LACK OF BINDING OF BACTERIAL LIPOPOLYSACCHARIDE TO MOUSE LUNG MACROPHAGES AND RESTORATION OF BINDING BY \( \gamma \) INTERFERON

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Lung macrophages (LM)\(^1\) are known to differ from peritoneal macrophages (PM) in many respects, such as their lysosomal enzymes, metabolic activity for energy requirement, morphology, and function (1–3). We reported that the expression of a glycosphingolipid, asialo GM1, on the cell surface is markedly different between LM and PM (4, 5).

In the present paper, we report that LM are unresponsive to activation for tumor cytotoxicity by bacterial lipopolysaccharide (LPS), while PM from the same strains of mice were responsive, because of the lack of binding with LPS on the cell surface of LM. The LPS-binding sites appear after treatment of LM in vitro with a lymphokine-rich supernatant or recombinant murine gamma interferon (IFN-\(\gamma\)), resulting in recovery of responsiveness to activation by LPS.

Materials and Methods

Animals. Female C3H/HeSlc, BALB/cSlc, and ICR/Slc mice were obtained from Shizuoka Experimental Animal Cooperative (Hamamatsu, Japan), and C3H/HeNcrj were from Charles River Japan, Inc. (Atsugi, Japan). Mice (6–8-wkold) were housed in a specific pathogen–free condition before use.

Reagents. LPS (phenol extract from *Escherichia coli* 055:B5) and LPS conjugated with fluorescein isothiocyanate (FITC-LPS) (molar ratio, 24:1) were obtained from List Biological Laboratories, Inc. (Campbell, CA). Insoluble concanavalin A (Con A–Sepharose) and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO). Polymixin B sulfate was obtained from P-L Biochemicals, Inc. (Milwaukee, WI).

Recombinant Murine IFN-\(\gamma\). Recombinant murine IFN-\(\gamma\), obtained from gene expression in *Escherichia coli* (6), was a gift from Toray Industries, Inc. (Tokyo, Japan). Amino acid sequence encoded by the IFN-\(\gamma\) gene used is identical to that of the natural murine IFN-\(\gamma\) gene (personal communication, Toray Industries, Inc.). Specific activity of the IFN-\(\gamma\)

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Abbreviations used in this paper: AC, alveolar cells; AM, alveolar macrophages; Con A, concanavalin A; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC-LPS, LPS conjugated with fluorescein isothiocyanate; IFN, interferon; LC, lung cells; LK, lymphokines; LM, lung macrophages; LPS, lipopolysaccharide; MAF, macrophage-activating factor; Nu-RPMI medium, RPMI 1640 with 10% Nusserum; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PEM, peritoneal exudate macrophages; PGE, prostaglandin E; PM, peritoneal macrophages; PRC, peritoneal resident cells; PRM, peritoneal resident macrophage.

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γ was 8.3 × 10^6 U/mg, and a stock solution of 1 × 10^5 U/ml contained <0.05 ng/ml endotoxin as determined by the Limulus amebocyte lysate test.

**Antiserum.** Rabbit anti-mouse IFN-γ and IFN-(α + β) antisera were obtained from Enzo Biochem, Inc. (New York, NY). The number of neutralizing units of the anti-mouse IFN-γ was 50,000 against mouse IFN-γ, and <16 against mouse IFN-α and -β. The number of neutralizing units of anti-mouse IFN-(α + β) was 28,000 against mouse IFN-(α + β) or mouse IFN-β, 10,000 against mouse IFN-α, and <16 against mouse IFN-γ.

**Culture Medium.** RPMI 1640 medium was prepared from powdered stock (Nissui Seiyaku Co., Ltd., Tokyo) and supplemented with 100 U/ml of penicillin G potassium (Banyu Seiyaku Kabushiki Kaisha, Tokyo) and 100 µg/ml of streptomycin sulfate (Meiji Seika Kaisha, Yokohama, Japan). Fetal bovine serum (FBS) (Lot 309, Gibco Laboratories, Grand Island, NY) was inactivated at 56°C for 30 min before use. The FBS contained 0.013 ng/ml of LPS according to the test with Limulus amebocyte lysate (Gibco Laboratories). RPMI 1640 medium was further supplemented with 5% FBS and used as culture medium unless otherwise stated (FBS-RPMI medium). For preparing lymphokines, RPMI 1640 medium was supplemented with 10% Nuserum (Collaborative Research, Inc., Lexington, MA) (Nu-RPMI medium).

