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# Liposomal encapsulation enhances antiviral efficacy of SPC3 against human immunodeficiency virus type-1 infection in human lymphocytes

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**Abstract**

Because encapsulation of antiviral drugs in liposomes resulted generally in improved activity against retroviral replication in vivo, the antiviral effects of free-SPC3 and liposome-associated SPC3 were compared in cultured human lymphocytes infected with HIV-1. SPC3 was entrapped in various liposomal formulations, either different in size (mean diameter of 100 and 250 nm), SPC3 concentration or cholesterol content. Liposome-associated SPC3 were tested for both inhibition of cell–cell fusion and infection with HIV-1 clones. SPC3 inhibited HIV-1-induced fusion at a micromolar concentration range. When associated with liposomes, SPC3 was found to be about 10-fold more potent than free SPC3 in inhibiting syncytium formation. Continuous treatment with free SPC3 also inhibited virus production in a dose-dependent manner, with inhibition of HIV infection of C8166 T-cells or human peripheral blood lymphocytes (PBLs) at micromolar concentrations. Liposomal entrapment was found to increase the antiviral efficacy of SPC3 by more than 10- and 5-fold in C8166 and PBLs, respectively. These data suggest that the liposome approach may be used to improve SPC3 antiviral efficacy. © 2002 Published by Elsevier Science B.V.

*Keywords:* Lymphocyte; Liposome; HIV; Antiviral; SPC3

*Abbreviations:* PBL, peripheral blood lymphocyte; DMEM, Dulbecco's modified Eagle's medium; Lipo, liposome; Chol, cholesterol; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; α-Toco, DL alpha-tocopherol; ddI, 2′,3′-dideoxyinosine; $CCID_{50}$, 50% cell culture infectious dose; IL-2, interleukin-2; TCLA, T-cell line adapted virus.

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## 1. Introduction

SPC3 is a synthetic polymeric peptide construction containing eight repetitive GPGRAF motifs. This sequence corresponds to the consensus pattern of the third variable domain (V3 loop) of human immunodeficiency virus type-1 (HIV-1) surface envelope glycoprotein gp120 (LaRosa et al., 1990). The V3 loop appears to play a key role in HIV-1 infection and pathogenesis; it is involved in viral cytopathogenicity and cell tropism (Palker et al., 1988; Shioda et al., 1991; Hwang et al., 1991; Westervelt et al., 1991; De Mareuil et al., 1995; Choe et al., 1996) and corresponds to a major immunodominant epitope for non-neutralizing antibodies (Nara et al., 1991). SPC3 is a potent antiviral drug, which blocks lymphocyte and macrophage infections with various HIV strains in vitro (Yahi et al., 1994, 1995). SPC3 has undergone preliminary phase II clinical trials in HIV-1-infected patients but showed only moderate effects on viral load. To improve the efficacy of SPC3 in vivo, new liposomal formulations were tested in vitro.

The liposome approach has been used successfully in the treatment of specific diseases in vivo to enhance drug targeting to the cells (Phillips et al., 1991; Papahadjopoulos et al., 1991; Girard et al., 1996; Gottlieb et al., 1997; Newell et al., 1998; Cheung et al., 1999; Nunez et al., 2000). Liposomes protect bioactive peptides from a rapid proteolytic degradation in the blood stream. Furthermore, liposomal entrapment is particularly valuable as anti-HIV drugs because liposomes accumulate in the reticulo-endothelial tissues in which HIV infection occurs. Thus, this formulation appears to be a promising route to improve the therapeutic index of antiviral drugs. In the present study, the antiviral efficacy of liposome-associated SPC3 was investigated.

Pharmacokinetic studies have shown that the size of the drug-containing liposome affects both their half-life and their relative distribution in the body tissues after intravenous injection. Increase of liposome size may result in a greater ability to retain the entrapped drug and in a superior plasma half-life (Abra and Hunt, 1981; Harvie et al., 1995). As the presence of cholesterol in liposomes has been shown to enhance their rigidity and body half-life, several liposomal formulations with or without cholesterol were also tested.

Therefore, several concentrations of SPC3 were entrapped in various types of liposomes differing in size and cholesterol content, and the antiviral effects of these formulations were compared with those obtained using free SPC3. The antiviral effects of these new SPC3 formulations were assessed on both cell-to-cell (syncytium formation) and virus-to-cell infections. Further to their potential clinical testing, the antiviral properties of the formulations of SPC3-containing liposomes were assessed on human peripheral blood lymphocytes (PBLs).

## 2. Materials and methods

### 2.1. Reagents

Chemical synthesis of SPC3 was performed by the solid-phase technique. The eight GPGRAF motifs were anchored on an uncharged lysine-based core matrix ($[GPGRAF]_8$-$[K]_4$-$[K]_2$-K-βA). SPC3 was characterized by electrospray mass spectrometry, amino acid analysis after acidolysis, and Edman sequencing, as described previously (Fantini et al., 1993; Sabatier et al., 1995). EPC (lot no. 105016-1/002) and EPG (lot no. 835014-1/03) were from Lipoid, cholesterol (lot no. 118H5231) from Sigma, and tocopherol (355472/1-24397) from Fluka.

