Tumoricidal Activity of Human Monocytes Activated In Vitro by Free and Liposome-encapsulated Human Lymphokines

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ABSTRACT Human peripheral blood mononuclear cells from normal donors obtained by separation on a Percoll gradiant were incubated with free or liposome-entrapped lymphokines produced from concanavalin A-stimulated lymphocytes and then were tested for cytotoxic activity against tumor cells. The treated monocytes lysed tumorigenic melanoma and glioblastoma target cells, but had no effect on three types of nontumorigenic target cells. The activation of monocytes to become tumoricidal was caused by macrophage-activating factor (MAF) and not by contamination with endotoxins, concanavalin A, or interferon. The endocytosis of liposomes containing MAF, but not of those containing control supernatants, led to the activation of cytotoxic properties in the monocytes. Activation by liposome-encapsulated MAF was very efficient and required <1/800th of the amount of free MAF necessary to achieve the same levels of cytotoxicity. Thus, the encapsulation of mitogen-induced MAF in liposomes could provide an effective approach for the activation of blood monocytes in situ.

INTRODUCTION

A major pathway for the activation of normal noncytotoxic cells of the macrophage-histiocyte series to become cytotoxic against tumor cells and/or microorganisms involves their interaction with soluble mediators (lymphokines) released by antigen- or mitogen-stimulated lymphocytes (1–7). The soluble lymphokine referred to as macrophage-activating factor (MAF)[1] specifically interacts with macrophages or monocytes bearing appropriate receptors (7). Macrophages rendered tumoricidal in vitro by MAF acquire the ability to recognize and destroy neoplastic cells both in vitro and in vivo by a mechanism that requires cell-to-cell contact. The ability of tumoricidal macrophages to recognize and selectively destroy neoplastic cells while leaving nonneoplastic cells unharmed has been demonstrated in a wide range of experimental systems (1, 2, 4, 7, 8).

Although intratumoral inoculation of lymphokine preparations containing MAF has been shown to induce partial regression of skin tumors (9, 10), the in vivo administration of lymphokines to bring about systemic activation of macrophages has not been accomplished. After injection into the circulation, lymphokines have a short half-life, because they rapidly bind to serum proteins (8). Moreover, many crude lymphokine preparations are antigenic and therefore their repeated administration may not be feasible. The efficient activation of macrophages in vivo therefore may require the use of a stable, nonimmunogenic, and nontoxic activating agents. Studies in our laboratories suggest that these requirements can be fulfilled by using liposomes as vehicles to deliver lymphokines to macrophages in situ. In several rodent systems, lymphokines encapsulated within liposomes have been shown to be highly effective in activating macrophages

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; FCS, fetal calf serum; [^{125}I]IUdR, [^{125}I]iododeoxyuridine; LPS, lipopolysaccharide; MAF, macrophage-activating factor; MLV, multilamellar vesicles; MNL, mononuclear leukocytes; NBD-PE, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; S-Con A, Sepharose-bound Con A.

in vitro (7, 8, 11–13) and in vivo (14–16). Furthermore, multiple intravenous injections of liposomes containing lymphokines into mice bearing well established spontaneous lung and lymph node metastases resulted in the activation of tumoricidal properties of lung macrophages and the eradication of the metastases (14, 15).

Although previous studies have reported the activation of human monocytes by soluble human lymphokines (4, 5), none has examined the possibility of activating human monocyte tumoricidal activity by using lymphokines encapsulated within multilamellar vesicles (MLV, liposomes). In the present studies, we defined a method for the production of MAF by mitogen-stimulated human peripheral blood mononuclear leukocytes (MNL). MAF activity was determined by assessing the ability of the lymphokine to activate human blood monocytes to the tumoricidal state. In addition, we also demonstrated that human blood monocytes can be rendered tumoricidal after the endocytosis of MLV with MAF entrapped within their aqueous interiors.

METHODS

Reagents. Roswell Park Memorial Institute (RPMI) 1640 medium, human AB serum, Hanks' balanced salt solution (HBSS), and fetal calf serum (FCS) were purchased from M. A. Bioproducts, Walkersville, MD. Sepharose-bound concanavalin A (S-Con A) was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. All reagents were free of endotoxins as determined by the *Limulus* amebocyte lysate assay (sensitivity limit of 0.125 ng/ml). *Salmonella typhosa* lipopolysaccharide (LPS) was purchased from Difco Laboratories (Detroit, MI).

Preparation of human MAF. Human MNL from the peripheral blood of normal donors were separated on lymphocyte separation medium (Litton Bionetics, Kensington, MD), washed twice in HBSS and then resuspended in RPMI 1640 medium supplemented with 5% human AB serum. The MNL were counted, adjusted to the desired concentrations ($1–5 \times 10^6$ MNL/ml), and cultured with 100 μg/ml S-Con A at 37°C in a 5% CO_2/95% air atmosphere. 48 h later, the cell cultures were centrifuged at 300 *g* for 10 min and the cell-free supernatants were filtered through a 0.45-μm filter (Millipore Corporation, Bedford, MA) to facilitate the removal of the insoluble S-Con A and stored at −70°C. In preliminary studies, we determined that the most active preparation of MAF (as determined by the ability of the lymphokine to activate blood monocytes) was produced from cultures of MNL at the concentration of 2.5×10^6 cells/ml. Therefore, we used this cell concentration for the routine production of lymphokines. For descriptive convenience and brevity, cell-free culture supernatants with MAF activity will be referred to simply as MAF throughout the remainder of this paper.

In several experiments, we also used control supernatants to determine whether Con A was directly responsible for the observed monocyte activation. The control supernatant fluids were (*a*) medium incubated with 100 μg/ml S-Con A for 48 h, (*b*) MNL incubated in medium for 48 h, and (*c*) MNL incubated in medium for 46 h and then with 100 μg/ml of S-Con A for 2 h.