**Preparation of Macrophages.** Peritoneal resident cells (PRC) were obtained by washing out normal mouse peritoneal cavities with physiologic saline. Peritoneal exudate cells (PEC) were prepared as previously described (4). Briefly, the lungs were chopped into 1–2-mm squares, after vascular perfusion with Ca++ and Mg++-free phosphate-buffered saline (PBS) containing 10 U/ml of heparin to avoid blood cell contamination, until the lung tissue was uniformly white. LC were washed out from the cut pieces with PBS containing 0.05% EDTA and the cells were filtered through a 200-mesh stainless steel sieve. Alveolar cells (AC) were harvested by bronchial lavage. The trachea was exposed and an 18-gauge catheter was inserted into the trachea between two rings of cartilage. The lavage fluid (PBS containing 0.05% EDTA) was introduced in 0.8-ml amounts from a 1 ml syringe connected to the catheter, the chest cavity was gently massaged, and the lavage fluid was withdrawn from the lungs into the syringe. A total of 2.4 ml of lavage fluid per mouse was used.

PRC, PEC, LC, and AC were washed three times by centrifugation and resuspended in ice-cold RPMI 1640 medium. Total cell counts were made with a hemocytometer, and cells were differentiated by using cell smears of a Diff-Quik Stain Set (Harleco, a division of American Hospital Supply Corp., Gibbstown, NJ). 0.1-ml quantities of cell suspension containing 2 × 10^6 macrophages/ml were pipetted into wells of 96-well flat-bottom tissue culture plates (Falcon No. 3072; Becton Dickinson & Co., Oxnard, CA), or, 80–90-µl quantities of cell suspension containing 2 × 10^6 macrophages/ml were spotted onto coverslips (15-mm diam). They were incubated at 37°C for 120 min, and nonadherent cells were removed by repeated washing with physiologic saline solution. At least 96% of the adherent cells were macrophages as judged by morphologic and phagocytic criteria (4, 5), and they were used as macrophage monolayers of peritoneal resident macrophages (PRM), peritoneal exudate macrophages (PEM), lung macrophages (LM), or alveolar macrophages (AM).

**Crude Lymphokines (LK).** Spleen cells from normal ICR/SIc mice were obtained by extrusion of the spleens through a 200-mesh stainless steel sieve and washed with physiologic saline solution three times. The cells were suspended in RPMI 1640 medium and treated with 3 vol of 0.5% ammonium chloride to lyse red blood cells. The cells were washed twice and suspended in Nu-RPMI medium. 10 ml of the spleen cell suspension (1 × 10^7 cells/ml) were incubated with 100 µg/ml of Con A-Sepharose at 37°C for 48 h in plastic tissue culture dishes (Falcon No. 3003) according to the method of Fidler et al. (7) with slight modification. The culture supernatant was harvested by centrifugation at 500 g for 20 min at 4°C, then filtered through a 0.45-µm Millipore filter (Millipore Corp., Bedford, MA) and stored at −70°C (crude LK). The culture supernatant of the spleen cells alone and Con A-Sepharose without cells were used as controls.

10 ml of crude LK were concentrated 10-fold by vacuum dialysis using Immersible CX-
10 (Millipore Corp.), and then fractionated on a 1.5 × 90 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) that was previously equilibrated with 0.15 M PBS (pH 7.2). The column was operated at a flow rate of 25 ml/h, and calibrated by using as markers, bovine serum albumin (67,000 mol wt), ovalbumin (43,000 mol wt), Blue Dextran 2000, catalase (232,000 mol wt) (Pharmacia Fine Chemicals), and cytochrome c (12,500 mol wt) (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany). Fractions were collected and protein was quantitated by measuring the absorption at 280 nm.

Activation of Macrophages. Procedures of macrophage activation with LK or LPS were described previously (5). Briefly, macrophage monolayers prepared in 96-well plates were preincubated for various time periods with 200 µl each of either LK or LPS at selected concentrations, and then washed twice with physiologic saline.

Assay for Tumor Cytotoxicity. The assay method was described previously (5). Briefly, cells (5 × 10⁶/0.5 ml) of an EL4 leukemia cell line, established in this laboratory, were incubated with 50 µCi of Na₂⁵¹CrO₄ (New England Nuclear, Boston, MA) for 50 min at 37°C and washed three times and suspended in FBS-RPMI medium. 200 µl of labeled EL4 cell suspension containing 1 × 10⁴ cells were added to the macrophage monolayers, and incubated for 20 h. The plates were then centrifuged for 10 min at 350 g and 100 µl of the supernatant was removed from each well for counting radioactivity on an automatic gamma spectrometer. All samples were assayed in triplicate. Cytotoxicity was expressed as percent specific ⁵¹Cr release calculated from the average of triplicate samples as follows: percent specific release = (experimental release - spontaneous release)/(maximum release - spontaneous release). Spontaneous release was taken as the amount of radioisotope released from target cells cultured alone, and maximal release was the amount obtained by treatment with 2% Nonidet P-40 (Sigma Chemical Co.).

