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Liposome targeting to human immunodeficiency virus type 1-infected cells via recombinant soluble CD4 and CD4 immunoadhesin (CD4-IgG)

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Abstract

HIV-infected cells producing virions express the viral envelope glycoprotein gp120/gp41 on their surface. We examined whether liposomes coupled to recombinant soluble CD4 (sCD4, the ectodomain of CD4 which binds gp120 with high affinity) could specifically bind to HIV-infected cells. sCD4 was chemically coupled by 2 different methods to liposomes containing rhodamine-phosphatidylethanolamine in their membrane as a fluorescent marker. In one method, sCD4 was thiolated with *N*-succinimidyl acetylthioacetate (SATA) and coupled to liposomes via a maleimide-derivatised phospholipid. In the other method, the oligosaccharides on sCD4 were coupled to a sulfhydryl-derivatised phospholipid, utilizing the bifunctional reagent, 4-(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH). The association of the liposomes with HIV-1-infected or uninfected cells was examined by flow cytometry. CD4-coupled liposomes associated specifically to chronically infected H9/HTLV-IIIB cells, but not to uninfected H9 cells. CD4-coupled liposomes also associated specifically with monocytic THP-1 cells chronically infected with HIV-1 (THP-1/HIV-1_{IIIB}). Control liposomes without coupled CD4 did not associate significantly with any of the cells, while free sCD4 could competitively inhibit the association of the CD4-coupled liposomes with the infected cells. The chimeric molecule CD4-immunoadhesin (CD4-IgG) could also be used as a ligand to target liposomes with covalently coupled Protein A (which binds the Fc region of the CD4-IgG) to H9/HTLV-IIIB cells. The CD4-liposomes inhibited the infectivity of HIV-1 in A3.01 cells, and also bound rgp120. Our results suggest that liposomes containing antiviral or cytotoxic agents may be targeted specifically to HIV-infected cells.

Key words: Liposome; CD4; HIV; Liposome targeting

1. Introduction

The transmembrane glycoprotein CD4, expressed mainly on the surface of helper/inducer T lymphocytes and monocyte/macrophages, is the primary high-affinity receptor for human immunodeficiency virus type 1 (HIV-1) [1,2]. Several therapeutic strategies which exploit the high affinity of CD4 for the HIV-1 envelope glycoprotein gp120 have been developed in recent years. One such strategy involves the use of the recombinant ectodomain of CD4 (soluble CD4, or sCD4), which retains high affinity binding to gp120, as a neutralizing agent to block HIV-1 infection and HIV-induced syncytium formation [3–7]. Treatment of simian immunodeficiency virus-infected rhesus monkeys with sCD4 produced some therapeutic effects [8]. CD4-immunoglubulin (CD4-IgG) chimeras that combine the V1 (D1) and V2 (D2) or the V1–V4 domains of CD4, and the constant region of IgG, have also been shown to inhibit the infection of T cells and monocytes, and to have a longer plasma half-life than sCD4 [9]. Pre-treatment of chimpanzees with CD4-IgG containing the V1 and V2 domains of CD4 prevented their infection with

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HIV-1 [10]. More recent constructs, such as red blood cells with electro-inserted full-length CD4 or covalently bound sCD4, have been shown to inhibit HIV-1 infectivity or HIV-1 binding to target cells [11–14]. However, the relative resistance of primary HIV isolates to neutralization of infectivity by diverse constructs of sCD4 [15], as well as the inability of sCD4 or CD4-IgG to reduce the viral load in AIDS patients in clinical trials [16–18], has shed serious doubt on the feasibility of using sCD4 as an HIV-1 neutralizing agent.

Another therapeutic approach has been the use of sCD4 to target effector molecules such as toxins to infected cells. These chemically or genetically coupled conjugates have been used to kill selectively HIV-infected cells in vitro, and to prevent the spreading of HIV from infected to uninfected cells [19–26]. However, the potential non-specific toxicity of such toxin constructs in vivo may limit their eventual use [27].

Liposomes have been employed as drug carriers in the treatment of numerous diseases, both in experimental animals and in humans [28,29]. One potential advantage of targeted liposomes over toxin conjugates is that they can enclose a diverse array of molecules in their internal aqueous space and in their lipid bilayer. In addition, some anti-HIV drugs that have limited ability to reach infected tissues, and others that have limited access to the cytoplasm of infected cells may benefit from delivery in liposome-encapsulated form. Liposome mediated delivery may significantly enhance the efficacy as well as reduce the potential toxicity of antiviral drugs [28,30,31]. Liposomes containing the complete CD4 molecule reconstituted in their membranes have been shown to interact selectively with HIV-infected cells [32]. A peptide corresponding to the CDR3-like region of CD4 has been coupled to dimyristylphosphatidylcholine-containing liposomes, and these liposomes have been shown to be selectively cytotoxic against HIV-infected cells [33].

In this study we have investigated the use of the 368-amino acid sCD4 [6,34] as a ligand to target liposomes to HIV-infected cells. We have employed two different methods to couple covalently sCD4 to liposomes and compared their ability to leave intact the gp120-binding capacity of sCD4. *N*-Succinimidyl acetylthioacetate (SATA) is used to attach sulfhydryl groups on proteins [35], which can subsequently be coupled to a maleimide-derivatized phospholipid [36]. However, since the reaction of SATA with proteins is through primary amines, it is possible that this reagent may derivatize residues on the V1 domain that may be involved in binding to gp120. In contrast to SATA, the novel cross-linking agent 4-(4-*N*-maleimidophenyl)-butyric acid hydrazide (MPBH) [37] is directed toward carbohydrate residues, which in the case of sCD4 are located within the V3 domain, away from the gp120-binding site. This is expected to leave the gp120-binding site undisturbed, as well as to enhance the likelihood of proper orientation of CD4 with respect to the liposome membrane, thereby increasing gp120 binding. In addition to covalent attachment of sCD4 to liposomes, we have utilized liposome-coupled Protein A to mediate the binding of the chimeric molecule CD4-IgG (immunoadhesin) to liposomes. CD4-IgG is predicted to bind to cell surface gp120 on infected cells via its CD4 moiety, and to Protein A via the Fc domain. Some of our results have been presented earlier in preliminary form [38].

