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Persistent SIV infection of a blood–brain barrier model

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In order to better model HIV infection of the brain, a dynamic, *in vitro* model of the blood–brain barrier (the DIV-BBB) was characterized. The model was composed of simian brain microvascular endothelial cells (MVEC) cocultured with human fetal astrocytes under conditions of media flow. Simian immunodeficiency virus (SIV) was introduced into the DIV-BBB model in order to determine whether SIV infection has an effect on the blood–brain barrier (BBB). The cells of the DIV-BBB model were maintained for 127 days, during which a low permeability to sucrose developed. SIV infection of the BBB model was readily accomplished with cell-free virus. Results from ELISA for viral p27 protein, s-MAGI assays, and coculture techniques indicate that SIV productively and persistently infected the BBB model. These studies indicate that SIV can persist in MVEC without overtly compromising BBB function, and suggest that the DIV-BBB will be a highly valuable and suitable model for studies of HIV neuropathogenesis. *Journal of NeuroVirology* (2002) **8,** 270–280.

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Introduction

The blood–brain barrier (BBB) is a morphological and functional barrier between molecules circulating in the blood and the brain parenchyma, and is composed of specialized microvascular endothelial cells (MVEC) in contact with astrocytes (Goldstein and Betz, 1983). In addition to controlling the blood-to-brain and brain-to-blood transport of electrolytes, nutrients, regulatory molecules, and peptides, the BBB also modulates interactions between the CNS and the immune system. Consequently, immune cells such as monocytes trafficking through the blood must come in contact with MVEC in order to cross the BBB. Several studies have demonstrated an increase in the expression of cellular adhesion molecules on the surface of MVEC in response to contact between endothelium and monocytes (Sasseville *et al*, 1995; Nottet *et al*, 1996; Persidsky *et al*, 1997), suggesting a potential route for monocytes to cross the BBB. Although HIV and SIV readily gain access to the CNS through as yet undefined mechanisms, many antiretroviral drugs cannot cross the blood–brain barrier. The failure of most antiretrovirals to gain access to the CNS through the BBB raises the potential for the CNS to act as a reservoir for virus, even in patients undergoing highly active antiretroviral therapy (HAART) (Pialoux *et al*, 1997; Cohen, 1998). In order to study BBB properties *in vitro*, several different models of BBB have been developed (reviewed in Grant *et al*, 1998; Janigro *et al*, 1999; Pardridge, 1999). Commonly, the models involve a static coculture of endothelial cells with astrocytes or with astrocyte-conditioned media and are characterized by relatively high sucrose permeability and lack some of the transporters demonstrated by the BBB *in vivo*. A dynamic, *in vitro* model of the BBB, called the DIV-BBB model, was recently developed (Stanness *et al*, 1996, 1997). In this model, brain MVEC are cocultured with astrocytes under pulsatile

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flow, which has been shown to induce ultrastructural differentiation of endothelial cells (Ott *et al*, 1995). The DIV-BBB model is characterized by a low permeability to sucrose, the expression of a BBB-like glucose transporter, the presence of tight interendothelial junctions and by the presence of stereospecific amino acid transporters (Stanness *et al*, 1996, 1997).

Infection with human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) often results in neurological dysfunction throughout the natural course of the disease. Virus appears to gain entry into the central nervous system (CNS) early during infection (Chakrabarti *et al*, 1991; Davis *et al*, 1992; Bell *et al*, 1993), although serious manifestations of neuropathology usually occur only after immunosuppression late during the disease course (Chiodi and Fenyo, 1991; Bell *et al*, 1993; Zink *et al*, 1997). Within infected brain tissues *in vivo*, macrophages and microglia are the major targets for both HIV-1 and SIV infection (Vazeux *et al*, 1987; Budka *et al*, 1991; Simon *et al*, 1992; Takahashi *et al*, 1996; Wiley *et al*, 1986). The advent of sensitive detection methods has made it apparent that other brain cell types are infected, among them astrocytes (Saito *et al*, 1994; Tornatore *et al*, 1994; An *et al*, 1999b), oligodendrocytes (Bagasra *et al*, 1996), and microvascular endothelial cells (MVEC) (Gabuzda *et al*, 1986; Koenig *et al*, 1986; Wiley *et al*, 1986; Ward *et al*, 1987; Mankowski *et al*, 1994; Bagasra *et al*, 1996; Flaherty *et al*, 1997; An *et al*, 1999a, 1999b). The susceptibility of MVEC to HIV infection *in vivo* is still controversial. However, MVEC are an integral part of the BBB, which can become compromised during HIV infection. MVEC are directly or indirectly affected by HIV during the development of neuropathology.

SIV infection of macaques provides a relevant model to examine HIV-induced neuropathogenesis (Desrosiers, 1990). SIV-infected macaques succumb to an AIDS-like disease course and demonstrate neurological dysfunctions similar to those seen in AIDS patients (Desrosiers, 1990; Chakrabarti *et al*, 1991; Lackner *et al*, 1991; Sharer *et al*, 1991; Simon *et al*, 1992; Prospero-Garcia *et al*, 1996; Horn *et al*, 1998), thereby providing a suitable model for studies of HIV-induced neuropathology. Like HIV, SIV infects cells of the macrophage/microglial lineage within the brains of SIV-infected macaques (Simon *et al*, 1992). In addition, SIV productively infects simian brain MVEC *in vivo* and *in vitro* (Mankowski *et al*, 1994; Flaherty *et al*, 1997; Strelow *et al*, 1998). BBB abnormalities have been reported for SIV-infected macaques (Smith *et al*, 1994; Prospero-Garcia *et al*, 1996), substantiating the utility of the SIV/macaque model for studies of virally induced CNS dysfunction. Initial animal studies demonstrated the presence of SIV within macaque brain as early as 7 days postinfection (Chakrabarti *et al*, 1991). In addition, it is possible to derive neurovirulent and neuroinvasive SIV variants via serial animal passage of SIV-infected brain-derived microglia or bone marrow cells (Sharma *et al*, 1992; Mankowski *et al*, 1994; Lane *et al*, 1995; Watry *et al*, 1995; Flaherty *et al*, 1997).

