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Efficient activation of human blood monocytes to a tumoricidal state by liposomes containing human recombinant gamma interferon

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Summary. *Human recombinant gamma interferon (INF-γ) activated human peripheral blood monocytes to a cytotoxic state capable of lysing adherent tumorigenic cells without harming normal cells. The efficiency of INF-γ activation of monocytes is enhanced by encapsulating INF-γ within liposomes: The minimum effective dose (MED) of free INF-γ for monocyte activation was found to be 1–10 U/ml, per 10^5 monocytes, whereas the minimum dose for INF-γ encapsulated in liposomes was less than 0.0025 U. Monocytes treated with liposome-encapsulated INF-γ retained their cytotoxic phenotype for much longer than do monocytes treated with free INF-γ. Since liposomes can be passively targeted to cells of the reticuloendothelial system following IV administration, these findings suggest that liposome-encapsulated INF-γ may have therapeutic potential that should be evaluated in vivo.*

Introduction

Cells of the monocyte-macrophage series can be activated by a variety of immunomodulatory compounds to a cytotoxic state that renders them capable of recognizing and destroying tumorigenic or virus-infected cells without any effect on nontumorigenic or uninfected cells [5, 7, 10, 23, 26, 32]. The plethora of agents capable of activating monocytes/macrophages to this cytotoxic state includes gamma interferon (INF-γ), a lymphokine with biochemical and functional properties quite distinct from alpha (INF-α) and beta (INF-β) interferons [3, 4, 8, 9, 25]. In addition to its macrophage-activating capability, INF-γ exhibits antiviral [36] and antiproliferative activities [31], augments natural killer cell functions [6], and enhances host antimicrobial activity against several intracellular pathogens [19, 28, 35]. These multiple activities have made this lymphokine a prime candidate for immunomodulation therapy in cancer and infectious diseases. However, several major obstacles to the successful use of lymphokines such as INF-γ for in vivo immunomodulatory therapy must be overcome. These include the rapid clearance of the agent from the circulation, toxicity from high-dosage regimens, and potential antigenicity.

Recent studies with phospholipid vesicles (liposomes) have demonstrated that these structures are effective vehicles for the delivery of immune modulators to cells of the monocyte-macrophage series in vitro and in vivo [33]. Liposomes consisting of phosphatidylcholine (PC) and phosphatidylserine (PS) rapidly bind to and are endocytosed by macrophages in vitro [34] and are cleared from the circulation by cells of the reticuloendothelial system, which include blood monocytes [30]. In addition, when macrophage-activating agents are entrapped within liposomes, cytotoxic macrophages can be generated in vitro and in situ [15, 29]. Liposome encapsulation of biologic response modifiers enhances the macrophage-activating capacity of these substances nearly 1,000-fold over that of the unencapsulated form, thereby providing a system for macrophage activation with diminished and less-toxic doses of immunomodulators [12]. Indeed, multiple IV administrations of liposomes containing lymphokines have been successfully used in therapy for spontaneous pulmonary metastases in murine systems [11, 14]. Clearly, the encapsulation of lymphokines within liposomes offers great potential for immunomodulatory therapy and overcomes several of the hurdles that impede the use of free lymphokines in vivo.

The recent cloning of the gene for human INF-γ and its expression in *Escherichia coli* and mammalian cells has made large quantitites of purified recombinant INF-γ available for the first time [16]. Human recombinant INF-γ activates macrophage oxidative metabolism and antimicrobial activity [27]. In addition, we have recently demonstrated that INF-γ encapsulated in liposomes activates human monocytes to discriminate and lyse herpes virus-infected cells while leaving uninfected cells unharmed [24]. The present study focuses on the ability of human recombinant INF-γ to activate human blood monocytes to a tumoricidal state; the encapsulation of recombinant INF-γ in liposomes, which allows doses as low as 0.0025 U to retain monocyte-activating capacity; the ability of monocytes activated with liposome-encapsulated INF-γ to retain their cytotoxic phenotype for a longer duration than monocytes activated with free INF-γ; and the selective lysis of tumor cells by monocytes activated with liposome-encapsulated INF-γ.

