JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 295–302
© 2001 The Authors
Received March 6, 2000
Accepted October 4, 2000
ISSN 0022-3573



# A novel and simple type of liposome carrier for recombinant interleukin-2

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**Abstract**

The strong interaction between recombinant interleukin-2 (IL-2) and liposome was characterized and its possible application to drug-delivery control considered. The liposomes were prepared with egg phosphatidylcholine, distearoyl-phosphatidylglycerol (DSPG), dipalmitoyl-phosphatidylcholine, dipalmitoyl-phosphatidylglycerol or distearoyl-phosphatidylcholine (DSPC). Small and hydrophobic liposomes were selected, which were composed of saturated and long-fatty-acid-chain phospholipids. When the composition and the mixture ratio of IL-2 and the liposome were optimized, more than 95 % of the lyophilized IL-2 (Imunace, 350 000 JRU) was adsorbed consistently onto the DSPC–DSPG liposome (molar ratio, 10 : 1; 25 μmol $mL^{-1}$; 30 nm in size). Merely mixing IL-2 lyophilized with liposome suspension is convenient pharmaceutically. After intravenous administration to mice, liposomal IL-2 was eliminated half as slowly from the systemic circulation as free IL-2, with more than 13 and 18 times more IL-2 being delivered to the liver and spleen, respectively. After subcutaneous administration of liposomal IL-2 to mice, the mean residence time of IL-2 in the systemic circulation was 8 times that of free IL-2. These results show that IL-2 consistently adsorbs onto the surface of liposomes after optimization of its composition and mixing ratio. Intravenous and subcutaneous administration to mice demonstrates the gradual release of IL-2. Further trials are warranted using these liposomes.

## Introduction

Recombinant interleukin-2 (IL-2) is a relatively small protein (MW 15 420). It has numerous immunoregulatory properties, including involvement in the response of the immune system to cancer (Ortald et al 1984; Mule & Rosenberg 1985; Terry 1993; Heys et al 1996). IL-2 is rapidly eliminated after intravenous administration and strong systemic toxicity has often been seen with high doses (Donohoue & Rosenberg 1983; Lotze et al 1985; Konrad et al 1990; Ohnishi et al 1990; Anderson & Sorenson 1994). Thus, much effort has been expended toward increasing the efficacy of IL-2 and reducing its side effects by polyethylene glycol (PEG) substitution, using mini-pellets, liposome encapsulation and covalent coupling (Karte et al 1987; Matsuoka et al 1990; Anderson et al 1992a; Wood et al 1993; Anderson & Sorenson 1994; Bernen et al 1995; Fleury et al 1995; Storm et al 1995; Konigsberg et al 1998). Combinations of liposome and IL-2 have been tried since liposome is considered a good, safe carrier for IL-2, which is a hydrophobic protein. In this

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report, we found that mixing IL-2 with liposome was effective, and that the pharmacokinetics of IL-2, after intravenous or subcutaneous administration in mice, were improved.

## Materials and Methods

### Materials

Bulk material and commercial formulation (Imunace 35, 350000 IU/vial) of IL-2 were products of Shionogi & Co. Ltd (Osaka, Japan). $^{125}$I-Labelled IL-2 was obtained by the chloramine T method modified by Nakamura et al (1991). Bovine serum albumin (BSA, globulin-free grade) was obtained from Sigma (St Louis, MO). Hydrogenated soy phosphatidylcholine (HSPC), distearoyl-L-$\alpha$-phosphatidylcholine (DSPC), dipalmitoyl-L-$\alpha$-phosphatidylcholine (DPPC), dimiristoyl-L-$\alpha$-phosphatidylcholine (DMPC), distearoyl-L-$\alpha$-phosphatidylglycerol (DSPG) and dipalmitoyl-L-$\alpha$-phosphatidylglycerol (DPPG) were obtained from Nichiyu Liposome Co. Ltd (Tokyo, Japan). Egg phosphatidylcholine (EggPC) was obtained from Asahi Chemical Industry Co. Ltd (Nobeoka, Japan). Tertiary butyl alcohol and maltose were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). NaCl, $NaH_2PO_4$ and $Na_2HPO_4.H_2O$ were purchased from Nacalai Tesque Inc. (Kyoto, Japan). These reagents were all of analytical grade. Dulbecco's phosphate-buffered saline (PBS; $Ca^{2+}$- and $Mg^{2+}$-free) was purchased from Nissui Pharmaceutical Co. Ltd (Tokyo, Japan). The water used was of injectable grade and obtained from Otsuka Pharmaceutical Co. Ltd (Tokyo, Japan).

