Modulation of Macrophage Ia Expression by Lipopolysaccharide: Stem Cell Requirements, Accessory Lymphocyte Involvement, and IA-Inducing Factor Production

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The mechanism of induction of murine macrophage Ia expression by lipopolysaccharide (LPS) was studied. Intraperitoneal injection of 1 µg of LPS resulted in a 3- to 10-fold increase in the number of IA-positive peritoneal macrophages (flow cytometry and immunofluorescence) and a 6- to 16-fold increase by radioimmunoassay. The isolated lipid A moiety of LPS was a potent inducer of macrophage Ia expression. Ia induction required a functional myelopoietic system as indicated by the finding that the response to LPS was eliminated in irradiated (900 rads) mice and reinstated by reconstitution with bone marrow cells. Comparison of LPS-induced Ia expression in normal and LPS-primed mice revealed a faster secondary response to LPS. The memory response could be adoptively transferred to normal mice with nonadherent spleen cells prepared 60 days after LPS injection. Spleen cells prepared 5 days after LPS injection caused Ia induction in LPS-nonresponder mice; such induction was not observed in irradiated (900 rads) recipients. The cell responsible for this phenomenon was identified as a Thy-1⁺, immunoglobulin-negative nonadherent cell. The biosynthesis and expression of Ia were not increased by direct exposure of macrophages to LPS *in vitro*. Small amounts of LPS inhibited Ia induction by gamma interferon. LPS showed positive regulatory effects on Ia expression by delaying the loss of Ia expression on cultured macrophages and by stimulating the production of Ia-inducing factors. Supernatants from cultured spleen cells stimulated with LPS *in vitro* contained antiviral and Ia-inducing activity that was acid labile, indicating that the active factor is gamma interferon. We conclude that induction of Ia expression by LPS *in vivo* is a bone-marrow-dependent, radiation-sensitive process which involves the stimulation of a gamma interferon-producing accessory lymphocyte and a delay in Ia turnover.

Lipopolysaccharide (LPS) is an integral component of the outer membrane of gram-negative bacteria and has extremely potent biological activity (17, 18). Our laboratory and others have shown that intraperitoneal (i.p.) injection of nanogram amounts of LPS results in a profound increase in peritoneal macrophage Ia expression (1, 32). In keeping with the augmented Ia expression, LPS-elicited macrophages were more effective than normal macrophages in presenting antigen to T lymphocytes (32).

When compared with other studies of Ia induction by gram-positive bacteria or adjuvants, the induction by LPS has several unique features. First, LPS is classically known as a T-cell-independent antigen (18). The LPS-mediated elaboration of lymphokines, usually associated with T-cell activation, has not been easily detected or well studied. Also, Ia induction by LPS can occur in athymic nude mice (1, 31). Second, LPS is a well-defined structure with the lipid portion having potent biological activities. As such, Ia induction in response to lipid A may involve a novel mechanism. Third, the cellular response to LPS is under *lps* gene control (32), which presents other questions and experimental avenues not relevant to other Ia-inducing agents. Fourth, the modulation of Ia by LPS occurs by distinctive kinetics: Ia expression is reduced early (3 days) after injection, but is augmented 7 to 14 days after injection (32). Thus, both negative and positive regulatory effects require integration into a complete mechanistic picture. Finally, recent evidence indicates that LPS is the major, if not only, Ia-

inducing agent active in the infectious disease process with viable pathogenic gram-negative bacteria (16). While the relationship between Ia induction and protective immune responses remains to be well defined, it is likely that regulation of Ia plays a key role in the lymphocyte responses required for the resolution of infection and establishment of protective immunity. For these reasons, a thorough understanding of the events involved in LPS-mediated Ia regulation is warranted.

In the present study, we have used a combination of approaches, both *in vivo* and *in vitro*, to address stem cell requirements, LPS-reactive accessory cell involvement, the production of Ia-inducing factor, and the direct effects of LPS on macrophage Ia expression. A model to account for the demonstrated LPS-mediated events is discussed.

MATERIALS AND METHODS

Much of the methodology used in this study was identical to that described previously. For details of methods concerning induction of peritoneal exudates, isolation and characterization of macrophages, quantitation of Ia by radioimmunoassay (RIA) and immunofluorescence (IF), and specificity of anti-Ia antibodies, see references 31 and 32.