In the present study, we characterized a dynamic BBB model (the DIV-BBB) composed of simian brain MVEC and human astrocytes cocultured under flow. The cells of the DIV-BBB model can be maintained for prolonged periods of time without significant loss of viability. In order to model HIV infection of the brain, an SIV infection of the BBB model was accomplished with cell-free virus. Our results demonstrate that SIV can productively and persistently infect a blood–brain barrier model, and suggest that SIV can persist in brain endothelial cells without overtly compromising BBB function. The DIV-BBB will be a useful and relevant model for future studies of HIV infection of the CNS.

Results

Characterization of the DIV-BBB model The DIV-BBB model is described in detail elsewhere (Stanness *et al*, 1996; Stanness *et al*, 1997; Janigro *et al*, 1998). Simian brain MVEC were isolated and assessed for purity at early passage by immunofluorescence for von Willebrand factor (vWF; factor VIII-related antigen) and CD31 expression. Only those cultures with greater than 95% purity and a homogeneous appearance at the time of seeding were utilized (Figure 1A). The presence of contaminating cells types was assessed by the absence of staining for GFAP (glial fibrillary acidic protein) and by the passage of cells prior to seeding to preclude macrophage contamination. Simian brain MVEC were inoculated into the luminal compartment of DIV-BBB cartridges, while normal human astrocytes (NHA) (Figure 1B) were seeded into the extracapillary space (ECS). The NHA utilized in these studies were obtained commercially (Clonetics), and expressed GFAP at low passage only; purity was therefore assessed at passage 4. A schematic of the DIV-BBB cartridges is shown in Figure 2. MVEC and NHA were cocultured under pulsatile flow and shear stress, resulting in the formation of a barrier to sucrose (Janigro *et al*, 1998). A functional barrier to sucrose will not form in MVEC in the absence of glial influences. The presence of a barrier to sucrose therefore indicates that the MVEC and NHA seeded into the DIV-BBB maintained their phenotypic properties throughout the culture period.

Glucose consumption, as measured by lactate production, was used as a measure of cell viability. The relatively constant rate of lactate production seen for the DIV-BBB (data not shown and Janigro *et al*, 1998) indicates that MVEC can be viably cocultured with astrocytes for prolonged periods of time in this model. In support of this observation, several DIV-BBB cartridges have been viably maintained for over 1 year (data not shown). The overall rate of lactate production also indicates that the majority of cells

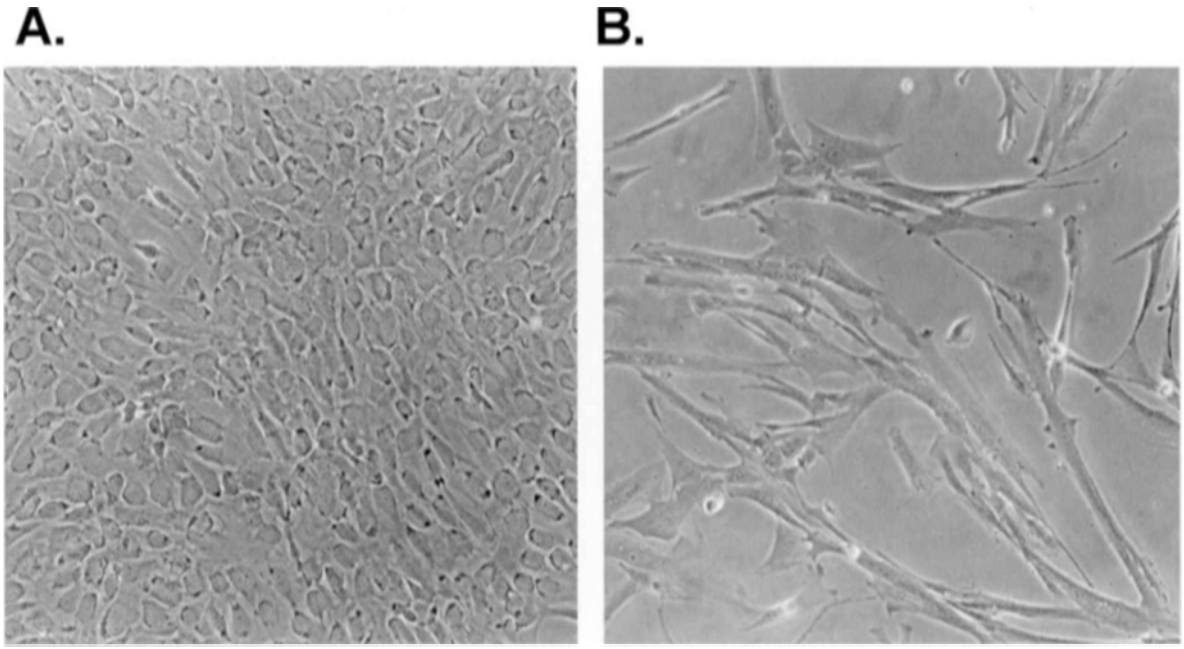

Figure 1 Cell characterization for the DIV-BBB. (**A**) Phase contrast microscopy of simian brain microvascular endothelial cells (MVEC) at passage 5, showing cobblestone morphology. (**B**) Phase contrast microscopy of normal human fetal astrocytes. Original magnification 100×.

are viable at late times post-seeding. Viable cells were shed from the artificial capillaries into the reservoir bottles (see Figure 6). The rate of lactate production remains constant, which suggests that there is a steady-state equilibrium reached, with cells dividing to fill gaps left by cells that are shed into the medium. Alternatively, the MVEC remaining in the capillaries may extend to fill in gaps left when cells are shed.

