SPC3, a V3 Loop-Derived Synthetic Peptide Inhibitor of HIV-1 Infection, Binds to Cell Surface Glycosphingolipids†

Olivier Delézay, Djilali Hammache, Jacques Fantini, and Nouara Yahi*

Laboratoire de Biochimie et Biologie de la Nutrition, URA-CNRS 1820, Faculté des Sciences de St Jérôme, 13397 Marseille Cedex 20, France

Received May 20, 1996; Revised Manuscript Received September 26, 1996⊗

ABSTRACT: Synthetic multibranched peptides derived from the V3 domain of human immunodeficiency virus type 1 (HIV-1) gp120 inhibit HIV-1 entry into $CD4^+$ and $CD4^-$ cells by two distinct mechanisms: competitive inhibition of HIV-1 binding to $CD4^-/GalCer^+$ colon cells and postbinding inhibition of HIV-1 fusion with $CD4^+$ lymphocytes. In the present study, we have characterized the cellular binding sites for the V3 peptide SPC3, which possesses eight V3 consensus motifs GPGRAF radially branched on a neutral polyLys core matrix. These binding sites are glycosphingolipids that share a common structural determinant, i.e., a terminal galactose residue with a free hydroxyl group in position 4: GalCer/sulfatide on $CD4^-/GalCer^+$ colon cells; LacCer and its sialosyl derivatives GM3 and GD3 on $CD4^+$ human lymphocytes. These data suggest that the V3 peptide binds to the GalCer/sulfatide receptor for HIV-1 gp120 on HT-29 cells and thus acts as a competitive inhibitor of virus binding to these $CD4^-$ cells, in full agreement with previously published virological data. In contrast, SPC3 does not bind to the CD4 receptor, in agreement with the data showing that the peptide inhibits HIV-1 infection of $CD4^+$ cells by acting at a postattachment step. The binding of SPC3 to LacCer, GM3, and GD3, expressed by $CD4^+$ lymphocytes, suggests a role for these glycosphingolipids in the fusion process between the viral envelope and the plasma membrane of $CD4^+$ cells. Since the multivalent peptide can theoretically bind to several of these glycosphingolipids, we hypothesize that the resulting cross-linking of membrane components may affect the fluidity of the plasma membrane and/or membrane curvature, altering the virus−cell fusion mechanism.

The third variable region (V3 loop) of HIV-1[1] gp120 plays an important role in the mechanisms controlling the fusion between the envelope of HIV-1 and the cell membrane of the target cells (Moore & Nara, 1991; Levy, 1993; Moore et al., 1993). In particular, the V3 loop has been considered as a fusion domain whose role would be to initiate the fusion process after an initial interaction between gp120 and CD4 on the surface of $CD4^+$ cells (Freed et al., 1991). According to this paradigm, the V3 loop might interact with an accessory molecule (a coreceptor) which is specifically expressed on human cells (Maddon et al., 1986; Clements et al., 1991; Dragic et al., 1992; Lorès et al., 1992; Broder et al., 1993). The recent identification of a family of β-chemokine receptors as fusion cofactors for primary and laboratory isolates of HIV-1 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dorantz et al., 1996; Dragic et al., 1996; Feng et al., 1996) will help to validate this model.

In addition, an alternative pathway of HIV-1 infection has been recently characterized in $CD4^-$ cells of neural and colonic origin (Harouse et al., 1991; Yahi et al., 1992). These cells express the glycosphingolipid galactosylceramide (GalCer), which acts as a high-affinity receptor for HIV-1 gp120 (Bhat et al., 1991; Long et al., 1994). The interaction between GalCer and gp120 appears to involve the V3 loop of the viral glycoprotein (Cook et al., 1994; Yahi et al., 1994a, 1995a, 1996).

The importance of the V3 loop for HIV-1 entry into both $CD4^-$ and $CD4^+$ cells prompted us to use V3-derived synthetic peptides as potential inhibitors of HIV-1 entry into both cell types. After several unsuccessful trials with various monomeric peptides, we have tested synthetic peptides in which the V3 motifs were radially branched on an uncharged polyLys core matrix, a procedure originally developed by Tam (1988) to enhance peptide immunogenicity. The prototype peptide in this series was SPC3, an octamer of the HIV-1 gp120 V3 consensus sequence GPGRAF (i.e., $[GPGRAF]_8$-$[K]_4$-$[K]_2$-K-βA). SPC3 inhibits HIV-1 infection in $CD4^+$ lymphocytes and macrophages (Yahi et al., 1994b) as well as in $CD4^-/GalCer^+$ epithelial cells (Yahi et al., 1995a). Interestingly, SPC3 was shown to affect HIV-1 infection by at least two distinct mechanisms including prevention of GalCer-mediated virus attachment to the surface of $CD4^-/GalCer^+$ cells and postbinding inhibition of virus entry into $CD4^+$ lymphocytes (Yahi et al., 1995b). In the latter case, the data were consistent with the recognition, by SPC3, of cell surface molecules involved in the fusion process.

In the present study, we have determined the nature of the cellular binding sites for SPC3 on both $CD4^-$ and $CD4^+$ cells. We show that SPC3 binds to cellular glycosphin-

† This work was supported by grants from the Agence Nationale de Recherche sur le SIDA (ANRS) and the Fondation pour la Recherche Médicale (SIDACTION). O.D. is the recipient of a SIDACTION fellowship. N.Y. is the recipient of an ANRS fellowship.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: CDH, ceramide dihexoside; CTH, ceramide trihexoside; GalCer, galactosylceramide; GluCer, glucosylceramide; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HIV, human immunodeficiency virus; HPTLC, high performance thin layer chromatography; LacCer, lactosylceramide; mAb, monoclonal antibody; MIP, macrophage inflammatory protein; PBMC, peripheral blood mononuclear cells; PBS, Ca^{2+}, Mg^{2+}-free phosphate buffered saline; RANTES, regulated on activation normal T-cell expressed and secreted.

S0006-2960(96)01205-6 CCC: $12.00

golipids that share a common structural feature, i.e., a free hydroxyl group in position 4 of the terminal galactose residue. These glycosphingolipids are (i) GalCer/sulfatide in $CD4^-$ intestinal cells and (ii) LacCer and its sialosyl derivatives GM3 and GD3 in $CD4^+$ lymphocytes. The potential role of these glycosphingolipids during the fusion process between HIV-1 and its target cells is discussed.

MATERIALS AND METHODS

Materials. SPC3, i.e., $[GPGRAF]_8$-$[K]_4$-$[K]_2$-K-βA, was generously provided by M. Mollard (Eurethics, Paris, France). The peptide was purified to homogeneity, and the amino acid analysis of the purified peptide agreed with the deduced amino acid ratios. [^{3}H]Suramin (49 Ci/mmol) was purchased from Isotopchim (Ganagobie-Peyruis, France). Lipids were from Sigma (St Louis, MO). Poly(vinyl chloride) multiwell plates (No. 3911) were from Falcon-Becton Dickinson (Le Pont de Claix, France). Immulon 1 multiwell plates were purchased from Poly Labo (Strasbourg, France). The anti-V3 mAb F5 was generously provided by F. Traincard (Institut Pasteur, Paris, France). Tissue culture media and fetal calf serum were from BioWhittaker (Gagny, France).

Cell Culture. Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors, activated with phytohemagglutinin, and cultured in RPMI 1640 medium containing 10% fetal calf serum and interleukin 2 as described (Yahi et al., 1994b). CD4-positive cells were purified from PBMCs with anti-CD4 antibodies attached on magnetic beads Dynabeads (Dynal, France). The human T-lymphoblastoid CEM cell line was grown in RPMI 1640 containing 10% fetal calf serum. Human colon epithelial cell lines HT-29-D4 and Caco-2/Cl2 (Fantini et al., 1993a) were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) with 10% fetal calf serum.

