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SYNERGISM OF RECOMBINANT HUMAN INTERFERON GAMMA WITH LIPOSOME-ENCAPSULATED MURAMYL TRIPEPTIDE IN ACTIVATION OF THE TUMORICIDAL PROPERTIES OF HUMAN MONOCYTES

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Freshly isolated human peripheral blood monocytes from healthy volunteers are not cytotoxic to allogeneic A375 melanoma cells, but they were rendered tumoricidal by incubation *in vitro* with either liposomes containing 5 μg/μmol phospholipid of muramyl tripeptide phosphatidylethanolamine (liposome-MTP-PE; optimal dose, 500 nmol/ml) or recombinant human interferon gamma (rIFN-γ; optimal dose, 100 U/ml). A combination of sub-threshold concentrations of liposome-MTP-PE (50 nmol/ml) and rIFN-γ (1 or 10 U/ml) also induced significant tumor-cell killing, indicating that the effects of rIFN-γ and liposome-MTP-PE in monocyte activation are synergistic. In contrast to rIFN-γ, recombinant INF-α and IFN-β had additive effects with liposome-MTP-PE in human monocyte activation. Since recombinant human IFN-γ has a synergistic effect with liposome-MTP-PE in monocyte activation, unlike IFN-α or IFN-β, and liposome-MTP-PE as well as rIFN-γ is available at standardized concentrations, this combination could be of clinical value in the treatment of disseminated malignant disease.

The important role of cells of the monocyte-macrophage series in host defense against cancer is now well recognized. Human monocytes and macrophages can be activated *in vitro* by various agents such as lymphokines, designated as MAF (Mantovani *et al.*, 1980; Kleinerman *et al.*, 1983), or bacterial preparations such as LPS (Sone *et al.*, 1982), and MDP (Sone *et al.*, 1982, 1984, 1985*a*). Interferon alpha (IFN-α) and interferon beta (IFN-β) have also been shown to induce human monocyte-mediated cytotoxicity (Jett *et al.*, 1980; Sone *et al.*, 1985*b*). Recently, much attention has been focused on the immunoregulating effect of IFN-γ in modulation of NK cell activity (Herberman *et al.*, 1980) and macrophage functions, such as expression of HLA-DR antigen (Basham and Merigan, 1983), increased expression of Fc receptor (Guyre *et al.*, 1983), activation of oxidative metabolism and antimicrobial activity (Nathan *et al.*, 1983), and potentiation of monocyte-mediated tumor cytotoxicity (Le *et al.*, 1983; Dean and Virelizier, 1983; Kleinerman and Herberman, 1984).

The activation of macrophages for tumor cytotoxicity is thought to require at least two activation stimuli with a defined sequence of stimulations. For example, lymphokines such as MAF or IFN-γ, and bacterial preparations such as LPS or MDP have been shown to have synergistic effects in rendering rodent alveolar and peritoneal macrophages tumoricidal *in vitro* (Ruco and Meltzer, 1978; Sone and Fidler, 1980; Pace *et al.*, 1983; Saiki and Fidler, 1985). Recently, we also reported the synergistic effects of IFN-γ and MDP or its analogue in activating human monocytes to the tumoricidal state (Saiki *et al.*, 1985; Utsugi and Sone, 1986).

Liposomes are now used as carriers to deliver more than a single agent to macrophages (Fidler, 1985). Indeed, when encapsulated in the same liposomes, MDP and MAF have been shown to cause synergistic activation of rat and mouse macrophages *in vitro* and *in vivo*, leading to the destruction of established disseminated metastases (Sone and Fidler, 1980; Fidler and Schroit, 1984). We also showed that when intact IFN-γ and MDP were encapsulated within the same preparation of MLV liposomes, they had synergistic effects in human monocyte activation (Saiki *et al.*, 1985). Nevertheless, the entrapment of soluble agents in the aqueous space of MLV liposomes poses problems in terms of efficient incorporation and subsequent retention of the agents (Schroit and Fidler, 1982). Recently, we demonstrated that a dried preparation of liposomes containing a lipophilic analogue of MDP (MTP-PE), which can be standardized, has a reproducible effect, like conventionally prepared liposome-MTP-PE, in activation of human monocytes to the tumoricidal state (Sone *et al.*, 1986). In the present study, since recombinant IFN-γ and synthetic liposome-MTP-PE can now be obtained in large quantities, we examined whether human rIFN-γ acted synergistically with liposome-encapsulated MTP-PE in activating human monocytes to the tumoricidal state; we also examined the effects of other IFNs (IFN-α and IFN-β) in combination with liposome-MTP-PE on activation of human monocytes.

MATERIAL AND METHODS

Cell cultures

A375 cells, derived from a human melanoma, were adapted to growth in culture (Sone and Tsubura, 1982; Sone *et al.*, 1984). All cultures were maintained on plastic in RPMI 1640 supplemented with 10% heat-inactivated FBS and gentamicin, designated CRPMI 1640, at 37°C in a humidified atmosphere of 5% CO_2 in air. Cytotoxicity assays were performed when the cultured target cells were in the exponential growth phase.