Mice. C3HeB/FeJ and C3H/HeJ female mice, ages 6 to 10 weeks, were purchased from Jackson Laboratory, Bar Harbor, Maine. BALB/c female nude mice with defined flora (6 to 8 weeks old) were acquired from Life Sciences, Inc., St. Petersburg, Fla. Nude and irradiated mice were maintained under rigorous sterile conditions including autoclaved food, water, cages, and bedding. The mice remained within a laminar flow containment hood throughout the experiment.

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Beginning several days prior to experimental manipulation, the mice were continuously treated with 1 g of tetracycline hydrochloride per liter dissolved in their drinking water.

LPS. The LPS utilized in these experiments was prepared from *Salmonella minnesota* R595 as described by Galanos et al. (9). LPS was generously supplied by David Morrison, Kansas University Medical Center, Kansas City, and was purchased from Ribi ImmunoChem, Hamilton, Mont. The LPS concentration was determined by weighing and, following reconstitution with phosphate-buffered saline (PBS), sterilized by boiling for 15 min. Purified lipid A (0.3% 2-keto-3-deoxyoctulosonic acid and 0.4% protein) derived from *S. minnesota* (Ribi ImmunoChem) was also used. The lipid A was reconstituted (5 mg/ml) with 95% ethanol and stored at 4°C.

Isolation of peritoneal macrophages. Peritoneal exudate cells (PECs) were harvested by peritoneal lavage, centrifuged, and suspended in culture medium (RPMI 1640 containing 5% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] buffer, 0.07% sodium bicarbonate, 0.5 mM sodium pyruvate, and 50 U/50 µg/ml of penicillin/streptomycin). Viable cells were plated in 96-well microdilution plates (usually 10^5 PECs per well) (Flow Laboratories, McLean, Va.) and cultured for 2 h at 37°C; nonadherent cells were removed by vigorous washing, and adherent cells were fixed (1% paraformaldehyde, 20 min, 24°C) and stored at 4°C until the RIA was done.

Quantitation of Ia. A.TH-anti-A.TL antiserum (Cedarlane Laboratories, Hornby, Ontario, Canada) and the 10-2.16, 11-5.2, MK-D6, and 14-4-4S monoclonal antibodies were used to detect Ia^k, IA^k (12), IA^k (Becton Dickinson and Co., Paramus, N.J.), IA^d (20), and IE^k/IE^d (22) antigens, respectively. Immunoglobulin G subclass 2b (IgG2b) myeloma protein and normal mouse serum were used as controls for nonspecific binding.

All incubations in the RIA were for 60 min at 20°C. Intervening washes and reagent dilutions were performed with PBS containing 0.1 mg of bovine serum albumin per ml. First, fixed macrophages were incubated with 50 µl of 1% bovine serum albumin per well to reduce nonspecific binding. Second, 25 µl of the various antibody preparations per well and corresponding controls were added. The third incubation was with 25 µl of a 1:300 dilution of rabbit anti-mouse IgG1, IgG2, IgG3, and IgM (Litton Bionetics, Kensington, Md.) per ml, and the final incubation was with 25 µl (10^5 cpm per well) of ¹²⁵I-labeled *Staphylococcus aureus* protein A (specific activity, 0.5 to 1.0 µCi/µg) per well. The plate was then washed three times and cut with a hot wire, and the radioactivity was determined. Experimental values are represented as the average of triplicates minus control values (Δ counts per minute). The number of adherent macrophages per well was confirmed by random field counting and determination of total protein (15, 32).

IF was performed as stated previously (32) by plating PECs (with latex beads) on glass cover slips in 16-mm tissue culture wells (2). The cover slips were incubated with anti-IA monoclonal antibody, washed, and incubated with fluorescein-conjugated F(ab')₂ rabbit anti-mouse IgG. Approximately 200 latex bead-ingesting macrophages were scored.

Analysis of IA by flow cytometry was performed with a FACS analyzer (Becton Dickinson, Mountain View, Calif.) equipped with appropriate computer hardware and software systems (Consort 30; Becton Dickinson). PECs (10^6) were stained with fluorescein-conjugated F(ab')₂ fragments of

goat anti-mouse IgG (Organon Teknika, Malvern, Pa.) for 30 to 45 min (4°C) after incubation (30 to 45 min, 4°C) with 1 µg of either monoclonal anti-IA (clone 11-5.2) or IgG2b myeloma protein control (MOPC 195; Litton Bionetics, Irving, Tex.). Following staining, cells were washed and fixed as described previously (32) and then analyzed (10^4 cells) for fluorescence (with logarithmic amplification) and volume. Macrophages were identified by size (larger cells). The identity of the larger cells as macrophages was confirmed by morphological studies (32) and by the finding that the removal of adherent cells resulted in an almost complete disappearance of the larger population.

