABSTRACT **Human endothelial cells isolated from hepatic sinusoids were infected *in vitro* with human immunodeficiency virus type 1 (HIV-1). An early sign of infection occurring in the culture was the formation of multinucleated cells. By double-labeling immunofluorescence, 5–15% of the cells recognized as endothelial cells owing to the presence of von Willebrand factor were found to contain HIV p24 and gp120 antigens after 2 weeks. Reverse transcriptase activity was released into the medium, and different steps in the process of viral budding were observed by electron microscopy. The virus produced by the endothelial cells was found to be infectious for CEM cells, a human T-cell line. CD4 molecules are present at the surface of the endothelial cells, as demonstrated by immunogold–silver staining and backscattered electron imaging. Treatment with an anti-CD4 antibody abolished productive infection of the sinusoidal endothelial cells. The possibility that endothelial cells of the liver sinusoid are infected *in vivo* with HIV remains to be clearly shown.**

Human immunodeficiency virus (HIV), the major etiological factor responsible for the acquired immunodeficiency syndrome (AIDS), has been shown to replicate predominantly in cells of the lymphoid and monocyte–macrophage lineages (1). Other nonhematopoietic cell types have, however, also been claimed to be susceptible targets for the virus, notably endothelial cells, although conflicting results have been obtained. In the brain, frequently targeted in AIDS, infection of endothelial cells has been revealed by immunocytochemistry and *in situ* hybridization in some patients (2–6). However, other studies, including electron microscopic examinations, were unable to demonstrate signs of virus replication in endothelial cells (7–21). In other tissues, such as lymph nodes (5, 22, 23), submucosa of the cervix (24), and villous tissue of the placenta (25), HIV has also been described in association with endothelial cells. A recent study has again emphasized the possible role of endothelial cells in HIV infection, given the high levels of von Willebrand factor (vWF; factor VIII-related antigen), an endothelial cell marker, regularly found in the plasma of patients at various stages of HIV infection (26).

Biopsies have shown that the involvement of the liver in AIDS patients is often restricted to opportunistic infections and to a few AIDS-related tumors, but alterations possibly due to HIV infection itself have also been observed (for review, see ref. 27). Abnormalities in the liver sinusoid such as Kupffer cell hyperplasia (28, 29), sinusoidal dilatation (28–30), and peliosis hepatis (28, 31, 32) have been reported. On the other hand, no conclusive evidence has been presented for HIV type 1 (HIV-1) in the liver of AIDS patients, be it in the hepatocytes, in the Kupffer cells, or along the sinusoids (5, 29, 33, 34, ||).

In the present study we demonstrate that primary cultures of human liver sinusoidal endothelial cells (SEC) are permissive for HIV-1 replication, as is the case for Kupffer cells (35).

MATERIALS AND METHODS

Isolation and Culture of Endothelial Cells from the Hepatic Sinusoid. Surgical samples were obtained from HIV-seronegative patients after partial hepatectomy for liver cancer. Sinusoidal cells were isolated by collagenase perfusion and centrifugal elutriation according to the method previously described (36). The endothelial cells of the liver sinusoid (SEC) were plated in either Falcon Primaria or collagen-coated 24-well Corning tissue culture trays. They were incubated in Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, 10 mM $NaHCO_3$, gentamycin at 50 μg/ml, and 20% heat-inactivated human type AB serum. Twelve liver samples were perfused to isolate the endothelial cells used in this study.

HIV Infection. Each culture was infected 3–8 days after cell isolation with 200 μl per well of HIV-1 Bru (37) or the HTLV-IIIB strain of HIV-1 (ref. 38; reverse transcriptase activity of 5×10^6 cpm/ml) for 1 hr, then extensively washed and fed with fresh medium. The medium was completely renewed twice a week. These infected primary cultures were maintained for 15–30 days.

To measure the release of virus by the cells, the culture medium was assayed for reverse transcriptase activity. Cell-free supernatants were ultracentrifuged at 350,000 × *g* for 10 min. The viral pellet was resuspended in 100 μl of RPMI medium 1640, and dissociated by addition of 15 μl of detergent solution (0.5% Triton X-100/0.75 M KCl/50 mM DL-dithiothreitol). The reverse transcriptase activity was measured according to T. O. Jonassen (Skatron application note, April 1986).

