MACROPHAGE STIMULATION BY BACTERIAL LIPOPOLYSACCHARIDES

I. Cytolytic Effect on Tumor Target Cells*

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Diverse stimuli induce macrophages to become nonspecifically cytotoxic for transformed cells in vitro (1). Bacterial infections of mice by Bacillus Calmette Guérin (BCG)[1] (2), protozoal infestations by *Toxoplasma gondii* or *Besnoitia jellisoni* (3), certain viral infections (4), and intraperitoneal inoculations of bacterial lipopolysaccharides (LPS) (5) produce macrophages that injure transformed cells in vitro. In addition, macrophages obtained directly from tumors nonspecifically lyse transformed target cells (6–9). Taken together, these data suggest a central role for stimulated macrophages both in host control of tumor cell proliferation and in the pathogenesis of the chronic inflammatory process. However, the mechanisms involved in the stimulation of macrophages, the generation of the lytic state, and the mediation of target cell injury are not understood. Recently, we and others have shown that cytolytic properties can be induced in peritoneal macrophages by using LPS (10, 11) thereby offering the prospect of examining how macrophages become stimulated, then injure tissues. The studies reported here investigate the nature of the interaction between LPS and murine macrophages that results in the generation of their capacity to kill tumor cells in a nonspecific manner.

Materials and Methods

Preparations of LPS. The heptose-deficient mutant from *Salmonella minnesota* (R595) was grown in trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.) under aerated conditions. Cells were harvested by centrifugation and washed twice with 0.85% sodium chloride. LPS from R595 was extracted and purified in our laboratory by Dr. D. C. Morrison using the phenol-petroleum ether-chloroform procedure of Galanos et al. (12). The water insoluble LPS extract was then sonicated and dissolved in 0.1% triethylamine. After extensive dialysis, the LPS preparation of R595 was stored at −70°C until required. LPS from *Escherichia coli* serotype 0111:B4 (ATCC 12015) was extracted by the phenol-water method of Westphal and Jann (13).

Media and Cell Lines. Eagle's minimal essential medium (MEM) was supplemented by

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[1] *Abbreviations used in this paper:* BCG, Bacillus Calmette Güerin; ^{51}Cr, sodium ^{51}chromate; FBS, fetal bovine serum; LPS, lipopolysaccharide; MEM, minimal essential medium; PEC, peritoneal exudate cells.

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glutamine (2 mM), sodium pyruvate (1 mM), Hepes buffer (20 mM), sodium bicarbonate 0.14%, heat inactivated (56°C, 30 min) fetal bovine serum (FBS, Flow Laboratories, Inc., Rockville, Md.). penicillin (100,000 U/liter), and streptomycin (100,000 μg/liter) (Grand Island Biological Company, Grand Island, N.Y.) for use in tissue culture. RPMI-1640 medium (Flow Laboratories, Inc.) supplemented with glutamine (2 mM), 5% FBS, and 2 mercaptoethanol (5×10^{-5} M) was used for studies of spleen cell mitogenesis.

The DBA_2 mouse mastocytoma line, P815, the C57 BL/6 mouse lymphoma line EL-4 obtained from the Salk Institute tissue bank, and the BALB/c mouse lymphoma line Bd-1 (a gift from Dr. C. J. Peters of Scripps Clinic and Research Foundation) were maintained in vitro by regular passage in Hepes MEM supplemtned with 10% FBS.

Spleen cells from C57 BL/6 mice were prepared by disaggregation on a wire mesh into ice-cold Hepes MEM. After washing by centrifugation at 1,200 rpm for 10 min at 4°C, a spleen cell count was performed on a sample of the cell suspension using a 1 in 10 dilution of 0.10% crystal violet acetic acid. Petri dishes containing 1×10^7 spleen cells in RPMI medium supplemented by 5% heat-inactivated FBS were incubated for 48 h at 37°C in 5% CO_2 in air before being used. Transformed B lymphocytes were produced by exposing the spleen cell suspensions to 10 μg/ml of LPS 0111:B4 fraction II during the 48-h incubation. Mouse erythrocytes from C57 BL/6 mice were washed three times by centrifugation at 4°C in Hepes MEM before being used as target cells.

Macrophage Monolayers. Female C57 BL/6 mice, 8- to 10-wk of age (from The Jackson Laboratory, Bar Harbor, Maine) were injected intraperitoneally with 2 ml of thioglycollate broth (BBL, Cockeysville, Md.) to be used as the source of elicited peritoneal macrophages (14). The resulting peritoneal exudate, which consisted of 80–90% macrophages, was harvested 5 days later by peritoneal lavage using Hepes MEM containing 10 U per ml of sodium heparin. Aliquots usually consisting of 1.25×10^6 peritoneal exudate cells (PEC) were placed in the 16-mm diameter wells of a Linbro plate (Flow Laboratories, Inc.). After 30 min at 37°C in 5% CO_2 air, adherent cell monolayers were washed once with Hepes MEM and then incubated overnight in Hepes MEM + 20% FBS. After two further washes and the addition of Hepes MEM + 20% FBS, the monolayers comprised over 95% of cells which were phagocytic for zymosan (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio) and which had the morphologic appearances of macrophages.

