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Lipid vesicles loaded with thymopentin: characterization and in vitro activity on tumoral cells

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Summary

The cytotoxic activity on tumoral cells of thymopentin (TP5), a synthetic pentapeptide corresponding to residues 32–36 of thymopoietin, was evaluated after inclusion in multilamellar liposomes. These were made by all neutral (zwitterionic) phospholipids as well as those containing positively (stearylamine, SA) or negatively charged (DPPS) lipids. Vesicles were also prepared containing cholesterol, and were characterized by light scattering dimensional analysis and differential scanning calorimetry. The effect of charged species and of the presence of cholesterol on TP5 incorporation was studied. For the different kinds of liposomes, loaded with sodium fluoresceinate, the rate of interaction after incubation with lymphocytes in a normal or tumoral (IM9 lymphoblastoid cells) state was also evaluated. TP5-loaded vesicles were assayed on two lines of tumoral cells (IM9 and K562) to assess the ability of liposomal carriers to potentiate the cell growth inhibitory activity of the drug. SA-containing positive liposomes appeared as the most efficacious, resulting in 87% inhibition on IM9 cells, with an amount of liposomes containing 100 ng of TP5.

Introduction

In a preceding study, we investigated the feasibility of liposomes as carriers for an immunomodulatory agent extracted from calf thymus, thymostimulin (Ts). Drug loaded vesicles were characterized for their encapsulation efficiency and stability (Panico et al., 1992) and their in vitro evaluation showed liposome-incorporated Ts to be more effective than the free drug in inhibiting growth of a tumoral cell line (Panico et al., 1991). In the last few years, biochemical research has led to the isolation and preparation of even smaller fragments of natural thymic hormones, which retain their efficacy and specificity. Among them, Goldstein and co-workers (Goldstein, 1974; Basch and Goldstein, 1974; Scheid et al., 1978) have characterized a pentapeptidic compound, corresponding to residues 32–36 of thymopoietin, named thymopentin or TP5. TP5 (Arg-Lys-Asp-

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Val-Tyr) elicits the biological activity of thymopoietin, i.e., its ability to induce T-cell differentiation and maturation and to affect immunoregulatory balance (Goldstein et al., 1979; Ho et al., 1983; Goldstein and Audhya, 1985) and thus was considered to represent its active site.

Preliminary clinical studies showed that treatment with TP5 restores the immunological responsiveness of patients with different diseases, such as primary immunodeficiences, chronic viral affections, dermatological diseases, rheumatoid arthritis and malignancies (Lau et al., 1982; Thrower et al., 1982; Aiuti et al., 1983; Davies et al., 1983; Przybilla et al., 1983; Szende et al., 1987; Nagel et al., 1988). Moreover, TP5 could be able to counteract the immunosuppressive side effects of anti-tumor chemotherapy (Mallmann and Krebs, 1991).

Within our current interest in liposomes as carriers and for cellular release of biologically active agents, in the present work we have prepared a series of liposomal formulations, with different lipid compositions and containing TP5, in order to assay their influence on TP5 activity. For this purpose, we planned to evaluate its in vitro inhibitory effect on cell growth, using two different cultures: a human erythroleukemic line (K562) (Lozzio, 1981) and a human lymphoblastoid cell line (IM9) (Fahey et al., 1977), with the aim of observing both the influence of different liposomes composition, e.g., the overall surface charge and the presence of cholesterol, and the ability of liposomal carriers in introducing TP5 into cells of different kinds.

In a preliminary step, we have also investigated the possibility of discriminating the rate of interaction of liposomes with different composition, charge, size and fluidity, with cells in a normal or tumoral state. Thus, multilamellar liposomes (MLVs) containing a high concentration of sodium fluorescinate (SF) were incubated with human peripheral blood lymphocytes and IM9 lymphoblastoid cells; the consequent distribution of fluorescence throughout the cytoplasm of cells enabled us to monitor transfer of the dye from vesicles to cells and thus to determine the rate of interaction between them (Weinstein et al., 1977). The intracellular distribution of released material was determined by conventional fluorescence microscopy.

Materials and Methods

1,2-Dimyristoyl-L-α-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-L-α-phosphatidylcholine (DPPC), 1,2-dipalmitoyl-DL-α-phosphatidylserine (DPPS), cholesterol (Chol) and stearylamine (SA) were purchased from Sigma-Aldrich S.r.l., Milan (Italy) and were used as received. The absence of lysophosphatides was checked by two-dimensional TLC.