To evaluate the infectivity of the virus propagated in SEC, supernatants from endothelial cell cultures (reverse tran-

Abbreviations: HIV, human immunodeficiency virus; HIV-1, HIV type 1; SEC, sinusoidal endothelial cells of the liver; SEM, scanning electron microscopy; TEM, transmission electron microscopy; vWF, von Willebrand factor; WPB, Weibel–Palade body or bodies.

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scriptase activity of 200,000 cpm/ml) were added to permissive CEM cells (a human T-cell line) at a 1:40 dilution for 1 hr. Reverse transcriptase activity in the CEM supernatant was assessed twice a week for a fortnight.

Electron Microscopy. The cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (75 mM sodium cacodylate, pH 7.3/4.5% sucrose/1 mM $MgCl_2$/1 mM $CaCl_2$) for 48 hr and postfixed with 1% OsO_4 before being either dried with hexamethyldisilazane for scanning electron microscopy (SEM) or embedded in LX112 (Ladd Research Industries, Burlington, VT) for transmission electron microscopy (TEM). The SEM samples were coated with gold/palladium and examined in a Hitachi S-800 electron microscope. Ultrathin sections were observed in a Philips EM 410 electron microscope.

Immunocytochemistry. Indirect immunofluorescence was performed on cells fixed in acetone/methanol at −20°C for 20 min. Endothelial cells were identified by the presence of vWF detected with a rabbit serum Clotimmun factor VIII-associated protein/factor VIII-related antigen (Behring; 1:50). Kupffer cells and Ito cells were characterized by the anti-macrophage monoclonal antibody M718 (DAKO, Carpinteria, CA; 1:50) and by the anti-desmin monoclonal antibody M724 (DAKO; 1:50), respectively. HIV-1 antigens were detected with mouse monoclonal antibodies (anti-gp120/160, clone M38; anti-p24, clone 1-HIV-p24; Clonatec Biosoft, Paris; 1:20). The second antibody was either a fluorescein-conjugated goat antibody to mouse or rabbit IgG (Pasteur Diagnostics, Marnes-La-Coquette, France; 1:100) or a rhodamine-conjugated goat antibody to mouse IgG (Caltag, South San Francisco, CA; 1:100). In double-labeling experiments, the cells were first immunostained with antibody to HIV antigens and with rhodamine conjugate and then by treatment with vWF antibodies and fluorescein conjugate. Photographs (Nikon Diaphot inverted microscope) were taken by using alternating fluorescein and rhodamine filters on the same microscopic field.

Detection of CD4 Molecules by Backscattered Electron Imaging. The presence of CD4 on noninfected endothelial cells after 7 days in culture was detected by indirect immunogold labeling and silver enhancement, using SEM and the backscattered electron mode. The cells were fixed in 0.1% glutaraldehyde in phosphate-buffered saline for 5 min. The immune labeling was performed with the Leu-3a + 3b antibodies preparation (Becton Dickinson; 1:20). The second antibody was a 5-nm gold-conjugated goat antibody to mouse IgG (AuroProbe EM GAM IgG G5 Janssen; Amersham; 1:50). Silver enhancement was performed with the Janssen IntenSE M kit. The cells were finally fixed in 2.5% glutaraldehyde and prepared for SEM as described above except for the gold/palladium coating, which was replaced by a carbon one. They were observed under SEM in both secondary and backscattered electron modes. As controls the primary anti-CD4 antibody was replaced either by phosphate-buffered saline or by the aforementioned anti-p24 monoclonal antibody, which is the same isotype as Leu-3a + 3b.

Blocking of CD4 Receptors. Blocking of the CD4 receptor was carried out by adding to the culture medium anti-CD4 monoclonal antibody OKT4A (Ortho Diagnostics; 1:10), dialyzed to remove sodium azide. After incubation with OKT4A for 1 hr, the cells were infected and virus adsorption was carried out for 1 hr. Thereafter, the virus was washed out and the cells were maintained in medium plus OKT4A, which was renewed twice a week. The effect of OKT4A on endothelial cell infection was studied (*i*) with double-labeling immunofluorescence for HIV-1 antigen and vWF, as described above; and (*ii*) by measuring the release of p24 antigen in the supernatant of SEC cultures with an ELISA test (HIVAG-1; Abbott). As a control, OKT4A was replaced by OKT3 (Ortho Diagnostics), which is the same isotype.