Phospholipids and liposome preparation. Chromatographically pure egg phosphatidylcholine (PC), beef brain phosphatidylserine (PS), and 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylethanolamine (NBD-PE) were purchased from Avanti Biochemicals, Inc., Birmingham, AL. MLV were prepared from a mixture of the appropriate lipids in chloroform. For encapsulation of material within MLV, lipids (10–20 μmol) were first evaporated to dryness by rotory evaporation in an atmosphere of nitrogen, resuspended in 1–5 ml of medium or MAF, and then mechanically agitated on a vortex mixer at 25°C. The liposomes were washed by centrifugation (15,000 *g* for 30 min) and resuspended at the appropriate concentrations. The MLV preparations were adjusted to 1 μmol of lipid/ml of medium and contained ~2.5 μl aqueous phase/μmol lipid (17). MLV were added to the monocyte cultures at a liposome dose of 100, 50, or 25 nmol lipid/10^5 cells per culture well.

Phagocytosis of liposomes. Phagocytosis of MLV by monocytes in suspension was assessed by incubating 20×10^6 cells with 2.4×10^{-6} mol total lipid containing 0.1% NBD-PE in 8 ml RPMI 1640 medium supplemented with 5% heat-inactivated human type AB serum at 37°C with constant gentle rocking. At various times, aliquots were removed and overlaid on 3 ml of FCS. The tubes were then centrifuged at 400 *g*. The upper phase containing nonphagocytosed liposomes was aspirated, and the monocyte pellet was diluted 10-fold with HBSS. The centrifugation step was then repeated with the washed pellet. Under these conditions, free MLV were not pelleted, as determined by fluorescence analysis, but remained at the buffer-serum interface. The pelleted monocytes were then treated with chloroform/methanol/0.1 N HCl (1:2:0.8) to extract phagocytosed MLV. Insoluble material was removed by centrifugation to be reextracted. 1 vol each of chloroform and water was added to the extracts, and the mixture was chilled and centrifuged to separate the aqueous and organic phases. The chloroform phase was removed and dried under a stream of nitrogen. The residue was dissolved in ethanol, and the relative fluorescence of the NBD-PE in the extracts was determined with a Farrand MK II spectrophotofluorometer. Excitation and emission wavelengths were 470 and 525 nm, respectively. NBD-PE fluorescence is directly proportional to lipid concentration and therefore was used to quantify the amount of internalized liposomes.

Isolation and culture of human monocytes from MNL. Peripheral blood monocytes were isolated from MNL by further separation on a preformed continuous Percoll gradient, as previously described (18). Briefly, MNL (40×10^6) were layered onto the gradients in 15-ml polycarbonate tubes and spun in swing-out buckets in a refrigerated centrifuge at 1,000 *g* for 20 min. Upon centrifugation, cell populations layered on top of the Percoll gradient separated on the basis of their relative densities into two distinct bands. The upper band was enriched for monocytes (70–90%) as determined by nonspecific esterase staining and examination of the morphology of the cells. The cells from this band were harvested, washed twice in HBSS, then resuspended in RPMI 1640 medium with 5% human AB serum, and the cell concentration was adjusted to 1×10^6 monocytes/ml. 1×10^5 monocytes were added to each well of a 96-well flat bottom Microtest II plate (Falcon Labware, Div. of Becton, Dickinson and Co., Oxnard, CA) that had been pretreated with FCS for 1 h at 37°C (the serum removed before the addition of monocytes). The monocytes were allowed to adhere for 1

h at 37°C. After incubation, the nonadherent cells were removed, and the plates washed thrice with RPMI 1640 medium. The purity of monocytes at this point was >99%, as assessed by the following criteria: india ink ingestion, morphology, nonspecific esterase staining, and the ability to stain 100% of the cells with monoclonal anti-human monocyte antibody 61D3 (Bethesda Research Laboratories, Inc., Rockville, MD). The plating efficiency was >90%.

In vitro activation of monocytes by MAF. Purified monocytes were incubated at 37°C for 18–24 h with 0.2 ml of control medium, culture supernatants with MAF activity, control supernatant, LPS (5 μg/ml), or medium with liposomes containing various materials. After this incubation period, the adherent monocyte cultures were washed with RPMI 1640 medium and [^{125}I]iododeoxyuridine ([^{125}I]IUdR)-labeled target cells were added as described below to determine monocyte-mediated cytotoxicity. There was no difference in the survival of monocytes cultured in medium alone, control supernatants, free MAF, or liposome-encapsulated MAF. In all culture conditions, monocyte viability exceeded 95%, as determined by trypan blue exclusion.

Target cell cultures. The cultured cell line A375, derived from a human melanoma (19) was provided by Dr. Raymond Ruddon (National Cancer Institute (NCI) Frederick Cancer Research Facility). Monolayer cultures were maintained on plastic in Eagle's complete minimal essential medium supplemented with 5% FCS, sodium pyruvate, nonessential amino acids, twofold concentrated vitamin solution, L-glutamine (M. A. Bioproducts) at 37°C in a humidified atmosphere containing 5% CO_2. The cell line NATUSCH, a human glioblastoma was obtained from Dr. Thomas Hoffman (NCI Frederick Cancer Research Facility). Monolayer cultures were maintained on plastic in McCoy's medium (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% FCS. The propagated, normal human cell lines purchased from Flow Laboratories were (*a*) Flow 1000, derived from embryonic skin and muscle; (*b*) Flow 2000, derived from embryonic lung; and (*c*) Flow 4000 derived from embryonic kidney. All cultures were free of *Mycoplasma* and pathogenic murine viruses. In our laboratory, the two tumor lines produced progressively growing tumors in nude mice, whereas the three nontumorigenic cell lines did not.