### Preparation of liposomes

Liposomes smaller than 100 nm were prepared using a Nanomizer (Sayama Trading, Tokyo, Japan) as reported previously (Hirano et al 1996; Kanaoka et al 1999), and those larger than 100 nm were prepared using an Extruder (Nichiyu Liposome Co. Ltd, Tokyo, Japan). After preparing multilamellar liposomes (MLVs) as reported previously, the MLVs were reduced in size to 100 nm and 1000 nm by the Extruder using a 100-nm and a 1000-nm Nuclepore membrane (Nomura Science Co. Ltd, Tokyo, Japan) in a water bath maintained above the phase-transition temperature of phospholipids. This step was repeated three times. The liposome size was determined by a quasi-elastic light-scattering method using a sub-micron particle analyser (Model N4, Coulter Co. Ltd, FL). The compositions and average sizes of typical liposomes prepared are listed in Table 1.

**Table 1** Liposome sizes.

| Liposome | Size (nm) |
|---|---|
| Liposome 30 | $37.3 \pm 23.0$ |
| Liposome 100 | $96.0 \pm 11.0$ |
| Liposome 1000 | $1080 \pm 400$ |

Values are expressed as average $\pm$ s.d. Liposome sizes were measured by a quasi-elastic light-scattering method using a Coulter N4. Liposome composition was DSPC–DSPG, 10:1.

### Assay of $^{125}$I-IL-2, IL-2 and phospholipids

The radioactivity of $^{125}$I-labelled IL-2 was measured by a liquid scintillation counter (Tri-calb 2000, Packard). Unlabelled IL-2 was measured by immunoassay performed using the ELISA system (BIOTRAK, human IL-2) obtained from Amersham Int. Plc. (Bucks, UK). The concentration of phosphatidylcholine was determined by an enzyme method using a phospholipid assay kit obtained from Nippon Shoji Co. Ltd (Osaka, Japan).

### Gel-filtration chromatography

IL-2 bound to liposomes and unbound IL-2 were separated by gel-filtration chromatography, using a Sephadex G-75 column ($0.9 \times 25$ cm) and an eluent composed of PBS containing 1% BSA, at a flow rate of 0.5 mL $min^{-1}$. The eluent was collected every minute and, concentrations of IL-2 and phosphatidylcholine in the liposomal fractions were measured. Fractions 6–10 contained the liposomal fraction, those from 10 onwards contained the IL-2 fractions (Figure 1).

### Percentage of IL-2 associated with liposomes

The strength of the interaction between IL-2 and liposomes was assessed by comparing the percentage of IL-2 associated with the liposomes. When the IL-2 bound to liposomes and unbound IL-2 were separated by gel-filtration chromatography, the ratio of amounts of IL-2 and liposome was almost equal in the liposomal fractions (data not shown). The percentage of IL-2 associated with liposome was calculated using equation 1.

