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Lipid Rafts and HIV Pathogenesis: Host Membrane Cholesterol Is Required for Infection by HIV Type 1

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ABSTRACT

In a previous study we showed that budding of HIV-1 particles occurs at highly specialized membrane microdomains known as lipid rafts. These microdomains are characterized by a distinct lipid composition that includes high concentrations of cholesterol, sphingolipids, and glycolipids. Since cholesterol is known to play a key role in the entry of some other viruses, our observation of HIV budding from lipid rafts led us to investigate the role in HIV-1 entry of cholesterol and lipid rafts in the plasma membrane of susceptible cells. We have used 2-OH-propyl-β-cyclodextrin (β-cyclodextrin) to deplete cellular cholesterol and disperse lipid rafts. Our results show that removal of cellular cholesterol rendered primary cells and cell lines highly resistant to HIV-1-mediated syncytium formation and to infection by both CXCR4- and CCR5-specific viruses. β-Cyclodextrin treatment of cells partially reduced HIV-1 binding, while rendering chemokine receptors highly sensitive to antibody-mediated internalization. There was no effect on CD4 expression. All of the above-described effects were readily reversed by incubating cholesterol-depleted cells with low concentrations of cholesterol-loaded β-cyclodextrin to restore cholesterol levels. Cholesterol depletion made cells resistant to SDF-1-induced binding to ICAM-1 through LFA-1. Since LFA-1 contributes significantly to cell binding by HIV-1, this latter effect may have contributed to the observed reduction in HIV-1 binding to cells after treatment with β-cyclodextrin. Our results indicate that cholesterol may be critical to the HIV-1 coreceptor function of chemokine receptors and is required for infection of cells by HIV-1.

INTRODUCTION

STUDIES OF CELL MEMBRANE composition and function have revealed the presence of membrane microdomains called lipid rafts. These specialized membrane regions have distinct lipid and protein compositions compared with other areas of the cell membrane. Cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins are enriched in lipid rafts (reviewed in Ref. 1). The high concentration of cholesterol and sphingolipids in lipid rafts results in a tightly packed, ordered lipid domain that is resistant to nonionic detergents at low temperature.[2] The structural protein caveolin causes formation of flask like invaginations (caveolae) in the cell membrane with a lipid composition similar to that of lipid rafts.[3] Signaling molecules including Lck, LAT (linker for activation of T cells), nitric oxide synthase (NOS), and G protein α subunit are localized to rafts on the intracellular side of the membrane, and are targeted by lipid modifications such as palmitylation, myristoylation, or both.[2, 4–8] Many transmembrane proteins do not show preferences for lipid rafts, for example, CD45 and E cadherin are excluded from these areas.[9, 10] Certain lipid-modified transmembrane proteins, such as the hemagglutinin (HA) molecule of influenza virus, do localize to lipid rafts.[11] Lipid rafts are believed to be important in vesicle transport, endocytosis, signaling through the T cell receptor, and other cellular functions, including regulating apoptosis.[12–15] New discoveries are linking lipid rafts to more and more cell functions, and tight junctions have been characterized as lipid rafts as well.[16]

Chemokine receptors (CRs), which serve as HIV coreceptors,[17] are G-coupled proteins with seven membrane-spanning domains and thus belong to the large family of serpentine receptors. Given this number of membrane-interacting domains, it is predicted that CRs can be more profoundly affected by the

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lipids in the surrounding milieu, compared with a single-pass transmembrane protein. For example, membrane cholesterol is essential in the binding of the neuropeptide galanin to its G-coupled seven membrane-spanning receptor, GalR2.[18] Precedence for cholesterol effects on transmembrane protein function has been established by Gimpl and others, who showed that cholesterol is required for ligand binding by two serpentine receptors, the oxytocin receptor and the brain cholecystokinin receptor.[19] The role of cholesterol in receptor function has been attributed to association of the oxytocin receptor with lipid rafts.[20] Similarly, the Semliki Forest virus (SFV) spike protein has also been shown to require cholesterol and sphingolipids on target membranes for infection.[21,22] Interestingly, the presence of CCR5 in lipid rafts on MCF7 cells correlates with its polarized distribution in chemotactic cells, but the functional correlation between CCR5 and lipid rafts has not been well studied.[23] A role for lipid rafts in CXCR4 signaling has not been established.

We have shown that the human immunodeficiency virus type 1 (HIV-1) buds selectively from lipid rafts of infected T cells.[24] These viruses have membrane compositions similar to lipid rafts, containing ganglioside GM1 and GPI-anchored proteins while excluding CD45. We predicted that virions budding from lipid rafts would have selective advantages over virions that do not bud from rafts. This process may be relevant and important for many other viruses as well. So far, measles, influenza, Semliki Forest, and polio viruses have been reported to assemble by raft association and, in the case of influenza virus, to bud from rafts.[10, 25–28]

We have further explored the involvement of lipid rafts in HIV-1 biology beyond its role in virus budding. In this study we show that partial depletion of cholesterol from cell membranes by exposing cells to β-cyclodextrin (BCD) inhibits HIV-1-induced syncytium formation in cell lines and primary T cells. BCD treatment of cells also increases CR internalization induced by monoclonal antibody (MAb) binding. Most significantly, both primary cells and cell lines are rendered resistant to infection with CXCR4- and CCR5-specific HIV-1 strains by treatment with BCD. The effects observed were not due to loss of cell viability after BCD treatment. These results indicate a requirement for intact lipid rafts and cholesterol in HIV-1 infection and syncytium formation.