Simian brain MVEC are productively infected by SIV Several studies have shown that SIV_{mac} infects simian brain MVEC both *in vitro* and *in vivo* (Mankowski *et al*, 1994; Flaherty *et al*, 1997; Strelow *et al*, 1998). It has been our observation that distinct lines of primary brain MVEC from separate animals respond differently to SIV infection *in vitro* (Strelow and Nelson, unpublished observations). We therefore

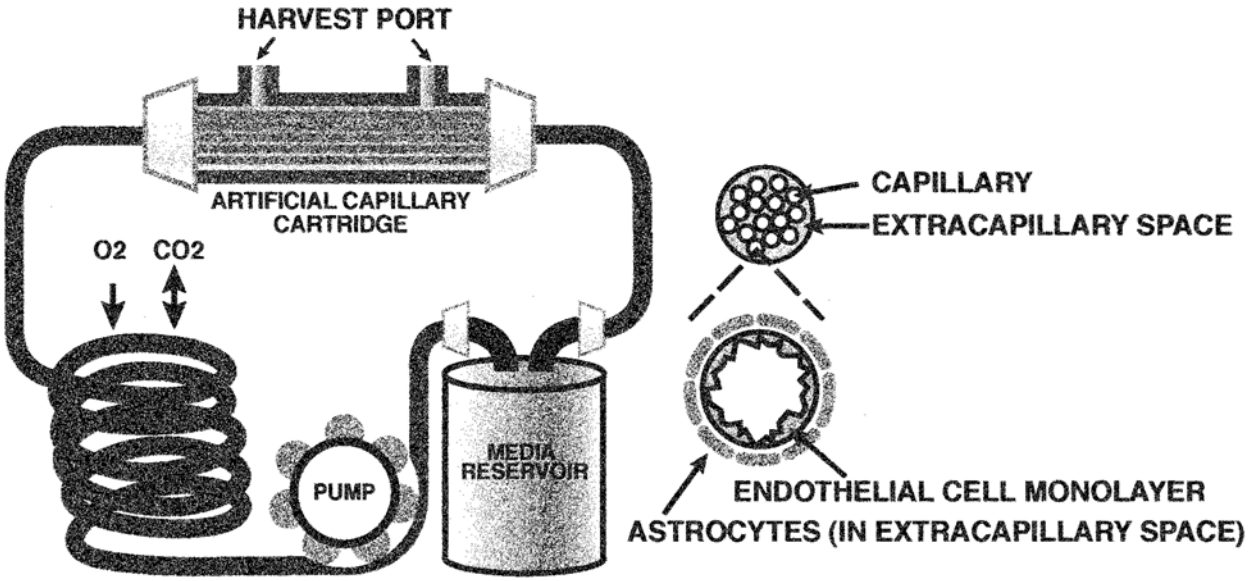

Figure 2 Schematic of the DIV-BBB model. Simian brain MVEC are seeded into cartridges intraluminally while astrocytes are seeded into the extracapillary space. The polypropylene artificial capillary fibers are permeable, with a pore size of 0.5 μm. Cells are grown under shear stress generated by pulsatile flow at a flow rate of approximately 7.5 ml/min. The cartridges are maintained in a humidified incubator at 37°C and at 7% CO_2 by gas permeable tubing.

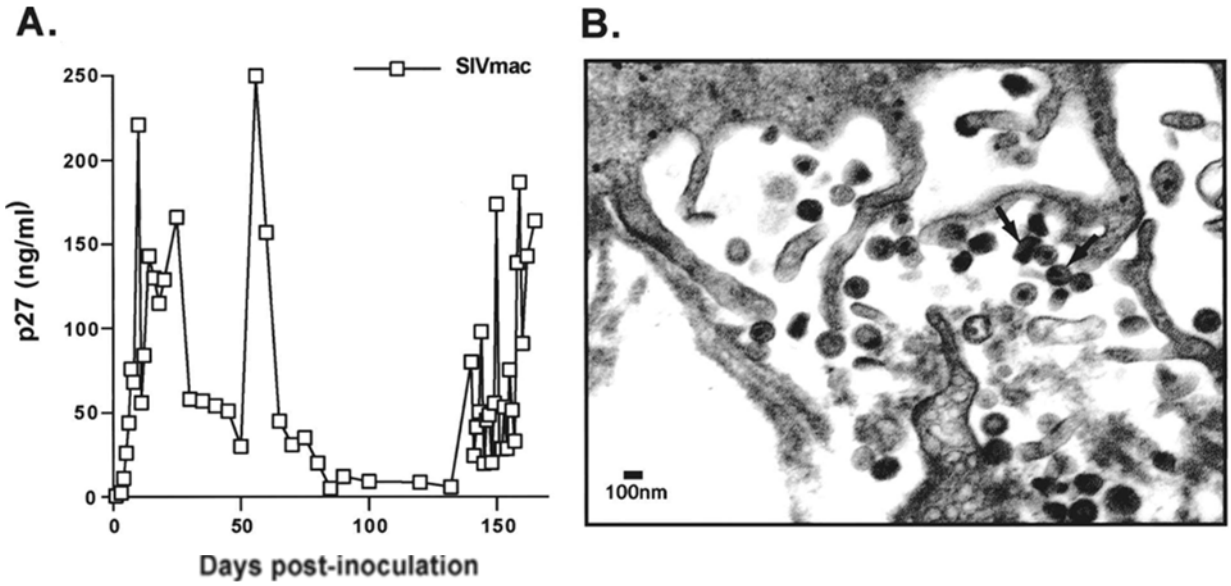

Figure 3 SIV infects MVEC in static culture. Simian MVEC were exposed to SIV at an MOI of 0.02 for 4 h, after which cultures were extensively washed. (**A**) Viral p27 antigen (as assayed by ELISA) in culture supernatants versus time postinoculation. (**B**) Electron micrograph showing lentiviral-like particles in MVEC at day 18 post-SIV infection. Arrows highlight typical lentiviral particles. Size bar = 100 nm; original magnification 25,000×.