Thymopentin acetate (TP5) was a gift from Cilag S.p.A., Cologno Monzese (Italy). Purity was greater than 99% (HPLC).

Sodium fluoresceinate (SF) was obtained from Carlo Erba (Milan, Italy). Other chemicals were of analytical grade and doubly distilled water was employed.

Tissue culture supplies are from Flow Laboratories (Rockville, MD, U.S.A.).

Preparation of TP5-loaded liposomes

MLVs were prepared following the film deposition technique (Bangham et al., 1974). The compositions as molar ratio of the preparations were as follows: DPPC/SA (9:1); DPPC/Chol/SA (4 : 3 : 1); DPPC/DPPS (9 : 1); DPPC/Chol/DPPS (4 : 3 : 1); DPPC/DMPC (1 : 1); DPPC/ DMPC/ Chol (1 : 1 : 1), DPPC/Chol (2:1), and DPPC alone.

Lipid mixtures (10 mg), were dissolved in chloroform and the solvent removed at 30°C under a nitrogen stream. The resulting film was kept overnight at 30°C under high vacuum. Liposomes were obtained by adding 100 μg of TP5 in 200 μl of 0.9% NaCl solution; the mixture was heated at a temperature (70°C) above that of its gel-to-liquid crystal phase transition (T_m) to allow the full hydration of the sample and then vortexed twice for 3 min. The liposomal suspension was centrifuged for 15 min at 30 000 $\times g$ and at 4°C, in a 50 Ti type rotor of a Beckman L8-60 M ultracentrifuge, in order to separate the incorporated TP5 from the free form. This washing step was repeated twice.

Unloaded MLVs, used as controls for biological assays, were obtained in the same manner, but using only saline (200 μl) for rehydratation of the lipid film.

Determination of TP5

The amount of unassociated TP5 was determined by reaction of supernatant solution with tetranitrobenzenesulfonic acid (TNBS) (Fields, 1972) and evaluation of the increase in absorbance at 420 nm (Varian 640 Spectrophometer). Blanks (empty liposomes) were used as reference standards in order to correct for turbidity effects. A standard working curve was constructed daily from known concentrations of TP5. The incorporation of TP5 was determined by difference from the initial amount of drug added and was expressed as entrapment (%), i.e., the fraction of encapsulated drug relative to the initial amount of the drug in the solution:

$$E(\%) = [\mathrm{TP5}]_{\mathrm{enc}}/[\mathrm{TP5}]_{\mathrm{tot}} \times 100$$

Moreover, according to Benita et al. (1984), the encapsulation capacity (EC) was also calculated from the following equation:

$$\mathrm{EC} = \frac{C_a}{C_0 \cdot C_l}$$

where C_a and C_0 are the amounts (mmol/ml) of liposome-associated and initially added drug, respectively, and C_l denotes the concentration (mmol/ml) of lipids in the vesicles.

Incorporation results for the prepared formulations are reported in Table 1.

Preparation of SF containing vesicles

Fluorescent liposomes were prepared by the same procedure as described above. The fluorescent dye was added as 200 μl of a stock solution containing 0.6 μmol in 10 ml of a 0.9% NaCl solution. Free SF was separated by washing with saline and centrifugation at 30 000 $\times g$ (4°C for 15 min). From the resulting solution, the amount of unincorporated SF was determined by measuring the absorbance at 493.5 nm on a Varian 640 spectrophotometer.

Morphology and size analysis

Liposomes were examined under a photomicroscope (Zeiss III RS, Germany) for morphological evaluation. The method employed gives rise to a rather homogeneous population of multilamellar vesicles; no cluster or formation of crystals was observed.