For immunofluorescence studies, macrophage monolayers were stained with $F(ab)_2$ preparations of fluoresceinated polyvalent goat antiserum against purified mouse myeloma proteins of all major immunoglobulin classes and subclasses and rhodamine conjugated antiserum against brain associated thymus antigen prepared and kindly provided by Dr. S. Russell (15). Washed macrophage monolayers and positive controls consisting of spleen cells adhered to a plastic surface treated with poly L lysine (16) were stained and examined by using methods previously reported (15).

LPS Stimulation of Macrophages. LPS was sonicated immediately before addition to macrophage monolayers at appropriate concentrations either 24 h before or at the same time as the target cells.

Cytolytic Assay. The cell lines P815, Bd-1, and EL-4 were labeled with 51 chromium (^{51}Cr) in an identical fashion. Approximately 1×10^7 cells were suspended in a 50-ml plastic centrifuge tube using 0.75 ml of Hepes MEM + 20% FBS. Aliquots usually consisting of 5×10^4 cells representing about 8,000–10,000 cpm were added to appropriate wells containing macrophage monolayers. Supernates were harvested at intervals after commencing incubation at 37°C in 5% CO_2 air, spun at 400 *g* for 10 min, and transferred to tubes for counting in a γ-scintillation counter (Searle Analytic Inc., Des Plaines, Ill.). Results were calculated as means of four replicates and then expressed as percent specific release by using the following calculation:

$$\text{specific release} = \frac{\text{experimental release} - \text{macrophage control release}}{\text{total releasable counts} - \text{macrophage control release}} \times 100.$$

Macrophage control release consisted of macrophage monolayers which were not treated with LPS. Morphological examination confirmed that specific release of ^{51}Cr was associated with overt destruction of target cells. Total releasable counts were obtained from the supernate after freezing and thawing of labeled cells placed in Linbro wells and comprised about 90% of the total amount of ^{51}Cr added to each well. Viability of macrophages after LPS stimulation was assessed by trypan blue staining (Grand Island Biological Co.).

Irradiation and LPS Stimulation of Macrophages and Spleen Cells. Mice were irradiated with

600–1,200 rads in a Gammacell 40 (Atomic Energy of Canada) using a Cesium 137 source calibrated to deliver 120 rads/min. Spleen cell suspensions from irradiated and control (unirradiated) mice diluted to a final concentration of 2.5×10^6/ml in RPMI medium 5% FBS containing 2-mercaptoethanol (5×10^{-5} M) and 200-μl aliquots placed in microtiter wells (Falcon Plastics, Oxnard, Calif.). Either the specific T-cell mitogen, concanavalin A (Pharmacia, Uppsala, Sweden) or the B-cell mitogen, LPS 0111:B4 was added to appropriate wells. After a 48-h incubation at 37°C in 5% CO_2 air, the wells were pulsed overnight with 1 μCi of [^{3}H]thymidine (sp act 1 mCi/16 μg) (New England Nuclear Corp., Boston, Mass.). Cells were harvested with a MASH apparatus (Microbiological Associates, Walkersville, Md.). Filters containing the labeled cells were added to counting vials containing 10 ml of a one to one mixture of Aquasol-toluene and radioactivity was counted in a β-scintillation counter (Searle Analytic Inc.).

Results

Cytolytic Effect of LPS-Stimulated Macrophages on Tumor Cells. To determine whether LPS stimulates mouse peritoneal macrophages to lyse tumor target cells in vitro, LPS at a final concentration of 25 μg/ml, was added to macrophage monolayers at the same time as labeled target cells and the kinetics of radioactivity released into the supernate was followed for 32 h. After 24 h incubation, there was significant specific ^{51}Cr release which increased to 40% over the next 8 h (Figs. 1 a and 1 b). However, when macrophages were incubated with LPS for 24 h before the addition of target cells, cytolysis was significant after an 8 h incubation and increased linearly over the next 8 h until a plateau occurred at about 24 h. P815 tumor target cells incubated with LPS or added to macrophage monolayers not treated with LPS, released radioactive counts with kinetics similar to those spontaneously lost from target cells alone (Fig. 1 a). Further experiments were performed to define the time required for the LPS to induce a cytolytic capacity in macrophages. In these experiments the preincubation times of LPS and macrophages varied from 32 to 4 h before the addition of target cells, and lysis was assessed at regular intervals. The earliest cytolysis was measured 4 h after the addition of labeled tumor cells when the preincubation period of LPS and macrophages had been 16 h or more (data not shown).

To examine the specificity, if any, of LPS-induced macrophage killing, macrophages preincubated with LPS were tested against the syngeneic lymphoma cell line EL-4 and results compared to those seen when the allogeneic tumor cell lines P815 and Bd-1 were used. All three cell lines were lysed and the kinetics for specific release were similar showing that LPS-stimulated C57 BL/6 macrophages can kill a syngeneic and several allogeneic tumor cell lines (Fig. 2).

