Interleukin-2-Containing Liposomes: Interaction of Interleukin-2 with Liposomal Bilayers and Preliminary Studies on Application in Cancer Vaccines

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Received January 6, 1993; accepted June 2, 1993

Interleukin-2 (IL-2) incorporation in liposomes was studied under different conditions. Information was obtained on the mechanism of interaction of glycosylated recombinant IL-2 with liposomal bilayers. This information was utilized to formulate liposomes with high levels of incorporated IL-2. Multilamellar vesicles were prepared by hydration of a lipid film with an IL-2 solution. The incorporation efficiency, measured with a bioassay after forced release of IL-2 from the vesicles, was strongly dependent on the charge of the liposomes and the pH and ionic strength of the hydration medium. Negatively charged liposomes composed of phosphatidylcholine/phosphatidylglycerol (9:1) and prepared with IL-2 dissolved in 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5, showed the highest incorporation efficiency (81%) among the investigated preparations. This type of liposome was selected for further study. Electrostatics play a crucial role in the process of IL-2 association with this type of liposome. Initial studies concerning induction of protective tumor immunity by immunization with reconstituted membranes with muramyl tripeptide phosphatidylethanolamine indicate that coinjection of IL-2-containing liposomes provided a significant enhancement of the immune response.

KEY WORDS: liposome; interleukin-2; interleukin-2/bilayer interaction; protein/bilayer interaction; cancer vaccine.

INTRODUCTION

Interleukin-2 (IL-2), a globular glycoprotein with a molecular weight of approximately 16 kD, has numerous immunoregulatory properties. The potential of both glycosylated and unglycosylated IL-2 for treatment of cancer and infectious diseases and as an adjuvant for vaccines has been under investigation for several years (1,2). For cancer treatment high systemic doses of IL-2 are usually used in order to achieve adequate antitumor activity (1). However, this causes severe side effects, whereas the therapeutic results remain limited. As IL-2 is rapidly cleared from the circulation (3), different attempts have been made to increase the efficacy of IL-2 and to avoid or reduce the serious side effects of the high systemic doses by time- and/or site controlled delivery of the drug (4–6).

One approach to optimize IL-2 delivery is the formulation of the protein in liposomes. Liposomes are biodegradable, relatively nontoxic, and versatile colloidal structures. They can serve as a depot system for the sustained release of associated drugs and alter the biodistribution of the drug. For IL-2 this should lead to prolonged exposure of target cells to IL-2 and to induction of enhanced immunostimulating effects. Some recent papers have already demonstrated that, compared to free IL-2, liposome-incorporated IL-2 can induce increased therapeutic efficacy against murine tumors (6–8). In addition, IL-2 liposomes have been successfully used as adjuvants for vaccines (9–11). Although several protocols for the preparation of IL-2 liposomes have been employed, little attention has been paid to the mode of IL-2 association with liposomes (bound to the bilayers of the vesicles by electrostatic and/or hydrophobic forces and/or entrapped in the aqueous interior of the liposomes) and to the physical and chemical properties of IL-2 liposomes.

In this study the mechanism of interaction of glycosylated recombinant IL-2 with liposomes is investigated in order to obtain stable liposomes with a high incorporation efficiency of active IL-2 for *in vivo* use. It was hypothesized that at pH values below the isoelectric point of IL-2 [7.0/7.6 (12)], electrostatic attraction between IL-2 and negatively charged bilayers would be sufficiently effective to achieve high incorporation efficiencies of IL-2 in liposomes. Therefore, the influence of liposome charge and buffer pH and ionic strength on the extent of incorporation of IL-2 into multilamellar vesicles (MLV) was investigated. In addition, initial findings are presented on the potential of IL-2 liposomes to improve immunization against cancer with reconstituted membranes prepared from tumor cell constituents.