Vesicle size was determined by photon correlation spectroscopy (PCS) light scattering analysis (Douglas et al., 1984). The apparatus consisted of an He-Ne Spectra Physic model 120 Laser (7 mW), a holding sample cell (PC8 Malvern) thermostated at 24°C by a Haake F3-R and equipped

TABLE 1

Composition, size analysis and TP5 entrapment of liposome formulations

Sample [a]	Composition [b]	Charge	Mean [c] size (μm)	P.I.	EC [d]	Entrapment (%) (±S.D.)
1	DPPC	neutral	3.1	1.1	8.44	56.0 (0.41)
2	DPPC/Chol, 2:1	neutral	–	–	8.74	70.3 (0.82)
3	DPPC/DMPC, 1:1	neutral	1.2	0.6	11.74	81.2 (0.31)
4	DPPC/DMPC/Chol, 1:1:1	neutral	–	–	8.70	72.6 (0.72)
5	DPPC/SA, 9:1	positive	1.1	0.5	11.55	88.0 (0.15)
6	DPPC/SA/Chol, 4:1:3	positive	2.5	0.2	9.98	89.8 (0.70)
7	DPPC/DPPS, 9:1	negative	1.1	0.3	10.01	68.8 (0.22)
8	DPPC/DPPS/Chol 4:1:3	negative	1.3	1.0	8.65	69.8 (0.25)

[a] Each liposome type was obtained in three separate preparations.
[b] Molar ratios.
[c] By light scattering analysis.
[d] ml/mmol.

with a Microcontrol precise mechanical goniometer and an optical system (Melles-Griot f.150); Hamamazu R 1333 and RCA 8852 photomultipliers were used. All the data from PCS analysis were correlated by a Malvern 4700 C particle analyzer connected to an Olivetti 240 computer. The scattering angles were 20 and 40°.

From the scattering behavior of vesicles, the quality parameter or polydispersity index (PI) (Pusey et al., 1974) was determined. This parameter, which can range from 0 to 9, for a monodisperse system shows values approaching 0 or for a polydisperse system higher values.

Size analysis results are gathered in Table 1.

Differential scanning calorimetry (DSC)

Calorimetric data were obtained with a Mettler TA 3000 differential scanning calorimeter, equipped with a DSC 30 cell and a TC 10 processor, using 120 μl aluminum pans. The plotting range, as full scale deflection, was set to 1.71 mW. Palmitic acid was used to calibrate the temperature scale and the ΔH. A pan containing 120 μl of Tris buffer (pH 7) was used as a reference. Samples and reference pans, placed in the DSC cell, were equilibrated at 5°C for 15 min. The heating scanning rate was 2.5°C min^{-1}. Enthalpy changes were calculated from the peak areas (Table 2).

TABLE 2

DSC results of the prepared vesicles

Sample	Empty vesicles		TP5-loaded vesicles	
	T_m (°C)	ΔH (kcal/mol)	T_m (°C)	ΔH (kcal/mol)
1	42.4	8.72	41.9	9.10
2	42.3	0.82	42.1	0.51
3	34.7	6.96	34.9	7.16
4	34.6	0.43	34.6	0.52
5	53.9	9.27	54.6	8.34
6	44.9	0.62	44.9	0.57
7	43.0	8.22	43.0	12.05
8	43.7	1.03	45.4	1.28