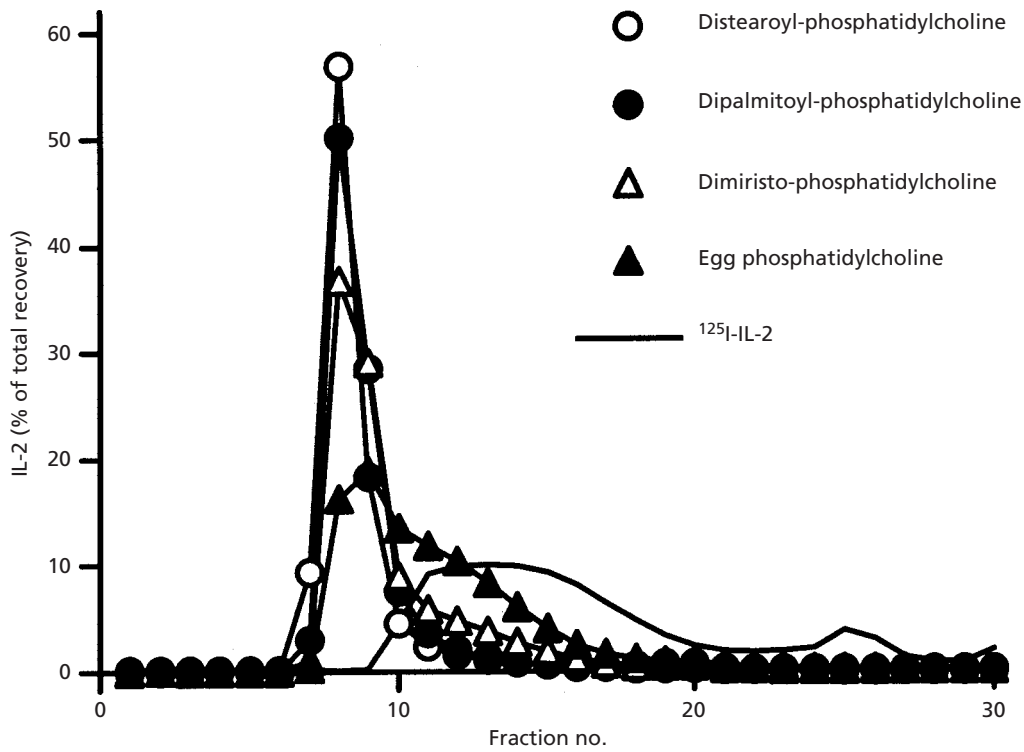


**Figure 1** Gel-filtration chromatography of $^{125}$I-IL-2 and its mixtures with four kinds of liposomes. The $^{125}$I-IL-2 in each fraction as a percentage of the total $^{125}$I-IL-2 recovery was plotted.

$$\text{Associated}\,\% = \frac{C_{\text{lipo,IL-2}}/C_{\text{lipo, liposome}}}{C_{\text{I,IL-2}}/C_{\text{I, liposome}}} \times 100 \qquad (1)$$

where $C_{\text{lipo, IL-2}}$, $C_{\text{lipo, liposome}}$, $C_{\text{I, IL-2}}$ and $C_{\text{I,liposome}}$ represent the concentrations of IL-2 and liposome in the liposomal fractions, and their concentrations in the initial mixture, respectively.

### Pharmacokinetics of IL-2 after intravenous or subcutaneous administration to mice

Five-week-old male mice (BDF1) were obtained from Japan SLC Inc. For intravenous studies IL-2 or liposomal IL-2 ($5 \times 10^5$ JRU kg$^{-1}$; 125 $\mu$mol lipid kg$^{-1}$; 0.2 mL) was administered into the tail vein of groups of 3 mice. For subcutaneous studies, IL-2 or liposomal IL-2 ($1.5 \sim 1.75 \times 10^6$ JRU kg$^{-1}$; 300 $\mu$mol lipid kg$^{-1}$; 0.2 mL) was administered subcutaneously into the mouse's back. The blood sample was diluted with PBS solution (containing Triton X (2% w/v) or centrifuged (3000 rev min$^{-1}$; 10 min) and the serum was used for IL-2 immunoassay. Organs were prepared as 10% homogenates with PBS solution (containing Triton X (2% w/v)) for IL-2 immunoassay. Pharmacokinetic parameters were calculated by a trapezoidal method.

## Results

### Interaction between $^{125}$I-IL-2 and liposome

$^{125}$I-IL-2 and liposomes (size about 100 nm) were mixed under physiological conditions (pH 7.4) with 1% of BSA, and any $^{125}$I-IL-2 not bound to liposome was separated by gel-filtration chromatography. Figure 1 shows the chromatograms for mixtures of $^{125}$I-IL-2 and four kinds of liposomes prepared with different phosphatidylcholines (EggPC, DMPC, DPPC, DSPC). Liposomes containing negatively charged phospholipid (DPPG) were also tested (data not shown). DSPC and DSPC–DPPG liposomes showed the strongest association with IL-2; free IL-2 was not detected. These findings suggest that the interaction is dependent not on a negative charge but on the liposomal hydrophobicity. Almost none of the IL-2 adsorbed to these liposomes was released overnight at 4°C in PBS (data not shown), showing its strong adsorption to the liposome surface.