Abbreviations: FBS, fetal bovine serum; MAF, macrophage-activating factor; MDP, muramyl dipeptide; MLV, multilamellar vesicles; MTP-PE, muramyl tripeptide phosphatidylethanolamine; norMDP, des-methyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine; liposome-N-Rh-PE, liposomes containing N-(lissamine rhodamine-B-sulfonyl)dioleoylphosphatidylethanolamine; LPS, lipopolysaccharide; rIFN, recombinant interferon; NK, natural killer.

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Reagents

FBS was purchased from M.A. Bioproducts, Walkersville, MD. Hydrophilic norMDP (des-methyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine) was a gift from Ciba-Geigy, Basel, Switzerland. LPS (*Escherichia coli* 055:B5) was obtained from Difco, Detroit, MI. rIFN-γ (lot G408062; specific activity, 5.36×10^6 U/mg protein) and human hybrid recombinant interferon alpha, referred to as rIFN-αA/D (lot NR-ADB-01, 1.5×10^8 U/mg protein) were kindly supplied by Nippon Roche, Tokyo, Japan. rIFN-β (lot 0623; 1.2×10^8 U/mg protein) was a gift from Toray, Tokyo, Japan. All reagents were free of endotoxins as determined by the *Limulus* amebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

IFN (antiviral activity) assay

The antiviral activity of IFN was determined by measuring reduction of the cytopathic effect of Sindbis virus on FL cells (for rIFN-β and rIFN-γ), or of vesicular stomatitis virus on bovine MDBK cells (for rIFN-αA/D).

Dried preparation of MLV liposomes containing MTP-PE

Dried MLV liposome-MTP-PE (CGP19835A) were prepared by Ciba-Geigy, Basel, Switzerland, and sent by air to this laboratory. The dried liposomes containing MTP-PE (5 μg/μmol phospholipid) were prepared by lyophilizing 250 mg of synthetic PC and PS; (molar ratio, 7:3) with 1 mg of MTP-PE in a 15-ml serum vial, and were stored at $-20°C$ until use. A liposome suspension was prepared by shaking the dried liposome preparations with 2.5 ml of PBS-buffer. The average diameter of the constituted liposomes varied between 2.0 and 3.5 μm, and at least 80% of the liposomes were larger than 1.5 μm (Sone *et al.*, 1986).

Isolation and culture of human peripheral blood monocytes

Leukocyte concentrates were collected from peripheral blood (200 ml) in a Kubota KR-400 centrifuge with a RS-6600 rotor, and mononuclear cells were separated from the leukocyte concentrates in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). Then monocytes were isolated from the mononuclear cell samples by centrifugal elutriation with a Hitachi SRR6Y elutriation rotor (Utsugi and Sone, 1986). A fraction containing more than 90% of the total monocyte population was obtained at a speed of 2,000 rpm and flow rate of 20 ml/min. More than 95% of these cells were monocytes as determined by non-specific esterase staining and morphological examination, and more than 97% were viable, as judged by the Trypan-blue dye exclusion test. This fraction was washed twice with BSS, and resuspended in CRPMI 1640 supplemented with 5% FBS, at a concentration of 5×10^5 monocytes per ml. These cells were then plated for 2 hr in 96-well Microtest III plates (Falcon, Oxnard, CA). Then the non-adherent cells were removed by washing with medium. At this point the purity of the monocytes was >99% as judged by their morphology and non-specific esterase staining.

In vitro *activation of monocytes*

Monocytes were incubated at 37°C in medium with or without various amounts of IFN and/or liposomes containing MTP-PE (5 μg/μmol phospholipid) suspended in CRPMI 1640. After 24 hr, the monocytes were washed thoroughly with medium and added to tumor target cells.

Monocyte-mediated cytotoxicity

Cytotoxicity was assayed by measuring release of radioactivity as described in detail previously (Sone *et al.*, 1984; Utsugi and Sone, 1986). Target cells in the exponential growth phase were incubated for 24 hr in CRPMI 1640 with 0.4 μCi/ml (^{125}I) iododeoxyuridine (specific activity 5 Ci/mg; Amersham International, Little Chalfont, UK). Then 10^4 target cells were plated in wells containing monocytes, and 16 hr later they were washed to remove non-adherent and dead cells and re-fed with fresh CRPMI 1640. After further incubation for 56 hr, the monocyte/target-cell cultures were washed twice with PBS; adherent, presumably viable, cells were lysed with 0.1 ml of 0.1 N NaOH, and their radioactivity was measured in a gamma counter.

The percentage cytotoxicity mediated by activated human monocytes was calculated as follows:

$$\text{Percentage specific cytotoxicity mediated by activated monocytes} = 100 \times \frac{A-B}{A}$$

where A represents cpm in cultures of untreated monocytes and target cells, and B represents cpm in cultures of test monocytes and target cells. The statistical significance of differences between test groups was analyzed by Student's *t*-test (two-tailed).