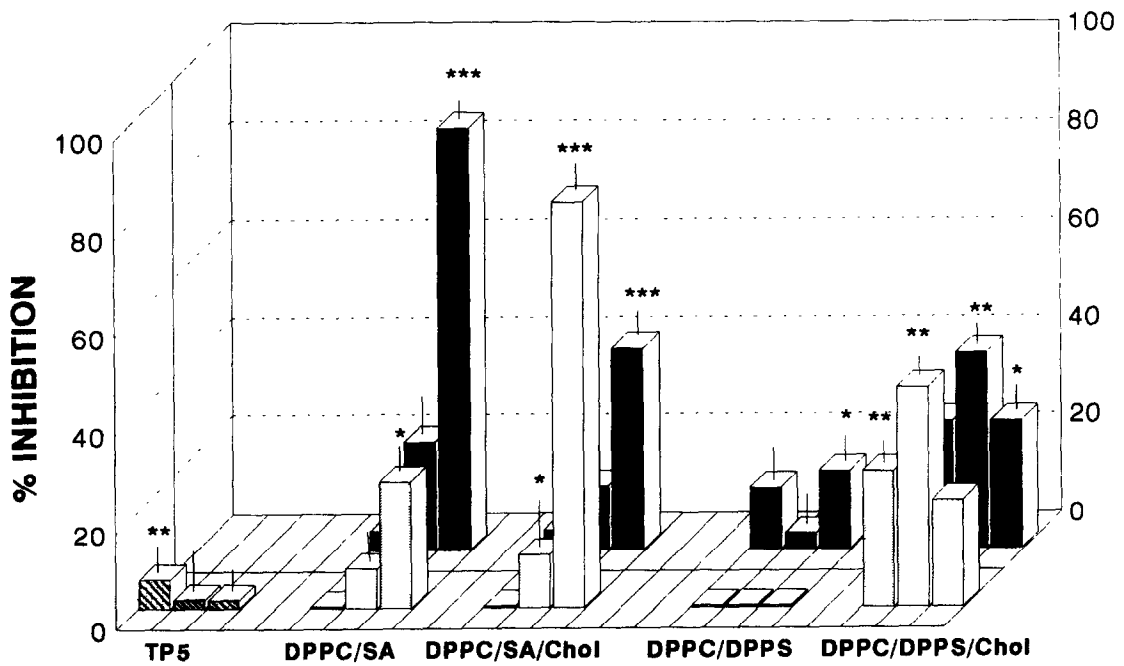

Fig. 1. In vitro activity on IM9 cells of positively and negatively charged TP5 liposomes: % inhibition (±S.D.) of cell growth vs controls. The absence of values indicates no inhibition or stimulation of cell proliferation. Each series of values refers to TP5 doses of 5, 50 and 100 ng, respectively. (Dotted bars) Empty vesicles, (filled bars) TP5-loaded vesicles. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Cells

Peripheral blood lymphocytes derived from normal donors were isolated on Ficoll-Hypaque gradients and cells were then washed three times and resuspended in RPMI 1640 medium (Gibco Labs, Grand island, NY, U.S.A.) with 10% heat inactivated fetal calf serum.

Aliquots of cell preparations were plated on 50 mm plastic Petri dishes at a concentration of 1.5×10^6 cells/ml and suspended in culture medium. Cells were incubated at 37°C for 60 min, and thereafter non-adherent cells were removed and used as pure lymphocytes.

K562 human erytrholeukemic cells and the IM9 human lymphoblastoid cell line were grown in 25 ml plastic flasks with 5 ml of RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum and 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamycin. Cells were cultured in a humidified atmosphere of 5% CO_2-air mixture. Cell transfer for subcultures was performed every 3 days.

Determination of cell proliferation rates

Cells (12×10^3) suspended in 0.1 ml of complete medium were set up in round bottomed microplates and kept in culture for 72 h. Three different doses (5, 50 or 100 ng/ml) of TP5, both free or encapsulated in the various liposomes, were added to microplates in 0.1 ml medium. For each dose of every compound to be tested, four replicates were performed. 6 h before the end of the culture, 1 μCi of [^{3}H]TdB was added to each microplate. Cells were harvested by a multiple cell-culture harvester (Flow Labs, Rockville, MD, U.S.A.) and radioactivity (cpm/min) was detected by liquid scintillation counting (Beckman Instruments, Nyon, Switzerland). Results are reported in Figs 1 and 2.

SF-liposome uptake studies

Both lymphocytes and IM9 cells were harvested by centrifugation and washed twice with 0.9% saline. Usually 1×10^8 cells were resuspended in 10 ml RPMI 1640 medium containing

