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Liposomes modulate human immunodeficiency virus infectivity

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We have investigated the effects of the fusion of liposomes with human immunodeficiency virus type 1 (HIV-1_{LVA}) on the ability of the virus to infect $CD4^+$ and $CD4^-$ cells. Fluorescence dequenching measurements indicated that HIV-1 fuses with liposomes composed of either cardiolipin (CL) or N-[2,3-(dioleyloxy) propyl]-*N,N,N*-trimethyl ammonium chloride (DOTMA) but not appreciably with dioleoylphosphatidylcholine (DOPC) liposomes. Pre-incubation of HIV-1 with DOTMA liposomes enhanced virus production (measured by p24 *gag* antigen production in the culture medium and *in situ*) in $CD4^+$ A3.01 and H9 cells in a concentration-dependent manner, but did not mediate the infection of the $CD4^-$ cell line, K562. Pre-incubation of HIV-1 with between 10 and 30 μM-DOTMA liposomes, and subsequent incubation with A3.01 cells, resulted in the production of about 30-fold greater levels of virus than controls. The presence of DOTMA liposomes during the incubation of A3.01 cells with HIV-1 enhanced the infectivity of the virus up to 90-fold compared to controls. Conversely, pre-incubation of HIV-1 with CL liposomes inhibited infection of A3.01 cells, dependent on the concentration of liposomes; DOPC liposomes did not alter the infectivity of the virus under any of the incubation conditions. Our results thus indicate that fusion of HIV-1 with liposomes alters the ability of the virus to infect its target cells.

Introduction

Human immunodeficiency virus type 1 (HIV-1) primarily infects $CD4^+$ T lymphocytes, monocytes and macrophages (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Gartner *et al.*, 1986; Ho *et al.*, 1986; McDougal *et al.*, 1986). Recent studies have shown, however, that cells that do not express CD4 can also be infected with HIV (Cheng-Mayer *et al.*, 1987; Clapham *et al.*, 1989; Harouse *et al.*, 1989; Tateno *et al.*, 1989). Furthermore, expression of the CD4 molecule on the cell surface may not be sufficient for HIV-1 infection (Maddon *et al.*, 1986; Chesebro *et al.*, 1990); fusion of HIV-1 with the target cell membrane appears necessary for productive infection. We have reported recently that HIV-1 and simian immunodeficiency virus (SIV_{mac}) can fuse with liposomes (phospholipid vesicles) that do not contain the CD4 molecule (Larsen *et al.*, 1989, 1990). SIV_{mac} fuses rapidly, at neutral pH, with negatively charged liposomes composed of cardiolipin (CL), CL and dioleoylphosphatidylcholine (DOPC) (3:7), or phosphatidylserine. We have also reported that HIV-1 fuses with CL liposomes. Here, we have investigated whether the products of the fusion of HIV-1 with CL liposomes alter the infectivity of the virus. We have also investigated the fusion of HIV-1 with liposomes composed of the positively charged synthetic lipid *N*-[2,3-(dioleyloxy) propyl]-*N,N,N*-trimethyl ammonium chloride (DOTMA) and the infectivity of the fusion product. We have found that DOTMA liposomes enhance the infectivity of the virus and CL liposomes inhibit it, whereas DOPC liposomes (which do not fuse appreciably with HIV-1) are ineffective.

Methods

Chemicals. DOPC and CL from bovine heart were obtained from Avanti Polar Lipids and stored in chloroform under argon in sealed ampoules at −70 °C. DOTMA, synthesized as described previously (Felgner & Ringold, 1989) and kindly provided by N. Dyson at Syntex Research, was stored in ethanol under argon at −70 °C. Octadecylrhodamine (R_{18}) was purchased from Molecular Probes and $C_{12}E_8$ detergent was from Calbiochem.

Cell lines. The cell lines A3.01, H9 and K562 were used for our studies. The chronically infected cells, H9/human T-cell lymphotropic virus type III (HTLV-III_B), were used as a positive control for the determination of viral p24 antigen inside the cells. The $CD4^+$ human

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lymphoblastoid cell line A3.01 was obtained from T. M. Folks (Centers for Disease Control, Atlanta, Ga., U.S.A.). The following cell lines were obtained through the AIDS Research and Reference Reagent Program NIAID: H9 cells from R. C. Gallo; the H9/HTLV-III_B cell clone from L. Arthur. The $CD4^-$ human chronic myelogenous leukaemia cell line, K562, was obtained from the American Type Culture Collection. Cells were cultured at 37 °C (7% CO_2). The A3.01, K562 and H9/HTLV-III_B cell lines were propagated in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin (50 units/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). H9 cells were maintained in Iscove's modified Dulbecco's medium with 20% FBS and antibiotics.