Quantitative analysis of liposome uptake by human monocytes

For this experiment, MLV liposomes containing 3 mol % of N-(lissamine rhodamine-B-sulfonyl) dioleoylphosphatidylethanolamine (N-Rh-PE, Avanti Polar-lipids, Birmingham, AL) were prepared from a mixture of PC and PS in a molar ratio of 7:3 (Avanti Polar-lipids) by mechanical agitation in a vortex mixer as described previously (Sone *et al.*, 1982, 1984). Human monocytes (10^5 cells/well) were plated for 60 min in wells of LAB-TEK tissue culture chamber slides in 1 ml of CRPMI 1640. Chamber cultures of human monocytes were incubated for 10 hr in 1 ml of medium with liposome-N-Rh-PE (250 nmol/ml) with or without rIFN-γ (10 U/ml). Then the chamber slides were washed twice with pre-warmed RPMI 1640, kept at 4°C for 30 min in buffered formalin, washed twice, dried and mounted in glycerine. Microfluorometry with a photon counter was used to quantitate the fluorescence intensity in single cells (Sone *et al.*, 1984). Excitation light at 535-550 nm was supplied by a high-pressure mercury lamp with compound filters, light at under 580 nm being cut off with a barrier filter. To measure the fluorescence intensity of the whole area of a single monocyte, a metal mask with a pinhole of 30-μm diameter was placed over the imaged field in the microfluorometer.

RESULTS

Synergism between rIFN-γ and liposome-MTP-PE in human monocyte activation

Human blood monocytes isolated by centrifugal elutriation were plated for 1 hr and washed with RPMI-1640 medium to obtain monocyte monolayers. These monocytes were then treated for 24 hr with mixtures of rIFN-γ and liposome-MTP-PE at the indicated concentrations before addition of labelled A375 melanoma cells (10^4). Untreated monocytes were not cytotoxic to A375 melanoma cells. Two types of experiment were performed as shown in Figures 1 and 2. In the first set

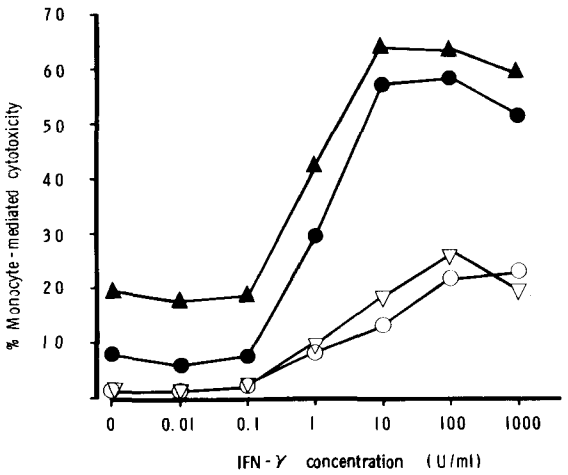

FIGURE 1 – *In vitro* tumoricidal activity of monocytes treated with different amounts of rIFN-γ (○) alone or in combination with 50 nmol/ml of empty liposomes (▽), or 50 nmol/ml (●) or 125 nmol/ml (▲) of liposome-MTP-PE for 24 hr. Cytotoxicity is shown as a percentage of that of control (untreated) monocytes at the same ratio as to A375 target cells. Results are mean values from triplicate cultures in 4 independent experiments. SDs in each experiment were consistently less than 10%.

of experiments, monocytes were incubated in medium containing rIFN-γ at concentrations of 0.001 to 1,000 U/ml with 50 or 125 nmol/ml of liposome-MTP-PE. The monocytes were incubated for 24 hr and then washed thoroughly before addition of labelled target A375 melanoma cells. Monocytes treated with more than 10 U/ml of rIFN-γ alone were significantly cytotoxic (33%, $p<0.01$). The addition of 50 nmol/ml of liposome-MTP-PE to the rIFN-γ greatly enhanced monocyte cytotoxicity. Even at a concentration of 1 U/ml of rIFN-γ, the addition of liposome-MTP-PE (50 nmol/ml) increased the monocyte cytotoxicity. The other set of parallel experiments (Fig. 2) also demonstrated the synergistic effects of rIFN-γ and liposome-MTP-PE in monocyte activation. In these experiments, liposome-MTP-PE at concentrations ranging from 5 to 500 nmol/ml was diluted in medium with 1 or 10 U/ml of rIFN-γ and added to monocyte cultures 24 hr before the addition of target cells. As shown in Figure 2, in the absence of rIFN-γ, at least 50 nmol/ml of liposome-MTP-PE was required to activate human monocytes to the anti-tumor state, but in the presence of either 1 or 10 U/ml of rIFN-γ, even 5

