Infection of Brain Cells by Diverse Human Immunodeficiency Virus Isolates: Role of CD4 as Receptor

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SUMMARY

Cell lines originally derived from malignant tumours of the brain were infected by diverse human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) isolates. By surface immunofluorescence it was shown that susceptible cells did not bear the CD4 antigen. They were also non-permissive for the formation of plaques by vesicular stomatitis virus pseudotypes and did not form syncytia with HIV-producing cells. Virus production was of low titre, and reverse transcriptase and the p24 antigen were consistently undetectable in the culture supernatants. Output virus could be detected by cocultivation with a sensitive T cell line, C8166, by the culture of supernatant medium with T cells and by detection of proviral HIV DNA after amplification. A higher multiplicity of input virus was required to establish a brain cell infection than was required for T lymphocytes or monocytes. Some HIV-susceptible brain cells contained mRNA for CD4 but infection was not blocked by anti-CD4 antibodies. Apparently HIV infection of these cells does not involve CD4 as the cellular receptor.

INTRODUCTION

The ability of human immunodeficiency virus (HIV) to infect cell lines derived from brain tumours has been reported by several investigators. Dewhurst et al. (1987a) reported that cell lines from malignant gliomas, U251 and U373, and a medulloblastoma cell line, TE671, could be infected by one isolate of HIV-1. These cell lines did not express viral antigen on the cell surface, but immunoblots with anti-HIV sera using these infected glial cells as the antigen showed the presence of bands representing the product of the viral gag gene. In long-term culture, viral gene transcripts were found, but no infectious virus was produced. Transfection of the whole HIV genome into the U251 MG cell line resulted in a productive viral infection (Dewhurst et al., 1988). These cells were all negative for CD4 antigen, shown by cell surface immunofluorescence (IF), but contained CD4 mRNA, demonstrated by Northern blotting (Dewhurst et al., 1987b). The cells did not exhibit CPE after HIV infection. The finding that the CD4 gene was expressed at a low level in these cells supported the role of CD4 as the cellular receptor for HIV in glial cells as well as in T lymphocytes and monocytes. Chiodi et al. (1987) showed that further CD4- glial cell lines derived from malignant gliomas, U138, U489 and U373, could be latently infected by HIV-1 (HTLV-III B and HTLV-III, RF isolates). No viral antigens were detected on infected cells, but cocultivation with a CD4+ cell line resulted in the detection of infectious virus production. These cells were not studied for CD4 mRNA, but were shown again by IF to be negative for surface CD4. Cheng-Mayer et al. (1987) showed that isolates of HIV-1 differed in their ability to infect cell lines from malignant gliomas (U343, U251), and primary early passage
brain cell cultures. However, the input infecting virus strains, although of high reverse transcriptase activity, were not strictly comparable in terms of tissue culture infectious doses (TCID). IF showed that these gloma cell lines all lacked CD4+; however, heterogeneous cell populations cannot be excluded in primary cultures, hence these cultures could have contained bone marrow-derived CD4+ macrophages. Christofinis et al. (1987) also demonstrated HIV-1 (HTLV-III) infection of primary embryonic brain cell cultures. The role of the CD4 antigen in HIV infection of non-haematopoetic cells was further elucidated by Adachi et al. (1987), in CD4+ colorectal cell lines. Cell lines of gut origin susceptible to HIV infection contained a full-length 3 kb mRNA species of the CD4 gene, whereas HIV-resistant colorectal cell lines had no detectable CD4 mRNA.

It seemed likely from these experiments that brain cell cultures susceptible to HIV were using a low concentration of CD4 antigen as the receptor for HIV entry; the low level of CD4 molecules made detection by cell surface IF impossible although mRNA could still be detected. The high virus input required for cell infection and the low virus productivity of the cells was related to the low level of CD4 expression (Asjo et al., 1987).