MATERIALS AND METHODS

Materials

Recombinant IL-2 (sp act, about 2.0×10^7 U/mg) was a gift from Sanofi (Toulouse, France). This IL-2 is produced by mammalian CHO-cells in which the human IL-2 gene was inserted (12). Consequently, this IL-2 is glycosylated, which renders the molecule more hydrophilic than the unglycosylated IL-2 produced by prokaryotic systems. The isoelectric point of the glycosylated IL-2 is 7.0 (disialylated form) and 7.6 (monosialylated form) (12). IL-2 was diluted with buffer containing 0.1% bovine serum albumin (BSA). Buffer changes were performed by gel filtration of the IL-2 solution on a PD-10 column (Pharmacia, Uppsala, Sweden). Egg L-α-phosphatidylcholine type V-E (PC), BSA, fluorescein isothiocyanate–dextran (molecular weight 19 kD) (FITC-dextran), and MEM nonessential amino acid solution (100×) were obtained from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylglycerol (PG) was a gift from Nattermann GmbH (Cologne, Germany). Muramyl tripeptide phosphatidylethanolamine (MTP-PE) was donated by Ciba-Geigy (Basle, Switzerland). Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY) and was purified by the method described by Ralston *et al.* (13). RPMI-1640 and

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fetal calf serum (FCS) were obtained from Flow Laboratories (McLean, VA).

Preparation of Liposomes

Multilamellar vesicles containing IL-2 were prepared using the "classical film" method. A solution of PC and PG in chloroform/methanol (9:1) was evaporated to dryness with a rotary evaporator. Care was taken to form a thin lipid film with a homogeneous appearance. The film was kept at the rotary evaporator at 45°C for at least 15 min, followed by evacuation (below 2 kPa) in a desiccator for at least 1 hr. Glass beads were added and the film was hydrated with the IL-2 solution. Buffers with different ionic strengths and pH's were used (see Results). At this stage of preparation the liposome dispersion contained 10^5 or 4×10^5 U IL-2/mL and 40 m*M* phospholipid (PL). After incubation for at least 2 hr at 4°C, nonincorporated IL-2 was removed by diluting the liposome dispersion about 10 times with buffer without BSA and subsequent by centrifugation at 100,000*g* for 30 min. The pellet was washed with buffer without BSA, and the final pellet was suspended in buffer with BSA.

Empty liposomes (with 0.1% BSA but without IL-2) and liposomes with 5 m*M* CF or with 0.5 m*M* FITC-dextran were prepared following the same procedure with the following exceptions: empty liposomes were centrifuged only one time, and removal of CF and FITC-dextran was performed as described by Talsma *et al.* (14) for "retention of CF after a freezing/thawing cycle." Fluorescence measurements were performed on a LS-50 luminescence spectrometer (Perkin-Elmer Corp., Norwalk, CT) at an excitation wavelength of 492 nm and an emission wavelength of 518 nm. The ζ potential of the liposomes was calculated from the electrophoretic mobility of liposomes with the Smoluchowski equation (15). Mobilities were measured with a Malvern Zetasizer 2C unit (Malvern Ltd., Malvern, UK) at 25°C using a PC-3 cell (for dispersions with an ionic strength of 0.15 *M*) or a PC-4 cell (for dispersions with an ionic strength of 0.01 *M*).

Determination of the Percentage IL-2 Activity in Liposomes

The activity of IL-2 incorporated in liposomes was measured after forced release in a standard bioassay using the IL-2-dependent murine T-cell line CTLL-16 (16). Forced release was realized by sonication of liposomes in 25 m*M* sodium dodecyl sulfate (SDS) at 45°C during 15 min. The clear or slightly opalescent dispersion was diluted with culture medium, consisting of RPMI-1640 medium supplemented with 10% fetal calf serum, 0.25 mg/mL penicillin, 0.05 mg/mL streptomycin, 25 m*M* Hepes, 2.5×10^{-2} m*M* 2-mercaptoethanol, 2 m*M* glutamine, and 1% MEM nonessential amino acid solution, until a SDS concentration of 0.2 m*M* was reached. Further dilutions were prepared with culture medium with 0.2 m*M* SDS. Consequently, the SDS concentration in the bioassay remained constant. To 100 μL of serial dilutions of IL-2 (in triplicate), 100 μL of 2×10^5 washed CTLL-16 cells was added. After incubation for 45 hr at 37°C, viability and proliferation of CTLL-16 cells were determined by the colorimetric MTT assay as described by Mosmann (17). The percentage IL-2 activity incorporated in the liposomes was assessed against a mixture of IL-2 and empty liposomes treated identically to the IL-2-containing liposomes. A standard curve of IL-2 (also sonicated in SDS) was included in each assay as the presence of liposomes may interfere with the IL-2 bioassay (18).