### 2.2. Liposomes

Oligolamellar vesicles were prepared by the film-rehydration method. Briefly, 10 ml of 60 mM phospholipids at a 9:1:0.1 EPC:EPG:α-Toco or 6:3:1:0.1 EPC:Chol:EPG:α-Toco molar ratios were dissolved in ethanol under moderate magnetic stirring and heating ($T = 37 \pm 2$ °C). This organic phase was poured into a round bottom flask and the organic solvent was then evaporated by rotary evaporation at $T = 37 \pm 2$ °C under reduced pressure and $N_2$ stream until ethanol was removed. The dried film was rehydrated with 10 ml of 300 mM-sucrose solution containing SPC3

at a concentration of 1, 4 or 16 mM and stirred until the phospholipid film was completely resuspended. The SPC3/lipid molar ratio ranged between 0.017 and 0.267. The liposome suspension was extruded through two stacked 400 nm isopore polycarbonate membranes (Millipore) in order to homogenize the liposome size. Liposome mean diameters of $250 \pm 50$ nm were obtained after five successive passages. To get liposomes of $100 \pm 30$ nm mean size, the suspension was further passaged through two stacked 200 and 100 nm isopore polycarbonate membranes, successively. The extruded liposome suspension was separated in two equal fractions. The first one was freeze-dried without further processing. The second fraction was dialyzed prior to lyophilization. The extruded liposome suspension was dialyzed against a 300 mM sucrose solution to remove free SPC3. The cut-off value of cellulose ester dialysis membrane ranged from 100 to 300 kDa (Spectra/Por CE, Biovalley, USA). This procedure eliminated about 90% of SPC3 which was presumably in a 'free' or 'weakly' liposome-associated state. We define the remaining 10% SPC3 as entrapped or 'strongly' liposome-associated peptide. In dialyzed SPC3-liposomes, the peptide concentration described throughout the text is the final SPC3 concentration after dialysis.

Aliquots (20 μl) of solutions containing free-SPC3, or SPC3-liposomes before and after dialysis, were used for peptide characterization and quantification. The molecular weight and purity of SPC3 (MW 5671.1 Da) were verified by electrospray mass spectrometry. The peptide quantities were assessed after SPC3 acidolysis (HCl 6N, 1% phenol for 48 h at 120 °C) and amino-acid content determination (Beckman 6300 apparatus). The quantity of SPC3 that was only weakly associated with the liposomes was estimated by calculating the difference in SPC3 concentration before and after dialysis. According to this procedure, we calculated that about 10% of total SPC3 remained associated to the liposomes after dialysis independently of the presence of cholesterol. The remaining SPC3 may be entrapped inside the liposome and/or 'strongly' associated to the membrane (i.e. not removable by dialysis). Peptide entrapment should occur considering the fact that the liposome formation was achieved in the presence of free SPC3.

The stability of dialyzed SPC3-liposomes, incubated in RPMI 1640 (containing 10% fetal calf serum), was assessed by using analytical liquid chromatography combined with mass spectrometry and amino-acid analysis for simultaneous qualitative and quantitative evaluation. Sample removal and analysis on a timely basis (each hour) showed that SPC3-liposomes were stable for 5–6 h in these experimental conditions (data not shown). After this time, free SPC3 could be detected in the medium, which indicated the occurrence of some leakage of the peptide from the liposomal preparation. This free SPC3 remained unaltered for ca. 16 h. After this period of time, some peptide degradation occurred as evidenced by mass spectrometry analysis. The presence of cholesterol in SPC3-liposomes did not significantly modify their stability (data not shown). SPC3-liposomes were not particularly sensitive to temperature variation (in the range of 4–37 °C).

### 2.3. Cells and virus

Human PBLs obtained from healthy HIV-seronegative donor (Etablissement Français du Sang, Marseille, France) were isolated by ficoll-Hypaque gradient centrifugation. Cells were cultured in R10 medium supplemented with 20 U/ml of interleukin-2 (IL-2, Proleukin, Chiron, The Netherlands). R10 medium consisted of RPMI 1640 supplemented with 2 mM ultraglutamine (BioWhittaker, Verviers, Belgium), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (BioWhittaker). Cells were first stimulated with phytohemagglutinin (20 μg/ml)-supplemented R10 (PHA P, DIFCO, Detroit, MI) for 3 days. Then, the medium was replaced with R10 supplemented with IL2 (20 U/ml), and subsequent cultures and experiments were carried out in this medium in a 37 °C humidified incubator with 5% $CO_2$.

C8166 (Salahuddin et al., 1983), H9 (from Q. Sattentau, Imperial College, London, UK) and CEM (Nara et al., 1987) T-cell lines were grown in R10.

Viral stocks of the TCLA X4 HIV-$1_{NL4-3}$ (obtained from I. Hirsh, INSERM U 372, Marseille, France) (Adachi et al., 1986; Barré-Sinoussi et al., 1983) were produced in permissive CEM cells. HIV-$1_{Hx10}$ and HIV-$1_{MN}$ (obtained from Q. Sattentau) were propagated in H9 cells. Cultured supernatants from infected cells were collected at the peak of maximal viral production as assessed by p24 assay, and residual cells were removed by centrifugation at 4 °C (2000 rpm/5 min). They were sampled and stored at −80 °C. The viral stock infectious titer (50% cell culture infectious dose, $CCID_{50}$) was established on C8166 cells.

Chronically infected H9 cells were prepared as follows. Briefly, H9 cells were infected for 2 h, washed extensively and propagated for 8–10 days. At this time post infection, there was neither detectable expression of the CD4 molecule at the cell surface nor syncytium formation in the cell culture. However, viral envelope was highly expressed as shown using gp120-specific monoclonal antibodies and flow cytometry analysis (Moulard et al., 2000).

### 2.4. Infection and treatment

#### 2.4.1. Fusion assay

A total of $2.5 \times 10^4$ H9 cells chronically infected by HIV-$1_{MN}$ were added to the target non-infected cells growing in suspension cultures at a ratio of 1:1 in the absence or presence of various concentrations and formulations of SPC3. The size of the SPC3-containing liposomes was either 100 or 250 nm. Co-cultivation of chronically infected H9 cells with non-infected C8166 cells resulted in cell–cell membrane fusion and generated multinucleated giant cells (syncytia) which could be easily evidenced by microscopy. Samples were visually inspected by phase contrast microscopy and the syncytia were scored after 24, 48 and 72 h incubation in 96-well flat-bottomed culture plates. Experiments were performed three times in duplicate.