MATERIALS AND METHODS

Cells and reagents

Jurkat, PM1, and CEM × 174 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained in complete medium (cRPMI), consisting of RPMI 1640 (GIBCO-BRL, Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum (HyClone, Logan, UT) and 10 mM HEPES. Peripheral blood mononuclear cells were isolated from leukapheresis buffy coats and stimulated with phytohemagglutinin (PHA) as previously described.[29] Control mouse myeloma IgG1 and rabbit anti-mouse IgG (Fc specific) were purchased from Jackson ImmunoResearch (West Grove, PA). CXCR4 MAb (12G5) was a kind gift from J. Hoxie (University of Pennsylvania, Philadelphia, PA). MAbs to MHC class I antigen (MHM.5), MHC class II antigen (MHM.33), CD4 (SIM.4), CXCR4 (FSN.NT.M3), and CD45 (H5A5) were produced in our laboratory and purified from ascites fluids. Primary HIV-1 strains were kind gifts from J. Margolick (Johns Hopkins School of Public Health, Baltimore, MD). Primary isolates of HIV-1 refers to low-passage (<p3) clinical isolates expanded by growth on pooled PHA blasts in the presence of interleukin 2 (IL-2). 2-OH-propyl-β-cyclodextrin (Trappsol) was obtained from Cyclodextrin Technologies Development (CTD, Gainesville, FL).

BCD treatment and virus production

PM1 cells chronically infected with HIV-1_{RF} were washed and treated with 20 mM BCD in cRPMI or with cRPMI alone for 1 hr at 37°C. The cells were then washed twice before resuspension at a density of 5×10^6/ml in cRPMI. The cells were incubated for 6 hr at 37°C, 5% CO_2 and pelleted by centrifugation. The virus-containing supernatants were collected and purified by centrifugation through a 20% sucrose cushion.[30] The virus pellets were taken up in cRPMI and titrated against LuSIV cells to determine infectivity. p24 was measured in a standard p24 enzyme-linked immunosorbent assay (ELISA).

Cholesterol measurement

Cellular cholesterol was measured with a sensitive cholesterol oxidase-based fluorometric assay (Amplex Red cholesterol kit) from Molecular Probes (Eugene, OR). Cholesterol content of cells was normalized to total cellular protein.

Syncytium assays

Syncytium assays were carried out essentially as previously described.[31] Briefly, cell lines or PHA blasts were treated with 20 mM BCD in RPMI 1640 or medium alone for 1 hr at 37°C before washing twice with phosphate-buffered saline (PBS). The treated cells were then mixed with HIV-1-infected cells, each at density of 2×10^6/ml, in cRPMI and incubated at 37°C. Syncytia were scored and photographed 3 to 6 hr after mixing. For free virus-mediated syncytium assays, HIV-1_{RF} from infected PM1 culture supernatants was clarified by 0.45-μm pore size filtration (p24 concentration greater than 500 ng/ml). BCD-treated and nontreated cell lines were added to the virus preparations and incubated at 37°C for 3 hr before counting syncytia.

Flow cytometry

Flow cytometry was performed as previously described.[32] Briefly, 2×10^5 BCD-treated or untreated cells in 100 μl of PBS containing 5% normal goat serum (NGS) were added to 100 μl of MAb (1–5 μg) and incubated for 30 min on ice. In some experiments cells were prefixed with 2% paraformaldehyde in PBS immediately after BCD treatment. Cells were washed with PBS, resuspended in 100 μl of PBS–5% NGS containing 2 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (FITC–GAM) and incubated for 1 hr on ice. Cells were then washed with PBS and fixed with 2% paraformaldehyde followed by analysis on an EPICS Profile II (Coulter, Hialeah, FL) flow cytometer.

Confocal microscopy

Cell surface staining of BCD-treated and untreated cells was performed under saturating conditions. BCD-treated and untreated PHA blasts (3×10^5) were washed in cold PBS and preincubated on ice for 15 min in 5% NGS–PBS. Cells were then incubated with MAb (1–5 μg in 5% NGS–PBS) for 45 min on ice, washed with PBS, and incubated with 2 μg of FITC–GAM in 5% NGS–PBS. The cells were fixed with 2% paraformaldehyde in PBS and spun onto poly-L-lysine-coated slides, using Cyto-funnels (Shandon, Pittsburgh, PA). The pellets were overlaid with 50 μl of 25% glycerol in PBS and coverslips were positioned over the droplets. The edges of the slides were sealed with nail polish before storing them at 4°C. This staining procedure was also carried out with cells prefixed with 2% paraformaldehyde in PBS prior to MAb staining. Viewing of slides was performed with an Olympus (Norwood, MA) IX50 confocal microscope under oil immersion at ×100 magnification. Micrographs were acquired on a Silicon Graphics (Mountain View, CA) workstation equipped with InterVision (Santa Clara, CA) software. Final images were enhanced on the Silicon Graphics workstation by two-dimensional deconvolution, and brightness and contrast were adjusted for viewing.

Free virus binding assay

Virus binding was measured through host cell antigen transfer as described.[30] Briefly, Jurkat cells (1×10^6) were washed with serum-free RPMI 1640 medium (pRPMI) before incubation in 20 mM BCD in iRPMI or iRPMI alone for 1 hr at 37°C. Cells were then washed twice with iRPMI before adding 100 μl of clarified HIV-1 supernatant (p24 at >10 ng/ml from PM1 cells) for 1 hr on ice. Excess virus was removed by washing twice with iRPMI. MAbs were then added at 20 μg/ml in 5% NGS–PBS and allowed to incubate for 1 hr on ice before washing with iRPMI. FITC–GAM (10 μg/ml) was then added for 45 min on ice before washing with iRPMI. Cells were then fixed with 2% paraformaldehyde followed by analysis on an EPICS Profile II flow cytometer.

Primary virus infection assay

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque centrifugation from buffy coats obtained from the Johns Hopkins Hemapheresis Center (Baltimore, MD). Cells were stimulated for 3 days with PHA (3 μg/ml in cRPMI), washed with iRPMI, and treated with 10 mM BCD in cRPMI for 1 hr. Cells were then washed twice with iRPMI and resuspended in cRPMI (1×10^6/ml) supplemented with IL-2 (50 U/ml) and containing primary HIV-1 strains at 20 ng of p24 per milliliter. Cells were incubated with virus for 24 hr at 37°C before washing twice with iRPMI. Cells were then resuspended in cRPMI supplemented with IL-2 (50 U/ml) and cultured for 6 days at 37°C. Supernatants were collected and p24 was quantitated by p24 capture ELISA.

