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# Characterization of a Novel Binding Site for the Human Immunodeficiency Virus Type 1 Envelope Protein gp120 on Human Fetal Astrocytes

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**$^{125}$I-labeled recombinant gp120 bound to primary cultures of human fetal astrocytes with a single class of 260-kDa binding sites, with a $K_d$ of 26 nM and maximal number of binding sites of 29.9 fmol/4 × $10^4$ cells. Neither CD4 nor galactocerebroside was detectable on astrocytes, and $^{125}$I-labeled recombinant gp120 binding to astrocytes was not blocked by antibodies against galactocerebroside or the gp120 binding domain of CD4.**

Infection with human immunodeficiency virus type 1 (HIV-1) frequently results in a dementing illness (25). Since HIV-1 encephalopathy was first recognized, the infection of resident cells with HIV-1 in the central nervous system and the toxic effects of the HIV-1 envelope protein gp120 on neural cell types have been topics of intense study. It has now been firmly established that glial cells (microglia and astrocytes) can be infected in vivo (9, 13, 17, 19, 23, 27, 28, 34) and in vitro (4, 5, 7, 8, 16, 35, 37). As it is now understood, gp120 is responsible for mediating virus entry in various cell types. Infection of microglia is mediated via gp120 binding to CD4 (16). The homologous receptor on astrocytes remains to be determined. It has been shown, however, that gp120 acts directly on astrocytes to (i) alter its transport functions, including $Na^+/H^+$ exchange, $K^+$ conductance, and glutamate efflux (2); (ii) induce tyrosine kinase activity (30); (iii) inhibit β-adrenergic regulation of astrocytic function (22); and (iv) inhibit glial fibrillary acidic protein expression (26).

gp120 binds to the CD4 molecule on lymphocytes and macrophages, a membrane-associated C-type lectin in placental cells (7), sulfated oligosaccharides (21, 32, 33), and sulfated glycoconjugates (36). It also binds to glycolipids, galactocerebroside C (Gal C), and sulfatide in neuroblastoma cell lines (3, 14, 15) and binds to a 180-kDa protein on a glioma cell line (18). In this study, we characterize the molecule on the surface of primary human fetal astrocytes responsible for interacting with gp120 and describe specific binding sites for iodinated recombinant gp120 ($^{125}$I-rgp120).

**Binding of $^{125}$I-rgp120 to astrocytes.** Human fetal brain tissue (gestational age of 13 to 16 weeks) was obtained with written consent from women undergoing elective termination of pregnancy and with approval of the University of Manitoba Ethics Committee. Brain tissue was dissected, and pure (>99%) cultures of astrocytes, as determined by positive staining for glial fibrillary acidic protein, were prepared as previously described (11). HeLa-T4 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 0.2 mg of G418 per ml, and 0.1 mg of hygromycin B per ml. SK-N-MC cells (American Type Culture Collection) were cultured in minimal essential medium with sodium pyruvate and 10% fetal bovine serum. Prior to the binding assay, 2 × $10^4$ astrocytes and 3 × $10^4$ HeLa-T4 cells and SK-N-MC cells were plated in each well of 96-well plates and maintained at 37°C for 18 h. The final cell number of astrocytes per well was approximately 4 × $10^4$ at the time of assay.