Binding of Anti-V3 mAb to SPC3. SPC3 (10 μM) was coated on Immulon 1 multiwell plates for 1 h at 37 °C. Non specific binding sites were saturated with PBS, containing 2% bovine serum albumin for 1 h at 37 °C. After washing, the peptide was incubated with the anti-V3 mAb F5 (1.25 μg/mL) in either the absence or presence of the indicated concentration of suramin. After 1 h at 37 °C, the wells were washed and the bound mAb was revealed with peroxidase-conjugated rabbit anti-mouse antibodies (1:1000) using *o*-phenylenediamine as substrate. The absorbance was measured at 490 nm with a Biotek EL 311 multiwell spectrophotometer (Osi, Les Ulis, France).

Binding of SPC3 to Lipids. Stock solutions of lipids were prepared in chloroform/methanol (1:1, vol/vol) at a concentration of 1 mg/mL. The indicated amounts of lipids (100 μL) were then allowed to adsorb on poly(vinyl chloride) multiwell plates by evaporation of the solvent under a chemical hood, according to Backenson et al. (1995). The plates were treated with PBS containing 2% bovine serum albumin overnight at 4 °C to reduce nonspecific binding and then incubated with 100 μL of 10 μM SPC3. After 1 h at 37 °C, the plates were rinsed six times with 200 μL of PBS and incubated with 100 μL of [^{3}H]suramin (1 μCi/mL). As previously reported, suramin binds specifically to the GPGRAF motif of SPC3 and not to unrelated peptides (Yahi et al., 1994a). After 1 h at 37 °C, the plates were washed seven times with 200 μL of PBS, each well was individualized, and the radioactivity was determined in a β scintillation counter (Beckman, Marseille, France).

Binding Studies on CD4. CD4 binding studies were performed on soluble CD4 immobilized strips from the gp120 capture ELISA kit (Intracel Corp., London, UK). The wells were incubated with either the anti-CD4 mAb MT151 (Boehringer Mannheim, Les Ulis, France) or SPC3 at the indicated concentrations. The binding of MT151 was revealed by ELISA using peroxidase-conjugated rabbit anti-mouse antibodies as reported (Delézay et al., 1996). SPC3 was revealed with [^{3}H]suramin after individualization and counting of each well in a β scintillation counter.

Incorporation of Sulfatide into Caco/Cl2 Cells. GalCer-negative human colon epithelial Caco-2/Cl2 cells were incubated for 2 h at 37 °C in DMEM/F12 culture medium containing 1 mg/mL sulfatide. After three washings with PBS, the presence of sulfatide was determined by indirect immunofluorescence with the anti-GalCer monoclonal antibody R-mAb (Rantsch et al., 1982) as previously reported (Delézay et al., 1996).

Binding of SPC3 to Live Cells. The cells were incubated with various concentrations of SPC3 in PBS with 2% bovine serum albumin for 1 h at 4 °C. After several washings with PBS, the cells were incubated with [^{3}H]suramin (1 μCi/mL) in PBS containing 0.5% gelatin for 20 min at 37 °C. The cells were then washed in PBS and disrupted with 0.1 N NaOH. The radioactivity associated with the cells was determined in a β scintillation counter.

Binding of Anti-CD4 mAbs to CEM Cells. CEM cells were resuspended in PBS containing 0.5% bovine serum albumin and incubated with saturating concentrations of various anti-CD4 mAbs in the presence of the indicated amount of SPC3. After washing in PBS, the cells were incubated with fluorescein-conjugated anti-mouse antibodies and analyzed by quantitative flow cytometry using the Qifikit assay (Biocytex, Marseille, France). The number of antibody molecules bound to the cells was determined according to a standard assay with antibody-conjugated beads (linear in the range of 4000−500 000 antigenic sites per bead).

Lipid Extraction and Analysis. PBMCs and immunopurified $CD4^+$ lymphocytes were metabolically labeled with [^{14}C]galactose (2 μCi/mL) for 24 h at 37 °C. Lipids were extracted with chloroform/methanol/water (4:8:3) and partitioned as described (Yahi et al., 1994c). The lipids contained in the Folch lower and upper phases were resolved on high-performance thin-layer chromatography (HPTLC) plates (silica gel 60; Merck, Darmstadt, Germany) and chromatographied in chloroform/methanol/water (60:35:8). The position of glycolipids was determined by orcinol staining of corresponding standards ran in parallel on the same plate. The chromatography plates were analyzed with a GS-505 phosphoimager (Biorad, Ivry sur Seine, France).

RESULTS

Specific Detection of SPC3 Using [^{3}H]Suramin. SPC3 is a synthetic polymeric peptide construction in which eight V3 loop consensus motifs (GPGRAF) from HIV-1 gp120 are radially branched on an uncharged core matrix (Yahi et al., 1995b). This multibranched peptide is recognized by F5, a neutralizing anti-gp120 mAb directed to the tip of the V3 loop. As shown in Figure 1, the binding of F5 to SPC3 is inhibited by increasing concentrations of suramin, a

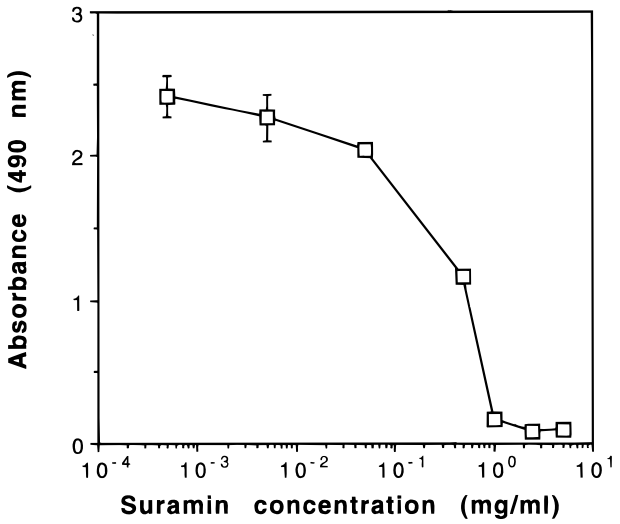

FIGURE 1: Effect of suramin on anti-V3 mAb binding to SPC3. The binding of F5, an anti-gp120 mAb recognizing the tip of the V3 domain, to SPC3 was measured by ELISA as described in Materials and Methods. Suramin was present at the indicated concentrations during the incubation with F5. The results are expressed as the mean of two experiments (±SD).

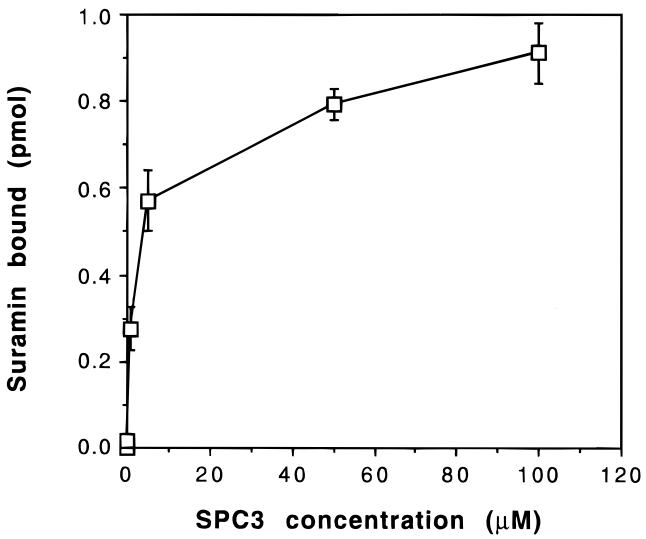

FIGURE 2: Binding of SPC3 to HT-29-D4 intestinal cells. $CD4^-$/$GalCer^+$ HT-29-D4 cells were incubated for 2 h with the indicated concentrations of SPC3. After three washes, the bound peptide was revealed with [^{3}H]suramin. Cell-associated radioactivity was measured with a scintillation counter. Results are expressed as the mean of three independent experiments (±SD).

polysulfonyl naphthylurea previously reported to bind to the V3 domain of HIV-1 gp120 (Yahi et al., 1994a). These data demonstrate that the F5 mAb and suramin recognize the same V3 loop GPGRAF motif. We took advantage of this property to develop a detection assay of SPC3 using radiolabeled suramin as ligand for the V3 peptide (Yahi et al., 1994a). In this assay, the binding of [^{3}H]suramin to SPC3 was dose-dependent and saturable. The suramin/SPC3 stoichiometry was found to be 2:1 as determined by the Hill plot method, indicating that SPC3, as coated on multiwell plates, displays two binding sites for suramin (data not shown). The interaction between SPC3 and [^{3}H]suramin was inhibited by unlabeled suramin, with a 50% effective concentration of 10 μM. The binding specificity of suramin for SPC3 was further demonstrated by using irrelevant multibranched peptides as well as SPC3 analogs with the same net charge as SPC3 but with a shorter motif (i.e., GPGR, GPGRA). As previously reported (Yahi et al., 1994a), [^{3}H]suramin did not bind to these peptides. Taken together, these data indicated that [^{3}H]suramin specifically recognizes the entire V3 consensus motif GPGRAF and that it could be used as a specific probe for SPC3 detection and quantitation.

