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Infection of Human Fetal Dorsal Root Ganglion Glial Cells with Human Immunodeficiency Virus Type 1 Involves an Entry Mechanism Independent of the CD4 T4A Epitope

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Human immunodeficiency virus type 1 (HIV-1) has been implicated in the generation of acquired immunodeficiency syndrome-associated neurological dysfunction, and it is believed that the presence of CD4 in the nervous system may be involved in the susceptibility of selected neural cell populations to HIV-1 infection. We previously demonstrated (B. Wigdahl, R. A. Guyton, and P. S. Sarin, Virology 159:440–445, 1987) that glial cells derived from human fetal dorsal root ganglion (DRG) are susceptible to HIV-1 infection and subsequently express at least a fraction of the virus genome. In contrast to HIV-1 infection of $CD4^+$ lymphocytes, which can be blocked by treatment with monoclonal antibodies directed against the HIV-1-binding region of CD4 (T4A epitope), treatment of human fetal DRG glial cells with similar antibodies resulted in only a slight reduction in HIV-1-specific *gag* antigen expression. In addition, preincubation of the HIV-1 inoculum prior to infection with HIV-1-neutralizing antiserum did not reduce HIV-1 *gag* antigen expression in these cells. Furthermore, we were unable to detect the synthesis or accumulation of the CD4 molecule in neural cell populations derived from DRG. However, a protected CD4-specific RNA fragment was detected in RNA isolated from human fetal DRG and spinal cord tissue by an RNase protection assay with a CD4-specific antisense RNA probe. RNA blot hybridization analysis of total cellular RNA isolated from human fetal DRG and spinal cord demonstrated specific hybridization to an RNA species that comigrated with the mature 3.0-kilobase CD4 mRNA as well as two unique CD4 RNA species with relative molecular sizes of approximately 5.3 and 6.7 kilobases. Furthermore, all three CD4-related RNA species were polyadenylated when isolated from human fetal spinal cord tissue. These data suggest that HIV-1 infection of human fetal DRG glial cells may proceed via a mechanism of viral entry independent of the T4A epitope of CD4.

Acquired immunodeficiency syndrome is now known to be caused by a retrovirus designated human immunodeficiency virus type 1 (HIV-1). It has become evident that infection with HIV-1 results in not only immunologic complications but also severe and progressive neurologic dysfunction in both adults and infants (3, 17, 29, 31, 33, 34, 40). There is considerable evidence demonstrating HIV-1 nucleic acid, protein, and virus particles in nervous system tissue from autopsied acquired immunodeficiency syndrome patients with neurologic complications (18, 25, 35, 36), and it is now evident that the primary cells harboring HIV-1 in brains of individuals with neurologic complications are those of the monocyte-macrophage lineage and possibly capillary endothelial cells (38, 43, 45). The observed HIV-1-induced neuropathology may occur indirectly after HIV-1 infection by the subsequent production of neurotoxic factors secreted by HIV-1-infected macrophages in the brain (14). Alternatively, Lee et al. (24) and Brenneman and co-workers (2) have demonstrated that the HIV-1 envelope glycoprotein gp120 may directly interfere with neuronal function by competing with neurotrophic factors normally present within the central nervous system.

Although the primary target of HIV-1 infection in the central nervous system of infected individuals does not appear to be glial cells or neurons (38, 43, 45), some evidence suggests that a low-level infection may occur in astrocytes and neurons (43, 45). In support of this observation, several investigators have demonstrated that HIV-1 can infect continuous human transformed cell lines of glial origin as well as low-passage neural cell populations isolated from both fetal and adult human nervous system tissues (4–6, 10, 22, 37, 39). Therefore, the possibility that neural cell types in the central and peripheral nervous systems, such as astrocytes, oligodendrocytes, Schwann cells, and neurons, are persistently or latently infected and are involved in the generation of neural cell abnormalities cannot be excluded.

If direct infection of human glial cell types by HIV-1 does occur, CD4 or a CD4-like molecule may be involved, since various studies have indicated that the CD4 protein directly interacts with HIV-1 gp120 (21, 23, 30, 44) and is at least a component of the receptor for HIV-1 in lymphocytes and monocytes (1, 8, 9, 20, 26, 30). Experimental evidence demonstrating the presence of CD4 in the nervous system includes (i) the detection of CD4-reactive cells with neuronal and glial cell morphology in human brain sections by immunohistochemical analysis (13, 16); (ii) the presence of both 3.0- and 1.8-kilobase (kb) CD4 mRNAs in RNA isolated from adult human brain sections (13, 26); (iii) the presence of L3T4 (the mouse homolog of CD4) mRNA in mouse brain (26, 28, 32); and (iv) the detection of CD4 RNA in a human glial cell line which is susceptible to HIV-1 infection (11). Although this evidence suggests that CD4 protein may be present in the adult human nervous system, in which it may potentially function as a receptor for HIV-1, attempts to detect CD4 protein in neural cell populations have been unsuccessful (4–6, 15) and little is known regarding the expression of CD4 in the developing nervous system. In addition, we as well as others (7, 15) have not been able to

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block HIV-1 infection of human neural cells in vitro with monoclonal antibodies (MAbs) with specificity for the HIV-1 gp120-binding region of the CD4 molecule. Consequently, the exact role of CD4 in HIV-1 neuropathogenesis is still unclear.