HIV-$1_{SF2}$ rgp120 (>90% pure as determined by Coomassie staining after polyacrylamide gel electrophoresis [PAGE]) was labeled with Na$^{125}$I by lactoperoxidase with Enzymobeads (Bio-Rad). The $^{125}$I-labeled protein was separated from free $^{125}$I by using a PD-10 column (Pharmacia). The specific activity of $^{125}$I-rgp120 was 3.9 × $10^4$ cpm/ng. Binding of $^{125}$I-rgp120 to cells was performed as described previously (31, 32), with the following modifications. Briefly, astrocytes and HeLa-T4 cells were incubated in binding medium (RPMI 1640, 0.5% bovine serum albumin [BSA], 50 μg of bacitracin per ml) containing $^{125}$I-rgp120 (1.0 nM) and unlabeled rgp120 (0 to 120 nM) at 4°C for 5 h. The cells were solubilized in 0.2 N NaOH and were counted in a gamma counter for cell-associated radioactivity. Astrocytes incubated with $^{125}$I-rgp120 showed maximal binding at 5 h at 4°C (data not shown), with or without 100-fold unlabeled rgp120. At 1.0 nM $^{125}$I-rgp120, the specific binding was 50% of the total binding (Fig. 1), similar to that seen on monocytes (10). Scatchard analysis of these data revealed that $^{125}$I-rgp120 bound to a single class of binding molecules on the astrocytes with an apparent $K_d$ of 26 nM and an apparent maximal number of binding sites of 29.9 fmol/4 × $10^4$ cells (4.5 × $10^5$ binding molecules per cell) (Fig. 1).

**$^{125}$I-rgp120 binding to astrocytes is CD4 and Gal C independent.** A monoclonal antibody against the gp120 binding site on CD4 (OKT4a) (Ortho Diagnostics) and rabbit anti-Gal C antibody (Chemicon) were used to determine if these two antibodies could block the binding of rgp120 to astrocytes. HeLa-T4 cells were used as a positive control with OKT4a and SK-N-MC cells as a positive control with anti-Gal C antibody (3, 14). The cells were preincubated with the respective antibodies for 60 min at 37°C, washed twice in ice-cold binding medium, and incubated with 1.0 nM $^{125}$I-rgp120 for 5 h at 4°C. The cells were washed and counted as described above. Sixty percent of the total binding on HeLa-T4 cells was inhibited by 1 μg of OKT4a per ml (Fig. 2A), and 70% $^{125}$I-rgp120 binding on SK-N-MC cells was seen with anti-Gal C antibody at a dilution of 1:20 (Fig. 2B). Neither of the antibodies (up to 4 μg of OKT4a per ml or 1:20 dilution of anti-Gal C antibody) (Fig. 2) nor recombinant soluble CD4 protein (up to 8 μg/ml) had

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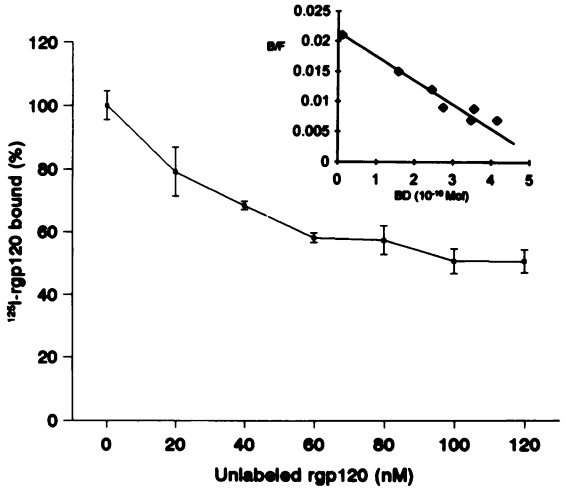


FIG. 1. Binding of $^{125}$I-rgp120 to astrocytes. Human fetal astrocytes ($4 \times 10^4$ cells per well) were incubated with 1 nM $^{125}$I-rgp120 alone or with increasing concentrations of unlabeled rgp120 ranging from 0 to 120 nM for 5 h at 4°C. The unbound radioactivity was removed by three washes in ice-cold PBS with 0.5% BSA. The cell-associated radioactivity was measured in a gamma counter after solubilization of the cells in 0.2 N NaOH. The values represent the mean ± standard error of two experiments, each done in triplicate. The insert presents a saturation isotherm plot of the data from above. A single binding site was revealed with $K_d$ of 26 nM and maximal number of binding sites of 29 fmol. B/F, bound/free ratio.