### Percentage of IL-2 associated with liposome

The interaction of unlabelled IL-2 and liposomes was tested. IL-2 and three kinds of liposomes (EggPC–

**Table 2** Percentage of IL-2 associated with liposome.

| Liposome | % |
|---|---|
| EggPC–DPPG, 3:1 | 2 |
| DPPC–DPPG, 10:1 | 54 |
| DSPC–DPPG, 10:1 | 85 |

The percentage of IL-2 associated with liposome was calculated from the results of gel-filtration chromatography using equation 1.

DPPG, 3:1; DPPC–DPPG, 10:1; DSPC–DPPG, 10:1; about 100 nm in size; 50 $\mu$mol mL$^{-1}$) were mixed at a ratio of 7.0 JRU (nmol lipid)$^{-1}$, and then IL-2 bound and unbound to liposome were separated by gel-filtration chromatography. The percentage of IL-2 associated with the liposome was calculated from these results using equation 1 (Table 2). Unlabelled IL-2 showed the strongest association with the DSPC–DPPG liposome; similar to the result with $^{125}$I-IL-2, the association percentage was 85.

### Effects of liposome size on the interaction with IL-2

The concentration of IL-2 associated with liposomes (DSPC–DSPG, 10:1) was measured, for the sizes of 30, 100 and 1000 nm. All mixtures were prepared at the same ratio, 7.0 JRU (nmol lipid)$^{-1}$. When the liposome was assumed to be multilamellar and spherical vesicles, the total surface area ($S$) of the liposome suspension could be expressed by equation 2.

$$S = V \times \frac{c \times 4\pi r^2}{\frac{4}{3}\pi r^3 \times d} \quad (2)$$

where $V$, $c$, $r$ and $d$ represent the volume, concentration, radius and density of liposome, respectively. When d is nearly equal at these three sizes of liposome, equation 2 can be simplified to equation 3.

$$S = k \times \frac{c}{r} \quad (3)$$

where $k$ is a constant. When the concentration of IL-2 associated with the DSPC–DSPG liposome was plotted with respect to $c/r$, it showed a linear relationship ($R^2 = 0.98$) (Figure 2). As the liposomal radius becomes small, the surface area increases. Therefore, it is clear that a smaller liposome is advantageous for IL-2 adsorption.

With the 30-nm DSPC–DSPG liposome, IL-2 was mixed at a ratio of 0.2–10.0 JRU (nmol lipid)$^{-1}$, and the concentrations of IL-2 associated with the liposome were calculated. Almost all of the IL-2 was associated with the DSPC–DSPG liposome when the mixture of IL-2 and liposome was prepared at a ratio of less than 10 JRU nmol$^{-1}$ (Figure 3). The percentage of IL-2 associated with the DSPC–DSPG liposome was calculated to be 93.4 $\pm$ 9.6.

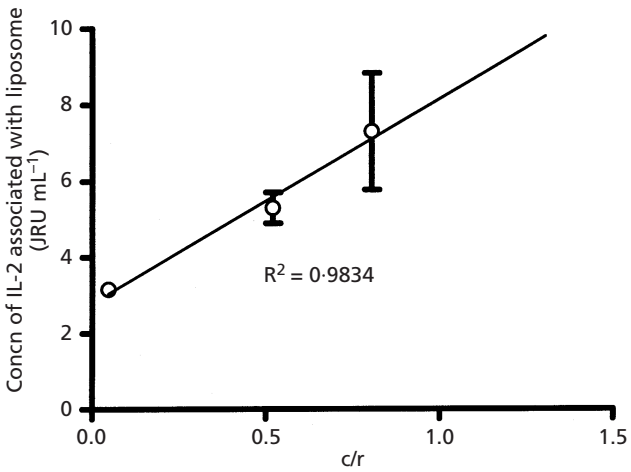


**Figure 2** Effects of liposome size on IL-2 association with liposome. The liposome composition was DSPC–DSPG, 10:1. IL-2:lipid ratio was 7 JRU nmol$^{-1}$. c and r are liposomal concentration and radius, respectively. The liposome sizes are shown in Table 1.