Virus. The virus and infected cell lines were handled in P-2 and P-3 facilities as recommended by the Centers for Disease Control (1988). For fusion experiments, HIV-1 (LAV, obtained from the Pasteur Institute), propagated in human lymphoblastic leukaemia (CEM) cells, was provided by M. Jennings (University of California, Davis, Ca., U.S.A.). The virus preparation and the cell line tested negative for *Mycoplasma* contamination. Cell-free medium from HIV-1-infected cell cultures was collected and the virus was pelleted by centrifugation and purified by sucrose density gradient centrifugation. The virus band was collected, assayed for protein (Bradford, 1976), diluted and stored at −70 °C. A standard laboratory strain of HIV-1_{LAV} was originally provided by T. M. Folks and was propagated in A3.01 cells. Virus supernatant was harvested at times of peak reverse transcriptase activity, divided into aliquots and stored at −80 °C. The reverse transcriptase activity of the virus stock was $5{\cdot}1 \times 10^5$ c.p.m./ml supernatant, as detected by the method of Hoffman *et al.* (1985). The ELISA-reactive p24 protein in the viral supernatant was present at a concentration of approximately 1·0 μg/ml. Viral inocula were standardized by their content of p24 antigen and cultures were infected with 1·0 or 2·0 ng of viral p24/10^6 cells in all experiments.

Virus labelling. A fluorescence assay was used to monitor the intermixing of viral and target membranes during fusion, using R_{18} as the fluorescent lipid marker (Hoekstra *et al.*, 1984; Loyter *et al.*, 1988). HIV-1 (100 μg) was labelled with a solution of R_{18} (final ethanol concentration less than 1%) for 0·5 to 1 h at room temperature. Previously reported HIV-1 lipid-to-protein ratios (Aloia *et al.*, 1988) were assumed for determining the amount of R_{18} with which to label the virus. The final concentration of label was 3 mol% of viral lipid. Free fluorophore was not present after labelling. An excess of unlabelled virus did not result in redistribution of R_{18} as measured by fluorescence dequenching (data not shown). In some experiments, the labelled virus was subjected to chromatography on Sephadex G-75 to eliminate any free dye, producing a lower yield of labelled virus; the fusion results obtained with this virus preparation were similar to those obtained with the virus that was not subjected to chromatography.

Preparation of liposomes. Large unilamellar vesicles composed of DOPC or CL were prepared in 150 mM-NaCl, 10 mM-TES (Sigma and Calbiochem) and 10 mM-citrate pH 7·5 ('fusion buffer') by a modification (Düzgüneş *et al.*, 1983) of the reverse-phase evaporation procedure (Szoka *et al.*, 1980). Vesicles were extruded three times under argon through double polycarbonate membranes of 0·1 μm pore diameter (Poretics) in a Lipex Biomembranes high pressure extrusion cell to achieve a uniform size distribution of vesicles (Szoka *et al.*, 1980). DOTMA:cholesterol (3:1) liposomes were prepared in fusion buffer by hydrating dried lipid (Bangham *et al.*, 1965) and extruded three times under argon through double polycarbonate membranes (0·4 μm pore diameter; Nuclepore). This procedure results in the production of unilamellar vesicles (Düzgüneş *et al.*,1983; Hope *et al.*, 1985). Pure DOTMA liposomes were prepared similarly, except that they were extruded through membranes of 0·2 μm pore diameter. The lipid concentration of phospholipids was determined by phosphate assay (Bartlett, 1959).

Fusion experiments. The dilution of R_{18} into the 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 fluorimeter, linked through a MacAdios analog:digital converter using Manager II software (GW Instruments), to a Macintosh SE/30 computer to collect raw data. The excitation wavelength was 555 nm and the emission monochromator was set at 595 nm, with a 590 nm high-pass cut-off filter. All experiments were performed at 37 °C in a stirred cuvette. Liposomes were rapidly injected into a temperature-equilibrated cuvette containing labelled virus in fusion buffer set to pH 7·5. The final reaction volume was 2·0 ml. The fluorescence scale was calibrated to 100% (complete dilution of the probe; i.e. complete fusion) by addition of 0·1 to 0·5% (final concentration) of $C_{12}E_8$ detergent to disperse the R_{18} completely. All data were collected within 6 months of virus preparation.

Detection of CD4. The presence of cell surface CD4 protein was examined by direct immunofluorescence assay, using fluorescein isothiocyanate (FITC)-conjugated Leu3 antibody (Becton-Dickinson) with FITC-conjugated mouse IgG as a control. The fluorescence distribution was analysed with a fluorescence-activated cell sorter (FACScan; Becton-Dickinson).

HIV infection of cells. Cells (2×10^7/ml) were incubated with HIV-1 for 2 h at 37 °C to permit adsorption of virus particles and then washed three times to remove unbound virus. Cells were then resuspended at 5×10^5 cells/ml in RPMI 1640/10% FBS or Iscove's modified Dulbecco's medium/20% FBS and cultured at 37 °C in 48-well culture plates (0·6 ml per well), or in 25 cm^2 culture flasks, and maintained in 10 ml of medium. Control cells were similarly treated but not exposed to the virus. Samples (400 μl) of the supernatant were collected every 3 or 4 days for subsequent screening by ELISA for HIV p24 antigen content; 400 μl of fresh medium was added to each well. The effect of liposomes on HIV infectivity was investigated by three different protocols: (i) the virus was pretreated with liposomes before addition to the cells, (ii) liposomes were added to the culture medium during the exposure of cells to virus, or (iii) liposomes were added after the cells had been exposed to virus and washed.