determined whether the line of MVEC used in these studies was susceptible to SIV infection in conventional static culture. As shown in Figure 3A, we were able to detect viral p27 antigen in supernatants from SIV-exposed MVEC by an antigen capture ELISA. In addition, lentiviral particles were present in electron micrographs of infected cells (Figure 3B). The presence of infectious virus indicates that the brain MVEC used in this study were capable of productive infection by SIV_{mac} 251. In addition, viral antigen was continuously present throughout 165 days of static MVEC infection, indicating that SIV infection of MVEC is persistent *in vitro*.

The DIV-BBB is persistently and productively infected Having established that simian MVEC were susceptible to SIV_{mac} 251 infection in static culture, we next examined SIV infection of cells in dynamic coculture with astrocytes in the context of the DIV-BBB model. Two DIV-BBB cartridges were infected with SIV_{mac} 251 molecular clone as described and monitored over time using several different criteria to detect viral infection. Two additional cartridges were exposed to mock inoculum only. As shown in Figure 5, SIV infection was persistent throughout the time course of the infected DIV-BBB cartridge [116 days postinfection (p.i.)]. SIV first detected at

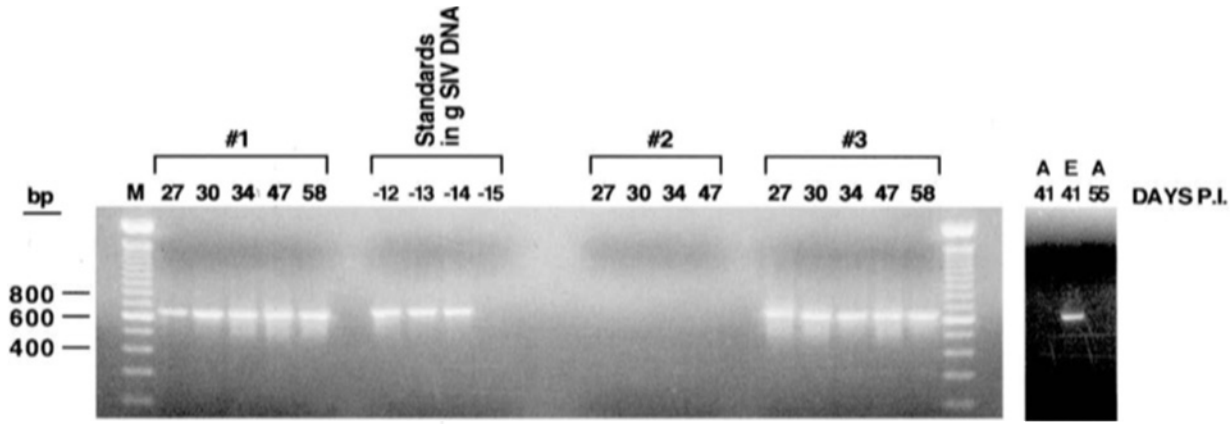

Figure 4 Presence of viral DNA in the DIV-BBB. DNA was prepared from cells that were shed from cartridges during the normal course of culturing. The DNA samples were subjected to a nested PCR for SIV LTR. The size of the second round PCR product is 631 base pairs (bp). M = DNA size marker, in bp. Cartridges 1 and 3 were SIV-exposed; cartridge 2 was exposed to mock inoculum only. Numbers at the top of lanes represents days p.i. Standards represent grams of SIV DNA. A = astrocytes; E = endothelial cells for cartridge 1.