Monocyte-mediated cytotoxicity. Cytotoxicity was assessed by a modification of a radioactive release assay previously described (20). Target cells in exponential growth phase were incubated for 24 h in the appropriate medium containing [^{125}I]IUdR (0.3 μCi/ml; sp act 200 mCi/μmol; New England Nuclear, Boston, MA). The cells were then washed twice to remove unbound radiolabel, harvested by a 1-min trypsination with 0.25% Difco trypsin and 0.02% EDTA and washed. The labeled cells were resuspended in supplemented complete minimal essential medium, and 10^4 cells were plated into the culture well to obtain an initial target-to-effector cell ratio of 1:10. Radiolabeled target cells were plated alone as an additional control group. After 24 h, the cultures were washed to remove the nonadherent cells, re-fed with fresh medium, and then cultured for an additional 2 d. Because we used an adherent cell assay in which cell-to-cell contact between effector and target cells is required to achieve killing, washing after 24 h removed the error introduced by cells that did not adhere but were not necessarily killed in the 3-d assay. In this assay, the initial plating efficiency of the target cells is >85% when plated alone or with either control or activated monocytes. Time-course studies have shown that when target cells are co-cultivated with activated monocytes, loss of radioactivity begins after 24 h and reaches a maximum at 72 h. Therefore, 72 h after the addition of tumor cells, the cultures were washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml of 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter and the cytotoxicity activity of the macrophages calculated as follows:

Percentage of spontaneous cytotoxicity = 100

$$-\left(\frac{\text{cpm in target cells cultured with control monocytes}}{\text{cpm in target cells alone}} \times 100\right)$$

Percentage of generated cytotoxicity = 100

$$-\left(\frac{\text{cpm in target cells cultured with test monocytes}}{\text{cpm in target cells cultured with control monocytes}} \times 100\right).$$

Statistical analysis. Experimental results were analyzed for their statistical significance by the *t* test.

RESULTS

Generation of tumoricidal activity in human blood monocytes by MAF. Endotoxin-free culture supernatant fluids of S-Con A-stimulated human peripheral MNL were assessed for their ability to activate blood monocytes to become tumoricidal. The cumulative data from 10 separate experiments performed with different batches of MAF and different donor monocytes are shown in Table I. In seven of 10 normal donors, monocytes cultured for 24 h with medium (devoid of MAF activity) were not significantly cytotoxic to the target cells. In sharp contrast, monocytes from all 10 donors were significantly cytotoxic to the tumor targets subsequent to incubation with MAF. In every instance, MAF-activated monocytes were significantly more reactive than monocytes incubated in control medium, indicating that the phenomenon was reproducible. In addition, the tumoricidal activity of MAF-activated monocytes was specific for the tumorigenic phenotype. The data shown in Table II demonstrate that MAF-activated monocytes lysed tumor targets (A375 melanoma, NATASCH glioblastoma), but were not cytotoxic to three nontumorigenic human cell lines (kidney, skin, and lung cells).

Evidence of lysis of tumor cells by activated monocytes. In all of the experiments reported here, the degree of monocyte-mediated cytotoxicity was assessed by the amount of radioactivity associated with the DNA of adherent viable target cells. Loss of radioactivity, however, could also be attributed to the detachment of viable target cells and not necessarily to lysis. We therefore examined the distribution of [^{125}I]IUdR from A375 cells after 3 d of interaction with MAF-activated monocytes. The supernatant fluids from these cultures were filtered through a 0.45-μm Millipore filter. In all of three experiments, >91% of radioactivity was associated with filterable material. A representative experiment is shown in Table III. These data validate the conclusion that the measure-

TABLE I

Activation of Human Blood Monocytes by MAF Supernatants from Lymphocytes Stimulated with S-Con A

Experiment	MAF batch‡	Residual adhering radioactivity*		
		Tumor cells alone	Tumor cells + control monocytes (% spontaneous cytotoxicity§)	Tumor cells + activated monocytes (% generated cytotoxicity‖)
			cpm±SD	
1	3	525±26	525±50 (0)	203±30 (61)
2	3	618±39	516±38 (16)	268±58 (48)
3	3	678±144	774±60 (−0.14)	469±82 (40)
4	4	1,619±154	1,203±53 (25)	776±65 (35)
5	4	3,034±9	2,938±26 (3)	2,118±83 (27)
6	5	2,097±106	1,790±445 (14)	1,007±72 (53)
7	5	2,924±90	2,301±78 (21)	1,225±97 (47)
8	5	1,611±122	1,531±138 (5)	1,095±93 (28)
9	5	2,285±284	1,192±145 (47)	693±114 (41)
10	6	1,293±144	1,197±44 (7)	291±38 (75)

All values $P < 0.005$.

* Residual adherent radioactivity was determined after 3 d of co-cultivation of monocytes with [^{125}I]IUdR-labeled A375 cells at the target/effector cell ratio of 1:10.

‡ Cultured supernatants containing MAF were prepared from different donors on separate days, harvested, filtered, frozen, and subsequently assayed on different days for activity.

§ Percentage of spontaneous cytotoxicity mediated by control monocytes as compared with that of tumor cells alone.

‖ Percentage of generated cytotoxicity mediated by activated monocytes compared with that of control monocytes.

ment of [^{125}I]IUdR in remaining adherent target cells is a reliable index of monocyte-induced target lysis. Moreover, this result was confirmed by the fact that we were unable to precipitate >10% of the total radioactivity associated with the unfiltered supernatants after centrifugation for 10 min at 250 *g* (data not shown). Finally, human monocytes do not reincorporate released ^{125}I activity; the addition of [^{125}I]IUdR

TABLE II

Spectrum of In Vitro Cytotoxicity Mediated by MAF-activated Human Monocytes

Target cell	Residual radioactivity*			
	Target cells alone	Target cells + control monocytes	Target cells + activated monocytes	Generated cytotoxicity‡
		mean cpm±SD		%
Nontumorigenic				
Kidney	1,490±127	1,554±88	1,612±100	−3.7
Skin	1,875±29	1,892±26	1,825±52	3
Lung	2,430±149	2,430±66	2,372±200	2
Tumorigenic				
A375	1,293±144	1,197±44	291±38	75§
NATUSCH	846±102	891±38	183±54	82§

* 10^4 target cells labeled with [^{125}I]IUdR were added to 10^5 monocytes 24 h after incubation with MAF or medium alone. Residual radioactivity was determined after 72 h of co-cultivation.