RESULTS

Characterization of Cultured SEC. SEC isolated from human liver were adherent and flattened out after 24 hr in culture. Their cytoplasm showed a strong immunoreactivity for vWF (Fig. 1*a*). Under SEM, their peripheral cytoplasm appeared markedly attenuated and displayed typical fenestrae (Fig. 1*b*). The presence of Weibel–Palade bodies (WPB), known to contain vWF (39), was considered to characterize the cells as SEC in thin sections (Fig. 1*c*). The percentage of SEC in the cultures varied with the individual liver samples from 70% to 85%, the other cells being mainly Kupffer cells; some fat-storing cells (Ito cells) and epithelial cells could also be found. During culture, the development of SEC clusters was suggestive of cell proliferation (Fig. 1*a*).

Search for CD4 Receptors. Expression of CD4 receptors, known to be present *in vivo* on liver endothelial cells (34, 40, 41), was shown by backscattered electron imaging under SEM of immunogold–silver stained samples (Fig. 2*a*). A fine, intense granular labeling corresponding to the immunogold–silver complexes was found on the surface of Leu-3a + 3b-treated cells but never on untreated SEC or on SEC treated with an anti-p24 monoclonal antibody of the same isotype. Examination of the labeled cells under secondary electron imaging mode confirmed that these cells were highly flattened SEC (Fig. 2*b*).

Infection of SEC with HIV. Cultures of endothelial cells isolated from 12 liver samples were used. They were infected with either HTLV-IIIB or HIV-1 Bru 3–7 days after isolation, when several SEC clusters were present in the culture. Syncytia could be observed as early as 7 days after infection. The origin of these multinucleated giant cells was ascertained by their labeling with anti-vWF antibodies (Fig. 3*a*). Reduced intensity and differences in the cellular distribution of the vWF labeling were sometimes noted during the course of infection (results not shown). Viral p24 and gp120 antigens could be detected in syncytia and in single cells. That infected cells were SEC was demonstrated by double-labeling immunofluorescence for vWF and viral antigens (Fig. 3 *b* and *c*). The proportion of infected SEC after 2 weeks was in the range of 5–15%, depending on the experiment.

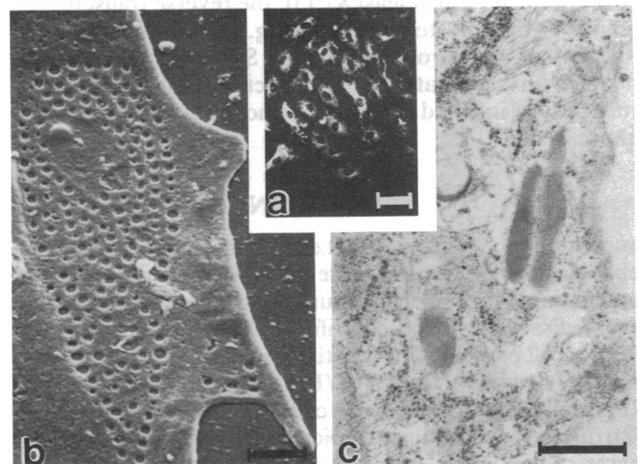

FIG. 1. Characterization of the cultured SEC. (*a*) Cells arranged in a cluster after proliferation are readily labeled with anti-vWF antibodies by indirect fluorescein isothiocyanate immunofluorescence. (*b*) Typical fenestrae arranged in sieve plates as seen under SEM. (*c*) Characteristic WPB are observed in the cytoplasm under TEM. (Bar in a = 50 μm; bars in b and c = 0.5 μm.)

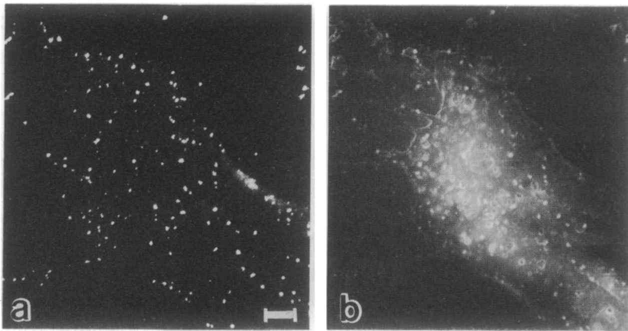

FIG. 2. Presence of CD4 receptors on SEC as shown by immunogold–silver staining under SEM. (*a*) In the backscattered electron mode, bright dots appear that reveal the presence of gold particles after silver enhancement. (*b*) In the secondary electron mode, the overall appearance of the SEC is noted. (Bar = 1 μm.)

