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Transferrin lipoplex-mediated suicide gene therapy of oral squamous cell carcinoma in an immunocompetent murine model and mechanisms involved in the antitumoral response

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Anionic liposomes inhibit human immunodeficiency virus type 1 (HIV-1) infectivity in CD4$^+$ A3.01 and H9 cells

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Summary

Immunodeficiency viruses undergo fusion with liposomes containing anionic phospholipids (Larsen *et al.*, 1990). We have investigated the effect of liposomes composed of cardiolipin, phosphatidylserine or phosphatidylinositol, on the infectivity of three strains of HIV-1 in A3.01 and H9 cells, measured by p24 (*gag*) production in the medium. The infectivity of HIV-1 in A3.01 or H9 cells was inhibited by the presence of cardiolipin liposomes during a 2h infection period, with IC_{50}'s of 23.0, 4.8, and 5.0 μM phospholipid, respectively, for the different strains. Liposomes composed of phosphatidylserine or phosphatidylinositol were ineffective under similar conditions. However, prolonged pre-incubation of the virus with these liposomes also inhibited infectivity. Inhibition of virus binding to cells could not account for the inhibition of infectivity. We propose that the fusion products of HIV-1 and anionic liposomes are impaired in their ability to fuse with the plasma membrane.

Introduction

Human immunodeficiency virus type 1 (HIV-1) primarily infects human T-lymphocytes, monocytes and macrophages, and other cell types that express the CD4 antigen on the cell surface (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Gartner *et al.*, 1985; Ho *et al.*, 1986). The CD4 protein also serves as the receptor for simian immunodeficiency virus (SIV_{mac}) (Kannagi *et al.*, 1985). However, cells that do not express detectable amounts of CD4 can also be infected with HIV-1 (Clapham *et al.*, 1989; Harouse *et al.*, 1989; Tateno *et al.*, 1989). Furthermore, expression of the human CD4 molecule on the cell surface via transfection may not be sufficient for HIV or SIV infection (Maddon *et al.*,1986; Clapham *et al.*, 1991).

We have reported previously that HIV-1 and SIV_{mac} can fuse with phospholipid membranes (liposomes) lacking the CD4 molecule (Konopka *et al.*, 1990; Larsen *et al.*, 1990, 1991). HIV-1 fuses both with negatively charged cardiolipin (CL, 1,3-bisphosphatidyl-*sn*-glycerol) liposomes and with positively charged liposomes composed of N-[2,3-(dioleyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride (DOTMA), but only very slowly with neutral liposomes of dioleoylphosphatidylcholine (DOPC) (Konopka *et al.*, 1990, 1991). The fusion of HIV-1 with CL liposomes is stimulated by reduced pH and the presence of calcium (Larsen *et al.*, 1990, 1991). Interestingly, the fusion of cationic or anionic liposomess with HIV-1 affects differentially the infectivity of the virus. Pre-incubation of HIV-1 with DOTMA liposomes or the addition of such liposomes during exposure of cells to the virus, enhances the infectivity of the virus (Konopka *et al.*, 1990, 1991). The enhancing effect of DOTMA liposomes on HIV-1 infectivity is CD4-dependent, and appears to involve both virus-liposome fusion and liposome binding to the cell surface (Konopka *et al.*, 1991). In contrast, preliminary results indicated that pre-incubation of HIV-1 with CL liposomes causes dose-dependent inhibition of HIV-1 replication in CD4$^+$ A3.01 cells, as measured by p24 (*gag*) antigen production (Konopka *et al.*, 1990, 1992). The mechanism(s) of the inhibitory effect of CL liposomes on HIV-1 infectivity is unclear. It is possible that both the ability to fuse with the virus as well as the negative charge of cardiolipin liposomes may contribute to the observed inhibition. It is known that various negatively charged but non-fusogenic compounds exert antiviral activity *in vitro* against a variety of viruses, including HIV-1 (Takemoto and Spicer, 1965; De Somer *et al.*, 1968; Baba *et al.*, 1988). Here, we examined whether the presence of CL liposomes during infection affected the infectivity of HIV-1 in two CD4$^+$ cell lines, and investigated the susceptibility of three different isolates to this treatment. We focused on whether CL liposomes affect the cell or the virus membrane. Since pretreatment of A3.01 cells with cationic DOTMA liposomes enhances HIV-1 infectivity (Konopka *et al.*, 1991),

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Table 1. Effect of medium composition on the fusion rate of HIV-1 with CL liposomes.

Reaction mixture	Serum content	Initial rate[a]
NaCl/TES	None	25
NaCl/TES (0.5 mM Ca^{2+})	None	73
RPMI 1640[b]	None	41
RPMI 1640	10% FBS	1.3

[a] Initial fusion rates were calculated from the slopes of fluorescence dequenching per unit time. Values are given as % F_{max} min^{-1}. Initial rates of less than 5 were averaged over the first min (the rates of fusion in these cases did not change appreciably with time). The others were averaged over the first 0.2 min. Reaction medium conditions were set prior to injection of CL liposomes (18.8 μM). Virus concentration was 0.75 μg ml^{-1}.
[b] The medium contained no phenol red.

we investigated if the interaction of CL liposomes with host cells affect HIV-1 infectivity. We determined whether the effect of CL liposomes could be explained by its effect on virus binding to cells. We also examined whether two other negatively charged but less fusogenic liposomes, phosphatidylserine (PS) and phosphatidylinositol (PI) (Larsen *et al.*, 1991), could inhibit the infectivity of HIV-1.