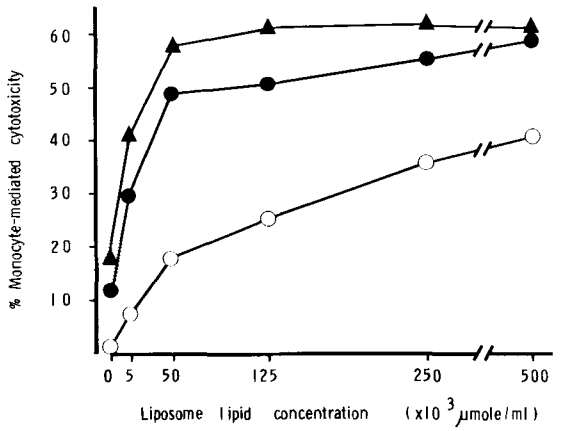

FIGURE 2 – *In vitro* cytotoxicity mediated by human monocytes after 24 hr incubation with different concentrations of liposome-MTP-PE (○) alone or in combination with 1 U/ml (●) or 10 U/ml (▲) of rIFN-γ. Data are means for 4 independent experiments. Experimental details are the same as for Figure 1.

nmol/ml of liposome-MTP-PE were sufficient to render monocytes tumoricidal. These two sets of experiments clearly demonstrate the synergistic effects of rIFN-γ and liposome-MTP-PE in activation of human monocytes to become cytotoxic to A375 melanoma cells. Moreover, human monocytes treated with rIFN-γ (100 U/ml), norMDP (50 ug/ml), liposome-MTP-PE (500 nmol/ml) or a mixture of rIFN-γ and norMDP or liposome-MTP-PE at sub-optimal concentrations were cytotoxic to A375 melanoma cells but not to normal fibroblast (Flow-7000) cells (data not shown).

Order of monocyte treatments and kinetics of activation by rIFN-γ and liposome-MTP-PE

The above experiments demonstrated the synergistic activation of monocytes by rIFN-γ and liposome-MTP-PE when both agents were added simultaneously. Next we examined the kinetics of monocyte activation and the order of treatments necessary for synergistic activation (*i.e.*, rIFN-γ then liposome-MTP-PE, or liposome-MTP-PE then rIFN-γ). The results showed that, for synergistic activation of the tumoricidal properties of monocytes, the cells had to be incubated first with rIFN-γ (1 U or 10 U/ml) and then with 25, 50 or 75 nmol/ml of liposome-MTP-PE (Table I). The treatment of monocytes first with liposome-MTP-PE and then with rIFN-γ (1 U/ml) resulted in little or no tumoricidal activity of monocytes.

Phagocytosis of MLV liposomes by monocytes treated with IFN-γ

Next, we examined phagocytosis of MLV liposomes containing fluorescent N-Rh-PE by monocytes, either untreated or treated with a sub-optimal amount of IFN-γ. For this, human monocytes were incubated for 10 hr in medium containing 250 nmol/ml of MLV liposomes with or without 10 U/ml of rIFN-γ. As shown in Table II, treatment of monocytes with IFN-γ resulted in a significant increase (approximately 2- to 3-fold) in phagocytosis of MLV liposomes.

TABLE I – EFFECT OF ORDER OF TREATMENTS WITH rIFN-γ AND LIPOSOME-MTP-PE IN ACTIVATION OF HUMAN MONOCYTES

rIFN-γ 4 hr	then	liposome-MTP-PE 20 hr	Monocyte-mediated cytotoxicity[1]	Liposome-MTP-PE 4 hr	then	rIFN-γ 20 hr	Monocyte-mediated cytotoxicity
U/ml		nmol/ml		U/ml		nmol/ml	
0		0	1,188±51[2]				
0		25	1,161±25	0		1	1,247±13
0		50	1,111±2	0		10	1,090±37
0		75	1,119±55	25		0	1,093±13
1		0	1,089±57	25		1	1,250±54
1		25	913±90 (23)[3]	25		10	1,020±36 (14)
1		50	827±20 (30)	50		0	1,103±35
1		75	627±69 (47)	50		1	1,180±76
10		0	991±76 (17)	50		10	1,007±26 (15)
10		25	583±4 (51)	75		0	1,028±21
10		50	414±80 (65)	75		1	1,111±85
10		75	284±25 (76)	75		10	906±6 (24)

[1]Human monocytes were treated for 4 hr with rIFN-γ or liposome-MTP-PE as indicated, then washed thoroughly and incubated in medium with or without liposome-MTP-PE or rIFN-γ before addition of labelled A375 melanoma cells. The assays were terminated 72 hr later. –[2]Cpm±SD for triplicate cultures. Representative data from 4 separate experiments.–[3]Cytotoxicity of treated monocytes as a percentage of that of untreated monocytes ($p<0.05$).