Materials and methods

Reagents. Roswell Park Memorial Institute (RPMI) 1640 medium, Eagle's minimum essential medium (MEM), human AB serum, and fetal bovine serum (FBS) were purchased from M.A. Bioproducts, (Walkersville, Md). Human recombinant gamma interferon (INF-γ, specific activity of 1.7×10^7 U/mg) was a generous gift from Genentech, Inc. (South San Francisko, Calif). All reagents were free of endotoxin (< 0.125 ng/ml) as measured by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass).

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Isolation of human monocytes. Human peripheral blood monocytes were separated from mononuclear leukocytes of normal healthy donors by Percoll gradient centrifugation followed by adherence as previously described [21]. Monocytes were adjusted to 5×10^5 cells/ml in RPMI-1640 medium containing 5% AB serum, and 10^5 monocytes were added to each 38-mm^2 well of a 96-well flat-bottom microtiter plate (Corning Glass Works, Corning, NY). Following 1-h adherence period, wells were washed with RPMI-1640 medium to remove nonadherent cells, and then monocyte activators were added. The purity of monocytes was >95% on the basis of morphological, functional, and biochemical criteria [21].

Preparation of liposomes. Chromatographically pure egg PC and beef brain PS were purchased from Avanti Polar Lipids, Inc. (Birmingham, Ala) and stored at −80° C in sealed ampoules. Multilamellar vesicles (MLV) were prepared by mixing PC and PS at a 7 : 3 molar ratio in chloroform, drying the lipids to a film by rotary evaporation, removing all traces of solvent by vacuum dessication, rehydrating with culture medium or INF-γ, and vortexing for 10 min at room temperature. Free INF-γ was separated from liposome-encapsulated INF-γ by centrifugation (15,000 *g* for 30 min), and liposomes were resuspended at 500 nmol lipid/ml. MLV were added to the monocyte cultures at 100, 50, or 10 nmol doses of lipid per 1×10^5 cells.

Activation of monocytes. Human monocytes were treated with 0.2 ml culture medium (RPMI-1640 plus 5% AB serum), unencapsulated INF-γ, or liposomes containing either culture medium or INF-γ, for 18 h at 37° C. Bacterial lipopolysaccharide (LPS) (*Escherichia coli*) obtained from Difco Laboratories (Detroit, Mich) was added at 100 pg/ml to all wells during the 18-h activation phase. This level of endotoxin was more than 2 $\log_{10}$ lower than the dose required to activate monocytes to a tumoricidal state (W. C. Koff unpublished observations) and served only as a second-signal triggering capacity for monocyte activation [37]. Lymphokine produced from concanavalin A-treated human mononuclear leukocytes [21] was always used as a positive control preparation for monocyte activation.

Target cell cultures. The following target cell lines were used throughout these studies: A-375 (human melanoma), Natusch (human glioblastoma); HT-29 (human colon carcinoma); and Flow 1000 (human embryonic skin). All cells were maintained in Eagle's MEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, vitamins, and L-glutamine. Target cells were labeled with [^{125}I]UdR (0.3 γCi/ml; specific activity 150−200 mCi/μmol; New England Nuclear, Boston, Mass) for 18−24 h prior to use in monocyte-mediated cytotoxicity assays. At that time the target cells were washed to remove unbound radiolabel and harvested by a 1-min trypsinization with 0.25% trypsin (Difco) and 0.02% EDTA.

Cytotoxicity assay. The quantitation of monocyte-mediated tumoricidal activity was carried out as described previously [21]. Briefly, following the 18-h monocyte activation period, activators were removed and 1×10^4 labeled target cells in 0.2 ml were added to assay wells to achieve an initial effector-to-target cell ratio of 10 : 1. Target cells were also added to empty wells to serve as controls for target cell plating efficiency and for quantitation of spontaneous cytotoxicity by untreated monocytes. Cultures were washed and refed with fresh medium at 24 h, and terminated at 72 h. At assay termination, cells were washed twice with Hank's balanced salt solution, and adherent cells were lysed with 0.1 *N* NaOH. Cotton swabs were used to remove the lysates, and the radioactivity was determined in a gamma counter. The percentage generated cytotoxicity was determined by the formula:

$$\text{Percent cytotoxicity} = \frac{A - B}{A} \times 100$$

where A is cpm in target cells cultured with control monocytes and B is cpm in target cells cultured with test monocytes.