Influence of LPS Concentration. The amount of LPS required to induce macrophages to lyse tumor cells was determined by preincubating macrophage monolayers for 24 h with concentrations of LPS ranging from 0.04 to 50 μg/ml. The lysis of labeled target cells was subsequently measured at three time points over a 32-h period (Fig. 3 a). 10 and 25 μg/ml LPS produced maximal cytolysis and showed similar kinetics over 32 h but significant effects were also seen at 1 and 5 μg/ml (Fig. 3 a). The highest LPS concentration, 50 μg/ml, was eventually toxic for macrophages, 40% of which failed to exclude trypan blue at the end of the 32-h incubation with target cells. By contrast, concentrations of LPS below 25 μg/ml had minimal toxic effect, less than 5% of macrophages being killed

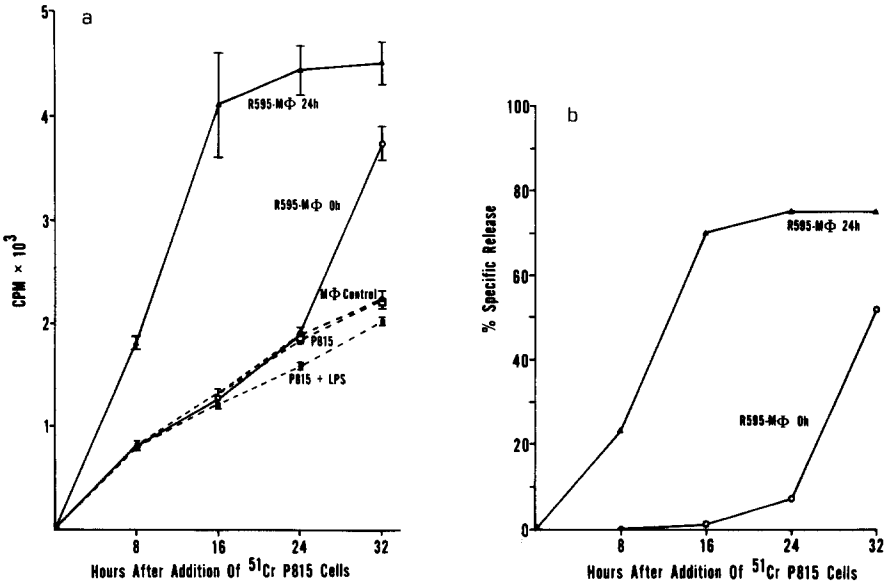

FIG. 1. Kinetics of cytolysis of ^{51}Cr-labeled P815 mastocytoma cells from DBA/2 mice by C57BL/6 peritoneal macrophages incubated with LPS-R595 at a concentration of 25 μg/ml. (a) Absolute radioactive counts released into supernate by 5×10^4 labeled P815 cells added to macrophages preincubated with LPS for 24 h (R595 MΦ 24 h, ▲), macrophages to which LPS was added at the same time as target cells (R595 MΦ 0 h, ○), untreated macrophage monolayers MΦ control, ●, medium (P815□) and medium + LPS-R595 (P815 + LPS, ■). Data expressed as means of quadruplicates ± 1 SD shown by vertical bars. Interrupted lines indicate kinetics for controls. (b) Data from Fig. 1 (a) expressed as percent specific release calculated as:

$$\frac{\text{experimental release} - \text{macrophage control release}}{\text{total releasable counts} - \text{macrophage control release}} \times 100.$$

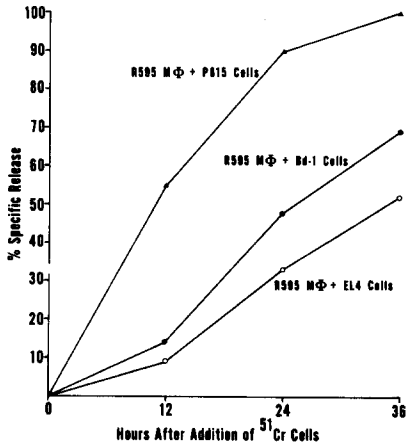

FIG. 2. Kinetics of cytolysis of the allogeneic target cell lines, P815 mastocytoma cells, Bd-1 lymphoma cells, and the syngeneic lymphoma cell line EL-4 by 1×10^6 peritoneal macrophages preincubated with LPS-R595. Data expressed as percent specific release from 5×10^4 ^{51}Cr-labeled target cells calculated from means of four replicates.

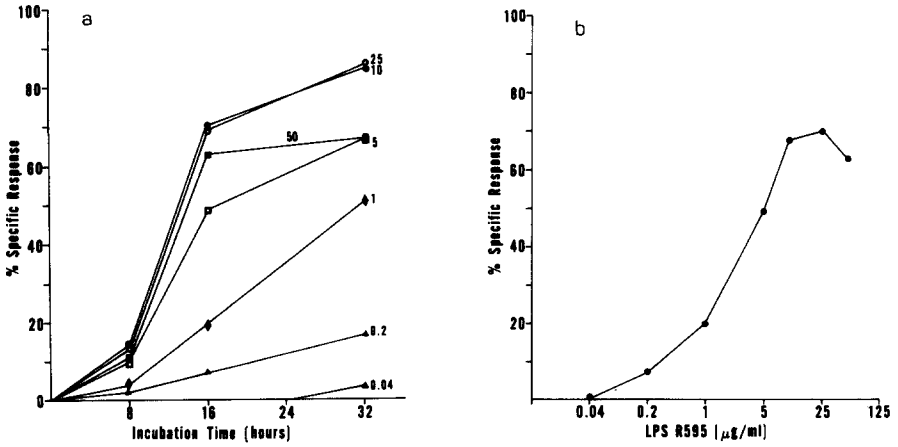

FIG. 3. Dose-response curve for LPS-R595 stimulation of macrophages assayed by their cytolytic effect on 5×10^4 ^{51}Cr-labeled P815 mastocytoma cells. All concentrations of LPS-R595 were preincubated with 1×10^6 macrophages for 24 h before the addition of target cells (a) kinetics over 32 h. Abscissa shows incubation time in hours after addition of P815 cells at time 0. (b) Cytolytic effect at concentrations of LPS-R595 from 0.04 to 50 μg/ml at 16 h after addition of P815 target cells.

