Feline immunodeficiency virus can productively infect cultured endothelial cells from cat brain microvessels

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Feline immunodeficiency virus (FIV) provokes a disease in cats characterized by histopathological lesions similar to those observed in AIDS patients. In order to determine whether endothelial cells from brain microvessels are involved in the central nervous system disease to the same extent as macrophages and microglia, cells were isolated from healthy cat brains, cultured and infected in vitro with the FIV Villefranche IFFA 1/88 strain. The isolated cells displayed typical endothelial cell ultrastructural features and were characterized further by von Willebrand factor-labelling and the binding of specific lectins such as *Ulex europaeus* lectin on their membrane. They were also able to take up acetylated low density lipoproteins. Two weeks after infection, significant amounts of FIV p24 antigen were detected by indirect immunofluorescence in syncytia and single cells. Concomitantly, the same antigen could be detected in the culture medium of the infected cells by an ELISA technique. Numerous viral particles as well as different steps in the process of viral budding were observed under transmission electron microscopy. The synthesis of FIV p24 antigens still occurred in cells in which replication was blocked in the G2 phase with taxol. Our results suggest the possibility of a productive infection of brain microvascular endothelial cells by FIV in vivo, which could lead to important perturbations in the functions of the blood–brain barrier.

Cats infected with the feline immunodeficiency virus (FIV) provide an interesting model for a physiopathological approach to human immunodeficiency virus (HIV) infection of the central nervous system (CNS), since FIV provokes encephalopathies in cats (Dow et al., 1990; Hurtrel et al., 1992; Gray et al., 1993) similar to those described in AIDS patients (Budka et al., 1987; Hall et al., 1991; Brüstle et al., 1992; Burns, 1992; Geleziunas et al., 1992; Rao et al., 1993) and simian immunodeficiency virus (SIV)-infected monkeys (Chakrabarti et al., 1991). Although changes in the CNS have been extensively described (for review see Sharer, 1992; Burns, 1992; Connor & Ho, 1992) the mechanism of entry of HIV and SIV into the CNS is still a matter of debate.

Given the strategic position of microvascular endothelial cells (EC) in forming the blood–brain barrier, and their numerous functions (Rosenblum, 1986; Engberg, 1989) the involvement of these cells in the dissemination of the virus has already been suggested. This could occur either by direct infection of the EC leading to viral spread into the CNS (Wiley et al., 1986; Rostad et al., 1987; Rao et al., 1993) or by cytokine-induced damage to the blood–brain barrier which may allow the passive entrance of infected cells or viruses (Genis et al., 1992). Vascular alterations have been observed in AIDS-related encephalopathies in human (Smith et al., 1990; Hall et al., 1991; Brüstle et al., 1992; Burns, 1992; Gray et al., 1993; Power et al., 1993) as well as in cats (Hurtrel et al., 1992). Several studies revealed the presence of HIV in different cell types, especially in macrophages and microglial cells (Koenig et al., 1986; Parravicini et al., 1989; Brüstle et al., 1992; Geleziunas et al., 1992), but also more rarely in astrocytes (Epstein et al., 1985; Mirra & del Rio, 1989), oligodendrocytes (Gyorkey et al., 1987) and EC (Wiley et al., 1986; Pumarola-Sune et al., 1987; Rostad et al., 1987; Ward et al., 1987; Wiley & Nelson, 1988; Smith et al., 1990). Most often, virus-infected microglial cells or macrophages were found in perivascular infiltrates in close contact with EC (Smith et al., 1990). In a recent study with cultured feline neural cells, Dow et al. (1992) found the astrocytes and microglial cells to be susceptible to FIV and the EC relatively resistant to the infection. On the other hand, we recently demonstrated that EC from the human liver are permissive for HIV in vitro (Steffan et al., 1992) and that the corresponding cells from the cat liver likewise support FIV growth in culture (Steffan et al., 1993).
Brain microvascular EC from healthy 5- to 6-month-old cats (IFFA-CREDO) were isolated according to the method described by Dorovini-Zis et al. (1991) for isolating EC from human brain microvessels. The whole brain was minced and incubated under agitation (280 r.p.m.) in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 20 mM-HEPES, 10 mM-NaHCO₃, 50 µg/ml gentamycin and 0.5% dispase (Boehringer Mannheim) for 3 h at 37 °C. The pellets obtained after centrifugation at 1000 g for 10 min were resuspended in the same medium containing 15% dextran (Sigma; average $M_r$ 70000). After another centrifugation at 5800 g for 10 min the pellets containing the microvessels were washed and collected by centrifugation and incubated in DMEM containing 1 mg/ml collagenase/dispase (Boehringer Mannheim) at 37 °C for 16 h. The microvessels were then washed, pelleted at 800 g for 10 min, suspended in 2 ml of medium and layered over a metrizamide cushion (22.3% w/v; density 1.089 g/ml; Nyegaard). After a centrifugation of 15 min at 1400 g the layer containing the EC was aspirated, washed in medium and centrifuged for 10 min at 1000 g. The purified cells were finally suspended in 15 ml of medium supplemented with 4% Ultrasol G (Sepracor; IBF) and cultured in collagen-coated 24-well Corning tissue culture trays (500 µl/well).