‡ Percent cytotoxicity as compared to control monocytes.

§ $P < 0.001$.

TABLE III
Evidence for the Actual Lysis of Tumor Target Cells by Activated Monocytes

Cell combination	Counts per minute*		
	Adherent cells	Filtered cells (residual cpm)	Filtrate (soluble cpm)
Tumor	38,630±3,157	—	—
Tumor + control monocytes	49,018±1,859	—	—
Tumor + activated monocytes	25,315±11	2,761±798	28,972±3,193
Percentage of total label	44	5	51

* After 72-h co-cultivation of activated monocytes with tumor cells, the supernatant fluids from the wells were collected and filtered through a 0.45-μm filter.

to control wells containing monocytes did not lead to retention of any detectable radioactivity (data not shown).

MAF activation of blood monocytes:control experiments. The activation of blood monocytes to the tumoricidal state by supernatant fluids with MAF activity was not caused by contamination with endotoxins, Con A, or interferon. The MAF preparations were devoid of contamination with LPS. Polymixin B is known to bind to the lipid A region of LPS and block several of its biological activities, including macrophage activation (11). We therefore incubated the MAF preparation with 10 μg/ml of polymixin B for 30 min and then added both to monocytes for 24 h. Treatment of MAF with polymixin B did not diminish the ability of the MAF preparation to activate monocyte cytotoxic properties (48.3 vs. 48.5%). Moreover, all supernatants used in our studies were examined for and found free of endotoxins (<0.125 ng/ml, as determined by the *Limulus* amebocyte lysate assay).

Several lines of evidence rule out the possibility that carryover of some Con A was responsible for the activation of the monocytes. Supernatant fluids from MNL cultured in medium alone for 48 h or those from MNL cultured in medium for 46 h and then for a final 2-h period with S-Con A were devoid of MAF activity. Similarly, no MAF activity was detected in supernatants of medium incubated for 48 h with S-Con A in the absence of MNL. In contrast, supernatant fluids of MNL cultured with S-Con A for 48 h rendered blood monocytes highly cytotoxic (Table IV). Because previous reports have shown that both human and rodent MAF is heat stable (6, 7, 21), whereas Con A is heat labile, we heated the MAF preparations to 100°C for 2 min to remove any remaining Con A that may have eluted off the Sepharose. Table IV shows that MAF activity was unchanged after treatment for 2 min at 100°C. Finally, the direct addition of soluble Con A (0.01–10 μg) to monocyte cultures did not result in their activation (data not shown). As a positive control, we incubated human monocytes with 1 μg LPS for 24 h before assaying for cytotoxic activity. As also shown in Table IV, 1 μg LPS activated human monocytes to become tumoricidal.

The culture supernatant fluid with MAF activity (human MNL cultured for 48 h with S-Con A) was found negative for interferon activity when evaluated with a virus neutralization assay using foreskin fibroblasts. (Interferon activity was $<7 \times 10^{-1}$ U/ml.)

Binding and phagocytosis of MLV by monocytes. For liposomes to serve as vehicles for the delivery of compounds to monocytes, these structures must avidly bind to and become endocytosed by the cells. Similar to phagocytosis by rodent alveolar and peritoneal macrophages (17), phagocytosis of liposomes by human blood monocytes was influenced by the chemical composition and surface charge of the liposomes. We used two different types of MLV, those composed exclusively of PC and those consisting of PC and PS admixed at a 7:3 mol ratio. As shown in Fig. 1, neutral PC

TABLE IV
Effect of Various Control Supernatants on Monocyte-mediated Cytotoxicity

Treatment of monocytes with culture supernatants from:*	Residual adhering radioactivity‡	Generated cytotoxicity§
	cpm±SD	*%*
Medium control	1,790±441	—
Medium and S-Con A	1,961±20	−0.09
MNL and medium	2,041±90	−0.014
MNL and S-Con A for 2 h	2,117±166	−0.018
MNL and S-Con A for 48 h	1,029±72	43
MNL and S-Con A for 48 h, then treated at 100°C for 2 min	1,007±72	44
Control: LPS (1 μg)	541±31	71

* 1×10^5 human monocytes were plated and incubated for 24 h with the indicated agents. The cells were washed and 1×10^4 [^{125}I]IUdR-labeled A375 cells added.

‡ Mean of triplicate cultures.

§ Cytotoxicity as compared with monocytes treated with medium.

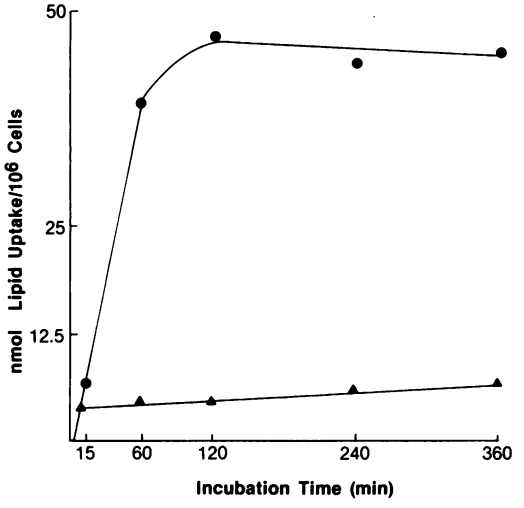

FIGURE 1 Phagocytosis of liposomes by human blood monocytes. MLV containing 0.1% NBD-PE were incubated with the monocytes for various times at 37°C. Cells were washed, and the lipids extracted and quantified. SEM was <10%. The MLV consisted of either PC (▲) or PC/PS at a 7:3 mole ratio (●). One of four experiments.