Luciferase-based infectivity assay

The effect of BCD on infectivity of HIV was measured in a luciferase-based single-cycle infection assay as previously described.[30] LuSIV cells were treated with 20 mM BCD in iRPMI or iRPMI alone for 1 hr at 37°C. Cells were then washed with iRPMI before resuspension in cRPMI at a density of 2×10^6/ml. Cells were mixed with cRPMI alone or with dilutions of virus supernatant (p24 at 62 to 500 pg/ml) from BCD-treated or untreated PM1 cells and allowed to incubate overnight (16 hr) at 37°C. The LuSIV cells were washed with PBS and lysed with 100 μl of reporter lysis buffer (Promega, Madison, WI). After centrifugation at 13,000 × g for 30 sec, 10 μl of lysate was added to 100 μl of luciferase reagent (Promega) in an opaque 96-well plate and luminescence was measured on a Packard (Downers Grove, IL) Lumicount luminometer.

SDF-1α-induced cell adhesion assay

Cell adhesion assays were carried out essentially as previously described.[30] The wells of 96-well plates were coated with recombinant intercellular adhesion molecule (ICAM)–Ig and blocked as described. Jurkat cells were labeled with horseradish peroxidase (HRP) and treated with either 20 mM BCD or medium alone as described above. The cells were then washed and resuspended in cRPMI at a density of 2×10^6/ml. One hundred microliters of cells was added to the wells alone with cRPMI alone or medium containing stromal cell-derived factor 1α (SDF-1α) at 10 ng/ml. The wells were incubated at 37°C for various times before washing to remove unbound cells. Bound cells were lysed and HRP measured as described previously. A standard curve was generated by from known numbers of labeled cells lysed and quantitated by measuring HRP.

RESULTS

BCD treatment blocks syncytium formation of primary cells and cell lines

Our observation that HIV-1 buds selectively from lipid rafts[24] led us to determine whether lipid rafts and cholesterol are involved in virus entry as well. We first examined the possible role of lipid rafts in the HIV-1 fusion process by treating $CD4^+$ HIV-susceptible target cells with BCD to deplete membrane cholesterol and disperse lipid rafts. In our studies we use 2-OH-propyl-β-CD, which has been shown to be less efficient than widely used methyl-β-CD in depleting cellular cholesterol and also much less toxic. We found that treatment of cells with 10 to 20 mM BCD (2-OH-propyl-β-CD) for 1 hr at 37°C, followed by washing to remove free BCD, depleted more than 70% of total cellular cholesterol without any loss in cell viability as measured by trypan blue exclusion (data not shown). Furthermore, treated cells continued to grow normally after BCD treatment when placed back into culture in cholesterol-containing medium (data not shown). A further indication of the nontoxicity of BCD treatment was the fact that after such treatment Jurkat cells still showed Ca^{2+} flux responses to anti-CD4 MAb (D.H. Nguyen, unpublished). $CD4^+$ SupT1 T cells form numerous large syncytia within 3 hr on addition of HIV-1_{MN}-infected H9 cells (Control, Fig. 1). BCD treatment of SupT1 cells completely inhibited syncytium formation with HIV-1_{MN}-infected H9 cells (BCD, Fig. 1). No syncytia were apparent in this culture for more than 15 hr, an observation that probably reflects the slow recovery of cholesterol in BCD-treated T cells. It is important to note that BCD is washed out after the 1-hr treatment and is not present during the coculti-

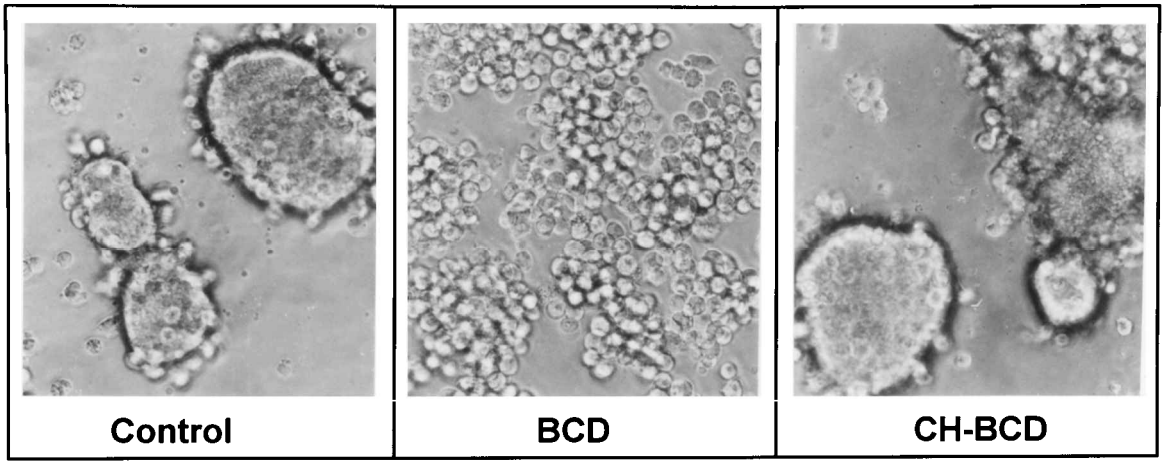

FIG. 1. BCD treatment of SupT1 T cells blocks syncytium formation with HIV-1-infected H9 cells. Uninfected SupT1 cells were treated with medium alone (Control), BCD in RPMI (BCD), or BCD preloaded with cholesterol (CH-BCD). To make preloaded BCD, cholesterol in powder form (Sigma, St. Louis, MO) was dissolved to saturation in a solution of 270 mM BCD in distilled H_2O to a final cholesterol concentration of 3.9 mM. This solution was then diluted in RPMI to approximately 1 mM BCD. The control and treated SupT1 cells were added to HIV-1_{MN}-infected H9 cells and incubated for 3 hr before photographs were obtained at an original magnification of ×40.