After 24 h of culture, numerous EC were seen escaping from clumps of microvessel cells and forming small colonies (Fig. 1 a). Cell divisions were observed and after 3 to 4 days the culture was almost confluent. The cells displayed a highly flattened-out cytoplasm and a smooth cell surface as seen under scanning electron microscopy (SEM; Fig. 1 b). Cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (75 mM-sodium cacodylate pH 7.4, 4.5% sucrose, 1 mM-MgCl₂, 1 mM-CaCl₂) for 48 h and post-fixed with 1% OsO₄ before being either dried with hexamethyldisilazane (Merck) for scanning transmission electron microscopy (STEM) or embedded in LX112 resin (Ladd Research Industries) for transmission electron microscopy (TEM). The SEM samples were coated with gold and palladium, and examined in a Hitachi S-800 electron microscope. Under TEM (Philips 410 electron microscope), numerous intracytoplasmic vacuoles were found (Fig. 1 c) together with caveolae on the side of the cell facing the culture support (Fig. 1 d). The cells displayed three properties commonly used to identify EC: (i) they could be labelled with a rabbit antiserum (1:50) to human factor VIII-related antigen (von Willebrand factor, vWF; Dako)
Fig. 2. Cultured endothelial cells, labelled with antibodies to vWF (a), take up AcLDL (b) and display receptors for UEA1 (c). Bar represents 25 μm.

detected with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Pasteur Diagnostics; 1:100; Fig. 2a); (ii) the cells were able to take up acetylated low-density lipoproteins (AcLDL; Voyta et al., 1984; Fig. 2b); (iii) they possessed receptors for *Ulex europaeus* agglutinin I (UEA I; Holthöfer et al., 1982) at their membrane (Fig. 2c). Fresh cells were incubated with AcLDL (Biomedical Technologies; 1:20) for 4 h, washed and observed under a Nikon Diaphot inverted microscope using a rhodamine filter set. Paraformaldehyde (2 %)-fixed cells were incubated with biotin-labelled UEA I at a concentration of 500 μg/ml (Sigma) for 1 h, which was revealed with streptavidin–Texas Red (Amersham; 1:100) using the same filter set.

Three days after plating, confluent monolayers of EC (2.5 × 10⁴/well) were infected with 150 μl of a suspension of the FIV strain Villefranche IFFA 1/88 (reverse transcriptase activity 400000 c.p.m./ml; propagated on chronically infected IRC4 feline kidney cells; Rhône Mérieux) at 37 °C for 3 h, washed and fed with fresh medium. As demonstrated by an immunofluorescence assay, IRC4 cells were negative for spumavirus and feline leukaemia virus (FeLV; data not shown); moreover they showed no Mn²⁺-dependent reverse transcriptase activity (Y. Giraud, Rhône-Mérieux, personal communication). As early as 7 days after infection, significant amounts of FIV p24 antigen were detected in the culture medium by ELISA performed on Nunc Maxisorp plates (Life Technologies) coated with an anti-FIV p24 monoclonal antibody (Rhône Mérieux; Fig. 3). The adsorbed FIV proteins contained in undiluted culture medium were revealed with an anti-FIV p24 monoclonal antibody labelled with peroxidase. After each sampling, the medium of the infected cultures was completely renewed.

High amounts of p24 antigen were released into the medium from 17 to 21 days after infection. Two weeks after infection, viral p24 antigen could be detected in numerous syncytia and in single cells by indirect immunofluorescence (Fig. 4). Up to 50% of EC displayed intense fluorescence. An indirect immunofluorescence assay was performed on cells fixed in acetone and methanol (1:1) at 20 °C for 20 min. They were incubated with an anti-FIV p24 monoclonal antibody, diluted 1:20 in PBS, for 1 h at room temperature. The second antibody was a FITC-conjugated anti-mouse IgG antibody (Pasteur Diagnostics), diluted 1:100. By TEM, clusters of viral particles were readily observed in the
Fig. 4. Cultures of endothelial cells infected for 2 weeks with FIV displaying syncytia, and single cells (arrows) containing high amounts of FIV p24 antigen as detected by immunofluorescence. Bar represents 20 μm.

vicinity of cells displaying endothelial features (Fig. 5a) or inside their intracytoplasmic vacuoles (Fig. 5b). Furthermore, budding viral particles confirmed the production of virus by these EC (Fig. 5c).

We wondered if the permissiveness of EC for FIV necessitated the division of the cells. Therefore, we blocked cell proliferation by treating the cultures with taxol (Sigma), a drug known to block the cell cycle in the G2 phase by generating non-functional microtubules. The drug was first dissolved in 10 mM-dimethyl-sulphoxide and further diluted with culture medium at a final concentration of 10 μM. The cells were treated 24 h before infection and the drug was maintained throughout the experiment. No proliferation was observed under light microscopy, but this did not preclude the detection of viral antigens in both syncytia and single cells (Fig. 6).