over the entire incubation period. Concentrations of LPS below 0.2 μg/ml did not induce macrophage monolayers to become cytolytic under these assay conditions (Fig. 3 a). 16 h after addition of labeled target cells, cytolysis curves for different concentrations of LPS showed maximal dose-response effects (Fig. 3 b).

Effect of Altering the Ratio between Macrophages and Target Cells. We used two methods to evaluate the relative importance of the ratio between macrophages and target cells to the lysis of target cells. In the first, a constant number of macrophages (1×10^6) was plated in wells to produce confluent monolayers and labeled Bd-1 cells were added to provide a macrophage to target cell ratio varying from 100:1 to 1:1. The Bd-1 lymphoma cell line was selected for these experiments because of its ability to grow exponentially at high cell densities. Despite a wide range in macrophage to tumor cell ratios, the curve describing specific release of radioactive counts showed significant killing at all ratios tested over 36 h (Fig. 4 a). The second method involved adding a constant number of target cells to wells containing varying numbers of macrophages. Here, a different relationship between macrophages and tumor cells was evident (Fig. 4 b), even though the ratios were the same as before (Fig. 4 a). When macrophage numbers greater than 6×10^5 per well were plated in the 16-mm wells, the monolayers were completely confluent and there was lysis over ratios varying from 11:1 (6×10^5 macrophages) to 40:1 (2×10^6 macrophages) (Fig. 4 b). When less than 6×10^5 macrophages were added to the wells, target cell killing was not measurable even at a macrophage to tumor cell ratio of 5 and 10 to 1, contrasting with the marked cytolysis seen when the same ratios were achieved on confluent macrophage monolayers (Fig. 4 a). These results indicate that the ratio of effector to target cell itself is less important to target cell lysis than the absolute number of macrophages suggesting that either confluence per se is important to achieve optimal killing of tumor cells or

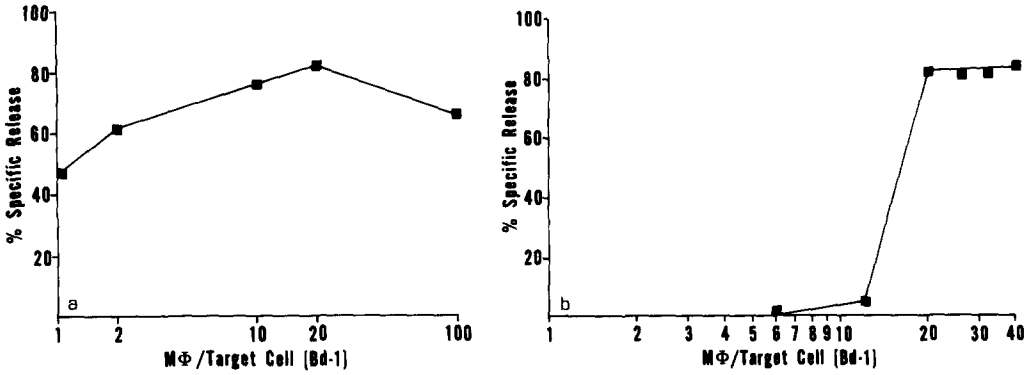

FIG. 4. Effect of macrophage to target cell ratio on LPS-stimulated cytolysis of ^{51}Cr-labeled Bd-1 lymphoma cells measured at 36 h after addition of target cells. (a) Specific release of radioactivity when ratios were achieved by adding varying numbers of target cells from 1×10^4 to 1×10^6 to confluent monolayers consisting of 1×10^6 macrophages. (b) Cytolytic effect produced by varying macrophage to target cell ratios using constant numbers of Bd-1 target cells (5×10^4) and varying the number of macrophages in monolayers from 0.3×10^6 to 2×10^6.

alternatively that a minimal number of macrophages is required for the generation of a soluble cytolytic factor or factors in sufficient concentration to kill target cells.

Effect of Conditioned Supernates from Macrophage Monolayers Incubated with LPS. The following experiment was designed to test whether the conditioned culture medium from LPS-treated macrophages lysed the tumor cells. Supernates from wells containing macrophages preincubated with an optimal dose of LPS for 24 h were centrifuged at 400 *g* for 10 min and added to wells containing 5×10^4 labeled P815 as targets. No lysis was seen after a 24-h period. By contrast, cytolysis was observed when target cells were added to control wells containing LPS stimulated macrophages (Table I). These results indicate that conditioned supernate alone is not capable of causing the cytolytic effect measured.