Results

HIV-1 fuses with cardiolipin liposomes

HIV-1 fuses rapidly with CL liposomes, as detected by the dequenching of R_{18} due to dilution of the probe within the fusion product. Fusion was also observed with both freeze-fracture and negative-stain electron microscopy (Larsen *et al.*, 1991). Fusion of HIV-1 with CL liposomes was dependent on the composition of the medium (Table 1). At pH 7.5, fusion was faster in the presence of RPMI than in NaCl/TES buffer alone. This may be due to the fact that RPMI contains 0.45 mM Ca^{2+} (Larsen *et al.*, 1991), but the other constituents of the medium may also play a role. Addition of 10% FBS significantly reduced, but did not completely eliminate HIV-1 fusion with CL liposomes. Thus, it is expected that the extent of fusion would reach significant levels following a 2 h or 24 h pre-incubation of the virus with CL liposomes in the presence of 10% FBS, as described below.

HIV-1 infectivity is inhibited by cardiolipin liposomes present during the initial infection of A3.01 or H9 cells

When A3.01 cells were infected in the presence of CL liposomes with HIV-1_{LAV}, at 10 ng of viral p24 per 10^6 cells, the production of p24 antigen in the culture supernatant was inhibited with an IC_{50} of ~23 μM (Fig. 1A). Over 90% inhibition was observed at a lipid concentration of 100 μM (p24 = 0.64 ± 0.003 and 88.1 ± 15.1 ng ml^{-1} compared to 9.9 ± 1.5 and 6390 ± 206.6 ng ml^{-1} for untreated controls, after 7 and 10 days p.i., respectively). Similar results were obtained with HIV-1_{LAV}, at 4 ng of viral p24 per 10^6 cells (IC_{50} of ~20 μM and ~14 μM, with p24 determinations after 7 and 10 days p.i., respectively; data not shown). CL liposomes present during exposure of A3.01 cells to HIV-$1_{LAV\text{-}BRU/P}$ reduced the infectivity of the virus to a greater extent (IC_{50} ~4.8 μM, with p24 determination after 7 days p.i.) than that of HIV-1_{LAV} (Fig. 1B). Complete inhibition of p24 production was achieved at concentrations of 25 to 100 μM. CL liposomes, at concentrations of 1–100 μM and under such treatment conditions (2×10^7 cells ml^{-1}; RPMI/10% FBS; 2 h at 37°C), were not cytotoxic, as determined by trypan blue exclusion (data not shown). The infectivity of purified HIV-$1_{LAV\text{-}BRU}$, used for the fusion experiments, was inhibited by CL liposomes to a similar extent as that observed with propagated virus (data not shown), although the overall production of p24 in A3.01 cells infected with the purified virus, at 4 ng of viral

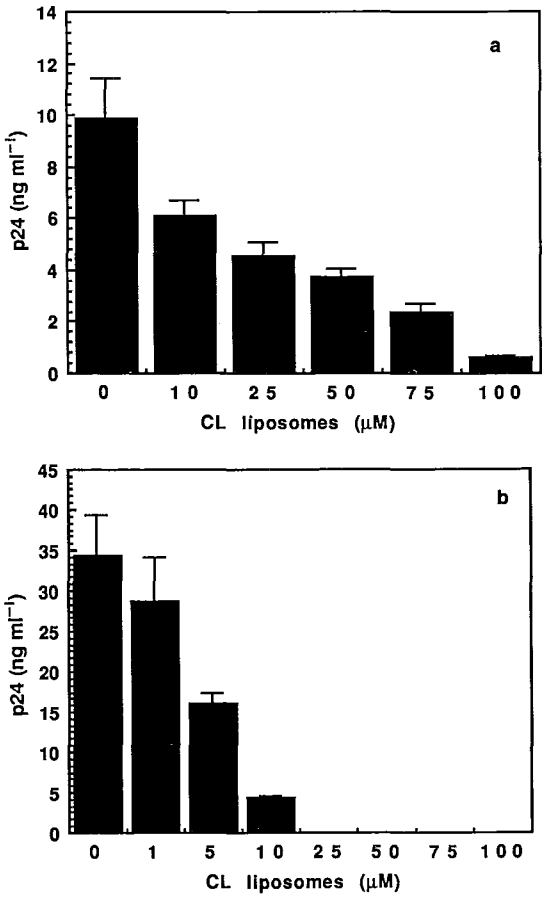

Fig. 1. Effect of CL liposomes on HIV-1 replication in A3.01 cells. CL liposomes were added during exposure of A3.01 cells to HIV-1_{LAV} at 10 ng of viral p24 per 10^6 cells (at an MOI of 0.0016) (a) or $HIV_{LAV\text{-}BRU/P}$ at 4 ng of viral p24 per 10^6 cells (at an MOI of 0.018) (b) as described in the text. Virus production was monitored by measuring HIV p24 in the culture supernatant on 7 day post-infection as described in Materials and Methods. Data represent the mean ± standard deviation of four replicate p24 determinations on a single well or duplicate wells (control infections).