Statistical analysis. Experimental results were evaluated for statistical significance using Student's *t*-test (two-tailed).

Results

The capacity of human recombinant INF-γ to activate human blood monocytes to become tumoricidal is shown in Table 1. INF-γ diluted in culture medium to 10^3 U/ml activated monocytes to lyse adherent A-375 human melanoma target cells (27%, $P < 0.01$), whereas treatment of monocytes with culture medium had no effect.

We determined the minimum effective dose (MED) of INF-γ required to activate blood monocytes to a cytotoxic state. A representative dose-response analysis is shown in Table 2. Throughout these studies, we observed an MED of 1−10 U/ml for INF-γ when it was administered to monocytes in free form. Although the percentage cytotoxicity levels were highly variable among individual donors, the MED remained reproducible in the 1−10 U/ml range.

Several recent reports have documented the efficacy of liposomes in targeting immunopotentiating agents to reticuloendothelial cells in vitro and in vivo, where they activate macrophages to become cytotoxic against tumor or virus-infected cells [15, 24, 29]. The results shown in Table 3 indicate the efficiency of human blood monocytes treated with liposomes containing INF-γ in lysing A-375 tumor cells. A 100-nmol dose of liposomes was routinely used to activate 10^5 monocytes in a 38-mm^2 culture well. The MLV contained approximately 2.5 μl aqueous phase/μmol phospholipid; thus, this dose of liposomes delivered approximately 0.25 μl INF-γ solution, compared with 200 μl free aqueous material deliv-

Table 1. Activation of human peripheral blood monocytes to a tumoricidal state by human recombinant gamma interferon

Monocyte treatment	cpm ± SD[c]
No monocytes, tumor cells alone	2,299 ± 403
Culture medium	2,358 ± 211
Human INF-γ[a]	1,723 ± 79 (27)[d]
Human lymphokine[b]	1,118 ± 179 (53)[d]

[a] 1,000 U/ml

[b] Supernatant from human lymphocytes stimulated with Sepharose-bound ConA

[c] Counts/min ± standard deviation of ^{125}I-labeled A375 cells remaining after 3 days of co-cultivation with human monocytes. All samples were assayed in triplicate. This is a representative of five replicate assays. Values in parentheses indicate percent cytotoxicity

[d] $P < 0.001$

ered. Thus, liposomes prepared with 10^3 U/ml INF-γ delivered approximately 0.25 U INF-γ to the 10^5 monocytes cultured in the microtiter wells. This mathematical exercise indicates the highly efficient capacity of liposomes containing INF-γ to activate monocytes to a tumoricidal state, compared with free unencapsulated INF-γ. The data in Table 4 show that concentrations of INF-γ as low as 0.0025 U (10 U/ml. 100 nmol) encapsulated in MLV consisting of PC : PS at a 7 : 3 mol ratio activated human monocytes to a cytotoxic state.

Monocytes activated with liposome-encapsulated INF-γ maintained their cytotoxic phenotype longer than monocytes treated with free INF-γ (Table 5). Monocytes treated with free INF-γ showed diminished cytotoxic capacity against A-375 cells by 24 h after activation, whereas the cytotoxic effects of

Table 2. Dose-response study of the activation of human peripheral blood monocytes by human INF-γ to a tumoricidal state[a]

Monocyte treatment	cpm ± SD
No monocytes, tumor cells alone	1,000 ± 115
Culture medium	1,147 ± 67
Human lymphokine	856 ± 91 (25)[b]
INF-γ 10^5 U/ml	743 ± 92 (35)[c]
INF-γ 10^4 U/ml	837 ± 80 (27)[c]
INF-γ 10^3 U/ml	812 ± 93 (29)[c]
INF-γ 10^2 U/ml	860 ± 121 (25)[b]
INF-γ 10^1 U/ml	976 ± 44 (15)[b]

[a] Assay conditions are described in *Materials and methods*. Values in parentheses indicate percent cytotoxicity. Targets were ^{125}I-labeled A-375 cells
[b] $P < 0.05$
[d] $P < 0.01$