SPC3 Binds to $GalCer^+$ but Not to $GalCer^-$ Epithelial Cells. SPC3 inhibits the GalCer-mediated entry of HIV-1 into the $CD4^-$/$GalCer^+$ intestinal epithelial cell line HT-29 (Yahi et al., 1995a). Based on the ability of SPC3 to inhibit the binding of recombinant gp120 to purified GalCer, it has been assumed that SPC3 was a competitive inhibitor of HIV-1 attachment to the GalCer receptor. Thus, we analyzed the binding of SPC3 to the surface of HT-29 cells. In this experiment, HT-29 cells were first incubated with various concentrations of SPC3, and the binding of the peptide was then revealed with [^{3}H]suramin as described in Materials and Methods (Figure 2). Since [^{3}H]suramin alone did not bind to HT-29 cells (Baghdiguian et al., 1996), the cell-associated radioactivity corresponded to the specific detection of SPC3 bound to the cells. This binding was dose-dependent, with a maximal binding capacity of 0.911 ± 0.069 pmol of suramin, corresponding to 137 105 ± 10 384 SPC3 binding sites per cell (considering that the SPC3/suramin stoichiometry was not affected by cellular binding of the peptide). In contrast, SPC3 did not significantly bind to Caco-2/Cl2, a $GalCer^-$ epithelial cell line (Figure 3).

Incorporation of GalCer-SO_3^- (Sulfatide) in Caco-2/Cl2 Plasma Membrane Results in SPC3 Binding. Some exogenously added glycosphingolipids can be incorporated in the plasma membrane of various cultured cells (Schwartzmann et al., 1983; Chigorno et al., 1985; Varani et al., 1994). The sulfated derivative of GalCer, i.e., 3-sulfoGalCer or sulfatide, which is far more soluble than GalCer in an aqueous medium, has been successfully introduced in the plasma membrane of human B-lymphocytes, rendering these cells able to bind recombinant gp120 (McAlarney et al., 1994). In the experiment presented in Figure 3, Caco-2/Cl2 cells were incubated with sulfatide and then evaluated for SPC3 binding. The incorporation of sulfatide resulted in a dramatic increase of SPC3 binding. These data confirmed the specificity of our binding assay for SPC3 and identified cell surface-associated GalCer/sulfatide as a receptor for the V3 peptide.

SPC3 Binds to Purified Sulfatide. We then analyzed the ability of SPC3 to recognize purified sulfatide. In this assay, increasing amounts of sulfatide were adsorbed on the surface of poly(vinyl chloride) multiwell plates and subsequently incubated with a unique concentration of SPC3. [^{3}H]suramin was used as a revealing agent to detect the V3 peptide. As shown in Figure 4, the binding of SPC3 to sulfatide was specific and dose-dependent. The minimal amount of sulfatide that was detected by SPC3 was 250 ng, while maximal binding was reached for 2 μg of sulfatide. These data confirm that sulfatide is specifically recognized by SPC3.

SPC3 Binds to Purified Human $CD4^+$ Lymphocytes. Next we investigated whether SPC3, which inhibits HIV-1 infection in $CD4^+$ lymphocytes (Yahi et al., 1994b), could bind to the surface of these cells. To this end, $CD4^+$ lymphocytes were purified from PBMC obtained from healthy volunteers using anti-CD4 antibody-coated magnetic beads. The percentage of $CD4^+$ cells obtained after purification was superior

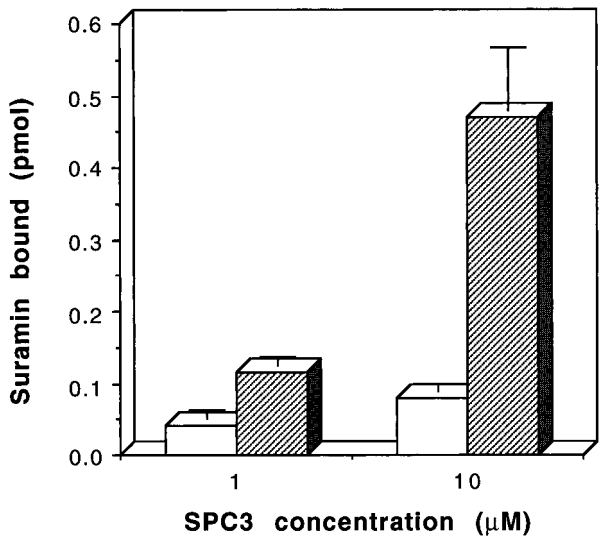

FIGURE 3: Binding of SPC3 to Caco-2/Cl2 cells after incorporation of sulfatide. $CD4^-$/$GalCer^-$ Caco-2/Cl2 cells were either treated (closed histograms) or not treated (open histograms) with sulfatide for 2 h at 37 °C. After thorough washings, the cells were incubated with the indicated concentration of SPC3 and the peptide bound to the cells was revealed with [^{3}H]suramin. Results are expressed as the mean of three independent experiments (±SD).

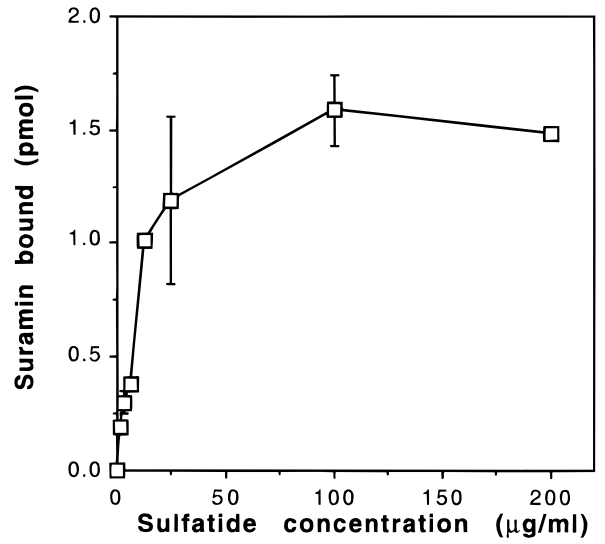

FIGURE 4: Binding of SPC3 to purified sulfatide. Sulfatide was dissolved in methanol at the indicated concentrations, distributed in 96-well multiwell plates (100 μL/well) and evaporated overnight under a chemical hood. After blocking of nonspecific binding with PBS containing 2% bovine serum albumin, the wells were serially incubated with 10 μM SPC3 and [^{3}H]suramin. After throrough washing in PBS, the radioactivity bound to the wells was counted in a scintillation counter. Results are means of two experiments performed in duplicate (± SD)

to 98%, as assessed by immunofluorescence studies with FITC-conjugated OKT4A monoclonal anti-CD4 antibody (data not shown). These $CD4^+$ lymphocytes were incubated with various concentrations of SPC3, and the peptide bound was detected with [^{3}H]suramin. The data in Figure 5 show that SPC3 bound to the surface of CD4 lymphocytes. The binding was dose-dependent, with a maximal binding capacity of 1.216 ± 0.027 pmol of [^{3}H]suramin, corresponding to 366 016 ± 8 127 SPC3 binding sites per cell. Therefore, the plasma membrane of $CD4^+$ lymphocytes displays a wide number of specific binding sites for SPC3.