The participation of CD4 in the infection of SEC was tested by blocking with OKT4A, a monoclonal antibody directed against the gp120 binding site on CD4, in three experiments. p24–gp120 antigens could not be detected in SEC cultures with OKT4A by double-labeling immunofluorescence assays (Fig. 3 *d* and *e*). The control cultures, infected and maintained in the presence of OKT3, a marker directed against CD3 antigen and belonging to the same isotype as OKT4A, or in the absence of any antibody, contained HIV-positive endothelial cells. Blockage of CD4 receptors for 14 days decreased the release of p24 antigen to 80 pg/ml in the presence of OKT4A (versus 6800 pg/ml in the control culture with OKT3).

By electron microscopy, clusters of viral particles were readily observed at the surface of either syncytia or single cells (Fig. 4*a*). Moreover, pictures of viral budding at the plasma membrane were observed (Fig. 4 *b* and *c*), thus proving that viral particles were produced in the endothelial cells. Budding occurred not only at the plasma membrane but also inside intracytoplasmic vacuoles (Fig. 4*d*). That the infected cells were SEC was ascertained by the presence of either typical fenestrae (Fig. 4*a*) or WPB (Fig. 4 *b* and *d*). WPB were more difficult to find in cells of infected cultures than in control cultures, which corroborates our observations of decreased vWF immunostaining in infected cells.

In three cultures with particularly high SEC contents (SEC to Kupffer cell ratio at least 85:15), the reverse transcriptase activity in the infected cultures (Fig. 5) was considered as indicative of virus production by the SEC. In transfer experiments with supernatants, the infectiousness of the virus produced by infected SEC was demonstrated on CEM cell cultures.

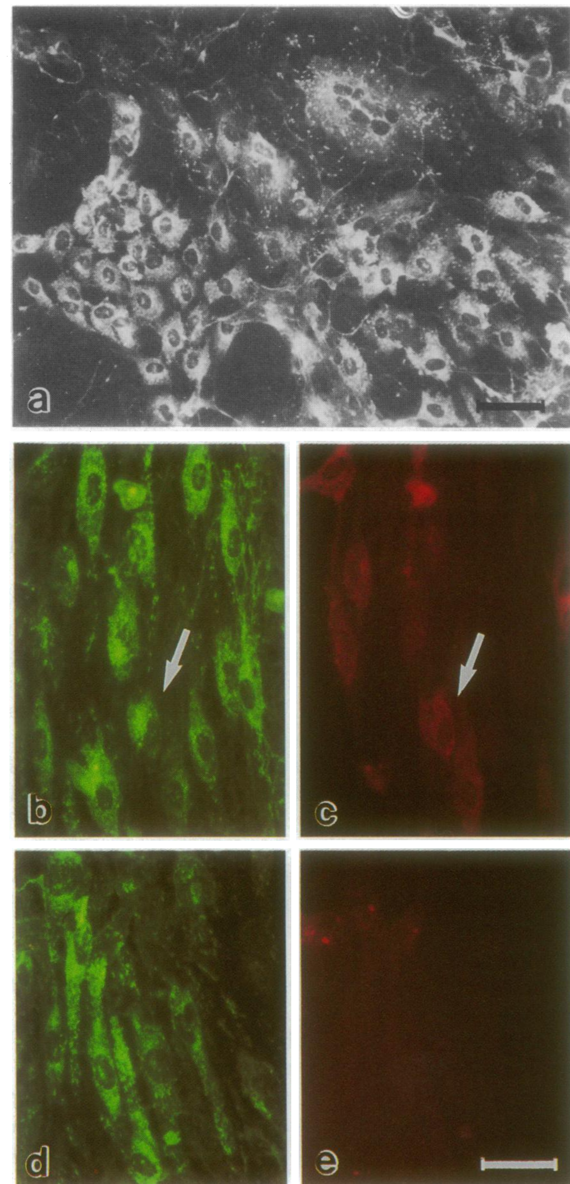

FIG. 3. Light microscopy study of HIV-infected SEC. (*a*) Presence of a syncytium in a culture infected for 7 days. It appears to contain vWF, as do the surrounding cells. (*b–e*) Double-labeling immunofluorescence 14 days after infection, revealing vWF with fluorescein labeling in *b* and *d* and HIV p24/gp120 with rhodamine in *c* and *e*. Among all the SEC present in *b*, some (arrow) are infected with HIV in *c*. This is no longer the case when the cells (*d*) are cultured in the presence of OKT4A monoclonal antibody (*e*). (Bar = 50 μm.)

