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Lecithinization of IL-6 Enhances Its Thrombopoietic Activity in Mice

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Abstract

This study was conducted to assess the merit of lecithinization of recombinant human interleukin-6 (IL-6) as a drug delivery system.

IL-6 was lecithinized by covalently binding it with a phosphatidylcholine (lecithin, PC) derivative. The in-vivo thrombopoietic potency of lecithinized IL-6 (PC-IL-6) was greater than that of native IL-6 when administered subcutaneously, although the in-vitro bioactivity of PC-IL-6 was markedly reduced by lecithinization. When PC-IL-6 and native IL-6 were given in doses that produced the same level of thrombopoietic activity, the former stimulated less production of IgG_1, a marker of the adverse effects of IL-6, than did the latter. Furthermore, PC-IL-6 persisted in the blood longer than native IL-6.

Based on the above, PC-IL-6 appears to be useful as a drug delivery system and may also be useful in the treatment of drug-induced thrombocytopenia.

Recently, many bioactive peptides have been produced using advanced biotechnology. Interleukin 6 (IL-6) has a potent thrombopoietic activity (Ishibashi et al 1989; Asano et al 1990; Hill et al 1990, 1991; McDonald et al 1991) and is expected to be used in the treatment of drug-induced thrombocytopenia which often arises as a haematologic complication of cancer chemotherapy (Hoagland 1982; Miescher 1973; Takatsuki et al 1990; Carrington et al 1992). However, the rapid metabolic clearance of exogeneous IL-6 (Castell et al 1988) and the low affinity for the target cell (megakaryocytes) limit its clinical application. Furthermore, when administered in large amounts, IL-6 induces several adverse effects including mesangial proliferative glomerulonephritis (Horii et al 1989) and IgG_1 plasmacytosis (Suematsu et al 1989). Therefore, an effective drug delivery system for IL-6 has been awaited.

We previously reported that conjugating IL-6 with polyethylene glycol (PEG) drastically increased its thrombopoietic activity (Tsutsumi et al 1995), however, very little of the PEG-modified IL-6 was distributed from the blood into the target tissues (such as bone-marrow, for instance). Additionally, it is well-known that pegylation of bioactive protein impairs the original cell affinity. We have studied the lecithinization of superoxide dismutase, and found that its pharmacological potency was much greater due to increased affinity for cell membranes and to prolongation of its elimination half-life (Igarashi et al 1992, 1994). In this paper, we synthesized lecithinized IL-6(PC-IL-6) by covalently binding phosphatidylcholine (lecithin, PC) derivative to IL-6, and evaluated its usefulness as a drug delivery system for IL-6.

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Materials and Methods

The protocol used in this study was approved by the animal experimentation committees of St. Marianna University and Osaka University.

Reagents

Recombinant human IL-6 (M.W. 21 000) was a kind gift from the Central Research of Ajinomoto Co., Inc.(Kawasaki, Japan). MH-60.BSF-2 cells were generously provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). *N*-Succinimidyl[2,3-^{3}H]propionate 2.16 Tbq mmol^{-1} was purchased from Du Pont NEN Products (Boston, MA, USA). Lysophosphatidylcholine palmitoyl (lysolecithin, lyso PC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). BUN kit and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals

Male C_3H/He mice (5 weeks old) were obtained from SLC Experimental Animals (Hamamatsu, Japan). The mice were housed at a constant temparature (23 ± 1°C) and relative humidity (50–60%) with free access to a standard diet and water. The animal room had a 12-h light/dark cycle (light on 0630 to 1800 h).

Synthesis of PC-IL-6

PC-IL-6 was synthesized according to the method previously reported with slight modification (Igarashi et al 1994). Namely, to lyso PC (2 g, 3·92 mmol) in chloroform/pyridine (80 mL/20 mL) was added glutaric anhydride (1·1 g, 9·81 mmol) and 4-dimethylaminopyridine(1·2 g, 9·81 mmol). The mixture was stirred at 50°C, and evaporated. The residue was applied to an open octadecylsilane column [Dowex 50w-X8 (10 × 10 cm)]. This process gave 1·9 g (yield 80%) of 2-(4-hydro-

xycarbonylbutyroyl)lysoPC (C_3PC). The rate of flow value of C_3PC was 0·1 when thin-layer chromatography was performed with a mobile phase of chloroform, methanol and water (65:25:4).

