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CYTOTOXICITY OF HUMAN MACROPHAGES FOR TUMOR CELLS: ENHANCEMENT BY BACTERIAL LIPOPOLYSACCHARIDES (LPS)[1]

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Bacterial lipopolysaccharide (LPS) stimulates human macrophages derived from peripheral blood monocytes to kill tumor cells *in vitro*. Maximum cytotoxicity was observed after 8 to 24 hr of incubation with LPS. However, if the macrophages are activated with LPS for 8 hr and then maintained in medium for an additional 16 hr before assay, their cytotoxic capacity is lost. In comparison to normal macrophages, LPS-activated macrophages were cytotoxic to the three malignant cell lines tested but had no effect on the three nonmalignant cell lines. Human macrophages can be made tumoricidal by the addition of ≥10 μg/ml LPS, and the effect is abolished in the presence of polymyxin B.

Macrophages can be activated for *in vitro* tumor cytotoxicity by a variety of *in vivo* or *in vitro* treatments. Peritoneal macrophages from mice with chronic Bacillus Calmette-Guerin (1), *Toxoplasma gondii* infection (2), or certain viral infections (3) can kill tumor cells *in vitro*. Macrophages can also be activated for tumor cytotoxicity by *in vitro* treatment with lymphokine-rich supernatants from antigen-stimulated lymphocyte cultures (4–6) or with bacterial lipopolysaccharide (LPS) (7, 8). However, the mechanisms by which these macrophages are activated and the mediation of target cell injury remain poorly understood.

Recently, we have shown that human macrophages, derived from peripheral blood monocytes, also acquire enhanced cytotoxicity for human target cells after incubation in mediator-rich supernatants obtained from antigen-stimulated lymphocytes (MAF)[3] (9). We have now extended our studies to show that LPS as well as MAF can stimulate human macrophages to kill human target cells.

Mouse macrophages respond to nanogram amounts of endotoxin (10, 11) and synergistically respond to combined treatment with threshold amounts of endotoxin and MAF (10, 11). In contrast to these findings, our results indicate that microgram amounts of endotoxin are required to induce cytotoxic human macrophages. Furthermore, the combined treatment with MAF and endotoxin does not result in enhancement of cytotoxicity.

MATERIALS AND METHODS

LPS preparations. LPS from *Escherichia coli* 026:B6 extracted with trichloroacetic acid by the Boivin method was purchased from Difco Laboratories (Detroit, Mich.) (12).

Polymyxin B treatment. Ten micrograms/ml of polymyxin B sulfate obtained from Sigma Chemical Co., St. Louis, Mo.) was incubated with 10 μg/ml of LPS for 30 min at 37°C before addition to the macrophage monolayers. Since LPS binds polymyxin B in a stoichiometric manner, these conditions provided polymyxin B excess (13). Control preparations contained 10 μg/ml of polymyxin B alone.

Preparation of lymphocyte supernatants. Human peripheral blood mononuclear cells obtained from normal donors undergoing leukophoresis were isolated on Ficoll-Hypaque gradients. The cells were washed twice in MEM containing 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per ml (MEM-PSA) and resuspended at a concentration of 2.5×10^6 cells/ml in MEM-PSA supplemented with 10% human AB^+ serum and 1% glutamine (Grand Island Biological Co., Grand Island, N. Y.).

Cells were incubated for 48 hr at 37°C with or without 50 units/ml of SK-SD (see Abbreviations) (Lederle, Pearl River, N. Y.). The cellfree supernatants were obtained by centrifugation and SK-SD in the original amount was added to the control supernatant.