To ascertain whether CD4 is involved in HIV-1 infection of the developing human nervous system in vitro, we examined the human fetal nervous system for the presence and tissue distribution of CD4 RNA and protein. Additionally, we attempted to identify the functional role of CD4 in HIV-1 infection of human fetal dorsal root ganglion (DRG) glial cells in vitro. Our results indicate that infection of human fetal DRG glial cells by HIV-1 is not inhibited by MAbs directed against the HIV-1-binding region of CD4 (the T4A epitope) or HIV-1-neutralizing antiserum. In addition, we identified two novel CD4-related RNA species in human fetal DRG and spinal cord tissue. These results suggest that HIV-1 infection of the developing human nervous system may proceed via a mechanism independent of the T4A epitope of the CD4 molecule.

MATERIALS AND METHODS

Cell culture and human fetal tissue isolation. The lymphocyte cell lines SupT1 (a generous gift from F. Gonzalez-Scarano, Departments of Neurology and Microbiology, University of Pennsylvania, Philadelphia), H9, and Raji were maintained at 37°C in 10% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Human fetal DRG and spinal cord tissue were removed from aborted human fetuses (between approximately 11 and 16 weeks of gestational age) by microscopic surgery between 2 and 6 h postmortem. After isolation, fetal tissues were either enzymatically dissociated to obtain single-cell suspensions for in vitro cell culturing or suspended in RNA extraction buffer for preparation of total cellular RNA.

Isolation and in vitro maintenance of human fetal DRG glial cells. Primary cultures of human fetal neural cells were obtained as previously described (12, 19, 39–42). Briefly, each DRG with associated peripheral nerve and spinal root was removed from aborted fetuses by microscopic surgery and enzymatically dissociated with collagenase type IIS (0.02%), bovine pancreatic DNase I (0.004%), and porcine pancreatic trypsin type II (0.025%). After gentle mechanical disruption by aspiration through an 18-gauge needle, the resultant single-cell suspension was washed and maintained in supplemented Eagle basal medium, plated on collagen-coated glass cover slips, and maintained at 37°C in 10% CO_2. This mixed neural cell population contained approximately 10 to 30% neurons, 20 to 25% Schwann cells, 40 to 65% fibroblastoid cells, and less than 10% astrocytes and oligodendrocytes (39).

HIV-1 infection of human fetal DRG neural cells. Within 18 to 24 h after surgical isolation, the cell monolayer (approximately 10^4 cells per cover slip) was exposed for 24 h to either a 1:500 dilution of a partially purified preparation of HIV-1 (strain III_B; kindly provided by P. Sarin, National Cancer Institute, Bethesda, Md.; 50% tissue culture infective dose, approximately 10^4 to 10^5 infectious units per ml; reverse transcriptase activity, approximately 3.0×10^6 cpm/ml) or an appropriate mock-infecting solution consisting of phosphate-buffered saline (PBS) diluted in medium. The virus inoculum was subsequently removed, and the cell monolayer was overlaid with fresh medium that was changed every 48 h.

Blocking of HIV-1 infection with CD4-specific MAbs. Human fetal DRG neural cell populations were isolated and maintained in vitro for 18 to 24 h before the addition of 5.0 μg of an appropriate antibody per ml (diluted in medium) for 1 h at 37°C. The cells were infected with HIV-1 in the presence of MAb (2.5 μg/ml) for 18 h, followed by the removal of the virus inoculum and the addition of fresh medium containing the same MAb (2.5 μg/ml). Three days after infection, the cells were washed twice with PBS and fixed for immunofluorescence analysis of HIV-1 p17 and p24 *gag* proteins. Similarly, 2×10^5 SupT1 cells were treated with the selected MAb (at concentrations ranging from 1.25 to 10.0 μg/ml) as described above. After infection with HIV-1, the cells were washed twice with PBS and suspended in 200 μl of medium. After 3 to 4 days in culture, the SupT1 cells were expanded to individual wells of a 24-well tissue culture plate (Costar, Cambridge, Mass.) and maintained at approximately 7×10^5 to 9×10^5 cells per ml. Supernatant p24 antigen, as detected by an enzyme-linked immunosorbent assay (Coulter Immunology, Hialeah, Fla.), and the appearance of cytopathic effects (syncytium formation) within the SupT1 cells were used to monitor HIV-1 infection at increasing times postinfection. OKT4, OKT4A, OKT8, and OKM1 were purchased from Ortho Diagnostic Systems, Inc., Raritan, N.J. Leu3a was purchased from Becton Dickinson and Co., Mountain View, Calif.

Treatment of HIV-1-infected human fetal DRG glial cell populations with HIV-1-neutralizing antiserum. Human fetal DRG neural cells were isolated and maintained in vitro for 18 to 24 h as previously described. Pooled HIV-1-neutralizing antiserum from multiple donors, single-donor HIV-1 neutralizing antiserum, or normal human serum was diluted 1:2 with a 1:250 dilution of HIV-1 stock and incubated for 1 h at 37°C. This mixture was used to infect human fetal DRG neural cell monolayers or 2×10^5 SupT1 cells. All HIV-1-neutralizing antisera were obtained from F. Gonzalez-Scarano and contained antibodies directed against gp160, gp120, gp41, and *gag* proteins, as determined by immunoblot analysis. The neutralizing titer for the pooled HIV-1 antiserum was 1,600, as determined with H9 cells (F. Gonzalez-Scarano, personal communication).