The C_3PC (1·9 g, 3·14 mmol) in 58 mL of methylene chloride was cooled to 0°C, added to dicyclohexylcarbodiimide (810 mg, 3·93 mmol) in a mixture of *n*-hydroxysuccinimide (360 mg, 3·93 mmol) and tetrazole (220 mg, 3·14 mmol), and stirred at 25°C for 12 h. The resulting precipitate was removed using cerite filtration, and the filtrate was evaporated under reduced pressure. The residue gave 3·1 mmol of activated C_3PC. The RF value of the activated C_3PC was 0·4 on thin layer chromatography with a mobile phase of chloroform/methanol/water (65:25:4).

The activated C_3PC ($14{\cdot}8 \times 10^{-4}$ mmol $(2{\cdot}5\,mL)^{-1}$ isopropyl alcohol) was dropped into IL-6 solution [$4{\cdot}8 \times 10^{-4}$ mmol $(10\,mL)^{-1}$ 11 mM phosphate buffered-saline (PBS) containing 1 mL of isopropyl alcohol], and after 5 min the reaction solution was dialysed into 10 mM PBS at 5°C for 2 h. PC-IL-6 was analysed by high performance liquid chromatography (TSK Phenyl-5PW RP, 0·8 mL min^{-1}, elution buffer 20% acetonitrile/0·1% TFA and 90% acetonitrile/0·075% TFA solution, detection: 220 nm).

In-vitro activity of PC-IL-6

The in-vitro bioactivity of PC-IL-6 and native IL-6 were measured by the MH-60.BSF-2 growth assay (Matsuda et al 1988), and the activity of PC-IL-6 relative to native IL-6 was calculated.

Elimination half-life

^3H labelling of IL-6 was performed according to the method previously reported by Kummer et al (1981). PC-[^{3}H]IL-6; $7{\cdot}0 \times 10^7$ dpm mg^{-1} and native [^{3}H]IL-6; $5{\cdot}2 \times 10^7$ dpm mg^{-1} were obtained. C_3H/He mice were given either PC-[^{3}H]IL-6 or native [^{3}H]IL-6, 10 μgEq IL-6 in 100 μL of 10 mM PBS via a tail vein or by subcutaneous injection. Fifty microlitres of blood was obtained from an ophthalmic vein using a heparinized capillary tube at 10 min, 1, 2, 4, 8, 24 h after administration. The radioactivity of the blood was counted using a liquid scintillation counter, and the IL-6 concentration in blood was calculated.

Thrombopoietic activity

Daily intravenous injection. PC-IL-6 and native IL-6 were diluted in PBS(-) containing 0·025% bovine serum albumin (dilution buffer) and administered intravenously at 4–25 μg every day for 7 days. The control mice were injected with dilution buffer or dilution buffer containing PC palmitoyl derivative equivalent to PC-IL-6 (lecithin control). Blood was sampled from a tail vein and the platelet count was measured with a Platelet Counter PL-110 (Toa Medical Electronics Co., Ltd., Osaka, Japan). The initial platelet level of day 1 was defined as 100%, and the increase percentage in the platelet was calculated on days 3, 5, 7, 9, 11, 13, 15.

Daily subcutaneous injection. PC-IL-6, 0·33–3·3 μg daily and native IL-6, 1·0–10 μg daily were administered subcutaneously every day for 7 days. Blood was sampled on day 1, 3, 5, 7, 9, 11 and 13. The other procedures were the same as by the intravenous route. The platelet count was also calculated.

Subcutaneous injection every other day. PC-IL-6, 0·8–12·5 μg per day and native IL-6, 12·5–50 μg per day were administered subcutaneously on days 1, 3, 5 and 7. Blood was sampled on day 1, 3, 5, 7, 9, 11, 13 and 15. The platelet count was calculated as described above.

Plasma IgG_1 level and blood urea nitrogen (BUN) measurements

Plasma IgG_1 level was measured by mouse IgG_1 enzyme linked immunosorbent assay (ELISA), and BUN using a WAKO BUN kit, on day 9 after subcutaneous injection of IL-6 every other day for 7 days.

Statistical analysis

Data are expressed as the mean ± s.d. Statistical analysis was performed by the Mann-Whitney *U*-test and the level of significance was set at $P < 0{\cdot}05$.