Table 2. Effect of CL liposomes present during infection on HIV-$1_{HTLV-IIIB}$ replication in A3.01 and H9 cells.

	A3.01 cells				H9 cells			
	7 days		10 days		7 days		10 days	
Treatment[a]	p24 (ng ml^{-1})[b]	%[c]	p24 (ng ml^{-1})	%	p24 (ng ml^{-1})	%	p24 (ng ml^{-1})	%
No liposomes (control)	0.26 ± 0.05	100	1.25 ± 0.09	100	0.23 ± 0.10	100	0.67 ± 0.20	100
1 μM CL	0.16 ± 0.07	61.5	0.69 ± 0.04	55.2	0.11 ± 0.02	47.8	0.18 ± 0.01	26.9
5 μM CL	0.12 ± 0.06	46.1	0.53 ± 0.09	42.4	0.12 ± 0.01	52.2	0.14 ± 0.01	21.0
10 μM CL	0.04 ± 0.04	15.4	0.27 ± 0.05	21.6	0.05 ± 0.04	21.7	0.05 ± 0.02	7.5
25 μM CL	0	0	0	0	0.04 ± 0.02	17.4	0.04 ± 0.01	6.0
50–100 μM CL	0	0	0	0	0	0	0	0

[a] CL liposomes were added during exposure of A3.01 or H9 cells to virus at 4 or 6 ng of viral p24 per 10^6 cells, respectively.
[b] p24 Antigen was determined in cell culture supernatant on 7 and 10 days post-infection. Data represent mean values ± S.D. (n = 4).
[c] Calculated as % of control infection (without liposomes).

p24/10^6 cells, was approximately 10 times lower than those infected with propagated HIV-$1_{LAV-BRU/P}$ (3.8 ± 0.1 vs. 34.4 ± 5.0 ng p24 ml^{-1}, respectively, on 7 day p.i.). Similar reduction of the production of p24 (IC_{50} ~5 μM) was also observed when CL liposomes were present during infection of A3.01 and H9 cells with HIV-$1_{HTLV-IIIB}$ (Table 2). The IC_{50} values were similar regardless of when p24 production was measured (7, 10, 14, or 17 days p.i.) (Table 2 and data not shown). The effect of CL liposomes on HIV-1_{LAV} infectivity of H9 cells was not tested.

The differential sensitivities of the three isolates of HIV-1 to the inhibitory effect of CL liposomes were not due to their different infectivities measured as $TCID_{50}$. For example HIV-$1_{LAV-BRU/P}$ and HIV-$1_{HTLV-IIIB}$ differed by about 100-fold in $TCID_{50}$ per 100 ng of viral p24 (4.6×10^5 and 4.8×10^3, respectively). However, CL liposomes inhibited the infectivity of the two strains to a similar extent (Fig. 1B and Table 2).

Presence of phosphatidylserine or phosphatidylinositol liposomes during the initial infection of A3.01 cells does not inhibit HIV-1 infectivity

To determine whether the inhibitory effect of CL liposomes on HIV-1 infectivity was due to the negative charge of the lipid, we tested the antiviral activity of two other negatively charged, but less fusogenic, liposomes. The rate of fusion of HIV-1 with negatively charged liposomes at neutral pH depends on the lipid composition, and decreases in the sequence pure CL > phosphatidylinositol (PI) > phosphatidylserine (PS) (Larsen *et al.*, 1991; and unpublished results). When compared to pure CL liposomes the initial rate of HIV-1 fusion with PI or PS liposomes was decreased by about 9- and 19-fold, respectively, in NaCl/TES buffer, pH 7.5, in the absence of Ca^{2+}, and by about 10-fold in the presence of 0.5 mM Ca^{2+} (Larsen *et al.*, 1991). Addition of serum to the NaCl/TES buffer reduced HIV-1 fusion with CL liposomes by 97% (Table 1). Thus, it is expected that the interaction of PI or PS liposomes with HIV-1 in the presence of 10% FBS would be very slow. In contrast to CL lipsomes, the addition of PI or PS liposomes during infection, at concentrations between 10 and 100 μM, did not affect p24 production in A3.01 cells (Fig. 2). The presence of PI or PS liposomes, at concentrations of 5 and 50 μM, also had no effect on HIV-1 infectivity in A3.01 cells when the serum concentration was reduced to 0.2% during infection (data not shown). To compare adequately the fusion of HIV-1 with different liposomes, and the ability of these liposomes to inhibit infectivity, we also examined the effect of liposomes on the infectivity of the purified virus preparation used in the fusion studies.