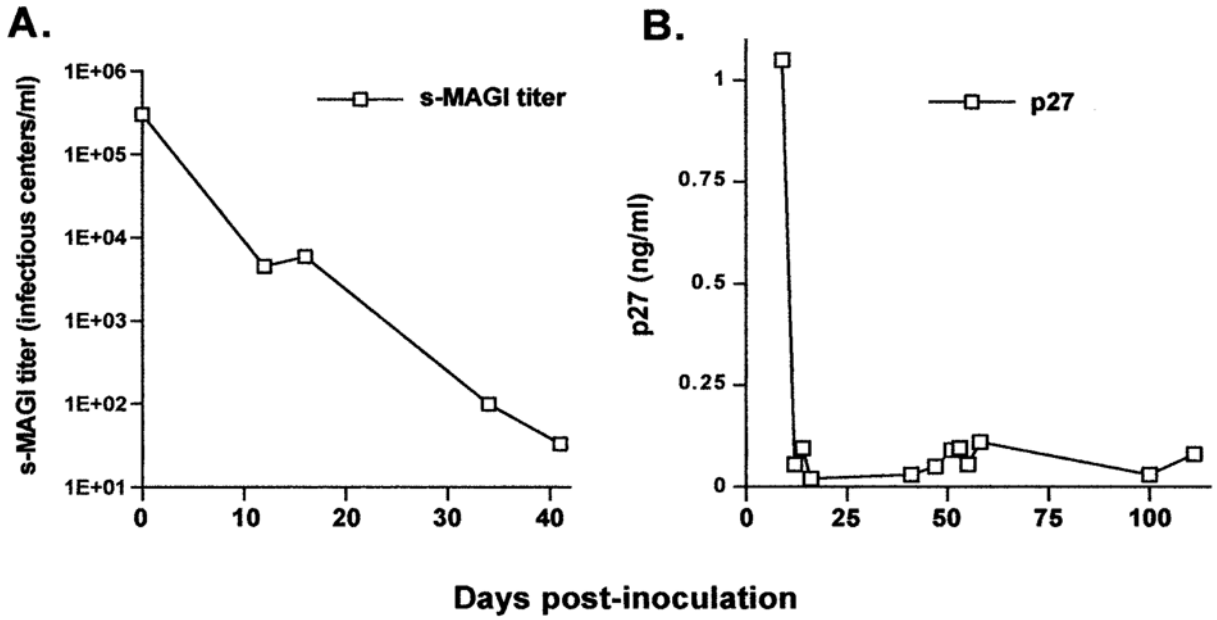

Figure 5 SIV infects the DIV-BBB model. DIV-BBB cartridges containing MVEC cocultured with astrocytes were exposed to 3×10^5 infectious centers/ml of SIV in medium containing 2 μg/ml polybrene at day 0. (**A**) Infectious titer of cell-free supernatants obtained from the DIV-BBB (s-MAGI assay). (**B**) SIV p27 antigen (ELISA) in DIV-BBB culture supernatants. Although levels are low, infectious virus was culturable at all times.

day 2 p.i. most likely represents residual input inoculum. Virus continued to be detected throughout the study. Although viral titers and p27 values decreased over time (Figure 5), infectious virus was present both in culture supernatants as well as in infected cells shed into the medium at late times postinfection (Figure 6).

Cells shed into the reservoir bottle from either SIV-inoculated or mock-inoculated cartridges at day 45, 73, or 91 p.i. were plated into Primaria dishes. One set of plates was washed and maintained, while another set was washed and had CEMX174 cells added to the cell monolayer. After coculture, the CEMX174 cells overlaid onto the SIV-inoculated cartridge MVEC layer demonstrated visible syncytia. Cell-free supernatants from the SIV-inoculated cartridge MVEC cultured in isolation were then added to CEMX174 cells and syncytia again resulted, demonstrating the presence of infectious virus within the MVEC of the DIV-BBB (Figure 6). Syncytia were never observed for plates seeded with cells shed from mock-infected cartridges, nor did supernatants from mock-infected cartridge cells induce syncytia in CEMX174 cells (data not shown). Cells shed into the reservoir bottle were routinely collected for PCR analysis of viral signal. In those samples where detectable levels of DNA were recovered, the samples from SIV-infected cartridges were positive for SIV LTR, whereas mock-inoculated cartridges gave no signal (Figure 4). Viral p27 protein was detectable in the extracapillary space (ECS) samples at day 16 p.i. (data not shown), although astrocytes could not be demonstrated to be productively infected (Figure 4). Thus, although ECS supernatants occasionally yielded infectious virus, astrocytes were viral DNA negative (Figure 4) and virus could not be cultured from isolated astrocytes cultures (data not shown). The detection of virus at all times postsinfection demonstrates that a persistent and productive SIV infection was accomplished in the DIV-BBB model.

Discussion

In this study we developed and characterized an *in vitro* model of simian blood–brain barrier. Simian MVEC and human astrocytes were cocultured under pulsatile flow in a dynamic model, the DIV-BBB, which demonstrates some of the characteristic and defining properties of the *in vivo* BBB. In this model, MVEC and astrocytes were viably cocultured for prolonged periods of time and were capable of sustaining a productive, persistent SIV infection. This model will be useful for further examination of the consequences of SIV or HIV infection of brain cells and the effects of viral infection on the blood–brain barrier.

The DIV-BBB model presented here is a physiologic model as compared to current models of BBB. The cell types utilized in the DIV-BBB are those found within the BBB *in situ*, namely, brain microvascular endothelial cells (MVEC) and astrocytes. Although infection of MVEC *in vivo* is still controversial, brain MVEC have been shown to be susceptible to HIV and SIV infection *in vitro*. Microvascular cells are susceptible to HIV *in vitro*, whereas endothelial cells from macrovascular sources are not (Moses *et al*, 1993). For simian cells, MVEC *in vitro* are infected by SIV via a CCR5-dependent mechanism (Edinger *et al*, 1997).