Determination of HIV-1 p17 and p24 accumulation by indirect immunofluorescence. To examine primary human fetal DRG neural cells for the presence of HIV-1 *gag* proteins, we washed the cells twice in PBS and fixed them in methanol-acetone (1:1) for 90 min at room temperature. After rehydration in 4% bovine serum albumin in PBS for 2 h at room temperature, the cells were exposed to a 1:50 dilution of a mixture of mouse MAbs specific for HIV-1 p17 and p24 proteins (provided by P. Sarin) for 60 min at 37°C. The cells were washed with PBS containing 0.1% Triton X-100 and subsequently exposed to a rhodamine-conjugated goat anti-mouse immunoglobulin G secondary antibody (diluted 1:75; Tago, Inc., Burlingame, Calif.) for 60 min at 37°C. The cells were washed free of the secondary antibody and mounted on glass cover slips for photomicroscopic analysis.

Fluorescence-activated flow cytometric analysis. Human fetal DRG were surgically removed and dissociated as previously described. Subsequent to obtaining a single-cell suspension by mechanical disruption, the dissociated DRG and H9 cells were washed twice in PBS containing 2% FCS (2% FCS–PBS) and incubated in 4% bovine serum albumin for 30 min. MAbs used included OKT4, OKT8, OKM1, and PAB-901 (kindly provided by S. Tevethia, Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey). The cells were incubated with a 1:50 dilution of the appropriate primary

antibody for 30 min and washed twice in 2% FCS–PBS containing 0.01% Tween 20 and twice in 2% FCS–PBS without Tween 20. The cells were incubated with a fluorescein-conjugated goat anti-mouse secondary antibody (Becton Dickinson) for 30 min. All incubations were performed at 4°C to prevent capping of the immune complexes on the live cell surface. The cells were washed twice in 2% FCS–PBS, fixed in 2% paraformaldehyde, and analyzed on an Epics IV flow cytometer. Relative fluorescence intensity was expressed as log integrated green fluorescence and plotted with respect to cell number.

Radioimmunoprecipitation analysis of CD4 protein. Cells (1×10^7 to 2×10^7) were metabolically labeled for 10 to 12 h in cysteine- and methionine-free RPMI 1640 medium containing 10% heat-inactivated dialyzed FCS supplemented with 50 μCi each of [^{35}S] cysteine and [^{35}S]methionine per ml. Labeled cells were washed twice in ice-cold PBS, and the resultant cell pellet was suspended in 1.0 ml of lysis buffer consisting of 10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride and kept on ice for 15 min. The cell lysate was cleared of cellular debris by centrifugation at 26,000 × *g* for 30 min. Aliquots of the precleared cell lysate were exposed overnight to protein A-Sepharose beads conjugated to MAb OKT4 or PAB-901. After four washes in lysis buffer plus 0.2% sodium dodecyl sulfate (SDS), the beads were suspended in SDS-polyacrylamide sample buffer (60 mM Tris hydrochloride [pH 6.8], 1.0% SDS, 1.0% 2-mercaptoethanol) and boiled for 3 min. The eluted proteins were subjected to electrophoresis through a denaturing SDS–10% polyacrylamide gel. The gel was processed for fluorography and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen.

Total RNA isolation and RNA blot hybridization analysis. Total cellular RNA was isolated from human cell lines or human fetal tissues approximately 2 to 6 h postmortem (following microscopic surgical removal with extensive washing in PBS) by homogenization in 4 M guanidine isothiocyanate solution containing 0.024 M sodium acetate (pH 6.0) and 0.12 M 2-mercaptoethanol. The lysate was subjected to centrifugation through a 5.7 M CsCl gradient, and the RNA pellet was suspended in 0.3 M sodium acetate and precipitated under ethanol.

Analysis of CD4-specific RNA was achieved by electrophoresis of 10 μg of total RNA or 1 μg of polyadenylated RNA through a 1.0% agarose gel containing 1.1 M formaldehyde. After transfer to nitrocellulose filters, the filters were baked in vacuo for 90 min at 80°C, prehybridized for 1 h at 55°C, and hybridized with 10^6 cpm of radiolabeled probe per ml overnight at 55°C. Prehybridization and hybridization mixtures contained 50% formamide, 5× Denhardt reagent (1× Denhardt reagent contains 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll 400), 0.5% SDS, 100 μg of salmon sperm DNA per ml, and 5× SSPE (1× SSPE contains 50 mM Tris, 50 mM boric acid, and 1 mM EDTA).

Preparation of radiolabeled probes. A 661-base-pair (bp) *Sst*I fragment or a 284-bp *Hin*dIII-*Sst*I fragment from a CD4 cDNA clone (obtained from Richard Fisher, Biogen Corp., Boston, Mass.) was subcloned into the pGEM-3Z vector (Promega Biotech, Madison, Wis.). Generation of single-stranded, ^{32}P-labeled sense and antisense RNA probes was achieved by linearization of the plasmid DNA, in vitro transcription with either T7 or SP6 polymerase, and phenol-chloroform extraction and ethanol precipitation of the radiolabeled RNA.

RNase protection analysis. Total RNA (10 μg) was hybridized for 16 h at 37°C with 10^6 cpm of a ^{32}P-labeled 284-bp CD4 RNA probe in 30 μl of hybridization buffer [80% formamide, 0.04 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.7), 0.4 M NaCl, 1 mM EDTA]. After termination of the hybridization with 300 μl of stop buffer (0.3 M NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA), the unhybridized RNA was digested with RNase A (5 μg/ml) and RNase T_1 (0.25 μg/ml) for 30 min at 30°C. RNase digestion was terminated by the addition of 50 μg of proteinase K and 20 μl of 10% SDS and incubation for 15 min at 37°C. After phenol-chloroform extraction, the hybridized RNA was precipitated under ethanol, suspended in loading buffer (95% formamide, 10 mM EDTA, 0.05% bromphenol blue), and denatured for 5 min at 85°C. The assay products were subjected to electrophoresis through a 3.5% polyacrylamide–7 M urea gel, and autoradiography was performed.