Immunoprotection Studies

Reconstituted membranes were prepared as described previously (19). Briefly, crude membranes of murine SL2 lymphosarcoma cells were solubilized by incubation with 150 m*M* octylglucoside. Nonsolubilized material was removed by centrifugation. MTP-PE was solubilized in the supernatant and reconstituted membranes were obtained by detergent removal.

Groups of male DBA/2 mice (IFFA-Credo, France; 8–12 weeks old) were immunized s.c. at two sites on the chest in a total volume of 0.2 mL at day −20 and day −10. Immunization injections consisted of 0.13 ± 0.02 mg reconstituted membrane proteins (obtained from about 2.5×10^7 SL2 cells) supplemented with 20 μg MTP-PE. Free IL-2, IL-2 incorporated in liposomes, or empty liposomes were coadministered with the second immunization with reconstituted membranes on day −10, followed by daily s.c. injections at the immunization sites for 4 days. On day 0 mice were challenged i.p. with 10^5 syngeneic viable SL2 cells. Survival of the animals was monitored daily. The nonparametric Wilcoxon rank-sum test (two-tailed) was used to test the significance of differences in survival times of groups of mice.

RESULTS

Assay to Determine the IL-2 Activity in Liposomes

The incorporation efficiency of IL-2 in liposomes was assessed with an IL-2 specific bioassay. This implies that incorporation of irreversibly inactivated IL-2 (due to the preparation procedure or to association with the vesicles) is not measured. Therefore, incorporation efficiencies refer to the percentage liposome association of active IL-2. To release IL-2 from the liposomes, IL-2 liposomes were sonicated in 25 m*M* SDS at 45°C. Subsequently, the dispersion was diluted 250 times with culture medium to reduce the SDS concentration to a nontoxic level for the bioassay. Initial studies showed that a SDS concentration of 0.1 m*M* in the culture medium did not significantly affect the IL-2-dependent proliferation of CTLL-16 cells (Table I). Sonication of free IL-2 in 25 m*M* SDS or a mixture of free IL-2 and empty liposomes in 25 m*M* SDS yielded (almost) complete recovery of the IL-2 activity (Table I).

Incorporation of IL-2 in Different Types of Liposomes

IL-2 liposomes were first prepared by hydrating a film composed of the zwitterionic lipid PC with 10^5 U IL-2/ml phosphate-buffered saline (PBS), pH 7.3, and 0.1% BSA. This resulted in incorporation of only 8% of the initial IL-2 activity (Table II). The IL-2 incorporation was almost doubled when a negatively charged PL (PG) was included in the bilayers. Attempts were made to increase the incorporation of IL-2 in liposomes by lowering the pH of the hydration medium. For negatively charged liposomes, electrostatic interactions would be more likely at pH values where the mean

Table I. Recovery of IL-2 Activity in the Assay Used to Determine the IL-2 Incorporation in Liposomes

Treatment	% IL-2 activity[a]
0.1 m*M* SDS in cell culture medium[b]	103 ± 15 (*n* = 3)
Sonication of IL-2 in 25 m*M* SDS[c]	103 ± 16 (*n* = 3)
Sonication of a mixture of IL-2 and empty liposomes in 25 m*M* SDS[d]	87 ± 12 (*n* = 41)

[a] Mean ± SD.
[b] Expressed in comparison to IL-2 without 0.1 m*M* SDS in culture medium.
[c] IL-2 in 25 m*M* SDS was sonicated at 45°C for 15 min.
[d] A mixture of IL-2 and empty liposomes was sonicated in 25 m*M* SDS at 45°C during 15 min. The subsequent steps to determine IL-2 activity are described under Materials and Methods. Combined results of the different types of liposome used in this paper are shown. The percentage IL-2 activity was compared with the activity of IL-2 sonicated in 25 m*M* SDS at 45°C during 15 min.