Here we demonstrate that HIV-1 and HIV-2 infection of brain cells correlates with CD4 mRNA expression, but that infection cannot be blocked by anti-CD4 polyclonal antibodies or monoclonal antibodies (Mabs). Brain cells susceptible to HIV infection do not appear to use the CD4 antigen as the cellular receptor.

**METHODS**

The cell lines U138, MG, U343a, MG, U251, SP, MG, U251. AG, MG and U87. MG (Westermark et al., 1973) derived from human malignant gliomas were grown as monolayers in Dulbecco's modification of Eagle's medium (DMEM) with 10% foetal calf serum (FCS) in 50 ml flasks, and passaged twice a week by trypsinization. Another cell line, TE671 (McAllister et al., 1977) was initially described as being of human medulloblastoma derivation, but evidence has subsequently been published which strongly suggests that these cells are indistinguishable from the RD rhabdomyosarcoma cell line (Stratton et al., 1989). These cell lines were initially cloned when derived, but it is possible that heterogeneity has developed over long periods in cell culture passage; they were not recloned for this study. An adherent subline of the parent CEM. CCRF T cell line, and the C8166 T cell line were grown in RPMI 1640 and 10% FCS. All HIV isolates were propagated in H9 cells, and supernatant medium was cleared of cells by centrifugation. Viral titres were assayed by infection of C8166 cells, to yield TCID values for C8166. Aliquots of virus supernatant stock were preserved in liquid nitrogen, and thus equivalent titres were used for all experiments. The brain cell lines were plated onto 24-well trays at 5 × 10^4 cells/well, and were infected with between 10^3 and 10^4 TCID of cell-free virus stock.

Infection of the brain cell lines was assayed by cocultivation of monolayers with an overlay of 5 × 10^5 C8166 cells, in 24-well trays. Alternatively, supernatant from the brain cell cultures was centrifuged at 1500 r.p.m., for 5 min, and added to uninfected C8166 cells. Infection of C8166 cells was detected by syncytium formation after incubation at 37 °C for 72 to 120 h (Dalgleish et al., 1984); specificity for HIV was confirmed by IF with an anti-p24 Mab (3D3, kindly supplied by R. Tedder; Ferszt et al., 1987). All culture supernatants were assayed for the presence of RNA-directed DNA polymerase activity (reverse transcriptase) by the method of Hoffman et al. (1985), and for the presence of the HIV p24 core antigen by the DuPont antigen capture immunoassay.

**Polymerase chain reaction (PCR).** DNA was extracted from the infected cell lines by standard methods, and 1 μg DNA was amplified using Taq polymerase (Cetus) according to Saiki et al. (1986) using primers from the sequence of the gag and env genes, HIV-1 (HTLV-III) clone BH10, at the following sites: primer SK68, AGCAGCAGGAAAG-GACATTGCT, env 7151 to 7170; SK69, CTGTTGCAACTCACAGTCTGG, env 7272 to 7292; GAGAGG- GAAGCTGAGAAATGGG, gag 730 to 748; GAG, GAGCATTTGACATTAAGACAAAGACC, gag 951 to 977. The PCR products were analysed by agarose gel electrophoresis.

**Immunofluorescence and other receptor studies.** Indirect IF was performed on live, unfixed cells for the detection of the CD4 antigen, using the anti-CD4 mouse polyclonal antibody (provided by Q. Sattentau), at a 200-fold dilution and the Leu3a anti-CD4 MAb at a 1000-fold dilution. Brain-derived cells and control adherent CEM. CCRF cell lines were grown in Petri dishes with sterile glass coverslips. Immediately before the cells had reached confluence, the coverslips were removed, washed twice in phosphate-buffered saline solution A (PBSA) with 1% FCS, and incubated with 30 μl of the antibody dilution for 30 min at 37 °C. The cells were washed with PBSA, and incubated with fluorescein isothiocyanate conjugated to a goat anti-mouse antibody (Miles Laboratories) for 30 min at 37 °C; after the final washing, the coverslips were mounted in Aquamount u.v.-inert mounting fluid, and examined by u.v. microscopy. IF examination for the presence of viral antigens was performed with the 3D3 anti-p24 HIV MAB using cells grown on coverslips as above, but with pre-fixing using
cells (data not shown).