Viability of LPS-Stimulated Macrophages after Killing Target Cells. Macrophages harvested from mice inoculated with allogenic tumor cells retain their lytic capacity in vitro for a sustained period and are capable of killing tumor target cells on repeated occasions (17). To determine whether LPS-stimulated macrophages behave similarly, we first incubated them with labeled target cells for 24 h, then aspirated the lysed target cells and supernate, and finally rechallenged the same monolayers of LPS-stimulated macrophages with a second aliquot of 5×10^4 target cells in fresh medium without LPS. Marked specific release was again seen after 24 h (Table II) indicating that LPS-stimulated macrophages are capable of repeated target cell killing over a sustained period in vitro.

Effect of Lysed LPS-Stimulated Macrophages on Tumor Cell Viability in Vitro. To exclude the possibility that lysis of tumor cells by LPS-stimulated macrophages was due to loss of toxic products from injured macrophages, rather than the function of stimulated, fully viable macrophages, monolayers optimally stimulated by LPS were sonicated to cause their complete dissolution into

TABLE I
Conditioned Supernates—Effects on Tumor Cell Viability

P815 Target cells added to	24 h Incubation		36 h Incubation	
	^{51}Cr cpm	SR	^{51}Cr cpm	SR
		%		%
SN from LPS-MΘ	1,561 ± 56	0	2,140 ± 194	0
SN from MΘ	1,615 ± 98	0	2,243 ± 100	0
LPS + MΘ	3,072 ± 282	54	4,045 ± 100	80
MΘ	1,222 ± 26	0	1,619 ± 160	0
LPS	1,559 ± 114	0	2,183 ± 113	0
Medium	1,600 ± 36	0	2,190 ± 50	0

Total releasable cpm 4,634 ± 214.
All cpm expressed as means ± 1 SD of quadruplicates.
Abbreviatons used in this table: MΘ, peritoneal macrophages elicited with thioglycollate; SR, specific release; SN, supernate.

TABLE II
Rechallenge of LPS-Stimulated MΘ with Tumor Target Cells

	First 24 h incubation		24 h Incubation with second challenge of P815 cells to same MΘ monolayers	
	cpm ^{51}Cr	SR	*cpm*	SR
LPS + MΘ + P815	3,072 ± 242	54%	5,572 ± 472	89%
MΘ + P815	1,222 ± 26	–	2,797 ± 92	–
P815	1,580 ± 74	–	2,937 ± 92	–
LPS + P815	1,622 ± 92	–	2,907 ± 306	–
Total releasable cpm	4,634 ± 214	–	5,922 ± 90	–

All cpm expressed as means ± 1 SD of quadruplicates.
Abbreviations used in this table: SR, specific release; MΘ, macrophages.

conditioned medium. When tumor cells were added to the sonicated macrophages and to parallel LPS-treated monolayers of intact macrophages, only the intact LPS-stimulated macrophages killed their targets (Table III).

The experiments outlined above suggest that LPS stimulates macrophages to develop a sustained capacity to lyse tumor cells and to retain full viability over a prolonged period of challenge with tumor cells. Moreover, the death or injury of stimulated macrophages does not appear relevant to the killing mechanism.

Evidence That LPS Acts Directly on the Macrophage to Induce Cytolysis. Since LPS is a potent mitogen for murine B cells (18, 19), it was important to establish whether the effect of LPS on macrophages was direct or a consequence of stimulating contaminating adherent B cells present in the monolayer. There was also the remote possibility that contaminating peritoneal T lymphocytes, sensitized in vivo to LPS, were contributing to the observed target cell lysis.

Therefore, the peritoneal exudate cells used as a source of macrophages were processed for adherence to plastic, washed, and examined for the presence of B and T lymphocytes. Over 95% of the cells comprising the monolayers had the

TABLE III
Effect of Macrophage Lysis on Tumor Cell Viability

P815 Target cells added to:	cpm ^{51}Cr	SR
		%
Sonicated LPS/MΘ	3,224 ± 62	0
LPS-MΘ	6,500 ± 466	51
MΘ	3,159 ± 154	–

All cpm expressed as means ± 1 SD of quadruplicates.
Abbreviations used in this table: SR, specific release; MΘ, macrophages.

morphologic appearance of macrophages when viewed by phase contrast microscopy, and after incubation with zymosan particles over 95% of the cells contained zymosan. When similarly prepared monolayers were stained with $F(ab)_2$ preparations of fluoresceinated rabbit anti-brain associated thymus antigen and rhodaminated anti-mouse immunoglobulin, no positively stained cells were seen in the 25 high power fields examined for each stain. Control preparations of adherent spleen cells, however, revealed the expected proportions of T and B lymphocytes when stained by the same reagents. Thus, there was no detectable contamination of the macrophage monolayers by adherent B or T lymphocytes.

A further experiment was conducted to determine whether macrophages from heavily irradiated mice could be stimulated by LPS even though the mitogenic responses of B and T lymphocytes had been abolished by the irradiation. LPS stimulated macrophages from mice subjected to whole body irradiation with 600, 1,200, and 2,400 rads 4 h before PEC were harvested, and induced the same percentage of target cell killing as macrophages from the unirradiated control group (Fig. 5). By contrast, the mitogenic responses of splenic T cells to concanavalin A and of splenic B cells to LPS from the same animals were almost completely ablated in those mice subjected to 600 rads and higher irradiation doses (Fig. 5). Taken together with the purity of the macrophage monolayers, these data suggest that LPS does not require the intermediary involvement of B or T lymphocytes to stimulate macrophages to become cytolytic for tumor cells.