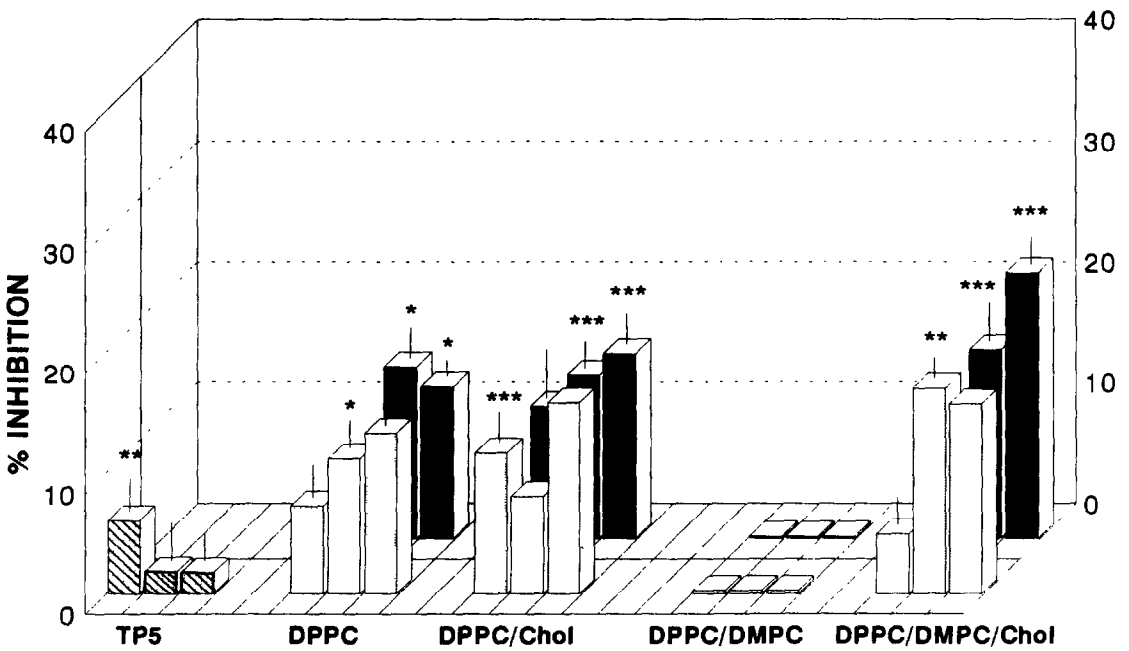

Fig. 2. In vitro activity on IM9 cells of neutral TP5 liposomes: % inhibition (±S.D.) of cell growth vs controls. The absence of values indicates no inhibition or stimulation of cell proliferation. Each series of values refers to TP5 doses of 5, 50 and 100 ng, respectively. (Dotted bars) Empty vesicles, (filled bars) TP5-loaded vesicles. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

10% heat inactivated fetal calf serum and incubated for 5 h at 37°C.

Liposomes containing SF were added to a final concentration of 1.2 μmol/ml. After 0.5, 1, 1.5 and 5 h, 500 μl of the suspension were removed and placed in 2 ml of ice-cold 0.9% saline. The samples were then centrifuged at $200 \times g$ and washed four times with 5 ml of cold saline. The final pellet was suspended in 0.5 ml saline. The intracellular distribution of released material was determined by conventional fluorescence microscopy. Results were expressed as the percentage of cells that became fluorescent (Figs 3 and 4).

Results and Discussion

Liposome preparation and features

Following the lipid 'film' method here adopted, a quite homogeneous population of multilamellar liposomes (MLVs) was obtained. Based on the components of the lipid mixture, positive ($MLVs^+$) or negative ($MLVs^-$) vesicles were prepared containing SA or DPPS, respectively, whereas uncharged liposomes ($MLVs^0$) were obtained from the zwitterionic DPPC and DMPC.

Light scattering analysis showed a mean size between 1.1 and 3.1 μm, with a very narrow dimensional distribution (< 1), indicating almost monodisperse systems (Table 1). The presence of any charge on the liposome surface seems to have no effect on their size, whereas Chol increases it to various extents.

As regards calorimetric analysis, DSC is known to represent a simple and precise methodology, useful for providing information about the level of interaction of a drug molecule with the membrane phospholipids (PLs). When applied to empty liposomes, DSC shows a main endothermal peak (T_m) corresponding to the 'gel-liquid crystal' phase transition. The associated enthalpy variation ΔH, describes the number of 'cooperative units', i.e., the number of PLs chains that change phase simultaneously, and thus takes account of the degree of penetration of the drug into the lipidic domain of bilayers (Lee, 1976).

For our formulations, T_m and ΔH values are reported in Table 2 for empty and TP5-loaded vesicles.

With respect to lipid composition and charge, vesicles containing positive or negative PLs all displayed a higher T_m than DPPC liposomes; the addition of Chol (33–37.5%, mol/mol), resulted in a depression of the transition peak, and thus in a strong reduction of ΔH, due to the well known 'fluidizing' effect of steroids on PL bilayers (Bach, 1984).

When TP5 was enclosed within liposome bilayers, in most cases neither T_m nor ΔH was observed to change markedly. A lack of modification of a PL transition temperature classically means a quite negligible interaction between the exogenous molecule and liposomes, i.e., the drug was located entirely within the interlamellar water spaces (Jain and Wu, 1977). The absence of significant ΔH variations confirmed this explanation.

Only in the case of DPPC/SA and DPPC/DPPS/Chol liposomes did the presence of TP5 induce a slight increase in T_m (about 0.7°C). Even if this behavior merits further investigation, it could be merely related to an interaction of the drug with the PL polar heads (Jain and Wu, 1977).