Pre-incubation of HIV-1 with cardiolipin, phosphatidylserine or phosphatidylinositol liposomes reduces virus infectivity

Since HIV-1 could fuse with PS or PI liposomes and since neither liposome type inhibited HIV-1 infectivity when present during infection, we investigated whether pre-incubation of the virus with these liposomes affected infectivity. HIV-$1_{LAV-BRU/P}$ was pre-incubated with CL, PS, or PI liposomes, for either 2 h or 24 h at 37°C and then incubated with A3.01 cells for 2 h. The amount of HIV-1 p24 antigen released into culture supernatants (Table 3) indicates that pre-incubation of HIV-1 with CL, PS, or PI liposomes resulted in a dose- and time-dependent inhibition of virus replication. The inhibition of p24 production in the supernatant of HIV-1-infected cells correlated with the fusogenic activity of negatively charged liposomes in NaCl/TES buffer alone. Both PS and PI liposomes had some inhibitory effect at 5 μM. At a concentration of 50 μM, PS liposomes, which fuse significantly slower with HIV-1 than CL or PI liposomes (Larsen *et al.*, 1991), inhibited p24 production 3 days p.i. by 22% and 70%, after 2 h and 24 h pre-incubation, respectively. Pre-incubation of HIV-1 with 50 μM PI liposomes for 2 h or 24 h reduced the infectivity of

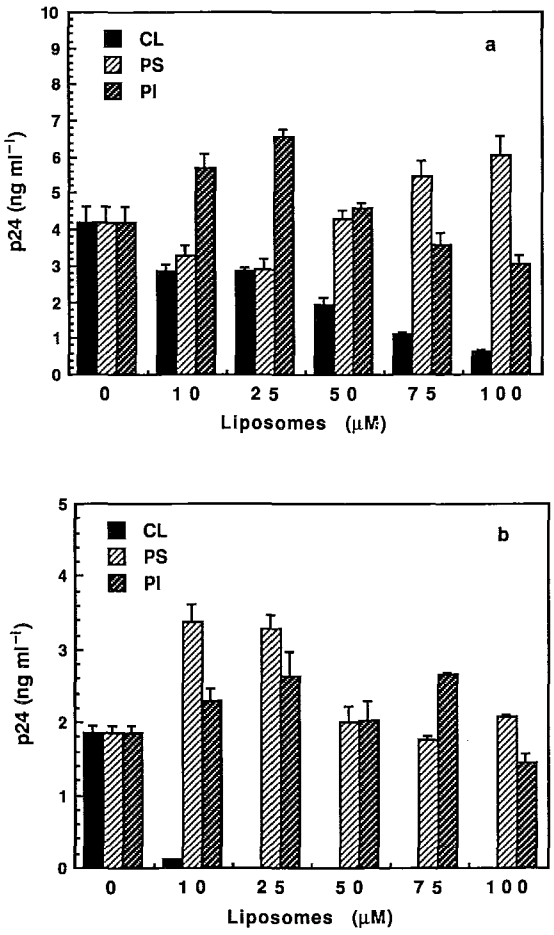

Fig. 2. Effect of CL, PS, and PI liposomes on HIV-1_{LAV} (a) and purified HIV-$1_{LAV\text{-}BRU}$ (b) replication in A3.01 cells. CL, PS, and PI liposomes were added during exposure of A3.01 cells to virus (4 ng of viral p24 per 10^6 cells) as described in the text. Virus production was monitored by measuring HIV p24 in the culture supernatant on 7 day post-infection as described in 'Materials and Experimental procedures'. Data represent the mean ± standard deviation of four replicate p24 determinations on a single well or duplicate wells (controls infections).

the virus by 46% and 100%, respectively. Under the same conditions, 5 and 50 μM CL liposomes inhibited viral replication by 99% and 100%, respectively, after a 2 h pre-incubation, and by 100% after a 24 h pre-incubation. Similar results wre obtained after 7 days post-infection (Table 3).

Pre-incubation of A3.01 cells with cardiolipoin liposomes does not affect HIV-1 infectivity

To determine whether the inhibitory effect of CL liposomes was due to binding to sites on the cell surface, A3.01 cells were pre-incubated with various concentrations of CL liposomes (10 to 100 μM lipid), both in the presence of 10% FBS and without serum. The cells were then washed and exposed to HIV-1_{LAV} at 4 ng viral p24/10^6 cells, as described in Methods, and cultured for 7 days. Under such treatment conditions CL liposomes showed no cytotoxicity to the cells in the presence of serum (data not shown). However, in the absence of serum cytotoxicity of CL liposomes was observed with a CD_{50} of ~25 μM. Pre-treatment of A3.01 cells with CL liposomes in the presence of 10% FBS did not affect p24 production when compared to untreated cells. For example, when A3.01 cells were pre-treated with 50 or 100 μM CL liposomes, the values of p24 determined in the supernatant after 7 days were 2.75 ± 0.21 and 2.35 ± 0.28 ng ml^{-1}, respectively, while for cells treated with RPMI medium alone (control) this value was 2.78 ± 0.59 ng ml^{-1} (the mean ± standard deviation, n = 4).