RESULTS

Effect of anti-CD4 MAbs and HIV-1-neutralizing antisera on HIV-1 infection of human fetal DRG glial cells. We reported elsewhere (C. Kunsch and B. Wigdahl, Virology, in press) the establishment of a nonproductive HIV-1 infection of primary human fetal DRG neural cells in vitro. Infection of this cell population (as determined by expression of viral RNA and viral *gag* proteins) is primarily limited to neural cells with a nonneuronal morphology (DRG glial cells), with little or no detectable *gag* protein in cells morphologically and biochemically defined as neurons (39). HIV-1 infection of this cell population results in a transient increase in expression of viral RNA and protein, with expression of the viral genome reaching a maximum by 3 days postinfection and declining to undetectable levels thereafter (Kunsch and Wigdahl, in press), with no detectable cytopathic damage to the infected cell population. Furthermore, proviral DNA is readily detectable during this time interval. This interaction ultimately results in a nonproductive or abortive infection, as demonstrated by the lack of infectious progeny virus production (Kunsch and Wigdahl, in press), with the possible establishment of virus latency within the human fetal DRG neural cell population.

To determine whether CD4 has a functional role in infection of human fetal DRG neural cells with HIV-1, we attempted to block HIV-1 infection of DRG neural cells and a CD4$^+$ T-lymphocyte cell line (SupT1) by pretreatment with MAbs directed against the CD4 protein. MAbs OKT4 and OKT4A bind to two distinct epitopes of the CD4 protein; however, only MAb OKT4A prevents the interaction of gp120 with CD4 (21, 23). Inhibition of HIV-1 infection of SupT1 cells was demonstrated by the lack of cytopathic effects (syncytium formation) and the lack of detectable HIV-1 p24 in the culture supernatants of SupT1 cells treated with MAb OKT4A but not MAb OKT4 (Table 1). Concentrations of OKT4A as low as 1.25 μl/ml were sufficient to prevent infection of this high-level CD4-expressing lymphocyte cell line. These results support previous observations that HIV-1 infection of T lymphocytes requires the T4A epitope of CD4 (9, 20, 30).

In the presence of MAb OKT4A, human fetal DRG glial cells were susceptible to HIV-1 infection, as demonstrated by only a slight reduction in the number of HIV-1 p17- and p24-positive DRG glial cells (Table 1) relative to the results for cells treated with PBS, OKT4, OKT8 (which is specific for the CD8 cell surface lymphocyte glycoprotein), or OKM1 (which is specific for a monocyte-macrophage cell surface marker [data not shown]). When MAb Leu3a (another MAb which is specific for the gp120-binding region of CD4) was

TABLE 1. Effect of anti-CD4 MAbs on HIV-1 infection of human fetal DRG glial cells and SupT1 cells[a]

MAb	% p17- and p24-positive DRG glial cells in expt[b]:		Cytopathic effects in SupT1 cultures[c]	p24 antigen in SupT1 supernatant fluids[d]
	1	2		
None (PBS)	70	79	+++	+
OKT8	69	88	ND[e]	ND
OKT4	ND	82	+++	+
OKT4A	45	71	−	−

[a] In the experiments with SupT1 cells, the indicated MAbs were used at 1.25 μg/ml.
[b] Determined 3 days postinfection by indirect immunofluorescence microscopy. An average of 10 microscopic fields with a total of >750 cells was counted for each experimental value. Mock-infected cultures consistently lacked detectable HIV-1 p17 and p24 immunoreactivity (39; Kunsch and Wigdahl, in press).
[c] Relative amount of cytopathic effects as indicated by syncytium formation in the SupT1 cultures at 14 days postinfection.
[d] Relative presence (+) or absence (−) of HIV-1 p24 antigen in the SupT1 supernatant fluids at 14 days postinfection.
[e] ND, Not determined.

used to inhibit HIV-1 infection of human fetal DRG glial cells, similar results were obtained (15). The slight reduction in the number of *gag*-positive DRG glial cells in samples treated with OKT4A (Table 1) was reproducible and may represent a population of cells that utilize a T4A-dependent entry pathway. Whether this is a functionally distinct cell population is not known. It is unlikely that the inability to block HIV-1 infection of human fetal DRG glial cells (Table 1) was due to insufficient concentrations of blocking MAb, since when fivefold-higher concentrations of antibody were used in similar experiments, no additional blocking was observed (data not shown).

Human serum with HIV-1-neutralizing activity from a single donor or pooled from multiple donors did not prevent HIV-1 infection of fetal DRG glial cells, as compared with cells treated with normal human serum (Table 2). However, pooled HIV-1 antiserum effectively prevented HIV-1 infection of SupT1 cells, as demonstrated by the lack of syncytium formation in the SupT1 cells and p24 antigen in the SupT1 supernatants (Table 2).