Preparation of macrophage monolayers. The mononuclear cells were washed twice in MEM-PSA and resuspended in MEM-PSA supplemented with 10% horse serum at a concentration of either 30, 20, 10, or 5×10^6 cells/ml. One-hundred-microliter aliquots of these mononuclear cell suspensions were added to Falcon microtiter plate wells (No. 3040, Oxnard, Calif.). After an incubation period of 1 hr at 37°C, the nonadherent cells were removed by gently washing two times with warm (37°C) MEM-PSA. Two hundred microliters of MEM-PSA containing 10% AB^+ serum and 1% L-glutamine (10% MEM-PSA) were then added to each well, and the monocyte preparations were allowed to develop into macrophages by incubating them in this medium for 5 days at 37°C (14). After the 5-day incubation period, the medium was removed and the macrophages were incubated for an additional 24 hr with the

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[2] Recipient of a Research Career Development Award No. 5 K04 CA 00116.
[3] Abbreviations used in this paper: MAF, macrophage-activating factor; MEM-PSA, Eagle's minimal essential medium that contained 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per ml; 10% MEM-PSA, Eagle's minimal essential medium supplemented with 100 units of penicillin, 100 μg of streptomycin, 0.25 μg of amphotericin B per ml, 10% AB+ serum and 1% L-glutamine; SK-SD, streptokinase-streptodornase varidase; MIF, migration inhibitory factor; TCM, Eagle's minimal essential medium supplemented with 100 units penicillin, 100 μg amphotericin B per ml, 15% heat-inactivated fetal calf serum and 1% L-glutamine.

appropriate concentrations of LPS or MAF. Control macrophages were incubated with 10% MEM-PSA. In several experiments, the macrophages were incubated in a subthreshold amount of MAF (1:15 dilution) and LPS (4 μg/ml) for 24 hr, or in MAF for 20 hr followed by LPS for 4 to 8 hr or in LPS for 4 to 8 hr followed by MAF for 20 hr.

Target cells

Tumor cell lines. Two adherent human cell lines were obtained from Dr. J. Fogh at Sloan Kettering Institute for Cancer Research. SK-BR-3 was derived from an adenocarcinoma of the breast, and HT-29 was derived from an adenocarcinoma of the colon (15). These two cell lines were grown in McCoy's medium supplemented with 15% heat-inactivated FCS (endotoxin free, Sterile Systems, Logan, Utah), 1% L-glutamine, 100 units of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B. MA-160, a long-term tissue culture line obtained from Microbiological Associates (Walkersville, Md.), was grown in MEM-PSA supplemented with 15% heat-inactivated FCS and 1% L-glutamine.

Normal cell lines. WI-38 and M-7, derived from diploid human embryonic lung cells, were grown in MEM-PSA supplemented with 1% L-glutamine and 1% heat-inactivated FCS. MA-349, derived from human embryonic intestinal cells, was grown in MEM-PSA supplemented with 10% heat-inactivated FCS and 1% L-glutamine.

Cytotoxic assay. The assay and preparation of target cells have been described in detail (9). Briefly, all target cells were cultured as monolayers. The target cells were prelabeled with ^{3}H-thymidine by adding 20 μCi of ^{3}H-thymidine (methyl-^{3}H-thymidine, Sp. act. 40–60 Ci/mmole, New England Nuclear, Boston, Mass.). After 24 incubation, the labeled target cells were trypsinized, washed, and added to activated or control macrophages at a concentration of 2.5×10^4 target cells/ml. After 5 days of maturation and additional day in mediator-rich or control supernatants, the macrophage monolayers were washed with warm (37°C) MEM-PSA, overlaid with 200 μl of the target cell suspension, and incubated for 24 hr in a moist atmosphere of 5% CO_2 in air. The amount of cytotoxicity was determined from the residual adhering radioactivity by using linear regression analysis as previously described (9). The formula utilized for determining cytotoxicity is:

$$\%\ \text{kill by activated macrophages} = \left[1 - \frac{\text{Mean CPM (tumor + activated macrophages)}}{\text{Mean CPM (tumor + control macrophages)}}\right] \times 100$$

RESULTS

Cytotoxic effect of LPS-stimulated macrophages on tumor cells. To determine whether LPS would stimulate normal human monocyte-derived macrophages to kill tumor cells *in vitro*, the macrophage monolayers were incubated for 24 hr with LPS concentrations ranging from 4 to 50 μg/ml. As shown in Table I, 10 and 25 μg/ml LPS produced maximal cytotoxicity. Macrophages treated with 4 μg/ml LPS were not cytotoxic. With the exception of two experiments, the cytotoxicity observed when the macrophages were treated with 50 μg/ml LPS was less pronounced than that seen when a dose of 10 to 25 μg/ml of LPS was utilized to activate the macrophages.