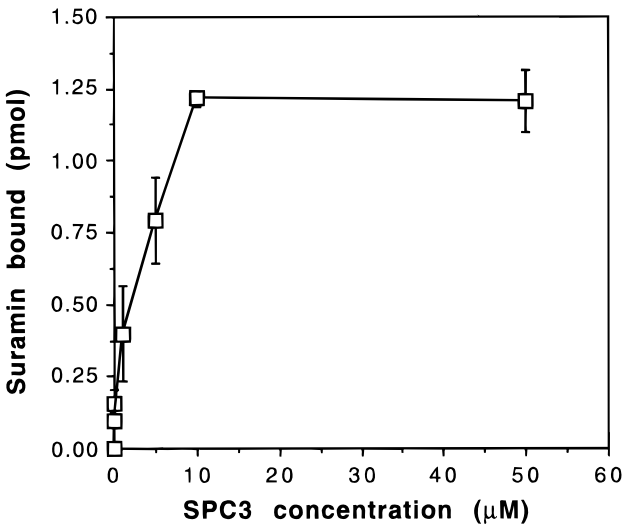

FIGURE 5: Binding of SPC3 to human $CD4^+$ lymphocytes. $CD4^+$ cells were purified from normal PBMC using anti-CD4 antibody-coated magnetic beads. The cells were incubated with the indicated concentration of SPC3 and the bound peptide was revealed with [^{3}H]suramin. Results are expressed as the mean of three independent experiments (±SD).

SPC3 Does Not Bind to CD4. In a first attempt to identify the nature of the SPC3 binding site(s) on $CD4^+$ lymphocytes, we studied the interaction between SPC3 and the CD4 molecule in a solid-phase assay. As shown in Figure 6B, CD4 adsorbed on ELISA plates was easily detectable by the anti-CD4 monoclonal antibody MT151. However, we did not detect any binding of SPC3 to CD4, even for concentrations of SPC3 up to 20 μM (Figure 6A). The peptide could however bind to sulfatide, which was used as a positive control for SPC3 binding (Figure 6A). Moreover, preincubation of CEM cells with concentrations of SPC3 up to 100 μM failed to inhibit the binding of any of five different anti-CD4 monoclonal antibodies (MT151, Q4120, 13B8-2, BL4, or ABT) (Table 1). Instead, an increased binding was observed for four out of the five anti-CD4 mAbs when tested in presence of the highest concentration of SPC3. Reciprocally, none of these antibodies were able to inhibit the binding of SPC3 to either CEM cells or $CD4^+$ lymphocytes (data not shown). Finally, treatment of CEM cells with 100 μg/mL proteinase K resulted in the loss of detection of CD4, while SPC3 binding was not significantly inhibited (data not shown). Taken together, these data do not support the hypothesis that SPC3 binds to CD4 (Benjouad et al., 1995) and rather suggest that the V3 peptide recognizes proteinase K-resistant molecules on the surface of $CD4^+$ lymphocytes.

SPC3 Binds to Glycosphingolipids Extracted from CD4 Lymphocytes. We thus considered the possibility that the SPC3 binding site(s) on $CD4^+$ lymphocytes could be nonproteic plasma membrane components. Among these molecules, glycosphingolipids have focused our attention since (i) SPC3 recognizes GalCer/sulfatide on the surface of $CD4^-$ intestinal cells and (ii) this class of molecules has been suggested to mediate the fusion induced by HIV-1 via an interaction with the V3 loop of gp120 (Lazaro et al., 1994; Dragic et al., 1995). Neutral and acidic glycosphingolipids were extracted from purified $CD4^+$ lymphocytes after metabolic labeling with [^{14}C]galactose and separated by HPTLC (Figure 7). In agreement with previous studies (Stein & Marcus, 1977; Macher et al., 1981; Symington & Hakomori, 1985; Yuasa et al., 1990; Reivinen et al., 1994),

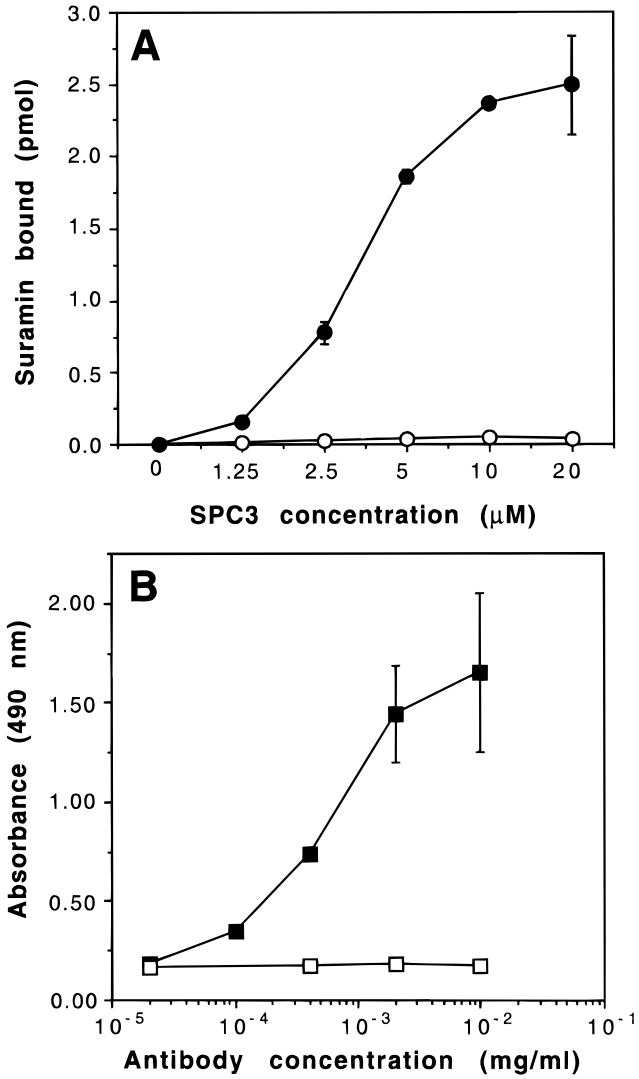

FIGURE 6: SPC3 does not bind to CD4. (A) SPC3 binding to sulfatide but not to CD4. Multiwell plates precoated with either soluble CD4 (open circles) or sulfatide (closed circles) were incubated with various concentrations of SPC3 and the bound peptide was then detected with [^{3}H]suramin. The data are expressed as the mean of duplicate experiments (±SD). (B) Detection of CD4 with the monoclonal anti-CD4 antibody MT151. Multiwell plates precoated with soluble CD4 were incubated with various concentrations of MT151 (closed squares) or of R-mAb as control (open squares). The bound antibodies were detected with peroxidase-conjugated rabbit anti-mouse antibodies. The data are expressed as the mean of duplicate experiments (±SD).

the Folch lower phase contained various neutral glycosphingolipids including GluCer (glucosylceramide) and LacCer (lactosylceramide), while the Folch upper phase contained several gangliosides including GD3 and GM3. Neither GalCer nor sulfatide could be evidenced in these extracts, as previously reported (Stein & Marcus, 1977). For comparison, the pattern of glycosphingolipids extracted from normal PBMC was also presented (Figure 7). Some interesting differences between these extracts could be seen, illustrating the efficiency of the purification process of $CD4^+$ lymphocytes. For instance, $CD4^+$ lymphocytes were enriched in ganglioside GD3, while ceramide trihexosides (CTHs) were more represented in PBMC. Lipid extracts from CEM cells were also characterized. Interestingly, these cells expressed mainly the ganglioside GM3 but not GD3 (not shown). Lipids from the Folch lower and upper phases of PBMC and CEM cells were adsorbed onto the surface of multiwell plates and probed with SPC3, using [^{3}H]suramin as revealing agent. SPC3 bound to these lipids with a maximal binding capacity of 0.348 ± 0.020 and 0.256 ± 0.002 pmol of SPC3/μg of, respectively, lower and upper phase lipids extracted from PBMC and 0.289 ± 0.017 and 0.265 ± 0.012 pmol of SPC3/μg of, respectively, lower and upper phase lipids extracted from CEM cells (Figure 8).