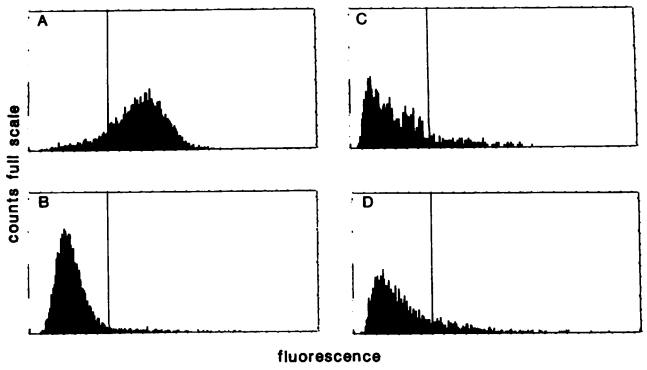


FIG. 3. Expression of surface molecules determined by flow cytometry. (A) As a positive control, CD4 was detected on HeLa-T4 cells by flow cytometry with DAKO-T4-FITC. (B) Background level of fluorescence on HeLa-T4 cells with goat anti-mouse IgG conjugated with FITC alone. (C) CD4 could not be detected on astrocytes by flow cytometry with DAKO-T4-FITC. (D) Background level of fluorescence on astrocytes with goat anti-mouse IgG conjugated with FITC alone.

any effect on the binding of $^{125}$I-rgp120 to astrocytes (data not shown).

The presence of CD4 molecules on the cell surface was determined by immunofluorescence flow cytometry (11). The astrocytes were trypsinized briefly (30 s) and removed from the monolayer primarily by mechanical force. Cells were incubated with a monoclonal antibody (1 μg/ml) against CD4 molecule conjugated to fluorescein isothiocyanate (FITC) (DAKO-T4-FITC) (Dakopatts). To ensure that trypsinization did not alter the epitope defined by DAKO-T4, HeLa-T4 cells, which also form adherent monolayers, were used as a positive control. HeLa-T4 cells exhibited strongly positive staining (Fig. 3A). Background levels of fluorescence were determined by using goat anti-mouse immunoglobulin G (IgG) conjugated to FITC (Boehringer Mannheim) (Fig. 3B and D). CD4 could not be detected on the cell surface of astrocytes (Fig. 3C). We simultaneously plated cells on glass coverslips, fixed them in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and stained them by an immunofluorescence technique (11) with OKT4a (0.5 μg/ml) and DAKO-T4 (1 μg/ml). Goat anti-mouse IgG conjugated with rhodamine (1:50) (Chemicon)