As regards the increase in ΔH induced by TP5 in DPPC/DPPS liposomes, as has been reported for many proteins (Papahadjopoulos et al., 1975), it could be explained in terms of a simple electrostatic interaction between the peptide and the negatively charged lipids, which stabilizes the bilayer without penetration of the drug into the liposome structure.

Encapsulation of TP5 into liposomes

The encapsulation capacity and percentage entrapment of TP5 in neutral or charged vesicles are given in Table 1.

EC is related to the fraction of aqueous volume entrapped in the vesicles (Benita et al., 1984), and can help in understanding the behavior of a hydrophilic molecule, like TP5, during liposome formation. In fact, polar compounds are unable to interact deeply with PL lipidic domains, and thus become located within interlamellar aqueous spaces.

The surface charge seems to affect the efficiency of TP5 entrapment: the presence of a charged species increases the spacing between the phospholipid bilayers, resulting in the enhancement of the volume of the aqueous compartments and the possibility of containing a greater amount of drug.

In particular, $MLVs^+$ displayed higher values of EC and incorporation. The presence of Chol in the matrix did not change the degree of TP5 entrapment: this could be explained by the fact that even if Chol modifies membrane fluidity, reducing the aqueous volume within the bilayers (lower ECs), the drug content is balanced by less leakage from the stabilized vesicles as well as by their greater sizes (Table 1), thereby confirming our previous findings (Panico et al., 1992).

As concerns DPPC liposomes, the relatively low EC and entrapment values, related to their greater size, could reflect the formation of a lower amount of liposomes but having a greater number of lamellae/vesicle and thus a reduced entrapped aqueous volume.

Finally, DPPC/DMPC vesicles show size, EC and entrapment values comparable to those of $MLVs^+$. If such results are related to the lower T_m of these vesicles, and consequently a greater fluidity which can favor inclusion of the drug during the formation of the liposomes, our previous data (Panico et al., 1992) anyway indicate this property to have an adverse influence on vesicle stability and drug retention.

However, it is worth noting that the values of EC and % drug incorporation obtained for TP5-loaded DPPC (EC = 8.44; entrapment = 56%) and DDPC/DMPC liposomes (EC = 11.74; entrapment = 81.2%) are very closely comparable to those found for the same formulations containing thymostimulin (DPPC: EC = 8.42, entrapment = 55%; DPPC/DMPC (1:1): EC = 12.16, entrapment = 83%) (Panico et al., 1992). Such results, besides providing evidence of the reproducibility of our liposome preparations, might indicate that compounds with a similar nature, regardless of size and molar weight, can behave in a quite similar manner, even from a quantitative view-