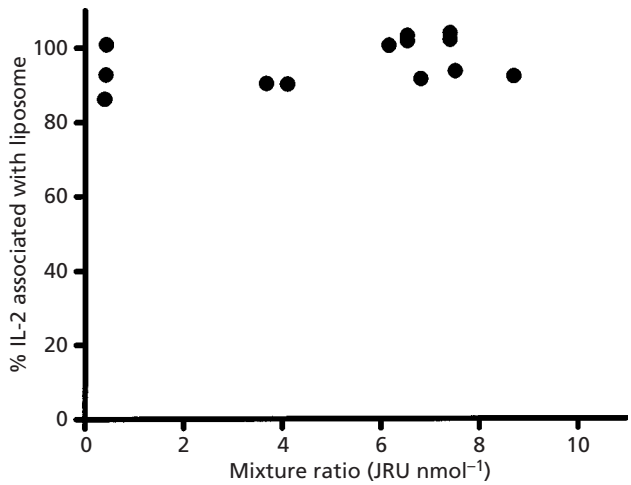


**Figure 3** Relationship of the percentage of IL-2 associated with liposome and the mixture ratio of IL-2 and liposome. The mixtures of IL-2 (Imunace) and liposome (DSPC–DSPG, 10:1) were prepared at various ratios (0.2 ~ 10 JRU nmol$^{-1}$). The percentage was calculated from the results of gel-filtration chromatography using equation 1.

### Interaction of an IL-2 commercial product and liposome

We tested the interaction between liposomes and a commercially available IL-2 product (Imunace 35), which contained not only albumin but also a surfactant (polysorbate 80, to stabilize IL-2). These additives might interfere with the adsorption of IL-2 onto the liposome. However, the mixture was prepared by optimizing the composition of liposome and the ratio of IL-2 to liposome, and it was found that almost all of the IL-2 was adsorbed onto the liposome. For example, one mixture was of 2 mL of the liposome suspension (DSPC–DSPG, 10:1; 25 $\mu$mol mL$^{-1}$; 30 $\pm$ 25 nm in size) and a vial of IL-2 (Imunace 35; 350 000 JRU). The percentage of IL-2 associated with the liposome was calculated to be 96.0 $\pm$ 9.9. The IL-2 associated with the liposome was biologically active in the CTLL-2 assay (data not shown) (Gillis et al 1978). Thus, this method offers a very easy and convenient way to use IL-2 adsorbed to liposome.

### Characteristics of liposomal IL-2 after administration to mice

*Elimination and distribution of liposomal IL-2 after intravenous administration*

IL-2 or its mixture with DSPC–DSPG liposomes was administered intravenously to mice ($5 \times 10^5$ JRU kg$^{-1}$; 125 $\mu$mol lipid kg$^{-1}$). The elimination from the systemic circulation and distribution of IL-2 in the liver, kidneys, lungs and spleen were investigated. The blood half-life

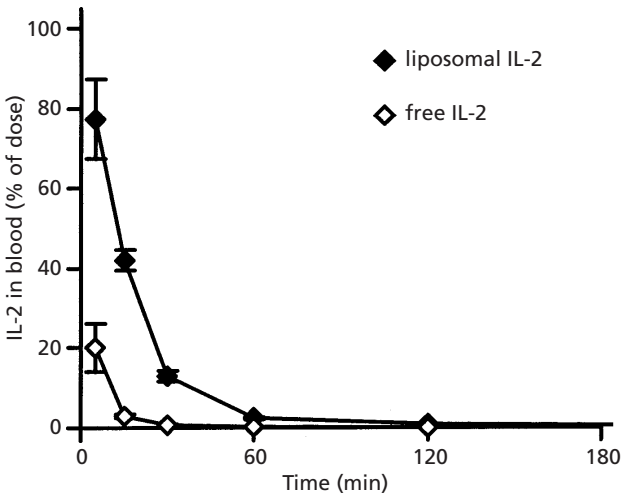


**Figure 4** Concentration of IL-2 in the blood after intravenous administration of free IL-2 and liposomal IL-2 to mice (n = 3). Dose of free and liposomal IL-2: $5 \times 10^5$ JRU kg$^{-1}$; 125 $\mu$mol lipid kg$^{-1}$; liposome: DSPC–DSPG, 10:1.