Table 1: Effect of SPC3 on the binding of anti-CD4 mAbs to CEM cells

antibody	specificity	SPC3 (μM)	sites/cell
BL4	D4 domain of CD4	0	29 726
		0.1	29 396
		1	28 846
		10	32 222
		100	47 149
ABT	D1 domain of CD4, region CDR2	0	29 769
		0.1	24 724
		1	30 067
		10	34 149
		100	46 955
13-B8−2	D1 domain of CD4, region CDR3	0	30 787
		0.1	30 762
		1	30 836
		10	30 886
		100	49 889
Q4120	D1 domain of CD4, region CDR2	0	28 433
		100	28 603
MT-151	D1 domain of CD4, region CDR3	0	29 869
		100	35 997
ALB12	CD45	0	81 867

[a] The number of sites per cell was determined for each antibody by quantitative immunocytometry using the Qifikit kit as described in Materials and Methods. The anti-CD45 mAb was used as a positive control. SPC3 was present at the indicated concentrations in competition with the antibodies.

Identification of the Glycosphingolipids Recognized by SPC3. In order to identify the glycosphingolipids recognized by SPC3 in the lymphocyte extracts, we first evaluated the ability of SPC3 to bind to a series of purified glycosphingolipids. In these experiments, each glycosphingolipid was adsorbed at various concentrations on poly(vinyl chloride) multiwell plates and then probed with SPC3. Typical curves are shown in Figure 9. The data are summarized in Table 2. The ceramide moiety of glycosphingolipids was not recognized by SPC3. Besides sulfatide, three glycosphingolipids displayed high binding capacity for the peptide: LacCer and its sialosylderivatives GM3 and GD3. The common feature of these glycolipids is a free hydroxyl group in position 4 of the terminal galactose residue. As a matter of fact, substitution of this group by a neutral sugar (asialo-GM2 or CTH) resulted in a significant decrease of binding. Interestingly, the additional presence of a sialic acid residue (e.g., in GM2) further decreased the binding capacity for SPC3. Moreover, glycolipids with longer sugar moieties (e.g., globoside, asialo-GM1, GM1, and GT1b) were not recognized. These data emphasized the specificity of the binding of SPC3 to glycosphingolipids.

On the basis of these results, one could hypothesize that the glycosphingolipids recognized by SPC3 in lymphocyte lipid extracts correspond to LacCer, GM3, and GD3. LacCer and GM3 were the major glycosphingolipid species found in, respectively, the Folch lower and upper phases of CEM extracts. These glycolipids were purified to homogeneity by preparative HPTLC and evaluated for SPC3 binding. As

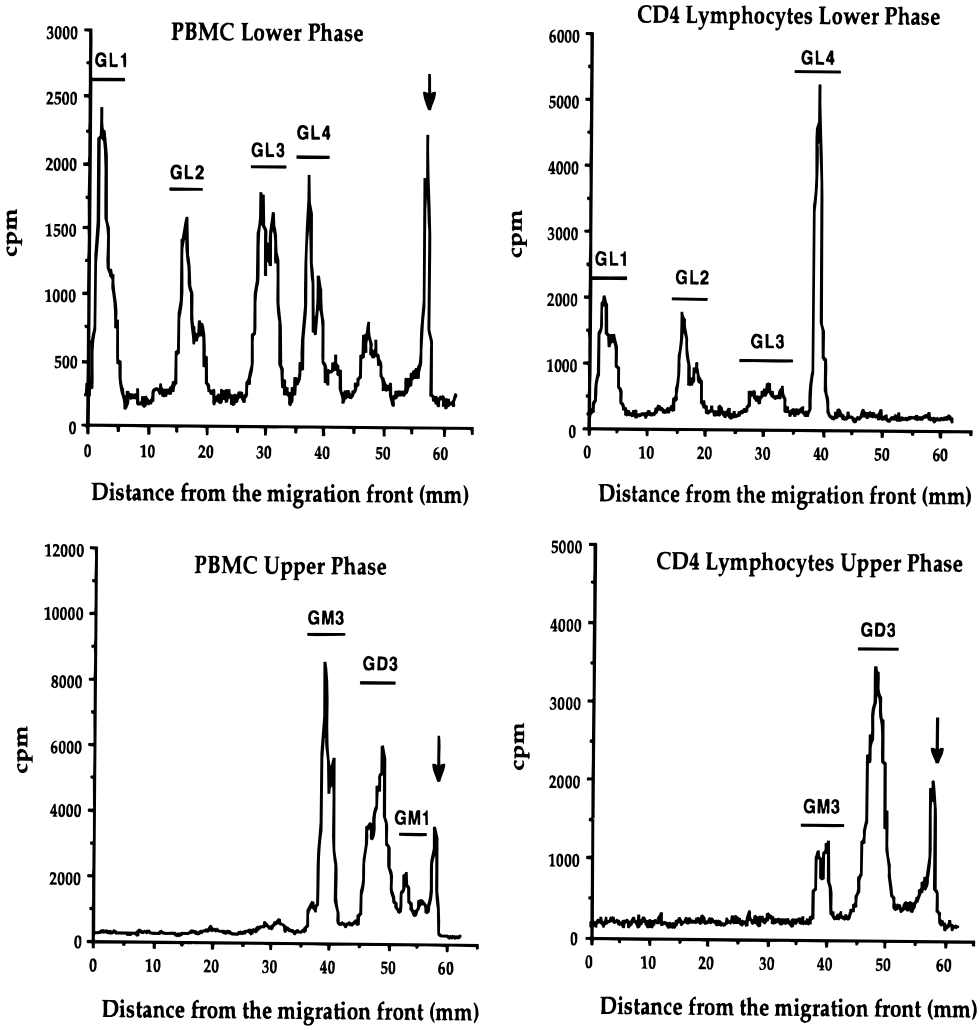

FIGURE 7: Analysis of the glycosphingolipid composition of PBMC and $CD4^+$ lymphocytes. Glycosphingolipids were extracted from normal PBMC and purified $CD4^+$ lymphocytes after metabolic labeling with [^{14}C]galactose (2 μCi/mL) for 24 h at 37 °C. The crude lipid extracts were partitioned according to the Folch procedure into a lower and an upper phase which were run on a HPTLC plate using chloroform/methanol/water (60:35:8) as the mobile phase. The position of migration of lower phase neutral glycosphingolipids is indicated as follows: GL1, ceramide monohexosides (GluCer); GL2, ceramide dihexosides (LacCer); GL3, ceramide trihexosides; GL4, tetraosyl ceramides. The position of migration of upper phase gangliosides (GM1, GM3, GD3) is indicated. The arrow refers to material at the depot point that has not migrated. The radioactive signals were quantified by phosphoimaging as indicated in Materials and Methods.

shown in Figure 10, both LacCer and GM3 purified from CEM cells were recognized by SPC3. These data strongly suggest that the proteinase K-resistant lymphocytic binding sites for SPC3 include LacCer and its monosialosyl derivative GM3. In addition, it is likely that GD3, i.e., the disialosyl LacCer derivative found in the upper phase of normal $CD4^+$ lymphocytes, is also recognized by SPC3.

DISCUSSION

The multibranched V3 loop-derived peptide SPC3 is a potent inhibitor of HIV infection in human lymphocytes and macrophages as well as in $CD4^-$ human colon epithelial cells (Fantini et al., 1993a; Yahi et al., 1994a, 1995a). Virological data suggested that the peptide is a competitive inhibitor of HIV-1 binding to the GalCer receptor of $CD4^-$ HT-29 cells, whereas it acts at a postbinding step in $CD4^+$ lymphocytes (Yahi et al., 1995b). The aim of the present study was to identify the cell surface molecules recognized by SPC3 on $CD4^-$ and $CD4^+$ HIV-1 target cells.