Kinetics of induction of cytotoxic macrophages. The cytotoxic capacity of human macrophages incubated with 10 μg/ml LPS for 4, 8, 24, or 48 hr was compared with that of human macrophages incubated in medium for the same period of time. Table II summarizes the results of three experiments. In all experiments, maximum cytotoxicity was obtained with cells activated for 8 to 24 hr. Macrophages activated for 4 hr were not cytotoxic for the target cells. After 48 hr activation, cytotoxicity was still observed but was less pronounced than at 8 or 24 hr.

TABLE I

Tumor cytotoxicity by human macrophages treated in vitro with LPS[a]

Expt. No.	LPS (μg/ml)			
	4	10	25	50
	% cytotoxicity			
1	0	22	23[b]	3
2	1	18[b]	4	4
3	−4	0	34[b]	34[b]
4	10	21[b]	19[b]	7
5	5	20	29[b]	29[b]
M ± S.E.	2.4 ± 2.4	16 ± 4.1	21 ± 5.1	15 ± 6.6

[a] Macrophages were activated with various doses of LPS for 24 hr and then co-cultivated with labeled target cells. Effector to target ratio, 20:1.
[b] $p \leq 0.05$.

TABLE II

Kinetics of enhancement of macrophage-mediated cytotoxicity by LPS[a]

Expt. No.	Activation			
	4 hr	8 hr	24 hr	48 hr
	% cytotoxicity			
1	0	20[b]	22[b]	6
2	0	21[b]	20[b]	9
3	0	15[b]	13	6
M ± S.E.	0	18.7 ± 1.8	18.3 ± 2.7	7.0 ± 1.0

[a] Macrophages were activated with LPS (10 μg/ml) for varying time intervals and then co-cultivated with labeled target cells. Effector to target ratio, 20:1.
[b] $p \leq 0.05$.

Duration of LPS-induced macrophage cytotoxicity. It is known that mediator-induced human macrophages become cytotoxic after 8 hr incubation with mediators, and the enhanced cytotoxicity persists for at least 40 hr after the lymphocyte mediators are removed (9). Therefore, we attempted to establish whether continued exposure to LPS was required to maintain the cytotoxic capacity of human macrophages. In several experiments, the macrophages were activated for 8 hr with LPS, washed, and cultured for an additional 16 hr in fresh tissue culture medium to determine if human macrophages retain the ability to kill tumor cells once the LPS has been removed. As shown in Table III, with the exception of one experiment, LPS activated human macrophages lost cytotoxic activity when the LPS was removed 16 hr before assay.

Effect of polymyxin B on LPS-enhanced tumor cell killing. Since polymyxin B is known to form a stable molecular complex with the lipid A region of LPS (13) and block several effects of LPS *in vitro* (16, 17) and *in vivo* (18, 19), we wanted to know if polymyxin B would inhibit the effect of LPS. When 10 μg/ml of polymyxin B was incubated with 10 μg/ml of LPS before its addition to the macrophage monolayers, the LPS was completely inhibited in its ability to make the human macrophages tumoricidal in four of five experiments (Table IV). Chapman and Hibbs (20) have shown that polymyxin B does not alter the effects of MAF. In two experiments (results not shown), we also have shown that the addition of polymyxin B (25 μg/ml) to

our MAF supernatants does not alter its ability to enhance macrophage-mediated cytotoxicity.

Cytotoxicity of activated macrophages for malignant cell lines but not for normal human target cells. Mediator-activated macrophages are cytotoxic for a variety of human target cell lines (9). Several of these targets have been utilized to determine if they are susceptible to LPS-induced macrophage-mediated cytotoxicity. SK-BR-3 and MA-160 showed the most susceptibility to macrophage-mediated cytotoxicity (Table V). These cell lines were killed in all experiments. The degree of cytotoxicity ranged from 13 to 37%. HT-29 was also quite susceptible to macrophage-mediated cytotoxicity. They were killed in three of four experiments. However, macrophages incubated with LPS were not cytotoxic for the three normal cell lines tested (Table V).