**Blocking experiments.** Parallel cultures of brain cell lines were established at an equivalent passage number at a concentration of 10^4 cells/well, and were pre-incubated with an anti-CD4 antibody at various concentrations for 1 h at 37 °C; an anti-CD8 MAb was used as a negative control. The anti-CD4 antibodies have all been previously shown to block HIV infection of T cells (Sattentau et al., 1986). These pre-incubated cells and untreated cells of equivalent passage number were then exposed to 5 × 10^4 TCID virus supernatant for 1 h at 37 °C. The cells were then washed three times with DMEM, and the pretreated cells were cultured in DMEM with 10% FCS and 2 μg/ml of anti-CD4 antibody for 24 h. All cells were subsequently grown in medium and 10% FCS alone. The cells were harvested at 48 h by trypsinization, and split every 3 days. Cells were examined initially at 8 days by cocultivation of both cells and supernatant with C8166. Cells were examined weekly if positive until they were no longer so, or until the cell line was lost.

**RESULTS**

Table 1 shows the characteristics of the cell lines, compared to the adherent CEM.CCRF cells, whose characteristics have been described (Dalgleish et al., 1984). None of the brain-derived cells expressed the CD4 antigen (which would be detectable by IF) on the cell surface or in the cytoplasm. None of the brain cells were permissive for the entry of the VSV(HIV) pseudotype viruses, and none formed syncytia with HIV-producing cells on cocultivation for 48 h. These results showed that, in contrast to the CEM.CCRF cells, the brain cells did not express detectable quantities of the receptor for HIV.

Table 2 shows the results 14 days after infection of the cell lines with 5 × 10^4 TCID of HIV-1 (HTLV-III. RF). The T cells (CEM.CCRF) became positive for HIV antigens (demonstrated by IF with an anti-p24 MAb), had detectable reverse transcriptase activity in the supernatant medium and p24 antigen could be detected in the supernatant. However in the brain cells there was no reverse transcriptase activity, nor was p24 antigen ever detected in the supernatant at 14 days post-infection (p.i.), nor at any other time. Although a small proportion of cells were occasionally positive for the p24 antigen by IF, this was not consistent. No c.p.e. was observed in the infected cell lines, and there was no decrease in cell number in the infected cell cultures (data not shown).

Table 3 shows the results of cocultivation with C8166 cells of the brain cell lines infected with diverse HIV isolates at 5 × 10^4 TCID, taken at day 14 p.i. Infected CEM.CCRF cells formed syncytia with C8166 within 12 h. However, brain cell lines frequently took up to 5 days to produce syncytia following cocultivation with C8166. Cells could be infected by diverse isolates of HIV-1 and HIV-2, and infection was most readily achieved by the HIV-1 HTLV-III. RF isolate. U87 cells could not be infected by any isolate, whereas TE671 cells were readily infected by all isolates.

Fig. 1 shows the production of infectious virus particles in the cell lines TE671, U138 and C8166. Supernatant medium from these cell cultures at 14 days p.i. (5 days p.i. for the C8166 cells) were serially diluted 10-fold and added to uninfected C8166 cells. The highest dilution to show syncytium production within 5 days of culture was taken as the productive virus titre.
Table 1. Cell surface characteristics of brain cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CD4*</th>
<th>GFAP†</th>
<th>VSV(HIV)‡</th>
<th>Syncytia§</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM.CCRF</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>TE671</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U138</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U343a</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U251</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U87</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indirect IF for CD4 antigen.
† IF for glial fibrillary acid protein.
‡ Plaque formation by pseudotype viruses with HIV envelope and VSV genome.
§ Multinucleated giant cells following cocultivation with the indicator cell line, C8166.