vesicles were inefficiently internalized by the monocytes, whereas the uptake of MLV consisting of PC and PS was >10-fold. The MLV were endocytosed by the monocytes, as verified by fluorescence microscopic studies. Monocytes were incubated with PC/PS MLV for 2 h, then thoroughly washed to remove unbound MLV and reincubated 2 h at 37°C. At this time, the monocytes were washed again and fixed. As shown in Fig. 2, all observable MLV were intracellular, and no MLV could be detected at the cell surface. This observation strongly suggests that human blood monocytes can rapidly internalize vesicles composed of PC and PS and that synthetic lipid vesicles can indeed be used as vehicles for the delivery of various compounds to these cells. Support for the conclusion that phagocytosis is the mechanism of liposome uptake by monocytes is provided by experiments in which pretreatment of macrophages with metabolic inhibitors that block phagocytosis significantly reduce the incorporation of MLV into the cytoplasm of the cells (11, 17).

Activation of monocytes by MAF encapsulated in liposomes. In the next set of experiments, we examined whether the endocytosis of MLV containing MAF would result in the activation of tumoricidal properties in blood monocytes. Solutions of undiluted MAF or various dilutions of MAF in medium were encapsulated within the aqueous interior of MLV. Blood monocytes were incubated for 18–24 h with the MLV, and then the cytotoxic function of the monocytes was assessed. Control cultures consisted of monocytes incubated in medium without MLV or monocytes incubated with MLV containing medium but suspended in free MAF at an equivalent concentration to that entrapped within liposomes. These studies were performed several times with monocytes from different donors and with different batches of MAF. Because the results were reproducible, we show a representative experiment in Table V. Monocytes treated with medium as well as those incubated with liposomes containing medium were not tumoricidal. In contrast, monocytes incubated with MLV containing MAF were highly cytotoxic to their tumor targets. The activation of the monocytes resulted from the internalization of MAF and not from MAF that leaked out of the liposomes, as shown by the finding that monocytes incubated with MLV containing medium but suspended in 0.25 μl of soluble MAF (the amount contained in 100 nmol of MLV) were not tumoricidal (Table V). We also found that the binding of MLV to the surface of the monocytes did not alter the monocyte surface in such a way as to render the monocytes more responsive to small amounts of MAF that may have leaked from the MLV.

As previously observed with monocytes treated with free MAF (Table II), the cytotoxic activity of monocytes activated with liposome-encapsulated MAF was specific for the tumorigenic phenotype. The data shown in Table VI demonstrate that monocytes incubated with liposome-encapsulated MAF lysed tumor targets (A375, NATUSCH), but were not cytotoxic to two nontumorigenic human cell lines (lung, kidney).

Relative activating efficiency of free and liposome-encapsulated MAF. In the next set of experiments, we determined the minimum amount of free or liposome-encapsulated MAF required to activate monocytes to become tumoricidal. These data are shown in Fig. 3. Monocytes incubated with dilutions of free MAF up to 1:32 were rendered tumoricidal. This experiment was carried out three times with different monocytes and with different batches of MAF with consistent results. Since the culture wells contained 200 μl of medium, a 1:32 dilution is equivalent to a total of 6 μl of the original lymphokine preparation.

Monocytes incubated with MLV containing MAF were highly tumoricidal. The amount of MAF solution contained in 100 nmol of phospholipids is 0.25 μl. A 1:8 dilution of MAF before encapsulation into MLV did not diminish the level of activation (Table V). Moreover, when the actual dose of the MLV placed into individual wells was titrated (100, 50, or 25 nmol MLV/well), MAF activity could be detected with as little as 0.008 μl of the lymphokine preparation (Table V). In three independent experiments, a comparison of the ability of free MAF and liposome-entrapped MAF to activate monocytes revealed that liposome-