charge of IL-2 is positive, that is, below the isoelectric point of IL-2. Indeed, PC/PG (9:1) liposomes prepared at pH 5 showed a higher incorporation of IL-2 than PC/PG liposomes prepared at pH 7.3 (Table II). Reduction of the ionic strength of the hydration medium from 0.15 to 0.01 *M* to promote electrostatic interactions resulted in a further increase in IL-2 incorporation in PC/PG liposomes. To maintain isoosmotic conditions, 270 m*M* glycerol was added to the hydration medium. Thus, in addition to the ionic strength-related changes in electrostatic effects, the presence of glycerol may affect the bilayer characteristics of these liposomes and thereby the extent of IL-2 association with the liposomes. These manipulations induced an increase in the IL-2 incorporation in PC liposomes as well, but to a lesser extent than in PC/PG liposomes. The highest level of IL-2 incorporation, 66 ± 6% (*n* = 4; mean ± SD), was found for PC/PG liposomes using a low-ionic strength buffer (10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA) of pH 5. Preparation of this type of liposome with 4×10^5 U/mL IL-2 instead of 10^5 U/mL yielded an incorporation efficiency of 81 ± 10% (*n* = 8).

The incorporation of CF (pH 7.3) and FITC-dextran (pH 5) in liposomes (in the presence of 0.1% BSA) was measured in order to determine the entrapped aqueous space. From Table II it can be derived that the IL-2 incorporation values for all types of liposomes were higher than those for CF or FITC-dextran. This indicates that a substantial part of the IL-2 is associated with the PL bilayers of the liposomes.

The experiments described above demonstrate that the charges on liposomes and IL-2 play an important role in the interaction process. Therefore, the effect of manipulation of electrostatic interactions on the incorporation process was studied in more detail.

The effect of varying the charge of the liposomes on the IL-2 incorporation at pH 5 is presented in Table III. The incorporation of IL-2 in liposomes was reduced from 66% for PC/PG (9:1) liposomes to less than 10% for liposomes composed of PC/PG (1:1) or of PG only. Inactivation of IL-2 could have been induced by the very low liposomal surface pH (pH 2–3), resulting from the high negative charge on the bilayers and the low ionic strength. However, when these liposomes were tested in the bioassay without removal of free, nonincorporated IL-2, 91% (PC/PG, 1:1) and 114% (PG) of the IL-2 activity could be recovered. This demonstrates that the low IL-2 activities were not caused by inactivation of IL-2 by the liposomes. Thus, a lower extent of IL-2 association with the liposomes is more likely. A possible reason for this phenomenon is that BSA in the hydration medium interferes with the IL-2 binding to the liposomes with high surface charge densities. Therefore, experiments were performed to gain insight into the possible effects of BSA on the liposomes. The ζ-potential differences between liposomes with and liposomes without BSA were small (Table III), indicating that BSA did not neutralize the charge of the liposomes. Although the absence of ζ-potential changes does not prove the absence of interactions of BSA with the liposomes, the results point out that interference of BSA with electrostatic interactions between IL-2 and liposomes is unlikely. When PC/PG (1:1) or PG liposomes were diluted 20 times with low-ionic strength buffer containing 0.1% BSA, a strong reduction in the turbidity (measured spectroscopically at 630 nm) was observed compared to liposomes diluted with buffer without BSA (data not shown). Such a reduction was not found for PC or PC/PG 9:1 liposomes. In addition, in the presence of 0.1% BSA in the hydration medium, FITC-dextran could not be incorporated in PC/PG (1:1) and PG liposomes (<1%). These results suggest that for PC/PG (1:1) and PG liposomes under those conditions, a BSA–PL interaction interferes with bilayer formation. This may account for the low degree of incorporation of IL-2 in the liposomes with a high PG content.