Inhibition of infectivity by cardiolipin liposomes is not due only to decreased virus binding to cells

We investigated whether decreased infectivity in the presence of CL liposomes was due to inhibition of virus

Table 3. Effect of preincubation of HIV-$1_{LAV\text{-}BRU/P}$ with CL, PI, and PS liposomes on HIV-1 infection of A3.01 cells.

	3 days p.i.				7 days p.i.			
	2 h pre-incubation		24 h pre-incubation		2 h pre-incubation		24 h pre-incubation	
Treatment[a]	p24 (ng ml^{-1})[b]	%[c]	p24 (ng ml^{-1})	%	p24 (ng ml^{-1})	%[c]	p24 (ng ml^{-1})	%
No liposomes (control)	5.2 ± 0.7	100	0.37 ± 0.13	100	260.1 ± 64.1	100	9.1 ± 0.4	100
5 μM CL	0.06 ± 0.0004	1.2	0	0	6.0 ± 0.4	9.9	0	0
50 μM CL	0	0	0	0	0	0	0	0
5 μM PI	4.5 ± 0.4	86.5	0.31 ± 0.02	83.8	218.8 ± 15.5	84.1	13.1 ± 1.3	143
50 μM PI	2.8 ± 0.1	53.8	0	0	120.4 ± 19.1	46.3	0.66 ± 0.05	7.2
5 μM PS	4.3 ± 0.2	82.7	0.37 ± 0.02	100	209.0 ± 19.0	80.4	12.7 ± 0.8	139
50 μM PS	4.0 ± 0.3	76.9	0.11 ± 0.01	29.7	92.0 ± 8.7	35.4	4.9 ± 0.3	53.7

[a] HIV-$1_{LAV\ BRU/P}$ was pre-incubated without or with CL, PI, or PS liposomes for 2 h or 24 h at 37°C. A3.01 cells were infected with pre-incubated HIV-$1_{LAV\text{-}BRU/P}$ (4 ng of viral p24 per 10^6 cells).

[b] p24 Antigen was determined in cell culture supernatant 3 and 7 days post-infection. Data represent the mean ± standard deviation of four replicate p24 determinations on a single well or duplicate wells (control infections).

[c] Calculated as % of control infection (pre-incubation of virus without liposomes).

binding to cells. Binding was measured at 0°C since HIV-1 is not expected to fuse at this temperature (Sinangil *et al.*, 1988; Orloff *et al.*, 1991). A3.01 cells were exposed to the virus at 0°C, in the absence or presence of CL liposomes. The liposomes and free virus were then removed by washing the cells. The presence of CL liposomes, at concentrations of 5–50 μM, reduced binding of HIV-1 by 29% and 32%, respectively (Fig. 3a). This bound virus was allowed to infect the cells by raising the temperature to 37°C and virus production in the supernatant was determined 3 days p.i. (Fig. 3B). Similar results were obtained after 5 days p.i. (data not shown). At 50 μM CL the amount of p24 was reduced to 3% of that produced by control cells treated identically except for the absence of liposomes. In a separate experiment 50 μM CL reduced binding by 56% while inhibiting infectivity by 88% (12% of p24 production in controls). Thus, infectivity was inhibited to a greater extent than binding.

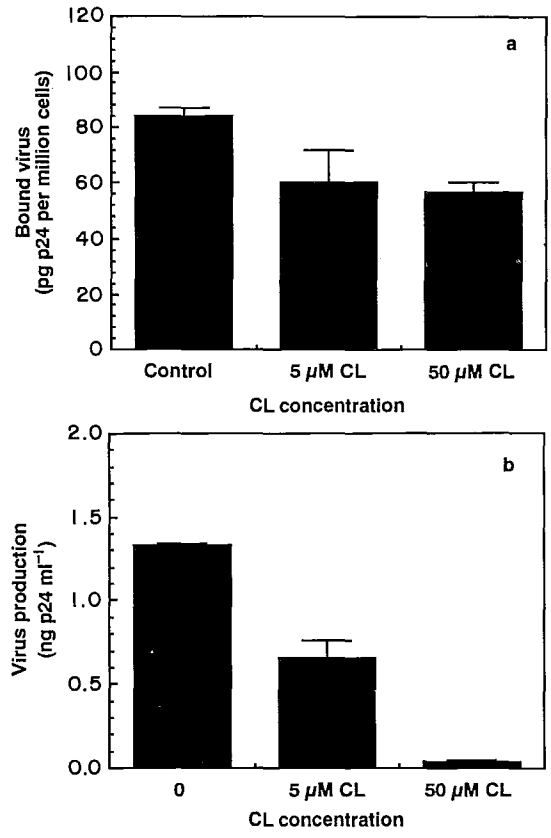

Fig. 3. Effect of CL liposomes on HIV-1 binding and infectivity. CL liposomes were added during exosure of A3.01 cells to HIV-$1_{LAV\text{-}BRU/P}$ at 100 ng of viral p24 per 10^6 cells (at an MOI of 0.46) as described in the text. Virus binding was monitored by measuring HIV p24 in the cell lysates (a) and virus replication was measured by p24 production in cell culture supernatants on 3 day post-infection (b) as described in 'Materials and Experimental procedures'. Data represent the mean ± standard deviation of two replicate p24 determinations in duplicate (n = 4).