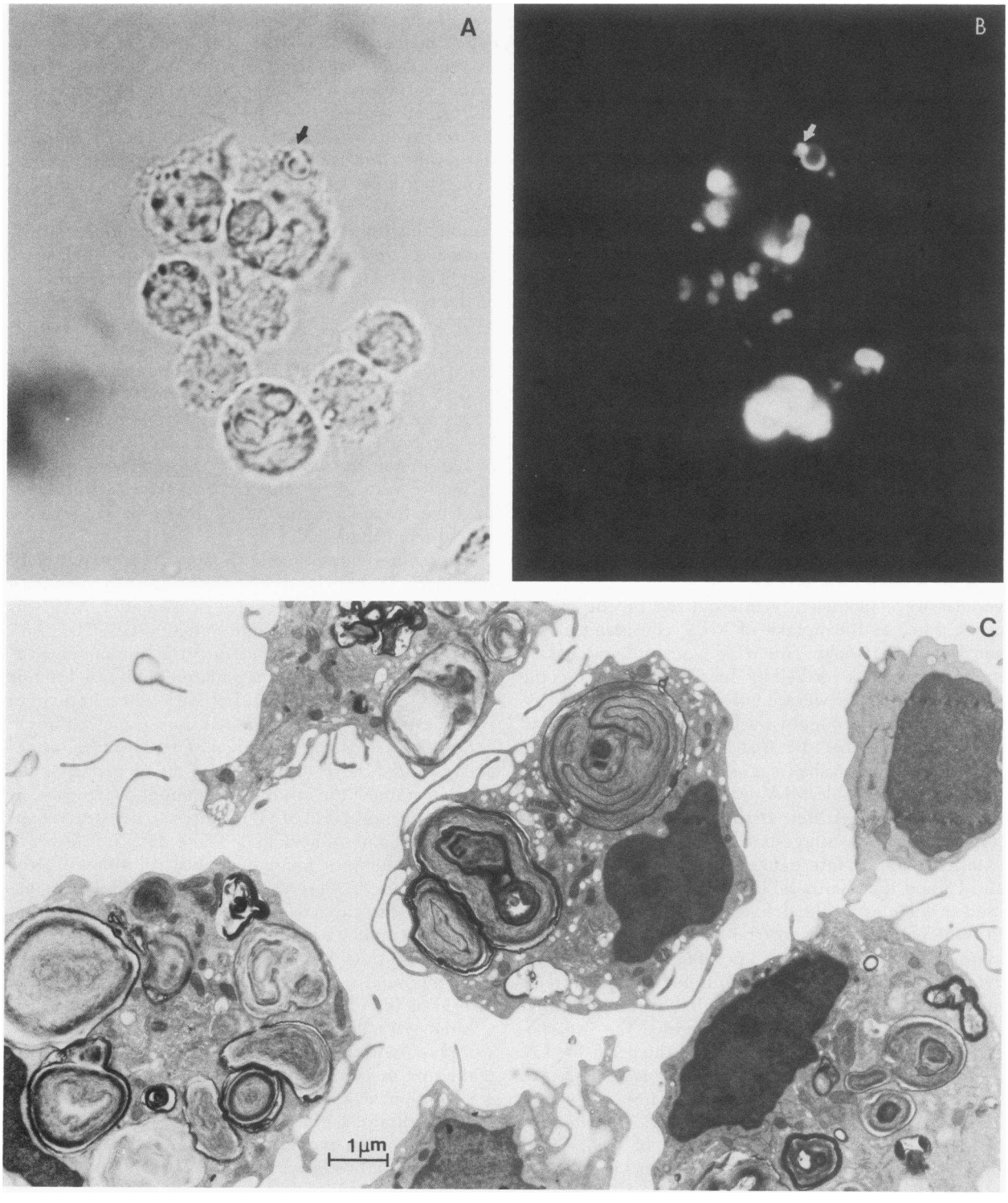

FIGURE 2 Phagocytosis of liposomes by human blood monocytes. (*A*) Phase, (*B*) fluorescent, and (*C*) transmission electron photomicrographs of human blood monocytes after 2-h incubation with PC/PS MLV containing 0.1% NBD-PE. Note fluorescent liposomes at arrow. Bar, 1 μm.

TABLE V

Liposome-encapsulated MAF Activation of Monocyte-mediated Cytotoxicity

Liposome concentration*	Content of liposome	Total MAF content/culture	Residual radioactivity‡	Generated cytotoxicity§
nmol phospholipid		*μl equivalent*	*cpm±SD*	%
No liposomes (media control)	—	0	1,458±220	—
No liposomes (unencapsulated MAF)	—	200	232±50	84
100	Medium	0.25‖	1,674±72	None
100	MAF	0.25	160±233	90
100	MAF 1:4	0.06	155±10	90
100	MAF 1:8	0.03	339±26	79
50	MAF	0.125	610±134	63
50	MAF 1:4	0.031	552±35	67
50	MAF 1:8	0.016	735±21	56
25	MAF	0.063	802±77	52
25	MAF 1:4	0.016	1,071±59	35
25	MAF 1:8	0.008	1,373±46	17

* 1×10^5 monocytes were incubated with medium control, MAF, or liposomes containing the indicated materials encapsulated within the aqueous interior of the liposome (capture volume = 2.5 μl/μmol lipid) for 24 h. The monocytes were then washed before addition of 10^4 [^{125}I]IUdR-labeled A375 cells.

‡ Mean of triplicate cultures.

§ Percentage cytotoxicity compared with monocytes treated with liposomes containing medium. (All values $P < 0.01$.)

‖ Liposomes containing medium and suspended in 0.25 μl MAF.

TABLE VI

Spectrum of In Vitro Cytotoxicity Mediated by Human Monocytes Activated with Liposomes Containing MAF

Target cell	Residual radioactivity*		
	Target cells alone	Target cells + control monocytes‡	Target cells + activated monocytes
	mean cpm±SD		
Nontumorigenic			
Lung	1,727±26	1,752±116	1,787±28
Kidney	1,422±14	1,466±60	1,462±51
Tumorigenic			
A375	1,901±96	1,615±70	1,086±145 (32)§
NATUSCH	2,978±223	2,879±51	1,767±152 (38)§

* 10^4 target cells labeled with [^{125}I]IUdR were added to 10^6 monocytes 24 h after incubation with 100 nmol control liposomes or liposomes containing MAF. Residual radioactivity was determined after 72 h of co-cultivation.

‡ Monocytes incubated with liposomes containing medium and suspended in 0.25 μl MAF (control liposomes).

§ Percentage of generated cytotoxicity as compared with tumor cells plus control monocytes ($P < 0.01$).

encapsulated MAF was at least 800 times more efficient in rendering monocytes cytotoxic against tumor cells in vitro. A representative comparison is shown in Fig. 3.