vation step. The effects seen are therefore not a result of a steric blockade by BCD, which does not bind to cells. To confirm that the effects of BCD on syncytium formation were due to cholesterol depletion, we treated cells with BCD that had been preloaded (saturated) with cholesterol (CH-BCD) and was therefore unable to deplete cellular cholesterol. As seen in Fig. 1 (CH-BCD), cells treated with cholesterol-loaded BCD fused to HIV-1-infected cells as efficiently as to control untreated cells. This result confirmed that BCD blocked HIV-induced fusion by depleting cholesterol. Similar results were obtained when primary cells (PHA-stimulated T cells) that had been treated with BCD and CH-BCD were used as fusion partners with HIV-infected cells (data not shown).

We also determined the effect of BCD treatment on HIV-induced fusion of several other cell lines as well. Four $CD4^+CXCR4^+$ cell lines, SupT1, H9, PM1, and MT2, were treated with BCD and tested for syncytium formation with HIV-1-infected cells as described above. In each case syncytium formation was completely blocked by depleting cellular cholesterol (Table 1). These results indicate that cholesterol and intact lipid rafts are required for HIV-induced syncytium formation between HIV-infected cells and uninfected HIV-susceptible cells.

The effect of cholesterol depletion on virus–cell fusion was also determined. When cells expressing high levels of CD4 and the appropriate chemokine receptor are exposed to high concentrations of fusion-competent HIV particles, sufficient HIV envelope glycoproteins are delivered into their membranes to initiate fusion and syncytium formation with neighboring cells. The kinetics (less than 3 hr) of this process preclude *de novo* synthesis and expression of HIV glycoproteins. This form of the syncytium assay can thus be used to indirectly measure HIV particle fusion to cell membranes. $CD4^+CXCR4^+$ cell lines (MT2, SupT1, and PM1) incubated for 3 hr at 37°C with free HIV-1_{RF} at p24 concentrations greater than 500 ng/ml showed extensive syncytium formation (>30 syncytia per high-power field [HPF]). Cells treated with BCD as described above showed no syncytium formation when exposed to virus under these conditions (data not shown). We cannot rule out low levels of fusion that do not proceed to gross syncytia. The results of this experiment showed that depletion of cellular cholesterol blocked HIV particle fusion to cells since syncytium formation under these conditions first requires extensive fusion of virus particles to cells in order to put HIV envelope proteins into cell membranes.

TABLE 1. β-CYCLODEXTRIN EFFECTS ON CD4 AND CXCR4 SURFACE EXPRESSION AND SYNCYTIUM FORMATION OF CELL LINES WITH HIV-INFECTED H9 CELLS

	Syncytia (no./HPF)		*CD4 (MCF)*		*CXCR4 (MCF)*	
Cell line	*Control*	*BCD*	*Control*	*BCD*	*Control*	*BCD*
MT2	55 ± 10	0	65.3	62.2	30.1	16.7
PM1	71 ± 5	0	78.6	85.9	16.2	3.1
H9	52 ± 3	0	21.2	20.9	29.9	6.7
SupT1	63 ± 8	0	140.1	188.3	44.9	15.0

Abbreviations: MCF, Mean channel fluorescence; BCD, β-cyclodextrin treatment at 20 mM in medium; HPF, high-power field.

TABLE 2. LACK OF EFFECT OF β-CYCLODEXTRIN TREATMENT ON CELL SURFACE EXPRESSION OF CXCR4: FLOW CYTOMETRY OF FIXED CELLS[a]

	CD4 (MCF)		*CXCR4 (MCF)*	
Cell line	*Control*	*BCD*	*Control*	*BCD*
PM1	146	129	63	64
H9	46	50	45	55
SupT1	216	213	94	91

Abbreviations: MCF, Mean channel fluorescence; BCD, β-cyclodextrin treatment (20 mM).

[a]Cells were fixed with 2% paraformaldehyde in PBS immediately after BCD treatment and before performing flow cytometry as described in Materials and Methods. The CD4 MAb SIM.4 recognizes the CD4a epitope. CXCR4 was detected with MAb 12G5.

Cholesterol depletion promotes CXCR4 downmodulation by MAb-induced internalization

One possible explanation for the inhibition of syncytium formation by cholesterol depletion is that CD4, CRs, or both are lost from the cell surface. The proteins could possibly be extruded from the membrane in vesicles after loss of cholesterol or they could be internalized. To explore these possibilities, we analyzed BCD-treated cells for expression of HIV receptors by flow cytometry. CD4 expression did not change after treatment with BCD in PHA blasts or any of the cell lines tested (Table 1). The anti-CD4 MAb used, SIM.4, recognizes the CD4a epitope and thus the results indicate that cholesterol depletion did not remove CD4 from the surface or mask its gp120-binding domain. In contrast, cell surface expression of CXCR4 was reduced by 50% or more in all of the cells after BCD treatment (Table 1). PM1 cells showed the most significant loss of CXCR4 expression, with a drop in total mean channel fluorescence (MCF) from 16.2 to 3.1. Primary T cells showed a similar reduction in CCR5 expression, from 19 to 8% of cells staining positive (data not shown). To determine whether the loss of CXCR4 expression was due to MAb-induced internalization, we fixed cells with 2% paraformaldehyde in PBS immediately after BCD treatment and before staining with MAbs for flow cytometry. Under these conditions both CD4 and CXCR4 expression remained unchanged on the cell surface (Table 2). BCD-treated cells fixed and permeabilized after the MAb binding step showed no significant reduction in anti-CXCR4 MAb staining compared with control cells (data not shown). The results of these studies indicate that CXCR4 remains on the surface after BCD treatment but appears to be rapidly internalized after MAb binding.