#### 2.4.2. Infection of C8166 cells

*2.4.2.1. HIV-$1_{Hx10}$ infection of C8166 cells.* Cells were infected with HIV-$1_{Hx10}$ for 2 h in the presence of various concentrations of peptide. Cells were grown in 96-well U-bottom plates and a final volume of 200 μl. Supernatants were assayed for p24 production as previously described (Chenine et al., 2000).

*2.4.2.2. HIV-$1_{NL4-3}$ infection of C8166 cells.* Samples of $3 \times 10^5$ C8166 cells were placed in 96-well plates in a volume of 100 μl of culture medium containing various concentrations of SPC3. After 1 h treatment at 37 °C, 100 μl of viral suspension of HIV-$1_{NL4-3}$ were added. The cells were exposed to the virus for 1 h at 37 °C at a multiplicity of infection of 1000 $CCID_{50}$ per ml. After thorough washing, cells were replaced with 1 ml of R10 in 24-well plates and cultured in a 37 °C incubator. C8166 culture medium was replaced at day 4 post infection. During this assay, treatment with SPC3 or the corresponding liposomes was permanent (before, during and after infection). Assays on C8166 cells were performed at least twice and in duplicate. For the study of time of action, the experimental conditions for cell infection and culture were similar except for the treatment performed: (i) only before and during the course of infection, or (ii) immediately post infection and during 7 days, or (iii) 4 h post infection and during 7 days. Toxicity was evaluated by daily cell count and trypan-blue exclusion assay. Infection of C8166 T-cells with HIV-$1_{NL4-3}$ was assessed by virus-induced cytopathic effects (syncytium formation) and by quantification of p24 viral protein in the culture supernatants. Measurements of HIV-1 $p24^{gag}$ concentrations in the culture supernatant were achieved by ELISA, with a detection cut-off of 5 pg/ml (p24 HIV kit, NEN Dupont, Belgium; Quanti-Kine software, RILAB, Genova, Italy).

#### 2.4.3. Infection of human peripheral blood lymphocytes

Samples of $10^6$ human PBLs were placed in 96-well plates in 100 μl of R10 containing various concentrations of SPC3. After 1 h treatment at 37 °C, 100 μl of viral suspension of HIV-$1_{NL4-3}$ were added. The cells were exposed to the virus for 1 h at 37 °C at a multiplicity of infection of 100 $CCID_{50}$ per ml. After thorough washing, cells

were replaced in 1 ml of culture medium in 24-well plates and cultured in a 37 °C incubator flushed with 5% $CO_2$. The PBL culture medium was replaced every 3–4 days. In this assay, treatment was permanent and the diameter of liposomes with or without cholesterol was 250 nm. The cell viability was assessed by cell counts and trypan-blue exclusion assay. The viral production in the culture supernatant was quantified by p24 ELISA test, as described earlier. All the experiments were done in a blinded fashion. Tests have been achieved on forty samples of SPC3-liposomes and in duplicate.

## 3. Results

### *3.1. Effects on antiviral properties of SPC3 entrapment in liposomes of different sizes*

#### *3.1.1. Inhibition of HIV-1-mediated cell-to-cell spread by free SPC3 or SPC3 entrapped in liposomes with different sizes*

Chronically virus-infected cells were obtained by infection of H9 lymphoblastoid cell line with HIV-$1_{MN}$. Ten to twelve days post infection, no additional syncytia could be observed by microscopy. Co-cultivation of chronically-infected H9 cells with non-infected C8166 cells generated large and numerous syncytia within the next 24 h.

The antifusogenic properties of various formulations of SPC3 were evaluated by co-cultivation assay. The effects were concentration-dependent and maximal at 10 μM for free SPC3 or below 1 μM for SPC3 entrapped in liposomes with a mean diameter of 250 nm (Table 1). Similar antifusogenic effects were observed using either free SPC3 or SPC3 entrapped in liposomes with a diameter of about 100 nm. Inhibition of fusion was observed during the 3 days of co-cultivation.

#### *3.1.2. Inhibition of HIV-1 infection by free SPC3 or SPC3 entrapped in different sizes of liposomes*

C8166 cells were exposed to HIV-$1_{Hx10}$ in the presence of a series of concentrations of free SPC3 or in liposomes of different sizes. The viral production was monitored by ELISA measurement of the p24 viral protein in the cell supernatants. Continuous treatment with 10 μM of SPC3-liposomes inhibited more than 99% of HIV production in the supernatants at days 3 and 4 post infection (Table 2). For instance, we only measured a production of 0.09 ng/ml of p24 by C8166 cells treated with 250 nm SPC3-liposomes at day 3 compared with 145 ng/ml of p24 in untreated control cells. There was no significant difference in the antiviral efficacy between 100 and 250 nm SPC3-liposomes except that some signs of infectivity remained in cells treated with 100 nm SPC3-liposomes, whereas p24 production was below the threshold value of infection for cells treated with 250 nm SPC3-liposomes. In any case, liposomal SPC3 was more efficient than free SPC3 since viral production was inhibited by 94.5% (8 ng/ml of p24) when using 10 μM free SPC3 at day 3.

Table 1
Inhibition of syncytium formation with free-SPC3 or SPC3 entrapped in liposomes of various sizes

| | Syncytium formation (Day 2 post cocultivation)[a] | |
|---|---|---|
| Dilution factor | 1 | 1/10 |
| SPC3-Lipo. (250 nm) | − | − |
| SPC3-Lipo. (100 nm) | − | ± |
| Free SPC3 | − | ± |
| Lipo. (250 nm) without SPC3 | +++ | +++ |
| Lipo. (100 nm) without SPC3 | +++ | +++ |
| No treatment | +++ | +++ |
| Control cells | − | − |

[a] A total of $2.5 \times 10^4$ H9 cells chronically-infected with HIV-$1_{MN}$ were co-cultivated with $2.5 \times 10^4$ non-infected C8166 cells in the absence or presence of various concentrations and formulations of SPC3. Liposomal SPC3, entrapped in vesicles of 100 or 250 nm diameter was not dialyzed. At 48 h after co-cultivation, the syncytia were scored using an optical microscope. SPC3 concentration at a dilution factor of 1 was 10 and 1 μM at a dilution factor of 1/10. Control cells were non-infected and non-treated. Symbols: +++, number of syncytia present in the well, similar to the number in the untreated well (15–40 syncytia per well); ±, 1–5 syncytia per well; −, no syncytium in the well.