2. Materials and methods

2.1. Materials

Recombinant soluble CD4 (sCD4) and immunoadhesin (CD4-IgG) were produced in CHO cells using a vector directing expression of dihydrofolate reductase and of sCD4 or CD4-IgG, as described previously [6,9]. The amino acid sequence and glycosylation sites of sCD4 have been described in detail [34]. 4-(*p*-Maleimidophenyl)butyrylphosphatidylethanolamine (MPB-PE), *N*-[3-(2-pyridyldithio)propionyl]dipalmitoylphosphatidylethanolamine (PDP-PE), egg phosphatidylcholine (PC), and *N*-(lissamine-rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (CH) was from Calbiochem (San Diego). *N*-Succinimidyl acetylthioacetate (SATA), and dimethylformamide (DMF) were from Pierce (Rockford, IL). All chemicals used in the buffers, hydroxylamine, dithiothreitol (DTT), metrizamide, sodium periodate ($NaIO_4$), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). 4-(4-*N*-Maleimidophenyl)-butyric acid hydrazide (MPBH) was synthesized according to Chamow et al. [37]. Protein A purified from *Staphylococcus aureus*, Cowan I strain, was purchased from Zymed Laboratories (South San Francisco, CA).

2.2. Coupling of sCD4 to liposomes using N-succinimidyl acetylthioacetate (SATA)

Soluble CD4 was coupled to liposomes using a modification of the method of Martin et al. [39]. The protein was thiolated using SATA [35,36], and coupled to liposomes containing the thiol-reactive lipid MPB-PE. A 54.1 mM solution of SATA in DMF was prepared immediately before use and added to 6 mg/ml sCD4 (in 20 mM Hepes, 188 mM NaCl (pH 7.0)) at room temperature, under argon, at molar ratios of 2.25:1 or 1.125:1 (SATA/sCD4). The reaction was carried out with gentle stirring for 1 h, followed by removal of unreacted SATA by dialysis (Spectrapor 2,

Spectrum Medical Industries, Los Angeles, CA) against three 660-fold volumes of Buffer A (20 mM Mes, 20 mM Mops, 125 mM NaCl, 1 mM EDTA (pH 6.7)), for 24 h at 4°C. Sulfhdryl residues were deprotected by reaction with 10 mM hydroxylamine at room temperature, under argon. Activated thiol groups were measured by reaction of the thiol-modified protein with DTNB against a cysteine standard [40,41].

Large unilamellar liposomes composed of a 9.7:5:0.15:0.2 molar ratio of PC/CH/Rh-PE/MPB-PE were prepared by reverse phase evaporation [42,43] in Buffer A saturated with argon. To obtain a uniform size distribution, the liposomes were extruded 4× through double polycarbonate membranes of 0.1 μm pore-diameter (Poretics, Pleasanton, CA) under argon pressure in a high-pressure stainless steel extruder [43–45]. This method has been shown previously to produce unilamellar liposomes of homogeneous size [44,45]. MPB-PE-containing liposomes were prepared immediately before the coupling reaction, at which time an aliquot of the liposomes was set aside to be used as an uncoupled liposome control.

Thiolated sCD4 was incubated with liposomes at final concentrations of 3.1 mg/ml sCD4 and 8 mM lipid (for the 2.25:1 molar ratio derivative), or 2.9 mg/ml sCD4 and 8 mM lipid (for the 1.125:1 molar ratio derivative). The coupling reactions proceeded at room temperature, under argon, with gentle stirring overnight.

2.3. Coupling of Protein A to liposomes using SATA

Modification of Protein A with SATA was achieved according to the method described above for the derivatization of sCD4, with the following exceptions: 54.1 mM SATA in DMF was added to 5 mg/ml Protein A in PBS/1 mM EDTA at a molar ratio of 9:1 SATA/Protein A. Unreacted SATA was removed from modified Protein A by dialysis against two 640-fold volumes of Buffer B (10 mM Hepes, 140 mM NaCl, 10 mM KCl, 1 mM EDTA (pH 7.0)).

Liposomes were prepared and coupled to Protein A as described above for sCD4-liposome coupling with the following modifications: Liposomes were prepared in argon-saturated Buffer A. Thiolated Protein A was incubated with liposomes at final concentrations of 2.2 mg/ml and 8 mM lipid, respectively.

2.4. Coupling of sCD4 to liposomes using 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH)

The oligosaccharide-directed heterobifunctional reagent MPBH [37] was used to derivatize sCD4. The protein (5.4 mg/ml in 0.1 M Na acetate buffer (pH 5.5)) was oxidized by reaction with 10 mM $NaIO_4$ for 30 min at room temperature. Excess $NaIO_4$ was removed by gel filtration on Sephadex G-25 equilibrated with 0.1 M Na acetate buffer (pH 5.5). MPBH (dissolved in DMF) was added to oxidized sCD4 to produce final concentrations of 1 mM and 2.7 mg/ml, respectively. The reaction proceeded for 2 h at room temperature, after which unreacted MPBH was removed by gel filtration on Sephadex G-25 equilibrated with 10 mM Na acetate/150 mM NaCl (pH 5.5). The sCD4-MPBH conjugate was concentrated to 10 mg/ml by ultrafiltration in a Centricon 10 concentrator (Amicon, Beverly, MA).

Liposomes composed of a 9.7:5:0.15:0.2 molar mixture of PC/CH/Rh-PE/PDP-PE were prepared by reverse phase evaporation as described above. An aliquot of the liposomes was removed to be used as an uncoupled liposome control. PDP-PE-containing liposomes were reduced to expose free thiol groups with 20-fold excess (12 mM) DTT at room temperature under argon for 1 h. Excess DTT was removed by gel filtration on Sephadex G-75 equilibrated with Buffer A under a constant stream of argon. Sulfydryl reduction of liposomes was measured by their reaction with DTNB.

Immediately prior to mixing with thiol-containing liposomes, the pH of the sCD4-MPBH solution was adjusted to 7.0 by titration with 500 mM Mes/500 mM Mops (pH 8.0). Coupling of sCD4 to liposomes was achieved using final concentrations of 2.4 mg/ml and 10 mM lipid, respectively, under the same conditions as described for SATA.