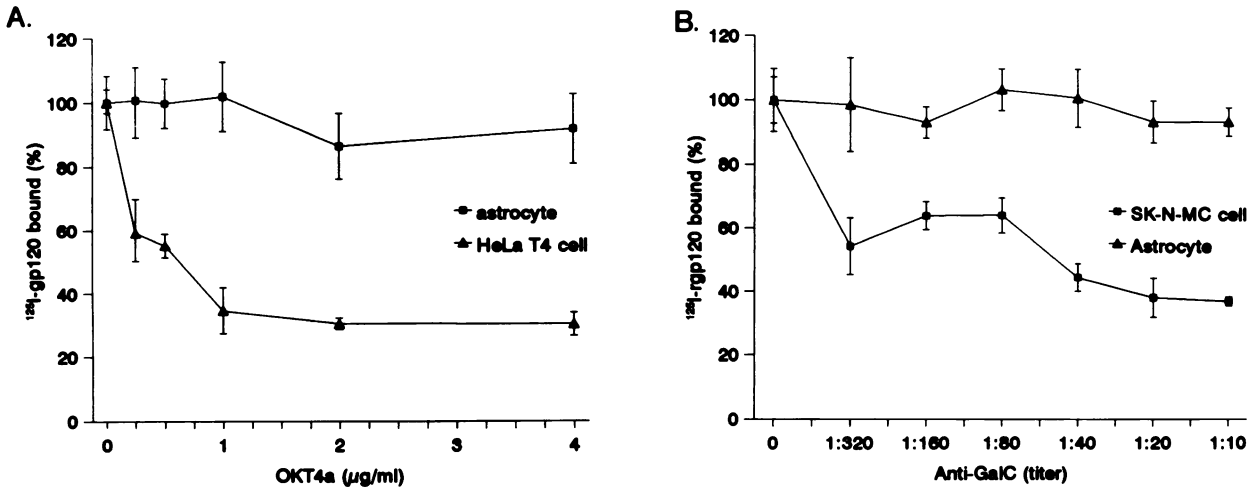


FIG. 2. (A) Effect of OKT4a on the binding of $^{125}$I-rgp120 to astrocytes (■) and HeLa-T4 cells (▲). The cells were preincubated with various concentrations of OKT4a for 60 min at 4°C. After removal of unbound antibody by two washes, 1 nM $^{125}$I-rgp120 was added for 5 h at 4°C. OKT4a was unable to block $^{125}$I-rgp120 binding to astrocytes. The data represent the mean ± standard error of two independent experiments done in duplicate. (B) Effect of anti-Gal C antibody on the binding of $^{125}$I-rgp120 to astrocytes (▲) and SK-N-MC cells (■). The cells were preincubated for 60 min at 4°C with various dilutions of anti-Gal C antibody. After removal of unbound antibody by two washes, 1 nM $^{125}$I-rgp120 was added and incubated for 5 h at 4°C. The data represent the mean ± standard error of two independent experiments done in duplicate.