Table 3. Activation of human monocytes by liposome encapsulated human INF-γ[a]

Monocyte treatment	cpm ± SD
No monocytes, tumor cells alone	2,663 ± 249
Culture medium	2,968 ± 110
Liposomes containing culture medium	2,810 ± 78 (5)
Liposomes containing INF-γ[b]	1,309 ± 28 (56)
INF-γ[c]	1,449 ± 99 (51)[d]

[a] Assay conditions are described in *Materials and methods*. Values in parentheses indicate percent cytotoxicity. This is a representative of several replicate experiments. Targets were ^{125}I-labeled A-375 cells
[b] 1,000 U/ml INF-γ encapsulated in liposomes
[c] 1,000 U/ml
[d] $P < 0.001$

Table 4. Dose-response study of the activation of human blood monocytes to a tumoricidal state by liposomes containing INF-γ

Monocyte treatment[a]	cpm ± SD[b]
No monocytes, tumor cells alone	2,662 ± 57
Culture medium	2,659 ± 62
MLV 100 nmol[c]	2,731 ± 57
MLV 50 nmol	2,731 ± 63
MLV 10 nmol	2,715 ± 100
MLV − INF-γ 10^2 U/ml; 100 nmol; 0.025 U[a]	1,598 ± 45 (39)[d]
MLV − INF-γ 10^2 U/ml; 50 nmol; 0.013 U[a]	1,822 ± 43 (31)[d]
MLV − INF-γ 10^2 U/ml; 10 nmol; 0.0025 U[a]	2,258 ± 90 (15)[d]
MLV − INF-γ 10 U/ml; 100 nmol; 0.0025 U[a]	2,107 ± 86 (20)[d]
MLV − INF-γ 10 U/ml; 50 nmol; 0.0013 U[a]	2,453 ± 77 (7.7)[f]
MLV − INF-γ 10 U/ml; 10 nmol; 0.00025 U[a]	2,746 ± 23
MLV + INF-γ 1 U/ml	2,601 ± 281
MLV + INF-γ U/ml 1 h mixture	2,789 ± 57
INF-γ 10^3 U/ml	2,037 ± 78 (23)[d]

[a] Monocytes were treated with activators for 18 h at 37° C. Targets were ^{125}I-labeled A-375 cells
[b] Values in parentheses indicate percent cytotoxicity
[c] nmol indicates nmol phospholipid delivered to monocytes during 18 h activation period
[d] $P < 0.001$
[e] $P < 0.01$
[f] $P < 0.05$
[g] Value indicates amount of INF-γ delivered to monocytes

Table 5. Duration of tumoricidal activity by human monocytes activated with free or liposome encapsulated INF-γ[a]

Monocyte treatment	cpm ± SD[b]			
	(0)[f]	(24)[f]	(48)[f]	(72)[f]
No monocytes; tumor cells alone	1,917 ± 46	2,323 ± 77	1,744 ± 58	2,706 ± 23
Culture medium	1,857 ± 7	2,117 ± 276	1,759 ± 38	2,765 ± 69
INF-γ 10^3 units/ml	1,281 ± 46 (31)[a]	1,799 ± 68 (15)	1,732 ± 53	2,754 ± 61
MLV	1,977 ± 20	2,287 ± 43	1,751 ± 45	2,725 ± 83
MLV − INF-γ[c]	1,389 ± 85 (25)[d]	1,488 ± 52 (29)[a]	1,513 ± 13 (14)[d]	2,452 ± 123 (11)[e]

[a] ^{125}I-labeled A-375 were added to monocytes at the indicated time (0, 24, 48, 72 h) following the 18-h activation period and cocultivated for 72 h before cultures were terminated
[b] Values in parentheses indicate percent cytotoxicity
[c] 1,000 U/ml INF-γ used for encapsulation in liposomes
[d] $P < 0.001$
[e] $P < 0.05$
[f] Value indicate time (h) of addition of labeled target cells following the monocyte activation period

Table 6. Human monocytes activated by liposomes containing recombinant gamma interferon lyse tumor but not normal cells[a]

Monocyte treatment	% Cytotoxicity			
	A-375	HT-29	Natusch	Skin
Culture	4	10	3	2
Liposomes containing culture medium	< 0	< 0	< 0	< 0
Liposomes containing human recombinant INF-γ[b]	47[c]	30[d]	36[c]	< 0

[a] Assay conditions are described in *Materials and methods*
[b] 1,000 U/ml INF-γ used for encapsulation in liposomes
[c] $P < 0.01$
[d] $P < 0.001$

monocytes activated with liposome-encapsulated INF-γ did not decline until 48 h after activation, and some cytotoxic capacity was still evident at 72 h after activation.