In order to confirm that the coupling reaction of maleimide-derivatized sCD4 with thiol-containing liposomes was specific, an aliquot of sCD4 was not oxidized by periodate prior to incubation with MPBH. Subsequently, this mock-derivatized sCD4 was incubated with thiol-containing liposomes according to the procedure described above.

2.5. Purification of protein-liposome conjugates

Protein A- and sCD4-liposomes were purified by flotation on a discontinuous metrizamide gradient as described by Heath [46] to remove uncoupled protein, followed by overnight dialysis (Spectrapor 2) against ≥ 1000-volumes of either Buffer A or Buffer B, both without EDTA, at 4°C, under argon. Conjugates were sterilized by passage through a 0.22 μm Millex filter (Millipore, Keene, NH) and assayed for lipid and protein concentration by the phosphate assay [47] and the method of Lowry et al. [48], respectively. Of the initial amount of phospholipid used for the different preparations, between 19–26% was recovered after the last filtration step. The amount of liposome-coupled protein recovered was between 8 and 20% of the initial free protein added to the liposomes.

2.6. gp120 binding

Binding of gp120 to CD4-liposomes was determined by radioimmunoassay, as described previously [49,50]. Microtiter wells were coated with anti-IgG antibody to immobilize CD4-IgG. Then, ^{125}I-labeled gp120 (HIV-1_{IIIB}) was added simultaneously with free CD4-IgG or liposome-coupled CD4 to determine the ability of the latter two compounds to compete with the immobilized CD4-IgG for binding to gp120.

2.7. Cells and virus

THP-1 cells were obtained from the American Type Culture Collection. A chronically HIV-infected cell line (designated THP-1/HIV-1_{IIIB}) was developed in our laboratory by infecting THP-1 cells with HIV-1_{IIIB} [51]. Chronically infected H9/HTLV-IIIB cells were obtained from J. Mills and T. El-Beik (San Francisco General Hospital, San Francisco, CA). H9 cells were obtained from R.C. Gallo, through the AIDS Research and Reference Reagent Program (NIH, Bethesda, MD). Human lymphoblastoid A3.01 cells were provided by T.M. Folks (CDC, Atlanta, GA). All cells were maintained at 37°C, under 5% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (both from Irvine Scientific, Santa Clara, CA), penicillin (50 units/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). The cells were passaged 1:5 or 1:6 every 3 or 4 days. The percentage of viable cells in each culture was measured by Trypan blue dye exclusion.

HIV-$1_{LAV\text{-}BRU}$ (previously designated as the LAV strain, and recently re-identified as LAI [52]), was obtained from Dr. F. Barré-Sinoussi, propagated in CEM cells, and purified as described in Larsen et al. [53]. The virus preparation was then propagated in A3.01 cells, and harvested at times of peak p24 production. The latter virus preparation was designated as HIV-$1_{LAV\text{-}BRU/P}$ [54], and used for the experiments on inhibition of infectivity by CD4-liposomes. The reverse transcriptase activity of the virus stock solutions was $2.45 \cdot 10^6$ cpm/ml, and the p24 concentration was 2.6 μg/ml. The infectious titer per 100 ng of p24 antigen was $4.6 \cdot 10^5$ tissue culture infectious dose, 50% endpoint ($TCID_{50}$), as determined in both H9 and A3.01 cells by the method of Johnson and Byington [55].

2.8. Effect of CD4-liposomes on HIV-1 infectivity

HIV-$1_{LAV\text{-}BRU/P}$ (0.4 μg p24/ml) was mixed with 0.1, 0.3, 1.0, or 3.0 μg sCD4, the same amount of CD4 in liposome-coupled form, or uncoupled liposomes (corresponding to the lipid concentration of 1.5 μg of CD4-MPBH liposomes) in a final volume of 50 μl RPMI/10% FBS. The mixture was incubated for 2 h at 37°C, after which 10 μl (= 4 ng viral p24) of each treated virus sample was added to 90 μl of (harvested and washed) A3.01 cells in RPMI/10% FBS, at a final infection density of $2.0 \cdot 10^7$ cells/ml (i.e., 2 ng viral p24/10^6 cells). Cells were incubated with virus for 2 h at 37°C to allow for viral adsorption and fusion. Cells were then washed 3 × with 2 ml RPMI/10% FBS to remove unbound virus and plated at a cell density of $5 \cdot 10^5$ cells/ml in 12-well cell culture plates (Corning, Corning, NY) (4 ml/well).

At 3 days post-infection, 2 ml of the culture medium was removed for analysis, and replaced with fresh medium until day 7. Day 3 and day 7 cell-free supernatants were subsequently analyzed for virus production by an ELISA for viral p24, as described previously [56], using a Molecular Devices (Menlo Park, CA) V_{max} microplate reader. The assay was calibrated with standards provided by a p24 ELISA kit from Coulter (Hialeah, FL), and a standard HIV-1 preparation was used for each determination. The assay could detect p24 levels of 0.038 ng/ml.

2.9. Effect of CD4-liposomes on HIV-1-induced syncytium formation

Chronically infected H9/HTLV-IIIB cells were mixed (1:1) with uninfected $CD4^+$ A3.01 cells ($5 \cdot 10^4$ each) in phosphate-buffered saline, and syncytium formation was scored 16–20 h later on a scale of '+++' for fully formed syncytia throughout the culture to '−' for no syncytium formation, with intermediate syncytia formation being assigned values of ++, + or ± [57].

2.10. Association of sCD4-liposome conjugates with HIV-infected cells

Liposomes were incubated with uninfected H9 or THP-1 cells or chronically infected H9/HTLV-IIIB or THP-1/HIV-1_{IIIB} cells for 1 h at 37°C (10^6 viable cells in a final volume of 100 μl of RPMI/0.2% BSA). The concentration of CD4 in liposome-conjugated form was 10 μg/ml. An equivalent lipid concentration of uncoupled liposomes was used as a control.