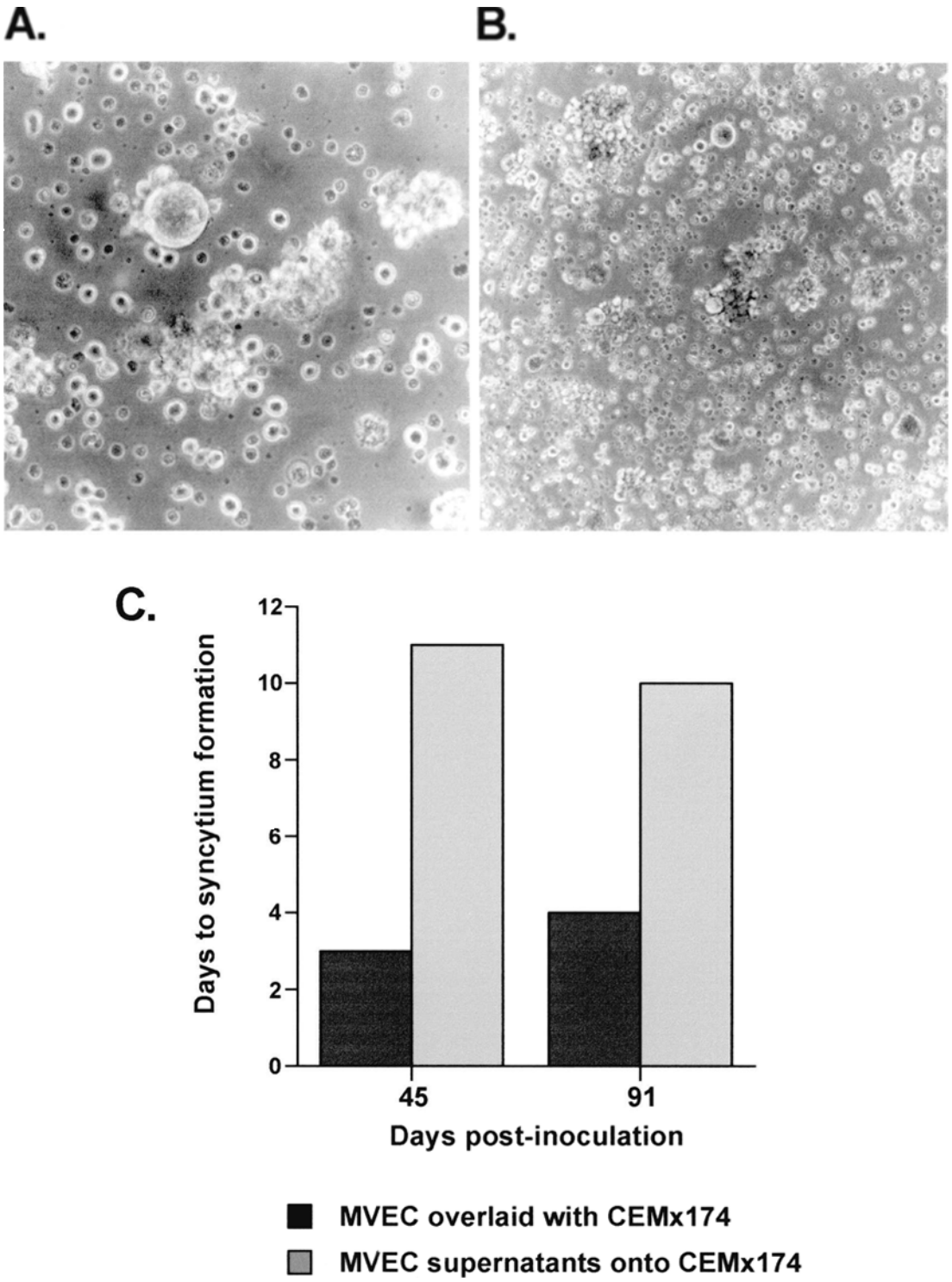

Figure 6 Infectious virus is present at late times post-infection. MVEC shed into the lumen of the DIV-BBB post-SIV infection were washed, plated, and maintained in static culture. At various times postplating, cell-free supernatants were collected and cocultured with indicator CEMX174 cells. Alternatively, CEMX174 cells were added directly to the MVEC monolayer. Cultures were subsequently monitored for the presence of syncytia. (**A**) MVEC 73 days in the DIV-BBB and cultured for 42 days before addition of CEMX174 cells. (**B**) MVEC 91 days in the DIV-BBB and cultured for 24 days before addition of CEMX174 cells. (**C**) Chart demonstrating time to syncytium formation.

Astrocytes are the second component of the DIV-BBB model. HIV infection of astrocytes also remains controversial, with some studies finding infection in pediatric patients *in vivo* (Saito *et al*, 1994; Tornatore *et al*, 1994) and others in fetal astrocytes *in vitro* (Conant *et al*, 1994; Hatch *et al*, 1994). Astrocytes were not detectably SIV-infected in the context of the DIV-BBB. The astrocytes used to induce barrier in the DIV-BBB were of human origin and therefore SIV infection may have been below our level of detection. Ongoing studies utilize simian astrocytes. The cells of the DIV-BBB were cultured under pulsatile flow. Culturing cells under these conditions, which mimics blood flow *in vivo*, has been shown to promote the ultrastructural differentiation of endothelial cells *in vitro* (Ott *et al*, 1995). This utilization of physiologic flow conditions is novel for BBB models.

We were able to demonstrate a productive and persistent infection of the DIV-BBB in which virus was capable of traversing the MVEC monolayer into the astrocyte layer, representing a luminal to abluminal passage of virus. SIV was first detected in the extracapillary space (ECS) at day 16 p.i. At this early time point, however, virus may have been able to traverse the BBB through a paracellular route, since virus was introduced into the DIV-BBB before the establishment of a functional barrier to sucrose (Janigro *et al*, 1998). Infectious virus was also isolated by coculture from the ECS at day 95 p.i. (data not shown), after the establishment of a barrier to sucrose, suggesting that SIV is able to cross the barrier from luminal to abluminal compartments.

Preliminary results suggest that, as expected, the virus grown within DIV-BBB MVEC infects simian MVEC more readily and with greater viral yields than the parental SIV_{mac} 251 clone. The SIV_{mac} 251 molecular clone used in these studies is not pathogenic in macaques. SIV_{mac} 251 clone is *infectious* in macaques, however (Edmonson *et al*, 1998), and infects MVEC *in vitro* (Strelow *et al*, 1998, and the current study). Initial studies utilized this molecular clone of SIV in order to ensure that the genotype of the input inoculum was known, as well as to utilize an SIV_{mac} strain that is known to infect MVEC. Our ongoing studies are utilizing other MVEC-tropic viruses that have been demonstrated to be pathogenic in a macaque model (SIV_{mac} 182; Watry *et al*, 1995; Strelow *et al*, 1998).