Since PC/PG (9:1) liposomes prepared with 4×10^5 U IL-2/mL in low-ionic strength buffer of pH 5 (10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA) showed the highest IL-2 incorporation efficiency, this type of liposome was used for further studies.

Stability of IL-2 Liposomes

Free IL-2 is claimed to be stable over a wide pH range (20). The liposomal IL-2 activity and the retention of liposome-associated IL-2 were assessed immediately after preparation and after storage at 4°C for 7 days (Table IV). Free IL-2 dissolved in 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA (pH 5) was indeed stable under the chosen conditions. No differences were found in the IL-2 incorporation efficiency determined on day 0 and on day 7, suggesting that IL-2 incorporated in liposomes retained its activity upon storage during 1 week. Furthermore, no IL-2 leakage was found during this period.

When IL-2 liposomes (10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5) were diluted 15 times with PBS, only 68 ± 9% (*n* = 4) of the incorporated IL-2 remained liposome associated. This indicates that these liposomes rapidly lose part of their content upon dilution with medium with a physiologic pH and ionic strength. For comparison, similar leakage experiments were carried out using liposomes with FITC-dextran instead of IL-2. Upon dilution of the FITC containing liposomes with PBS, 96% of the incorporated FITC-dextran was retained in these liposomes.

In Vitro Activity of IL-2 Liposomes

A representative example of the ability of IL-2 lipo-

Table II. Incorporation of IL-2 in Liposomes: Effect of Liposome Charge and of pH and Ionic Strength of the Hydration Medium

Hydration medium	pH	Liposomal IL-2 activity (%)[a] PC	Liposomal IL-2 activity (%)[a] PC/PG	Entrapped volume (%)[b] PC	Entrapped volume (%)[b] PC/PG
Phosphate-buffered saline	7.3	8 ± 1	15 ± 1	1	5
4.1 m*M* phosphate/270 m*M* glycerol	7.3	16 ± 4	27 ± 3	2	10
10 m*M* NaAc/140 m*M* NaCl	5.0	15 ± 2	28 ± 2	3	9
10 m*M* NaAc/270 m*M* glycerol	5.0	26 ± 4	66 ± 6	8	13

[a] Hydration medium contained 10^5 U IL-2/mL of the indicated buffer supplemented with 0.1% BSA. IL-2 was released from the liposomes by treatment with 25 m*M* SDS and sonication. The IL-2 activity was compared with that of a mixture of empty liposomes with IL-2 treated identically to the IL-2-containing liposomes. The activity was determined using the IL-2-dependent CTLL-16 cells. Mean ± SD of three or four experiments.

[b] Entrapped volumes of liposomes were determined with CF (pH 7.3) or FITC-dextran (pH 5). Mean value of at least two experiments; individual and mean values differ <2%.

somes to stimulate CTLL-16 cells in the bioassay (without SDS) is shown in Fig. 1. Compared to the activity of free IL-2, IL-2 liposomes were less effective (47 ± 6%; $n = 7$). Incubation of empty liposomes and IL-2 in the bioassay resulted in similar curves as free IL-2.

Preliminary Studies on the *in Vivo* Activity of IL-2 Liposomes

The potential of IL-2 liposomes to improve the protective tumor immunity induced by two immunization injections with reconstituted membranes with MTP-PE is shown in Fig. 2. Empty liposomes, free IL-2, or liposome-incorporated IL-2 was administered for 5 days, starting at the day of the second immunization with reconstituted membranes with MTP-PE. Mice were challenged i.p. with 10^5 SL2 cells 10 days after the second immunization. Nonimmunized mice all died within 20 days as a result of tumor growth. Immunization with reconstituted membranes with MTP-PE and empty liposomes induced a slight, but significant ($P < 0.05$) increase in survival time. Administration of five injections with 5×10^3 U or 2×10^4 U liposome-incorporated IL-2 instead of empty liposomes significantly improved the survival (both P's < 0.05). However, no significant differences at the 5% level were found between the survival of mice treated with free IL-2 and that of mice treated with liposome-incorporated IL-2 at either of the two dose levels tested. Furthermore, no dose dependence was observed, as comparing the two doses of either free or liposomal IL-2 (5×10^3 with 2×10^4 U/injection) did not show significant differences.

