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In vitro cytostatic effect of TNF (Tumor Necrosis Factor) entrapped in immunoliposomes on cells normally insensitive to TNF

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The cytostatic activity of TNF entrapped in novel immunoliposomes with a specific antibody against target cells is described. A two step conjugation method was used for the preparation of these targeted immunoliposomes. In the first step, liposomes containing *N*-4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine (MPB-PE) were conjugated with a goat anti-mouse IgG Fab' fragment which recognizes the Fc portion of a mouse antibody against the target cell markers. In the second step, the mouse antibody against human tumor cells was conjugated to the liposomes. Using these targeted immunoliposomes, we demonstrated that cells usually insensitive to TNF such as Daudi cells, MT-2 cells and T-24 cells could become sensitive to TNF in vitro. The cytostatic activity of these immunoliposomes was blocked by the addition of a lysosomotropic agent such as NH_4Cl or chloroquine. Significant uptake of ^{125}I-TNF into T-24 cells was observed when these immunoliposomes were used, and this uptake of TNF was inhibited by cytochalasin B or chloroquine. Free ^{125}I-TNF was not taken up by these cells.

Introduction

Tumor necrosis factor (TNF) [1] is a macrophage/monocyte-derived monokine which has many biological functions such as cytotoxic activity on tumor cells [2], immune modulating activities [3–5], and various other activities [6–8].

Immunoliposomes are known as specific drug carriers to target cells in vitro and in vivo [9–12]. Many methods of immunoliposome preparation have been reported [13–16]. For instance, Hashimoto et al. and Sunamoto et al. took advantage of the presence of the SH-bearing subunit in IgM [10,13] and Papahadjopoulos et al. and Nässander et al. prepared immunoliposomes using the Fab' fragment of IgG conjugated with *N*-4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine (MPB-PE) molecules [11,14]. Huang's group used palmitoyl IgG [15] while Leserman's group used IgG modified with *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate molecules [16]. Herein, we prepared immunoliposomes with two antibodies; the second one being anti-tumor-cell antibody. We report that by trapping TNF in our immunoliposomes, TNF became an effective cytostatic agent against cells otherwise insensitive to TNF. These findings suggest that the clinical effectiveness of TNF can be improved by entrapping TNF in these immunoliposomes. These findings have been reported preliminarily elsewhere [17].

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Abbreviations: TNF, recombinant human tumor necrosis factor-α; PC, egg phosphatidylcholine; PE, phosphatidylethanolamine; TCA, trichloroacetic acid; MPB-PE, *N*-4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine; DTT, dithiothreitol; Chol, cholesterol; PBS(-), phosphate buffered saline; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; Bq, Bequerel; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Materials and Methods

Materials

Egg phosphatidylcholine (PC) was purchased from Nichiyu Liposome (Tokyo, Japan). Cholesterol (Chol), dithiothreitol (DTT) and Ficoll 400 were purchased from Wako Pure Chemicals (Osaka, Japan). *N*-4-(*p*-Maleimidophenyl)butyryl phosphatidylethanolamine (MPB-PE) was prepared by the method of Martin [14]. Egg phosphatidylethanolamine (PE) was purchased

from Avanti Polar Lipids (Alabaster, AL, USA) and succinimidyl 4-(*p*-maleiminidophenyl)butyrate (SMPB) was from Pierce (Rockford, IL, USA). Bolton and Hunter reagent and ^{125}I-labelled recombinant human TNF-α (^{125}I-TNF, 23 TBq/mmol) which was prepared from recombinant human TNF-α by an oxidation method using Na^{125}I were obtained from Amersham (Buckinghamshire, UK). TNF is stable to the iodination process [18]. Recombinant human TNF-α (TNF, $1.3 \cdot 10^8$ units of TNF/mg protein) was prepared in our laboratory [19], and cytotoxic activity of TNF was determined by measuring actinomycin-D treated murine L929 cellular metabolic activity with the MTT assay using an automatic micro ELISA reader [20,21].

Cell lines and antibodies

Daudi cells (human Burkit lymphoma) and T-24 cells (human bladder carcinoma) were obtained from American Type Culture Collection (Rockville, MD, USA). MT-2 cells (human T cell leukemia) were kindly provided by Prof. Y. Hinuma of Kyoto University (Sakyo-ku, Kyoto, Japan). Daudi cells and MT-2 cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, v/v), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). T-24 cells were grown in Eagle Minimum Essential Medium (MEM, Gibco) containing the same concentration of FCS, penicillin and streptomycin as mentioned above. All cell lines were cultured at 37°C in 5% CO_2.

Monoclonal mouse antibody (OKT-4, IgG) was obtained from Ortho Diagnostic Systems (Raritan, NJ, USA). This antibody recognizes the CD-4 protein expressed on the MT-2 cell surface. Purified polyclonal goat F(ab')$_2$ anti-mouse IgG antibody which recognizes the Fc portion of IgG and purified polyclonal mouse anti-human IgG(H + L) which recognizes the cell surface IgG of Daudi cells [22] were purchased from Jackson Immunoresearch Lab. (Avondale, PA, USA). Monoclonal mouse anti T-24 cell antibody (IgG) [23] was a generous gift from Prof. Y. Hashimoto of Tohoku University (Sendai, Japan).

Preparation of MPB-PE liposomes

Liposomes containing MPB-PE molecules, which are binding sites for antibodies were prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos with minor modification [24]. This liposome is called MPB-PE liposome here. In brief, a lipid mixture (PC/Chol/MPB-PE (10:10:1, molar ratio)) containing 10 μmol of PC was dissolved in 1 ml of diethyl ether followed by the addition of 100 μl of PBS(-) solution (140 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing $2.8 \cdot 10^6$ units of TNF and a trace amount of ^{125}I-TNF (3.2 kBq). The mixture was vortexed for 1 min at room temperature and sonicated for 15 s at 4°C under argon gas. Immediately after sonication, diethyl ether was evaporated at 23°C by a rotary evaporator. The exposure of TNF to diethyl ether under these conditions did not inactivate TNF at all. The influence of diethyl ether treatment on cytotoxic activity of TNF was studied. The TNF solution (200 μl, $6.7 \cdot 10^7$ unit of TNF/ml) was added to 2 ml of diethyl ether in a test tube. The mixture was vortexed for 1 min at room temperature and sonicated for 15 s at 4°C. Immediately after sonication, diethyl ether was evaporated at 23°C by a rotary evaporator and then PBS(-) was added to the TNF solution to a final volume of 200 μl. The cytotoxic activity of the thus treated TNF was $6.6 \cdot 10^7$ unit/ml measured as described above. This indicates that TNF activity was not influenced by diethyl ether treatment. The residual TNF which was not encapsulated was separated from the liposomes by the Ficoll flotation method [25]. Briefly, 100 μl of the above mixture containing 10 μmol of PC was suspended in 1 ml of 20% Ficoll solution in PBS(-) and centrifuged for 5 min at $10000 \times g$ at room temperature. The lower layer containing free TNF and Ficoll was sucked up and the liposomes in the top layer were saved. The liposomes were mixed with 200 ml of PBS(-) and suspended well, and then 1 ml of 20% Ficoll was added again followed by centrifugation. These steps were repeated three times, and the MPB-PE liposomes containing TNF thus obtained were dispersed in 250 μl of PBS(-).