HIV-1 (0·4 μg viral p24/ml) was preincubated with various concentrations of liposomes for 2 h at 37 °C and then diluted with a cell suspension to give 1 or 2 ng of viral p24 protein/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 and cultured in 48-well culture plates. Following incubation, the number of viable cells was determined by trypan blue exclusion and p24 antigen was measured in duplicate in supernatant collected independently from two or three wells.

Detection of HIV infection. HIV-specific antigens were detected in cell culture supernatants using an HIV-1 p24 antigen capture assay. The expression of HIV antigen in the infected and mock-infected cells was determined using a mouse anti-p24 monoclonal antibody and soluble complexes of calf intestinal alkaline phosphatase and mouse anti-alkaline phosphatase monoclonal antibodies (APAAP; Dako). After the cells were washed with phosphate-buffered saline (PBS; Zymed Laboratories), they were suspended in PBS, placed on 10-well slides and fixed for between 3 and 5 min in acetone. The slides were overlaid with a mouse monoclonal antibody to HIV-1 p24 protein, rabbit anti-mouse immunoglobulins and APAAP and stained using the Alkaline Substrate Kit (Vector Laboratories). The uninfected cells and H9/HTLV-III_B cells were used as negative and positive controls, respectively.

p24 antigen capture assay. Cell-free supernatant fluids were assayed in duplicate by an HIV-1 p24 antigen capture assay with two mouse monoclonal antibodies specific for the HIV p24 *gag* protein, recognizing different epitopes. ELISA plates (96-well; Corning) were coated overnight with the capture antibody, incubated with 0·1% bovine serum albumin in PBS for 1 h at room temperature and washed three times with PBS–Tween 20 (0·05%). Next, supernatant (100 µl) was added and samples were incubated for 2 h at 37 °C. The biotinylated detector antibody was subsequently added and after 1 h incubation at 37 °C the antibody was bound with avidin–horseradish peroxidase. Colour develops from the reaction of the peroxidase with hydrogen peroxide in the presence of *o*-phenylene diamine (Dakopatts); the absorbance was read at 490 nm. The p24 antigen was quantified from serial dilutions of a positive control preparation containing a known amount of antigen. A culture supernatant containing a known amount of p24, calibrated with the lyophilized Antigen Reagent (Coulter HIV Ag Assay) (Coulter Immunology), was used as a standard. The sensitivity of detection was routinely 38 pg p24/ml of cell culture supernatant.

Detection of HIV DNA sequences by the polymerase chain reaction (PCR). K562 cells (either uninfected or exposed to HIV-1 preincubated in the presence or absence of 10 µM-DOTMA liposomes) were maintained as described above. After 3 and 10 days of culture, K562 cells at a density of 5×10^6/ml were treated for 1 h at 56 °C in 1 × amplification buffer (50 mM-KCl, 10 mM-Tris–HCl pH 8·3, 2·5 mM-$MgCl_2$, 0·1 mg/ml gelatin) containing 0·45% NP40, 0·45% Tween 20 and 60 µg/ml proteinase K; proteinase K was subsequently inactivated at 100 °C for 10 min. Aliquots of lysate containing 10^5 cell equivalents were amplified for 35 cycles using HIV *env* primers SK68/69, essentially according to Ou *et al.* (1988). Detection of *env*-specific amplified sequences was performed by liquid hybridization with a ^{32}P-labelled internal probe (SK70) (Ou *et al.*, 1988), polyacrylamide gel electrophoresis and autoradiography. The reactions were performed together with negative-DNA samples, as a control for carryover, and with a dilution series of HIV-infected cells (8E5 cells, one HIV proviral DNA copy per cell) (Folks *et al.*, 1986) to enable quantification of HIV DNA sequences present in a given sample.

Blocking experiments. A3.01 cells (2×10^7 cells/ml) were preincubated with the anti-CD4 antibody Leu3a at a concentration of 10 µg/ml for 1 h at 37 °C (Weber *et al.*, 1989). Leu3a antibody has been shown to block HIV infection of T cells (Sattentau *et al.*, 1986). The cells preincubated with anti-Leu3a and untreated cells were then exposed to HIV-1 at 1 ng viral p24/10^6 cells for 2 h at 37 °C, in the presence of 5 µM-DOTMA or in the absence of liposomes. The cells were then washed three times and cultured for 7 days.

Statistical comparisons were performed using a Student two-sided unpaired *t*-test.

Results

Fusion of HIV-1 with liposomes is dependent on membrane composition

Fusion of HIV-1 with liposomes at neutral pH was dependent on the lipid composition of the liposomes (Fig. 1). Fusion was fastest and most extensive with liposomes composed of DOTMA:cholesterol (3:1). Fusion of the virus with CL liposomes, although fairly dramatic, was somewhat slower than with DOTMA:cholesterol (3:1) liposomes. The virus did not fuse appreciably with pure DOPC membranes, with only 3% fluorescence dequenching after 4 min incubation.