Table 2
Inhibition of HIV-$1_{Hx10}$ infection in C8166 cells with free SPC3 or SPC3 entrapped in liposomes of various sizes

| | p24 production (ng/ml) | |
|---|---|---|
| Days post infection | 3 | 4 |
| SPC3-Lipo. (250 nm) | $0.090 \pm 0.035$ | $0.002 \pm 0.002$ |
| SPC3-Lipo. (100 nm) | $0.699 \pm 0.033$ | $1.279 \pm 0.051$ |
| Free SPC3 | $7.907 \pm 0.620$ | $8.533 \pm 0.415$ |
| Lipo. (250 nm) without SPC3 | $128.883 \pm 15.335$ | $136.275 \pm 17.432$ |
| Lipo. (100 nm) without SPC3 | $142.233 \pm 13.840$ | $151.324 \pm 22.305$ |
| Cells without virus | – | – |
| Cells without peptide | $145.724 \pm 13.502$ | $159.348 \pm 20.789$ |

C8166 cells were infected with HIV-$1_{Hx10}$ in the presence of 10 μM SPC3 [either free (Free SPC3) or entrapped in 100 nm diameter liposomes (SPC3-Lipo. (100 nm)) or 250 nm diameter liposomes (SPC3-Lipo. (250 nm))]. Control liposomes were of similar size and phospholipid content as liposomal-SPC3. Symbols: –, no p24 production detected. Data are representative of three independent tests.

### *3.2. Effects of cholesterol incorporation in liposomes on the inhibition of HIV-1 infection of C8166 cells*

SPC3 was tested for antiviral effects either free or entrapped in various formulations of liposomes that varied in their cholesterol content. The cells were exposed to HIV-$1_{NL4-3}$ in the presence of a series of peptide concentrations. The peptide antiviral efficacy was assessed by (i) de visu cytopathic effects in the C8166 human T-cell line cultures exposed to HIV-$1_{NL4-3}$ (syncytium formation), and (ii) production of viral p24 antigen in culture supernatants. In the absence of any antiviral treatment, we observed the HIV-1-induced formation of $25 \pm 5$ and $100 \pm 21$ syncytia/well at days 4 and 7, respectively. In these conditions, 0.5 μM liposomal SPC3 with and without cholesterol fully inhibited syncytium formation. Similar inhibition by free SPC3 was obtained at a concentration of 5 μM, whereas 2 μM free SPC3 was only partially active at day 7 post infection with $20 \pm 3$ syncytia scored. At day 4 and 7 post infection, the cell-free supernatants were also monitored for viral replication by measuring the amount of p24 antigen. The HIV-1 infection was inhibited by more than 97.7% in the presence of 2 μM of free SPC3 ($230 \pm 10$ pg/ml of p24 vs. $10\,128 \pm 1011$ pg/ml of p24 at day 7 post infection) (Fig. 1). At a concentration of 0.5 μM liposomal SPC3 with cholesterol, and at a concentration of 5 μM free SPC3 totally inhibited the infection (undetectable levels of p24, i.e. $p24 < 5$ pg/ml vs. 10 128 pg/ml). SPC3 entrapped in liposomes with or without cholesterol exhibited similar antiviral efficacy. Thus, SPC3 entrapment in liposomes enhanced the antiviral efficacy by 10-fold under our experimental conditions. In addition, the p24 antigen production using the control peptide-free liposomes was similar to that obtained with control samples without peptide (data not shown).

### *3.3. Effects of dialysis of SPC3-liposomes on C8166 HIV-1 infection*

We have evaluated the potential effects of free SPC3 in the prepared SPC3-containing liposomes. For this purpose, samples of the various SPC3-associated liposomes were dialyzed to remove free SPC3, and were tested for antiviral activity. The efficacy of inhibition of HIV-1 infection by SPC3 entrapped in liposomes with or without cholesterol was not significantly different before and after dialysis (blocking the infection at 0.5 μM SPC3 (final peptide concentration)) (Fig. 2). Equivalent results were obtained in the antifusogenic studies in which similar concentrations of dialyzed or non-dialyzed SPC3-liposome solutions were necessary to inhibit the fusion and this independently of the size of the liposomes (data not shown). The equivalent antiviral efficacy of dialyzed and non-dialyzed SPC3-liposomes at 0.5 μM raises two different possibilities with regard to the observation that, during dialysis, 90% of the total SPC3 can be removed (i.e. a major fraction of SPC3 that is either 'free' or 'weakly' liposome-associated). First, if one considers that 90% of SPC3 is in a free form in non dialyzed SPC3-liposomes then free SPC3 in the absence of liposomes is expected to be as active as entrapped SPC3. Second, if both free SPC3 in the absence of liposomes

and liposomal entrapped SPC3 have different antiviral efficacies, then, in the presence of liposomes, non trapped SPC3 is likely to be in a 'weakly' liposome-associated state and probably gains greater efficacy. We tested these two possibilities in the next set of experiments.