Our results demonstrate that FIV replicates intensively in EC from cat brain microvessels in vitro. A similar in vivo permissiveness would be a determinant for lentiviral-induced neurological disease since EC are the first cells of the brain to encounter potentially infected circulating lymphocytes and monocytes or viral particles. Moreover,

Fig. 5. TEM study of brain endothelial cells 2 weeks after infection. (a) Numerous mature FIV particles are found in the vicinity of endothelial cells. (b) FIV particles are also observed inside intracytoplasmic vacuoles. (c) FIV budding occurs at the plasma membrane. Bars represent 1 μm in (a) and 0.1 μm in (b) and (c).

Fig. 6. Detection of FIV p24 antigen by immunofluorescence in endothelial cells infected for 2 weeks with FIV in the presence of taxol. Bar represents 20 μm.
EC initiate and regulate the traffic of circulating blood cells across the blood–brain barrier. However, up to now, no study has demonstrated the involvement of cerebral EC in FIV infection in vivo. In HIV infection, viral antigens or viral DNA have mostly been found inside microglial cells, resident macrophages or monocytes and macrophages only. These cells have been claimed to be the principal targets for productive infection by HIV and the initiators of pathological processes (Koenig et al., 1986; Epstein & Gendelman, 1993). Although striking changes in the microvasculature, such as hypertrophy and hyperplasia of EC, increased thickness of the vascular wall and vascular mineralization have been reported (Smith et al., 1990; Brüstle et al., 1992; Burns, 1992), only a few authors have provided evidence of infection of human brain EC in AIDS patients (Wiley et al., 1986; Pumarola-Sune et al., 1987; Rostad et al., 1987; Ward et al., 1987; Wiley & Nelson, 1988; Smith et al., 1990) or of small vessel cells in the CNS of rhesus monkeys infected with SIV (Lackner et al., 1991). In most of these studies the infected cells were characterized as endothelial based on their location. On the other hand, when EC were characterized histochemically, viral products could not be detected (Kure et al., 1990) and the staining for HIV-1 antigens found in the microvessel walls could not be attributed with certainty to EC (Smith et al., 1990). The involvement of EC in HIV and SIV infections has also been considered, given the modification of concentrations of vWF and protein S in the plasma of AIDS patients (Drouet et al., 1990; Lafeuillade et al., 1992), the impairment of immune functions (Teitel et al., 1989) and the activation of endothelial-related vascular cell adhesion molecule 1 in brains of infected monkeys (Sasseville et al., 1992). It is worth noting that, in neurological diseases provoked by retroviruses such as Rous associated virus 1 in chickens (Ewert et al., 1990) or murine leukemia virus (MuLV) in mice (Kay et al., 1991; Parke et al., 1993, 1994), brain EC were identified as the main target cell containing viral particles or antigens. Moreover, a correlation between the efficiency of MuLV replication in EC in vitro and its ability to cause neurological disease in vivo has been established (Masuda et al., 1993).

Cell lines and primary cultures derived from human brain tissue have been extensively studied for their susceptibility to HIV infection (Popovic et al., 1988; Clapham et al., 1989; for review see Gonzales-Scarano et al., 1992) and correlations with the in vivo situation have been discussed. Microglial cells (Brinkmann et al., 1992) and also tumour cell lines of neuronal origin (Li et al., 1990) were found to be permissive for HIV. However, until now there has been only two reports that accord with our findings, namely the infection of primary cultures of human brain capillary EC by HIV (Moses et al., 1993) and of EC, isolated from simian cerebral white matter, by SIV (Brooks et al., 1991).

In cats experimentally infected with FIV, the virus has been recovered from several brain regions but no infection of EC has been reported (Dow et al., 1990). Moreover, isolated brain EC have been found to be rather resistant to FIV infection, in contrast to astrocytes and microglia (Dow et al., 1992). This clearly differs from the present study where up to 50% of the EC showed signs of infection, although the amount of FIV p24 antigen in the supernatant of EC was 10 to 20 times lower than that observed with chronically infected IRC4 cells (data not shown). This discrepancy may be attributable either to the use of a different virus strain or to differences in the cells used (primary cultures versus passaged cells). Early-passage feline brain cells (Kawaguchi et al., 1992) and a fetal feline brain cell line (Phillips et al., 1990) could be productively infected with different strains of FIV without characterization of the cell type involved.

The EC in our cultures were able to divide, but their permissiveness for FIV was independent of this property, as demonstrated in the experiments with taxol. Our results thus demonstrate that FIV, like HIV in HeLa cells bearing CD4 receptors, can replicate in non-dividing cells blocked in the G2 phase, in contrast to animal oncoretroviruses such as MuLV (Lewis et al., 1992). The productive infection by FIV of isolated cat brain EC reported here clearly does not provide evidence for a role of those cells in vivo. Brain cells do not appear to be special targets for FIV in the most recent in vivo study (Beebe et al., 1994). It has been shown for other viruses, such as FeLV, that tropism for cerebral EC has been acquired by recombinants generated between exogenous replication-competent and endogenous defective proviruses (Chakrabarti et al., 1994). Further in vitro investigations, carried out concomitantly with in vivo studies of experimentally infected cats should be helpful in understanding the basic mechanisms underlying FIV-induced CNS injury.

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References


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