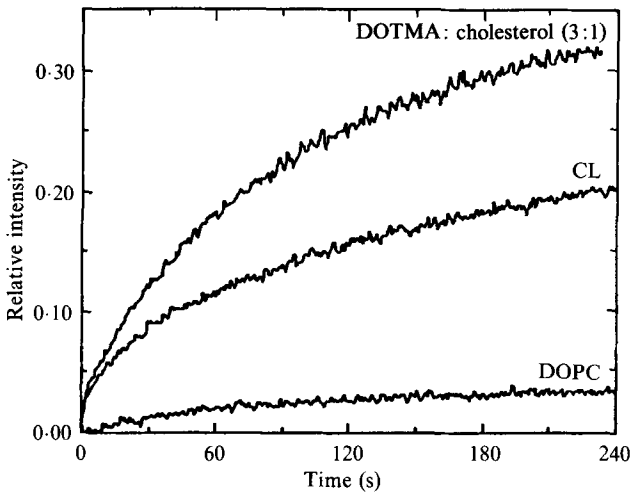

Fig. 1. Kinetics of fusion between HIV-1 and liposomes of various composition. Fusion between R_{18}-labelled HIV-1 (1 µg/ml) and liposomes (50 µM-lipid) (which corresponds to an approximate 100:1 liposome:virus lipid ratio) was monitored continuously at pH 7.5 as the increase of R_{18} fluorescence due to lipid mixing.

Treatment with cationic DOTMA liposomes enhances the replication of HIV in A3.01 cells

The CD4 molecule functions as a receptor for the HIV envelope glycoprotein gp120 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984) and the susceptibility of the cells to HIV infection is associated with the expression of CD4 on their surface (Sodroski *et al.*, 1986). FACS analysis indicated that 90% of the A3.01 cells used in our studies were CD4$^+$ (data not shown). To determine the effect of the concentration of DOTMA liposomes on HIV-1 infectivity, virus was preincubated with varying concentrations of DOTMA liposomes (0·1 to 50 µM-lipid). The mixture was then diluted and incubated with A3.01 cells for 2 h and the cells were cultured for 7 days. This treatment enhanced virus infectivity over a broad range of liposome concentrations, with an optimum between 10 and 30 µM-lipid (Fig. 2). In this concentration range, virus infectivity was enhanced by about 30-fold. Infection was also followed by immunohistochemical staining (APAAP assay) of infected and mock-infected cells. The expression of p24 antigen in HIV-1-infected cells correlated well with the level of production of p24 antigen, determined in the supernatant (data not shown). Despite the increased production of p24 protein, no loss of cellular viability was noted.

To study the kinetics of viral replication, A3.01 cells were infected with HIV-1 preincubated with 10 µM-DOTMA liposomes or with untreated virus. The

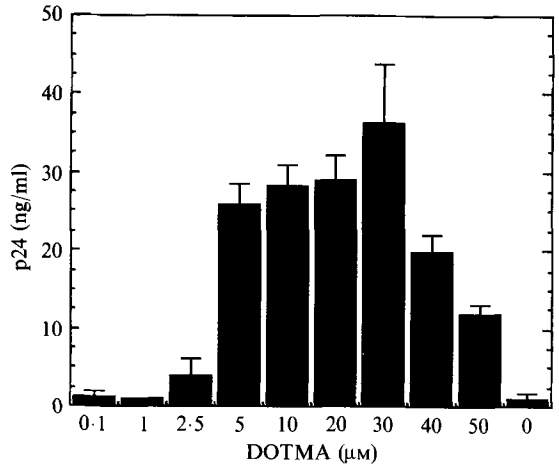

Fig. 2. Effect of preincubation of HIV-1 with various concentrations of DOTMA liposomes (0·1 to 50 μM-lipid) on HIV-1 replication in A3.01 cells. p24 antigen was detected in the cell culture supernatant 7 days p.i. Data represent mean values ± S.D. ($n = 4$).

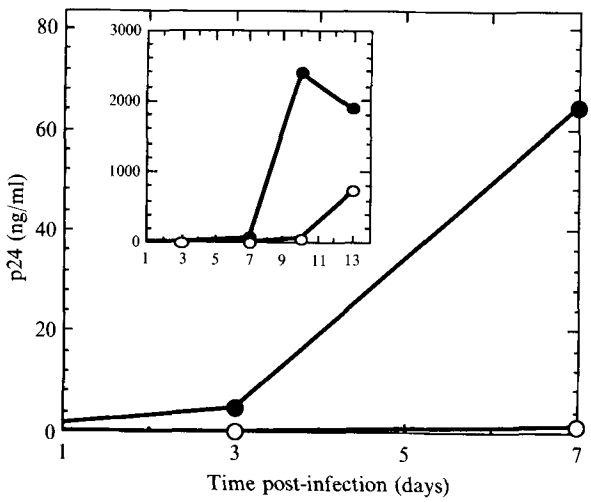

Fig. 3. Kinetics of p24 antigen production in the culture supernatant of A3.01 cells infected with HIV-1 preincubated with DOTMA liposomes (10 μM-lipid) (●), or with untreated virus (○). Supernatants were collected on 3, 7,10 and 13 days p.i. and assayed for p24 antigen. Data represent mean values ($n = 6$).