Immunostaining and confocal microscopy of BCD-treated PHA blasts and control cells showed that CXCR4 was not significantly redistributed on the cell surface after cholesterol depletion. Patchy staining of CXCR4 persisted after BCD treatment whether the cells were fixed before or after MAb staining (Fig. 2A). Consistent with flow cytometry data, overall staining was reduced in BCD-treated cells that were not fixed before the MAb staining procedure. The distribution of CD4 and CD45 was unchanged on the cell surface after BCD treatment (Fig. 2B and C, respectively). These results indicate that the overall membrane expression and distribution of critical HIV receptors were essentially unchanged after BCD treatment and suggest that the cholesterol content of the cell membrane is a critical factor in HIV-induced membrane fusion.

BCD treatment reduces HIV-1 binding

We examined the possibility that BCD treatment might affect virus binding or interactions between gp120 and CD4 or CRs. To measure virus binding to cells we used a flow cytometry assay that measures the transfer of host cell class II MHC proteins to class II MHC-negative cells by HIV virions, which incorporate large numbers of these proteins into their lipid envelopes.[30,32,33] We have previously used this approach to demonstrate adhesion molecule-mediated binding of HIV to cells.[30] Class II MHC-negative Jurkat cells were used as target cells in the HIV binding assay. As a positive control for flow cytometry analysis, we probed for class I MHC on the Jurkat cells, which were stained equally well with MAb against this protein before and after BCD treatment (Table 3). When HIV-1_{RF} from class II MHC-positive PM1 cells was added to untreated Jurkat cells, class II MHC MAb mean channel fluorescence increased from 1.0 to 7.8 relative fluorescence units, while the percentage of positive cells increased from 3.0 to 55.4% (Table 3). BCD treatment of the cells reduced virus binding by 70%, as determined by mean channel fluorescence of the anti-class II MHC MAb. These results indicate that HIV-1 is still capable of measurable attachment after cholesterol depletion of target cells but at much lower levels.

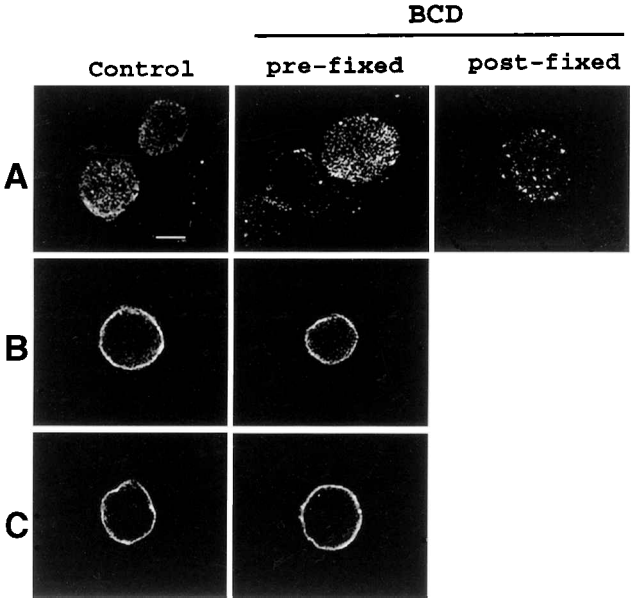

FIG. 2. Confocal immunomicroscopy of BCD-treated primary T cells. Uninfected primary T cells were treated with medium alone (column 1) or 20 mM BCD (columns 2 and 3). Cells were prepared for microscopy as described in Materials and Methods and stained with anti-CXCR4 (12G5) (**A**), anti-CD4 (SIM.4) (**B**), or anti-CD45 (H5A5) MAb (**C**). In columns 2 and 3, cells were fixed with 2% paraformaldehyde in PBS before or after staining with MAbs, respectively. Bar: 5 um.

TABLE 3. EFFECT OF β-CYCLODEXTRIN ON BINDING OF HIV-1 TO TARGET CELLS: MHC CLASS II TRANSFER ASSAY[a]

	IgG1 MCF (% Pos)		*MHM.5 MCF (% Pos)*		*MHM.33 MCF (% Pos)*	
	Control	*BCD*	*Control*	*BCD*	*Control*	*BCD*
No virus	0.9 (2.4)	0.7 (3.4)	158.6 (100)	165 (100)	1.0 (3.0)	0.7 (3.3)
HIV_{RF}	ND	ND	164 (99.9)	167.8 (100)	7.8 (55.4)	2.4 (26.0)

Abbreviations: MCF, Mean channel fluorescence; % Pos, percent positive cells; ND, not determined.
[a]HIV binding to Jurkat cells, control and BCD treated, was measured by transfer of class II MHC molecules as described in Materials and Methods. MAbs used were MHM.5 (anti-class I MHC), positive control; and MHM.33 (anti-class II MHC).

BCD treatment blocks CR-induced LFA-1 function

Results of the above-described studies suggested that intact lipid rafts may be required for stable membrane expression of CXCR4. Loss of CXCR function in regulating LFA-1 leukocyte function-associated antigen 1) could also explain the lower binding of HIV-1 to BCD-treated cells based on our previous studies.[30,32] As before, the treatment with BCD had no effect on cell viability. We therefore determined whether BCD treatment of cells affected control of LFA-1 function by CXCR4. Jurkat cells were treated with BCD or medium alone as described above. The cells were then added to the wells of culture plates coated with soluble recombinant ICAM–Ig. SDF-1, a CXCR4-specific chemokine that triggers LFA-1 function, was added to trigger binding of LFA-1 to ICAM-1 as previously described.[34] As seen in Fig. 3, control cells responded to SDF-1 and, as expected, bound well to ICAM–Ig. In contrast, the BCD-treated Jurkat cells showed no binding to ICAM–Ig after exposure to SDF-1. These results are consistent with previous reports showing disruption of integrin function by cholesterol depletion.[35–37] In a previous study we showed that CXCR4-specific gp120 could trigger the same responses through CXCR4 as SDF-1.[38] Thus HIV-1 particles may not trigger LFA-1 function on BCD-treated cells and this may partially explain lower virus binding to such cells.