Assay of Direct Toxic Effect of LPS on Macrophages. The killing effect of LPS on macrophages was assayed by the method of Glode et al. (8). Briefly, macrophage monolayers prepared in 96-well plates were incubated with FBS-RPMI medium containing various concentrations of LPS in the presence or absence of crude LK (1:10 dilution) for 40 h. The supernatants were decanted and replaced with 0.1 ml of 0.15 M saline containing 0.3% trypan blue dye. 200 cells were scored for viability on the basis of trypan blue exclusion, and the mean of triplicate cultures was obtained. The results are expressed as the percentage of dead macrophages relative to total cells.

Measurement of Binding of FITC-LPS to Macrophages. Binding of FITC-LPS to macrophages was determined by a fluorescence microscope or by flow cytometry. 40 µl of FITC-LPS (100 µg/ml) suspended in PBS containing 0.1% NaN₃ was spotted on the macrophage monolayers prepared on coverslips. The coverslips were held at 0°C for 60 min, washed, and then dipped into wells containing PBS supplemented with 1% paraformaldehyde. In some experiments, FITC-LPS was preincubated with various concentrations of polymyxin B for 30 min at 37°C. The number of cells stained with FITC-LPS was determined with a fluorescence microscope. The results were expressed as the percentage of cells stained with FITC-LPS among a total of 200 cells.

For the flow cytometry analysis, PC and LC (2 × 10⁶) were mixed with 300 µl of FITC-LPS (100 µg/ml) suspended in PBS containing 0.1% NaN₃, held at 0°C for 60 min, washed three times with ice-cold PBS, and resuspended in PBS. Cell-bound fluorescence was analyzed on a fluorescence-activated cell sorter (FACS 440; Becton Dickinson Immunocytometry Systems, Mountain View, CA) using an argon ion laser (model 164-06, 400 mW; Spectra-Physics, Inc., Mountain View, CA) at 488 nm excitation. Fluorescence was measured through a 530-nm-long pass filter. The fluorescence data of LM in LC and of PRM in PC were obtained by the use of the dual scatter (0° and 90° scatter) gate to exclude most cellular debris, dead cells, erythrocytes, and lymphocytes and to maximize macrophage detection. Fluorescence intensity was expressed on linear scale. For each experiment, at least 1 × 10⁴ cells were analyzed to yield the histogram shown.
Results

LPS-induced Tumor Cell Killing. PRM, PEM, LM, and AM from C3H/HeSlc mice were incubated at 37°C for 20 h either with various concentrations of LPS or with medium alone, washed, and then assayed for tumor cytotoxicity. As shown in Fig. 1, PEM preincubated with LPS showed strong cytotoxicity; 0.1 μg/ml of LPS was sufficient to induce a high level of cytotoxicity. PRM preincubated with LPS showed less but significant levels of cytotoxicity, which was dependent upon the dose of LPS. LM and AM, in contrast, did not show any cytotoxicity after incubation with LPS at any concentration. LM and AM were incubated with LPS at increasing doses up to 100 μg/ml, for various incubation periods from 4 to 48 h, and then cytotoxicity was tested at increasing ratios of effector-to-target cells up to 30. However, these LM and AM showed no cytotoxicity (data not shown). In one experiment, LM were incubated with 10 μg/ml of LPS for 20 h and then tested for cytotoxicity in the presence of 10 μg/ml of LPS, but no cytotoxic activity was observed.

Effect of Indomethacin on Activation of LM by LPS. Prostaglandins of the E series (PGE) are known to inhibit macrophage activation (9, 10). It is possible that the unresponsiveness of LM or AM to LPS is caused by a large amount of PGE produced by LPS-stimulated LM or AM. To test this possibility, a PGE inhibitor, indomethacin, was added together with LPS to LM or PRM. As shown in Table I, indomethacin (10^-6 M) greatly augmented the cytotoxicity of PRM stimulated with LPS. In contrast, LM stimulated with LPS showed no cytotoxicity, regardless of the presence of indomethacin. These results exclude the possibility mentioned above.