**Table 3** Distribution of IL-2 after intravenous administration of free IL-2 and liposomal IL-2 to mice.

| Organ | AUC$_{(0-4h)}$ (% dose·h mL$^{-1}$) | | AUC ratio |
|---|---|---|---|
| | Free IL-2 | Liposomal IL-2 | Liposomal IL-2:free IL-2 |
| Blood | 2.84 | 15.48 | 5.44 |
| Lung | 0.96 | 2.30 | 2.40 |
| Liver | 0.26 | 3.37 | 13.18 |
| Spleen | 0.60 | 10.59 | 17.73 |
| Kidney | 8.65 | 6.42 | 0.74 |

Distribution of IL-2 in lung, liver, spleen and kidney are compared, after intravenous administration of free IL-2 and liposomal IL-2 ($5 \times 10^5$ JRU kg$^{-1}$; 125 µmol lipid kg$^{-1}$; liposome: DSPC–DSPG, 10:1). The area under the curves (0–4 h) was calculated by a trapezoidal method. The values represent the calculated values from the mean of 3 mice.

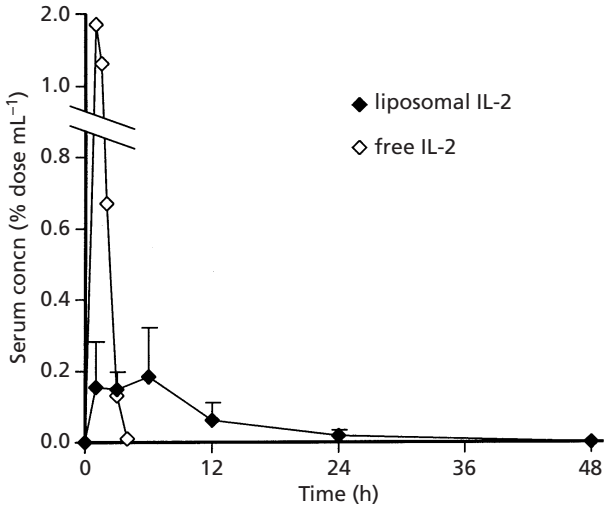


**Figure 5** Concentration of IL-2 in serum after subcutaneous administration of free IL-2 and liposomal IL-2 to mice. Dose of free IL-2: $1.5 \times 10^6$ JRU kg$^{-1}$, n = 2; dose of liposomal IL-2: $1.75 \times 10^6$ JRU kg$^{-1}$; 300 $\mu$mol lipid kg$^{-1}$; liposome: DSPC–DSPG, 10:1, n = 3.

of liposomal IL-2 was prolonged compared with that of free IL-2 (Figure 4). The calculated mean residence times (MRT) for liposomal IL-2 and free IL-2 were 0.2 h and 0.7 h, respectively. The distribution of liposomal IL-2 to the liver and spleen was 13 and 18 times greater, respectively, than that of IL-2 alone, while distribution to the kidneys was 0.74 times less (Table 3). These findings suggest that the interaction between IL-2 and the surface of DSPC–DSPG liposomes is so strong

**Table 4** Pharmacokinetic parameters of IL-2 and liposomal IL-2 in serum after subcutaneous administration to mice.

| Sample | $C_{max}$ (% dose $mL^{-1}$) | $T_{max}$ (h) | AUC (% dose·h $mL^{-1}$) | MRT (h) |
|---|---|---|---|---|
| Free IL-2 | 1.88 | 1.00 | 2.71 | 1.71 |
| Liposomal IL-2 | $0.27 \pm 0.06$ | $4.33 \pm 2.89$ | $2.29 \pm 1.32$ | $12.8 \pm 6.3$ |

Pharmacokinetic parameters of IL-2 were calculated, after subcutaneous administration of free IL-2 ($1.5 \times 10^6$ JRU $kg^{-1}$) and liposomal IL-2 ($1.75 \times 10^6$ JRU $kg^{-1}$) to mice (300 μmol lipid $kg^{-1}$; liposome: DSPC–DSPG, 10:1). The values represent the mean of 2 mice (free IL-2) and the mean ± s.d. of 3 mice (liposomal IL-2).

that IL-2 is gradually released in the systemic circulation, and IL-2 is delivered to the liver and spleen with liposome.