Table 2. Infection of brain cell lines with $5 \times 10^4$ TCID HIV-1 (HTLV-III.RF), assayed on day 14

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IF*</th>
<th>RT†</th>
<th>p24 Ag‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM.CCRF</td>
<td>90</td>
<td>120</td>
<td>&gt;100</td>
</tr>
<tr>
<td>TE671</td>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>U138</td>
<td>1 to 2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>U343a</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>U87</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Percentage of cells fluorescing with HIV p24 MAb.
† Reverse transcriptase assay in c.p.m. $\times 10^{-3}$.
‡ p24 antigen (Dupont assay) in pg/ml.

Table 3. Syncytium production following cocultivation of C8166 cells with brain cell lines infected with diverse HIV isolates

<table>
<thead>
<tr>
<th>HIV isolate*</th>
<th>IIIB</th>
<th>RF</th>
<th>CBL-4</th>
<th>CBL-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM.CCRF</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>TE671</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U138</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U251</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U87</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* IIIB, HIV-1 HTLV-III.B New York isolate; RF, HIV-1 HTLV-III.RF Haiti isolate; CBL-4, HIV-1 CBL-4 Tanzania isolate; CBL-20, HIV-2 CBL-20 Gambia isolate.
† Presence or absence of syncytia at 72 to 120 h, except for those of CEM.CCRF cells which were recorded at 48 h.

Infection of U138 could not be achieved with less than $10^4$ TCID of virus and TE671 was only slightly more sensitive to infection, and was infected inconsistently at $5 \times 10^3$ TCID. Similarly the virus output of TE671 was higher than that of U138, but both these cell lines were poorly productive and 1000-fold less sensitive to infection, in comparison to the C8166 T cell line. Infected U138 cells could be maintained for up to 90 days, over which time the titre of virus output fell gradually and no culture produced infectious virus after 90 days.

Fig. 2 shows an ethidium bromide-stained 1% agarose gel containing 25 cycles of PCR-amplified DNA from uninfected and infected cells. The infected TE671 cells, but not the exposed U87 cells, show evidence of HIV proviral DNA, thus indicating complete correlation between the detection of viral DNA and the biological assay of cocultivation.
Fig. 1. Production of infectious virus particles from cell lines TE671 (□), U138 (○) and C8166 (△), plotted against dose of infecting virus (recorded as TCID for C8166 cells).

Fig. 2. Ethidium bromide-stained 1% agarose gel of DNA amplified in 25 cycles of the PCR. Source of DNA: lanes 1 and 7, HIV infectious clone NY5/LAV-1; lanes 2 and 8, uninfected TE671; lanes 3 and 9, HIV-1 RF-infected TE671; lanes 4 and 10, uninfected U87; lanes 5 and 11, HIV-1 RF-infected U87. Lane 6 was not used. Oligonucleotide primers are shown by arrowheads: a, env 141 bp band (lanes 1 to 5) and b, gag 247 bp band (lanes 7 to 11).

Fig. 3 shows the Northern blot of RNA from uninfected brain cells, and from the Jurkat T cell line probed with a cDNA probe for CD4. It can be seen that U138 and U343 produced a faint positive mRNA hybridization with the full-length 3.1 kb CD4 transcript; however, the U251 sample did not hybridize to this transcript.

Table 4 shows the ability of various anti-CD4 antibodies to block infection by HIV. Pre-
Table 4. Effect of blocking the infection of brain cell lines by HIV-1 (HTLV-III, RF) with anti-CD4 antibodies and with a neutralizing antiserum

<table>
<thead>
<tr>
<th>Antibody to block infection*</th>
<th>CD8 (2 μg/ml)</th>
<th>pCD4 (1:200)</th>
<th>F101.5 (2 μg/ml)</th>
<th>Leu3a (2 μg/ml)</th>
<th>Leu3a (20 μg/ml)</th>
<th>No. 6 (1:50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8166</td>
<td>++ ++ ++ †</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TE671</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>U138</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>U87</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* CD8, MAAb directed against CD8; pCD4, mouse polyclonal antibody against CD4; F101.5 and Leu3a, MAAbs against CD4; No. 6, human polyclonal HIV-1 neutralizing serum.
† Presence or absence of syncytia on cocultivation with C8166.