### 3.4. Dose–response effects of free SPC3, and dialyzed SPC3-liposomes with or without cholesterol on C8166 HIV-1 infection

Fig. 3 illustrates that SPC3 entrapment into liposomes improves its antiviral efficacy by 6 (SPC3-Lipo) to 23-fold (SPC3-Lipo-Chol) as determined by 50% inhibition of p24 production in cell supernatants. Interestingly, the addition of cholesterol did not markedly improve the antiviral efficacy of the liposomal formulation. This suggests that liposome stability is not a crucial factor for the improvement of the antiviral properties brought by the SPC3 liposomal entrapment. Overall, these experiments apparently validate the second possibility that free SPC3 is not as active as liposome-entrapped SPC3 or SPC3 'weakly' associated to liposomes. This observation was further evidenced by the next set of experiments in

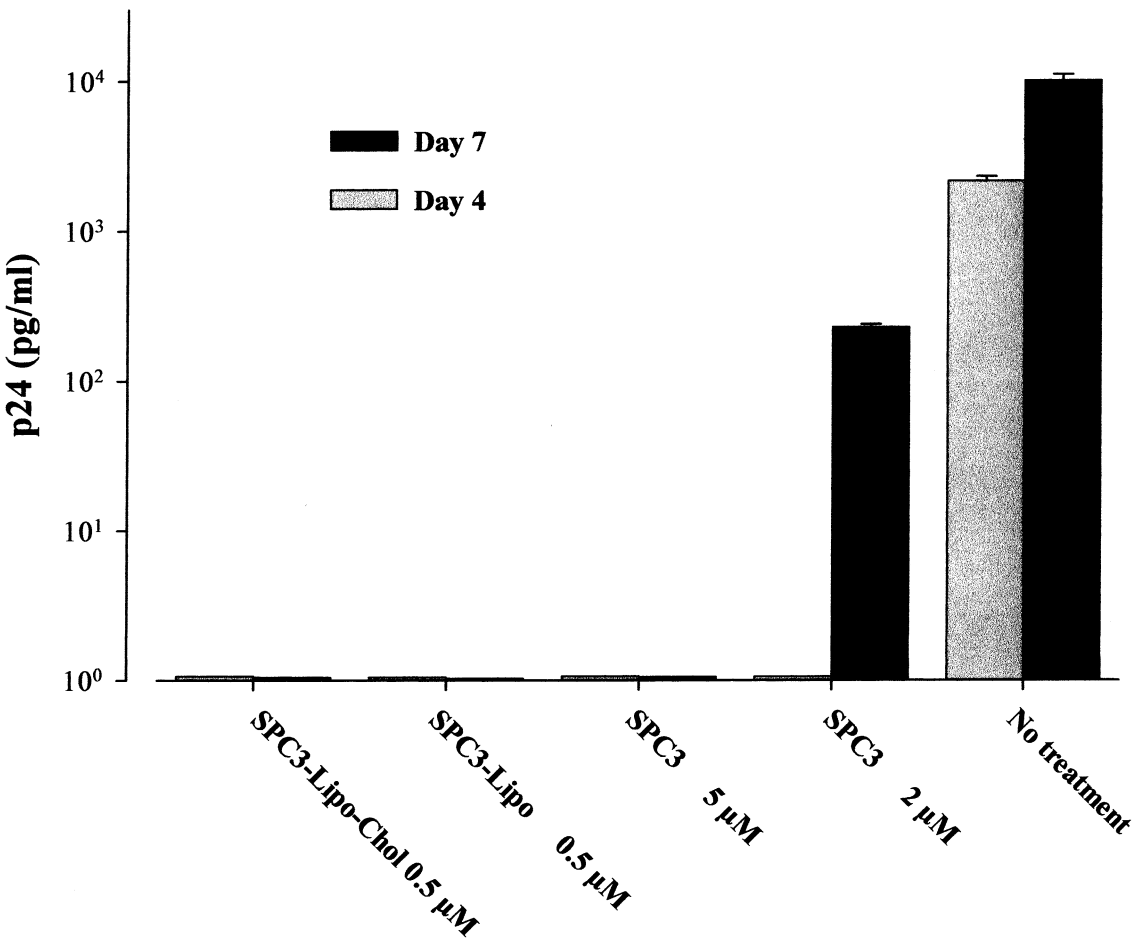



Fig. 1. Inhibition of HIV-1 infection in C8166 cells with free SPC3 or SPC3 entrapped in liposomes containing or not cholesterol. C8166 cells were exposed to HIV-$1_{NL4-3}$ in the presence of various concentrations of free-SPC3 or SPC3 entrapped in liposomes with (SPC3-Lipo-Chol) or without (SPC3-Lipo) cholesterol, as described in Section 2. HIV production was assessed by measurements of HIV-1 $p24^{gag}$ in the culture supernatants at days 4 and 7 post infection. No toxicity was detected at the peptide concentrations tested. Experiments were performed with non-dialyzed liposomes.

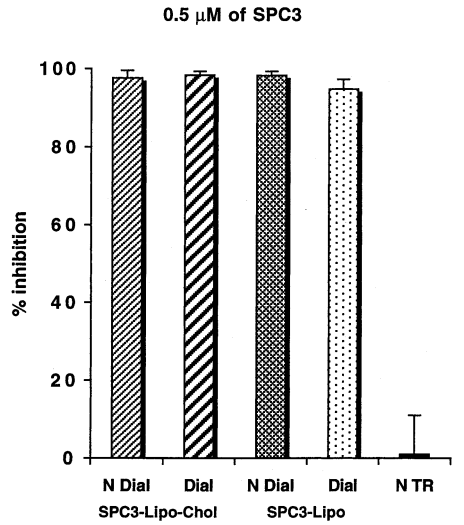



Fig. 2. Effect of dialysis on antiviral properties of liposome-associated SPC3. C8166 cells were exposed to HIV-$1_{NL4-3}$ in the presence of 0.5 μM SPC3 entrapped in liposomes containing either cholesterol (SPC3-Lipo-Chol) or not (SPC3-Lipo). The antiviral effects of liposomes containing SPC3 were measured before (N Dial) and after dialysis (Dial). The SPC3 concentrations mentioned are final ones (i.e. before and after dialysis). At day 7 post infection, viral p24 in the culture supernatants was quantified and the ratios between treated and non-treated (NTR) samples were plotted as percentage of virus inhibition. P24 production in the untreated samples was $10.1 \pm 1.0$ ng/ml. The experiment was performed in duplicate.

which SPC3 (1 mM) and SPC3-free liposomes (60 mM phospholipids) were incubated in 300 mM sucrose, and where the antiviral effects of serial dilutions were assessed. The preparations containing 2 μM of SPC3 were able to totally block the viral infection of C8166 cells. These results suggest that the antiviral effects observed in non-dialyzed SPC3-liposome preparations were mostly associated with SPC3 non-covalently attached to the liposome surface.