BCD treatment blocks virus infection

HIV-1 is capable of spreading in cell cultures without necessarily exerting cytopathic effects. Thus inhibition of syncytium formation by cholesterol depletion and lipid raft dispersion does not necessarily mean that HIV infection by free virus would also be blocked. To test the effects of cholesterol depletion on HIV-1 infection of primary T cells by free virus, we treated PHA blasts with 10 mM BCD or medium alone as described above and exposed them to HIV-1 for 2 hr before washing to remove input virus. As indicated previously, viability and growth of the PHA blasts were not affected by treatment with BCD under the conditions used. p24 release was measured after an additional 6 days of culture. We tested two primary strains of HIV-1, 97.099 and 97.534, macrophage (M)-tropic (R5) and dual-tropic (X4R5), respectively. The results were identical to those obtained in syncytium formation assays. BCD treatment of PHA blasts completely inhibited infection by HIV isolate 97.099 while infection by isolate 97.534 was inhibited by more than 70% (Fig. 4).

We also measured the effects of BCD treatment on HIV infectivity in a sensitive single-cycle infection assay based on a cell line transfected with an LTR-luciferase cassette.[30] These $CD4^+$ CEM × 174 (LuSIV) cells possess a modified SIV long terminal repeat (LTR) viral promoter linked to the luciferase gene. Quantitative measurements of single-round infection can be obtained with this assay system.[39] Viability of LuSIV cells as determined by trypan blue exclusion and proliferation was not affected by BCD treatment. Figure 5 compares $HIV\text{-}1_{RF}$ infection of control and BCD-treated LuSIV cells. BCD treatment of LuSIV cells reduced HIV infection by almost 100% and the effects were readily seen at all viral input levels. The effect of BCD treatment of LuSIV cells on HIV infection was completely reversed by exposing the BCD-treated cells to cholesterol-loaded BCD (cholesterol at 48 μg/ml) for 1 hr to restore membrane cholesterol before exposing the cells to HIV (data not

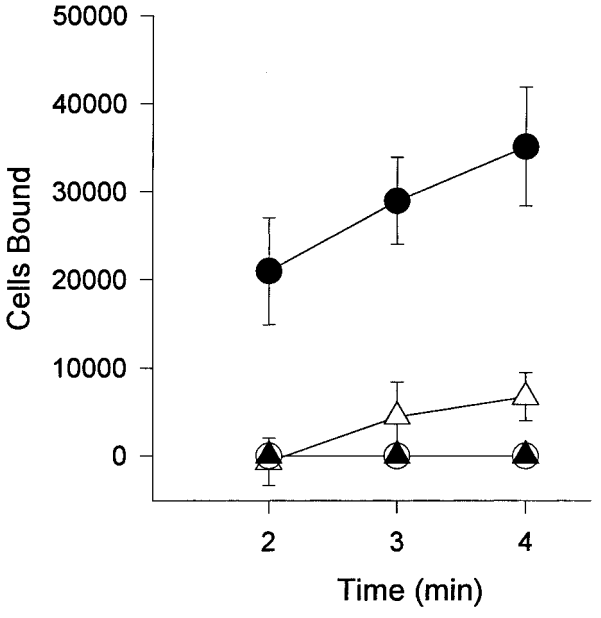

FIG. 3. BCD treatment blocks SDF-1-induced cell binding to ICAM-1. Jurkat cells were labeled with HRP and treated with medium or BCD. The cells were then added to wells coated with ICAM–Ig in the presence or absence of SDF-1 and incubated at 37°C. At the indicated times, the wells were washed to remove unbound cells and bound cells were assayed. Closed circles, control cells plus SDF-1; open circles, BCD-treated cells plus SDF-1; closed triangles, control cells plus medium; open triangles, BCD-treated cells plus medium. For BCD-treated cells the binding was below the limit of detection.

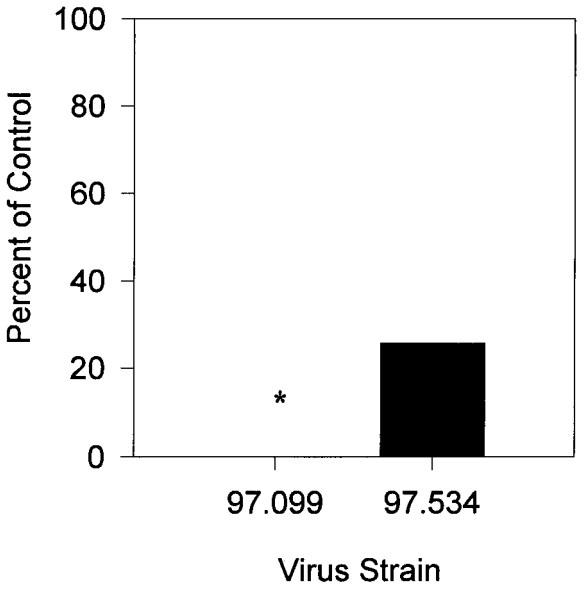

FIG. 4. BCD treatment of primary cells reduces infection by primary strains of HIV-1. After a 1-hr BCD treatment, PBMCs were washed and then exposed to virus (p24 at 20 ng/ml) for 24 hr. Free virus was then washed away and the cells were allowed to culture for 6 days. Supernatants were collected and p24 was quantitated by standard p24 ELISA. Untreated cells produced a total p24 concentration of 8.5 ng/ml for 97.099 and 94.1 ng/ml for 97.534. Data are expressed as p24 produced by BCD-treated cells as a percentage of p24 produced by untreated cells. (*p24 levels were below the limit of detection for 97.099 infection of BCD-treated cells.)

shown). These assays were repeated with identical results with HIV-1 particles that had been pelleted through a 20% sucrose cushion (data not shown). This ruled out any affects on the BCD-treated cells of soluble factors in virus preparations such as chemokines that may have contributed to the results obtained in the infectivity assays. The results of these studies show that cholesterol in the membrane of HIV susceptible cells is required for infection by free virus.