DISCUSSION

A biological assay was used to determine the IL-2 activity of the liposome incorporated protein, as it is important that the biological activity of IL-2 is preserved upon incorporation in liposomes. Using a simple preparation method—hydration of a PL film with IL-2 dissolved in 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5—81% of the IL-2 activity could be incorporated into negatively charged MLV. IL-2 incorporation efficiencies were less than 15% when PBS (pH 7.3) was employed as hydration buffer. Incorporation efficiencies reported in the literature for IL-2 in MLV prepared

Table III. Effect of Liposome Charge on the Incorporation of IL-2 at pH 5

Lipid composition (molar ratio)	Ionic strength (M)[a]	ζ potential of empty liposomes (mV) With BSA[b]	ζ potential of empty liposomes (mV) Without BSA	Liposomal IL-2 activity (%)[c]
PC	0.01	1	0	26 ± 4
PC/PG (9:1)	0.01	−43	−45	66 ± 6
PC/PG (1:1)	0.01	−65*	−86	9[d]
PG	0.01	−81*	−88	8 ± 1
PG	0.15	−54	−58	25[d]

[a] Hydration medium consisted of 10^5 U IL-2/mL 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA (ionic strength, 0.01 *M*) or of 10^5 U IL-2/mL 10 m*M* NaAc/140 m*M* NaCl, 0.1% BSA (ionic strength, 0.15 *M*).

[b] To prevent desorption of BSA from the liposomes, the dilutions required for ζ-potential measurements were prepared with buffer with 0.1% BSA. However, dilution of liposome dispersions marked with a superscript asterisk resulted in disruption of the liposomal bilayer structure. Therefore, these dispersions were diluted with buffer containing no BSA.

[c] IL-2 was released from the liposomes by treatment with 25 m*M* SDS and sonication; the IL-2 activity was measured against that of a mixture of empty liposomes with IL-2 which was treated identically to the IL-2-containing liposomes. Mean ± SD of three or four experiments.

[d] Mean value of two experiments; individual and mean values differ <1%.

Table IV. Stability of IL-2 Liposomes Stored at 4°C

	% IL-2 activity at[a]	
	Day 0	Day 7
1. "Free" IL-2[b]	103	102
2. Activity of IL-2 liposomes[c]	81	81
3. Retention of incorporated IL-2[d]	104	101

[a] Mean value of two experiments; individual and mean values differed <12%.

[b] IL-2 solutions in 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5, were stored at 4°C. The percentage IL-2 activity was compared with IL-2 stored at −20°C.

[c] PC/PG (9:1) liposomes were prepared with 4 × 10^5 U IL-2/mL 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5. Nonincorporated IL-2 was removed by washing the liposomes two times, and the percentage IL-2 activity of the liposomes was determined after forced release at day 0 and day 7.

[d] Liposomes (2) were washed for a third time. The percentage activity was determined after forced release and compared with that of the liposomes under 2 (above).

by standard aqueous hydration are 10–50% (7), 2% (8), and 15–19% (6). High levels of IL-2 incorporation (>75%) were reported for liposomes prepared by repeating cycles of freezing/thawing and bath sonication of MLV at very high PL concentrations (300 mg/ml) (7) and for dehydration–rehydration vesicles (9). Both procedures result in high entrapment of added solute (21,22) and probably yield large liposomes with most of the IL-2 entrapped in the aqueous interior of the liposomes. However, incorporation efficiencies in terms of associated IL-2/mol PL that can be reached with these preparation methods are generally low as high PL concentrations are required.