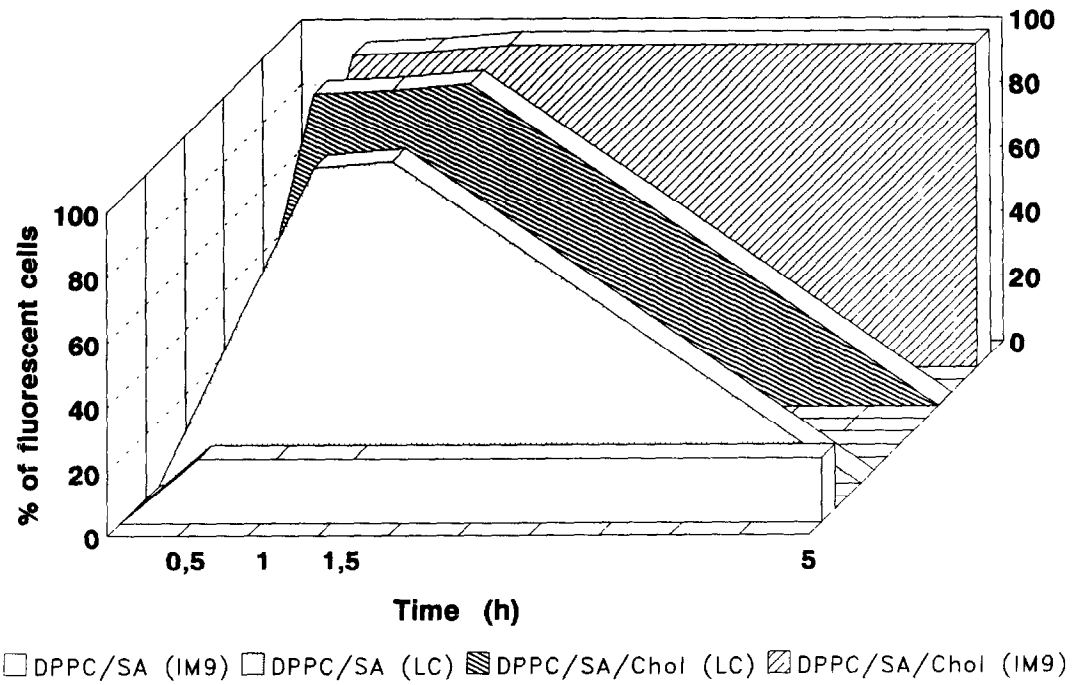

Fig. 3. Uptake of sodium fluoresceinate from positively charged vesicles by lymphocytes (LC) and IM9 cells.

point, when interacting with the same PL bilayers.

Cellular uptake of SF-loaded liposomes

We investigated the effect of changes in composition on cellular liposome uptake, using SF as a probe molecule and fluorescence microscopy as the method to evaluate the intracellular release of vesicle content. Data in Figs 3 and 4 show the dependence of positive charge in relation to the composition of the liposomes used on lymphocytes and IM9 cells.

Charged liposome species showed a time-dependent increase in internalized SF. Almost all the dye was taken up after 1 h of incubation.

Cells incubated with SF vesicles showed a generalized distibution of dye fluorescence, almost certainly indicating a release into the cytoplasmic space. Some cells also showed localized accumulation, and there were moderate differences in fluorescence intensity from cell to cell within a population.

Mixtures containing SA ($MLVs^+$) showed a lower affinity to IM9 than lymphocytes (Fig. 3); after 5 h, an evident death of lymphocytes, but not of IM9, was registered. The presence of cholesterol, on the contrary, makes both the cell lines able to totally take up the SF, but did not prevent toxicity of phospholipids upon lymphocytes.

DPPS-containing vesicles ($MLVs^-$) showed a higher rate of interaction for IM9 (60–100% of fluorescent cells) than for lymphocytes (0–40%) (Fig. 4); the introduction of Chol into the lipid matrix displayed a positive effect upon lymphocytes only, in that it reversed the selectivity ratio (IM9 vs lymphocytes) shown by DPPC/DPPS liposomes.

In the case of neutral DPPC and DPPC/DMPC vesicles, no interaction could be detected up to 5 h, between both types of cells and fluorescent vesicles (data not shown).

A similar trend, i.e., charged vesicles display a greater cellular uptake than neutral ones, has also been reported in the literature for murine macrophages (Hsu and Juliano, 1982; Stevenson et al., 1984), and could be explained as being due to the insufficient rate of cell association of lipo-