DISCUSSION

From this study, it appears that endothelial cells of the human hepatic sinusoid are permissive for HIV-1 *in vitro*. This was demonstrated on primary cultures of cells isolated by collagenase dissociation and centrifugal elutriation of liver samples from 12 patients. The identification of SEC as such relied on (*i*) the demonstration of vWF (42, 43); (*ii*) the presence of WPB as previously reported in cultured liver endothelial cells (42); (*iii*) the observation of typical fenestrae under SEM. The degree of cell purity obtained was variable, depending to a large extent on the tissue sample. Given the thorough washing of the cultures before use, most of the contamination should be imputable to cells able to adhere. Accordingly, three additional cell types could be present in our cultures: (*i*) Kupffer cells, the so-called liver macrophages; (*ii*) Ito cells (fat-storing cells), found in a perisinusoidal location between the SEC and the hepatocytes; and (*iii*) epithelial cells deriving from bile ducts.

Our demonstration of the permissiveness of SEC for HIV is based on the following observations: (*i*) the appearance of vWF-positive syncytia in clusters of SEC; (*ii*) the localization of HIV antigens in cells characterized as SEC by the presence of vWF (Double-labeling immunocytochemistry revealed that not only syncytia but also single SEC contained viral antigens.); (*iii*) the budding and maturation of virus particles in cells identified in TEM as SEC by their content of WPB; (*iv*) the release of reverse transcriptase-containing particles into the medium, part of which, at least, are infectious; and (*v*) the reduction in SEC infection in the presence of an anti-CD4 antibody.

The syncytia undoubtedly include SEC, since they always showed immunoreactivity for vWF. Also, the number of

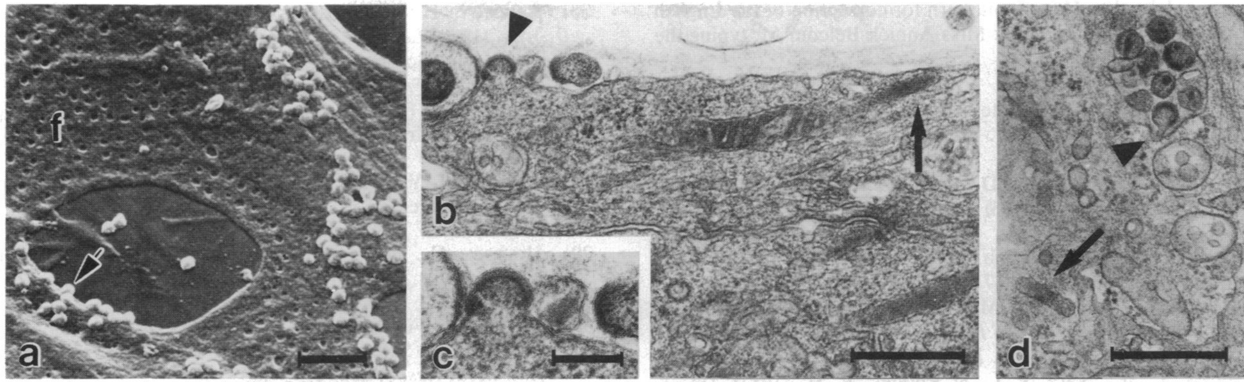

FIG. 4. Electron microscopy study on HIV-infected liver endothelial cells. (*a*) Numerous viral particles arranged on the surface of an SEC identified by its typical fenestrae (f). Pictures suggestive of viral budding may be observed (arrow). (*b*) Viral particle (arrowhead) budding from a cell containing a typical WPB (arrow). (*c*) Higher magnification of the budding shown in *b*. (*d*) Budding (arrowhead) into a vacuole of a cell containing a WPB (arrow). (Bars in *a*, *b*, and *d* = 0.5 μm; bar in *c* = 0.2 μm.)

contaminant Kupffer cells is too low and the cells are too scattered throughout the culture to allow formation of Kupffer cell syncytia. Furthermore, neither syncytia nor any sign of virus replication has ever been observed in pure cultures of Ito cells or in cocultures of hepatocytes and epithelial cells derived from the bile duct infected with HIV-1 (unpublished results). However, it cannot be excluded that, occasionally, syncytia may be formed between SEC and other cells in the cultures.