infectivity of the virus preincubated with DOTMA liposomes was strongly increased; p24 appeared in the supernatant 3 days post-infection (p.i.) (Fig. 3) and reached a peak value of approximately 2 μg p24/ml after 10 days of culture (Fig. 3, inset). In contrast, when A3.01 cells were infected with untreated HIV-1, the production of p24 was not detectable before 7 days p.i. and, by 10 days, reached only 2·2% of the value obtained by inoculation with DOTMA-pretreated virus (Fig. 3, inset).

The stimulatory effect of DOTMA liposomes on HIV-1 infection of A3.01 cells was dependent on the time of addition of DOTMA. When 5 μM-DOTMA liposomes

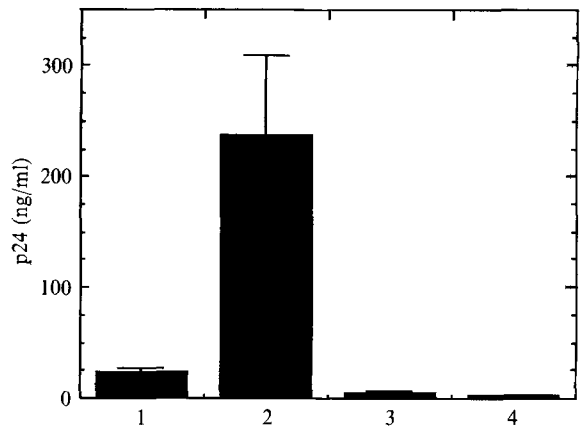

Fig. 4. Effect of the time of the addition of DOTMA liposomes on HIV-1 replication in A3.01 cells. 1. HIV-1 was preincubated with DOTMA liposomes (5 μM-lipid) for 2 h at 37 °C before infection of the cells (1 ng viral p24/1 × 10^6 cells). 2. Cells were incubated with HIV-1 in the presence of 5 μM-DOTMA for 2 h, then washed and maintained as above. 3. Cells were infected with HIV-1, then washed and resuspended in medium containing 5 μM-DOTMA at 5 × 10^5 cells/ml. Samples (400 μl) of the culture supernatant were removed 3 days p.i. and fresh medium with 5 μM-DOTMA was added to each well. 4. Cells were infected with HIV-1 and cultured in the absence of DOTMA. In all cases, p24 antigen was detected in cell culture supernatant on day 7 p.i. Data represent mean values ± S.D. ($n = 6$).

were present during infection, HIV-1 replication was increased by about 10-fold compared to cells exposed to HIV-1 preincubated with the same concentration of DOTMA (Fig. 4). This amounts to a 90-fold enhancement of infectivity compared to untreated HIV-1. A3.01 cells incubated with untreated virus and then cultured in the presence of 5 μM-DOTMA liposomes showed a small but statistically significant ($P < 0.0005$) increase in p24 antigen production from 2·63 ± 0·45 to 5·41 ± 0·55 ng/ml observed on day 7 p.i. (Fig. 4). The presence of 5 μM-DOTMA in the culture medium for 7 days following infection did not affect the viability of A3.01 cells.

Effect of DOTMA liposomes on HIV infection of H9 and K562 cells

Enhancement of HIV-1 infection by DOTMA liposomes was also tested in another $CD4^+$ cell line, H9. Of these cells, 52% were $CD4^+$ (data not shown). The effect of DOTMA liposomes was determined under three experimental conditions: H9 cells were incubated either with HIV-1 preincubated with 5 μM-DOTMA, or with HIV-1 in the presence of DOTMA liposomes; alternatively, cells inoculated with untreated virus were cultured in the presence of 5 μM-DOTMA liposomes for 3 days and then cultured in the absence of liposomes (Table 1). The results indicate that the first two conditions enhanced viral infectivity to a similar extent. A fivefold enhance-

Table 1. *Effect of DOTMA liposomes on HIV-1 replication in H9 cells*

Time p.i. (days)	Treatment: DOTMA-pretreated HIV + H9 cells	DOTMA + H9 cells + HIV	Infected H9 cells + DOTMA	H9 cells + HIV (control)
7	2·6 ± 0·4*	1·2 ± 0·1	0·5 ± 0·15	0·5 ± 0·2
10	6·5 ± 0·9	5·3 ± 1·2	2·3 ± 0·6	1·2 ± 0·2
14	388·0 ± 67·8	377·0 ± 90·2	201·5 ± 77·3	71·5 ± 20·9

* p24 Antigen was determined in cell culture supernatants on 7, 10 and 14 days p.i. Data represent mean p24 ng/ml ± S.D. ($n = 6$).

ment of p24 production was observed after 10 and 14 days when compared to H9 cells infected in the absence of DOTMA (Table 1). By comparison, the presence of DOTMA liposomes during the adsorption of virus to A3.01 cells resulted in a much higher stimulation of infection, with a 90-fold enhancement of p24 production 7 days p.i. (Fig. 4). Addition of 5 μM-DOTMA liposomes to A3.01 or H9 cells previously infected with HIV-1 resulted in a similar enhancement of p24 production (approximately twofold compared to untreated controls; Fig. 4 and Table 1).