Results

Synthesis of PC-IL-6

Activated phosphatidylcholine (0·22 mol Eq of the 14 lysine groups in native IL-6) was used in this reaction. Stoichiometrically, PC-IL-6 has 1–3 molecules of a PC derivative bound to each IL-6 molecule. Isoelectric focussing of PC-IL-6 was performed electrophoretically. The isoelectric point of PC-IL-6 was approximately 6·0 and that of native IL-6 was approximately 6·5.

In-vitro activity of PC-IL-6

The results of the IL-6-dependent MH-60.BSF-2 cell growth assay are shown in Fig. 1. Optical density at 595 nm (OD_{595}) shows the enzymatic activity of the cells. OD_{595} is used as the index of the cell growth. The concentration which showed 50% cell growth (EC50) was 8·0 pg mL^{-1} for PC-IL-6 and 1·0 pg mL^{-1} for native IL-6. The in-vitro bioactivity was $1{\cdot}25 \times 10^5$ U mg^{-1} for PC-IL-6 and 10^6 U mg^{-1} for native

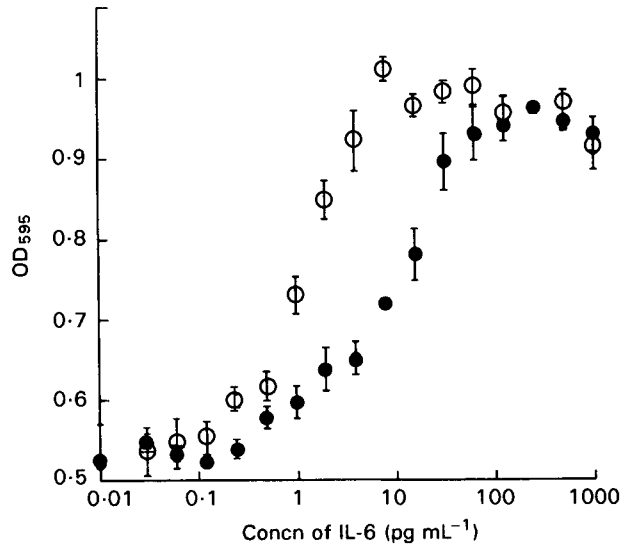

FIG. 1. In-vitro cell-growth activity of PC-IL-6. The data show the means ± s.d. (n = 4). ● PC-IL-6, ○ native IL-6.

IL-6. Thus activity of PC-IL-6 relative to native IL-6 was 12·5%.

Elimination half-life

The blood elimination half-life of PC-IL-6 was longer than that of native IL-6, both by intravenous and subcutaneous injection (Fig. 2). The AUC_{0-24h} was 1·53 $\mu g\,h\,mL^{-1}$ for PC-IL-6 and 1·13 $\mu g\,h\,mL^{-1}$ for native IL-6 by subcutaneous injection, and was 3·72 $\mu g\,h\,mL^{-1}$ for PC-IL-6 and 2·31 $\mu g\,h\,mL^{-1}$ for native IL-6 by the intravenous route.

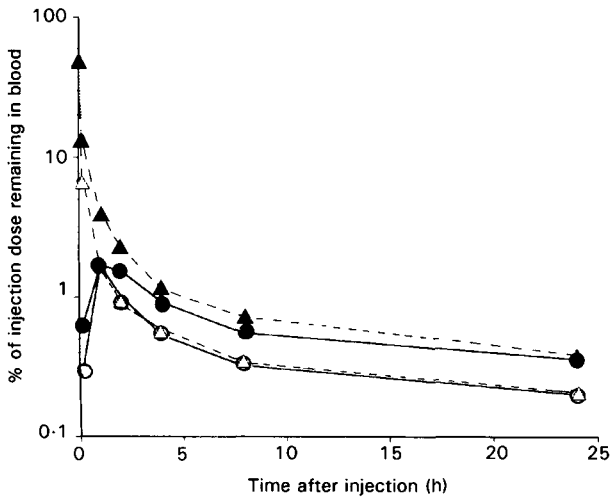

FIG. 2. Blood elimination of PC-IL-6. The data show means (n = 5). ● Subcutaneous PC-IL-6, ▲ intravenous PC-IL-6, ○ subcutaneous native IL-6, △ intravenous native IL-6.

Thrombopoietic activity

Daily intravenous administration. The platelet counts were slightly increased at highest dose of native IL-6 (25 μg daily). The administration of PC-IL-6 did not show thrombopoietic activity (Table 1).