The entrapping efficiency of TNF into MPB-PE liposome was determined by counting the radioactivity of the ^{125}I-TNF which behaves identically to the native TNF [26]. Radio activities of the unentrapped TNF fraction and liposomal TNF fraction which were separated by the Ficoll flotation method were measured by a γ-counter (Beckman, Irvine, CA, USA). The entrapping efficiency of TNF into the liposomes thus found was 63.7% (S.D. = 2.5, $n = 4$).

The size of the liposomes was determined by the Coulter Counter model ZM (Coulter, Luton, Beds, UK). 30 μl of MPB-PE liposomes solution (10 μmol of PC/ml) were added to 30 ml of saline and distribution of liposome size was measured. The saline was filtered through 0.22 μm pore size filter three times prior to use. The average diameter of the liposomes was 450 nm. Fig. 1 shows the size distribution of the liposome prepared. Whether or not Fab' coupling influences the particle size is unknown. There was no apparent aggregation (detected microscopic observation) during storage of the immunoliposome.

Preparation of immunoliposomes

The Fab' fragment of anti mouse IgG F(ab')$_2$, which recognizes the Fc portion of a mouse antibody against target cell markers, was prepared by the method of

Martin et al. with slight modifications [14] (Fig. 2). In brief, 3.1 mg of ^{125}I-labelled goat F(ab')$_2$ anti-mouse IgG (8.5 kBq/mg of protein) prepared by the method of Bolton and Hunter [27] were dissolved in 125 μl of PBS(-) containing 20 mM DTT (Martin et al.) was performed at pH 5.5. The solution was incubated for reduction of F(ab')$_2$ for 2h at 25°C and the mixture was subjected to Sephadex G-50 column chromatography with citric acid buffer (20 mM citric acid, 35 mM disodium phosphate, 108 mM NaCl, 1 mM EDTA (pH 5.0)) to separate Fab' fragment from DTT.

TNF entrapped MPB-PE liposomes containing 6 μmol of PC in 150 μl of PBS(-) were mixed with 210 μl of the citric acid buffer containing 1.0 mg of Fab' fragment of the goat anti-mouse IgG as prepared above. The pH was adjusted to 6.5 with 1 M NaOH and the reaction mixture was stirred under argon gas for 18 h at room temperature. Immunoliposomes conjugated with the first antibody thus obtained were separated from the unconjugated antibody fragments by the Ficoll flotation method. The radioactivities of the Fab' liposome and Ficoll layer were 4278 Bq and 2667 Bq, respectively, indicating that 61.6% of ^{125}I-Fab' were incorporated into MPB-PE liposome (103 μg Fab'/μmol PC). The amount of phosphatidylcholine was determined by using [^{3}H]dipalmitoylphosphatidylcholine as tracer. Since $2.4 \cdot 10^{11}$ vesicles of liposomes (450 nm diameter) can be obtained from 1 μmol of phospholipid [17], approx. 5200 molecules of Fab' fragment were conjugated per vesicle of MPB-PE liposome when the diameter of liposome is 450 nm. The number

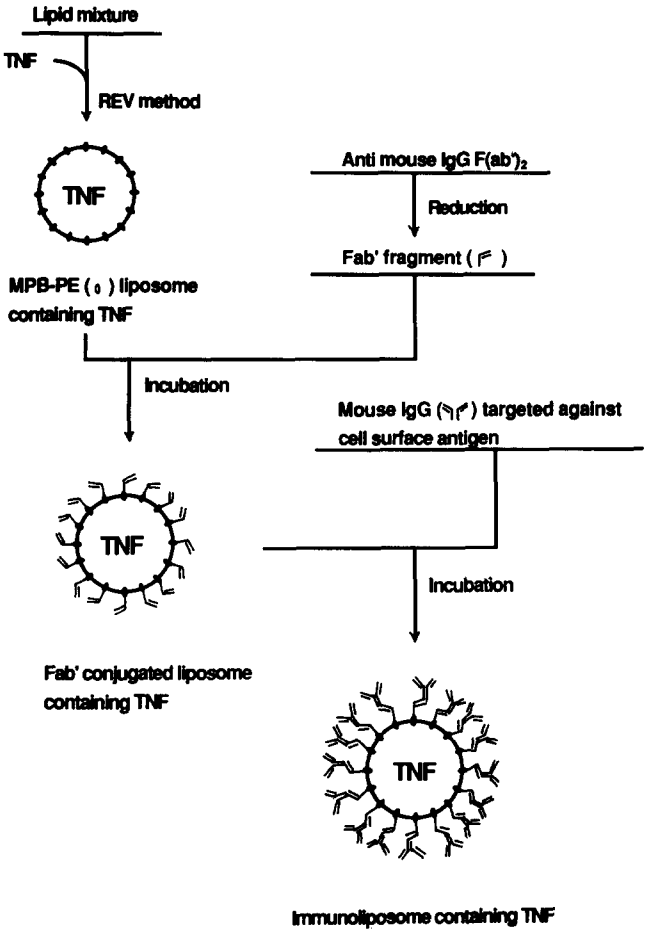

Fig. 2. Procedure for preparation of immunoliposomes containing TNF.

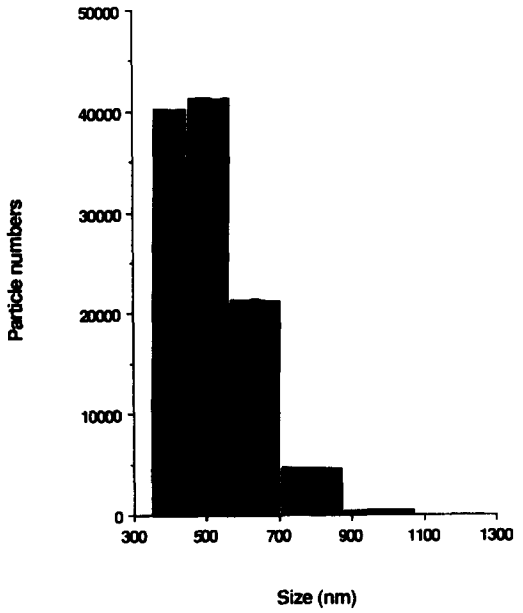

Fig. 1. Size distribution of MPB-PE liposome. The number of particles was counted for 5 s in a Coulter Counter. Each bar shows the average of quadruplicate assays (mean ± S.D.).

of Fab' fragments conjugated to the liposome surface depended on the particle size of liposome.