DISCUSSION

We demonstrated that lipid rafts play an important role in HIV biology by serving as sites for virus budding.[24] Since cholesterol is high concentrated in lipid rafts and cholesterol has been implicated in the entry of other viruses, we were interested in determining the effects of lipid raft dispersion by cholesterol depletion on HIV-1 infection and syncytium formation. Our results show that cholesterol is required for both HIV-1-induced cell–cell fusion as well as infection by free virus particles.

Similar to our findings with HIV-1, Semliki Forest virus also requires cholesterol for viral fusion.[40] In the case of Semliki Forest virus, it appears that the cholesterol dependence can be attributed to the envelope spike protein.[22,41] Another alphavirus, sindbis virus, also requires cholesterol in target membranes for infection.[42] *In vitro* assays determined that cholesterol and sphingolipids are required in liposomes for fusion with sindbis virus at low pH, even in the absence of receptor.[43] The studies referred to above established a clear requirement for cholesterol in membrane fusion for these viruses and our results indicate a similar role for cholesterol in HIV-1 fusion. The importance of cholesterol for HIV-induced membrane fusion is also supported by studies showing that cholesterol in large unilamellar vesicles enhanced the membrane fusion activity of an HIV-1 gp41-derived peptide.[44]

Glycolipids are important components of lipid rafts and the role of host glycolipids in HIV infection has begun to receive critical attention. Inhibition of sphingolipid synthesis by inhibitors such as PPMP (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol) has been shown to reduce HIV infection of $CD4^+$ human cells by 50%.[45] Moreover, $CD4^+$ nonhuman cells were made susceptible to gp120–gp41-mediated cell fusion by the addition of human erythrocyte glycolipids.[45] Hammache and others have demonstrated CD4-induced binding of gp120 to glycosphingolipids Gb3 and GM3 from reconstituted lipid raft microdomains.[46] Their results imply that glycolipids, which are enriched in lipid rafts, may also serve as cofactors in determining viral tropism. Indeed, Hug *et al.* obtained data suggesting that glycolipid-enriched membrane domains (lipid rafts) may serve as platforms for organizing CD4, CRs, and gp120/41 into fusion complexes.[47] Our findings support this model for preferential HIV-1 interactions with lipid rafts as sites for virus entry. Simian virus 40 also enters cells at lipid rafts (caveolae), even though its receptor appears to be MHC class I. The virus may bind to other regions of the cell membrane but translocates to caveolae for entry.[48] It is interesting to note that several bac-

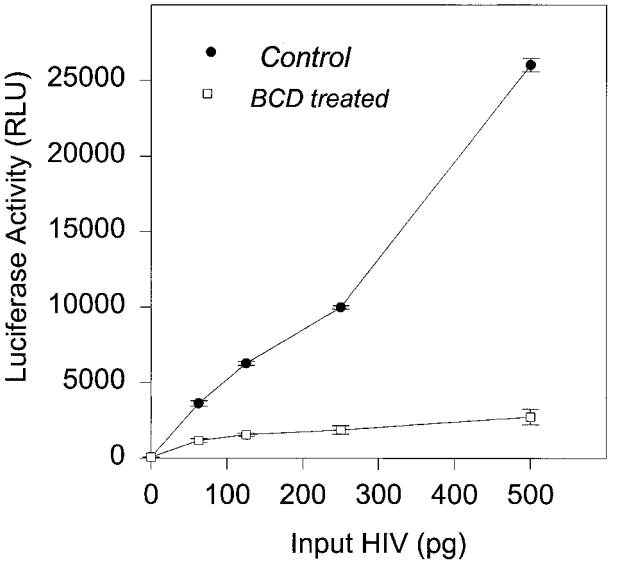

FIG. 5. BCD treatment of LuSIV cells inhibits infection by HIV-1. LuSIV cells were treated with or without 20 mM BCD in RPMI. $HIV\text{-}1_{RF}$ at various input concentrations of p24 was incubated with the cells overnight. Cells were then lysed and analyzed for luciferase activity (expressed as relative light units, RLU. Error bars represent the standard deviation of duplicate wells. The experiment was performed three times with similar results.

terial toxins target lipid rafts as well. For example, the bacterial toxins aerolysin and *Clostridium septicum* alpha toxin bind to GPI-anchored proteins, which are highly enriched in lipid rafts,[49,50] while *Vibrio cholerae* toxin binds to GM1.[51] Cholera toxin oligomerization and pore formation in liposomes have been found to be promoted by cholesterol and sphingolipids.[52] We previously demonstrated that the host derived GPI-anchored proteins acquired by HIV-1 budding from lipid rafts renders the virus susceptible to neutralization by aerolysin.[53]

The reduction of virus binding to cells treated with BCD (Table 3) is likely to involve more than BCD effects on CRs since adhesion molecules play a role in virus binding to cells.[30,31,54,55] The affinities of adhesion molecules, including integrins LFA-1 and $\alpha_V\beta_3$, for their ligands have been found to be diminished by treatment of cells with BCD.[34,35] Conversely, the addition of cholesterol was found to increase binding of $\alpha_5\beta_1$ integrin to fibronectin, as well as increasing its localization to focal adhesions and interactions with the cytoskeleton. [36] In the current study we showed that cholesterol depletion rendered CXCR4 sensitive to MAb-induced internalization not seen on control cells. This result implied that cholesterol may play a role in maintaining stable expression of CXCR4. Chemokine receptor sensitivity to MAb-induced internalization on BCD-treated cells raised the possibility that chemokines may induce internalization of these receptors as well. Such an effect by chemokines could not account for the results obtained in our studies, however, since BCD-treated cells also resisted infection by HIV pelleted through sucrose to remove soluble factors. Cells treated with BCD did not respond to SDF-1 in LFA-1-mediated cell adhesion assays. This result showed that CXCR4, which normally regulates LFA-1 function,[34] did not do so after cholesterol depletion. Thus, disruption of integrin function on BCD-treated cells could significantly diminish virus binding given the demonstrated role of these molecules in HIV binding to cells.[30,55,56]