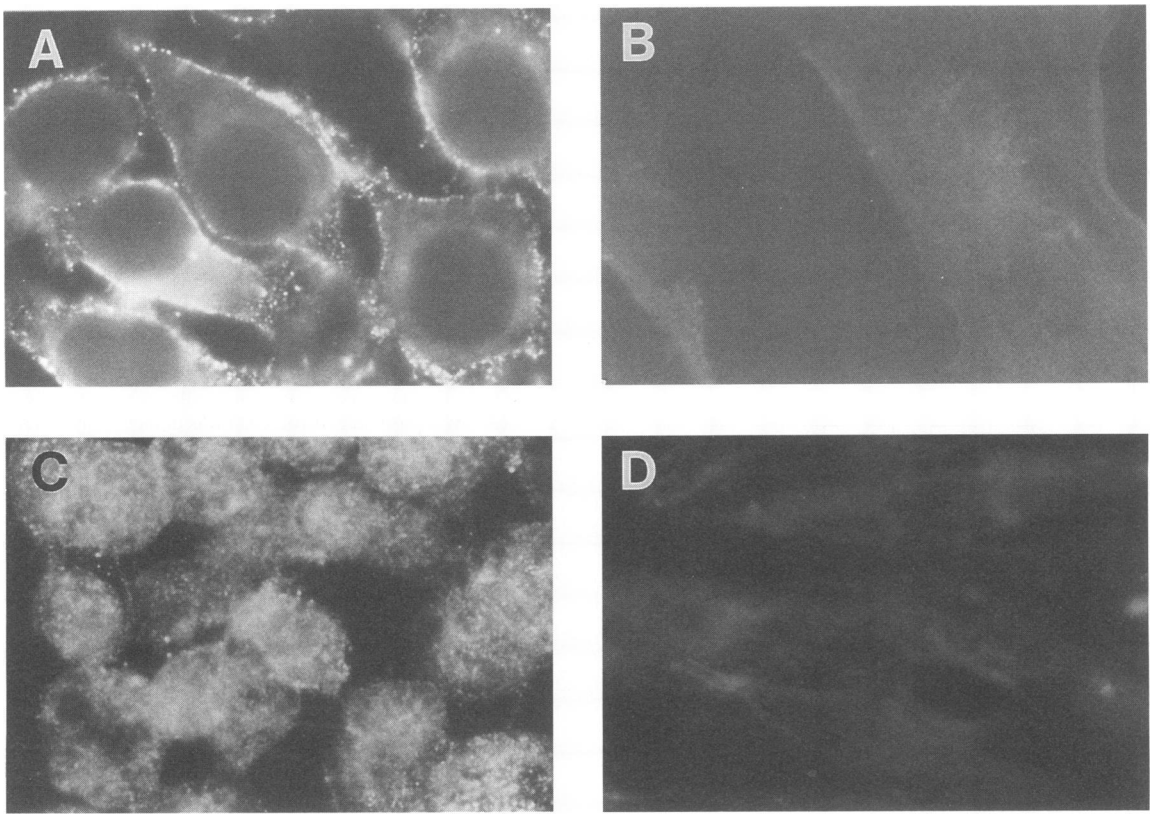


FIG. 4. Immunocytochemistry. (A) HeLa-T4 cells show staining for CD4 on cell membrane. (B) Astrocytes could not be stained with OKT4a. (C) Gal C was detected on SK-N-MC cells by staining with anti-Gal C antibody. (D) Astrocytes were Gal C negative.

was used as a secondary antibody. Secondary antibody alone without incubation in primary antibody was used as a negative control. HeLa-T4 cells were used as a positive control. The CD4 molecule was present on the cell membrane of HeLa-T4 cells (Fig. 4A). However, no specific staining on astrocytes was observed compared with that on controls (Fig. 4B). Similarly, astrocytes and SK-N-MC cells were stained with anti-Gal C antibody (1:100) by using goat-anti-rabbit IgG conjugated with rhodamine (1:50) (Chemicon) as a secondary antibody. SK-N-MC cells showed cell membrane staining with anti-Gal C antibody (Fig. 4C), while astrocytes showed only background levels of fluorescence (Fig. 4D).

**Immunoprecipitation of rgp120 binding molecules.** Astrocytes were surface radioiodinated by the lactoperoxidase technique (1). Briefly, $5 \times 10^7$ cells were suspended in 1 ml of PBS containing 1 mCi of $Na^{125}I$ and 20 μg of lactoperoxidase, and after 0, 1, 5, and 10 min, 10 μl of 0.03% $H_2O_2$ was added. Reactions were stopped by adding 5 ml of PBS containing 10 mM NaI. Labeled cells were incubated with $^{125}I$-rgp120 (0.1 μg) or rgp120 (1 μg) for 5 h at 4°C. The labeled cells without rgp120 were used as a negative control. The cell pellets were lysed by adding detergent lysing buffer, and the nuclei were removed by centrifugation (24). Antiserum from an HIV-1-infected patient predetermined to have a high titer of antibody against gp120 or normal human sera were each bound to protein A-agarose. All lysates were absorbed with the beads for 2 h at room temperature, washed, solubilized, and resolved by 4 to 15% polyacrylamide gradient sodium dodecyl sulfate gel electrophoresis (SDS-PAGE). Dried gels were exposed to X-ray film (Kodak, Rochester, N.Y.) for 5 to 7 days.

A single distinct band at 260 kDa was identified as a putative gp120 binding protein. This protein could not be immunoprecipitated when normal serum was substituted for anti-gp120 serum. It was also absent in cells not treated with rgp120 (Fig. 5A). Because the binding kinetics were determined with $^{125}I$-rgp120, we treated $^{125}I$-labeled astrocytes with $^{125}I$-rgp120 and immunoprecipitated them with anti-gp120 sera. The same 260-kDa protein was again observed, confirming that iodination of gp120 did not alter its binding properties. When $^{125}I$-rgp120 was incubated with the anti-gp120–protein A-agarose conjugate and analyzed by SDS-PAGE, a single major band of 120 kDa was seen (Fig. 5B).