Comparison of the incorporation efficiency of IL-2 and CF/FITC-dextran in the liposomes (Table II) suggests that a substantial part of the IL-2 is associated with the PL bilayers of the liposomes. The importance of electrostatic interac-

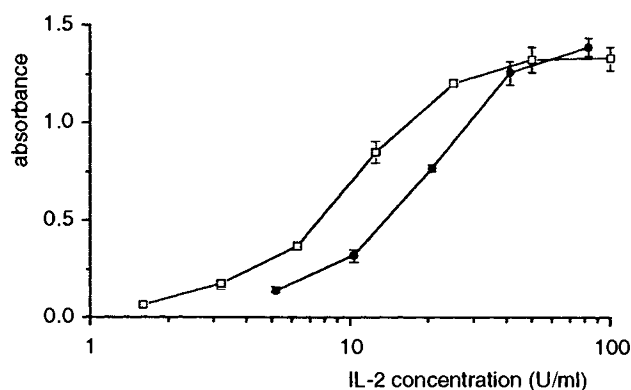

Fig. 1. Effect of free and liposome-incorporated IL-2 on *in vitro* proliferation of CTLL-16 cells. PC/PG (9:1) liposomes were prepared with 4 × 10^5 U IL-2/mL 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5. CTLL-16 cells were incubated with the indicated concentrations of IL-2 (——□——) or IL-2 incorporated in liposomes (——●——) for 45 hr. Cell proliferation was measured with the colorimetric MTT assay. A representative experiment of a series of seven is shown. Error bars indicate standard deviations of triplicate determinations. Small standard deviations are not shown.

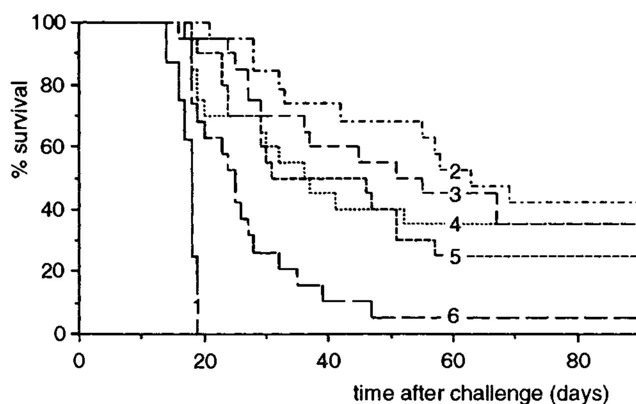

Fig. 2. Effect of free and liposome-incorporated IL-2 on the protective tumor immunity induced by immunization with reconstituted membranes. PC/PG (9:1) liposomes were prepared with 4 × 10^5 U IL-2/mL 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5. Mice were immunized with 0.13 mg reconstituted membranes with 20 μg MTP-PE on day −20 and day −10. From day −10 to day −6 mice were injected daily with 2 × 10^4 free IL-2 (2; --------), 5 × 10^3 U free IL-2 (3; ---), 2 × 10^4 U liposomal IL-2 (4; ·········), 5 × 10^3 U liposomal IL-2 (5; ------), or empty liposomes (6; ———) at the s.c. immunization sites. On day 0 mice were challenged i.p. with 10^5 SL2 cells. (1; ——) No immunization. Nineteen or twenty mice per group.

tions for IL-2 incorporation in PC/PG liposomes prepared at pH 5 is indicated by the following observations (Table II).

(a) Lowering the pH of the IL-2 containing hydration medium from 7.3 to 5 doubled the IL-2 incorporation in liposomes. With this pH drop, the mean positive charge of IL-2 increases (isoelectric point of IL-2 is 7.0 and 7.6). This may result in enhanced electrostatic attraction between opposite charges on IL-2 and the liposomes or in diminished charge–charge repulsions between equal (negative) charges on IL-2 and the liposomes.

(b) At pH 5 more IL-2 was incorporated in negatively charged liposomes than in liposomes composed of zwitterionic PL.

(c) At low ionic strength, more IL-2 was incorporated in liposomes than at high ionic strength.

None of these differences in IL-2 incorporation efficiency could be explained by differences in entrapped aqueous space (Table II).

To what extent hydrophobic interactions are involved in the IL-2 association with liposomes is not clear. Electrostatics may also contribute to the association of IL-2 with neutral PC liposomes. Although the net charge of the polar head group of PC is zero, the involvement of electrostatic interactions in the binding of some proteins (e.g., polylysine and apoA-II protein) to PC liposomes via charged moieties on the protein and the positively charged choline group and/or the negatively charged phosphate group of PC has been described (23).