Adoptive transfer of secondary response to LPS. C3HeB/FeJ mice were injected i.p. with 10^4 live *Listeria monocytogenes*, 1 µg of R595 LPS, or PBS in 0.5-ml volumes 60 to 70 days prior to use. Spleen cells were removed, washed, and cultured (10^7 /ml) for 4 h at 37°C to remove adherent cells. Nonadherent cells were then recovered, washed twice with PBS, and adjusted to a concentration of 2×10^7 viable cells per ml prior to i.p. injection of 0.5 ml into C3HeB/FeJ mice. Separate injections (0.5 ml per mouse) of either 1 µg of R595 LPS or PBS were given within 10 min. Five days after cell transfer, PECs were harvested and Ia was measured.

Adoptive transfer of LPS-activated spleen cells. The spleen cells of C3HeB/FeJ mice were harvested 5 days following i.p. injection of 1 µg of LPS. Nonadherent cells (adherent cells were removed as stated above) were washed three times with PBS and diluted to 2×10^7 cells per ml, and 1.0 ml was injected i.p. into C3HeB/HeJ LPS-nonresponder mice. After 5 days, PECs were harvested and macrophages were analyzed for Ia expression.

The LPS-activated cell(s) responsible for Ia induction following adoptive transfer was characterized by negative selection with antibody and complement. RPMI 1640 medium containing 0.3% bovine serum albumin was used for washing cells and diluting reagents; sodium azide (0.02%) was present during treatment to prevent capping of surface antigens (7). Spleen cells (5×10^7 /ml) were treated with either monoclonal anti-Thy-1.2 antibody (HO-13 hybridoma culture supernatant) or rabbit anti-mouse IgG1, IgG2, IgG3, and IgM (1:10 dilution). After 60 min (4°C), the cells were pelleted and suspended in an equivalent volume of Low-Tox-M Rabbit complement (1:15 dilution) for 60 min at 37°C. Such treatments resulted in approximately 40 to 50% total cell lysis (trypan blue), a 50% decrease in the response to LPS (anti-immunoglobulin plus complement), and a 95% reduction in the response to concanavalin A (ConA; anti-Thy plus complement) as tested by mitogenesis in vitro. The cells were then washed three times with PBS, adjusted to 2×10^7 viable cells per ml, and injected i.p. into recipient mice.

Irradiation and cell reconstitution experiments. Mice (maintained under sterile conditions as described above) were irradiated (900 rads) and then injected (0.2 ml per mouse intravenously) with syngeneic bone marrow (6×10^6 per mouse) or spleen cells (10^6 per mouse). Each group was then given an i.p. injection of either 1 µg of R595 LPS or PBS, and Ia levels were determined after 7 days.

Generation and quantitation of Ia-inducing factors. Washed single cell suspensions of activated or normal spleen cells (harvested 7 days following injection) were cultured (10^7 /ml) for 48 h at 37°C in medium containing (i) 1 µg of R595 LPS per ml, (ii) 2 µg of ConA per ml, or (iii) no additional agents. The spleen cells were sedimented and cell-free supernatants were collected to test for Ia-inducing activity. In some experiments, spleen cells were precultured for 0 or 48 h

before either addition of 10 μg of R595 LPS or 2 μg of ConA per ml or no addition. To assess Ia-inducing activity of the supernatants, peptone-elicited, predominantly Ia⁺ macrophages (C3HeB/FeJ and C3HeB/HeJ) were used. PECs were plated at 1.0×10^5 or 1.5×10^5 cells per well, washed with warm culture medium to remove nonadherent cells, and then cultured for 6 days with several dilutions of the supernatants. Unless stated otherwise, all cultures were performed in the presence of indomethacin (5 $\mu\text{g}/\text{ml}$) to eliminate inhibition of Ia expression by prostaglandins (24). Following culture, macrophages were fixed and Ia expression was measured.