In the case of Protein A-liposomes, H9/HTLV-IIIB cells were first pre-incubated for 1 h on ice, either in the presence or absence of 10 μg/ml CD4-IgG. Cells were then washed 3 × with PBS/0.2% BSA, and incubated for 30 min on ice and 30 min at 37°C in the presence of either Protein A-liposomes (100 μg protein/ml) or an equivalent lipid concentration of uncoupled liposomes.

Cells were washed 4 times with cold PBS/0.2% BSA to remove unassociated liposomes and were then fixed in 2% paraformaldehyde. Flow cytometric analysis was performed using a custom designed flow cytometer composed of Becton Dickinson FACS IV parts

[58], equipped with three argon lasers (Spectra Physics). The system was driven by a Consort 40 Computer and Software (Becton Dickinson). Samples were analyzed for lissamine rhodamine, with excitation at 528 nm and resulting emission at 575 nm, using a band-pass filter of 575/25 nm. Forward scatter energy was used to trigger the signal collection. Ten thousand events were recorded for each sample. Forward and side scatter signals were used to gate the cell subset of interest and to eliminate debris and cell aggregates.

Table 1
Preparation of liposome-conjugates

Conjugate	Coupling reagent	Protein/liposome	
		μg/μmol phospholipid	molecules/liposome
CD4-liposome	SATA	142 [a]	280 [a]
		114 [b]	224 [b]
CD4-liposome	MPBH	142	280
Protein A-liposome	SATA	105	200

[a] SATA/sCD4 2.25:1.
[b] SATA/sCD4 1.125:1.

3. Results

3.1. Conjugation of sCD4 and Protein A to liposomes

We first determined the optimal molar ratio of sCD4/SATA during the derivatization reaction, required for both efficient coupling of SATA and retention of the gp120 binding capacity of CD4. Soluble CD4 was thiolated with SATA using [molar] ratios of 9:1, 4.5:1, and 2.25:1 (SATA/sCD4). Using these preparations of thiolated sCD4, we obtained coupling ratios of 27, 89.4, and 53 μg sCD4/μmol phospholipid, respectively. At all ratios of SATA to sCD4, this method of coupling adversely affected the gp120-binding ability of the resulting CD4-liposome conjugate, Indeed, only the CD4-liposome conjugate obtained by 2.25-fold molar excess of SATA was able to both bind gp120 and inhibit HIV-induced syncytium formation. This CD4-liposome conjugate inhibited the binding of gp120 to immobilized CD4-IgG with an IC_{50} of 4.6 μg/ml, corresponding to a 20-fold decrease in gp120 binding ability relative to a CD4-IgG control. Syncytium formation between chronically HIV-1 infected H9/HTLV-IIIB and $CD4^+$ A3.01 cells was also inhibited most effectively by the 2.25:1 conjugate. These CD4-liposomes (at a concentration of 10 μg protein/ml) inhibited syncytium formation to + (from the control value of + + +), while free sCD4 at the same concentration inhibited syncytium formation completely (−). The liposomes with the 4.5:1 conjugate inhibited syncytium formation only slightly (+ +), while the 9:1 conjugate appeared to be toxic. These results suggested that the higher incubation ratios of SATA/sCD4, which yielded a greater number of thiols introduced per sCD4 molecule (data not shown), resulted in a loss of gp120 binding activity. Therefore, subsequent experiments were carried out with conjugation ratios of 2.25:1 and 1.125:1.

Table 2
The effect of liposome-coupled CD4 and sCD4 on the infectivity of $HIV\text{-}1_{BRU}$ in A3.01 cells [a]

Reagent	Concentration (μg CD4/ml)	Viral p24 (ng/ml)		% Inhibition at 7 days
		3 days	7 days	
Control	0	0.96 ± 0.06	52.8 ± 7.1	0
CD4-MPBH liposomes	0.1	0.74 ± 0.10	12.5 ± 1.0	76.3
	0.3	0.36 ± 0.13	9.8 ± 1.3	81.4
	1	0	1.8 ± 0.1	96.6
	3	0	0	100
CD4-SATA liposomes	0.1	0.72 ± 0.14	32.1 ± 6.2	39.2
	0.3	0.48 ± 0.06	16.9 ± 3.2	68
	1	0.12 ± 0.01	5.1 ± 0.6	90.3
	3	0	0	100
sCD4	0.1	0.10 ± 0.03	2.73 ± 1.29	94.8
	0.3	0	0.22 ± 0.13	99.6
	1	0	0	100
	3	0	0	100
Plain liposomes [b]	0	0.42 ± 0.04	23.2 ± 8.4	56.1

[a] $HIV\text{-}1_{BRU}$ (0.4 μg p24/ml) was pre-incubated with liposome-coupled CD4 or sCD4 at the indicated concentrations for 2 h at 37°C. $CD4^+$ A3.01 cells were infected at 2 ng p24/10^6 cells. Data represent the mean and standard deviation of p24 values obtained from duplicate determinations from duplicate wells.
[b] The lipid concentration of the liposomes (10 μM) corresponds to that of CD4-MPBH liposomes containing 1.5 μg/ml coupled CD4.

We calculated protein to lipid ratios for each preparation of liposome conjugates (Table 1). Molar ratios of 2.25:1 and 1.125:1 (SATA/sCD4) were employed during the initial incubation of sCD4 with SATA, and yielded coupling ratios of 142 μg sCD4/μmol phospholipid and 114 μg sCD4/μmol phospholipid, respectively. sCD4 activated with MPBH and incubated with thiol-containing liposomes yielded a coupling ratio of 142 μg sCD4/μmol phospholipid. The corresponding numbers of CD4 molecules per liposome for the different preparations are also given in Table 1.

In order to determine the specificity of the coupling reaction between MPBH-derivatized sCD4 and thiol-containing liposomes, mock-derivatized sCD4 was prepared and incubated with liposomes. For this control, sCD4, which was not oxidized by periodate prior to incubation with MPBH, did not incorporate the cross-linker. Subsequent incubation with maleimide-reactive liposomes resulted in liposomes without sCD4 coupled to their surface.

SATA-derivatized Protein A was coupled to thiol-reactive liposomes under conditions similar to sCD4 (except that a SATA/Protein A incubation ratio of 9:1 was used), yielding a coupling ratio of 105 μg Protein A/μmol phospholipid (Table 1).