![Figure 1](image-url) Figure 1. Tumor cell killing by LM, AM, PRM, and PEM incubated with LPS. Monolayers of LM (△), AM (○), PRM (●), and PEM (□) were incubated with various concentrations of LPS for 20 h at 37°C, washed, and assayed for cytotoxicity against ^51Cr-labeled EL4 cells.
Synergistic Effect of Crude LK and LPS on Activation of LM. It has been reported (11-13) that PEM from C3H/HeJ, A/J, and P/J mice fail to develop tumor cytotoxicity after LPS stimulation, but that they develop cytotoxicity when they are pretreated with LPS plus LK. Therefore, we tested the effect of crude LK on activation of LM by LPS. Monolayers of LM were incubated with various concentrations of LPS in the presence or absence of various dilutions of crude LK for 20 h at 37°C. The cells were washed and then assayed for their cytotoxicity. Incubation of LM with any concentrations of the crude LK alone or of LPS alone did not evoke cytotoxicity (Fig. 2). However, concurrent incubation with crude LK and LPS rendered LM cytotoxic in a dose-dependent fashion. Increasing the concentration of crude LK caused a progressive shifting of the LPS dose response curve toward lower LPS concentrations. The minimum concentration of LPS required for cooperation with crude LK (1:10 dilution) was as small as 1 ng/ml. The culture supernatants of spleen cells alone and Con A-Sepharose alone, used as controls, did not show such an effect (data not shown). Similar to LM, AM incubated with LPS and crude LK concurrently evoked cytotoxicity (data not shown).

The activation of LM was found to be critically dependent on the sequence of treatment with LK and LPS. Monolayers of LM were incubated with either crude LK (1:10 dilution), LPS (1 μg/ml), crude LK plus LPS, or medium alone for 14 h at 37°C, and washed. They were incubated further with either of these agents for 10 h at 37°C. The cells were washed and then assayed for cytotoxicity. LM treated first with crude LK and then with LPS were cytotoxic (Table II), as were LM treated with crude LK plus LPS for 24 h. LM treated with medium alone first and then treated with crude LK plus LPS showed almost the same degree of cytotoxicity. In contrast, LM treated first with LPS and then with crude LK showed no cytotoxicity. Thus, LM exposed to crude LK were not cytotoxic but became sensitive to subsequent stimulation with LPS. The reverse
Synergistic effect of crude LK and LPS on tumor cytotoxicity of LM. Monolayers of LM were incubated with various concentrations of LPS in the presence of various dilutions of crude LK (O, 1:10 dilution; A, 1:30 dilution; □, 1:90 dilution) or medium (●) for 20 h at 37°C, washed, and then assayed for cytotoxicity against ⁵¹Cr-labeled EL4 cells.

**TABLE II**

**Effect of Sequence of Treatment with Crude LK and LPS on the Activation of LM for Cytotoxicity**

| Treatment       | Cytotoxicity (mean ± SD) |
|-----------------|--------------------------|
|                 | First 14 h                | Second 10 h                |
| Medium          | Medium                   | -2.0 ± 0.8                 |
| LK              | LK                       | 1.0 ± 1.5                  |
| LPS             | LPS                      | 1.2 ± 2.3                  |
| LK plus LPS     | LK plus LPS              | 23.0 ± 2.1                 |
| LK              | LPS                      | 17.7 ± 2.6                 |
| LPS             | LK                       | -2.0 ± 3.1                 |
| Medium          | LK                       | -1.6 ± 2.5                 |
| Medium          | LPS                      | 0.1 ± 0.8                  |
| Medium          | LK plus LPS              | 21.6 ± 2.9                 |

LM were incubated with either crude LK (1:10 dilution), LPS (1 μg/ml), or medium alone for 14 h, and washed. The LM were incubated further with either of these agents for 10 h. Those cells were washed and assayed for cytotoxicity. The results are expressed as the mean percentage of triplicate cultures ± standard deviation.

sequence of treatment of LM with LPS and crude LK was completely ineffective. These experiments were repeated with PRM and LM from BALB/cSlc, ICR/Slc, and C3H/HeNCrj mice; results were similar to those obtained with C3H/HeSlc mice (data not shown).
TABLE III

| LM incubated with:         | Cytotoxicity % |
|----------------------------|----------------|
| Medium                     | 0.9 ± 1.3      |
| LPS                        | 0.8 ± 1.6      |
| LK                         | −6.3 ± 3.5     |
| LK treated with anti-IFN-γ | −2.6 ± 1.9     |
| LK treated with anti-IFN-(α + β) | −4.9 ± 2.8 |
| LPS plus LK                | 33.9 ± 1.8     |
| LPS plus LK treated with anti-IFN-γ | 3.3 ± 3.2 |
| LPS plus LK treated with anti-IFN-(α + β) | 25.6 ± 3.5 |

Crude LK was preincubated with either rabbit anti-IFN-γ or -(α + β) antiserum or medium alone for 60 min at 37°C. LM were incubated with either of these preincubated crude LK (1:20 dilution) together with or without LPS (1 μg/ml) at 37°C for 20 h, washed, and assayed for cytotoxicity. The results are expressed as the mean percentage of triplicate cultures ± standard deviation.