Reagents for in vitro experiments. ConA supernatant was generated as described previously (26). These preparations, containing 85 to 1,400 U of gamma interferon (IFN- γ) per ml, were termed lymphokine. Indomethacin (Sigma Chemical Co., St. Louis, Mo.) was diluted to a concentration of 10 mg/ml in 95% ethanol, stored at -20°C , and used at a final concentration of 5 $\mu\text{g}/\text{ml}$ in culture media. Recombinant IFN- γ (rat) was purchased from Amgen Biologicals, Thousand Oaks, Calif. The interferon was reconstituted with sterile distilled water (10^6 U/ml), aliquoted, and stored at -70°C .

Assay for antiviral activity. Antiviral activity was measured by LEE BioMolecular Research Laboratories, Inc., San Diego, Calif., using L929 murine fibroblasts challenged with encephalomyocarditis virus by the technique of Havell and Vilcek (11). Results were normalized to a National Institutes of Health murine interferon reference reagent (G-002-904-511) and expressed as international reference units per milliliter. Acid lability of antiviral and Ia-inducing activity was tested by acidifying the supernatants (pH 2, overnight, 4°C) and then neutralizing to pH 7 prior to testing.

RESULTS

FACS analysis of Ia induction by LPS. The expression of IA by normal and LPS-elicited PECs was studied by using flow cytometry (Fig. 1). PECs contained a population of larger (upper quadrants) and smaller (lower quadrants) cells corresponding to macrophages and lymphocytes, respectively. (Cells were identified as described in Materials and Methods and reference 32). In contrast to normal PECs, the PECs elicited by LPS showed a predominance of larger cells with increased fluorescent intensity; 43% of the PECs were in the upper right-hand quadrant. These results suggest that LPS causes a nearly complete shift in the IA expression of the peritoneal macrophage population. There was also a slight increase in staining of the smaller cell populations after LPS injection; the identity of such cells is not known.

Induction of IA by lipid A. LPS-mediated induction of Ia expression was due to the lipid A moiety. Figure 2 shows representative dose-response curves for the induction of IA and IE expression following injection of purified lipid A. The response to an optimum dose (1 μg) of R595 LPS is also shown for comparative purposes. As little as 1 ng of lipid A caused significant Ia induction. In this and in other experiments not shown (32), the optimal induction by lipid A was always greater than that with LPS, and comparison of dose-response curves showed that the purified lipid A (1,797 molecular weight) was at least 10-fold more active (weight basis) than LPS (2,860 molecular weight). Thus, the Ia-inductive effect of R595 LPS is most likely due to the lipid A component.

Secondary responses to LPS. We compared Ia induction in response to both primary and secondary challenge with LPS.

An enhanced secondary response was observed 5 to 7 days following challenge injection (Fig. 3). In four experiments of this type in which Ia expression was monitored over a 12-day period, the peak primary response was observed on days 7 to 9, with secondary response peaks occurring on days 5 to 7 (data not shown).

In a separate experiment, C3HeB/FeJ mice were injected with LPS, live *L. monocytogenes*, or PBS as primary or secondary injections or both, and macrophages were harvested after 5 days (Table 1). As measured by both RIA and IF, a secondary response to LPS was observed in LPS-primed mice, but not in *L. monocytogenes*-primed mice given LPS as a challenge injection. In the combined results of four experiments examining Ia expression at 5 days after injection, a primary response to LPS resulted in $60.7 \pm 48.6\%$ increase in Ia expression, while a secondary response showed a $235 \pm 67.5\%$ increase (mean $\pm$ standard deviation; $n = 4$). The secondary response was significantly greater than the primary response ($P = 0.02$; Student's *t* test). These results indicated that the increase in Ia caused by LPS has the characteristics of a typical memory response: rapidity and specificity.

The memory response to LPS was also examined through adoptive transfer studies. In the experiment shown in Table 2, nonadherent spleen cells from mice injected with LPS or PBS (2 months prior to isolation) were transferred into syngeneic recipient mice in conjunction with various challenge injections. As can be seen, LPS-primed spleen cells showed a greater induction than spleen cells from PBS-injected donors. Furthermore, we found that the adoptively transferred secondary response to LPS was reduced significantly (30 to 40% decrease) by treatment of the donor spleen cells with anti-Thy plus complement, as indicated by the following results: normal spleen cells (PBS-injected donor) transferred with PBS = $1,563 \pm 237$; normal spleen cells transferred with LPS = $3,127 \pm 432$; LPS-primed spleen cells transferred with LPS = $5,532 \pm 487$; and LPS-primed cells treated with anti-Thy plus complement prior to transfer with LPS = $3,301 \pm 126$.