3.2. Inhibition of infectivity of HIV-1_{BRU} in A3.01 cells

The effect of CD4-liposomes on the infectivity of HIV-1_{BRU} in A3.01 cells was examined (Table 2). For sCD4 as a positive control, a concentration of 1.0 μg/ml was effective in completely inhibiting infection in target cells, as measured by the production of viral p24 core antigen in the culture medium after 3 or 7 days. On day 7 of culture, 0.1 μg/ml sCD4 reduced virus production to 5.2% of the untreated control. Both CD4-MPBH liposomes and CD4-SATA liposomes required a higher concentration (3.0 μg sCD4/ml) to effect complete inhibition of p24 production on day 7. Control liposomes at a lipid concentration equivalent to that of CD4-liposomes at 1.5 μg CD4/ml, also had some inhibitory effect. CD4-coupled liposomes, however, inhibited infectivity to a considerably greater extent, even at a 5-fold lower concentration than the control liposomes. At the lowest concentration tested (0.1 μg/ml), sCD4-MPBH liposomes and sCD4-SATA liposomes reduced virus production to 23.7% and 60.8% of the untreated control, respectively.

3.3. CD4-liposome conjugates associate specifically with chronically infected THP-1 / HIV-1_{IIIB} cells

To determine whether CD4-liposomes could associate specifically with HIV-infected cells, rhodamine-PE-containing control liposomes and CD4-coupled liposomes were incubated with uninfected and HIV-1

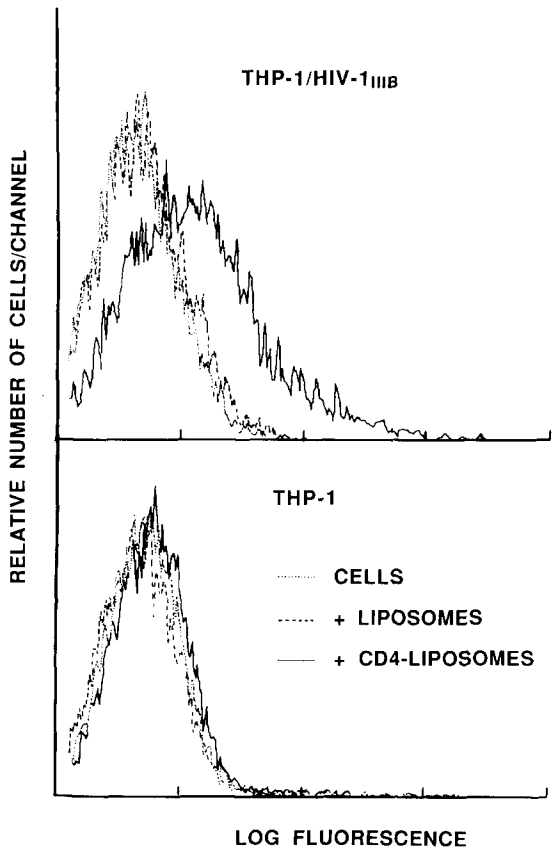

Fig. 1. Flow cytometry scans of chronically infected THP-1/HIV-1_{IIIB} cells (upper panel) or uninfected THP-1 cells (lower panel), incubated with CD4-MPBH-coupled liposomes or control liposomes for 1 h at 37°C, washed and fixed, as described in Materials and methods. Dotted lines: cells only. Solid lines: cells incubated with CD4-MPBH-coupled liposomes. Dashed lines: cells incubated with control liposomes.

infected THP-1 cells at 37°C, and the fixed cells were analyzed by flow cytometry. A representative experiment is shown in Fig. 1. Association of sCD4-MPBH-liposomes with HIV-1 infected cells is represented by a significant fluorescence shift relative to cell autofluorescence (Fig. 1, upper panel). Specific association of sCD4-liposomes with infected cells was also accompanied by a widening of the fluorescence peak, suggesting some degree of heterogeneity with respect to the level of gp120 expression on the surface of those cells. Control liposomes not containing sCD4 did not associate significantly with infected cells, as indicated by the absence of a shift in fluorescence intensity. The specificity of interaction of sCD4-liposomes with infected cells was further demonstrated by the absence of a significant increase in fluorescence intensity after incubating uninfected THP-1 cells with either sCD4-coupled or control liposomes (Fig. 1, lower panel). In this experiment, cell association of liposomes was induced by CD4-gp120 binding; however, this event may also be

followed by endocytosis and possible fusion, since the incubations were carried out at 37°C.

3.4. CD4-liposome conjugates associate specifically with chronically infected H9 / HTLV-IIIB cells

The association of CD4-coupled or control liposomes with the chronically infected T-cell line H9/HTLV-IIIB was also examined (Fig. 2). A significant fluorescence shift in flow cytometry scans was observed when the cells were incubated with CD4-liposomes (Fig. 2, upper panel, solid line). However, little or no liposome association was observed for infected cells incubated with control liposomes. No appreciable association was measured for uninfected H9 cells incubated with either CD4-coupled or control liposomes (Fig. 2, lower panel).