*Depot of IL-2 after subcutaneous administration of liposomal IL-2 in mice*

Free IL-2 or liposomal IL-2 was administered subcutaneously into the back of mice ($1.5 \sim 1.75 \times 10^6$ JRU $kg^{-1}$; 300 $\mu$mol lipid $kg^{-1}$), and the resulting IL-2 serum concentration is shown in Figure 5. Concentrations differed significantly when IL-2 was administered with liposome from when it was given alone. Though the area under the respective serum concentration curves (AUC) were almost equal, the mean residence time (MRT) was 12.8 h for liposomal IL-2 and 1.7 h for free IL-2. Liposome can decrease the peak level of IL-2, and increase the duration time. Clearly, an IL-2 depot was maintained with controlled release into the systemic circulation. Table 4 shows these pharmacokinetic parameters.

## Discussion

Many trials have been carried out using liposome as a carrier of IL-2, since it is known that IL-2 can be incorporated effectively into liposome vesicles because of its hydrophobic character (Kroon et al 1990; Bergers et al 1993a, b; Koppenhagen et al 1998). However, no studies have focused on the utility of a mixture of IL-2 and liposome.

In this study, we found a strong association between IL-2 and small (30 nm) hydrophobic liposomes. The DSPC liposome was highly bound to IL-2, but the EggPC liposome was not. The percentage of IL-2 associated with liposome increased in relation to the surface area of the liposome. According to these findings, the best liposomal composition for combination with Imunace 35 was selected (DSPC–DSPG, 10:1; 30 nm in size; 25 $\mu$mol $mL^{-1}$). When 2 mL of this liposome suspension was mixed with a vial of Imunace 35 (350 000 JRU), the percentage of IL-2 associated with the liposome was consistently about 95. IL-2 remains biologically active after mixing with liposomes. The production of liposomal IL-2 might be difficult, because IL-2 is stable in acidic conditions but may undergo self-aggregation under physiological conditions. The commercial product of IL-2 is, however, of guaranteed quality. Therefore, using separate supplies of IL-2 and liposome is very convenient. The liposome suspension selected was stable for more than a year (data not shown).

Kedar et al (1994) reported on the characterization of IL-2 incorporated into a long-circulating liposome, the MRT of which was 76 min after intravenous administration. Konigsberg et al (1998) investigated the covalent effect of the conjugation of IL-2 and phospholipid. IL-2 is quantitated biologically after coupling, and can be expected to remain in the systemic circulation for a long period. In our study, after intravenous and subcutaneous administrations of liposomal IL-2 to mice, the MRT of IL-2 in the systemic circulation was 40 min and 770 min, respectively, which was twice and 8 times as long as with free IL-2. The AUC ratios (liposomal IL-2 : free IL-2) for blood, liver and spleen after intravenous administration were 5.4, 13 and 18, respectively. From these results, we think the IL-2 bound to liposome available for the increase of the effect of IL-2 in intravenous and subcutaneous administration.

IL-2 has a special characteristic of increasing the immune response. The combination of IL-2 with antitumour drugs has been studied (Hirohata et al 1993) with the aim of increasing the efficacy of each drug. IL-2 could be added to other drugs encapsulated within a liposome, and may expected to produce a better effect than when used alone. IL-2 is administered by the

pulmonary route (Anderson 1992b; Khanna 1997a, b). When IL-2 associated with liposome was nebulized, it was stable (data not shown). Liposomes are likely to be taken up by the many macrophages found in the lungs. The activated macrophage may result in a greater efficiency.

The mixture of IL-2 and liposome offers several merits in clinical use: the mixture is very easy to prepare; the stability of IL-2 is assured; combinations with other drugs are possible; and IL-2 adsorbed to liposome is biologically active.

### Conclusion

We were able to obtain an effective liposomal IL-2 mixture by selecting the liposome composition and the mixture ratio: 7 JRU of IL-2 (Imunace, 350000 JRU) per nmol of liposome (DSPC–DSPG 10:1; 30 nm in size). IL-2 was consistently adsorbed onto the liposomal surface. Intravenous administration to mice enabled controlled release of IL-2 and its delivery to the liver and spleen. Further trials are warranted using these liposomes.

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