Overall, these results suggest that although the T4A epitope may be involved in HIV-1 infection of human fetal DRG glial cells to a small extent (as evidenced by the slight reduction in the number of *gag*-positive DRG glial cells), another mechanism independent of the T4A epitope appears to predominate. At present, it is unclear which exact cell

TABLE 2. Effect of HIV-1-neutralizing antiserum on HIV-1 infection of human fetal DRG glial cells and SupT1 cells

Human anti-HIV-1 serum	% p17- and p24-positive DRG glial cells in expt[a]:		Cytopathic effects in SupT1 cultures[b]	p24 antigen in SupT1 supernatant fluids[c]
	1	2		
Normal	64	63	+++	+
Pooled donor	51	68	±	−
Single donor	58	58	ND[d]	ND

[a] See Table 1, footnote *b*.
[b] See Table 1, footnote *c*.
[c] See Table 1, footnote *d*.
[d] See Table 1, footnote *e*.

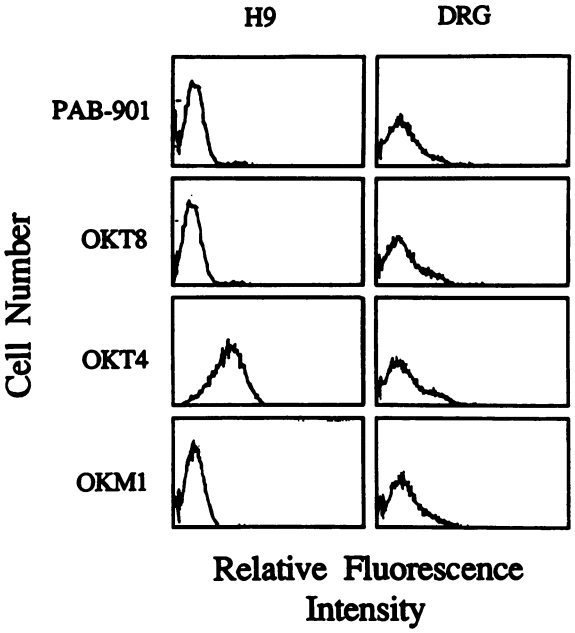

FIG. 1. Fluorescence-activated flow cytometric analysis of surface CD4 protein. Dissociated human fetal DRG cell populations and H9 cells were reacted with the indicated mouse MAb, incubated with a fluorescein-conjugated goat anti-mouse secondary antibody, fixed with 2% paraformaldehyde, and analyzed on an Epics IV flow cytometer. All primary MAbs were used at a 1:50 dilution. Antibodies included PAB-901 (an irrelevant immunoglobulin subclass-matched MAb specific for the simian virus 40 large T antigen), OKT8 (anti-CD8 [CD8 is a cytotoxic T-cell marker]), OKT4 (anti-CD4), and OKM1 (specific for a monocyte-macrophage cell surface marker).

type(s) within the DRG glial cell population uses a T4A-dependent or T4A-independent mechanism or whether there may be multiple entry mechanisms operating within a single cell type. In addition, the ability of HIV-1-neutralizing antiserum to block HIV-1 infection of SupT1 cells but not fetal DRG glial cells suggests that the region(s) of HIV-1 surface proteins (in particular, gp120) responsible for HIV-1 infection of lymphocytes may be different from that responsible for HIV-1 infection of human fetal DRG glial cells.

Radioimmunoprecipitation and fluorescence-activated flow cytometric analyses of CD4 in human fetal DRG glial cells. The surface expression of CD4 on human fetal neural cells after isolation from the DRG was examined by indirect immunofluorescence analysis with anti-CD4 MAbs in conjunction with flow cytometric analysis. H9 cells expressed detectable surface CD4 when reacted with anti-CD4 MAb OKT4, as evidenced by the increase in the relative fluorescence intensity (Fig. 1). However, for DRG cells, there was no significant increase in the relative fluorescence intensity in cell samples reacted with anti-CD4 MAb OKT4 (Fig. 1) or OKT4A (data not shown), as compared with cells treated with MAb PAB-901 (directed against the large T antigen of simian virus 40), OKT8, or OKM1. To control for the possibility that dissociation of the DRG may have removed cell surface protein, we used flow cytometric analysis with MAb to the major histocompatibility complex class I antigen to demonstrate that this cell surface molecule was retained at the surface of DRG neural cells (data not shown).

In addition to examination of the surface expression of the CD4 molecule on cell populations obtained from dissociated