Kinetics of monocyte activation by free and liposome encapsulated MAF. In the above experiments, the enhancement of tumoricidal activity of human monocytes was observed subsequent to 18–24 h incubation with free or liposome-encapsulated MAF. To determine whether activation could be achieved by a shorter exposure to MAF, we incubated monocytes with either free MAF or MAF encapsulated within MLV for 1, 2, 4, 8, or 24 h. The monocytes were then washed three times with medium and then incubated in medium for 23, 22, 20, 16, or 0 h respectively. Thus, all cultures were incubated for a total of 24 h before the addition of radiolabeled tumor cells took place. As shown in Table VII, series 1, no cytotoxicity developed after 1–4 h of incubation with free MAF. A minimum of 8 h interaction of monocytes with free MAF was required to achieve significant cytotoxic properties (Table VII, series 1) and these data closely agree with a previous report (4). In contrast, only 1–2 h incubation of monocytes with liposome encapsulated MAF was

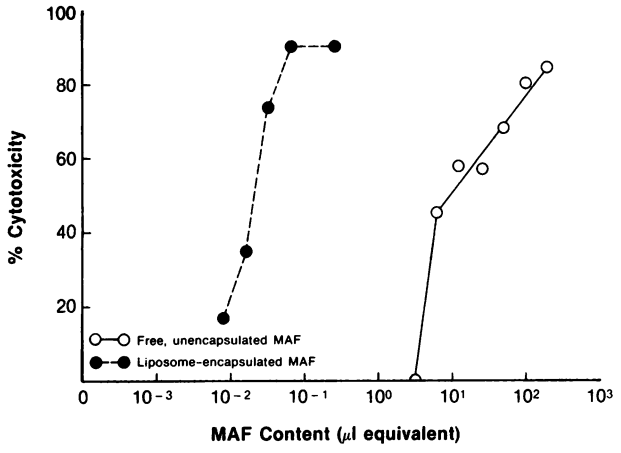

FIGURE 3 Efficiency of free, unencapsulated MAF vs. liposome-encapsulated MAF in generating monocyte-mediated cytotoxicity. Human monocytes were treated with free unencapsulated MAF or liposome-encapsulated MAF for 24 h, washed, and assayed for cytotoxicity against [^{125}I]IUdR-labeled A375 target cells at a target-to-effector cell ratio of 1:10.

required to generate appreciable levels of cytotoxicity (Table VII, series 1). Optimal levels of activation by liposome-entrapped MAF was achieved by 8 h of incubation with monocytes (53%), whereas free MAF required more than 8 h of continuous interaction with the monocytes to achieve similar levels of cytotoxicity (41%; Table VII, series 1). With free MAF, only 16% cytotoxicity was observed after an 8-h incubation.

Although incubation of monocytes with free MAF for 8 h is sufficient to begin to see cytotoxic activity as discussed above, expression of tumoricidal activity required an additional lag period. We base this conclusion on data obtained in experiments where monocytes treated first with medium for 16 h and then with free MAF for 8 h were not cytotoxic against the radiolabeled A375 melanoma targets (Table VII, series 2). If only an 8-h incubation were necessary to see cytotoxic activity, this incubation pattern should also render monocytes tumoricidal. Monocytes first incubated in medium for 8 h then in MAF for 8 h and finally in medium for 8 h were however, as cytotoxic against their targets (19%; Table VII, series 3) as monocytes incubated in MAF for 8 h and then medium for 16 h (16%; Table VII, series 1). Incubation of the monocytes for 8 h in medium and then for 16 h in MAF did not increase their level of cytotoxicity (18%; Table VII, series 2). This suggests that development of tumoricidal competence requires a lag period of at least 8 h after an initial 8-h exposure to free MAF. During this lag phase, the presence of MAF is not essential. In contrast, monocytes incubated with medium for 20 h and then liposome-encapsulated MAF for only 4 h were cytotoxic when tumor cells were added immediately (Table VII, series 2). No cytotoxicity was observed when monocytes were incubated with medium for 22 h and then with liposome-encapsulated MAF for the final 2 h. This suggests that a brief lag period is also required for the development of tumoricidal competence in monocytes exposed to liposome-encapsulated MAF. Again, these studies were performed several times with monocytes from different donors and with different batches of MAF. In each case, the data presented in Table VII were reproducible.

TABLE VII
Kinetics of Human Monocyte Activation by Free and Liposome-encapsulated MAF

Duration and sequence of monocyte treatment*		Residual adhering radioactivity‡	
		Free MAF	MLV-MAF
h		*cpm±SD*	
1. MAF then medium			
1	23	4,043±29	3,493±62 (14)§
2	22	4,102±39	3,252±30 (20)
4	20	4,100±28	2,706±30 (34)
8	16	3,427±21 (16)	1,926±26 (53)
24	0	2,359±29 (41)	1,469±21 (64)
2. Medium then MAF‖			
8	16	3,354±24 (18)	2,799±45 (31)
16	8	4,004±53	2,888±50 (29)
20	4	4,033±57	3,412±83 (16)
22	2	4,035±36	3,786±2 (7)
23	1	4,081±38	4,205±49
3. Medium 8 h, MAF 8 h, Medium 8 h¶		3,297±28 (19)	2,575±64 (37)

* Human monocytes were treated with free or MLV encapsulated MAF for the indicated times and then washed and incubated in fresh medium for the remainder of the 24-h period. 10^4 [^{125}I]IUdR-labeled A375 target cells were then added.

‡ Residual adherent radioactivity was determined after 3 d of cocultivation at the target:effector cell ratio of 1:10. Tumor cells alone, 4132±60; tumor cells + control monocytes, 4,075±11.

§ Percentage of generated cytotoxicity as compared with tumor plus control monocytes ($P < 0.01$).

‖ Human monocytes were incubated with medium for the indicated times, washed, and then treated with free of MLV-encapsulated MAF for the remainder of the 24-h period. 10^4 [^{125}I]IUdR-labeled A375 target cells were then added.

¶ Human monocytes were incubated for 8 h in medium alone, then treated for 8 h with free or MLV-encapsulated MAF, washed, and again incubated for 8 h in medium. 10^4 [^{125}I]IUdR-labeled A375 target cells were then added.

DISCUSSION

The present results demonstrate that mitogen-stimulated peripheral blood MNL produce a lymphokine with MAF activity for peripheral blood monocytes. They also provide new information on the use of liposomes as vehicles to deliver MAF to human monocytes. The experiments presented here show that incubation of human monocytes with MAF in either free form or encapsulated within the aqueous interior of liposomes rendered blood monocytes tumoricidal in vitro. However, the encapsulation of MAF in liposomes was shown to be a more efficient procedure for achieving macrophage activation.