Lack of cytotoxicity by macrophages treated with subthreshold doses of MAF and LPS. It is known that tumoricidal activity of mouse macrophages incubated with LPS and subthreshold doses of lymphokines is significantly greater than that of cells cultured in lymphokines or LPS alone (11). Therefore, we attempted to establish whether a synergistic effect of LPS on cytotoxicity by lymphokine-activated macrophages could be demonstrated in the human system. In Table VI, it can be seen that human macrophages incubated with MAF and LPS for 24 hr were not cytotoxic for the target cells, whereas at an optimal LPS dose, 10 μg/ml, macrophage-mediated cytotoxicity was observed.

Since Ruco and Meltzer (11) showed that this synergistic effect occurred only when LPS was simultaneously given with or after lymphokine treatment, we treated our human macrophages with MAF for 20 hr followed by LPS for 4 to 8 hr. However, once again no enhanced cytotoxicity could be demonstrated when MAF and LPS were added to the macrophage monolayers sequentially (Table VII). However, we did notice that macrophages incubated in MAF for 20 hr followed by LPS for 4 hr showed decreased cytotoxicity as compared to macrophages treated with MAF and LPS for 24 hr. The converse experiment, namely with LPS for the first 4 to 8 hr of incubation followed by MAF for 16 to 20 hr before assay, also failed to yield enhancement of cytotoxicity (Table VII).

TABLE III

Duration of macrophage-mediated cytotoxicity[a]

Expt. No.	LPS (10 μg/ml)	8 hr LPS (10 μg/ml) + 16 hr Fresh Medium
	% cytotoxicity	
1	21[b]	22[b]
2	34[b]	0
3	25	16[b]
4	16	4
5	14[b]	0
M ± S.E.	22 ± 3.6	8.4 ± 4.5

[a] Macrophages were activated for various time periods and then co-cultivated with target cells for 24 hr. Effector to target ratio, 20:1.
[b] $p \leq 0.05$.

TABLE IV

Effect of polymyxin B on LPS-enhanced tumor cell killing

Expt. No.	LPS (10 μg/ml)	LPS (10 μg/ml) + Polymyxin B (10 μg/ml)
	% cytotoxicity[a]	
1	21[b]	10
2	20[b]	25[b]
3	18[b]	0
4	34[b]	3
5	37[b]	−12
M ± S.E.	26 ± 3.9	5.2 ± 6.1

[a] Macrophages were activated with μg of endotoxin and then tested for cytotoxicity by co-cultivation with target cells for 24 hr.
[b] $p \leq 0.05$.

DISCUSSION

There is considerable evidence from animal studies that LPS activates macrophages to kill tumor cells *in vitro* (7, 8, 10, 11). Furthermore, Doe and Henson (8) have shown that LPS-stimulated murine macrophages become cytotoxic for syngeneic and allogeneic tumors but have no cytotoxicity for normal targets.

The experiments presented in this paper demonstrate that LPS can also stimulate human monocyte-derived macrophages to kill tumor cells *in vitro* and that cytotoxic macrophages produced by endotoxin have a quantitatively greater effect on malignant targets than they do on normal targets.

TABLE VI

Tumor cytotoxicity by human macrophages treated in vitro with lymphokine and LPS[a]

Expt. No.	(4 μg/ml)	MAF (1:15)	LPS (4 μg) + MAF (1:15)	LPS (10 μg/ml)
1	0	0	0	18[b]
2	0	0	9	34[b]
3	1	11	11	24[b]
4	10	0	13	34[b]
5	10	21	30[b]	24
M ± S.E.	4.2 ± 2.4	6.4 ± 4.2	12.6 ± 4 9	27.0 ± 3.0

[a] Macrophages were activated with LPS or MAF with and without 4 μg/ml LPS for 24 hr and then co-cultivated with target cells. Effector to target ratio, 20:1.
[b] $p \leq 0.05$.