Active Fraction in Crude LK. Gel filtration of crude LK by Sephacryl S-200 column chromatography was performed. LM were incubated for 20 h at 37°C with some of the fractions eluted from the column, in the presence of 1 μg/ml of LPS, and then assayed for their cytotoxicity. The results indicated that the molecular weights of active components were distributed broadly, ranging from ~40,000 to 70,000 (data not shown). The activity of the macrophage-activating factor (MAF) contained in the same fractions was determined by using PEM. PEM were incubated for 20 h with those fractions in the presence of 3 ng/ml of LPS and assayed for their cytotoxicity. The fractions showing MAF activity were also distributed broadly, ranging from about 40,000 to 70,000 mol wt (data not shown).

Recent studies indicate that IFN-γ has potent MAF activity (14–16). Therefore, crude LK was incubated with rabbit anti-mouse IFN-γ or IFN-(α + β) for 60 min at 37°C. The IFN titer of the crude LK was 159 U/ml before the incubation, though the type of IFN was not determined. Anti-IFN-γ and anti-IFN-(α + β) were able to neutralize 200 U of IFN-γ and IFN-β, respectively. After incubation, all the samples were diluted (1:20) and tested for their ability to render LM cytotoxic in the presence of LPS (1 μg/ml). LM incubated with the crude LK pretreated with anti-IFN-(α + β) plus LPS showed strong cytotoxicity (Table III), as did LM incubated with LPS plus nontreated crude LK. However, LM incubated with crude LK pretreated with anti-IFN-γ plus LPS showed no cytotoxicity.

Effect of Treatment with Recombinant Murine IFN-γ on Activation of LM by LPS. The results described above suggest that the molecule contained in crude LK that renders LM responsive to LPS is IFN-γ. Since the anti-IFN-γ antiserum used was polyclonal, we tested the effect of recombinant murine IFN-γ instead of crude LK. LM were incubated with 1 to 1 × 10³ U/ml of recombinant murine IFN-γ in the presence or absence of various concentrations of LPS, for 20 h, washed, and then assayed for their cytotoxicity. LM treated with the IFN-γ alone
Effect of recombinant murine IFN-γ on activation of LM by LPS. Monolayers of LM were incubated with various concentrations of LPS in the presence of various concentrations of recombinant murine IFN-γ (○, 10³ U/ml; Δ, 10² U/ml; ▲, 10 U/ml; □, 1 U/ml) or medium (●) for 20 h at 37°C, washed, and then assayed for cytotoxicity against ³¹Cr-labeled EL4 cells.

Figure 3. Effect of recombinant murine IFN-γ on activation of LM by LPS. Monolayers of LM were incubated with various concentrations of LPS in the presence of various concentrations of recombinant murine IFN-γ (○, 10³ U/ml; Δ, 10² U/ml; ▲, 10 U/ml; □, 1 U/ml) or medium (●) for 20 h at 37°C, washed, and then assayed for cytotoxicity against ³¹Cr-labeled EL4 cells.

Lethal Toxicity of LPS for PRM, PEM, and LM. Lethal toxicity of LPS for LM, PRM, and PEM was investigated by incubating these cells with 10–100 μg/ml of LPS for 40 h at 37°C. As indicated in Table IV, PRM and PEM were highly sensitive to LPS toxicity; 21.0% of PRM and 16.4% of PEM died when incubated with 10 μg/ml of LPS. On the other hand, LM were resistant to LPS toxicity, and only a negligible population of LM died from as high a concentration of LPS as 100 μg/ml. However, when LM were incubated with crude LK or recombinant murine IFN-γ, LM became sensitive to LPS toxicity. PRM and PEM treated with crude LK also became hyperreactive to LPS toxicity. Crude LK or the IFN-γ alone were not toxic to these macrophages.

Binding of FITC-LPS on the Surface of LM, AM, PRM, and PEM. To test the possibility that the unresponsiveness of LM to LPS was due to the lack of LPS-binding sites, we examined the binding of FITC-LPS to LM. The test results by fluorescence microscopy are shown in Table V. >90% of PRM and PEM were stained with FITC-LPS, whereas <5% of LM and AM were stained. The intensity of fluorescence of PEM was weaker than that of PRM. To exclude the possibility
that the binding of LPS to the cells is nonspecific, we examined FITC-LPS
preincubated with polymyxin B for 30 min at 37°C. The percentage of PRM or
PEM stained with FITC-LPS decreased markedly depending upon the dose of
polymyxin B (Table V).