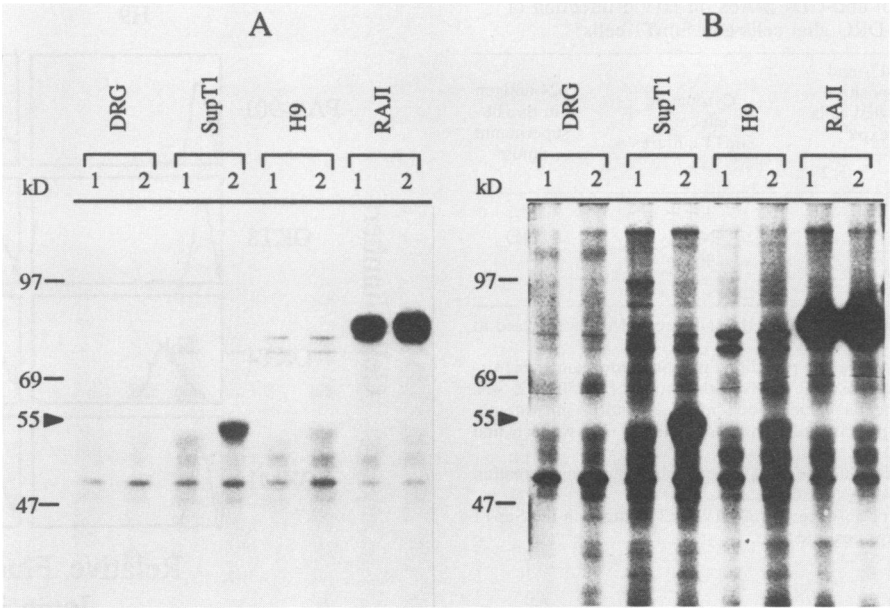

FIG. 2. Radioimmunoprecipitation analysis of CD4 protein. Human fetal DRG neural cells (DRG), SupT1 ($CD4^+$) cells, H9 ($CD4^+$) cells, and Raji ($CD4^-$) cells were metabolically labeled for 10 to 12 h, and the cell lysates were immunoprecipitated with an irrelevant immunoglobulin subclass-matched mouse MAb specific for the simian virus 40 large T antigen (PAB-901, lanes 1) or an MAb specific for CD4 (OKT4, lanes 2). The immune complexes were washed, boiled in gel loading buffer, and subjected to electrophoresis through an SDS–10% polyacrylamide gel, and autoradiography was performed. The gels were exposed for 2 days (A) and 10 days (B). kD, Kilodaltons.

DRG, immunoprecipitation of radiolabeled cell extracts and SDS-polyacrylamide gel electrophoresis were performed to determine whether these cells synthesized CD4. Cells were metabolically labeled for 12 h in cysteine- and methionine-free RPMI 1640 medium and immunoprecipitated with MAb OKT4 or PAB-901. The synthesis of CD4 was readily apparent in SupT1 cells and H9 cells (although to a much lesser extent), as demonstrated by a protein with a molecular size of approximately 55 kilodaltons in samples immunoprecipitated with OKT4 but not PAB-901 (Fig. 2). However, we were unable to demonstrate the synthesis of CD4 in control Raji cells (a $CD4^-$ B-cell line) or in cell populations of dissociated human fetal DRG after immunoprecipitation with OKT4, even with a fivefold-longer exposure (Fig. 2B). This result does not rule out the possibility, however, that the CD4 protein may have been synthesized in the DRG cell population at a level below our limits of detection.

Analysis of CD4 RNA in human fetal DRG and spinal cord. Although we were unable to detect CD4 in neural cell populations isolated from human fetal DRG (Fig. 1 and 2), the possibility remains that there may have been low-level expression of CD4 (or a CD4-related) protein that was undetectable by the assay methods used. This possibility is supported by the observation that a small percentage of fetal DRG glial cells appeared to be protected from HIV-1 infection by MAb OKT4A (Table 1), suggesting a low-level involvement of CD4 or a CD4-related protein. A majority of our effort focused on analyzing selected human fetal nervous system tissues for the presence and distribution of CD4 RNA, since it is conceivable that CD4 mRNA exists in the developing nervous system with the potential to code for an undetectable level of CD4 protein. To address this possibility, we examined human fetal DRG and spinal cord tissue for CD4-specific RNA by an RNase protection assay with a 284-bp CD4-specific antisense RNA probe (Fig. 3). This probe is a *Hin*dIII-*Sst*I recombinant subclone of the CD4 cDNA originally described by Maddon et al. (27). A 284-bp protected CD4-specific fragment was detected in the $CD4^+$ SupT1 cells but not in the $CD4^-$ Raji cells (Fig. 4). In addition, human fetal spinal cord and DRG also contained CD4-specific RNAs in approximately equal amounts but less than that observed in SupT1 cells. When a sense RNA probe was used in the RNase protection assay, no protected fragment was detected, demonstrating the specificity of the observed protected fragment (data not shown).

Having demonstrated the presence of CD4-related RNA in both human fetal spinal cord and DRG by RNase protection, we examined the size of the CD4 RNA by blot hybridization with a 661-bp CD4-specific antisense RNA probe (Fig. 3). In addition to detectable levels of 3.0-kb CD4 RNA in total cellular RNA isolated from human fetal DRG and spinal cord, we also demonstrated two unique CD4-related RNA species that migrated with relative molecular sizes of approximately 5.3 and 6.7 kb (Fig. 5). Interestingly, the 3.0-, 5.3-, and 6.7-kb CD4 RNA species were present in the polyadenylated fraction of human fetal spinal cord RNA, suggesting that they may code for CD4 and/or CD4-related proteins. The 3.0-kb RNA, which was also found in $CD4^+$ SupT1 and H9 cells but not in Raji cells (Fig. 5), is the mature mRNA for CD4 protein (27). The 5.3- and 6.7-kb CD4 RNA species were not found in total or polyadenylated RNA isolated from SupT1, H9, or Raji cells (Fig. 5) (C. Kunsch and B. Wigdahl, unpublished observations). The hybridization observed in the 2.0- and 5.1-kb regions in Fig. 5 represents nonspecific cross-hybridization of the RNA probe to the 18S and 28S rRNAs and was observed in all samples. Similar RNA blot hybridization analyses were done with various different RNA preparations from human fetal DRG and spinal cord tissue, although the relative hybridizations signals of the 5.3- and 6.7-kb species varied slightly between preparations. Since the 5.3- and 6.7-kb CD4-related RNA species detected in human fetal spinal cord are polyadenylated, it is likely that they are polyadenylated in human fetal DRG as well. From these preliminary data, it is