We found that SPC3 binds to cell surface glycosphingolipids that share a common structural feature, i.e., a free hydroxyl group in position 4 of a terminal galactose residue: GalCer/sulfatide, LacCer, GM3, and GD3. One of these glycolipids, i.e., GalCer/sulfatide, has been previously characterized as an alternative receptor allowing HIV-1 entry into $CD4^-$ neural and colonic cells (Harouse et al., 1991; Fantini et al., 1994). The surface envelope glycoprotein gp120 binds to GalCer/sulfatide with high affinity, and the infection of $CD4^-/GalCer^+$ cells is blocked by anti-GalCer antibodies (Yahi et al., 1992; Harouse et al., 1995). Thus, it can be reasonably assumed that SPC3 inhibits HIV-1 infection of $GalCer^+$ HT-29 cells by competing for the occupation of GalCer/sulfatide binding sites. This hypothesis is in full agreement with the virological data showing that SPC3 is actually a competitive inhibitor of HIV-1 infection of these cells (Yahi et al., 1995a).

In contrast, SPC3 does not inhibit the binding of HIV-1 to $CD4^+$ lymphocytes, and the presence of the peptide only during HIV-1 exposure does not inhibit the infection of these cells (Yahi et al., 1995b). In agreement with these findings, we could not demonstrate any SPC3 binding to the CD4 receptor, either in cell-free assays or on live cells. Confirming these data, digestion of cell surface CD4 with proteinase

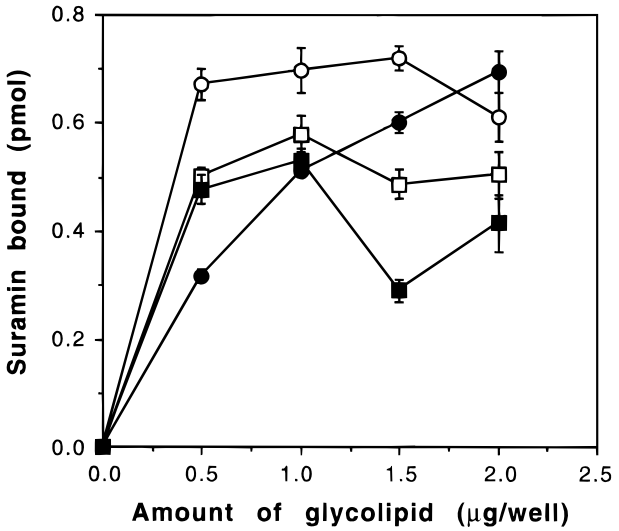

FIGURE 8: Binding of SPC3 to PBMC and CEM lipids. Total glycosphingolipids contained in the Folch lower (open symbols) and upper phases (closed symbols) from normal PBMC (circles) and CEM (squares) extracts were coated at the indicated concentrations in 96 multiwell plates. After evaporation of the solvent, the plates were saturated with PBS containing 2% bovine serum albumin and subsequently analyzed for SPC3 binding. The peptide bound was revealed with [^{3}H]suramin. The data are expressed as the mean of duplicate experiments (±SD).

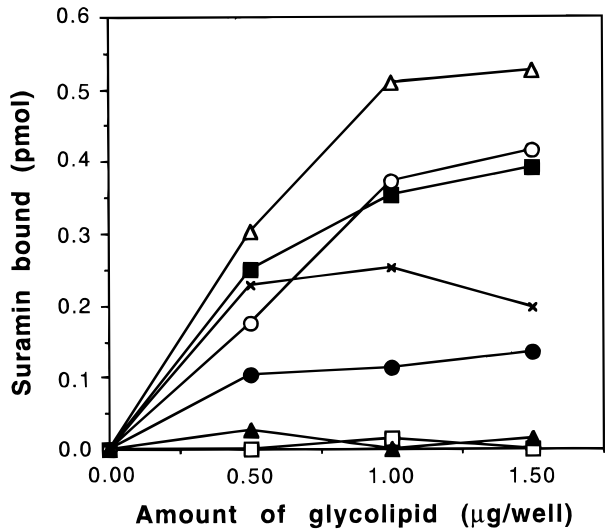

FIGURE 9: Binding of SPC3 to purified lipids. GT1b (□), GM1 (▲), GM2 (●), asialo-GM2 (×), LacCer (■), GD3 (○), and GM3 (△) were coated on poly(vinyl chloride) multiwell plates and assayed for SPC3 binding. The peptide bound was revealed with [^{3}H]suramin. The results are expressed as the mean of two independent experiments. Error bars were omitted for clarity.

K did not affect SPC3 binding to these cells. Therefore, we could not confirm the results reported by Benjouad et al. (1995) suggesting a possible interaction between SPC3 and the CDR-3 domain of CD4. Our data, however, are consistent with recent studies that have challenged the involvement of the CDR-3 region of CD4 during HIV-1 fusion (Broder & Berger, 1993; Moore, 1993). In addition, our virological data suggested that SPC3 acts at a postbinding step on CD4$^+$ lymphocytes, probably by interacting with cell surface components involved in the fusion process (Yahi et al., 1995b). The requirement of a human specific accessory binding site or fusion "coreceptor", probably recognized by the V3 loop of gp120, has been considered following the observation that CD4 by itself was not sufficient to allow HIV-1 entry into mouse fibroblasts transfected with the human CD4 cDNA (Maddon et al., 1986).

The data reported in the present study show that SPC3 recognizes at least three glycosphingolipids expressed by CD4$^+$ lymphocytes, i.e., LacCer, GM3, and GD3. Based on the ability of gp120 to recognize several related glycolipids including GalCer (Harouse et al., 1991), sulfatide (McAlarney et al., 1994), LacCer (Long et al., 1994), lactosyl sulfatide (Furuta et al., 1994), or seminolipid (Brogi et al., 1996), it is tempting to speculate that the HIV-1 coreceptor is a member of the glycosphingolipid family. However, the glycosphingolipids recognized by SPC3 cannot be considered as coreceptors for HIV-1, for the following reasons. On the one hand, these molecules are not specific to human cells and, in particular, are expressed by mouse cells (Yuasa et al., 1990). Therefore, the presence of these glycosphingolipids is not sufficient to support HIV-1 fusion with mouse fibroblasts transfected with the human CD4 gene (Maddon et al., 1986). On the other hand, the incorporation of these glycosphingolipids into Caco-2/CD4$^+$ cells, which are resistant to HIV-1 entry (Fantini et al., 1994), did not allow virus fusion (data not shown). Finally, G-protein-coupled seven-transmembrane domain receptors belonging to the family of β-chemokine receptors have been recently identified as fusion cofactors allowing the entry of primary and laboratory HIV-1 isolates into CD4$^+$ cells (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dorantz et al., 1996; Dragic et al., 1996; Feng et al., 1996). These data are consistent with the previous finding that some of these chemokines (RANTES, MIP-1α, MIP-1β) could inhibit HIV-1 infection at an early stage of HIV-1 cycle (Cocchi et al., 1995). Although the exact role of β-chemokine receptors in HIV-1 fusion remains to be elucidated, it is tempting to speculate that they could mediate V3 loop binding to the plasma membrane of the target cell (Choe et al., 1996). This consideration raised the interesting possibility that SPC3 could also act through binding to β-chemokine receptors. However, we believe that the main binding sites for SPC3 on CD4$^+$ lymphocytes are glycosphingolipids for the following reasons: (i) the important number of SPC3 binding sites on human CD4$^+$ lymphocytes, i.e., more than 350 000 sites/cell, is not compatible with a chemokine receptor (Samson et al., 1996); (ii) in contrast with SPC3, the presence of RANTES, MIP-1α, and MIP-1β *during* HIV-1 exposure resulted in a significant inhibition of HIV-1 entry into CD4$^+$ cells (Dragic et al., 1996), showing that the peptide and the chemokines act through distinct mechanisms; (iii) SPC3 inhibits the infection of CD4$^+$ lymphocytes and macrophages by various isolates of HIV-1 (clades B and D) and HIV-2 exhibiting different phenotype and tropism characteristics (Fantini et al., 1993b, Yahi et al., 1994). Since phenotypically distinct isolates differ in coreceptor usage, it is likely that a large family of fusion cofactors may exist (Weiss & Clapham, 1996). Thus, it is difficult to reconcile the anti-HIV properties of SPC3 with the recognition of a wide variety of coreceptors. Finally, high concentrations (up to 2 μg/mL) of RANTES, which binds to CC-CKR1, CC-CKR3, CC-CKR4, and CC-CKR5 β-chemokine receptors (Samson et al., 1996), did not affect SPC3 binding to human PBMC (data not shown). In conclusion, it can be reasonably assumed that SPC3 binds mainly to LacCer, GM3, and GD3 and that these glycosphingolipids have to be distinguished from the HIV-1 coreceptors.