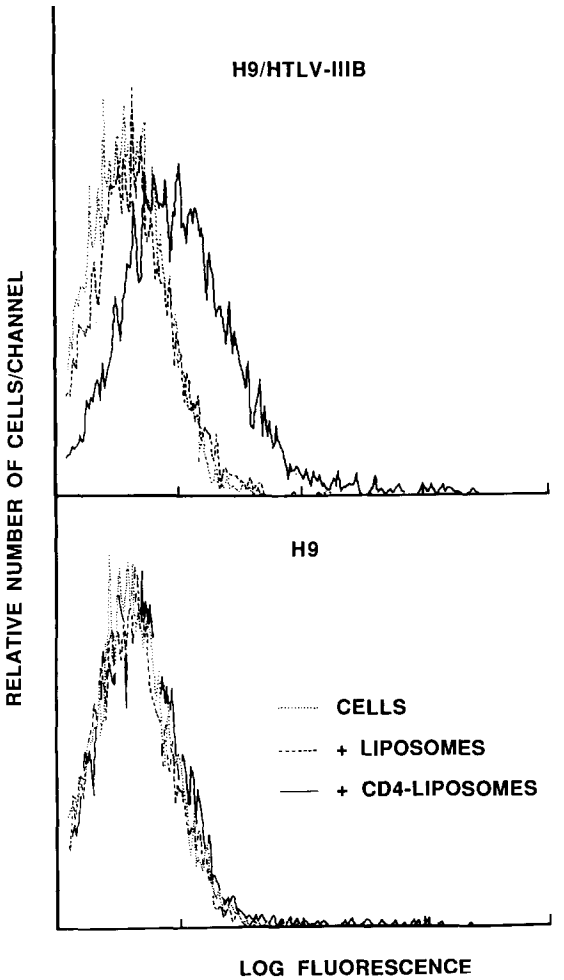

Fig. 2. Flow cytometry scans of chronically infected H9/HTLV-IIIB cells (upper panel) or uninfected H9 cells (lower panel), incubated with CD4-MPBH-coupled liposomes or control liposomes for 1 h at 37°C, washed and fixed, as described in Materials and methods. Dotted lines: cells only. Solid lines: cells incubated with CD4-MPBH-coupled liposomes. Dashed lines: cells incubated with control liposomes.

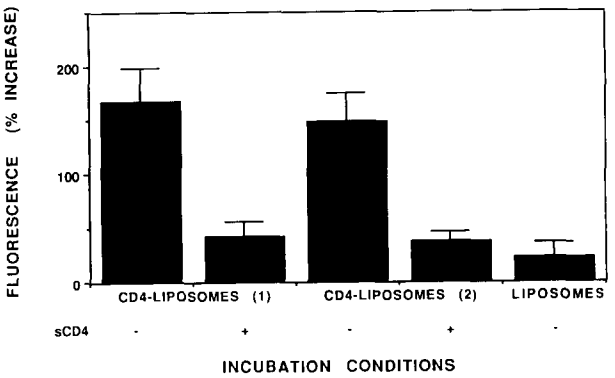

Fig. 3. Association of CD4-SATA-coupled and control (uncoupled) liposomes with H9/HTLV-IIIB cells, and the effect of sCD4, measured by flow cytometry. Cells and liposomes were incubated for 1 h at 37°C, washed and fixed, as described in Materials and methods. Cell-association of Rh-PE-labelled liposomes is expressed as the % fluorescence increase relative to the autofluorescence of H9/HTLV-IIIB cells (mean ± standard deviation of multiple flow cytometric determinations of duplicate samples for the CD4-liposomes, and mean ± range of duplicate samples for the control liposomes and sCD4 competition experiments). 'CD4-liposomes (1)' refers to liposomes coupled to the 1.125:1 (SATA-sCD4) conjugate, and 'CD4-liposomes (2)' refers to liposomes coupled to the 2.25:1 (SATA-sCD4) conjugate. 'Liposomes' refers to control liposomes of the same lipid composition. The presence or absence of sCD4 (10 μg/ml) in the incubation mixture is indicated by the '+' and '−' signs, respectively.

3.5. Effect of the coupling method on sCD4-liposome association with chronically infected cells

Two different ratios of SATA/sCD4 were used in coupling sCD4 to liposomes (Table 1). The calculated amount of sCD4 incorporated at these ratios was not markedly different, and these sCD4-coupled liposomes associated with H9/HTLV-IIIB cells to similar extents (Fig. 3). Identical ratios of sCD4/phospholipid were generated using either SATA (2.25:1 ratio) or MPBH as the coupling reagent (Table 1), thus allowing a direct comparison of the effect of these two coupling methods on the ability of the sCD4-liposomes to bind to HIV-1 infected cells. sCD4-liposomes obtained using either MPBH or SATA cross-linkers associated significantly and to a similar extent with infected THP-1/HIV-1_{IIIB} (Fig. 4) and H9/HTLV-IIIB cell lines (see CD4-SATA liposomes in Fig. 3 and CD4-MPBH liposomes in Fig. 5). A low extent of association (% fluorescence increase in the range 0–40%) was obtained with control liposomes. Control thiol-containing liposomes bound to a lesser extent to either cell line than did maleimide-containing liposomes (Fig. 4; see control liposomes in Fig. 3 and Fig. 5), suggesting lesser non-specific interaction of CD4-MPBH liposomes with infected cells. In the case of either coupling method, both sCD4-coupled and control liposomes bound to a greater degree to THP-1/HIV-1_{IIIB} cells

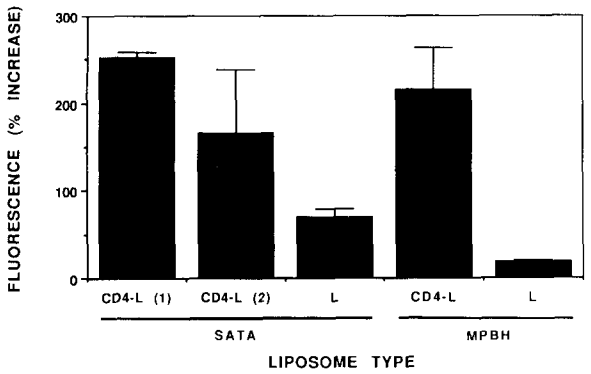

Fig. 4. Comparison of CD4-MPBH or CD4-SATA-coupled liposomes in their ability to associate with THP-1/HIV-1_{IIIB} cells, measured by flow cytometry. Cells and liposomes were incubated for 1 h at 37°C, washed and fixed, as described in Materials and methods. CD4-L (1) and CD4-L (2) correspond to the designation of liposomes in the legend to Fig. 3. The cell-association of control liposomes (L) used for sCD4 coupling in the two different methods is also shown. Cell-association of Rh-PE-labelled liposomes is expressed as the % fluorescence increase relative to the autofluorescence of THP-1/HIV-1_{IIIB} cells (mean ± standard deviation of multiple flow cytometric determinations of duplicate samples for the CD4-liposomes, and mean ± range of duplicate samples for the control liposomes).