For preparation of immunoliposomes conjugated with the second antibody, 48 μl of the PBS(-) solution containing the first antibody conjugated immunoliposomes (1.6 μmol of PC, 1141 Bq) as prepared above were mixed with 132 μl of PBS(-) solution containing purified 0.6 mg of ^{125}I-mouse anti-human IgG(H + L) (18.6 kBq, for Daudi cells) or 0.6 mg of mouse monoclonal anti T-24 (for T-24 cells) or 0.05 mg of mouse monoclonal anti-human CD4 (OKT-4, for MT-2 cells) as secondary antibodies. After incubation of the mixture for 3 h at 4°C, an unconjugated antibody was separated from the liposome-antibody complex by the Ficoll flotation method. The radioactivity of the immunoliposomes thus obtained was 8350 Bq and the Ficoll layer representing the unreacted antibody was 8724 Bq. Since the radioactivity of the Daudi immunoliposome is the sum of the radioactivity of the first and the second antibodies, the radioactivity of the second antibody in the immunoliposome was calculated to be 7363 Bq, indicating that approx. 46% of the added second antibody was conjugated with the liposome

containing the first antibody. This indicates that 163 μg of the second antibody was conjugated to the Fab' liposome with 1 μmol of PC. Therefore, 2717 molecules of the second antibody were calculated to be present per vesicle. Fig. 2 shows the scheme for preparation of these immunoliposomes. In the final preparation of liposomes, 176 units of TNF were encapsulated per nmol of PC.

The composition of our liposomes described here was obtained after many trial experiments (data not shown). The change in the lipid composition reported here has so far resulted in poorer liposomes, which are toxic or unstable. For example, positively charged liposomes were more toxic than the neutrally charged liposomes used in this study. Similarly Oku et al. [28], reported that their negatively charged liposomes leaked entrapped TNF materials. Our liposome has the advantage in that it can be prepared at room temperature, and the diethyl ether used for preparation can be easily removed from the liposome suspension.

Determination of cytotoxic effect of immunoliposomes in vitro

Daudi cells ($5 \cdot 10^3$ cells/well), MT-2 cells ($9 \cdot 10^3$ cells/well) and T-24 cells ($1 \cdot 10^4$ cells per well) were seeded into 96-wells tissue culture plates (100 μl/well) on day 0. All cell lines were cultured at 37°C. Liposomal TNF or free TNF was added to these cell cultures 3 h after the cell seeding, and the cells were cultured for 72 h. Cytotoxic activity was assayed by counting the number of cells by the Trypan blue dye exclusion method [29] on day 3. Since T-24 cells attached to the well, the number of viable cells for this strain was determined after treatment with 0.25% trypsin in PBS(-).

Measurement of ^{125}I-TNF uptake by T-24 cells

24 μl of PBS(-) buffer containing immunoliposomes (280 nmol of PC) with ^{125}I-TNF (1.3 kBq/374 ng of TNF) was added to T-24 cells ($1.5 \cdot 10^6$ cells/ml per well). As a control, 24 μl of PBS(-) containing non-targeted immunoliposomes (the same as above except that anti-Daudi antibodies were used instead of anti T-24 antibodies), or 1.8 μl of PBS(-) solution containing free ^{125}I-TNF (1.4 kBq/385 ng of TNF) were used. In some cases chloroquine or cytochalasin B was added as described in each experiment. For measuring the uptake of ^{125}I-TNF, the media were removed and the wells were washed with 1 ml of ice-cold PBS(-) three times. Cells were then suspended in 1 ml of ice-cold PBS(-), and were centrifuged for 5 min at 700 $\times$g. The pelleted cells were further washed three times with 1 ml of ice-cold PBS(-) and were disrupted in 70 μl of PBS(-) containing 0.6% SDS. The suspension was placed on Whatman 3MM paper with 2.4 cm in diameter. To collect the residual cell extract, an additional 70 μl of PBS(-) was added to the tube, and the wash was placed on the same Whatman 3MM paper. The radioactivity insoluble in 5% trichloroacetic acid (TCA) at 4°C was measured.

Assay of TNF activity within liposomes

MPB-PE liposomes containing TNF ($4.5 \cdot 10^5$ units of TNF/5 μmol of PC) were prepared by the method described above. 500 μl of PBS(-) solution containing the MPB-PE liposomes with TNF (5 μmol of PC) were mixed with an equal volume of 0.5% of trypsin (Gibco) in PBS(-) and incubated for 20 min at 37°C to eliminate the TNF on the liposome surface. Trypsin was removed by the Ficoll flotation method, and the liposome solution was adjusted to 500 μl with PBS(-). The liposome solution (50 μl) was then mixed with 4 μl of 5% Triton X-100 and 46 μl of PBS(-), and then stood for 30 min at room temperature. The TNF activity of the resulting solution was measured by the MTT assay which is a rapid colorimetric assay for cellular growth and survival as described above [22,23]. Triton X-100 and Ficoll did not interfere with the MTT assay under this experimental condition. The cytotoxic activity of the liposome fraction treated by trypsin was $3.5 \cdot 10^5$ units and represented 122% recovery of the TNF activity, and the entrapping efficiency of TNF into the liposome was 64%. The entrapping efficiency thus obtained was the same value as that calculated from the entrapped radioactive ^{125}I-TNF. These findings show that the biological activity of TNF was not reduced by entrapment into liposomes, and also suggested that TNF was not present on the outer surface of the liposome membrane, but inside of the liposome vesicle.

Results

Sensitivity of various tumor cells to TNF entrapped in immunoliposomes

Fig. 3 shows the time course of T-24 cell growth in the presence of 732 units of liposomal TNF containing 4.2 nmol of PC or 840 units of free TNF. Clearly, TNF was effective only when it was entrapped in the immunoliposomes. The rate of cell growth and the final level of the cell growth were reduced by the TNF entrapped in the immunoliposomes. All other controls including free TNF and empty immunoliposomes had practically no effect. The antibody attached to the immunoliposomes must be an antibody specifically against the target cells. Thus, TNF entrapped in the immunoliposomes against Daudi cells was not effective against T-24 cells. Since there was no apparent decrease in the number of cells due to the addition of the TNF liposomes, we concluded that TNF exhibited only a cytostatic effect [30] rather than a cytocidal effect [31] under the present experimental conditions.

The specific nature of the cytostatic effect of the immunoliposomes containing TNF was further demonstrated using other cell lines such as Daudi cells and MT-2 cells. Fig. 4 shows that the cytostatic effect was observed only with TNF entrapped in appropriate liposomes with the correct antibody against each cell. Approx. 90% inhibition of cell growth was observed when the immunoliposomes containing 300 units of TNF were added. The reason why 100% inhibition was not observed could be due to the existence of a resistant cell population and/or to the limited amount of TNF uptake through the immunoliposome mechanism. These findings showed that TNF in the immunoliposomes was very effective while TNF in conventional liposomes was completely inactive. As shown in Fig. 4C, TNF entrapped in immunoliposomes which recognize the surface marker of Daudi cells but not that of T-24 cells did not have a cytostatic effect on T-24 cells further indicating the specific nature of the immunoliposome action.