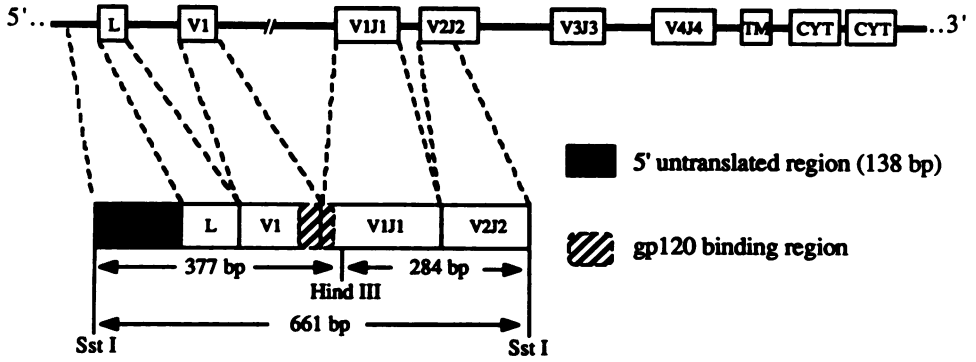

FIG. 3. Structure of the CD4 gene and cDNA probe. The leader (L), V-like (V), J-like (J), transmembrane (TM), and cytoplasmic (CYT) exon regions of the CD4 gene are designated by boxes along the chromosomal DNA. The CD4 cDNA clone (obtained from Richard Fisher, Biogen Corp.) is a 661-bp *Sst*I fragment of the CD4 cDNA originally described by Maddon et al. (27) and spans the L, V1, and V1J1 exons and approximately one-half of the V2J2 exon. This cDNA and a 284-bp *Hin*dIII-*Sst*I fragment were subcloned into the pGEM-3Z cloning vector and used to generate CD4-specific sense and antisense radiolabeled RNAs for use as molecular probes.

tempting to speculate that these two unique CD4-specific RNA species may potentially code for CD4 or CD4-related peptides that may function as biological receptors for HIV-1.

DISCUSSION

The studies reported here demonstrated that HIV-1 can infect human fetal DRG glial cell populations in vitro by a route independent from that of lymphoid cells. Although we were not able to demonstrate the synthesis or presence of CD4 on the cell surface of human fetal DRG neural cells, we identified two unique CD4-related RNA species approximately 5.3 and 6.7 kb in size, in addition to the 3.0-kb CD4 RNA, in human fetal DRG and spinal cord tissue.

In lymphocytes and monocytes, the CD4 glycoprotein is the only receptor molecule known for HIV-1 (1, 8, 9, 20, 26, 30). The suggestion that CD4 may act as the receptor for HIV-1 in other cells types, including those of the nervous system, has centered on the observation of CD4 RNA and protein in adult human brain sections (13, 16, 26) and in a human glioblastoma cell line (11). However, direct evidence for the involvement of CD4 in HIV-1 infection of neural cells is lacking. Recently, two reports suggested that HIV-1 infection of a human glioblastoma cell line does not depend on the CD4 receptor, as demonstrated by the inability to block HIV-1 infection with anti-CD4 MAb (7, 15) or with a soluble form of CD4 (7). In addition, we demonstrated that HIV-1 infection of human fetal DRG glial cells cannot be blocked by pretreatment with antibodies to the T4A epitope of CD4 or by preincubation of the infectious HIV-1 inoculum with HIV-1-neutralizing antiserum. Therefore, the T4A epitope of the CD4 molecule does not appear to be involved

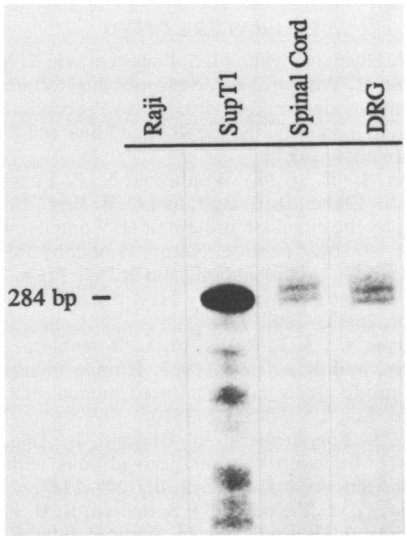

FIG. 4. Total RNA analysis of CD4 RNA by an RNase protection assay. Total RNA (10 μg) was hybridized to a 284-bp ^{32}P-labeled antisense CD4-specific RNA probe (Fig. 3), and the remaining single-stranded RNA was digested with RNase. After polyacrylamide gel electrophoresis, the amount of CD4 probe protected by each RNA sample was visualized by autoradiography. The relative amounts of protected probe are shown for Raji (CD4$^-$) cells, SupT1 (CD4$^+$) cells, and human fetal DRG and spinal cord tissue.