### *3.5. Effects of time of addition of SPC3 and SPC3-liposomes on C8166 HIV-1 infection*

To investigate whether the liposomal formulation could affect the mode of action of SPC3, comparisons of free SPC3 and SPC3 entrapped in liposome were performed with regard to the virucidal or antiviral effects obtained when drugs were added at different times of infection. Antiviral activity of SPC3-liposomes, and of free SPC3, was not associated with cytotoxicity or virucidal effects (data not shown). In the case of liposomal SPC3, we did not notice any significant decrease or increase in cellular toxicity as compared with free SPC3. Toxicity to relevant cells (e.g. human PBL, C8166) was not detected to any significant extent at SPC3 concentrations below 20 μM (free SPC3 or SPC3 entrapped in liposomes) (see also Yahi et al., 1994). Also, pre-incubation of the cell with SPC3 or SPC3-liposomes did not affect viral infectivity (data not shown). Results concerning the time of action clearly showed that SPC3 was more active when added post infection with HIV-$1_{NL4-3}$ (Fig. 4). Similar antiviral effects of free SPC3 or SPC3-liposomes were obtained when the drug was added from 0 to 4 h post infection.

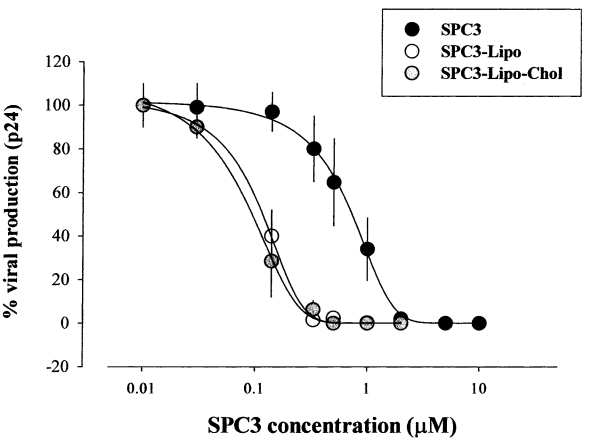
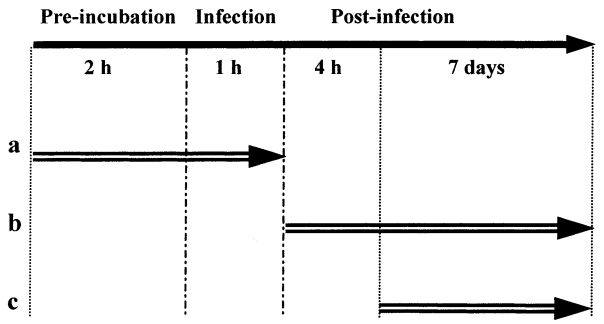



Fig. 3. Dose–response effect of free SPC3, and dialyzed SPC3-liposomes with or without cholesterol. C8166 cells were exposed to HIV-$1_{NL4-3}$ in the presence of various concentrations of free SPC3 (SPC3) or dialyzed SPC3-liposomes with (SPC3-Lipo-Chol) or without (SPC3-Lipo) cholesterol. At day 7 post infection, viral p24 in the culture supernatants was quantified by ELISA. P24 production in the untreated samples was $10.1 \pm 1.0$ ng/ml. Half-inhibition of p24 production occurred at SPC3 concentrations of 0.51 μM (SPC3), 0.08 μM (SPC3-Lipo) and 0.03 μM (SPC3-Lipo-Chol). The experiment was performed in duplicate.




| HIV production (pg/ml) | |
|---|---|
| SPC3-Lipo 2 μM | SPC3 10 μM |
| 16,661 ± 2,965 | 12,488 ± 2,440 |
| 0 | 777 ± 527 |
| 3.5 ± 3.5 | 28 ± 7 |

Fig. 4. Effect of time of addition of free-SPC3 and dialyzed SPC3-liposomes on HIV-1 infection in C8166 cells. The arrows indicate the time and duration of treatment of C8166 cells with 10 μM free SPC3 or 2 μM liposome-associated SPC3. Period of treatment: (a) 2 h before infection and at the time of infection, or (b) immediately after infection and during 7 days of culture, or (c) 4 h after infection and during 7 days of culture. The state of infection of the cells was assessed at day 7 after infection by measurement of viral p24 antigen in the culture supernatants of treated and non-treated samples. This experiment is representative of $n = 2$ experiments performed in duplicate.

### *3.6. Inhibition of HIV-1 infection of human peripheral blood lymphocytes (PBLs) with SPC3 or SPC3-liposomes*

We next determined whether SPC3 in liposomes inhibited HIV-1 infection of human PBLs. Human PBLs were exposed to the virus in the absence or presence of various concentrations of peptide. The peptide was present during the course of infection and the following days of cell culture. No toxic effects on cells were detected as measured by daily cell count, trypan blue exclusion and XTT assays. The SPC3-liposome-induced inhibition of viral infection was dose-dependent, with complete inhibition obtained at 2 μM peptide concentration (Fig. 5).

SPC3 entrapped in liposomes with cholesterol showed greater anti-HIV potency than the free form of SPC3. When 2 μM of SPC3 either associated with liposomes or not was used in the assay, the viral inhibition was complete at day 14 post infection only for SPC3-liposomes, contrary to free SPC3 (Fig. 6). Liposomes without SPC3 (control liposomes) did not affect viral production after infection of the PBLs (Fig. 6). Concentrations of SPC3 required for a complete inhibition of PBLs infection were in the range of 10 μM for free SPC3 and 2 μM for SPC3 associated with liposomes. This 5-fold gain in antiviral efficacy was also observed when comparing partial inhibition of HIV infection.