With regard to the possibility that virus may be produced by non-SEC, Kupffer cells must be considered, since their permissiveness for HIV in culture has been demonstrated (35). However, their relatively low number and the slower replication of HIV in Kupffer cells compared with SEC are probably reflected by a minor contribution to the reverse transcriptase and p24 antigen measurements in the present culture system. Thus, 20 days after infection the reverse transcriptase activity in the supernatant is already declining for SEC cultures, whereas it has not yet reached its peak in Kupffer cell cultures (35). Nevertheless, we cannot exclude that contaminating cells could be the source of cellular factors that may play an indirect role in the infection of SEC.

As also described by others (42), SEC in culture proliferate and form cell clusters (Figs. 1*a* and 3*a*), which may explain their higher permissiveness compared with the noncycling Kupffer cells, since cell multiplication may promote HIV replication, as shown to be the case for lymphocytes (44).

The ability of HIV to replicate in SEC was seen in two closely related virus strains characterized as lymphotropic, indicating that no specific tropism is needed for HIV to infect

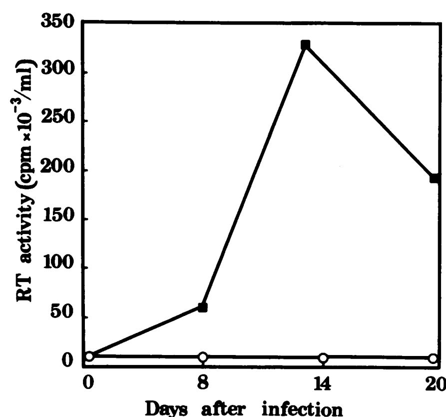

FIG. 5. Release of reverse transcriptase (RT) activity into the culture medium of SEC infected (■) or not infected (○) with HIV.

endothelial cells. Interestingly enough, the virus produced was still infectious for lymphoid cells such as CEM.

As is the case *in vivo* (34, 40, 41), CD4 receptors are expressed by cultured SEC as shown here by immunoelectron microscopy with backscattered electron imaging. This technique was chosen for its sensitiveness and the ease of correlation with morphology, SEC being identified by their overall appearance and the presence of fenestrae. The blanching by Leu-3a + 3b showed that CD4 receptors of SEC are of functional importance for the infection to occur. However, endothelial cells isolated from human adipose tissue, although permissive for HIV, appear to be CD4 negative (45).

Although some endothelial cells have been found to be infected in various organs, the significance of this observation remains unclear. The virus produced by endothelial cells could either be released into the bloodstream, thus causing viremia, or infect underlying cells, thereby promoting further dissemination of the virus. Moreover, infection of endothelial cells could result in their injury, with vascular consequences.

In AIDS, the involvement of the endothelial cells has also been considered in Kaposi sarcoma, a neoplasm frequently found in patients. Accordingly, the sarcoma cells, which have sometimes been found infected by HIV, are generally regarded as of endothelial origin (46).

Endothelial cells have also been reported susceptible to infection with other retroviruses. Antigens of SIV/Mne, a simian immunodeficiency virus closely related to HIV-2, have been found in bone marrow endothelial cells (47), and murine neurotropic retroviruses have been observed to bud from brain endothelial cells in the mouse (48, 49). On the other hand, since productive infection of cultured endothelial cells from the human umbilical vein by human T-lymphotropic virus type I has been reported (50, 51), it is worth noting that the same cells were found nonpermissive for HIV (unpublished results).

These studies show that endothelial cells may play a role in retroviral infections, as is known to be the case for some other viral infections (46). Our results obviously raise the question as to whether the endothelial cells of the liver sinusoid may be infected by HIV *in vivo*. This remains to be established, although such a possibility has been suspected by Housset and coworkers (29, 34).

Finally, it is noteworthy that two main cell types of the liver sinusoid, namely the endothelial cells and the Kupffer cells, appear, at least *in vitro* and possibly *in vivo*, to be permissive for HIV. This deserves further investigation to evaluate the role of the liver in HIV infection and in the development of the disease.

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