Preincubation of LM with crude LK or recombinant murine IFN-γ for 20 h
rendered LM able to bind with FITC-LPS (Table VI). Pretreatment of crude
LK with anti-IFN-γ destroyed the activity of crude LK, while pretreatment with

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**Table IV**

*Lethal Toxicity of LPS for LM, PRM, and PEM*

| Treatment | Quantity | Dead cells |
|-----------|----------|------------|
|           | µg/ml    | LM PRM PEM |
| Medium    | 1.7      | 2.7 2.1    |
| LK        | 1.7      | 3.4 2.9    |
| LPS 100   | 2.4      | 47.5 28.1  |
| LPS 50    | 2.0      | 26.5 23.4  |
| LPS 10    | 1.7      | 21.0 16.4  |
| LK + LPS 100 | 15.8 | 60.3 42.6 |
| LK + LPS 50 | 17.8 | 60.1 50.5 |
| LK + LPS 10 | 17.8 | 72.5 51.3 |
| IFN-γ     | 2.4      | 2.6 ND     |
| IFN-γ + LPS 100 | 29.0 | ND ND     |
| IFN-γ + LPS 50 | 33.1 | ND ND     |
| IFN-γ + LPS 25 | 25.5 | 56.9 ND   |

LM, PRM, and PEM were incubated with LPS, crude LK (1:10 dilution),
recombinant murine IFN-γ (100 U/ml), or LPS plus either crude LK or
IFN-γ, for 40 h. Cell death was measured by the trypan blue dye exclusion
test. ND, not determined. 200 cells per well were examined for viability,
and the results are expressed as the mean percentage of dead cells in three
wells.

**Table V**

*Binding of FITC-LPS With or Without Pretreatment With Polymyxin B on the Surface of LM, AM, PRM, and PEM*

| Cells | Pretreatment of FITC-LPS with polymyxin B | Cells stained with FITC-LPS |
|-------|------------------------------------------|----------------------------|
|       | %                                        |                            |
| LM    | -                                        | 3.9                        |
| AM    | -                                        | 4.6                        |
| PRM   | + (50 µg/ml)                             | 89.8                       |
|       | + (100 µg/ml)                            | 39.2                       |
|       | + (200 µg/ml)                            | 4.7                        |
| PEM   | -                                        | 90.9                       |
|       | + (200 µg/ml)                            | 7.5                        |

Macrophages on coverslips were incubated with FITC-LPS (100 µg/ml)
suspended in PBS in the presence or absence of various concentrations of
polymyxin B for 60 min at 0°C. 200 cells on one coverslip were examined,
and the results are expressed as the mean percentage of cells stained with
FITC-LPS out of 600 cells.
TABLE VI

Binding of LPS on the Surface of LM Treated with Crude LK or Recombinant Murine IFN-γ

| Preincubation of LM with:         | Cells stained with FITC-LPS |
|----------------------------------|----------------------------|
| Medium                           | 3.5                        |
| Crude LK                         | 69.5                       |
| Crude LK pretreated with anti-IFN-γ | 10.3                      |
| Crude LK pretreated with anti-IFN-(α + β) | 62.9                   |
| IFN-γ                            | 60.8                       |

LM were incubated with crude LK (1:5 dilution), IFN-γ (100 U/ml), or medium alone for 20 h, washed, and stained with FITC-LPS (100 μg/ml) for 60 min at 0°C. Crude LK was preincubated with either rabbit anti-IFN-γ or rabbit anti-IFN-(α + β) for 60 min at 37°C. Anti-IFN-γ and anti-IFN-(α + β) were able to neutralize 200 U of IFN-γ and IFN-(α + β), respectively. 200 cells per coverslip were examined, and the results are expressed as the mean percentage of cells stained with FITC-LPS out of 600 cells.

TABLE VII

Binding of FITC-LPS in the Presence and Absence of Polymyxin B on the Surface of LM Preincubated with Recombinant Murine IFN-γ

| Pretreatment of FITC-LPS with polymyxin B | Cells stained with FITC-LPS |
|------------------------------------------|----------------------------|
| LM preincubated with:                   | %                          |
| Medium                                   | 3.5                        |
| IFN-γ                                    | 59.1                       |

LM were incubated with recombinant murine IFN-γ (100 U/ml) or medium alone for 20 h, washed, and stained with FITC-LPS (100 μg/ml) suspended in PBS in the presence or absence of various concentrations of polymyxin B for 60 min at 0°C. 200 cells per culture were examined, and the results are expressed as the mean percentage of cells stained with FITC-LPS of triplicate cultures.

anti-IFN-(α + β) did not change it. The fluorescence intensity of LM treated with crude LK or IFN-γ was weaker than that of PRM. In the presence of polymyxin B, the binding of FITC-LPS to the surface of IFN-γ-treated LM was completely abolished (Table VII). In addition, FITC-LPS binding to IFN-γ-treated LM was inhibited in the presence of unlabeled LPS to an equal or greater degree than was binding to PRM or PEM (Table VIII).