Induction of Ia by transfer of LPS-activated spleen cells. We also tested the activity of adoptively transferred cells in another protocol. Unlike the preceding experiments in which responsiveness was noted with cells prepared 2 months after LPS injection, in these studies cells were prepared 5 days after LPS injection and transferred into C3HeB/HeJ LPS-nonresponder mice without further challenge with LPS. A representative experiment is shown in Fig. 4. In comparison to transfer of normal spleen cells, transfer of LPS-activated cells caused increased Ia expression. Analysis of the results from seven separate experiments showed that the difference in induction with LPS-activated spleen cells (150% increase over PBS injection) compared with normal spleen cells (75% increase) was significant ($P = 0.0026$). This inductive effect was not observed in irradiated mice (Fig. 4), indicating an active role by the host (possibly by providing an intact myelopoietic system). This active role and the fact that adherent cells were removed prior to transfer confirm that increased Ia was not due simply to the transfer of adherent Ia-positive cells present in the donor spleen cell population. The induction was also not due to LPS carry-over because LPS-nonresponder mice were used as recipients.

The cell responsible for induction was characterized by depletion of cells bearing the Thy-1.2 and surface immunoglobulin markers prior to adoptive transfer (Table 3). As mentioned above, the difference between induction following adoptive transfer of LPS-activated spleen cells versus

FIG. 1. Quantitation of IA expression by FACS analysis. Normal (left panels) and LPS-elicited (7 days after LPS i.p.) (right panels) PECs (10^4) from C3HeB/FeJ mice were treated with anti-IA (bottom row) or an IgG2b antibody control (top row). Each sample was then treated with fluorescein-conjugated goat anti-mouse IgG and evaluated for fluorescence (F1, fluorescence channel 1) and relative volume by a FACS analyzer as described in the Materials and Methods. Large cells with high fluorescence (i.e., IA⁺ macrophages) are located in the upper right-hand quadrant of each panel. The percentage of total PECs in each quadrant is indicated in upper right-hand corners.

normal spleen cells was significant (experiment 1, $P = 0.0010$; experiment 2, $P = 0.0094$; experiment 3, $P = 0.0019$; experiment 4, $P = 0.0061$). Depletion of immunoglobulin-bearing cells (anti-immunoglobulin plus complement) did not decrease the adoptive transfer effect. In contrast, treatment with anti-Thy plus complement reduced the Ia-inductive effect (32 to 58% decrease) to background levels observed with normal spleen cells. The difference between induction

with untreated LPS-activated spleen cells and that with cells treated with anti-Thy plus complement was significant in the two representative experiments shown (experiment 1, $P = 0.0019$; experiment 2, $P = 0.0015$). Thus, a nonadherent Thy-1⁺ surface immunoglobulin-negative cell is responsible for induction.

Irradiation and reconstitution. To determine the stem cell requirements for the LPS response, C3HeB/FeJ mice were

FIG. 2. Induction of Ia expression with lipid A. C3HeB/FeJ mice were given an i.p. injection of various concentrations of purified lipid A as indicated. After 7 days, peritoneal macrophages were harvested and plated at 1.5×10^5 per well. The levels of macrophage IA and IE expression were determined by RIA ($n = 2$). Macrophage IA and IE expression following injection of an optimal 1- μ g dose of R595 LPS is also shown for comparison.

irradiated, reconstituted with syngeneic bone marrow or spleen cells, and tested for responsiveness to LPS. Ia induction was observed in normal, but not irradiated, mice (Fig. 5). Reconstitution with bone marrow, but not spleen cells, restored the response. These data indicate that LPS-induced Ia expression is both radiation sensitive and bone marrow dependent.

Effects of LPS in vitro. In Fig. 6, the effects of lymphokine and LPS on macrophage IA expression are illustrated over an 8-day culture period. In the presence of lymphokine, added at the beginning of culture or at day 2, IA expression steadily increased with time. In the presence of LPS, expression did not increase above the initial level at day 0. Consistent with the lack of induction of Ia expression by LPS in vitro, LPS did not induce macrophage biosynthesis in vitro (32). In contrast, LPS did have a direct effect on Ia

FIG. 3. Kinetics of the secondary response to LPS. C3HeB/FeJ mice were given a primary injection of either PBS or 1 μ g of R595 LPS. After 60 days, a challenge injection of either PBS or 1 μ g of LPS was administered, and peritoneal macrophages were harvested on the indicated days. PECs were plated at 1.5×10^5 per well, and IA expression was determined by RIA. Primary response is indicated by a PBS/LPS protocol, and secondary response is shown by an LPS/LPS protocol.