Comparison of the effects of IFN-α, IFN-β and IFN-γ with liposome-MTP-PE in monocyte activation

Finally, we examined whether other types of human interferon such as rIFN-α and rIFN-β could act synergistically with liposome-MTP-PE to render monocytes tumoricidal. For this purpose, human monocytes were incubated for 24 hr in medium with or without mixture of different types of IFN and liposome-MTP-PE (125 nmol/ml) and then labelled A375 melanoma cells were added. As shown in Figure 3, IFN-αA/D and rIFN-β acted additively rather than synergistically with liposome-MTP-PE in activation of human monocytes to the tumoricidal state.

TABLE II – PHAGOCYTOSIS OF LIPOSOMES BY HUMAN MONOCYTES TREATED WITH RECOMBINANT HUMAN IFN-γ

Number of experiment	Treatment of monocytes[1]	Uptake of liposomes containing N-Rh-PE
1	Medium	85,576±6,010[2]
	rIFN-γ	187,739±9,167[3] (2.2)[4]
2	Medium	58,194±4,736
	rIFN-γ	101,818±5,220[3] (1.7)
3	Medium	24,092±2,423
	rIFN-γ	83,386±6,501[3] (3.5)

[1]Monocyte monolayers were incubated in medium containing 250 nmol/ml of liposome-PE-rhodamine with or without 10 U/ml of rIFN-γ. After 10 hr, the cultures were washed and their fluorescence intensities were determined as described in "Material and Methods".–[2]Fluorescence intensity per monocyte mean±SE for 260 monocytes.–[3]Significantly different from value for monocytes incubated in medium alone ($p<0.001$). –[4]Values in parentheses show stimulation indices (ratio of uptake by treated monocytes to that by untreated monocytes).

DISCUSSION

The present study indicates that human blood monocytes obtained by centrifugal elutriation from healthy donors can be rendered tumoricidal by the synergistic actions of human rIFN-γ and liposome-entrapped MTP-PE at sub-optimal concentrations.

It was recently suggested that small numbers of adherent NK cells contaminating monocyte monolayers respond to stimulation by rIFN-γ and are responsible for the observed cytotoxicity (Freundlich *et al.*, 1984), and in fact the A375 melanoma cells used as target cells in the present study are susceptible to cytotoxicity mediated by NK cells (Kleinerman *et al.*, 1984). However, we previously showed that the cytotoxic effector cells in the cytotoxicity assay used routinely were monocytes, not NK cells (Sone *et al.*, 1985*b*; Utsugi and Sone, 1986).

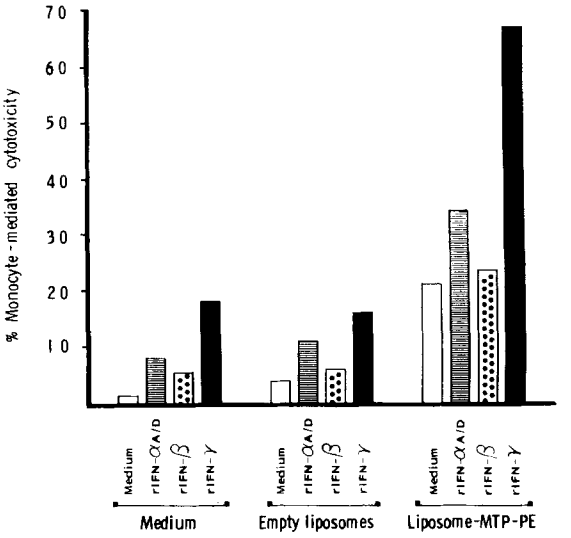

FIGURE 3 – Additive effects of rIFN-αA/D and rIFN-β with liposome-MTP-PE in human monocyte activation. Human monocytes were incubated for 24 hr in medium containing a mixture of IFN-αA/D (10 U/ml), IFN-β (10 U/ml) or rIFN-γ (1 U/ml) and liposome-MTP-PE before incubation with labelled A375 melanoma cells (10^4) for 72 hr. Cytotoxicity is shown as a percentage of that of untreated monocytes. SDs were consistently less than 10%. Data are a summary of those in 4 separate experiments.

The present finding that the synergism between rIFN-γ and liposome-MTP-PE in monocyte activation requires incubation of the cells first with rIFN-γ and then with liposome-MTP-PE is consistent with our previous observations (Utsugi and Sone, 1986) and those of others (Pace *et al.*, 1983; Kleinerman *et al.*, 1984) that IFN-γ must prime monocyte-macrophages,