TABLE V

Cytotoxicity of in vitro activated macrophage monolayers for various tumor and normal cells[a]

Expt. No.	Nonmalignant target cells			Malignant target cells		
	M-7	WI-38	MA-349	SK-BR-3	MA-160	HT-29
			% cytotoxicity			
1	−6			22[b]	18[b]	20[b]
2	10	9	5	36[b]	24[b]	17
3	12	9		13[b]	34[b]	5
4		0	0		37[b]	25[b]
M ± S.E.	5.3 ± 5.7	6 ± 3.0	2.5 ± 2.5	23.6 ± 6.7	28.2 ± 4.4	16.7 ± 4.2

[a] Macrophages were activated with 10 μg of endotoxin, and then tested for cytotoxicity by co-cultivation with target cells for 24 hr.
[b] $p \leq 0.05$.

TABLE VII

Tumor cytotoxicity by lymphokine-activated macrophages: effect of time in culture with LPS before and after lymphokine treatment

Expt. No.	LPS (4 μg/ml)	MAF (1:15)	MAF (1:15) + LPS (4 μg/ml)[a]		LPS (4 μg/ml + MAF (1:15)[b]		24 hr LPS + MAF	LPS (10 μg/ml)
			20 hr MAF + 4 hr LPS	20 hr MAF + 8 hr LPS	4 hr LPS + 20 hr LPS	8 hr LPS + 16 hr MAF		
1	0	16					4	22
2	10	21	−6				30[c]	24
3	10	0	−1	12			13	34[c]
4	−11	17[c]	−35	9			12	20[c]
5	1	11	3	11			11	24[c]
6	0	0			0		0	18[c]
7	0	0			8	12	9	34[c]
8	0	0			10	0	0	25
9	0	0			0	8	0	16[c]
M ± S.E.	1.1 ± 2.1	7.2 ± 3.0	−10.3 ± 2	10.7 ± 0.9	4.5 ± 2.6	6.7 ± 3.5	8.8 ± 3.2	24.1 ± 2.1

[a] Macrophages were incubated with MAF for 20 hr. LPS was then added to the wells for an additional 4 to 8 hr.

[b] LPS (4 μg/ml) was added to the macrophage monolayers for 4 or 8 hr. The LPS was removed and the macrophages were washed once with MEM. The macrophages were then incubated with MAF for the additional 16 to 20 hr before assay.

[c] $p \leq 0.05$.

With regard to the effect of LPS on macrophage cytotoxicity, there appear to be two major differences between mouse and human systems. First, less than 10 ng/ml of bacterial LPS is needed to convert the noncytotoxic murine macrophages into cytotoxic macrophages (10, 11), whereas more than 4 μg/ml of LPS is needed in the human system. Second, in the mouse system, there appears to be a synergistic effect between LPS and MAF. When LPS is added to the macrophage monolayers simultaneously or after lymphokine treatment, the macrophages kill the tumor cells more effectively than if treated with either agent alone (10, 11). In contrast, we have not been able to demonstrate a synergistic effect of LPS and MAF on the enhancement of human macrophage-mediated cytotoxicity. Maximum enhancement of human monocyte-derived macrophage cytotoxicity by LPS is observed at 8 to 24 hr of incubation, whereas in the mouse, macrophages activated for 8 hr with LPS and then maintained in TCM for an additional 16 hr before assay lose their cytotoxic capacity for tumor cells. Enhancement of human macrophage-mediated cytotoxicity by MAF was also observed after 8 hr of incubation. However, the enhanced cytotoxicity induced by MAF lasted for at least 40 hr after removal of the mediator from the macrophages (9).

Because microgram amounts of endotoxin are required to enhance macrophage-mediated cytotoxicity in humans, it is unlikely that endotoxin, contaminating reagents or tissue culture medium, contributes to enhancement of macrophage cytotoxicity by other means such as treatment with MAF. We have further attempted to exclude the possible role of contaminating endotoxin in several ways. First, as judged by the Limulus assay, the mediators, fetal calf serum and human AB^+ serum, were free of endotoxin. Second, we carried out experiments to see if polymyxin B would inhibit the effect of MAF and found that MAF supernatants treated with 25 μg/ml of polymyxin B still enhanced macrophage-mediated cytotoxicity as well as the untreated supernatants. Thus, it is unlikely that endotoxin in our mediators or reagents contributes to the enhancement of macrophage-mediated cytotoxicity observed after treatment with MAF.