Discussion

Our results indicate that CL liposomes are inhibitory to HIV-1 infection of two $CD4^+$ T-cell lines, A3.01 and H9. Similar results were obtained with a human monocytic leukaemia cell line, THP-1 (unpublished observations). The inhibitory effect of CL liposomes on HIV-1 infectivity does not appear to be merely due to the negative charge of the lipid, since two other negatively charged liposomes composed of PI or PS affected HIV-1 infectivity only after prolonged pre-incubation with the virus. Although the initial rate of fusion between HIV-1 and CL liposomes was reduced after addition of serum to RPMI medium (Table 1), virus and liposomes can fuse with each other to a significant extent during a 2 h incubation. One reason for the lack of anti-HIV-1 activity of PI and PS liposomes present during the infection period may be their lower fusion activity compared to CL liposomes. Although all these lipids carry 1 net negative charge per 2 acyl chains, the molecular arrangements and nature of the charged groups are different. PS has carboxy, phosphate and amino groups connected to 2 acyl chains, while CL has two phosphate groups connected to 4 acyl chains via a glycerol backbone, and PI contains a phosphate moiety shielded by the inositol residue.

Earlier reports have indicated that various negatively charged compounds exert antiviral activity against a variety of viruses *in vitro* (Takemoto and Spicer, 1965; De Somer *et al.*, 1968; Baba *et al.*, 1988) but the exact mechanism(s) of their inhibitory effect remains obscure. Sulphonated compounds such as suramin (bis-naphthalenetrisulphonic acid), suramin analogues, and Evans blue inhibit HIV replication (Balzarini *et al.*, 1986a, 1986b; Mohan *et al.*, 1991). Aurin tricarboxylic acid is thought to inhibit HIV adsorption to host cells by binding to lysine and/or arginine residues on CD4 involved in gp120 binding (Schols *et al.*, 1989). Sulphated polysaccharides (dextran sulphate, pentosan polysulphate, heparin) and modified, negatively charged, human serum albumins appear to inhibit HIV infection by interfering with virus attachment and fusion (Baba *et al.*, 1988; Mitsuya *et al.*, 1988; Jansen *et al.*, 1991). Recently, it was proposed that dextran sulphate interacts with positively charged amino acids concentrated in the V3 region of gp120 (Callahan *et al.*, 1991). It is possible that interactions with this region are also responsible for the observed inhibitory effect of CL liposomes. Negatively charged molecules can also affect cell surface CD4. Gangliosides, negatively charged glycolipids containing sialic acids, are known to down-modulate surface CD4 (Offner *et al.*, 1987) and the CD4/Thy-1 fusion protein, which is anchored to the membrane by a glycosylphosphatidylinositol tail (Jasin *et al.*, 1991). Sulphation of gangliosides enhances their inhibitory effect

compared to unsulphated analogues even in the presence of 10% FBS (Handa *et al.*, 1991). Liposomes containing sulphated galactocerebroside (sulphatide) or cholesterol sulphate also inhibit syncytium formation induced by bovine immunodeficiency virus (BIV) infected cells (Watari *et al.*, 1990). The inhibitory effect of sulphatide alone, not incorporated into liposomes, is low and the fusion of liposomes containing sulphated lipids with the membranes of BIV-infected and/or -uninfected cells has been suggested as an important step in the antiviral activity (Watari *et al.*, 1990).

CL liposomes inhibited the original step of infection, HIV-1 binding to the cell surface, to a certain extent. However, this level of inhibition (43%) could not account for the extent of inhibition of the subsequent infection (97%). The ability of the fusion product to fuse with fresh CL liposomes is indeed diminished, as demonstrated by R_{18} fluorescence dequenching experiments (C. E. Larsen *et al.*, personal communication). Future studies will examine whether CL liposomes inhibit the initial fusion step, by assaying virus-cell membrane lipid mixing and the intracellular delivery of p24, and monitoring the reverse transcription step by means of PCR analysis.

Our studies indicate that the antiviral effects of CL liposomes vary quantitatively among different strains of HIV-1 propagated in different cell lines. These findings were not unexpected, since strain specific antiviral effects of dextran sulphate (Busso and Resnick, 1990; Schols *et al.*, 1992) and rsCD4 (Daar *et al.*, 1990) have also been reported. Passage of HIV-1 isolates through various human cell lines can alter the cellular host range tropism (Cheng-Mayer *et al.*, 1991; Peden *et al.*, 1991), because of differences in the cell-type-dependent glycosylation of the same viral envelope protein (Cheng-Mayer *et al.*, 1991). It has been also suggested that the genotypes of some viruses *in vitro* are not stable and that selection for growth causes the appearance of variants with increased growth potential and expanded host range in the absence of immune selection of the host (Peden *et al.*, 1991). Therefore, it will be of interest to examine the effect of anionic liposomes on the infectivity of well characterized isolates that differ extensively with respect to sequence and biological properties.