Daily subcutaneous administration. When IL-6 was administered, the platelet counts were highest on day 7. As shown in Table 2, the increase in platelets seen with PC-IL-6 3·1 μg daily was almost the same as that with native IL-6 10 μg daily. The activity of PC-IL-6 was about 3 times more potent compared with native IL-6.

Subcutaneous administration every other day. The platelet counts were highest on days 7–9. The increase seen with PC-IL-6 3·1 μg daily was almost the same as that at native IL-6 50 μg daily (Table 3, Fig. 3). The activity of PC-IL-6 was over 15 times more potent than that of native IL-6.

Plasma IgG_1 level and BUN

Neither PC-IL-6 nor native IL-6 markedly influenced the BUN level at any of the doses tested (Table 4). The IgG_1 level in heparinized blood was increased by both PC-IL-6 and native IL-6. The increase of IgG_1 level caused by PC-IL-6 3·1 μg every other day was lower than that caused by native IL-6 50 μg every other day (Table 4) even though these doses produced the same thrombopoietic activity (Fig. 3, Table 3).

Discussion

Which is the most effective drug delivery system for IL-6? For IL-2: liposomal IL-2 (Kedar et al 1994), PEG-conjugated IL-2

Table 1. The platelet percentage increase after intravenous injection of IL-6 every day.

Group	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
PBS control	100	87·6 ± 10·9	96·0 ± 15·4	81·6 ± 8·7	82·3 ± 11·5	85·8 ± 16·8	83·9 ± 16·5	92·9 ± 15·5
Native IL-6								
4 μg	100	78·5 ± 4·9	95·5 ± 10·6	99·0 ± 14·1	79·5 ± 16·3	70·5 ± 10·6	83·0 ± 11·3	91·5 ± 10·6
10 μg	100	74·5 ± 2·1	94·0 ± 5·7	114·0 ± 7·1*	118·5 ± 19·1	97·5 ± 20·5	88·0 ± 9·9	90·5 ± 29·0
25 μg	100	84·5 ± 3·5	105·5 ± 16·3	108·0 ± 19·8	119·5 ± 10·6*	92·0 ± 24·0	98·0 ± 29·7	96·0 ± 26·9
Lecithin control	100	90·2 ± 24·5	87·4 ± 11·7	93·4 ± 21·7	88·2 ± 12·8	93·2 ± 14·9	82·2 ± 16·0	100·8 ± 11·1
PC-IL-6								
4 μg	100	96·0 ± 14·7	99·0 ± 14·7	109·3 ± 13·6	109·3 ± 14·4	101·7 ± 5·5	89·0 ± 13·1	92·3 ± 4·7
10 μg	100	100·5 ± 16·3	93·5 ± 7·8	110·5 ± 4·9	114·5 ± 0·7	123·0 ± 24·0	105·0 ± 15·6	107·0 ± 1·4
25 μg	100	103·5 ± 12·0	97·5 ± 23·3	101·0 ± 29·7	100·5 ± 30·4	108·5 ± 31·8	93·5 ± 24·7	91·0 ± 17·0

Values represent mean ± s.d. The initial platelet level was defined as 100%. *$P < 0·05$ vs PBS control.

Table 2. The platelet percentage increase after subcutaneous injection of IL-6 every day.

Group	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
Control	100	96·4 ± 12·1	93·8 ± 16·1	92·2 ± 16·1	83·4 ± 8·1	82·4 ± 7·6	108·2 ± 12·6
Native IL-6							
1·0 μg	100	98·8 ± 15·6	97·0 ± 18·2	105·2 ± 15·7	102·6 ± 7·4**	86·8 ± 11·0	103·6 ± 16·1
3·3 μg	100	95·6 ± 13·7	99·0 ± 27·1	118·4 ± 15·9	103·6 ± 12·2*	83·8 ± 4·4	90·8 ± 10·5
10·0 μg	100	98·4 ± 10·1	92·6 ± 13·0	131·0 ± 25·8*	104·8 ± 20·4	93·2 ± 15·0	112·2 ± 7·2
PC-IL-6							
0·3 μg	100	110·4 ± 10·1	122·8 ± 23·7	93·6 ± 8·3	94·4 ± 15·0	96·0 ± 12·6	112·2 ± 27·4
1·0 μg	100	106·6 ± 3·8	115·0 ± 6·2*	118·2 ± 10·6*	111·4 ± 9·4**	97·0 ± 19·7	106·4 ± 8·1
3·3 μg	100	113·8 ± 12·0	109·2 ± 20·0	130·6 ± 20·3	117·6 ± 13·5**	107·8 ± 8·7**	117·4 ± 17·2

Control = lecithin + BSA. Values represent mean ± s.d. The initial platelet level was defined as 100%. *$P < 0·05$ vs **$P < 0·01$ vs control.