## 4. Discussion

The goal of this work was to determine whether liposomal entrapment of the anti-HIV peptide SPC3 affects its antiviral properties in vitro. We studied the effects of the size and composition of liposomes that were likely to enhance the antiviral effects of SPC3 in vivo. We found that liposomal entrapment of SPC3 enhanced its ability to inhibit in vitro (i) the HIV-1 cell-to-cell transmission (human lymphocyte T-cell line C8166), as well as (ii) the C8166 cell infection with different strains of HIV-1. We then demonstrated that an increased antiviral effect was also observed during infection of human PBLs.

In cell cultures, the difference in antiviral efficacy observed between 100 and 250 nm SPC3-liposomes is not statistically significant. However, treatment with 250-nm SPC3-liposomes suppressed all infectivity, suggesting that they may possess a superior antiviral potency. This apparent effect may simply be due to a better settling of the liposomes next to the lymphocytes in culture

and may not necessarily translate to a greater antiviral efficacy in vivo. Obviously, an in vivo study of the antiviral efficacy of diverse liposome sizes would nicely complement the present analysis. The insight gained from preclinical and clinical studies of different liposome drug delivery systems evidenced that the biodistribution and disposition in vivo may vary depending on the route of administration, the vesicle size, its surface charge, and lipid composition (Allen and Hansen, 1991; Allen et al., 1991, 1993; Litzinger et al., 1994; Lian and Ho, 2001). The liposome mean diameter was found to greatly influence the lifetime and tissue targeting of liposome-encapsulated drugs. For example, the rate of liposome uptake by cells of the reticulo-endothelial system was reported to increase with the size of the vesicles (Hwang, 1987). When the size of the lipid vesicles diminished, the drug retention in vitro and in vivo was also found to be reduced for liposomes encapsulated with 2′,3′-dideoxyinosine (Harvie et al., 1995, 1996). Finally, liposomal size may also affect both lymphatic uptake and lymph node localization when administered subcutaneously (Oussoren et al., 1997). However, it should be noted that the effects of liposome size may vary with changes in lipid composition and/or drug content of the liposomal preparations (Lian and Ho, 2001).

Liposomes with cholesterol are more stable in vitro and in vivo and are therefore better candidate for therapeutic use than liposomes without cholesterol (Kirby et al., 1980). The fraction of cholesterol that is included in the lipid formulation increases vesicle rigidity in a similar way as the addition of sphingomyelin, and reduces serum-induced instability caused by the binding of

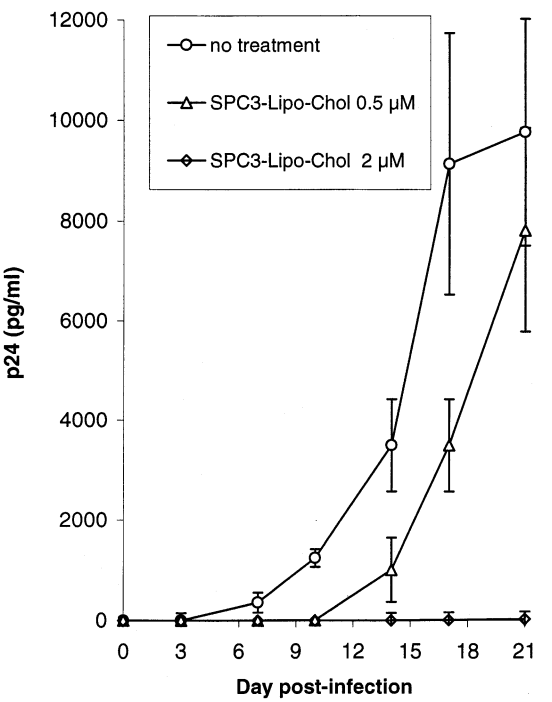



Fig. 5. Inhibition of HIV-1 infection in human PBLs with dialyzed SPC3 cholesterol-containing liposomes. Human PBLs were infected in the absence (no treatment) or presence of various concentrations of SPC3 entrapped in cholesterol-containing liposomes (SPC3-Lipo-Chol). Experiments were performed in duplicate and no cell toxicity was detected at the peptide concentrations tested.

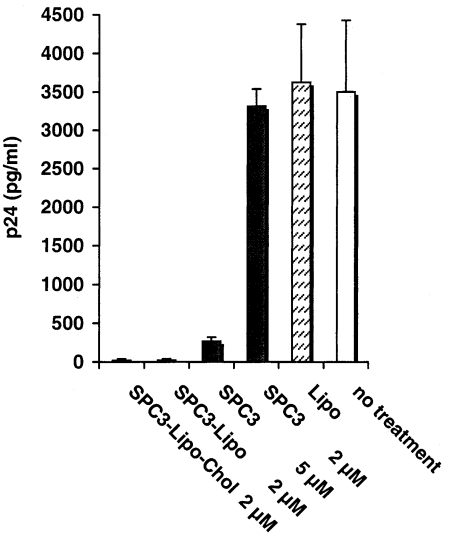



Fig. 6. Inhibition of HIV-1 infection in human peripheral blood lymphocytes (PBLs) with SPC3 or dialyzed SPC3-liposomes. PBLs were infected with HIV-$1_{NL4-3}$ in the absence or presence of the following SPC3 formulations: free-SPC3 (SPC3); SPC3 entrapped in cholesterol-free (SPC3-Lipo); or in cholesterol-containing liposomes (SPC3-Lipo-Chol). Methods of infection and treatment are described in Section 2. HIV production at day 14 post infection with treatments, using the indicated concentrations of SPC3, was determined by monitoring of HIV-1 $p24^{gag}$ in the culture supernatants (NEN Dupont kit; cut-off 5 pg/ml). Control was performed with liposomes without SPC3 (Lipo). $N = 2$ experiments performed in duplicate.