The binding of FITC-LPS to LM or PEM was analyzed by flow cytometry. Fig. 4 shows the histogram of LM and PRM. Almost all of PRM were stained with FITC-LPS, while LM contained few fluorescent cells. The intensity of autofluorescence of LM incubated with PBS alone was significantly higher than that of PRM. Analysis of the labeled cell fractions (channels beyond 94) indicated
TABLE VIII
Binding of FITC-LPS in the Presence or Absence of Unlabeled LPS on the Surfaces of LM Treated with Recombinant Murine IFN-γ, and of PRM and PEM

| Cells             | Presence of unlabeled LPS | Cells stained with FITC-LPS % |
|-------------------|---------------------------|-------------------------------|
| LM treated with IFN-γ | −                         | 60.8                          |
|                   | +                         | 3.0                           |
| PRM               | −                         | 98.1                          |
|                   | +                         | 22.0                          |
| PEM               | −                         | 85.6                          |
|                   | +                         | 18.9                          |

Macrophages on the coverslips were stained with FITC-LPS (100 μg/ml) suspended in PBS in the presence or absence of cold LPS (10 μg/ml) for 60 min at 0°C. LM were preincubated with recombinant murine IFN-γ (100 U/ml) for 20 h at 37°C. 200 cells per culture were examined, and the data are expressed as the mean percentage of cells stained with FITC-LPS of triplicate cultures.

that the percentage of labeled cells was 90% for PRM and 5.1% for LM, respectively. These results indicate that PRM and PEM bound LPS on their cell surface, while LM and AM did not, and that in vitro treatment of LM with crude LK or recombinant murine IFN-γ induced binding sites for LPS on the cell surface.

Kinetics of the effect of IFN-γ on the appearance of LPS-binding sites on LM was examined. During the first 6 h after incubating with IFN-γ (100 U/ml), binding of FITC-LPS did not increase; but, after 8 h, 30.3% of the cells were stained (Fig. 5). Thereafter, the percentage of the cells stained with FITC-LPS increased gradually and reached a maximum after 48 h.

Discussion

LM are known to differ from PM in various characteristics (1–5). The present data reveal that, under assay conditions in which 10 μg/ml of LPS rendered either PRM or PEM strongly cytotoxic, LM and AM never responded to LPS. Experimental manipulation in effector-to-target cell ratios, increasing doses of LPS to 100 μg/ml, or various periods of incubation with LPS from 4 to 48 h, never evoked cytotoxic activity.

It has been reported (9, 10) that the cytotoxicity of PM induced with LPS is augmented by indomethacin, because indomethacin prevented the synthesis of PGE produced by the LPS-stimulated PM. However, this was not the case with LM; the addition of indomethacin (10⁻⁶ M), which markedly augmented the cytotoxicity of LPS-stimulated PRM, had no influence on the unresponsiveness of LM to LPS (Table I). The amounts of PGE produced in the culture supernatants of LM and PRM stimulated by LPS were measured by the radioimmunoassay technique. The results (unpublished data) showed that LM incubated with LPS produced a negligible amount of PGE, while PRM incubated with LPS produced a very large amount of PGE.
It has been reported that there are some particular mouse strains (C3H/HeJ, A/J, and P/J) whose PEM cannot be activated by LPS (11-13). In contrast, we observed no difference in LPS unresponsiveness of LM from the mouse strains tested (C3H/HeSlc, BALB/cSlc, ICR/Slc, and C3H/HeNCrj). On the other hand, PEM from C3H/HeJ, A/J, and P/J mice developed cytotoxic activity if the cells were incubated with LPS plus LK (11-13). PEM from an LPS-responsive mouse strain, C3H/HeN, showed a similar synergistic effect of LK and LPS on macrophage activation (17-19). As shown in the text, we found a remarkable synergistic effect of LPS and crude LK on the activation of LM for tumor cytotoxicity. In addition, we found that the sequence of treatment of LM with LK and LPS was critical, as was also true for PEM from C3H/HeJ, A/J, and P/J mice. Only LM treated first with crude LK and then with LPS were activated to kill tumor cells. It has been suggested that the genetic basis for the defect of tumoricidal capacity of PEM of C3H/HeJ is either closely linked or identical to the Lps gene (11, 21), but the defects of PEM from A/J and P/J appear to be independent of the Lps gene (12, 22). The PEM of C3H/HeJ are not killed by...
LPS in vitro (8) whereas PEM from A/J and P/J were killed. In this point, LM are phenotypically similar to PEM of C3H/HeJ mice (Table IV). However, the unresponsiveness of C3H/HeJ PEM is not due to the lack of LPS binding (23, 24), in contrast to LM.