One of the limitations of the DIV-BBB is the inability to accurately determine absolute numbers of SIV-infected cells. As shown in Figure 5, the viral titers and p27 antigen levels appear to decrease over time. The reason for the decrease could be due to infected cells dying, to a non-productive infection, or to the virus going into a quiescent state. Levels of p27 antigen do not necessarily correlate to the amount of replication-competent virus (see, for example, the levels of p27 and corresponding infectious viral titers in Figure 5). In static culture, viral p27 values appear to fluctuate over time during extended infection. Initial levels of p27 in SIV-exposed MVEC cultures were seen to decrease slightly over time before increasing at late times postinfection (Figure 3A). In addition, there is a dilution factor involved with the DIV-BBB in that the reservoir holds 100 ml of media, whereas the total volume of the infected capillary compartment from which p27 is shed is 5 ml. These observations suggest that the fluctuations in p27 antigen levels most likely result from adaptation of SIV to MVEC, and that the decrease in viral titer/p27 antigen out to day 111 is probably not indicative of a loss of virally infected cells or of virus infection. The s-MAGI assay for infectious viral titer was performed initially to assess the infectious viral load at early times. At late times postinfection, infectivity and viral persistence were assessed by supernatant culture with CEMX174 cells. Additionally, cells shed into the reservoir bottle and cultured in isolation yielded infectious virus (Figure 6), indicating the persistent presence of infectious virus at late times p.i., when titer and/or p27 levels were low.

Preliminary evidence from the DIV-BBB indicates that cell-cell contact (as in MVEC with T-cells) may be important to viral dissemination. Cell-free culture supernatants taken from shed MVEC that were cultured in isolation were able to induce syncytia in CEMX174 indicator cells after 10–11 days of coculture, whereas CEMX174 cells added directly to similar cultures demonstrated syncytia in as little as 3 days (Figure 6C). Several studies have outlined the importance of MVEC-induced monocyte adherence for HIV transmission (Dhawan *et al*, 1995; Sasseville *et al*, 1995; Nottet *et al*, 1996; Persidsky *et al*, 1997; Weiss *et al*, 1999). The DIV-BBB will be useful to assess whether infected monocytes/macrophages pulsed through the lumen can initiate a productive infection in MVEC as well as to examine the trafficking patterns of infected cells through the BBB.

There are several models for the transmigration of HIV across brain endothelium of the BBB *in vivo* (reviewed in Moses and Nelson, 1995; Strelow *et al*, 2001). The DIV-BBB model demonstrates properties that closely approximate the *in vivo* BBB. As such, the model will be useful to examine mechanisms of viral persistence and activation as well as to examine viral entry into the CNS through the BBB. In addition, the DIV-BBB can be used to study viral interference with tight junction formation or with transporter function associated with a functional barrier. The model can be used to test the delivery of antiretroviral drugs across the BBB in the presence or absence of HIV/SIV infection. The DIV-BBB model will also be useful to test the effectiveness of antiretroviral therapy and other therapeutics on HIV/SIV persistence in both the BBB itself as well as within the CNS. Finally, reservoir studies of viral rebound after cessation of highly active antiretroviral therapy can be initiated with this model. The majority of conventional, static culture models of BBB lack neurons in the coculture system. A recent report by Stanness *et al* (1999) has

demonstrated the ability to coculture neuronal cells with endothelial and glial cells in the context of the DIV-BBB. This result demonstrates the suitability of the DIV-BBB model for studies of diverse CNS cell types. As such, the DIV-BBB model promises to be of great utility for studies of HIV and SIV neuropathogenesis.

Materials and methods

Cells The human T-B cell hybrid line CEMX174 (NIH AIDS Research and Reference Reagent Program 272) was cultured in complete RPMI medium (RPMI supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). 293T cells were cultured in complete DMEM (DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). s-MAGI cells (Chackerian *et al*, 1995) were maintained under G418 (0.2 mg/ml) and hygromycin (50 U/ml) selection in complete DMEM. Normal human astrocytes (NHA) were obtained commercially (Clonetics) and maintained as recommended. Macaque brain microvascular endothelial cells (MVEC) were isolated as previously described (Strelow *et al*, 1998) from cortex of SIV-, simian foamy virus-, herpesvirus B-, and simian type D retrovirus-negative rhesus macaques (*M. mulatta*). Simian brain MVEC were maintained in complete endothelial medium (EBM (Clonetics) supplemented with 10% human AB serum (Sigma), 25 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products), 40 μg/ml heparin (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) and grown in Primaria (Falcon) tissue culture plates. Heparin was not added to the medium when SIV was present. Purity of MVEC and NHA cultures was assessed by immunofluorescence at early time points, before cellular markers (vWF, CD31, GFAP; all antibodies from DAKO, Carpinteria, CA) were lost.

Virus stocks 5×10^5 293T cells were transfected with 2 μg of SIV_{mac} 251 phage DNA (molecular clone; NIH AIDS Research and Reference Reagent Program 213) using a modified calcium chloride transfection protocol (Bartz *et al*, 1996). Transfected cells were cultured in complete DMEM for 3 days, after which cell-free virus was further amplified through CEMX174 cells. Infection was monitored by supernatant p27 levels as measured by commercial ELISA (Coulter Corporation, Miami, FL). Pooled supernatant virus obtained at days 8 and 10 p.i. was concentrated by centrifugation and titered by s-MAGI assay as described (Chackerian *et al*, 1995).

DIV-BBB maintenance The dynamic, *in vitro* blood–brain barrier (DIV-BBB) model is described in detail elsewhere (Stanness *et al*, 1996; Stanness *et al*, 1997; Janigro *et al*, 1998). In brief, primary simian brain microvascular endothelial cells (MVEC) were isolated as described (Strelow *et al*, 1998). MVEC at passage 5 were inoculated into the luminal compartment of each DIV-BBB cartridge at between $1–2 \times 10^6$ cells per seeding. MVEC were seeded at days 0 and 9. On day 9, 2×10^6 normal human astrocytes (Clonetics, San Diego, CA) were seeded into the extracapillary space (ECS). The cartridges were maintained in a humidified incubator at 37°C and at 7% CO_2 by gas-permeable tubing. Cell viability was monitored via quantitation of nonadhered cells as well as by production of lactate using a commercial kit (Sigma, St. Louis, MO). The reservoir bottles supplying each DIV-BBB cartridge were supplemented with fresh endothelial medium according to lactate values; complete medium changes occurred whenever lactate levels rose to 400 mg/L or above. The cells were maintained under pulsatile flow at a rate of 7.5 ml/minute. Samples were obtained periodically from both luminal and ECS compartments for lactate measurements and virus isolation. Sampling was done via ports for the ECS and via the reservoir bottle for the luminal compartment.