LPS-Stimulated Macrophage Killing is Tumor Specific. As shown in Fig. 2, LPS-stimulated macrophages kill a variety of tumor target cells, both syngeneic and allogeneic. The question then arose as to whether LPS-induced macrophage killing was tumor specific, as reported for macrophages stimulated in vivo by a variety of other agents (1), or whether target cells devoid of neoplastic potential were also susceptible. Macrophage monolayers were optimally stimulated by LPS R595 and then ^{51}Cr-labeled normal spleen cells, spleen cells containing B lymphoblasts, mouse erythrocytes, or P815 cells at effector to target cell ratios of 1:1, 10:1, and 100:1 were added. While the P815 cells were lysed by the LPS-stimulated macrophage monolayers to produce specific releases of 44, 54, and 48%, respectively, no detectable specific release of ^{51}Cr was found for normal spleen cells, spleen cells containing B lymphoblasts, or erythrocytes. These results, therefore, suggest that the cytolytic properties induced in macrophages by incubation with LPS are tumor cell specific and that nonneoplastic cells are not susceptible to lysis.

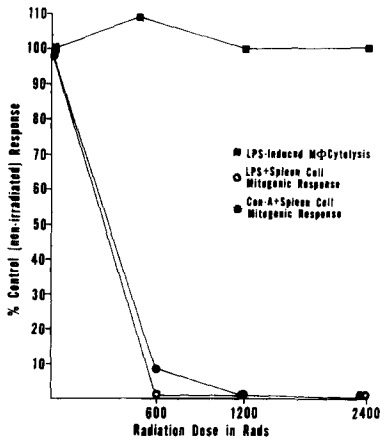

FIG. 5. Whole body irradiation of mice. Effect on LPS-induced macrophage cytolytic effect when 5×10^4 ^{51}Cr-labeled P815 cells are added to 1×10^6 macrophages from mice irradiated with 0–2,400 rads. LPS-treated monolayers were preincubated for 24 h with LPS R595 μg/ml (■). Results expressed as percent of control where 100% represents specific release by LPS-treated macrophages from mice which were not irradiated. Uptake of [^{3}H]thymidine pulse by spleen cells from irradiated mice stimulated by LPS-0111:B4 (●). Results expressed as percent of control where 100% represents uptake of [^{3}H]thymidine by 5×10^5 spleen cells stimulated by optimal concentrations of LPS-0111:B4 (1 μg/well) or concanavalin A (0.5 μg/well) achieved by constructing dose-response curves for both mitogens.

Discussion

The experiments presented in this report detail the nature of the interaction by which LPS stimulates mouse peritoneal macrophages to kill tumor cells in vitro. Macrophage stimulation by LPS seems to be independent of other cell types and results in the capacity to lyse both syngeneic and allogeneic tumor cell lines. In the accompanying paper we show that such stimulation of macrophages is a general property of LPS extracted from Gram-negative bacterial cell walls (20). Lysis of target cells by LPS stimulated macrophages is dependent upon the absolute number of macrophages required to produce confluent monolayers.

The kinetics of the LPS-induced macrophage cytolytic effect indicate two distinguishable components; a latent period after LPS exposure before target cell killing is demonstrable and a second delay for maximal target cell lysis to occur. The existence of a latent period of more than 16 h (Figs. 1 a and b) suggests that this period may be required for the elaboration of cytocidal function in macrophages and demands study using appropriate metabolic inhibitors. An additional time lag occurs before macrophages stimulated by LPS effect maximal lysis of tumor target cells. Even when labeled target cells are exposed to fully stimulated macrophages there is a linear increase in specific release over the first 16–20 h, the time required for maximal lysis. These kinetics closely resemble those seen in identical assay conditions using macrophages stimulated by intraperitoneally inoculating mice with allogenic tumor cells, W. F. Doe, unpublished observations. Moreover, similar cytolytic time curves have been demonstrated for in vitro tumor cell killing by macrophages harvested directly from solid neoplasms (9). Thus, it seems likely that tumor

cell lysis by LPS stimulated macrophages may involve the same or a very similar lytic mechanism to that of macrophages stimulated in vivo.

LPS-stimulated macrophages were nonspecifically cytolytic for tumor cells, killing both syngeneic (EL-4) and allogeneic (Bd-1) lymphoma lines and the allogeneic mastocytoma cell line, P815. Although the degree of killing varies for these different target cells, the time curves for lysis are very similar suggesting that variations in specific release between cell lines mainly reflect their sensitivities to lysis per se. Only target cells with neoplastic potential were susceptible to lysis by LPS-stimulated macrophages. By contrast, when a variety of non-neoplastic cells, including normal spleen cells, spleen cells containing a high proportion of B lymphoblasts or erythrocytes were offered as targets, no lysis was observed. These findings indicate that LPS treatment of macrophages in vitro induces a cytolytic state similar to that reported for macrophages activated in vivo by BCG, protozoa, or tumor cells (1–3, 6–9). The nature of the non-immunologic recognition mechanism which determines susceptibility to or protection from killer macrophages, however, remains to be elucidated.