Table 2: Binding of SPC3 to Sphingolipids

sphingolipid	structure[a]	binding[b]
ceramide	Cer	−
sulfatide	*Gal(3-SO$_4$)*β1-1Cer	+++
LacCer	*Galβ1*-4Glcβ1-1Cer	+++
ceramide trihexoside	Galα1-4Galβ1-4Glcβ1-1Cer	++
globoside	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	−
GM1	Galβ1-3GalNAcβ1-4[NeuAcα2-3]4Galβ1-4Glcβ1-1Cer	−
asialo-GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	−
GM2	GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glcβ1-1Cer	+
asialo-GM2	GalNAcβ1-4Galβ1-4Glcβ1-1Cer	++
GM3	NeuAcα2-3*Galβ1*-4Glcβ1-1Cer	+++
GD3	NeuAcα2-8NeuAcα2-3*Galβ1*-4Glcβ1-1Cer	+++
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4[NeuAcα2-8NeuAcα2-3]Galβ1-4Glcβ1-1Cer	−

[a] Italics indicate the minimum motif required for optimal binding, with special emphasis on a free hydroxyl group in position 4′ of galactose. [b] −, no binding to 2 μg of lipid; +, positive binding to 2 μg of lipid; ++, positive binding to 0.5 μg of lipid; +++, positive binding to less than 0.5 μg of lipid.

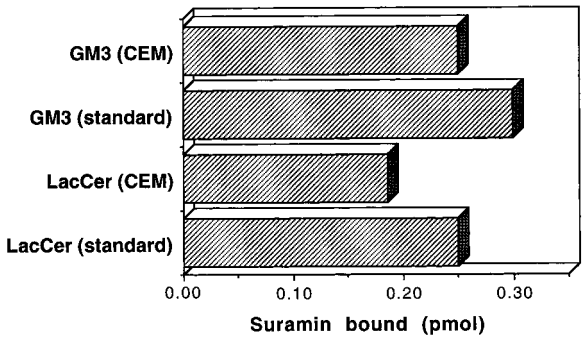

FIGURE 10: Binding of SPC3 to LacCer and GM3 purified from CEM cells. LacCer and GM3 were purified by preparative thin-layer chromatography and assayed for SPC3 binding with [^{3}H]-suramin as revealing agent. For comparison, the experiment was also done with standard LacCer and GM3 purified from bovine brain and canine blood, respectively. The results of a single experiment, which was repeated three times with similar results, are shown.

Since the functional part of SPC3, i.e., the GPGRAF motif, mimics the tip of the V3 loop of HIV-1 gp120 (both are recognized by a neutralizing anti-gp120 mAb), the data reported here suggest a role for glycosphingolipids in HIV-1 fusion. The recent report that L-cycloserine, an inhibitor of glycosphingolipid biosynthesis (Sundaram & Lev, 1984), could block HIV-1 infection (Mizrachi et al., 1996) is in agreement with this hypothesis. Indeed, an important requirement for induction of fusion is an increase in the hydrophobicity of the membrane, which can be achieved either by increasing the lateral membrane tension or by increasing the curvature of the membrane (Monck & Fernandez, 1996). For these reasons, the lipid composition of the plasma membrane of the target cell can determine the fusion capacity of various viruses, including HIV-1 (Larsen et al., 1993). GalCer, for instance, has been shown to promote membrane fusion of Semliki Forest virus (Nieva et al., 1994), while neutral glycolipids terminating in galactose (e.g., GalCer & LacCer) were found to mediate myxovirus-induced membrane fusion (Huang, 1983). Moreover, the presence of gangliosides in the target cell membrane may accelerate the formation of an active fusion complex and enhance the rate of the fusion process (Wilschut and Bron, 1993). Finally, HIV-1 infection and/or cytopathic effect can be inhibited by GM3 (Matsuda et al., 1993) and sulfated gangliosides (Handa et al., 1991). Taken together, these data suggest that specific gangliosides, especially GM3, are involved in the fusion process driven by HIV-1.

There are several mechanisms by which glycosphingolipids may influence viral fusion. The physical behavior of glycolipids in microdomains of the plasma membrane (Thompson & Tillack, 1985), together with their local association with sphingomyelin and cholesterol (Brown & Rose, 1992), may confer an adequate membrane fluidity compatible with the fusion event. By cross-linking glycosphingolipids such as LacCer, GM3, and/or GD3 in these microdomains, SPC3 may alter membrane fluidity, leading to the inhibition of membrane fusion. Although this interpretation needs experimental confirmation, it could explain why the GPGRAF monomeric peptide, which is unable to cross-link plasma membrane glycolipids, has no antiviral activity. Indeed, the anti-HIV effect requires at least two GPGRAF motifs branched on the same core (Yahi et al., 1994b). The cross-linking of glycosphingolipids by SPC3 could also interfere with the membrane distribution of proteins involved in the fusion process. For instance, we found that high concentrations of SPC3 increased the binding of anti-CD4 antibodies to CD4 on the surface of CEM cells (Table 1). Although this observation cannot account for the antiviral effect of SPC3 (which occurs at much lower concentrations), it may suggest a physical association between CD4 and specific glycosphingolipids. SPC3 binding to these glycosphingolipids could thus interfere with the packing of CD4. In this respect, it would be interesting to determine whether chemokines receptors (i.e., HIV-1 coreceptors), or other proteins involved in HIV-1 fusion, are associated with specific glycosphingolipids. Alternatively, it has been proposed that glycosphingolipids may activate the fusion process through induction of a conformational change in the viral fusion glycoprotein (Nieva et al., 1994). Current studies of the molecular interactions between HIV-1 gp120 and GalCer/sulfatide will help to clarify this point.

ACKNOWLEDGMENT

We thank M. Mollard (Eurethics, Paris, France) for the generous gift of the SPC3 peptide and F. Traincard (Institut Pasteur, Paris) for the F5 antibody. We also thank Biocytex (Marseille, France) for the Qifikit analysis.

REFERENCES

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., & Berger, E. A. (1996) *Science 272*, 1955−1958.

Backenson, P. B., Coleman, J. L., & Benach, J. L. (1995) *Infect. Immunol. 63*, 2811−2817.