incubation of T cells with a 200-fold dilution of polyclonal anti-CD4 antibody or 2 μg/ml of Leu3a would repeatedly and consistently block HIV infection. However, neither of these antibodies nor the F101.5 MAAb block HIV infection with either the HTLV-IIIB (data not shown) or the HTLV-III, RF isolates of the brain-derived cells. Twenty μg/ml of Leu3a gave incomplete blocking, and a toxic effect on the cells was observed at this concentration. A human polyclonal neutralizing anti-HIV serum was also used to block HIV infection of these cell lines; this serum blocked infection in all cell lines.

**DISCUSSION**

Human brain- and muscle-derived cells were infected by diverse HIV isolates, but only when an m.o.i. 1000-fold higher than was required for infection of T lymphocyte lines was used. Cell lines remained productively infected after multiple passages for up to 3 months, but not indefinitely; there was no evidence of input virus carry-over as a source of error. In addition,
HIV infection of brain cells

proviral DNA was detected by PCR in established, multiply passaged, infected cell lines, but could not be detected in exposed U87 cells, which were also shown by other assays to be resistant to infection. Diverse HIV-1 and HIV-2 isolates were able to infect these cell lines. Unfortunately, we were unable to obtain any of the putative neurotropic or central nervous system (CNS) isolates of HIV for comparative infectivity studies; it is possible that one of these non-lymphotropic isolates may have led to higher titres of viral production at a lower m.o.i. in glial cells.

Although there were no detectable CD4 molecules on the surfaces of these cells, small quantities of CD4 mRNA were detected, and correlated with infectivity in the brain cells. Thus it is possible to consider that the expression of the CD4 antigen at a level below IF detection, perhaps 1000 receptors/cell, might be sufficient for HIV infection. However our experiments show that antibodies to CD4 which consistently block HIV infection of T cells and monocytes do not block infection of brain-derived cells, whereas a neutralizing antiviral serum blocks infection of all cell lines. It is possible that at such low concentrations anti-CD4 antibodies may bind to only one antigen molecule, giving a lower avidity for blocking HIV than CD4-rich haematopoetic cells. However we have also found that infectious HIV, pre-incubated with a soluble recombinant CD4 antigen, which neutralizes HIV infectivity for lymphocytes and monocytes, can still infect brain cells (Clapham et al., 1989). Thus the brain cells, requiring larger quantities of virus input to lead to infection, do not appear to use CD4 as their receptor. Despite expressing small quantities of the CD4 mRNA, there is no evidence that CD4 was expressed on the cell surface, or in the cytoplasm. The inability of the cell lines to form plaques with the VSV(HIV) pseudotypes at a titre comparable to the TCID of HIV further supports the apparent absence of the surface antigen CD4.

The role of direct infection by HIV of glial cells in the CNS is still unresolved. Published data on post-mortem examination by in situ hybridization and immunocytochemistry reveals that the dominant CNS cell types infected by HIV are the CD4+ monocyte/macrophage and the microglial cells (Shaw et al., 1985; Koenig et al., 1986; Wiley et al., 1986; Vazeur et al., 1987). It is believed that the microglial cell is in fact a bone marrow-derived macrophage (Hickey & Kimura, 1988). It is not possible to extend observations in vitro to the clinical situation, but it is important to note that the glial cells infected in vitro do not express HIV antigen on their cell surface and the scarcity of infected cells would render them undetectable by the methods cited above.

Thus the mechanism of entry of HIV into these CNS cells remains to be elucidated. The higher dose of virus required suggests that a non-specific mechanism may operate. However some cell lines such as U87 could not be infected even at the highest virus input; therefore the presence of a second, low-affinity, receptor for HIV entry remains possible. As it has been demonstrated that murine cells transfected with human CD4 may bind HIV, but do not internalize the virus, it is possible that a second component of the receptor may be allowing HIV entry into these glial cells (Maddon et al., 1986).

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