TABLE 1. Secondary response to LPS: macrophage IA expression as measured by RIA and indirect IF

Injection ^a		IA expression	
Primary	Secondary	IA (Δ cpm $\pm$ SEM) ^b	% IA ⁺ cells ^c
PBS	PBS	1,331 $\pm$ 203	7.0
PBS	LPS	2,547 $\pm$ 205	17.2
LPS	LPS	5,969 $\pm$ 384	26.4
LPS	PBS	1,685 $\pm$ 236	4.4
LM	LPS	3,017 $\pm$ 107	15.3

^a R595 LPS (1 μ g), live *L. monocytogenes* (LM; 10^4), or PBS (0.5 ml) was injected i.p. into C3HeB/FeJ mice. The interval between primary and secondary injections was >60 days.

^b Values represent the IA content (RIA) of 1.5×10^5 PECs per well harvested 5 days after the secondary injection.

^c Values represent the percentage of cells judged to be IA positive (IA⁺) in macrophage populations harvested 5 days after secondary injection (measured by indirect IF).

turnover. The decrease in IA expression observed in medium was slowed in the presence of LPS; this was consistently observed when LPS was added at either day 0 or 2. This "delaying effect" of LPS was noted in macrophages elicited with LPS, ConA, peptone, or *L. monocytogenes* and was apparent with LPS concentrations ranging from 0.1 to 100 μ g/ml (data not shown).

Inhibitory effect of LPS on IFN- γ -mediated IA induction. While 0.01 U of IFN- γ per ml caused IA induction in the absence of LPS, 100-fold more IFN- γ was required to give similar induction in the presence of LPS (Fig. 7). In the presence of as little as 200 ng of LPS per ml, at least 1 to 10 U of IFN- γ was required to detect Ia induction. With larger amounts of LPS, even more IFN- γ appears to be required (Table 4; data not shown). Thus, LPS can mask the presence of IFN- γ when Ia-inducing activity is tested. In contrast, the presence of LPS (200 ng/ml) did not interfere with the detection of either high (310 U/ml) or low (35 U/ml) concentrations of IFN- γ as measured by antiviral activity (data not shown).

In a recent study, LPS was shown to induce IFN- γ production upon culture with normal spleen cells in vitro, but only if the spleen cells were precultured prior to stimulation with LPS (5). We have confirmed and extended this

TABLE 2. Adoptive transfer of secondary response to LPS: macrophage IA expression as measured by RIA and indirect IF

Injection of donor animals ^a		Challenge injection ^b		IA expression	
				IA (Δ cpm $\pm$ SEM) ^c	% IA ⁺ cells ^d
PBS	PBS			2,300 $\pm$ 83	3.9
PBS	LPS			5,442 $\pm$ 238	15.5
LPS	PBS			3,827 $\pm$ 245	7.5
LPS	LPS			7,891 $\pm$ 345	25.5

^a C3HeB/FeJ mice used as spleen cell donors were injected with 1 μ g of R595 LPS or 0.5 ml of PBS at least 60 days before adoptive transfer (see Materials and Methods).

^b Syngeneic recipient mice received challenge injections of either 1 μ g of R595 LPS or 0.5 μ l of PBS immediately after injection with 10^7 nonadherent spleen cells.

^c Values represent the IA content (RIA) of 1.5×10^5 PECs per well harvested 5 days after the adoptive transfer and challenge injection.

^d Values represent the percentage of cells scored as IA⁺ (indirect IF) in macrophage populations harvested 5 days after adoptive transfer and challenge injection.