Serum or serum albumin is generally included in the dilution medium of IL-2 to prevent nonspecific adsorption and to stabilize IL-2. The presence of albumin, however, may change the properties of the liposomes (24). Increased leakage rates of liposome-incorporated glucose upon addi-

tion of BSA have been reported in literature (25). This has been observed especially at low pH values, at which BSA undergoes conformational changes involving the exposure of hydrophobic areas of the protein to the solvent. The interaction of proteins with liposomes may ultimately lead to disruption of the liposome structure. The fall in turbidity upon dilution of liposomes composed of PC/PG (1:1) or only PG with BSA-containing buffer and the inability to entrap FITC-dextran in these types of "liposomes" clearly indicate a strong albumin–PG interaction under those conditions and disruption of the liposomal bilayer structure.

Incubation of IL-2 liposomes (PC/PG, 9:1) prepared in 10 m*M* NaAc/270 m*M* glycerol, 0.1% BSA, pH 5, with CTLL-16 cells during 45 hr induced a less effective proliferation of the cells than free IL-2 (Fig. 1). A mixture of empty liposomes and IL-2 was as effective as free IL-2 in stimulating CTLL-16 cells. This suggests that part of the IL-2 did not leak from the liposomes in the bioassay and is, thus, not available for stimulation of CTLL-16 cell growth. However, dilution of the IL-2 liposome dispersion with PBS resulted in substantial release of incorporated IL-2 (about 32%). Similar leakage experiments with FITC-dextran as marker for the internal aqueous phase of the liposomes indicate that in these circumstances, large molecules were retained in the liposomes. Therefore, the observed IL-2 release upon dilution of the liposomes with PBS is most likely due to IL-2 weakly bound to the exterior lamellae of the liposomes at low ionic strength, pH 5. If this presence of IL-2 on the outside of liposomes is undesired, e.g., because it creates a two-step release pattern, it will be necessary to remove it prior to administration. Apparently, the released IL-2 still exerts a biological effect and is not denaturated as was reported for other globular proteins of similar size (26; J. J. Bergers *et al.,* submitted for publication).

Initial immunoprotection experiments using reconstituted membranes prepared from SL2 tumor cell membranes with MTP-PE indicated that the immune response could be enhanced by IL-2 liposomes to almost similar levels as by free IL-2. MTP-PE was included in the immunization scheme, as earlier studies showed synergism between IL-2 and MTP-PE (J. J. Bergers *et al,* submitted for publication). No dose dependence of IL-2 could be demonstrated in the experimental range; 5×10^3 and 2×10^4 U of either free or liposomal IL-2 induced approximately similar levels of protection. Therefore, it is impossible to draw conclusions about the exact role of liposome-incorporated IL-2 in the immunoprotection experiments. The observed immune stimulation by IL-2 liposomes may be induced by IL-2 that is rapidly released from the liposomes, as indicated by the dilution experiments of the liposomes with PBS. More detailed studies on the desired IL-2 kinetics and on the leakage pattern of IL-2 from liposomes are required. Dose–response curves and different dose schedules should give the opportunity to maximize the therapeutic advantages of IL-2 liposomes.

In conclusion, the incorporation of IL-2 in liposomes is strongly dependent on the pH and ionic strength of the hydration medium and the charge of the liposomes. BSA, used in the dilution buffers of IL-2, affected the liposomal bilayer structure of the PG-rich dispersions prepared at pH 5 at low ionic strength. High IL-2 incorporation efficiencies in negatively charged liposomes were achieved by lowering the pH from 7.3 to 5 and by decreasing the ionic strength from 0.15 to 0.01 *M*. Electrostatics play a crucial role in the process of IL-2 association with this type of liposome.

ACKNOWLEDGMENTS

The authors wish to thank Louis van Bloois for technical assistance and Drs. Peter A. Steerenberg and Yechezkel Barenholz for stimulating discussions.

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