Association of ^{125}I-TNF in immunoliposomes with the target cells

Table I shows the association of ^{125}I-TNF with T-24 cells upon treatment with anti T-24 liposome containing ^{125}I-TNF. The maximum association of TNF with T-24 cells was observed with the anti T-24 immunoliposomes containing ^{125}I-TNF. When TNF was entrapped in the immunoliposomes, as much as 86% of the total liposomal TNF added to the culture medium was associated with T-24 cells. This represents an approx. 400-fold higher efficiency level compared to the association of free TNF. The association was relatively rapid, and at 40 min after the incubation, the association was already at the plateau. Endocytosis is probably involved in this association since the association of ^{125}I-TNF was significantly inhibited by cytochalasin B (an endocytosis inhibitor) [32] and chloroquine (an uptake inhibitor) [33]. The specificity of the immunoliposomes was further confirmed by the observation that the association of TNF in the nontargeted immunoliposomes was the same level as the association of TNF in the conventional liposomes.

Effect of endocytosis inhibitors on cytostatic activity of TNF entrapped in the immunoliposomes

As expected from Table I, chloroquine (lysosomotropic agent) would be an inhibitory agent to the cytostatic effect of TNF encapsulated in the immunoliposomes. This can be seen from Fig. 5 which indicates the dose response effect of TNF on the growth of T-24 cells. As much as a 40% decrease of the effect of TNF was observed with chloroquine at various doses of immunoliposomal TNF. In addition, this figure shows that NH_4Cl, which is known to raise the pH of the lysosomes [34], had a marked inhibitory effect (almost 70% decrease of its inhibitory effect) on the cytostatic activity of TNF entrapped in the immunoliposomes. Since the efficacy of TNF entrapped immunoliposomes was dependent on the specific antibody against the target cells, one expects the effect of this TNF in the immunoliposomes to be decreased by the presence of the same antibody against these cells in the culture. The antibody would compete for the cellular binding site with the immunoliposomes. As can be seen from this figure, an excess amount of the free antibody in the culture significantly inhibited the cytostatic effect of TNF entrapped in the immunoliposomes (almost 70% decrease). These findings strengthen the notion that the cytostatic effect of TNF entrapped in the immunoliposomes involves lysosomes as well as the specific attachment of these liposomes on the cell surface.

The antibody against the target cells has to be conjugated for the immunoliposome to be effective

The experiment described in Fig. 6, shows that the antibody against the target cell marker must be conjugated to the liposomes. As noted in Fig. 6, the free

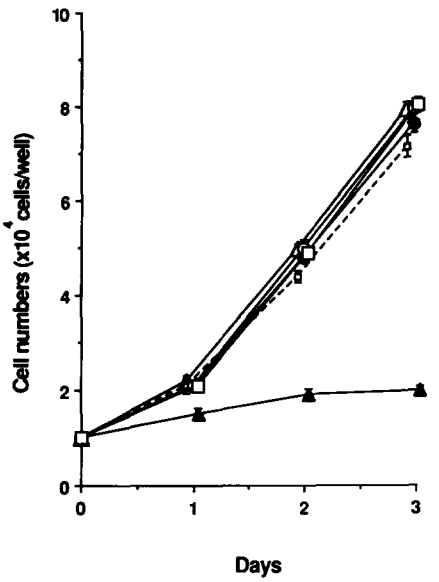

Fig. 3. Growth inhibition of T-24 cells by immunoliposomes containing TNF. T-24 $1 \cdot 10^4$ cells/100 μl/well were seeded and incubated for 3 h. Samples were added at this point and the increase in number of cells was followed. The amount of liposomal phosphatidylcholine added was 4.2 nmol/well. ○———○, control cells without TNF or liposomes; ●———●, 1.3 μl of PBS(-) containing MPB-PE liposomes (4.2 nmol PC equivalent) with 732 units of entrapped TNF; △———△, 1.3 μl of PBS(-) containing empty immunoliposomes (4.2 nmol PC equivalent); ▲———▲, 1.3 μl of PBS(-) containing immunoliposomes (4.2 nmol PC equivalent) with 732 units of entrapped TNF; □------□, 1.3 μl of PBS(-) containing non-targeted immunoliposomes (4.2 nmol PC equivalent, mouse anti-human IgG as secondary antibody) with 732 units of entrapped TNF; □———□, 0.6 μl of PBS(-) containing 840 units of free TNF. Each point is the average of quadruplicate assays (mean ± S.D.).

antibody against target cells together with TNF in the MPB-PE liposomes did not have any cytostatic or the cytotoxic activity against Daudi cells, MT-2 cells or T-24 cells (Figs. 6A–6C). In addition, in a similar fashion to the experiment described in Fig. 5, the cytostatic activity of the immunoliposomal TNF was blocked by the addition of a 35-fold excess of free antibody against T-24.

Since TNF molecules are known to be sticky, a portion of TNF can be suggested to exist on the outer surface of the liposomes as well as inside. However, this possibility was ruled out by the observation that a mixture of empty immunoliposomes and free TNF had no effect against target cells (Figs. 6A–6C). In a separate experiment, the liposomal TNF activity remained after trypsin treatment of the surface as described above. From these observations, we concluded that TNF molecules outside the liposomes are ineffective