In our studies, we obtained preparations of blood monocytes with a high degree of purity (>99%) that in most cases exhibited a low level of spontaneous cytotoxicity (Table I). We attribute our success in obtaining noncytotoxic blood monocytes to the two-step purification procedure of separation on Percoll gradients followed by adherence. Several studies have reported that human peripheral blood monocytes are spontaneously cytotoxic to tumor targets (5, 22–27). In most of these studies, the monocytes were first isolated by adherence and then harvested by mechanical scraping or treatment with chelating agents such as EDTA, manipulations that can perturb the cell membrane and thus alter the state of activation. Moreover, the present studies (Table IV) as well as others (28) illustrate that endotoxins can activate the cytotoxic properties of human macrophages. Human alveolar macrophages have been shown to be activated by as little as 1 ng of LPS (28). Although in our present studies a dose-response test with LPS was not performed, the expression of spontaneous cytotoxic properties by monocytes could also be caused by contamination of the media (or serum) with such agents. In our assays, once adherent, monocytes were not disturbed and all reagents were free of endotoxins. Although Table I shows that in three of 10 experiments spontaneous monocyte-mediated cytotoxicity was observed, these examples were selected to show some of the rare cases of spontaneous cytotoxicity we encountered. However, our overall experience with >60 assays has shown that significant spontaneous cytotoxicity in our assay only occurred in <10% of the donors.

In the studies presented here, we obtained lymphokines containing MAF from culture supernatants of peripheral blood MNL stimulated for 48 h with S-Con A. The MAF activity of the lymphokine preparations was not caused by contamination by endotoxins or Con A. Moreover, neither culture supernatant fluids of MNL alone nor Con A and medium alone demonstrated any MAF activity. MAF was obtained only when MNL were incubated for 48 h with the mitogen. The MAF activity was heat stable (2 min at 100°C), in agreement with previously published reports of rodent (7, 22) and human (6) MAF, and demonstrated no antiviral activity in a viral neutralization assay. Although the viral neutralization assay using foreskin fibroblasts is not sufficiently sensitive to measure gamma interferon, the stability of our MAF preparations at 100°C suggests that macrophage activation was probably not solely attributable to gamma interferon. Future experiments that use specific antibodies to various interferons should provide more definitive results.

Although many investigators have successfully activated macrophages in vitro with MAF, similar efforts to augment the tumoricidal activity of monocytes-macrophages in vivo by systemic administration of crude lymphokine preparations have not been successful. This failure may be related to the dilution of injected lymphokines, to the rapid undesirable clearance from the circulation, and to the observation that a minimum of 8 h interaction between human monocytes and free MAF is required to render the cells cytotoxic in vitro (Table VI and reference 4).

To achieve activation of monocytes in vivo, we have therefore focused on the use of MLV to deliver substances such as MAF to phagocytic cells in the monocyte-macrophage series (17, 29). MLV provide an efficient vehicle for the delivery of biologically active materials to mononuclear phagocytes in vivo. This targeting of agents should prevent not only the elicitation of undesirable side effects since only the targeted cell interacts with the substance, but also should help in the problems of lymphokine dilution and undesirable clearance from the circulation. MAF encapsulated within liposomes is highly effective in activating rodent macrophages in vitro or in vivo. The present results indicate that MLV containing MAF can also activate normal human monocytes to kill allogeneic tumor cells in vitro. Human monocytes were activated by 800–1,000 times less liposome-encapsulated MAF than free MAF (Fig. 3). Moreover, the exposure time of monocytes to liposome-encapsulated MAF (1–2 h) as well as the lag period for activation was significantly shorter than that required by free MAF (Table VII). In addition to the lower doses required for equivalent activation in rodent systems, liposome-encapsulated MAF has been shown to activate macrophages lacking the functional receptors for MAF (30). Moreover, in these rodent systems, MAF entrapped within liposomes had the advantage of being able to induce activation of subpopulations of histiocytes or tumor-associated macrophages that were completely refractory to activation by free unencapsulated MAF (30).

The phagocytosis of MLV by human monocytes was influenced by the chemical composition and the surface charge of the liposomes. Thus negatively charged PC/PS MLV (7:3 molar ratio) bound to and were more rapidly endocytosed by blood monocytes than neutral MLV consisting of PC alone (Fig. 1) and these MLV are therefore better suited to delivering agents intracellularly to the human monocytes. The interaction in vitro of MLV containing MAF with monocytes led to activation of the monocytes to lyse allogenic tumor cells but not normal cells in vitro (Table VI). This activation by encapsulated MAF could not have been caused by free MAF that was released from leaky liposomes, as shown by the finding that incubation of monocytes with liposomes containing medium and suspended in the same amount of free MAF as contained within the liposomes did not activate monocyte tumoricidal activity. This finding also demonstrated that the mere binding of MLV to the monocyte surface did not render the cells responsive to lower amounts of MAF leaking out of the liposomes. In addition, our studies show that liposome-encapsulated MAF activated tumoricidal activity of normal monocytes, thus supporting the hypothesis that monocyte-mediated cytotoxicity can be induced by the interaction of MAF with intracellular sites (11).

Previous studies have reported that the monocytes of cancer patients have impaired chemotactic responses (31) and impaired cytotoxic properties (32, 33). The possibility of stimulating monocyte-macrophages in situ by the administration of immunomodulators (such as MAF) encapsulated within liposomes could be an attractive approach for augmenting the host's natural defense mechanism. The passive localization of liposomes to phagocytic cells in vivo could provide a highly effective mechanism for targeting liposome-encapsulated materials to macrophages (14). Although in the present studies we have not determined the outcome of macrophage-liposome interaction in situ, the experiments reported here are encouraging and demonstrate that, at least in vitro, human monocytes respond well to activation by allogeneic MAF entrapped within liposomes.

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