Even at favorable ratios between effector and target cells, a threshold number of macrophages was needed for cytolysis, apparently related to the number required to produce confluent monolayers. Subconfluent monolayers were ineffective at lysing target cells at ratios as high as 10:1. Yet, at similar or lower ratios of target cells added to confluent monolayers, significant target cell killing occurred. Several interpretations of these experiments are possible. First, if membrane apposition between macrophages and tumor cells is essential to target cell lysis, subconfluent macrophage monolayers may offer a reduced opportunity for such contact. Second, confluence may be necessary for information transfer between macrophages, perhaps representing a mechanism by which a subpopulation of primed macrophages stimulates an entire monolayer. A third alternative is that a minimal number of macrophages may be required for the generation of the suprathreshold concentration of cytolytic factor(s) required to lyse tumor cells.

We then showed that viable LPS-stimulated macrophages were entirely responsible for lysing the tumor cells they contacted by showing that other possible factors did not participate. Conditioned supernates from wells containing LPS-stimulated macrophage monolayers did not lyse target cells, nor were disrupted LPS-stimulated macrophages capable of inducing lysis. Moreover, stimulated macrophages could kill on more than one occasion over a sustained period without further stimulation by additional LPS. These findings support the view that cell contact between effector and target cell is essential to achieve target cell lysis and suggest that if a soluble lytic factor is secreted by the stimulated macrophage, it requires the microenvironment within close membrane apposition to be effective, and is not sufficiently active, or not present at adequate concentration for the conditioned supernate alone to have lytic properties. Alexander and Evans reported similar findings for LPS stimulated macrophages in a cytostatic assay in which they failed to detect a growth inhibitory factor in supernates (5). Other workers have shown that only tumor cells in actual contact with macrophages stimulated by BCG (21), toxoplasma (3), or tumor cells (22) were prevented from growing. In addition, Hibbs has

suggested that the mechanism of macrophage cytotoxicity involves direct transfer of subcellular components across the fused membranes of a stimulated macrophage and adherent target cell (23). However, a number of soluble macrophage cytotoxic factors with widely differing properties and specificities have been reported. Currie and Basham describe a factor nonspecifically cytolytic for tumor cells and inhibited by FBS in supernates from LPS stimulated macrophages (10). This finding, together with those of soluble factors which are immunologically specific (24) or lytic for erythrocytes (25) may be reconciled with our results if they reflect molecular events occurring at the interacting surfaces of stimulated killer cells and target cells.

Our experimental evidence indicates that LPS stimulates macrophages directly and does not require the participation of B or T lymphocytes. Immunofluorescence staining of macrophage monolayers did not detect contamination by adherent B or T lymphocytes. Moreover, high doses of irradiation did not affect the LPS induced macrophage cytolytic capacity even though the mitogenic response of similarly irradiated spleen cells to LPS and to Concanavalin A was completely abolished. In addition, Bona has shown that labeled LPS binds to, and is taken up by, macrophages (26). It seems likely, therefore, that LPS interacts directly with macrophages to stimulate their differentiation into killer cells. These findings contrast with those of Wilton et al. who reported a requirement for B lymphocytes or supernates from stimulated B lymphocytes, in LPS stimulation of guinea pig macrophages using uptake of ^{14}C glucosamine as an assay for stimulation (27). This difference may result from the fact that ^{14}C glucosamine uptake does not reflect the same functional state as a cytolytic assay or from a species difference in macrophage response to LPS.

We have shown that thioglycollate elicited macrophages do not affect the viability of tumor cells in vitro unless the macrophage monolayer is stimulated by LPS (Fig. 1 a). However, elicited macrophages differ significantly from the resident peritoneal macrophage population in their morphologic, biochemical, and functional attributes and apparently represent a more differentiated cell population (14, 28–32). In this report, we have demonstrated that LPS stimulates further differentiation in elicited macrophages resulting in cells capable of killing tumor cells. Studies are therefore being undertaken to define the steps involved in the differentiation of macrophages stimulated by LPS. In the accompanying paper we examine the biochemical nature of the stimulation signal delivered by LPS.

Summary

Bacterial lipopolysaccharides (LPS) stimulate mouse peritoneal macrophages to kill tumor cells in vitro. Lysis is confined to tumor cells where it is nonspecific; both allogeneic and syngeneic cells being susceptible. Stimulation by LPS appears to be due to direct interaction between LPS and macrophages and does not involve participation by lymphocytes. After exposure to LPS, a latent period must elapse before macrophages can lyse tumor cells. The cytolytic mechanism requires contact between target cells and viable effector cells which maintain their lytic capacity for a sustained period and can kill on repeated occasions. The generation of a macrophage cytolytic effect by LPS is critically

dependent upon the absolute number of macrophages which must be suffcient to produce confluent monolayers. These findings indicate that LPS stimulation of macrophages in vitro represents a valuable model system for the study of the mechanisms of macrophage stimulation and of the mediation of tumor cell death.