and that these effector cells are then triggered by a second stimulus such as LPS or MDP or their analogues. Moreover, we recently reported that IFN-γ and MDP showed synergism when they were encapsulated in the aqueous space of MLV liposomes, and that when these two agents were incorporated into liposomes, they were effective at far lower concentrations than when they were added directly to the medium (Saiki *et al.*, 1985). Nevertheless, the use of these liposomes poses problems, such as leakage of encapsulated soluble agents from the liposomes and instability of the preparations (Schroit and Fidler, 1982). We previously reported that these problems can be overcome by inserting a lipophilic MTP-PE into the membrane bilayers of liposomes (Fidler *et al.*, 1982; Sone *et al.*, 1984). In fact, lipophilic MTP-PE in liposomes was more effective for macrophage activation *in vitro* and for tumor cytotoxicity *in situ* than the free forms of MDP encapsulated in liposomes (Fidler *et al.*, 1982; Key *et al.*, 1982). Moreover, in murine systems, encapsulation of lipophilic MDP derivatives within MLV liposomes was also effective for eradication of pulmonary metastases (Fidler *et al.*, 1985; Key *et al.*, 1982; Lopez-Berestein *et al.*, 1984; Phillips *et al.*, 1985). Thus, the present finding suggests that a combination of free rIFN-γ and liposome-MTP-PE is more effective for synergistic activation of human monocytes than liposomes containing both rIFN-γ and soluble MDP in the aqueous space.

This synergistic activation of blood monocytes by rIFN-γ and liposome-MTP-PE for tumoricidal activity has several important implications. First, rIFN-γ and the dried preparations of liposome-MTP-PE used in the present studies are now produced in pure and standardized forms. Second, the low levels (1 to 10 U/ml) of rIFN-γ necessary for significant synergistic activation of monocytes to the tumoricidal state are unlikely to be toxic *in vivo*. In fact, a serum level of 10 U/ml of rIFN-γ can be obtained clinically by administration of approximately 10^6 U of rIFN-γ (Kurzrock *et al.*, 1985; van der Burg *et al.*, 1985). Moreover, we previously reported that a dried preparation of liposomes (composed of synthetic PC and PS) containing lipophilic MTP-PE, which allows the preparation and long-term storage of reproducible liposome formulations, was a potent inducer of human monocyte-mediated cytotoxicity (Sone *et al.*, 1986). Thus, use of combinations of rIFN-γ and liposomes containing MTP-PE has a marked advantage over the use of each singly.

The present finding that recombinant IFN-αA/D and IFN-β had additive effects with liposome-MTP-PE in activation of human monocytes to the anti-tumor state, confirms and extends our previous observations on the additive effects of IFN-α and IFN-β respectively with soluble norMDP. Similarly, IFN-α and IFN-β have recently been shown to have different effects from IFN-γ on the expression of Ia antigen of monocytes (Ling *et al.*, 1985) and the interleukin-1 (IL-1) secretory potential of human monocytes (Arenzana-Seisdedos *et al.*, 1985), suggesting a quantitative difference between IFN-α or IFN-β and IFN-γ.

We (Sone *et al.*, 1984) and others (Schroit and Fidler, 1982) previously showed that phagocytosis of liposomes by monocyte-macrophages is a pre-requisite for induction of tumoricidal activity by liposomes containing MDP and its lipophilic analogue. The present finding that rIFN-γ significantly increases the uptake of liposomes by monocytes suggests that, although the mechanism of the synergistic action of IFN-γ and MTP-PE in liposomes may be very complex, one major factor contributing to development of the observed synergism in monocyte activation is a significant increase in phagocytosis of liposomes by rIFN-γ-treated monocytes. It is not known whether receptor sites for MDP and its analogue exist in the cytoplasm, but possibly cytoplasmic receptors for a second activator (liposome-MTP-PE) may increase on interaction of the cells with rIFN-γ and, if so, this may be involved in the synergism between IFN-γ and liposome-incorporated MTP-PE. This possibility is supported by the following findings: (1) In murine systems, IFN-γ was found to increase the number and affinity of binding sites for LPS on the surface of macrophages and so have a synergistic effect with sub-optimal doses of LPS in activation of macrophages to the tumoricidal state (Akagawa and Tokunaga, 1985); (2) the expression of tumoricidal activity in monocyte-macrophages treated with liposome-entrapped macrophage activators required intracellular interaction between macrophage activators and cytoplasmic receptor sites (Fidler *et al.*, 1981, 1985). Further studies are required to elucidate the events occurring in monocytes after phagocytosis of liposome-MTP-PE.

This study clearly indicates synergistic activation of the tumoricidal properties of human blood monocytes by rIFN-γ and liposome-MTP-PE at sub-optimal concentrations. These compounds can both be produced on a large scale and in standardized form. For this reason, *in vivo* activation of monocyte-macrophages by systemic administration of a combination of these agents (rIFN-γ and liposome-MTP-PE) should be tested in treatment of disseminated disease in humans.

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REFERENCES

AKAGAWA, K.S., and TOKUNAGA, T., Lack of binding of bacterial lipopolysaccharide to mouse lung macrophages and restoration of binding by γ interferon. *J. exp. Med.*, **162,** 1444-1459 (1985).

ARENZANA-SEISDEDOS, F., VIRELIZIER, J.L., and FIERS, W., Interferons as macrophage-activating factors. III. Preferential effects of interferon-γ on the interleukin-1 secretory potential of fresh or aged human monocytes. *J. Immunol.*, **134,** 2444-2448 (1985).