some serum proteins (e.g. albumin, lipoproteins) to the liposomal membrane (Allen et al., 1991; Lian and Ho, 2001). Also, recent data have shown that incorporation of cholesterol decreases the rate of transport of organic cations across the liposome bilayers (Yan and Eisenthal, 2000). In spite of the effect of cholesterol on liposomal stability, there was no significant difference between the effects of the positively-charged SPC3 peptide entrapped in liposomes with or without cholesterol. Therefore, these data suggest that liposomal stability in vitro has little, if any, effect on the antiviral efficacy of entrapped SPC3. In fact, the stability of the liposomal formulation did not exceed more than a few hours (5–6 h) at 37 °C in cell culture medium. Nevertheless, the improved antiviral efficacy of liposomal SPC3 as compared with free SPC3 is not put into question by stability considerations. First, it is unlikely that improved efficacy would result from SPC3 leakage from degraded liposomes, since liposomal SPC3 by itself is more efficient than free SPC3. Second, previous reports focusing on the antiviral efficacy of SPC3 have shown that SPC3 was effective when added for 2 h post infection as when present for 7 days (Yahi et al., 1995; Fantini et al., 1996). If, as expected, this property is applicable to liposomes, these data would suggest that the maximal antiviral efficacy of liposomal SPC3 would be concordant with full liposome integrity.

Similar antiviral effects were obtained when free SPC3 or SPC3 entrapped in liposomes were added at different times of the infection cycle, indicating that liposomal entrapment did not apparently alter the mechanism of action of SPC3. It has been reported that SPC3-induced inhibition occurs just after infection (Yahi et al., 1995), which is in agreement with our data. The increased efficacy of liposomal SPC3, as compared with free peptide, raises the question of the mechanism of action of SPC3. Yahi et al. (1994, 1995) have suggested that SPC3 may be directed against cell surface components and may interfere with fusogenic effects and viral fusion. In such a mechanism of action, improved efficacy of liposomal SPC3 would depend on SPC3 when 'strongly' associated to the external surface of the liposomes. Although most of the studies on liposome-encapsulated drugs were achieved with molecules acting on intracellular targets, a recent study has shown an enhanced activity of liposomal IL-2 on its extracellular target in vitro (Kedar et al., 2000). This observation suggests that liposomal formulations may not only be beneficial to intracellular drug delivery but could also improve membrane receptor targeting. Two alternative mechanisms of improved antiviral efficacy of liposomal SPC3 can be evoked: (i) peptide entrapment in the liposomes contributes to a protection from a degradation in the cell culture medium, or (ii) improved intracellular delivery of SPC3. With regard to the greater antiviral efficacy of both free and liposomal SPC3 when added post infection, several explanations can be provided. First, it is possible that the continuous activation of G protein-coupled CXCR4 receptors may induce some intracellular signaling cascade(s) that directly or indirectly interfere with viral replication. Indeed, recent data suggest that CXCR4 expression and SPC3-induced receptor activation in *Xenopus* oocytes, are followed by a phospholipase $A_2$-dependent signaling cascade itself triggering a chloride current (Carlier et al., 2000). Here, the SPC3 target would thus be a membrane-associated chemokine receptor such as CXCR4 (Moulard et al., 1999). A second possibility relies on the progressive intracellular accumulation of SPC3 to block viral replication (Barbouche et al., 1998). Following such a mechanism, SPC3 could interfere with the normal HIV replication cycle at (a) step(s) involving the SPC3-like V3 loop of the gp120 protein during virus biosynthesis.

Different results have been obtained regarding the effect of drug encapsulation in liposomes to improve drug delivery into lymphocyte. Liposome-entrapped L-689 502, an inhibitor of HIV-1 viral protease, was less active than the free drug in chronically infected H9 cells, but was more active in monocyte-derived macrophages (Pretzer et al., 1997). Thus, in agreement with our results, liposome entrapment of active molecules may enhance their effects on lymphocytes. The liposomal interleukin-2 (IL-2) formulations were more efficient than soluble IL-2 in stimulating spleen cell proliferation and lymphokine-activated killer (LAK) cells (Kedar et al., 2000). The most striking effect was obtained with antisense oligonucleotides di-

rected against the *rev* regulatory gene of HIV-1. A lipidic formulation was found to improve the inhibitory activity in acutely infected primary cells by 1000-fold and in chronically infected H9 lymphoblastoid T-cells by 1 500 000-fold (Lavigne and Thierry, 1997).

Inhibition of macrophage infection, by free or liposome-encapsulated anti-HIV drugs, has also shown different results. The antiviral efficacy of liposome-encapsulated 2′,3′-dideoxyinosine (ddI) was lower than that of the free drug in the HIV-1-infected U937 cell line (Desormeaux et al., 1994). More recently, the viral protease inhibitor L-689 502 was shown to be more effective when it was encapsulated in liposomes during a prolonged treatment of macrophages (Pretzer et al., 1997). Our preliminary data indicate that liposome-associated SPC3 completely inhibits HIV-1 infection of human monocyte-derived macrophages at concentrations below the micromolar range. Thus, regarding a potential SPC3-based anti-HIV treatment with liposomes, the two major target cell types, i.e. lymphocytes and macrophages, should be efficiently protected.

Liposomal delivery of drugs may improve their pharmacokinetic properties and bioactivities in vivo (Bakker-Woudenberg et al., 1990). Studies on liposome-encapsulated reverse transcriptase inhibitors have shown the superiority of such liposome formulations. The plasma half-life of liposome-encapsulated ddI was shown to be 46 times higher than that of the free drug (Harvie et al., 1995). The systemic clearances of ddI and foscarnet were, respectively, 180 and 77 times slower than those of the free drugs (Harvie et al., 1995; Dusserre et al., 1995).

The application of liposome-associated SPC3 in human monocyte-derived macrophages, and the effects of this formulation on infection with HIV clinical isolates will be the matter of further studies in our laboratory.

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