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References

1. Evans, R., and P. Alexander. 1975. Nucleated Cell Killing. *In* Immunobiology of the Macrophage. D. Nelson, editor. Academic Press, Inc., New York. p. 535.
2. Hibbs, J. B. 1973. Macrophage nonimmunologic recognition: target cell factors related to contact inhibition. *Science (Wash. D.C.).* **180**:868.
3. Hibbs, J. B., L. H. Lambert, and J. S. Remington. 1972. Possible role of macrophage mediated nonspecific cytotoxicity in tumor resistance. *Nat. New Biol.* **235**:48.
4. Stott, E. J., M. Probert, and L. H. Thomas. 1975. Cytotoxicity of alveolar macrophages for virus-infected cells. *Nature (Lond.).* **255**:710.
5. Alexander, P., and R. Evans. 1971. Endotoxin and double stranded RNA render macrophages cytotoxic. *Nat. New Biol.* **232**:76.
6. Evans, R. 1973. Macrophages and the tumor bearing host. *Br. J. Cancer.* **28**:19.
7. Haskill, J. S., J. W. Proctor, and Y. Yamamura. 1975. Host responses within solid tumors monocytic effector cells within rat sarcomas. *J. Natl. Cancer Inst.* **54**:387.
8. Holden, H. T., J. J. Haskill, H. Kichner, and R. B. Herberman. 1976. Two functionally distinct anti-tumor effector cells isolated from primary murine sarcoma virus-induced tumors. *J. Immunol.* **117**:440.
9. Russell, S. W., G. Y. Gillespie, and A. T. McIntosh. 1977. Inflammatory cells in solid murine neoplasms in cytotoxicity mediated in vitro by macrophages recovered from disaggregated regressing Moloney sarcomas. *J. Immunol.* **118**:1574.
10. Currie, G. A., and C. Basham. 1975. Activated macrophages release a factor which lyses malignant cells but not normal cells. *J. Exp. Med.* **142**:1600.
11. Doe, W. F., D. C. Morrison, and P. Henson. 1976. Lipopolysaccharide induced macrophage activation. *Fed. Proc.* **35**:336.
12. Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245.
13. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. *In* Methods in Carbohydrate Chemistry. R. C. Whistler, editor. Academic Press, Inc., New York. **5**:83.
14. Argyris, B. F. 1967. Role of macrophages in antibody production. Immune response to sheep red blood cells. *J. Immunol.* **99**:744.
15. Russell, S. W., W. F. Doe, R. G. Hoskins, and C. G. Cochrane. 1976. Inflammatory cells (IC) in solid murine neoplasms. I. Tumor disaggregation and identification of constituent IC. *Int. J. Cancer.* **18**:322.
16. Kennedy, J. C., and M. A. Axelrad. 1971. An improved asay for haemolytic plaque-forming cells. *Immunology.* **20**:253.
17. Lohmann-Matthes, M. C., H. Schipper, and H. Fischer. 1972. Macrophage-mediated cytotoxicity against allogeneic target cells *in vitro*. *Eur. J. Immunol.* **2**:45.
18. Andersson, J., G. Möller, and O. Sjoberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell. Immunol.* **4**:381.

19. Gery, I., J. Kruger, and S. Z. Spiesel. 1972. Stimulation of B lymphocytes by endotoxin. Reactions of thymus-deprived mice and karyotypic analysis of dividing cells in mice bearing T6T6 thymus grafts. *J. Immunol.* **108**:1088.
20. Doe, W. F., S. T. Yang, D. C. Morrison, S. Betz, and P. M. Henson. 1978. Macrophage stimulation by bacterial lipopolysaccharides. II. Evidence for differentiation signals delivered by lipid A and by a protein rich fraction of LPS. *J. Exp. Med.* **148**:557.
21. Cleveland, R. P., M. S. Meltzer, and B. Zbar. 1974. Tumor cytotoxicity *in vitro* by macrophages from mice infected with mycobacterium bovis strain BCG. *J. Natl. Cancer Inst.* **52**:1887.
22. Den Otter, W., R. Evans, and P. Alexander. 1972. Cytotoxicity of murine peritoneal macrophages in tumor allograft immunity. *Transplantation (Baltimore).* **14**:220.
23. Hibbs, J. R. 1976. Heterocytolysis by macrophages activated by Bacillus Calmette-Guerin: lysosome exocytosis into tumor cells. *Science (Wash. D.C.).* **184**:468.
24. McIvor, K. L., and R. S. Weiser. 1971. Mechanism of target cell destruction by alloimmune peritoneal macrophages. II. Release of a specific cytotoxin from interacting cells. *Immunology.* **20**:315.
25. Melsom, H., G. Kearny, S. Gruca, and R. Seljelid. 1974. Evidence for a cytolytic factor released by macrophages. *J. Exp. Med.* **140**:1085.
26. Bona, C. A. 1973. Fate of endotoxin in macrophages: biological and ultrastructural aspects. *J. Infect. Dis.* **128**(Suppl.):66.
27. Wilton, J. M., D. L. Rosenstreich, and J. J. Oppenheim. 1975. Activation of guinea pig macrophages by bacterial lipopolysaccharide requires bone-marrow derived lymphocytes. *J. Immunol.* **114**:388.
28. Edelson, P. J., R. Zweibel, and Z. A. Cohn. 1975. The pinocytotic rate of activated macrophages. *J. Exp. Med.* **142**:1150.
29. Bianco, C., F. M. Griffin, and S. C. Silverstein. 1975. Studies of the macrophage complement receptor alteration of receptor function upon macrophage activation. *J. Exp. Med.* **141**:1278.
30. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139**:834.
31. Werb, Z., and S. Gordon. 1975. Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142**:346.
32. Werb, Z., and S. Gordon. 1975. Elastase secretion by stimulated macrophages. *J. Exp. Med.* **142**:361.