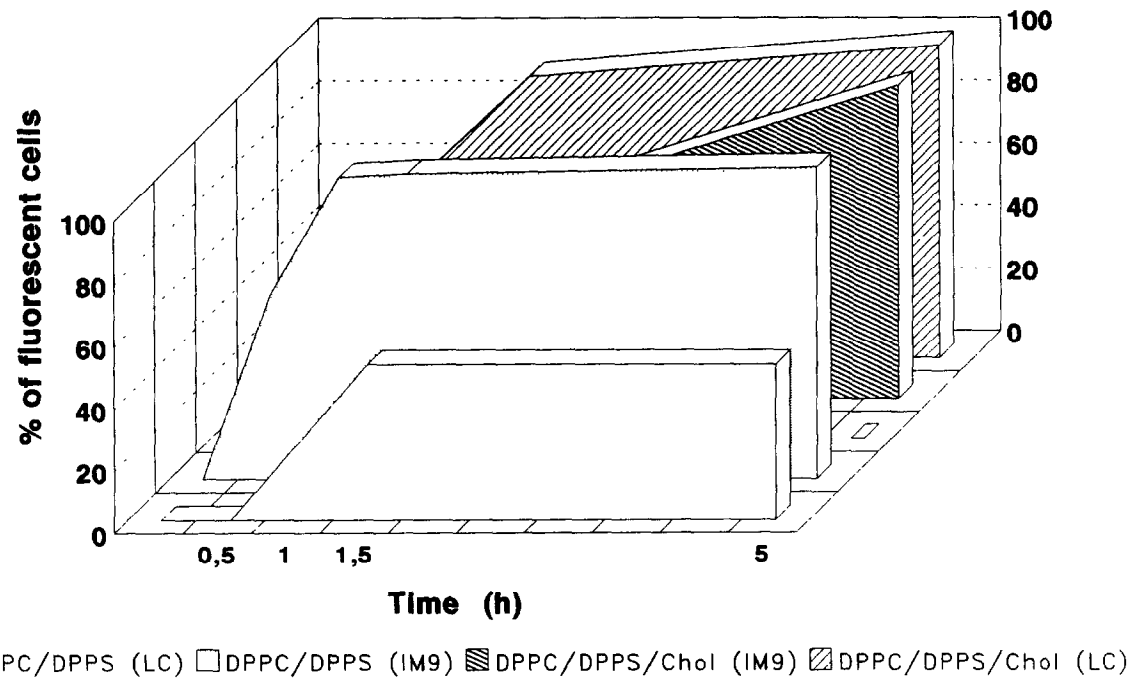

Fig. 4. Uptake of sodium fluoresceinate from negatively charged vesicles by lymphocytes (LC) and IM9 cells.

somes, the step that precedes their internalization.

In vitro activity of TP5-liposomes on cell growth

DPPC/SA liposomes appear to be the only type able to potentiate the cell growth inhibitory effect of TP5 on the IM9 cell line. At the higher dose (100 ng), liposome-associated TP5 showed 87% inhibition, considerably larger than that of the free drug (1.77%) and the empty vesicles (26%) (Fig. 1). At decreasing doses, TP5 was always more active when carried by liposomes, and at 5 ng dosage it remained doubly active compared to the free peptide. At all the doses, however, SA-containing formulations possess a cytotoxic effect on IM9 cells, implying the presence of SA in the lipid mixture, as confirmed by incubation of cells with empty vesicles.

Conflicting data exist in the literature concerning the cytotoxicity of SA-containing cationic liposomes. Although the toxic effects of SA must be related to its concentration in the culture medium, a value which was not always specified in the earlier papers (in our experiments, SA was present in the range 5–7 nmol/10^4 cells), the different sensitivity of various cell lines is evident. Thus, SA was toxic on leukemic L1210 cells (Panzner and Jansons, 1979), HeLa (Magee et al., 1974), and EMT-6 cells (Dunnick et al., 1976), whereas no inhibition of proliferation of human leukemic HL-60 cells was reported (Alpar et al., 1990). Also in our case, cationic MLVs inhibited IM9 cell growth, but were devoid of toxicity on erythroleukemic K562 cells (see below).

In the case of MLVs$^-$, TP5 activity was only slightly enhanced by liposomes with respect to the free drug (Fig. 1). In fact, the results derived from empty and TP5-loaded vesicles are quite identical, particularly in the presence of Chol. This indicates that the observed degree of inhibition must be ascribed mainly to the cytotoxic effect of PLs. Moreover, the low activity of TP5 liposomes containing the anionic DPPS could be related to the fact that their negative charge results in their repulsion by the overall negative charge on the cell surface.

For both positive and negative formulations, however, the introduction of Chol into the vesicle composition seems to reduce the activity of loaded liposomes, but not the cytotoxicity of phospholipids. This agrees with other observations, i.e., that since Chol decreases the fluidity of PLs bilayers, it is possible that the resulting vesicles do not fuse with the cells as readily as the more fluid liposomes. They were not so efficient in delivering the drug to the cellular cytoplasm.

Neutral DPPC/DMPC liposomes did not show any cytotoxicity on IM9 cells, but did not appear to be able to potentiate TP5 antitumoral activity (Fig. 2). Both empty and drug-loaded vesicles, in fact, even stimulated cell growth, as compared to free TP5 and untreated cultures. Addition of 33% Chol to these formulations reversed such an effect, thus inducing a rise in the cytotoxicity of PLs (Fig. 2). Also, for DPPC and DPPC/Chol mixtures, no increase in TP5 activity was observed, since the cell inhibition observed can be ascribed to the toxic effect of the lipids (Fig. 2).

On the K562 cell line, TP5 did not show any growth inhibitory activity up to highest concentration used (100 ng), either when used free or in the liposomal suspension (data not shown). In the meantime, no inhibition of the viability and proliferation of these cells due to all the assayed vesicles was observed.

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