BASHAM, T.Y., and MERIGAN, T.C., Recombinant interferon γ increases HLA-DR synthesis and expression. *J. Immunol.*, **130,** 1492-1494 (1983).

DEAN, R.T., and VIRELIZIER, J.L., Interferon as a macrophage-activating factor. I. Enhancement of cytotoxicity by fresh and matured human monocytes in the absence of other soluble signals. *Clin. exp. Immunol.*, **51,** 501-510 (1983).

FIDLER, I.J., Macrophages and metastases—a biological approach to cancer therapy. Presidential address. *Cancer Res.*, **45,** 4714-4726 (1985).

FIDLER, I.J., FOGLER, W.E., KLEINERMAN, E.S., and SAIKI, I., Abrogation of species specificity for activation of tumoricidal properties in macrophages by recombinant mouse or human interferon-r encapsulated in liposomes. *J. Immunol.*, **135,** 4289-4296 (1985).

FIDLER, I.J., RAZ, A., FOGLER, W.E., HOYER, L.C., and POSTE, G., The role of plasma membrane receptors and the kinetics of macrophage activation by lymphokines encapsulated in liposomes. *Cancer Res.*, **41,** 495-504 (1981).

FIDLER, I.J., and SCHROIT, A., Synergism between lymphokines and muramyl dipeptide encapsulated in liposomes: *in situ* activation of macrophages and therapy of spontaneous cancer metastases. *J. Immunol.*, **133,** 515-518 (1984).

FIDLER, I.J., SONE, S., FOGLER, W.E., SMITH, D., BRAUN, D.G., TARCSAY, L., GISLER, R.J., and SCHROIT, A.J., Efficacy of liposomes containing a lipophilic muramyl dipeptide derivative for activating the tumoricidal properties of alveolar macrophages *in vivo*. *J. biol. Resp. Modif.*, **1,** 43-55 (1982).

FREUNDLICH, B., TRINCHIERI, G., PERUSIA, B., and ZURIER, R.B., The cytotoxic effector cells in preparations of adherent mononuclear cells from human peripheral blood. *J. Immunol.*, **132,** 1255-1260 (1984).

GUYRE, P.M., MORGANELLI, P.M., and MILLER, R., Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *J. clin. Invest.*, **72,** 393-397 (1983).

HERBERMAN, R.B., ORTALDO, J.R., DJEU, J.Y., HOLDEN, H.T., JETT, J., LANG, N.P., RUBINSTEIN, M., and PESTKA, S., Role of interferon in regulation of cytoxic activity by natural killer cells and macrophages. *Ann. N.Y. Acad. Sci.*, **350,** 63-71 (1980).

JETT, J.R., MANTOVANI, A., and HERBERMAN, R.B., Augmentation of human monocyte-mediated cytolysis by interferon. *Cell. Immunol.*, **54,** 425-434 (1980).

KEY, M.E., TALMADGE, J.E., FOGLER, W.E., BUCANA, C., and FIDLER, I.J., Isolation of tumoricidal macrophages from lung melanoma metastases of mice treated systematically with liposomes containing a lipophilic derivative of muramyl dipeptide. *J. nat. Cancer Inst.*, **69,** 1189-1198 (1982).

KLEINERMAN, E.S., and HERBERMAN, R.B., Tumoricidal activity of human monocytes: evidence for cytolytic function distinct from that of NK cells. *J. Immunol.*, **133,** 4-6 (1984).

KLEINERMAN, E.S., SCHROIT, A., FOGLER, W.E., and FIDLER, I.J., Tumoricidal activity of human monocytes activated *in vitro* by free and liposome-encapsulated human lymphokines. *J. clin. Invest.*, **72,** 304-315 (1983).

KLEINERMAN, E.S., ZICHT, R., SARIN, R.S., GALLO, R.C., and FIDLER, I.J., Constitutive production and release of a lymphokine with macrophage-activating factor activity distinct from γ-interferon by a human T-cell leukemia virus-positive cell line. *Cancer Res.*, **44,** 4470-4475 (1984).

KURZOCK, R., ROSENBLUM, M.G., SHERWIN, S., RIOS, A., TALPAZ, M., QUESADA, J.R., and GUTTERMAN, J.U., Pharmacokinetics, single-dose tolerance, and biological activity of recombinant γ-interferon in cancer patients. *Cancer Res.*, **45,** 2866-2872 (1985).

LE, J., PRENSKY, W., YIP, Y.K., CHANG, Z., HOFFMAN, T., STEVENSON, H.C., BALAZS, I., SADLIK, J.R., and VILCEK, J., Activation of human monocyte cytotoxicity by natural and recombinant immune interferon. *J. Immunol.*, **131,** 2821-2826 (1983).