$CD4^-$ K562 cells were not susceptible to infection by either DOTMA-pretreated or untreated virus (2 ng viral $p24/10^6$ cells), as determined by the absence of p24 in the cell culture supernatant (data not shown). We have been able to detect by PCR amplification a very low amount of HIV proviral DNA (between 2 and 10 copies per 10^5 cells) in K562 cells, infected either with untreated HIV-1 or with HIV-1 pretreated with 10 μM-DOTMA liposomes, incubated for 3 days following the 2 h infection period; HIV-1 DNA was not detectable on day 10 p.i. (data not shown). At the present time we are not able to distinguish whether this low level HIV DNA represents newly synthesized HIV proviral DNA (by reverse transcription) or contaminating viral DNA from infected cells in the virus inoculum. The level of HIV proviral DNA was not increased after exposure of K562 cells to DOTMA-pretreated HIV-1, indicating that the presence of DOTMA liposomes did not enhance HIV-1 infection and replication in $CD4^-$ K562 cells.

Anti-Leu3a antibody inhibits the enhancement of HIV-1 infectivity by DOTMA liposomes

To ascertain whether the enhancement of HIV-1 infectivity by DOTMA liposomes required the CD4 receptor, we used anti-Leu3a antibodies that recognize the epitope on CD4 involved in binding to the HIV

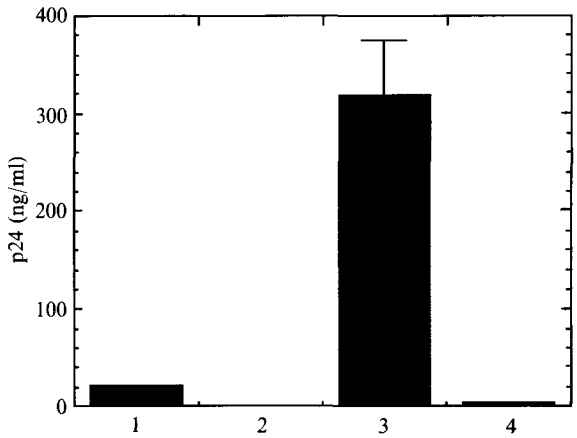

Fig. 5. Effect of the anti-CD4 antibody, anti-Leu3a, on HIV-1 infection of A3.01 cells in the presence and absence of DOTMA liposomes. Cells were preincubated with anti-Leu3a (1 and 2) and then exposed to HIV-1 as described in Methods. 1. HIV-1 was added in the presence of antibody and 5 μM-DOTMA liposomes. 2. HIV-1 was added in the presence of antibody. 3. HIV-1 was added in the absence of antibody and the presence of 5 μM-DOTMA liposomes. 4. HIV-1 alone was added. p24 antigen was detected in the cell culture supernatant 7 days p.i. Data represent mean values ± S.D. ($n = 4$). The S.D. values for 1 and 4 are too small to be shown at this scale, and are given in the text.

envelope glycoprotein, gp120 (Sattentau *et al.*, 1986). HIV-1-infected A3.01 cells produced 3·07 ± 0·22 ng/ml p24 in 7 days (Fig. 5). Preincubation of A3.01 cells with Leu3a antibody completely abolished the infectivity of HIV-1 (bar 2 in Fig. 5). When HIV-1 was added to Leu3a antibody-pretreated cells in the presence of DOTMA liposomes, the amount of p24 produced by these cells was reduced to 7% (21·1 ± 0·9 ng/ml) of the value produced by cells infected with HIV-1 in the presence of liposomes without antibody pretreatment (319 ± 56 ng/ml).

Anti-HIV activity of CL liposomes

It was of interest to examine whether the fusion product of HIV-1 and CL liposomes would retain the infectivity of the virus towards $CD4^+$ cells. The antiviral effect of CL liposomes was evaluated in experiments in which A3.01 cells, highly susceptible to infection, were infected with HIV-1 that had been preincubated with various concentrations of these liposomes. The results (Table 2) indicate that preincubation of HIV-1 with CL liposomes resulted in a dose-dependent inhibition of virus replication. The expression of p24 protein in HIV-1-infected cells correlated well with the production of p24 determined in the supernatant (data not shown). The concentrations of CL liposomes at which inhibition of infectivity was observed were not toxic to the cells.