Infection of simian MVEC in static culture Triplicate cultures of primary simian MVEC at passage 5 were exposed to SIV_{mac} 251 inoculum containing 2 μg/ml polybrene (Sigma) at an MOI of 0.02 at 37°C. Viral inoculum was removed after 4 h by washing three times with Hank's balanced salt solution and the medium replaced with complete endothelial medium. Supernatants were harvested daily for the determination of cell-free virus. Cultures were washed three times to ensure the measurement of daily virus production. Culture supernatants from SIV-exposed MVEC were assayed for viral p27 antigen by commercial ELISA (Coulter).

Infection of DIV-BBB DIV-BBB cartridges were inoculated on day 11 post-MVEC seeding with SIV_{mac} 251 molecular clone prepared as described above. Six ml of virus stock titered at 3×10^5 infectious centers/ml and containing 2 μg/ml polybrene was injected into the luminal compartment of each DIV-BBB cartridge and allowed to adsorb for 2 h at 37°C with no flow. The virus stock was removed and stored on ice while the cells were exposed to flow for 1 h at 37°C. Inoculum stored on ice was supplemented with 1 ml freshly thawed stock and re-inoculated for an additional 2 h as described previously. After the second exposure to virus, viral inoculum was removed, substituted with fresh endothelial medium, and the cartridges replaced under flow. Two mock-exposed cartridges were treated exactly the same as the virus-exposed cartridges, using as inoculum medium containing 2 μg/ml polybrene only. Mock and SIV-exposed DIV-BBB cartridges were sampled at various time points from both the luminal and ECS compartments and were fed at regular intervals based on lactate production. Productive viral infection was determined by several

criteria: levels of supernatant viral p27 as measured by commercial ELISA assay (Coulter), titration of infectious virus via s-MAGI assay (Chackerian *et al*, 1995) at early time points, and by syncytium formation in cocultures with CEMX174 cells. In addition, viable cells shed from the lumen of artificial capillaries into the reservoir bottle were plated into Primaria plates and assayed for virus as outlined previously. DIV-BBB cartridges were terminated at day 127 post-MVEC plating (day 116 postinfection) or earlier.

Electron microscopy Simian MVEC infected with SIV_{mac} 251 for 18 days were pelleted and resuspended in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 2% trinitrophenol (picric acid) in 0.1 M sodium Cacodylate buffer). Cacodylate buffer is 0.4 M sodium cacodylate, 2 mM HCl, pH 7.2. After 1 h of fixation, cell pellets were rinsed in buffer for 10 min and placed into 1% OsO_4 in 0.1 M Na Cacodylate buffer with 0.8% $K_3Fe(CN)_6$ for 1 h. The osmium was replaced with a distilled water rinse for 20 min. Cell pellets were prestained with 4% uranyl acetate for 1 h, and then rinsed with distilled water for 20 min. Cell pellets were dehydrated through a graded series of acetone as follows: 50% for 20 min, 70% for 10 min, and 90% for 10 min, with two changes of absolute acetone. Cell pellets were then infiltrated with a 50/50 mix of acetone and Epon 812 for 24 h. The infiltration solution was replaced with 100% Epon 812 and placed in an oven at 60°C for 24 h. After polymerization, 2 micron thick sections were cut with a MT 5000 Ultramicrotome and stained with Methylene blue azure II. Light microscopy was used to assess cell density and proper fixation. Then, 60–80 nanometer thin sections were subsequently cut and mounted on 300-mesh copper grids. The sections were then stained with uranyl acetate and lead citrate. Sections were examined on either a Philips CM120 or a Philips 300 Transmission Electron microscope and photographed.

DNA PCR Cells shed from the DIV-BBB during the course of culturing were collected from the reservoir bottle and cellular DNA prepared utilizing a kit per manufacturer's instructions (Qiagen, Valencia, CA). 100 ng of DNA was subjected to nested PCR for SIV LTR. PCR reactions contained per 100 μl reaction: 1× AmpliTaq buffer (Perkin Elmer, Branchburg, NJ), 200 μM each dNTP, 2 mM $MgCl_2$, 50 pmol each primer, and 2.5 U AmpliTaq enzyme. Primers for the first round are as follows: forward 5′ TGG AAG GGA TTT ATT ACA GTG CAA G 3′ and reverse 5′ GAG TAC CGA GTT GAC CAG GCG G 3′. Internal primers for the second round are as follows: forward 5′ CCA GAT TGG CAG GAT TAC ACC TCA GGA CCA GG 3′ and reverse 5′ GAG AGA TGG GAA CAC ACA CTG GCT TA 3′. The first round yields a product of 736 bp and the second round a product of 631 bp (for SIV_{mac} 251). 1/100th of the first round product was used in the second round reaction. 20 μl of second round product was subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. A set of standards was included in each round. SIV genomic DNA was diluted to 1×10^{-12}, 1×10^{-13}, 1×10^{-14}, or 1×10^{-15} grams/μl and 1 μl of each standard run in the PCR reactions.

Coculture assays At various times post-infection, viable cells shed into the media reservoir bottle were collected by centrifugation and plated into Primaria (Falcon) dishes in complete endothelial medium. Cultures thus obtained were stably maintained for extended times outside the context of the DIV-BBB. At various times post-culturing, cell-free culture supernatants were collected, cultured with indicator CEMX174 cells, and monitored for the presence of syncytia for at least 21 days. Negative results were confirmed by DNA PCR. Alternatively, CEMX174 cells were added directly to the MVEC monolayers and assayed as above for the presence of syncytia.

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