Our observations with CL liposomes raise the possibility of using such liposomes for therapeutic purposes. The concentrations of liposomes necessary for complete inhibition of infectivity are up to 100-fold lower than those injectable intravenously into experimental animals. Moreover, liposomes with long circulation times have been developed recently (Gabizon and Papahadjopoulos, 1988). It will be of interest to examine whether similar liposomes containing relatively low mole fractions of CL will inhibit infectivity and retain their ability to circulate for long periods in serum. HIV infectivity may not only be inhibited by liposomes themselves but also by encapsulated antiviral drugs which can enter the virus interior via fusion. Additionally, the covalent coupling of rsCD4 to the liposomes may render the liposomes highly specific for binding to and fusion with HIV-1 or HIV-infected cells (Düzgüneş *et al.*, 1992). These studies are in progress in our laboratory.

Materials and Experimental procedures

Compounds

Phospholipids (CL, PI, PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Octadecylrhodamine (R_{18}) was purchased from Molecular Probes, Inc. (Eugene, OR). Octaethyleneglycol dodecyl ether ($C_{12}E_8$) detergent was from Calbiochem, Inc. (La Jolla, CA).

Cell lines

The $CD4^+$ human lymphoblastoid cell line, A3.01, was obtained from T. Folks, and the H9 cell line was obtained from R. C. Gallo, through the AIDS Research and Reference Reagent Programme (Division of AIDS, NIAID, NIH, Bethesda, MD). H9/HTLV-IIIB cells were kindly provided by J. Mills (San Francisco General Hospital). The cells were maintained in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Irvine Scientific), penicillin (50 units ml^{-1}), streptomycin (50 $\mu g\ ml^{-1}$), and L-glutamine (2 mM) at 37°C. The cells were split 1:6 and fresh medium was added every 3–4 days.

Fusion experiments

To determine the fusion of HIV-1 with liposomes, the virus (LAV-BRU, now designated as LAV-LAI [Wain-Hobson *et al.*, 1991] [Pasteur Institute, Paris, France]) was propagated in human lymphoblastoid leukaemia (CEM) cells, purified as described (Larsen *et al.*, 1990) and stored at −70°C. This preparation was provided by M. Jennings (Department of Medical Pathology, UC Davis). Purified HIV-$1_{LAV\text{-}BRU}$ contained all viral antigens, including gp41 and gp120 (detected by Western blot), and was infectious as determined by its ability to induce syncytia formation and cell-free p24 antigen production in A3.01 cells. HIV-$1_{LAV\text{-}BRU}$ (100 μg) was labelled with octadecylrhodamine (R_{18}) at 3 mole% of the total viral lipid, by incubating the virus with an ethanolic solution of the fluorophore (final [ethanol] <1%) for 1 h at room temperature (Hoekstra *et al.*, 1984; Larsen *et al.*, 1990). It was necessary to use purified virus for these experiments to avoid labelling of other membranous material or serum components in cell culture supernatant. Free fluorophore was not present (an excess of added unlabelled virus did not result in redistribution of R_{18}; labelled virus chromatographed on Sephadex G-50 to eliminate any free R_{18} gave similar results). R_{18}-labelled HIV-1 remained infectious as determined by the methods above. The dilution of R_{18} into target membranes was measured by following the increase of rhodamine fluorescence, which results from the relief of self-quenching upon membrane fusion (Hoekstra *et al.*, 1984). Fluorescence measurements were performed with a Perkin-Elmer LS-5B fluorometer, as described earlier (Larsen *et*

al., 1990). Maximal fluorescence (F_{max} = 100%) was set by lysing the virus and liposome suspension with $C_{12}E_8$ (final concentration: 0.1–0.5% [w/v]), which completely disperses the fluorophore.

Virus stocks

The LAV strain of HIV-1, designated as HIV-1_{LAV}, was obtained from Dr T. M. Folks and propagated in A3.01 cells. The purified HIV-1 (labelled as HIV-$1_{LAV\text{-}BRU}$) was also propagated in A3.01 cells, and the propagated virus was designated as HIV-$1_{LAV\text{-}BRU/P}$. The supernatant of chronically infected H9/HTLV-IIIB cells was used as a source of the HIV-1 strain, HTLV-IIIB. The culture supernatants were harvested at times of peak p24 production, filtered through Millipore filters of 0.22 μm pore diameter, and stored in 1-ml aliquots at −80°C. The reverse transcriptase activity of the virus stock solutions was 8.8×10^5, 2.5×10^6, and 3.9×10^6 c.p.m. ml^{-1} and the ELISA-reactive p24 protein yielded 1.0, 2.6, and 0.3 μg ml^{-1} for HIV-1_{LAV}, HIV-$1_{LAV\text{-}BRU/P}$ and HIV-$1_{HTLV\text{-}IIIB}$, respectively.