Table 2. *Effect of preincubation of HIV-1 with CL liposomes on HIV-1 replication in A3.01 cells*

Treatment*	p24 (ng/ml)†		
	7 days p.i.	10 days p.i.	13 days p.i.
+ HIV (control)	7·8 ± 2·1	813·2 ± 131·0	1359·0 ± 216·4
10 μM-CL	5·7 ± 1·0	810·8 ± 220·5	1193·3 ± 288·5
25 μM-CL	3·3 ± 0·9	249·0 ± 74·9	679·0 ± 271·0
50 μM-CL	1·7 ± 1·0	52·0 ± 8·9	366·5 ± 17·6
75 μM-CL	0·9 ± 0·5	22·0 ± 10·4	210·0 ± 22·2
100 μM-CL	0·03 ± 0·02	0·7 ± 0·6	13·3 ± 7·8

* HIV-1 was preincubated with CL liposomes then added to A3.01 cells (2 ng viral p24/10^6 cells), as described in Methods. Samples (400 μl) were collected from three wells on 7, 10 and 13 days p.i.

† Data represent mean values ± S.D. ($n = 6$).

Effect of DOPC liposomes on HIV-1 infectivity

To confirm that the effects on HIV replication observed after preincubation of the virus with DOTMA and CL liposomes were due to the interaction of liposomes with the viral membrane, experiments were performed with DOPC liposomes. HIV-1 did not fuse with DOPC liposomes to a significant level (Fig. 1). Exposure of A3.01 cells to DOPC-pretreated virus did not affect p24 production when compared to cells incubated with untreated virus. When A3.01 cells were infected with virus preincubated with 10 or 100 μM-DOPC liposomes or with RPMI medium alone (control), the values of p24 determined in the supernatant after 7 days were 1·33 ± 0·38, 1·61 ± 0·18 and 1·31 ± 0·51 ng/ml (mean ± S.D., $n = 4$), respectively.

Discussion

Our studies, utilizing the R_{18} fluorescence assay for virus fusion, have shown that HIV-1 fuses with both negatively charged CL liposomes and positively charged DOTMA-containing liposomes. The preferential fusion of HIV-1 with negatively charged liposomes over uncharged liposomes at neutral pH (CL has a net negative charge and DOPC is neutral) was qualitatively similar to that seen with other lipid-enveloped viruses, including SIV_{mac} (Larsen, *et al.*, 1989, 1990), Sendai virus (Klappe *et al.*, 1986) and influenza virus (Stegmann *et al.*, 1985, 1989). Fusion of lipid-enveloped viruses with liposomes containing DOTMA has not been reported previously. In this study we examined the infectivity of the fusion product of HIV-1 with either CL or DOTMA liposomes and the effect of the presence of these liposomes during incubation of HIV-1 and target cells. We found that the infectivity of HIV-1 was significantly enhanced by DOTMA liposomes and markedly inhibited by CL liposomes, whereas DOPC liposomes had no effect.

Positively charged DOTMA liposomes interact spontaneously with DNA or mRNA, resulting in a liposome/polynucleotide complex that can facilitate transfection of a wide variety of tissue culture cells (Felgner *et al.*, 1987; Felgner & Ringold, 1989). In the presence of serum, DOTMA-mediated transfection is essentially blocked, presumably due to attachment of the DNA/lipid complexes to serum components (Felgner & Ringold, 1989). DOTMA liposomes have also been used to deliver DNA-binding proteins to the nucleus of cultured cells (Debs *et al.*, 1990). The mechanism of DOTMA-mediated delivery of polynucleotides or proteins into cells is unknown, although direct fusion with the cell membrane has been suggested (Felgner *et al.*, 1987; Felgner & Ringold, 1989). Liposomes composed of DOTMA and zwitterionic phospholipids have been shown to fuse with negatively charged liposomes, or with each other in the presence of multivalent anions (Düzgüneş *et al.*, 1989).

Our results demonstrate that HIV-1 infection can be enhanced after fusion of the virus membrane with DOTMA liposomes and imply that the fusion product delivers a functionally active virion core into the cytoplasm with greater efficiency than untreated virus. The enhancing effects of DOTMA liposomes were greatest when the liposomes were present during the exposure of $CD4^+$ cells to H1V-l (infection phase), when compared to their addition after virus inoculation (transmission phase) (Fig. 4 and Table 1). During the transmission period, cells infected with virus become competent to infect other $CD4^+$ cells by the release of viral progeny or by cell fusion (Nara *et al.*, 1989). The positive charge of DOTMA appeared to be necessary for increased virus infectivity because the enhancement could be abolished by the presence of polyanionic oligo(dN) or non-specific DNA (unpublished data).