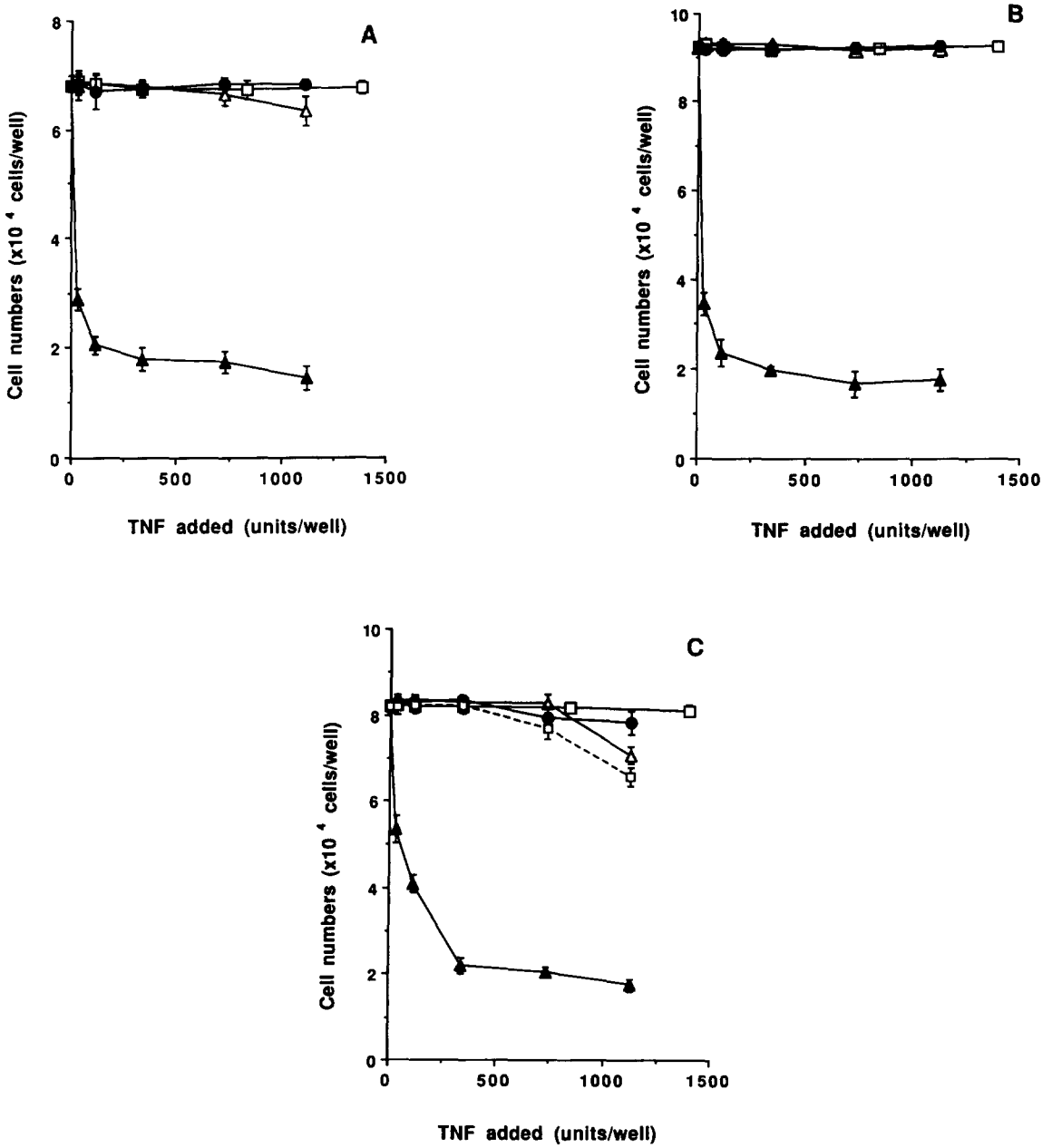

Fig. 4. Cytostatic activity of immunoliposomes containing TNF – Effect on Daudi, MT-2 and T-24 cells. Daudi cells ($5 \cdot 10^3$/100 μl per well) (A), MT-2 cells ($9 \cdot 10^3$/100 μl per well) (B) and T-24 cells ($1 \cdot 10^4$/100 μl per well) (C) were seeded and incubated for 3 h. Samples were then added at this point and cultures were further incubated for 72 h at 37°C and number of cells was counted. Each bar expresses mean ± S.D. (quadruplicate assays). ●———●, MPB-PE liposomes containing TNF; △———△, empty immunoliposomes; ▲———▲, appropriate immunoliposomes containing TNF against target cells; □———□, free TNF; □------□, nontargeted immunoliposomes containing TNF which have anti-human IgG(H + L) as secondary antibody.

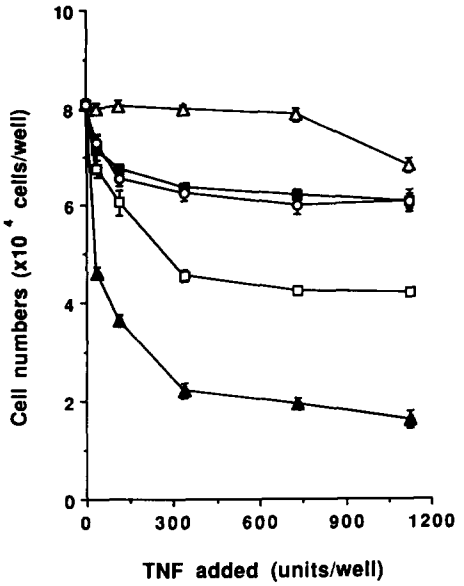

Fig. 5. Effect of lysosomotropic agents and free T-24 antibody on cytostatic activity of immunoliposomes containing TNF against T-24 cells. T-24 cells ($1 \cdot 10^4$/100 μl per well) were seeded and incubated for 3 h. Samples were added at this point to each well and the cultures were further incubated for 72 h at 37°C, and the number of cells per well was plotted against the amount of TNF added. Number of cells in the control culture without liposomes or TNF was $8.1 \cdot 10^4$ cells/well after 72 h incubation. △——△, empty immunoliposome (3.2 nmol of PC/μl of liposome stock solution); ▲——▲, immunoliposomes containing TNF (563 unit of TNF/3.2 nmol of PC/μl of liposome stock solution); ■——■, NH_4Cl (final concentration 50 μM) and immunoliposomes containing TNF; ○——○, 35-fold excess amount of free anti T-24 antibody (5.7 μg of IgG/nmol of PC) and immunoliposomes containing TNF; □——□, chloroquine (final concentration 50 μM) and immunoliposomes containing TNF. Each point is the average of quadruplicate assays (mean ± S.D.).

against these cells and TNF must be entrapped in the immunoliposomes to be effective against these cells. Fig. 6D shows the strong cytotoxic effect of the TNF used in this experiment on L929 cells, as a control.

Discussion

TNF is a protein which caused hemorrhagic necrosis of murine tumors [1]. Using recombinant human TNF, many phase 1 and 2 clinical tests have been performed on cancer patients. During these tests, various side effects such as hypotension, fever, nausea, headache, and tachycardia were observed with the systemic administration of TNF [35–38]. On the other hand, when TNF was administered locally to superficial tumors, complete regression or partial regression were observed with very few side effects except for fever and rigors [39]. Therefore, it appears possible to eliminate the side effects by targeting TNF specifically to the tumor tissues. It is for this reason that we applied TNF specifically to tumors using targeted immunoliposomes [11,12]. The second reason that TNF was not as effective as expected is that some tumor cells are not sensitive to TNF [40,41]. One possible reason for TNF resistance of certain tumor cells is the lack of TNF receptors on the cell surface. Such insensitive cells may be converted to sensitive cells by delivering TNF by means not depending on the TNF receptors. The immunoliposomes appeared to serve this purpose well because they are guided to the targeted cells through a specific antigen-antibody reaction on the surface rather than TNF receptors. In our in vitro experiment, 2-step