(Fig. 4) than to H9/HTLV-IIIB cells (Figs. 3 and 5). This observation suggests that the increase in binding to THP-1/HIV-1_{IIIB} cells is at least in part non-specific or mediated by the PDP-PE component in the liposomes. The increase may also be due to a higher level of expression of viral gp120 on the surface of THP-1/HIV-1_{IIIB} cells compared to the H9/HTLV-IIIB cells, consistent with the higher level of virus production by THP-1/HIV-1_{IIIB} cells.

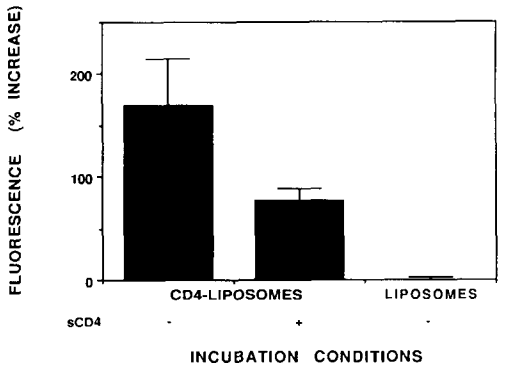

Fig. 5. Association of CD4-MPBH-coupled and control liposomes with H9/HTLV-IIIB cells, and the effect of sCD4, measured by flow cytometry. Cells and liposomes were incubated for 1 h at 37°C, washed and fixed, as described in Materials and methods. Cell-association of Rh-PE-labelled liposomes is expressed as the % fluorescence increase relative to the autofluorescence of H9/HTLV-IIIB cells. The error bars were determined as in Fig. 3. The presence or absence of sCD4 (10 μg/ml) in the incubation mixture is indicated by the '+' and '−' signs, respectively.

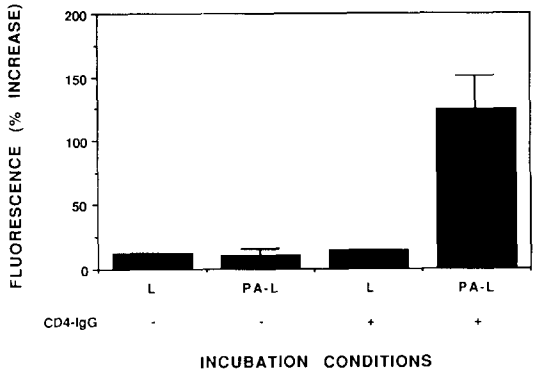

Fig. 6. The effect of CD4-IgG on the association of Protein A-coupled (PA-L) and control liposomes (L) with H9/HTLV-IIIB cells, measured by flow cytometry. Cells were pre-incubated with (+) or without (−) CD4-IgG for 1 h on ice, washed, incubated with liposomes for 30 min on ice and 30 min at 37°C, and fixed, as described in Materials and methods. Cell-association of Rh-PE-labelled liposomes is expressed as the % fluorescence increase relative to the autofluorescence of H9/HTLV-IIIB cells. The error bars indicate the range of duplicate samples for PA-L without CD4-IgG, and the standard deviation of multiple determinations on duplicate samples for PA-L with CD4-IgG.

3.6. Association of sCD4-liposomes with THP-1/HIV-1_{IIIB} or H9/HTLV-IIIB cells is inhibited by free sCD4

Co-incubation of free sCD4 (10 μg/ml) and sCD4-SATA liposomes with H9/HTLV-IIIB cells resulted in about 76% inhibition of the association of the sCD4-SATA liposome conjugate with these cells (Fig. 3), indicating that the greater gp120-binding affinity of unmodified sCD4 renders it an effective competitor in its specific interaction with gp120 expressed on the surface of cell membranes. In contrast, free sCD4 was less effective in inhibiting the association of CD4-MPBH liposomes with H9/HTLV-IIIB (Fig. 5) or THP-1/HIV-1_{IIIB} cells (data not shown). sCD4 reduced the association of CD4-MPBH liposomes with these two cell lines by about 55% and 29%, respectively, suggesting that the gp120-binding site on MPBH-derivatized sCD4 may be less compromised by this coupling method than by the SATA method.

3.7. CD4-Immunoadhesin mediates specific association of Protein A-coupled liposomes to H9/HTLV-IIIB cells

CD4-IgG binds to gp120 expressed on infected cells, and may thus be able to mediate the association of Protein A-coupled liposomes with these cells. Fig. 6 shows that the incubation of Protein A-liposomes with H9/HTLV-IIIB cells pre-incubated with CD4-IgG resulted in a significant fluorescence shift. In contrast, there was very little association of liposomes with these cells upon incubation of the infected cells with control liposomes alone, Protein A-coupled liposomes alone, or control liposomes after treatment of the cells with

CD4-IgG. These results demonstrate that the association of Protein A-liposomes with infected cells was mediated by CD4-IgG.

4. Discussion

We have utilized two covalent coupling methods to couple sCD4, and one non-covalent attachment technique to bind CD4-IgG, to liposomes, as a means to target liposomes to HIV-infected cells expressing the viral envelope glycoprotein gp120/gp41 on their surface. MPBH and SATA are heterobifunctional cross-linking agents that generate thio-ether bonds which cannot be cleaved readily, thereby conferring stability to the conjugate in physiological milieu. These two reagents were compared in their efficiency to couple sCD4 to liposomes and in the ability of the resulting conjugate to bind to infected cells.

Because the endogenous thiols on sCD4 are involved in disulfide bonds that stabilize its domain structure, we have used SATA to add exogenous thiols to sCD4, to avoid the possible loss of gp120-binding function resulting from disruption of disulfide bridges. SATA reacts with primary amines, and at least one lysine on the V1 domain of CD4 is thought to be involved in binding to gp120 [59]. Thus, derivatization of this primary amine may reduce sCD4 binding to gp120, which is consistent with our binding results. A similar finding was reported by Chamow et al. [37] in attempts to crosslink sCD4 via amino groups. Because primary amines are located throughout the sCD4 molecule, the orientation of sCD4 on the liposome surface is predicted to be heterogeneous, and some orientations may sterically hinder sCD4 binding to gp120. In contrast, MPBH is directed to carbohydrate residues in the V3 domain which are far removed from the gp120 binding site. Coupling of MPBH-derivatized sCD4 to liposomes is thus less likely to affect the V1 domain adversely. Moreover, because there are only 2 oligosaccharide chains in sCD4 and both are localized to the V3 domain, MPBH chemistry is less likely to limit the number of orientations of sCD4 on the liposome surface. The gp120-binding affinity of sCD4, in turn, would be compromised less by MPBH derivatization than by SATA derivatization. The higher inhibition of HIV-1 infectivity by sCD4-MPBH-liposomes than sCD4-SATA-liposomes (Table 2) supports this hypothesis. Our results on HIV-1 infectivity also suggest that sCD4 inhibits infectivity somewhat less effectively when conjugated to liposomes, than when free in solution. In these experiments, control liposomes at a relatively high concentration also had some inhibitory effect, perhaps because they bind non-specifically to and fuse with some virions, as observed previously for other liposome compositions [53].