The primary receptor for HIV-1 is CD4 (29, 31). In the brain, the infection of microglia has been shown to be mediated by CD4 (16, 37). However, HIV-1 infection of astrocytes and other nervous system-derived cell lines may proceed via an entry mechanism independent of CD4 (3, 4, 7, 8, 14, 18, 35). Furthermore, gp120 can bind to CD4-negative glial cells to activate tyrosine kinase (30) and to human astrocytes, altering ion exchange (2). Thus, it has been suggested that additional HIV-1 receptors are present on neural cells. We were unable to demonstrate the presence of CD4 or Gal C on human fetal astrocytes, and it is doubtful that this was due to CD4 or Gal C expression at levels below the sensitivity of the techniques used, because the binding of $^{125}I$-rgp120 to the astrocyte membrane was not inhibited by OKT4a, recombinant soluble CD4, or anti-Gal C antibody. Furthermore, Gal C and sulfatide could not be detected in lipid extracts of human fetal astrocytes by high-performance thin-layer chromatography (12).

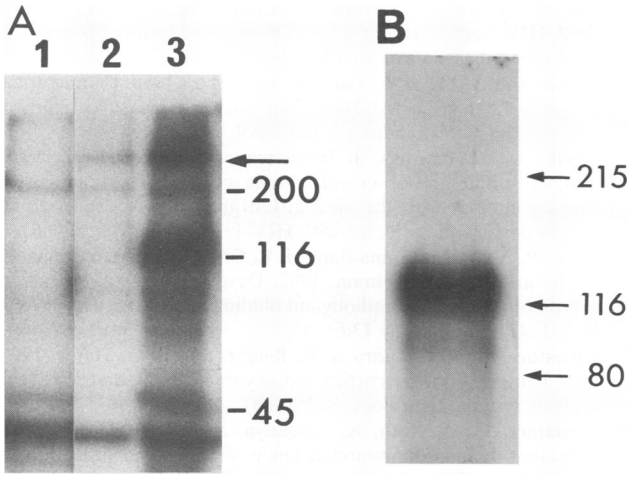


FIG. 5. Immunoprecipitation of gp120 binding sites. (A). Astrocytes were surface labeled with $^{125}$I and then incubated with rgp120 or $^{125}$I-rgp120 prior to detergent extraction and immunoprecipitation with anti-gp120–protein A-agarose. Samples were analyzed by SDS-PAGE in 4 to 15% polyacrylamide gradient gels followed by autoradiography. Lane 1, $^{125}$I-labeled astrocytes only. Lanes 2 and 3, $^{125}$I-labeled astrocytes incubated with rgp120 or $^{125}$I-rgp120, respectively. A 260-kDa band is seen in lanes 2 and 3 only (arrow). Fivefold more protein was added to lane 3 to detect any minor bands in addition to the 260-kDa molecule. (B). $^{125}$I-rgp120 incubated with anti-gp120–protein A-agarose conjugate and analyzed by SDS-PAGE (6% polyacrylamide). A single prominent band at 120 kDa and two minor bands at 95 and 66 kDa representing a breakdown product of gp120 are shown. No polymers of gp120 could be detected.

The $K_d$ of $^{125}$I-rgp120 binding to human fetal astrocytes (26 nM) is greater than those of the gp120-CD4 (2 to 5 nM) (20) and gp120-Gal C (12 nM) (13) interactions. These findings suggest that the affinity of gp120 for astrocytes is slightly lower than that for lymphocytes or Gal C-expressing cells. Nevertheless, gp120 at comparable concentrations has been shown to produce biochemical and morphological changes in astrocytes (26). Our finding of $4.5 \times 10^5$ binding sites on each astrocyte compares favorably to $0.5 \times 10^4$ to $1 \times 10^4$ CD4 receptors per lymphoid cell (7) when the fact that astrocytes are typically 10 to 20 times larger than lymphoid cells is taken into account. The size of the gp120 binding site on the astrocytes with an observed molecular mass of 260 kDa was much greater than that of the gp120 binding site to the CD4 molecule, which is a 58-kDa protein. Thus, several lines of evidence suggest that the rgp120 binding site on human fetal astrocytes is distinct from the CD4 molecule and Gal C. However, it remains to be determined if the binding site of HIV-1 gp120 on human astrocytes can act as a receptor for HIV-1.

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