LING, P.D., WARREN, M.K., and VOGEL, S.N., Antagonistic effect of interferon B on the interferon-γ-induced expression of Ia antigen in murine macrophages. *J. Immunol.*, **135,** 1857-1863 (1985).

LOPEZ-BERESTEIN, G., MILAS, L., HUNTER, N., MEHTA, K., EPPSTEIN, D., VANDERPAS, M.A., MATHEWS, T.R., and HERSH, E.M., Prophylaxis and treatment of experimental lung metastases in mice after treatment with liposome-encapsulated 6-O-stearoyl-N-acetyl muramyl-L-aminobutyryl-D-isoglutamine. *Clin. exp. Metast.*, **2,** 366-367 (1984).

MANTOVANI, A., DEAN, J.H., JERRELLS, T.R., and HERBERMAN, R.B., Augmentation of tumoricidal activity of human monocytes and macrophages by lymphokines. *Int. J. Cancer*, **25,** 691-699 (1980).

NATHAN, C.F., MURRAY, H.W., WIEBE, M.E., and RUBIN, B.Y., Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. exp. Med.*, **158,** 670-689 (1983).

PACE, J.L., RUSSELL, S.W., TORRES, B.A., JOHNSON, H.M., and GRAY, P.W., Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. *J. Immunol.*, **130,** 2011-2013 (1983).

PHILLIPS, N.C., MORA, M.L., CHEDID, L., LEFRANCIER, P., and BERNARD, J.M., Activation of tumoricidal activity and eradication of experimental metastases by freeze-dried liposomes containing a new lipophilic muramyl dipeptide derivative. *Cancer Res.*, **45,** 128-134 (1985).

RUCO, L.P., and MELTZER, M.S., Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxicity activity requires completion of a sequence of short-lived intermediary reactions. *J. Immunol.*, **121,** 2035-2042 (1978).

SAIKI, I., and FIDLER, I.J., Synergistic activation by recombinant mouse interferon-γ and muramyl dipeptide of tumoricidal properties in mouse macrophages. *J. Immunol.*, **135,** 684-688 (1985).

SAIKI, I., SONE, S., FOGLER, W.E., KLEINERMAN, E.S., LOPEZ-BERESTEIN, G., and FIDLER, I.J., Synergism between human recombinant γ-interferon and muramyl dipeptide encapsulated in liposomes for activation of antitumor properties in human blood monocytes. *Cancer Res.*, **45,** 6188-6193 (1985).

SCHROIT, A.J., and FIDLER, I.J., Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. *Cancer Res.*, **42,** 161-167 (1982).

SONE, S., and FIDLER, I.J., Synergistic activation by lymphokines and muramyl dipeptide of tumoricidal properties in rat alveolar macrophages. *J. Immunol.*, **125,** 2454-2460 (1980).

SONE, S., LOPEZ-BERESTEIN, G., and FIDLER, I.J., Kinetics and function of tumor cytotoxic factor(s) produced by human blood monocytes activated to the tumoricidal state. *J. nat. Cancer Inst.*, **74,** 583-590 (1985*a*).

SONE, S., MORIGUCHI, S., SHIMIZU, E., OGUSHI, F., and TSUBURA, E., *In vitro* generation of tumoricidal properties in human alveolar macrophages following interaction with endotoxin. *Cancer Res.* **42,** 2227-2231 (1982).

SONE, S., MUTSUURA, S., OGAWARA, M., and TSUBURA, E., Potentiating effect of muramyl dipeptide and its lipophilic analog encapsulated in liposomes on tumor cell killing by human monocytes. *J. Immunol.*, **132,** 2105-2110 (1984).

SONE, S., and TSUBURA, E., Human alveolar macrophages: potentiation of their tumoricidal activity by liposome-encapsulated muramyl dipeptide. *J. Immunol.*, **129,** 1313-1317 (1982).

SONE, S., UTSUGI, T., SHIRAHAMA, T., ISHII, K., MUTSUURA, S., and OGAWARA, M., Induction by interferon-α of tumoricidal activity of adherent mononuclear cells from human blood: monocytes as responder and effector cells. *J. biol. Resp. Modif.*, **4,** 134-140 (1985*b*).

SONE, S., UTSUGI, T., TANDON, P., and OGAWARA, M., A dried preparation of liposomes containing muramyl tripeptide phosphatidyl-ethanolamine as a potent activator of human blood monocytes to the antitumor state. *Cancer Immunol. Immunother.*, in press (1986).

UTSUGI, T., and SONE, S., Comparative analysis of the priming effect of human interferon gamma, alpha and beta on synergism with muramyl dipeptide analog for antitumor expression of human blood monocytes. *J. Immunol.*, **136,** 1117-1122 (1986).

VAN DER BURG, M., EDELSTEIN, M., GERLIS, L., LIANG, C-M., HIRSCHI, M., and DAWSON, A., Recombinant interferon-γ (immuneron): results of a phase-I trial in patients with cancer. *J. biol. Resp. Modif.*, **4,** 264-272 (1985).