Table 3. The platelet percentage increase after subcutaneous injection of IL-6 every other day.

Group	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
PBS control	100	96·2 ± 4·0	101·2 ± 12·3	91·6 ± 3·2	94·2 ± 8·8	75·8 ± 12·9	89·2 ± 16·4	108·8 ± 9·9
Native IL-6								
12·5 μg	100	105·4 ± 16·1	108·6 ± 15·0	103·1 ± 20·3	98·4 ± 9·8	99·4 ± 15·0*	94·3 ± 19·6	98·8 ± 10·7
25 μg	100	108·3 ± 18·8	118·6 ± 8·9*	118·9 ± 13·3**	114·6 ± 9·1**	106·6 ± 24·0*	104·0 ± 29·1	100·4 ± 16·9
50 μg	100	107·0 ± 9·2*	130·5 ± 19·8*	129·8 ± 14·6**	140·4 ± 10·2**	115·6 ± 30·4*	101·6 ± 27·2	95·4 ± 23·9
Lecithin control	100	94·6 ± 4·0	100·4 ± 11·5	93·8 ± 6·3	95·0 ± 13·4	68·8 ± 6·3	88·4 ± 9·6	104·6 ± 12·3
PC-IL-6								
0·8 μg	100	100·6 ± 6·2	111·4 ± 7·9	118·2 ± 14·0#	94·8 ± 13·3	110·0 ± 12·3##	121·6 ± 17·3##	120·0 ± 12·6
3·1 μg	100	107·0 ± 7·8#	123·4 ± 17·9	156·6 ± 6·6##	145·0 ± 15·0##	122·8 ± 6·2##	106·0 ± 14·4	119·0 ± 17·0
12·5 μg	100	115·6 ± 11·1##	152·4 ± 21·5##	195·6 ± 53·6##	194·6 ± 49·0##	117·4 ± 30·2##	103·2 ± 14·5	123·0 ± 12·9#

Values represent mean ± s.d. The initial platelet level was defined as 100%. *$P < 0.05$, **$P < 0.01$ vs PBS control, #$P < 0.05$, ##$P < 0.01$ vs lecithin control.

(Katre et al 1987) and IL-2 immunocomplexed with antibody (Sato et al 1994) were reported to exert a greater antitumour effect than native IL-2. It is important that a drug delivery system for IL-6 can deliver IL-6 to megakaryocytes, the target cells of IL-6 (Navarro et al 1991; Taga 1992), and that it can sustain the stimulating action of platelet production (Geissler et al 1992). The megakaryocytes are primarily located in the marrow and pulmonary vessels (Pedersen 1974; Lichtman et al 1978). Therefore, for a drug delivery system to be effective, it must prolong the elimination half-life of IL-6. It is possible that the observed enhancement of thrombopoietic potency of PEG-IL-6 is due to the longer elimination half-life (Tsutsumi et al 1995). What enhanced the thrombopoietic activity of PC-IL-6?

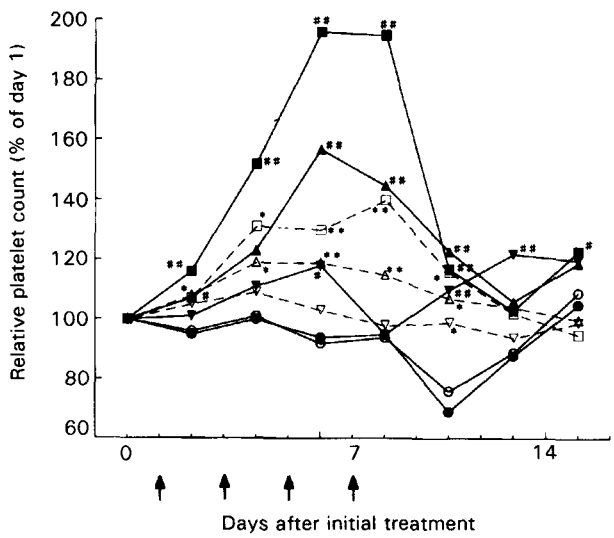

FIG. 3. Peripheral platelet level after subcutaneous injection every other day for 7 days. The initial platelet level was defined as 100%. The data show the mean (n = 5–8). ● lecithin control; PC-IL-6: ■ 12·5 μg, ▲ 3·1 μg, ▼ 0·8 μg; ○ PBS control; native IL-6: □ 50 μg, △ 25 μg, ▽ 12·5 μg. #$P < 0.05$, ##$P < 0.01$ vs lecithin control, * $P < 0.05$, ** $P < 0.01$ vs PBS control. Arrows show the day of subcutaneous injection.