Responsiveness to LPS, like MAF, appears to correlate with the stage of macrophage differentiation. When peripheral blood monocytes were tested immediately after explanation and activation with LPS, enhanced cytotoxicity was not observed. However, the same monocyte populations became responsive to endotoxin after differentiation for 5 days in culture. We previously showed that these monocytes also could not be activated with MAF immediately after explanation, but became responsive after a 5-day period of *in vitro* differentiation (9). Furthermore, in contrast to the significant enhancement of cytotoxicity observed in human macrophages pretreated with surface esterase inhibitors,[4] human monocytes pretreated with surface esterase inhibitors showed only a slight enhancement in their response to human MIF (see Abbreviations) (21). Thus, it is possible that the capacity to respond to esterase inhibitors, MAF and LPS, might be functions developed during the period of *in vitro* culture.

The mechanisms by which LPS affects the macrophage is unknown. LPS binds to macrophage membranes and is ultimately endocytosed by macrophages (22). Whether LPS enhances the macrophage tumoricidal effect by direct action on macrophages or by inducing MAF secretion from contaminating B cells is not yet established. LPS is a B cell mitogen that can elicit MIF production from B cells (23). Although MIF from purified B cells has not been directly tested for MAF activity, it is likely that these supernatants would contain MAF activity as well (24). LPS also stimulates B cells to release a factor that enhances glucosamine incorporation by macrophages (25). Enhanced incorporation of glucosamine is associated with macrophage activation and results in part from increased glucose utilization by activated macrophages (26–28). Hence, enhanced glucosamine utilization may correlate with enhanced macrophages cytotoxicity as well. In contrast to evidence that LPS requires B cells to enhance glucosamine incorporation by macrophages (25), the studies of Doe and Henson (8) indicate that LPS stimulates murine macrophages directly and does not require lymphocyte participation. Recently, Weinberg *et al.* (10) have shown, by utilizing pure populations of cloned murine macrophages as effector cells, that LPS can act in the absence of contaminating lymphocytes to induce macrophage-mediated cytotoxicity. However, this demonstration of a direct effect of LPS on macrophages still does not exclude the possibility that LPS could also enhance macrophage-mediated cytotoxicity through intermediate MAF-producing B lymphocytes when they are present. We are attempting to minimize the possible role of lymphocytes by starting with adherent monocytes that have been washed free of nonadherent lymphocytes. However,

[4] Cameron, D. J., and Churchill, W. H. Chemical modification of macrophages enhances their response to human macrophage activation factor (MAF). Submitted for publication.

these conditions clearly do not remove lymphocytes with the property of adherence.

It has been suggested that the lipid region of LPS is responsible for the LPS effect on macrophage-mediated cytotoxicity (10). Lipid A produced by acid hydrolysis of phenol-extracted LPS has the same effect as the intact LPS molecule, whereas base hydrolysis of LPS, which is known to destroy the mitogenicity of LPS, abolishes the ability of LPS to produce cytotoxic macrophages (29). Furthermore, polymyxin B, which binds to the lipid A region of LPS (13), markedly inhibits the effect of LPS on macrophage-mediated cytotoxicity.

Endotoxin is known to modify a number of immune functions in humans. It serves as an adjuvant for antibody formation (30) and as a mitogen for B cell proliferation (31). It is highly immunogenic as an antigen (32, 33) and can enhance macrophage-mediated cytotoxicity of tumor cells. It can stimulate properdin levels, enhance resistance to infection (34–36), and may contribute to regression of malignant disease in patients treated with bacterial extracts (37, 38). Further studies will be required to determine whether endotoxin-induced alteration in macrophage functions contributes to these changes in host immune function.

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