TABLE I

Association of T-24 cells and ^{125}I-TNF in liposome

Additions	Incubation time (min)	Cell associated counts ± S.D. (cpm)	% uptake
Free TNF	40	153 ± 2	0.2
	160	147 ± 11	0.2
MPB-PE liposomes containing TNF	40	4609 ± 215	5.9
	160	5029 ± 337	6.4
Targeted immunoliposomes	40	65539 ± 1269	83.7
containing TNF	160	67540 ± 1829	86.2
Chloroquine and targeted immuno-	40	28571 ± 1816	36.5
liposomes containing TNF	160	36908 ± 626	47.1
Cytochalasin B and targeted	40	15123 ± 22	19.3
immunoloposomes containing TNF	160	23066 ± 132	29.5
Nontargeted immunoliposomes	40	3223 ± 220	4.1
containing TNF *	160	3847 ± 107	4.9

Amounts of added ^{125}I-TNF to the system were 83006 cpm (free TNF) and 78309 cpm (TNF entrapped into the liposomes (280 nmol of PC)). Each experiment was carried out in duplicate. The final concentrations of chloroquine and cytochalasin B were 50 μM and 50 μg/well respectively.

* Nontargeted immunoliposomes have anti-human IgG(H + L) as secondary antibody on the surface of liposomes.

conjugated immunoliposomes were effective cytostatic agents when used with TNF, and these immunoliposomes could convert TNF into efficient cytostatic agents against cells which were otherwise insensitive to TNF. These observations suggest that such immunoliposomes can be used clinically to improve the efficiency of TNF activity. Indeed, in our preliminary experiments, our immunoliposomes exhibited marked antitumor activity in nude mice bearing TNF insensitive human tumor cells (will be reported elsewhere).

One obvious advantage of our immunoliposome is its ease of preparation. If the purified first antibody is available, one does not have to purify the second antibody which is targeted against the tumor cells. In addition, the fact that our immunoliposomes use a divalent rather than a monovalent antibody appears to be an advantage. This is because the affinity of the divalent antibody to target cells is higher than that of the monovalent antibody [42,43]. However, whether or not the unreacted first antibody on the liposome modi-

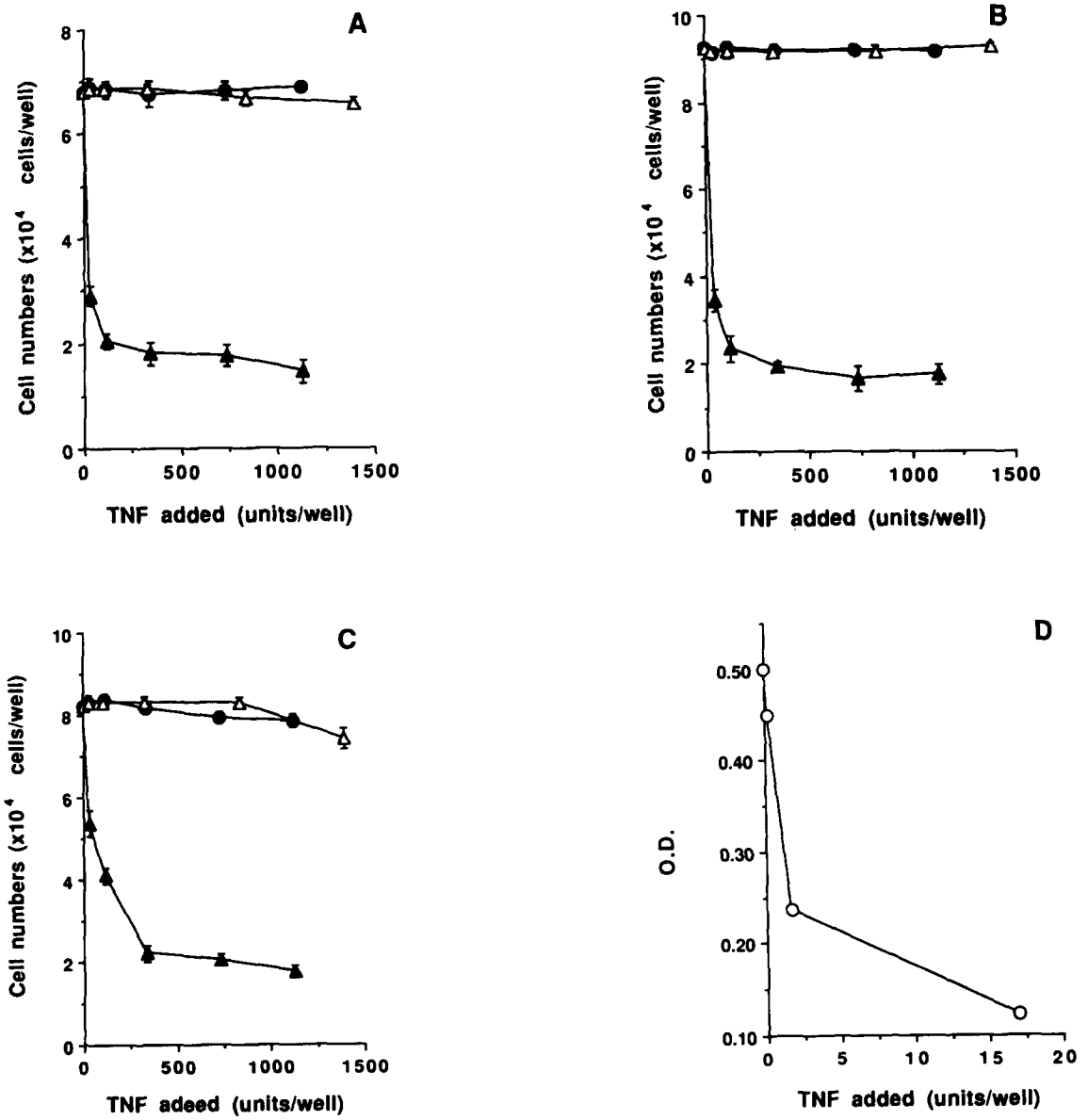

Fig. 6. The antibody against the target cells has to be bound to the liposome to be effective. Daudi cells ($5 \cdot 10^3$/well) (A), MT-2 cells ($9 \cdot 10^3$/well) (B) and T-24 cells ($1 \cdot 10^4$/well) (C) were seeded and incubated for 3 h. Samples were added at this point and the cultures were further incubated for 72 h at 37°C, and number of viable cells per well was counted. The free second antibodies (191 ng of IgG per 1 nmol of PC (A,C) or 31 ng of IgG per 1 nmol of PC (B)) were added. Each point is the average of quadruplicate assays (mean ± S.D.). ▲——▲, immunoliposomes containing TNF (data taken from Fig. 3, 176 unit of TNF/nmol of PC); ●——●, MPB-PE liposomes containing TNF and free antibody (A, mouse anti-human IgG (H+L); B, mouse anti-human CD-4 antibody; C, mouse anti T-24 antibody); △——△, free TNF and empty immunoliposomes (219 unit of TNF/nmol of PC). (D) Cytotoxic activity of free TNF on L929 cells. Viable cells were measured with the MTT assay method and plotted against the amount of TNF added. The optical density (540 nm) of the blank well was 0.113.

fies the function of immunoliposomes in vivo remains unknown. The fact that the Fc region is masked has an advantage [13] but liposomes without Fc are still trapped by the reticuloendothelial system [44]. Thus, no experimental evidence is available that the 2-step targeted immunoliposome is more effective in vivo than the conventional 1-step immunoliposome at the present moment. As described above, our preliminary in vivo studies gave encouraging results and the above unanswered questions will be dealt with in detail in future studies together with the use of sterically stabilized liposomes [45–47].