Polycationic reagents such as polybrene or DEAE–dextran have frequently been used to increase the efficiency of retrovirus infection *in vitro* (Toyoshima & Vogt, 1969; Cheng-Mayer *et al.*, 1987; Chesebro & Wehrly, 1988; Tateno *et al.*, 1989), although variability of effectiveness was observed when these compounds were used with different subgroups of avian retroviruses (Toyoshima & Vogt, 1969) or with different HIV isolates (Chesebro & Wehrly, 1988). The polycation-mediated enhancement is at least partially due to an increased adsorption of virus to the cell but these compounds may also facilitate virus penetration (Toyoshima & Vogt, 1969). The enhancing effect of polycations is due to their multiple positive charge, because the addition of

polyanions abolished the polycation-mediated enhancement of viral adsorption (Toyoshima & Vogt, 1969). Effective concentrations of the polycations ranged from 2 to 25 μg/ml. The infectivity was potentiated when target cells were pretreated with polycations or exposure to virus was performed in the presence of polycations. Pretreatment of Rous sarcoma virus with polybrene at a concentration of 16 μg/ml failed to enhance infectivity (Toyoshima & Vogt, 1969), suggesting an absence of a strong interaction between virus and polycations. In contrast, preincubation of HIV-1 with DOTMA enhanced the infectivity of the virus, suggesting that DOTMA-mediated enhancement may involve fusion in addition to the polycation effect.

It is likely that the fusion product of HIV-1 with DOTMA containing the surface protein, gp120, can still bind to CD4 and use CD4 as the receptor for cellular entry. Our data indicate that the sensitivity of the cells to the DOTMA-mediated enhancement correlated with the expression of CD4 on their surface; enhancement was most pronounced in the highly $CD4^+$ A3.01 cells and was lower in H9 cells, whereas $CD4^-$ K562 cells were not detectably infected. This observation contrasts with the results of Innes *et al.* (1990) who recently reported that lipofectin (a commercial preparation containing DOTMA) did not cause any enhancement of the infectivity of murine leukaemia virus in cells containing the viral receptor, it mediated the infection of non-permissive cells by murine leukaemia virus and there was a concentration-dependent inhibition of the lipofectin-mediated infectivity by serum.

Blocking of infection with Leu3a antibody has been taken as an indication that HIV-1 entry occurs predominantly via the CD4 receptor (Sattentau *et al.*, 1986; Weber *et al.*, 1989); presumably the antibody inhibits infection by binding at or near a gp120-binding epitope of the CD4 molecule (McDougal *et al.*, 1986; Sattentau *et al.*,1986). Weber *et al.* (1989) have reported that the infection of some brain cells by HIV-1 is not blocked by Leu3a antibodies and have concluded that infection of these cells does not appear to involve CD4 as the receptor. They have suggested that a non-specific mechanism may be involved, because higher doses of virus were required to infect these cells, and that a second, low-affinity, receptor for HIV entry may be used. Our observation that, in A3.01 cells, Leu3a antibody blocks 93% of the infectivity of HIV-1 in the presence of DOTMA liposomes, indicates that the enhancement of infectivity is largely via the CD4 receptor. A small percentage of the infection in these cells appears to proceed independently of CD4. It is likely that some of the fusion products of HIV-1 and DOTMA liposomes fuse with the plasma membrane because DOTMA liposomes appear to fuse with the plasma membrane of cultured cells (Felgner *et al.*, 1987; N. Düzgüneş & L. Stamatatos, unpublished data) and with erythrocyte ghost membranes (N. Düzgüneş & J. Goldstein, unpublished data).

Infectivity of HIV-1 was inhibited by prior treatment with CL liposomes; this may be caused by either decreased binding or decreased fusion capacity of the fusion product. Since preincubation of HIV-1 with CL liposomes is even more inhibitory to infection than the presence of CL during inoculation (data not shown), the fusion of HIV-1 with CL liposomes may also alter the conformation of the viral envelope protein and hence its fusogenic capacity. Alternatively, the CL molecules that are part of the fusion product may bind cell surface molecules and prevent the interaction of HIV-1 with its receptor.

Schlegel *et al.* (1983) have shown that vesicular stomatitis virus infection of Vero cells can be blocked by preincubation of the virus with phosphatidylserine vesicles and have suggested that this phospholipid may be a receptor for the virus. Activated peripheral blood mononuclear cells are stained with anti-CL antibodies (Misra *et al.*, 1989) and, because such cells are particularly prone to infection with HIV-1 (Gowda *et al.*, 1988), it is possible that HIV-1 interacts with molecules on the cell surface that mimic CL. This hypothesis is currently being investigated.

Our observations with CL liposomes raise the possibility of using such liposomes for therapeutic purposes. Doxorubicin-loaded CL-containing liposomes are already in phase II clinical trials for cancer chemotherapy (Treat *et al.*, 1989) and liposomes with prolonged circulation times have been developed recently (Gabizon & Papahadjopoulos, 1988). HIV infectivity may not only be inhibited by liposomes themselves but also by encapsulated antisense oligodeoxynucleotides or reverse transcriptase inhibitors which could enter the virus interior via fusion. Hence, any virus–liposome fusion product that does manage to inject its genome into the cytoplasm of target cells would also introduce these inhibitors. The encapsulation of proteases and nucleases in liposomes may degrade the virus and its genome upon fusion with the viral membrane and confer further protection against HIV infection. The covalent coupling of rsCD4 to the liposomes may render the liposomes highly specific for binding and fusion with HIV-1. These studies are in progress in our laboratory.

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