Fluorescence microscopic studies showed that neither LM nor AM were stained with FITC-LPS, while >90% of PRM and PEM were stained (Table V). In addition, FACS analysis showed that LM do not express binding sites with LPS. The binding of FITC-LPS onto LM was restored by treatment with either crude LK or IFN-γ (Table VI and VII). Given that no binding of FITC-LPS onto PRM-, PEM-, and IFN-γ-treated LM was seen in the presence of polymyxin B, which is known to form a stable molecular complex with the lipid A region of LPS (25) and to block the effect of LPS (26), and given that binding was inhibited also by the presence of cold LPS, it seems that the binding of FITC-LPS to these cells is not due to nonspecific attachment but rather to binding to the LPS receptor. Confirmation of this requires the quantification of binding sites by, for example, Scatchard analysis using an isotopically labeled LPS.

The molecular weight of the factor in the crude LK that rendered LM responsive to LPS was roughly estimated by the elution profile of Sephacryl S-200 chromatography as ~40,000–70,000. MAF activity was detected in almost the same fractions, in agreement with other findings (18). Recently (14–16), it was indicated that IFN-γ has MAF activity. In our experiment, the activity of crude LK rendering LM responsive to LPS was neutralized by rabbit anti-INF-γ antiserum but not by rabbit anti-INF-(α + β) antiserum. This suggested that the active molecule in the crude LK was IFN-γ. This was confirmed by the fact that IFN-γ could replace crude LK to render LM responsive to LPS (Fig. 3).
Recently (manuscript in preparation), we found that not only IFN-\(\gamma\) but also murine IFN-\(\beta\) and recombinant human IFN-\(\alpha\) A/D are able to induce the responsiveness of LM to LPS. The mechanism by which the expression of LPS-binding sites on LM is triggered by IFN-\(\gamma\) is unclear. The fact that LM lack expression of LPS-binding sites does not exclude the possibility that LM possess other defect(s) in tumoricidal capacity, such as in the pathway of signal transmission after LPS binding. Further studies are required on the events occurring in LM within 8 h after IFN treatment (Fig. 5).

The responsiveness of LM to LPS has been found to vary in different animal species. Our results are consistent with a report that demonstrated a lack of specific binding of LPS to rabbit AM (27). LM from rats (28) or humans (29) can be activated by LPS. We observed (unpublished data) that LM from SD rats were activated by LPS and that \(\sim 70\%\) of them were stained with FITC-LPS. In mice, our results are incompatible with those of Fogler et al. (24), which demonstrated that AM from C3H/HeN mice can be stained by FITC-LPS equally as well as can PEM, and that AM can be activated for tumor cytotoxicity by LPS stimulation alone. The reason for this discrepancy is unclear.

In the present report, we have described a marked difference between LM and PM in the expression of LPS-binding sites on the cell surface. The biologic role played by the lack of LPS binding to LM in host defense mechanisms and the its mode of acquisition are under study.

Summary

Although peritoneal resident macrophages (PRM) or peritoneal exudate macrophages (PEM) were activated by lipopolysaccharide (LPS) to kill tumor cells in vitro, lung macrophages (LM) obtained by mincing lung tissues or by harvesting bronchial lavage were not activated by LPS under any experimental conditions, i.e., different LPS concentrations, incubation times and cytotoxicity assay methods. The unresponsiveness of LM to LPS was seen in all of the mouse strains tested. Treatment of LM with indomethacin did not affect the unresponsiveness, although it greatly augmented the cytotoxicity of PRM stimulated with LPS. LM treated in vitro with crude lymphokines (LK) did not show cytotoxicity, but became sensitive to LPS and cytotoxic for tumor cells. LM treated first with crude LK and then with LPS were cytotoxic, but LM treated first with LPS and then with crude LK were not. The ability of crude LK to render LM responsive to LPS was neutralized by rabbit anti-mouse \(\gamma\) interferon (IFN-\(\gamma\)) antiserum but not by anti-mouse IFN-(\(\alpha + \beta\)) antiserum. LM treated with recombinant murine IFN-\(\gamma\) became responsive to LPS and showed cytotoxicity. LM were resistant to direct toxicity of LPS under conditions in which significant populations of PRM and PEM died. However, LM became sensitive to direct toxicity of LPS by treatment with crude LK or recombinant murine IFN-\(\gamma\). Fluorescence microscopy showed that almost all PRM and PEM were stained with fluorescein isothiocyanate (FITC)-LPS, while \(< 5\%\) of the LM were stained. Instead, \(\sim 60\%\) of the LM treated with the crude LK or recombinant IFN-\(\gamma\) for 20 h were stained with FITC-LPS. Fluorescence-activated cell sorter (FACS) analysis confirmed this result. The staining of IFN-\(\gamma\) treated LM with FITC-LPS was inhibited by polymyxin B or unlabeled LPS. These results suggest that the
defective responsiveness of LM to LPS is due to the lack or very low expression of LPS-binding sites on the cell surface and that in vitro treatment with IFN-γ brings about the expression of them and renders LM responsive to LPS.

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