There are two modes of TNF action. One is to influence the cells simply through the TNF receptor without internalization [48,49], and the second is to influence the cells after internalization by endocytosis or receptor mediated mechanism [50,51]. Our experiments suggest that TNF must be internalized into target cells through the endocytosis mechanism. This conclusion was derived from the fact that with these cells free TNF had no effect and the uptake of TNF entrapped in our immunoliposome was inhibited markedly by cytochalasin B, an endocytosis inhibitor. In addition, the cytostatic activity of immunoliposomal TNF was inhibited by chroloquine, another endocytosis inhibitor [33]. Our antibody on the immunoliposomes did not exert any cytocidal effect by itself indicating that these antibodies were not acting through the TNF receptor (data not shown). The notion that TNF is the actual effector on the cells, rather than the antibody on the liposome or liposome itself, was further strengthened by the finding that the following combinations did not exert any effects; (1) free TNF, (2) TNF entrapped in liposome without antibody plus free second antibody against target cells, and (3) empty immunoliposome plus free TNF.

Various mechanisms of TNF action have been postulated: activation of phospholipase A_2 [52,53], production of superoxide anion [54,55], fragmentation of DNA through possible endonuclease activation [56,57] and damage by the lysosomal enzymes [28,58,59]. It is not known at the present moment which cytotoxic mechanism described above operates with TNF entrapped in our immunoliposomes after internalization. However, the fact that lysosomotropic agent such as NH_4Cl inhibited the cytostatic activity of liposomal TNF suggests that lysosomes or lysosomal enzymes are involved. Moreover, the action of TNF entrapped in the immunoliposome was cytostatic rather than cytotoxic. In this connection, it is important to point out that TNF introduced through the microinjection technique into cells also exerts a cytostatic effect [60]. This suggests that a similar mechanism operates when the TNF is introduced into the cells through an unnatural means.

The amount of liposomal TNF bound with target cells was far greater than that of TNF when free TNF was added to the cells. Since there are approx. 1500 TNF receptor per T-24 cell [61], the amount of cell bound TNF was approx. 10-fold larger than the number of receptors even when free TNF was added to these cells (Table I). This suggests that the observed association of free TNF is mostly by physical attachment rather than attachment through the receptor. The association of TNF via immunoliposomes represented approx. 4800-fold more bound TNF than one would expect from the uptake through the receptor. Therefore, an entirely different mechanism of TNF action may take place in our case. Resistant cells often contain some cytoplasmic components such as protective proteins which prevent cytocidal action of TNF [54,55,60,62–64]. We postulate that a massive amount of TNF introduced by immunoliposome may overcome the effect of these protective proteins. However, such a massive amount of TNF may not be necessary for this effect because TNF injected through a microinjection technique represents only three times more than the amount of TNF uptake through the receptor mechanism [60]. In conclusion, a successful introduction of a large amount of TNF into the target cells by immunoliposomes further strengthens the possibility of applying this method for clinical use of TNF.

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References

1 Carswell, E.A., Old, L.J., Green, S., Fiore, N. and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 3666–3670.
2 Baglioni, C. (1992) in Tumor Necrosis Factors (Beutler, B., eds.), pp. 425–438, Raven Press, New York.
3 Ioannides, C.G., Fisk, B., Tomasovic, B., Pandita, R., Aggarwal, B.B. and Freedman, R.S. (1992) Cancer Immunol. Immunother. 35, 83–91.
4 Cicco, N.A., Lindemann, A., Content, J., Vandenbussche, P., Lübbert, M., Gauss, J., Mertelsmann, R. and Herrmann, F. (1990) Blood 75, 2049–2052.
5 Dinarello, C.A., Cannon, J.G., Wolff, S.M., Bernheim, H.A., Beutler, B., Cerami, A., Figari, I.S., Palladino, M.A., Jr. and O'Connor, J.V. (1986) J. Exp. Med. 163, 1433–1450.
6 Takahashi, K., Ando, K., Ono, A., Shimosawa, T., Ogata, E. and Fujita, T. (1992) Life Sci. 50, 1437–1444.
7 Darling, G., Fraker, D.L., Jensen, J.C., Gorschboth, C.M. and Norton, J.A. (1990) Cancer Res. 50, 4008–4013.
8 Stephens, K.E., Ishizaka, A., Larrick, J.W. and Raffin, T.A. (1988) Am. Rev. Resp. Dis. 137, 1364–1370.
9 Litzinger, D.C. and Huang, L. (1992) Biochim. Biophys. Acta 1113, 201–227.
10 Hirota, M., Fukushima, K., Hiratani, K., Kadota, J., Kawano, K., Oka, M., Tomonaga, A., Hara, K., Sato, T. and Sunamoto, J. (1988–1989) J. Liposome Res. 1, 15–33.