Finally, human monocyts activated by liposome-encapsulated INF-γ selectively lysed tumor cells without killing normal cells. A-375 (melanoma), HT-29 (colon carcinoma), and Natusch (glioblastoma) target cells were all lysed by monocytes treated with liposomes containing INF-γ, whereas human embryonic skin cultures were unaffected (Table 6).

Discussion

This study has demonstrated that human INF-γ, when encapsulated in liposomes, was a potent and efficient immunomodulator capable of activating human blood monocytes to a tumoricidal state. Monocytes activated by liposome-encapsulated INF-γ retained their cytotoxic phenotype longer than those activated with free INF-γ and selectively lysed tumorigenic cells. This demonstrated ability of liposome-encapsulated INF-γ to activate monocytes to a cytotoxic state has therapeutic potential and should be evaluated in vivo.

For this study, we defined activation as the rendering of normal, noncytotoxic monocytes cytotoxic against tumorigenic target cells. In our assays blood monocytes did not exhibit spontaneous cytotoxicity, probably because of the methods of collection and isolation [21]. As specified in *Materials and methods*, all reagents used here, including separation gradients, were screened for absence of endotoxins. When blood monocytes are not spontaneously cytotoxic the addition of a small quantity (0.1 ng/ml) of LPS is mandatory before INF-γ can activate their tumoricidal properties [22]. If human blood monocytes already exhibit antitumor activity, augmentation of such activity can be accomplished with INF-γ alone [8, 25]. Because the monocytes we used were not spontaneously cytotoxic, we always admixed 0.1 ng LPS/ml to the INF-γ.

Some of the major problems associated with therapeutic trials of immunomodulators have been the high-dosage regimens required for significant therapeutic efficacy, toxicity of these high-dosage schedules, and the inability to direct the biologic response modifier to the cellular sites of action. One way to overcome these problems is to encapsulate the immunomodulator in liposomes, which have been successfully used as drug carriers for the treatment of systemic parasitic [1], fungal [17], viral [20], and neoplastic diseases [11, 14]. In this report we have shown that when INF-γ is encapsulated in liposomes, the MED for activating monocytes to a tumoricidal state can be reduced from 1–10 U/ml to 0.0125 U/ml. This 2–3 $\log_{10}$ reduction in dosage, coupled with the natural targeting of liposomes to monocytes in vivo [30], suggests that liposome-encapsulated INF-γ may be efficacious for the activation of macrophages to a cytotoxic state in vivo.

Several reports have shown that human INF-γ binds to cell surface receptors with high affinity and that the cell surface interaction is a prerequisite for the induction of an antiviral state [2, 4]. Recently, Anderson et al. [2] demonstrated that human INF-γ is rapidly internalized following receptor binding and suggested that, by internalization, INF-γ might be delivered to an intracellular target required for induction of biological activity. It is unclear at present whether receptor binding is a prerequisite for the other actions of INF-γ, i.e., macrophage activation [25], antiproliferative activity [31], or stimulation of NK-cell activity [6]. Since INF-γ encapsulated within MLV would bypass cell surface receptors and be internalized by endocytosis or fusion with cell membranes [12, 13], these studies indirectly suggest that cell surface binding is not a requirement for the activation of human blood monocytes by INF-γ. In addition, since liposomes are slowly degraded intracellularly [18], the longer duration of the monocyte's cytotoxic phenotype when treated with liposome-encapsulated INF-γ than with free INF-γ further supports the hypothesis [2] that INF-γ must be delivered to an intracellular target to induce biological activity. Thus, in addition to the potential therapeutic efficacy, liposome encapsulation of INF-γ offers a model system for further exploration of the mechanisms by which INF-γ induces its biological effects on the host cell.

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