The tissue culture infectious dose, 50% endpoint ($TCID_{50}$), was determined by inoculating replicate microtitre wells of A3.01 or H9 cells (2×10^4 cells $well^{-1}$) in 96-well flat-bottom plates (Corning) with serial dilutions of cell-free supernatants (Johnson and Byington, 1990). Each well was scored daily for evidence of virus-induced cytopathic effect (CPE) (the presence of syncytia, ballooning cytoplasm, etc.). The cells were split 1:2 twice weekly and fed with fresh medium, until peak infection was noted, usually after 9 or 10 days. The infectious titre per 100 ng p24 antigen was: HIV-1_{LAV}, 1.64×10^4 $TCID_{50}$; HIV-$1_{LAV\text{-}BRU/P}$, 4.6×10^5 $TCID_{50}$ (both on A3.01 and H9 cells); and HIV-$1_{HTLV\text{-}IIIB}$, 4.8×10^3 $TCID_{50}$, on H9 cells. Viral inocula were standardized by their p24 content, and cultures were infected at a concentration of p24 between 4 and 10 ng p24 per 10^6 cells, as described in the Figure and Table legends. Viral p24 antigen was determined in cell-free supernatant by the HIV-1 p24 antigen capture assay described previously (Konopka *et al.*, 1990).

Preparation of liposomes

Liposomes were prepared in 150 mM NaCl, 10 mM N-(*Tris*[hydroxymethyl]methyl)-2-aminoethane sulphonic acid (TES), pH 7.5, either by reverse phase evaporation (Szoka *et al.*, 1980) or by hydrating dried lipid (Bangham *et al.*, 1965), and extruded three times under argon, through double polycarbonate membranes of 0.08 μm pore diameter in a Lipex Biomembranes (Vancouver, BC) high pressure extrusion apparatus, to achieve a uniform size distribution (Szoka *et al.*, 1980). This procedure results in the production of predominantly unilamellar vesicles (Düzgüneş *et al.*, 1983; Hope *et al.*, 1985). The lipid concentration was determined by phosphate assay (Bartlett, 1959).

HIV infection of cells

Cells (2×10^7 ml^{-1}) were exposed to HIV-1 for 2 h at 37°C, washed three times to remove unbound virus, resuspended at 5×10^5 cells ml^{-1} in RPMI-1640/10% FBS and cultured at 37°C in 12-well culture plates (4 ml per well). Control cells were treated similarly but not exposed to the virus. Every 3 or 4 days, 3 ml of the supernatant was removed and fresh medium was added. Following incubation for various time periods (e.g. 3, 7, 10 days), the number of viable cells was determined by Trypan Blue exclusion, and p24 antigen in the harvested samples of cell-free supernatant was measured by ELISA (Konopka *et al.*, 1990).

The effect of liposomes on HIV-1 infectivity was investigated using three different protocols: (1) the cell-free virus was pre-treated with liposomes before addition to the cells, (2) liposomes were added to the culture medium during exposure of cells to virus, or (3) the cells were pre-treated with liposomes before addition of virus.

HIV-$1_{LAV\text{-}BRU/P}$ (0.4 μg viral p24 ml^{-1}) was pre-incubated with various concentrations of liposomes for 2 h or 24 h at 37°C and then diluted with a cell suspension to give 4 ng of viral p24 10^{-6} cells. Control virus was pre-incubated without liposomes.

A3.01 cells (2×10^7 cells ml^{-1}) were pre-incubated with various concentrations of CL liposomes for 1 h at 37°C, washed three times to remove unbound CL and then exposed to HIV-1_{LAV} at 4 ng of viral p24 10^{-6} cells. After virus adsorption for 2 h at 37°C, the cells were washed three times, resuspended at 5×10^5 cells ml^{-1} and cultured in 12-well culture plates. Following incubation, cell viability was assessed visually both with and without the use of Trypan Blue, and p24 antigen was measured in duplicate in the supernatant.

Quantitation of HIV-1 binding to A3.01 cells

HIV-$1_{LAV\text{-}BRU/P}$ was added to A3.01 cells at 200 ng of viral p24 per 2×10^6 cells in 0.2 ml, at a multiplicity of infection (MOI) of 0.46 infectious virus/target cell. The virus-cell mixture, in the absence or presence of CL liposomes, was maintained at 0°C for 60 min to allow binding without internalization. The cells were washed to remove unbound virus and divided into two portions, each containing 1×10^6 cells. The first portion of cells was lysed with 1% Triton X-100. The lysate, representing cell-bound and/or cell-internalized viral antigen, was centrifuged at 13000 × g for 10 min and p24 antigen was measured in the supernatant. The second portion was resuspended at 5×10^5 cells ml^{-1} and cultured in 24-well culture plates (2 ml $well^{-1}$) at 37°C, and on days 3 and 5 of culture, 1 ml of the supernatant was removed and fresh medium was added. The harvested samples of cell-free supernatant were tested for p24 antigen.

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