11 Nässander, U.K., Steerenberg, P.A., Poppe, H., Storm, G., Poels, L.G., Jong, W.H.D. and Crommelin, D.J.A. (1992) Cancer Res. 52, 646–653.
12 Mori, A. and Huang, L. (1993) in Liposome Technology, (Gregoriadis, G., ed.), Vol. 3, pp. 153–162, CRC Press, Boca Raton.
13 Hashimoto, Y., Sugawara, M. and Endoh, H. (1983) J. Immunol. Methods 62, 155–162.
14 Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
15 Wright, S.E. and Huang, L. (1992) J. Liposome Res. 2, 257–273.
16 Barbet, J., Machy, P. and Leserman, L.D. (1981) J. Supramol. Struct. Cell. Biochem. 16, 243–258.
17 Morishige, H., Ohkuma, T. and Kaji, A. (1989) The Fourth Princeton Liposome Conference, Princeton, NJ, USA, May 15–16.
18 Kull, F.C., Jr., Jacobs, S. and Cuatrecasas, P. (1985) Proc. Natl. Acad. Sci. USA 82, 5756–5760.
19 Ito, R., Matsumoto, H., Uchida, K., Kubo, T., Tsukii, Y., Endo, T. and Kaji, A. (1991) Biochim. Biophys. Acta 1096, 245–252.
20 Creasey, A.A., Doyle, L.V., Reynolds, T., Jung, T., Lin, L.S. and Vitt, C.R. (1987) Cancer Res. 47, 145–149.
21 Mosmann, T. (1983) J. Immunol. Methods 65, 55–63.
22 Huber, C.H., Sundström, C., Nilsson, K. and Wigzell, H. (1976) Clin. Exp. Immunol 25, 367–376.
23 Masuko, T., Abe, J., Yagita, H. and Hashimoto, Y. (1985) Jpn. J. Cancer Res. 76, 386–394.
24 Szoka, F., Jr. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
25 Singh, M., Ghose, T., Faulkner, G., Kralovec, J. and Mezei, M. (1989) Cancer Res. 49, 3976–3984.
26 Kull. F.C., Jr., Jacobs, S. and Cuatrecasas, P. (1985) Proc. Natl. Acad. Sci. USA 82, 5756–5760.
27 Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
28 Oku, N., Araki, R., Araki, H., Shibamoto, S., Ito, F., Nishihara, T. and Tsujimoto, M. (1987) J. Biochem. 102, 1303–1310.
29 Snoeij, N.J., Iersel, A.A.J.V., Penninks, A.H. and Seinen, W. (1986) Toxicology 39, 71–83.
30 Onozaki, K., Urawa, H., Tamatani, T., Iwamura, Y., Hashimoto, T., Baba, T., Suzuki, H., Yamada, M., Yamamoto, S., Oppenheim, J.J. and Matsushima, K. (1988) J. Immunol. 140, 112–119.
31 Palombella, V.J. and Vilcek, J. (1989) J. Biol. Chem. 264, 18128–18136.
32 Coombs, K., Mann, E., Edwards, J. and Brown, D.T. (1981) J. Virol. 37, 1060–1065.
33 Noriega, A.G., Grubb, J.H., Talkad, V. and Sly, W.S. (1980) J. Cell Biol. 85, 839–852.
34 Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA 75, 3327–3331.
35 Spriggs, D.R. and Yates, W. (1992) in Tumor Necrosis Factors (Beutler, B., ed.), pp. 383–406, Raven Press, New York.
36 Blick, M., Sherwin, S.A., Rosenblum, M. and Gutterman, J. (1987) Cancer Res. 47, 2986–2989.
37 Bartsch, H.H. and Nagel, G.A. (1988) Mol. Biother. 1, 21–29.
38 Lenk, H., Tanneberger, S.T., Müller, U., Ebert, J. and Shiga, T. (1989) Cancer Chemother. Pharmacol. 24, 391–392.
39 Pfreundschuh, M.G., Steinmetz, H.T., Tüschen, R., Schenk, V., Diehl, V. and Schaadt, M. (1989) Eur. J. Cancer Clin. Oncol. 25, 379–388.
40 McIntosh, J.K., Mulé, J.J., Travis, W.D. and Rosenberg, S.A. (1990) Cancer Res. 50, 2463–2469.
41 Asher, A., Mulé, J.J., Reichert, C.M., Shiloni, E. and Rosenberg, S.A. (1987) J. Immunol. 138, 963–974.
42 Derocq, J.M., Casellas, P., Laurent, G., Ravel, S., Vidal, H. and Jansen, F. (1988) J. Immunol. 141, 2837–2843.
43 Better, M., Bernhard, S.L., Lei, S.-P., Fishwild, D.M., Lane, J.A., Carroll, S.F. and Horwitz, A.H. (1993) Proc. Natl. Acad. Sci. USA 90, 457–461.
44 Singhal, A. and Gupta, C.M. (1986) FEBS Lett. 201, 321–326.
45 Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biopys. Acta 1113, 171–199.
46 Lasic, D.D., Martin, F.J., Gabison, A., Huang, S.K. and Papahadjopoulos, D. (1991) Biochim. Biophys. Acta 1070, 187–192.
47 Mori, A., Kilbanov, A.L., Torchilin, V.P. and Huang, L. (1991) FEBS Lett. 284, 263–266.
48 Dressler, K.A., Mathias, S. and Kolesnick, R.N. (1992) Science 255, 1715–1718.
49 Espevik, T., Brockhaus, M., Loetscher, H., Nonstad, U. and Shalaby, R. (1990) J. Exp. Med. 171, 415–426.
50 Ohsawa, F. and Natori, S. (1987) Cancer Res. 47, 42–46.
51 Schall, T.J., Lewis, M., Koller, K.J., Lee, A., Rice, G.C., Wong, G.H.W., Gatanaga, T., Granger, G.A., Lentz, R., Raab, H., Kohr, W.J. and Goeddel, D.V. (1990) Cell 61, 361–370.
52 Suffys, P., Beyaert, R., Valck, D.D., Vanhaesebroeck, B., Roy, F.V. and Fiers, W. (1991) Eur. J. Biochem. 195, 465–475.
53 Higuchi, M., Higashi, N., Nishimura, Y., Toyoshima, S. and Osawa, T. (1991) Mol. Immunol. 28, 1039–1044.
54 Wong, G.H.W., Elwell, J.H., Oberley, L.W. and Goeddel, D.V. (1989) Cell 58, 923–931.
55 Himeno, T., Watanabe, N., Yamauchi, N., Maeda, M., Okamoto, T., Tsuji, N., Tsuji, Y., Akiyama, S., Sasaki, H. and Niitsu, Y. (1992) Int. J. Cancer 50, 458–462.
56 Kyprianou, N., Alexander, R.B. and Isaacs, J.T. (1991) J. Natl. Cancer Inst. 83, 346–350.
57 White, E., Sabbatini, P., Debbas, M., Wold, W.S.M., Kusher, D.I. and Gooding, L.R. (1992) Mol. Cell. Biol. 12, 2570–2580.
58 Ohsawa, F. and Natori, S. (1988) J. Biochem. 103, 730–734.
59 Liddil, J.D., Dorr, R.T. and Scuderi, P. (1989) Cancer Res. 49, 2722–2728.
60 Smith, M.R., Munger, W.E., Kung, H.-F., Takacs, L. and Durum, S.K. (1990) J. Immunol. 144, 162–169.
61 Sugarman, B.J., Aggarwal, B.B., Hass, P.E., Figari, I.S., Palladino, M.A., Jr. and Shepard, H.M. (1985) Science 230, 943–945.
62 Seckinger, P., Isaaz, S. and Dayer, J.-M. (1989) J. Biol. Chem. 264, 11966–11973.
63 Watanabe, N., Neda, H., Yamauchi, N., Maeda, M., Sone, H., Kuriyama, H. and Niitsu, Y. (1988) Immunopharmacol. Immunotoxicol. 10, 479–499.
64 Nophar, Y., Holtmann, H., Ber, R. and Wallach, D. (1988) J. Immunol. 140, 3456–3460.