For the MPBH chemistry, coupling of sCD4 to liposomes proceeded via a specific coupling reaction. sCD4 that was not previously oxidixed for cross-linking to MPBH was not coupled to liposomes, demonstrating that MPBH coupled to sCD4 only at sites of oxidation, and that non-specific adsorption of sCD4 to liposomes did not occur. We did not perform a similar control to determine directly that SATA chemistry was specific. However, given that parameters similar to those used to effect the coupling of CD4-MPBH to liposomes were also used for SATA-derivatized sCD4 (including the mol% of reactive lipid and the sCD4:liposome ratio and concentration), the observation that the number of sCD4 molecules coupled per liposome was similar using either cross-linker suggests that the SATA-mediated cross-linking was also specific.

CD4-liposomes specifically recognized HIV-infected cells, as demonstrated by (i) the inability of the liposomes to bind significantly to uninfected control cells, and (ii) the inhibition by free sCD4 of sCD4-liposome binding to infected cells. Two observations support our hypothesis that the binding of sCD4-liposomes to infected cells was mediated by CD4: (i) control liposomes do not associate significantly, or associate only at a low level, with infected cells (Figs. 1–5), and (ii) liposomes coupled to a protein (i.e., Protein A) that does not bind directly to cell surface gp120 do not bind to infected cells to any significant degree (Fig. 6). Our results so far do not enable us to differentiate between liposomes bound to the cell surface and those internalized by endocytosis, or even possibly fused with the plasma membrane, since our experiments were performed at 37°C and not restricted to incubation at 4°C. Whether the internal aqueous contents of liposomes are delivered to the cytoplasm of the target cells, or whether the membranes of the liposomes and cells undergo fusion will be the subject of future investigations. In this respect, the observations of Cudd et al. [32] suggest that large liposomes with whole (transmembrane) CD4 reconstituted in their membranes deliver their contents into the cytoplasm of HIV-infected H9-HT cells, and not into uninfected cells. However, that study did not report the percentage of cells in culture with which these observations were made, nor the extent of contents delivery. In comparison with CD4-coupled liposomes, the use of transmembrane CD4 may present problems in the large-scale preparation of the membrane protein. Additional problems may arise in the encapsulation of low molecular weight antiviral agents during the detergent dialysis procedure. An alternative approach may be the insertion into preformed liposomes of glycosylphosphatidylinositol-anchored CD4 molecules [60], analogous to the use of similarly anchored gp120 to target liposomes to CD4-expressing cells [61]. Plasma membrane vesicles from CD4-expressing HeLa cells have been shown to fuse with cells

expressing gp120/gp41, and to inhibit viral infectivity and syncytia formation [62]. Although a useful experimental system to study gp120/gp41-mediated membrane fusion with CD4-containing membranes, such vesicles are unlikely to be used for therapeutic purposes because of the involvement of cancer cells for their preparation. While adaptation of such a procedure to cells derived from individual patients might be envisioned, this would appear to be a highly labor intensive and a pharmaceutically rather unfeasible preparation.

Several studies have demonstrated the usefulness of targeting toxins to HIV-infected cells via sCD4 [19–26]. CD4-targeted liposomes offer a potential vehicle for delivering a variety of antiviral agents to HIV-infected cells. The use of antiviral drugs rather than highly potent toxins may prove to be less toxic to the host. Liposomes provide a means to deliver a large number of such molecules to infected cells, provided the contents are delivered efficiently into the cytoplasm. Liposomes may also function as a carrier matrix in which highly hydrophobic HIV-protease inhibitors can be targeted to infected cells. This may be a particularly effective means of delivering inhibitors of HIV protease, since this enzyme appears to act at the plasma membrane during the last stages of viral assembly or subsequent to virion formation [63]. We have indeed observed that the HIV protease inhibitor L-689,502 can be encapsulated in liposomes, and that this liposome formulation effectively inhibits virus production in macrophages (N. Düzgüneş, E. Pretzer and D. Flasher, unpublished data).

Clinical trials have indicated that sCD4 and CD4-IgG are not toxic in patients with AIDS or AIDS-related complex [16–18,64]. Moreover, CD4-toxin conjugates do not inhibit normal T- and B-cell functions, and do not interfere with MHC class II-dependent functions [23–25]. Although these considerations are likely to apply to the CD4-liposome conjugates as well, the possibility that multivalent interaction of liposomal CD4 with MHC class II molecules may affect the function of the latter should be investigated.

Recent evidence that sCD4 itself is less effective in inhibiting patient isolates of HIV-1 [15] may not apply to the CD4-mediated targeting of toxins to infected cells, since sCD4-toxin conjugates are equally, if not more cytotoxic against cells infected with primary HIV isolates as against cells infected with laboratory adapted strains [65]. The results of Kennedy et al. [65], as well as observations that sCD4 binds to gp120 from primary isolates and laboratory strains with equal affinity [66–68], suggest that CD4 may have greater potential as a targeting agent than as a soluble inhibitor. As a targeting agent, sCD4 incorporated into liposomes may be useful in targeting antiviral agents to actively infected cells in vivo. To this end, the use of liposome compositions that confer properties of prolonged circulation and extravasation into tissues including lymph nodes [69,70], may further enhance the usefulness of sCD4-coupled liposomes, since it appears that infected cells in the lymph nodes continuously produce virions during the latent stages of disease [71,72].

Acknowledgements

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