Table 4. Plasma BUN and IgG_1 levels on day 9 after subcutaneous injection every other day for 7 days.

	BUN (μg mL^{-1})	IgG_1 (μg mL^{-1})
PBS control	262·6 ± 34·6	165·4 ± 62·2
Lecithin control	349·6 ± 44·7	153·9 ± 29·5
Native IL-6		
50 μg	307·1 ± 41·2	256·4 ± 40·7
25 μg	291·9 ± 9·4	185·3 ± 50·6
12·5 μg	304·2 ± 9·0*	169·6 ± 24·7
PC-IL-6		
12·5 μg	257·2 ± 35·4#	212·1 ± 20·9#
3·1 μg	293·9 ± 30·8	216·8 ± 33·1
0·8 μg	261·3 ± 24·6	184·1 ± 70·5

Data represent means ± s.d. (n = 3–5). *$P < 0.05$ vs PBS control; #$P < 0.05$ vs lecithin control.

The in-vitro IL-6-dependent MH-60.BSF-2 growth assay showed decreased receptor binding activity as a result of lecithinization of IL-6. Although data were not shown, PC-IL-6 exhibited lower binding affinity for human and murine blood cells than native did IL-6 and this also accounted for difference in receptor binding activity between PC-IL-6 and native IL-6, as manifest in the in-vitro MH-60.BSF-2 growth activity. IL-6 exerts its activity by binding with the high molecular weight IL-6 receptor, and this activity would theoretically be reduced by steric hindrance following chemical modification such as lecithinization of IL-6, particularly modification of Lys171 or Arg168 which are crucial for IL-6 activity (Ida et al 1989; Fontaine et al 1994).

Although in-vitro activity of PC-IL-6 was 12·5% of that of native IL-6 as described above, the thrombopoietic activity in-vivo of PC-IL-6 was over 15 times more potent than native IL-6 by subcutaneous injection every other day. In the blood elimination study, the AUC of PC-IL-6 was about 1·5 times that of native IL-6. It is considered that radioactivity is present in the conjugated product, because the covalent bond between PC and amino terminal of IL-6 is not hydrolysed easily in serum (data not shown). Although the elimination curve for intravenous injection of PC-IL-6 was almost same as that for subcutaneous injection, subcutaneous administration showed more effective thrombopoietic activity. Therefore, prolongation of the elimination half-life does not fully explain the increased potency seen with PC-IL-6. It also may have arisen from improved delivery of IL-6 to the marrow and spleen following lecithinization. In a preliminary tissue distribution study, PC-[^{3}H]IL-6 accumulated in murine organs including spleen and bone marrow 2 ~ 3 times more than [^{3}H]IL-6. Nevertheless, the mechanism of enhancement of thrombopoietic activity of PC-IL-6 is still poorly understood.

BUN, a marker of IL-6-induced mesangial proliferative glomerulonephritis (Horii et al 1989), did not markedly

increase following administration of either PC-IL-6 or native IL-6 at any of the doses tested in this study. Concerning IgG plasmacytosis, the plasma IgG_1 level observed following administration of PC-IL-6 3·1 μg every other day was lower than that following native IL-6 50 μg every other day even though the thrombopoietic activity at these doses was the same.

In conclusion, the lecithinization of IL-6 enhanced the pharmacological potency and decreased the adverse effects. Thus, lecithinization appears to be an effective drug delivery system for IL-6. We may be able to prepare an even more effective PC-IL-6 by changing the site of modification and the binding lecithin number.

Recently, thrombopoietin, a specific megakaryocyte growth and development factor, was isolated (Bartley et al 1994; Wendling et al 1994; Kaushansky et al 1994; Sauvage et al 1994; Lok et al 1994). Lecithinization may potentially be an effective drug delivery system for thrombopoietin.

Acknowledgement

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