Multiple extracellular loop domains of both CXCR4 and CCR5 are believed to be involved in CR–gp120 binding and the subsequent conformational changes that lead to HIV-1 fusion. Mouse CCR5 extracellular loop (ECL) loop swapping with human CCR5 revealed that all three loops are involved in functional interaction with the HIV-1 envelope.[57] Env interactions with multiple ECLs of the coreceptor implies that binding may occur in a groove or pocket at the level of the plasma membrane. Accordingly, a small molecule has been found to block gp120 interaction with CCR5 in a pocket[58] formed between transmembrane helices 1, 2, 3, and 7. For CXCR4, antagonist peptide T22 has been shown to block HIV-1 infection by interacting with the N terminus and at least ECL1 and ECL3.[59] Since CRs can project no further than a few nanometers above the plane of the membrane, gp120–CR interactions probably bring their respective membranes into close opposition to each other. Close membrane contact is required for lipid intermixing between the two membranes after the triggering of conformational changes in gp41. The requirement for conformational integrity of CR TM domains is evidenced by the finding that structural analogs of TM domains of CXCR4 and CCR5 have been used to inhibit signaling and HIV infection.[60] The insertion of these peptides is believed to disrupt the interactions of the transmembrane helices in the CR, knocking out both its ability to transmit signals and support HIV fusion. Thus changes in CR conformation in either their TM domains or ECLs may profoundly affect their ability to serve as HIV-1 coreceptors. Our results imply that cholesterol may be important in maintaining functional conformations of both CCR5 and CXCR4. Strong support for this model comes from work on other serpentine receptors, the oxytocin and cholecystokinin receptors. Gimpl and colleagues have demonstrated that the receptor function of these molecules is strictly cholesterol dependent[19] and their studies of ligand binding suggest that depletion of cholesterol from the cell membrane probably alters the conformation of these receptors.

Clustering of CXCR4 by cytoskeletal rearrangements appears to be important in HIV-1 cell entry and promoting chemotaxis of CD4 and CD8 cells.[38,61] Lipid raft aggregation induced by a chemotactic stimulation produces similar cellular rearrangements.[23] Redistribution of proteins, including CCR5 and the T cell receptor, into lipid rafts appears to be a critical trigger for cell function and this idea is supported by the finding that the removal of cholesterol inhibits chemotaxis and cell polarization mediated through CCR5.[62,63] Inhibition of HIV infection by cholesterol depletion may reflect a similar requirement for these processes in HIV-1 infection.

We observed enhanced MAb-induced internalization of CR after BCD depletion of cellular cholesterol. Interestingly, the opposite effect has been seen in other studies of the transferrin and epidermal growth factor receptors, where BCD treatment reduced the rate of internalization through clathrin-coated pits.[64,65] Previous studies of CXCR2 and CXCR4 internalization induced by SDF-1α and phorbol myristate acetate (PMA) stimulation have shown that this process may be mediated by clathrin-coated vesicles.[66–68] Since BCD depletion of cholesterol appears to inhibit coated vesicle internalization, MAb-induced CR internalization in BCD-treated cells probably occurs through a distinct pathway. This phenomenon could be similar to the displacement of caveolin from caveolae to the Golgi apparatus seen after cholesterol oxidase treatment of cells, which produces membrane effects similar to cholesterol removal.[69] Cholesterol depletion may also alter CR interactions with other proteins at the cell membrane that are necessary for stable membrane expression.

We have previously provided evidence of the budding of HIV-1 from lipid rafts, and in this article have further demonstrated the significance of lipid rafts, cholesterol in particular, in the biology of HIV-1. CRs appear to require cholesterol to support HIV-1 fusion and infection. Whether cholesterol is needed for conformational stability, stable membrane expression, lipid raft-mediated cell signaling, or all of the above is not yet clear. We note that cholesterol removal does not strictly affect lipid rafts alone, but it will have effects on cell signaling and other cellular functions. However, our studies of HIV-1 induced syncytium formation, which only requires expression of envelope protein and viral coreceptors at appropriate levels, strongly suggest that intact lipid rafts and cholesterol play a critical role in the early steps of virus binding and fusion. Our results confirm and extend those of Manes *et al.*, who also demonstrated fully reversible inhibition of HIV infection by depleting cholesterol from susceptible cells with BCD.[70] Manes

et al. based their studies primarily on transfected epithelial cell lines (293 and HeLa) and did not examine the effect of BCD treatment on LFA-1, CD4, or CR expression. In contrast to our findings, Manes *et al.* did not see any reduction in HIV binding after BCD treatment. However, since they used LFA-1-negative cells in their binding assay a reduction in binding would not necessarily be seen in their system. Our data are entirely consistent with and support earlier findings by Hug *et al.*, who showed that glycosphingolipids, highly enriched components of lipid rafts, play a critical role in the formation of membrane fusion complexes by CD4, gp120/41, and CRs.[47] Our results clearly point to an important role for cholesterol in HIV infection and syncytium formation. Since cholesterol depletion is known to disperse lipid rafts, our data and those from other studies noted above clearly point to the importance of lipid rafts in the biology of HIV-1. However, definitive delineation of the exact role of lipid rafts in HIV infection of cells will require further study. Future studies that provide a better understanding of how HIV-1 utilizes cholesterol and lipid rafts may allow us to improve anti-HIV therapeutics, develop novel microbicides, or attenuate the pathogenic effects of HIV infection.

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