FIG. 4. Adoptive transfer of LPS-activated spleen cells into normal and irradiated hosts. Either 1 ml of PBS, 2×10^7 C3HeB/FeJ normal nonadherent spleen cells, or 2×10^7 LPS-activated nonadherent spleen cells from C3HeB/FeJ mice (spleens harvested 5 days following i.p. injection of 1 μ g of R595 LPS) were injected i.p. into normal or irradiated (900 rads) C3HeB/HeJ LPS-nonresponder mice. PECs were harvested 5 days following adoptive transfer and analyzed for macrophage Ia expression by RIA.

observation (Table 4). Cell-free supernatants were tested for antiviral activity, Ia-inducing activity, and pH 2 sensitivity. As expected, spleen cells stimulated in vitro with ConA produced IFN- γ which demonstrated antiviral and Ia-inducing activity that was acid labile. Normal spleen cells, cultured for 48 h prior to the addition of LPS, also produced a supernatant with substantial antiviral and Ia-inducing activity. In contrast, the factor generated by LPS-activated spleen cells cultured in medium alone displayed little antiviral activity, but showed a significant Ia-inducing activity. The Ia-inducing activity of these factors was sensitive to pH 2 treatment; in a typical experiment, the 3.5-fold increase (relative to medium) in Ia expression caused by the factor was completely eliminated by pH 2 treatment.

DISCUSSION

The present study represents an ongoing investigation of the mechanism by which LPS increases the Ia expression of peritoneal macrophages (31, 32). Both the potent activity of

FIG. 5. Ia-inductive response to LPS: a radiation-sensitive bone marrow-requiring process. Four experimental groups of C3HeB/FeJ mice were tested for a Ia-inductive response to R595 LPS: (i) normal mice; (ii) irradiated mice; (iii) irradiated mice reconstituted with syngeneic bone marrow cells; and (iv) irradiated mice reconstituted with syngeneic spleen cells. Each group was then given an i.p. injection of either R595 LPS (1 μ g) or PBS. Seven days later, peritoneal macrophages were evaluated for Ia expression by RIA.

isolated lipid A (Fig. 2) and the lack of responsiveness in LPS-nonresponder mice (32) indicate that the lipid A moiety is responsible for Ia induction. However, lipid A was 10- to 100-fold more active on a weight basis than R595 LPS, and this increased activity is greater than predicted by the lipid A content of the R595 LPS. It is possible, therefore, that the presence of some nonlipid component may have an inhibitory effect as reported previously (29).

Several observations indicate that increased Ia is not due solely to a direct LPS interaction with peritoneal macrophages. These include (i) the radiation sensitivity and bone marrow reconstitution of the inductive event (Fig. 5); (ii) the secondary memory response to LPS and adoptive transfer of secondary responsiveness with nonadherent spleen cells (Fig. 3; Tables 1 and 2); (iii) the ability to induce Ia in nonresponder mice with i.p. injections of LPS-activated nonadherent cells (Fig. 4 and Table 3); and (iv) the lack of Ia induction in vitro upon culture of isolated peritoneal macrophages with LPS (Fig. 6). It appears, therefore, that a more complex mechanism is operative.

The role of an intact myelopoietic system is suggested by

TABLE 3. Adoptive transfer of LPS-activated spleen cells treated with antibody and complement

Injection ^a	$\Delta\text{cpm} \pm \text{SEM}^b$			
	Expt 1	Expt 2	Expt 3	Expt 4
PBS	257 $\pm$ 151	2,144 $\pm$ 438	5,018 $\pm$ 285	3,518 $\pm$ 1,336
LPS	510 $\pm$ 91	ND	2,355 $\pm$ 122	2,809 $\pm$ 659
Normal spleen cells	1,521 $\pm$ 173	2,087 $\pm$ 300	5,196 $\pm$ 285	3,266 $\pm$ 307
LPS spleen cells	2,640 $\pm$ 147	3,017 $\pm$ 167	8,058 $\pm$ 244	5,090 $\pm$ 315
LPS spleen cells + complement	ND	ND	8,559 $\pm$ 744	ND
LPS spleen cells + anti-Thy + complement	1,117 $\pm$ 136	2,046 $\pm$ 174	ND	ND
LPS spleen cells + anti-immunoglobulin + complement	ND	ND	7,847 $\pm$ 166	4,527 $\pm$ 297

^a C3HeB/HeJ LPS-nonresponder mice were injected i.p. with PBS (1.0 ml), R595 LPS (1 μ g), or either normal or LPS-activated spleen cells (2×10^7) from C3HeB/FeJ mice (cells harvested 5 days after injection of 1 μ g of R595 LPS). Following removal of nonadherent cells, some cells were treated with HO13 monoclonal antibody plus complement (anti-Thy + complement), rabbit anti-mouse IgG/IgM antiserum plus complement (anti-immunoglobulin + complement), A.TH-anti-A.TL antiserum plus complement (anti-Ia + complement), or complement alone prior to adoptive transfer (refer to Materials and Methods). Depletion techniques resulted in approximately 40 to 50% cell lysis.