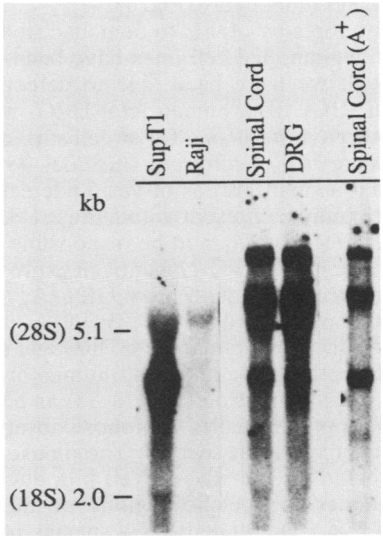

FIG. 5. Analysis of CD4 RNA by blot hybridization. Total RNA (10 μg) or polyadenylated RNA (1 μg) was subjected to electrophoresis and transferred to nitrocellulose. The blot was hybridized with a 661-bp ^{32}P-labeled antisense CD4-specific RNA probe (Fig. 3). Size markers are indicated on the left. The results are shown for total RNA preparations isolated from Raji (CD4$^-$), H9 (CD4$^+$), SupT1 (CD4$^+$), and human fetal DRG and spinal cord tissue and for polyadenylated RNA from human fetal spinal cord tissue [Spinal Cord (A$^+$)].

in HIV-1 infection of a majority of the human fetal DRG glial cell population, suggesting an alternative entry mechanism.

The observation that HIV-1-neutralizing antiserum was effective at neutralizing virus infection of SupT1 lymphocytes (Table 2) and H9 cells (data not shown) suggests that the anti-HIV-1 antibodies in this antiserum recognize regions of a viral surface protein (presumably gp120 and/or gp41) that are critical for HIV-1 infection of lymphocytes. Since the HIV-1-neutralizing antiserum did not inhibit infection of DRG glial cells, the specific regions of the viral protein that are critical for infection of lymphocytes in vitro may not be the same regions that are necessary for HIV-1 infection of fetal DRG glial cells. It is not known whether HIV-1 entry into neural cells occurs either by a specific neural cell receptor in conjunction with direct fusion of the viral envelope with the plasma membrane or by virus-cell fusion in the absence of a specific receptor molecule. Regions of the HIV-1 gp41 glycoprotein have been proposed to mediate fusion of the viral envelope with the plasma membrane; whether this fusion can occur in the absence of gp120-CD4 interactions is unclear. In any case, regions of CD4 that interact with gp120 do not appear to be necessary for HIV-1 infection of human fetal DRG neural cells.

At present, the exact human fetal nervous system cell types that are susceptible to HIV-1 infection and sensitive or refractile to treatment with the anti-T4A antibodies are unknown. Because the primary DRG glial cell populations are derived from the peripheral nervous system, astrocytes and oligodendrocytes (central nervous system cell types) do not constitute more than a small percentage of this mixed cell population. Other potential targets for HIV-1 infection in the human fetal DRG cell population include Schwann cells or partially differentiated nonneuronal fibroblastoid glial cells. Great care was taken to minimize the introduction of non-DRG tissue in the surgical isolation of the DRG; thus, it is unlikely that contaminating lymphocytic or monocytic cell types were present in this population. Furthermore, primary DRG cultures were negative for surface markers of both lymphocytes and monocytes (Fig. 1).

Previous attempts by others to demonstrate CD4 in HIV-1-susceptible human glial cell lines have been unsuccessful (4–6). Although we have been able to detect 3.0-kb CD4 RNA in total RNA from human fetal DRG, we have been unable to detect synthesis of CD4 or cell surface expression of CD4 in primary DRG cultures. This does not rule out the possibility that a small but, as-of-yet, undetectable amount of CD4 protein may be present within these cells. Low-level expression of CD4 may, in fact, be responsible for the slight reduction (Table 1) of HIV-1 *gag* antigen expression in DRG glial cells after treatment with MAb OKT4A.

The detection of 5.3- and 6.7-kb CD4 RNA species in total RNA preparations from human fetal DRG and spinal cord is of interest. In both mouse and adult human brains, various sizes of CD4 mRNA are produced (28, 32); in both cases, the variant transcripts are smaller than those observed in $CD4^+$ T lymphocytes from each species. The mouse CD4 (L3T4) gene is expressed (at the RNA level) in a developmentally regulated manner in specific regions of the brain (28). Whether the two CD4-related RNA species present in human fetal DRG and spinal cord arise via differential splicing, processing, or both and are developmentally regulated remains to be determined. Alternatively, it is possible that these two unique CD4-related RNA species represent gene products from an entirely different gene with significant nucleotide sequence similarity to the CD4 gene. This is less likely, however, since fairly stringent hybridization conditions were used (Fig. 5) and the two unique CD4 RNA species were present when hybridizations were performed under even more stringent conditions (data not shown). In light of the fact that HIV-1 appears to use an alternative entry pathway for infection of human neural cells (7, 15; Tables 1 and 2), it will be of interest to determine whether the 5.3- and 6.7-kb CD4 RNA species are involved in the T4A-independent infection mechanism by possibly coding for CD4 or CD4-related proteins.

In conclusion, the studies reported here demonstrate that HIV-1 can infect human fetal DRG glial cell populations by a route independent of the T4A epitope of the CD4 molecule and independent of regions of gp120 that are important for HIV-1 infection of T lymphocytes. Although we have not yet demonstrated the synthesis or physical presence of CD4 in the human fetal neural cell population, we have demonstrated, in addition to 3.0-kb CD4 mRNA, two unique CD4-related RNA species with relative molecular masses of 5.3 and 6.7 kb in human fetal DRG and spinal cord. However, the relative contribution of the 3.0-kb species as well as the two unique high-molecular-mass species to the coding of potential receptor proteins is currently under investigation. In addition, their functional significance, with respect to HIV-1 infection, has yet to be defined. These results may have long-range implications regarding therapeutic approaches aimed at controlling HIV-1 infection of the developing human nervous system.

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