Baghdiguian, S., Boudier, J. L., Boudier, J. A., & Fantini, J. (1996) *Eur. J. Cancer 32A*, 525−532.
Benjouad, A., Chapuis, F., Fenouillet, E., & Gluckman, J. C. (1995) *Virology 206*, 457−464.
Bhat, S., Spitalnik, S. L., Gonzalez-Scarano, F., & Silberberg, D. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7131−7134.
Broder, C. C., & Berger, E. A. (1993) *J. Virol. 67*, 913−926.
Broder, C. C., Dimitrov, D. S., Blumenthal, R., & Berger, E. A. (1993) *Virology 193*, 483−491.
Brogi, A., Presentini, R., Solazzo, D., Piomboni, P., & Costantino-Ceccarini, E. (1996) *AIDS Res. Hum. Retroviruses 12*, 483−489.
Brown, D. A., & Rose, J. K. (1992) *Cell 68*, 533−544.
Chigorno, V., Pitto, M., Cardace, G., Acquotti, D., Kirschner, G., Sonnino, S., Ghidoni, R., & Tettamanti, G. (1985) *Glycoconjugate J. 2*, 279−291.
Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Donath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., & Sodroski, J. (1996) *Cell 85*, 1135−1148.
Clements, G. J., Price-Jones, M. J., Stephens, P. E., Sutton, C., Schultz, T. F., Clapham, P. R., McKeating, J. A., McClure, M. O., Thompson, S., Marsh, M., Kay, J., Weiss, R. A., & Moore, J. P. (1991) *AIDS Res. Hum. Retroviruses 7*, 3−16.
Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., & Lusso, P. (1995) *Science 270*, 1811−1815.
Cook, D. G., Fantini, J., Spitalnik, S. L., & Gonzalez-Scarano, F. (1994) *Virology 201*, 206−214.
Delézay, O., Yahi, N., & Fantini, J. (1996) *Persp. Drug Discovery Des. 5*, 192−202.
Deng, H., Liu, R., Ellmeier, W., Choe, S., Unumatz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T.J., Littman, D. R., & Landau, N. R. (1996) *Nature 381*, 661−666.
Dorantz, B. M., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., & Doms, R. W. (1996) *Cell 85*, 1149−1158.
Dragic, T., Charneau, P., Clavel, F., & Alizon, M. (1992) *J. Virol. 66*, 4794−4802.
Dragic, T., Picard, L., & Alizon, M. (1995) *J. Virol. 69*, 1013−1018.
Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., & Paxton, W. A. (1996) *Nature 381*, 667−673.
Fantini, J., Cook, D. G., Nathanson, N., Spitalnik, S. L., & Gonzalez-Scarano, F. (1993a) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2700−2704.
Fantini, J., Yahi, N., Mabrouk, K., Van Rietschoten, J., Rochat, H., & Sabatier, M. (1993b) *C.R. Acad. Sci. Paris 316*, 1381−1387.
Fantini, J., Yahi, N., Delézay, O., & Gonzalez-Scarano, F. (1994) *AIDS 8*, 1347−1348.
Feng, Y., Broder, C. C., Kennedy, P. E., & Berger, E. A. (1996) *Science 272*, 872−877.
Freed, E. O., Myers, D. J., & Risser, R. (1991) *J. Virol. 65*, 190−194.
Furuta, Y., Eriksson, K., Svennerholm, B., Fredman, P., Horal, P., Jeansson, S., Vahlen, A., Holmgren, J., & Czerkinsky, C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12559−12563.
Handa, A., Hoshino, H., Nakajima, K., Adachi, M., Ikeda, K., Achiwa, K., Itoh, T., & Suzuki, Y. (1991) *Biochem. Biophys. Res. Commun. 175*, 1−9.
Harouse, J. M., Bhat, S., Spitalnik, S. L., Laughlin, M., Stefano, K., Silberberg, D. H., & Gonzalez-Scarano, F. (1991) *Science 253*, 320−323.
Harouse, J. M., Collman, R. G., & Gonzalez-Scarano, F. (1995) *J. Virol. 69*, 7383−7390.
Huang, R. T. C. (1983) *Lipids 18*, 489−492.
Larsen, C. E., Nir, S., Alford, D. R., Jennings, M., Lee, K. D., & Düzgünes, N. (1993) *Biochim. Biophys. Acta 1147*, 223−236.
Lazaro, I., Naniche, D., Signoret, N., Bernard, A. M., Marguet, D., Klatzmann, D., Dragic, T., Alizon, M., & Sattentau, Q. (1994) *J. Virol. 68*, 6535−6546.
Levy, J. A. (1993) *Microbiol. Rev. 57*, 183−289.
Long, D., Berson, J. F., Cook, D. G., & Doms, R. W. (1994) *J. Virol. 68*, 5890−5898.
Lorès, P., Boucher, V., MacKay, C., Pla, M., Von Boehmer, H., Jami, J., Barré-Sinoussi, F., & Weill, J. L. (1992) *AIDS Res. Hum. Retroviruses 8*, 2063−2071.
Macher, B. A., Klock, J. C., Fukuda, M. N., & Fukuda, M. (1981) *J. Biol. Chem. 256*, 1968−1974.
Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A., & Axel, R. (1986) *Cell 47*, 333−348.
Matsuda, K., Taki, T., Hamanaka, S., Kasama, T., Robukawa, C., Handa, S., & Yamamoto, N. (1993) *Biochim. Biophys. Acta 1168*, 123−129.
McAlarney, T., Apostolski, S., Lederman, S., & Latov, N. (1994) *J. Neurosci. Res. 37*, 453−460.
Mizrachi, Y., Lev, M., Harish, Z., Sundaram, S. K., & Rubinstein, A. (1996) *J. Acquired Immune Defic. Syndr. Hum. Retrovirol. 11*, 137−141.
Monck, J. R., & Fernandez, J. M. (1996) *Curr. Opin. Cell Biol. 8*, 524−533.
Moore, J. P. (1993) *J. Virol. 67*, 3656−3659.
Moore, J. P., & Nara, P. L. (1991) *AIDS 5*, S21−S33.
Moore, J. P., Bradford, A., Jameson, A., Weiss, R. A., & Sattentau, Q. J. (1993) in *Viral Fusion Mechanisms* (Bentz, J., Ed.) pp 233−289, CRC Press, Inc., Boca Raton, FL.
Nieva, J. L., Bron, R., Corver, J., & Wilschut, J. (1994) *EMBO J. 13*, 2797−2804.
Rantsch, B., Clapshaw, P. A., Price, J., Noble, M., & Seifert, W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2709−2713.
Reivinen, J., Holthöfer, H., & Miettinen, A. (1994) *Int. Immunol. 6*, 1409−1416.
Samson, M., Labbe, O., Mollereau, C., Vassart, G., & Parmentier, M. (1996) *Biochemistry 35*, 3362−3367.
Schwartzmann, G., Hoffmann-Bleihauer, P., Shubert, J., Sandhoff, K., & Marsh, D. (1983) *Biochemistry 22*, 5041−5048.
Stein, K. E., & Marcus, D. M. (1977) *Biochemistry 16*, 5285−5291.
Sundaram, S. K., & Lev, M. (1984) *J. Neurochem. 42*, 577−581.
Symington, F. W., & Hakomori, S. I. (1985) *Lymphokines 12*, 201−238.
Tam, J. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5409−5413.
Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Chem. 14*, 387−417.
Varani, I., Terzaghi, A., Donati, L., Masserini, M., & Tettamanti, G. (1994) *Arch. Dermatol. Res. 286*, 481−483.
Weiss, R. A., & Clapham, P. R. (1996) *Nature 381*, 647−648.
Wilschut, J., & Bron, R. (1993) in *Viral Fusion Mechanisms* (Bentz, J., Ed.) pp 133−161, CRC Press, Inc., Boca Raton, FL.
Yahi, N., Baghdiguian, S., Moreau, H., & Fantini, J. (1992) *J. Virol. 66*, 4848−4854.
Yahi, N., Sabatier, J. M., Nickel, P., Mabrouk, K., Gonzalez-Scarano, F., & Fantini, J. (1994a) *J. Biol. Chem. 269*, 24349−24353.
Yahi, N., Fantini, J., Mabrouk, K., Tamalet, C., De Micco, P., Van Rietschoten, J., Rochat, H., & Sabatier, J. M. (1994b) *J. Virol. 68*, 5714−5720.
Yahi, N., Spitalnik, S. L., Stefano, K. A., De Micco, P., Gonzalez-Scarano, F., & Fantini, J. (1994c) *Virology 204*, 550−557.
Yahi, N., Sabatier, J. M., Baghdiguian, S., Gonzalez-Scarano, F., & Fantini, J. (1995a) *J. Virol. 69*, 320−325.
Yahi, N., Fantini, J., Baghdiguian, S., Mabrouk, K., Tamalet, C., Rochat, H., Van Rietschoten, J., & Sabatier, J. M. (1995b) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4867−4871.
Yahi, N., Ratner, L., Harouse, J. M., Gonzalez-Scarano, F., & Fantini, J. (1996) *Perspect. Drug Discovery Des. 5*, 161−168.
Yuasa, H., Scheinberg, D. A., & Houghton, A. N. (1990) *Tissue Antigens 36*, 47−56.

BI961205G