^b Values were determined by RIA and represent the Ia expression of 10^5 PECs per well harvested 5 days following adoptive transfer. The RIA in each experiment was performed separately. ND, Not determined.

FIG. 6. Effect of lymphokine and LPS on macrophage IA in vitro. C3HeB/FeJ mice were injected with 1.5 ml of peptone, and PECs were harvested after 3 days and plated at 10^5 per well as described in Materials and Methods (see section on quantitation of Ia-inducing factors). Macrophages were cultured for the indicated number of days in the presence of medium, lymphokine (ConA supernatant, 1:10 dilution of stock), or LPS (1 μ g/ml). Culturing for 8 days in the presence of these agents is indicated by bold lines. Macrophages were also cultured in medium for 2 days before the addition of LPS or lymphokine (add-day 2, dotted lines). IA expression was determined by RIA. Standard deviations were $\leq 5\%$ of the mean of triplicate samples. Symbols: $\bigcirc$ — $\bigcirc$, medium; $\bullet$ — $\bullet$, lymphokine; $\times$ — $\times$, LPS; $\bigcirc$ — $\bigcirc$, lymphokine (add-day 2); $\times$ — $\times$, LPS (add-day 2).

the finding that the response to LPS could be eliminated by irradiation and then reconstituted with bone marrow cells (Fig. 5). Thus, as observed with the Ia-inductive response to bacteria and Ia-inducing factor (23), the LPS-induced Ia expression in vivo was found to be a radiation-sensitive, bone-marrow-requiring process. The finding that the response to LPS was not reinstated by reconstitution with spleen cells underlines the importance of a bone marrow stem cell source. The lack of induction in irradiated animals may be due to the effects of irradiation on the development of a cell (i) directly responsive to LPS; (ii) capable of

FIG. 7. Inhibition of IFN- γ -mediated IA induction by LPS. Peptone-elicited macrophages (C3HeB/FeJ mice injected with 1.5 ml of peptone 4 days prior to harvest) were cultured (10^5 cells per well) with 0 to 100 U of IFN- γ per ml as indicated, in the presence or absence of 0.2 μ g of R595 LPS per ml. Macrophages were fixed after 6 days, and a RIA was performed to quantitate IA expression.

TABLE 4. Antiviral and Ia-inducing activities of spleen cell culture supernatants

Spleen cells ^a	Preincubation time (h) ^b	Stimulation in vitro ^c	Reference units of antiviral activity per ml ^d		Ia-inducing activity (Δ cpm $\pm$ SEM) ^e
			Un-treated	pH 2 treated	
Normal	0	Medium	<5	≤ 15	0 ± 116
		LPS	59	≤ 15	84 ± 181
		ConA	190	≤ 15	$1,382 \pm 154$
	48	Medium	<5	≤ 15	0 ± 428
		LPS	120	≤ 15	$2,835 \pm 484$
		ConA	120	≤ 15	$2,413 \pm 740$
LPS activated	0	Medium	<5	≤ 15	11 ± 193
		LPS	53	≤ 15	47 ± 195
		ConA	340	≤ 15	$1,728 \pm 262$
	48	Medium	<5	≤ 15	$2,343 \pm 653$
		LPS	70	≤ 15	741 ± 443
		ConA	100	≤ 15	$1,284 \pm 502$

^a C3HeB/FeJ mice were either not injected (normal spleen cells) or injected with 1 μ g of R595 LPS (LPS-activated spleen cells). After 7 days, spleen cells were harvested and plated at 10^7 cells per ml.

^b Spleen cells were cultured for 0 or 48 h in medium alone prior to the addition of in vitro stimulants.

^c Spleenocytes were cultured for an additional 24 h in the presence of medium, 10 μ g of R595 LPS per ml, or 2 μ g of ConA per ml. Cell-free culture supernatants were then collected and tested (1:10 dilution) for Ia-inducing capacity as described in Materials and Methods.

^d Untreated and pH 2-treated (overnight, 4°C) factors were tested for antiviral activity on L929 murine fibroblasts challenged with encephalomyocarditis virus, using National Institutes of Health murine IFN as a standard.

^e Ia-inducing activity was determined by culturing peptone-elicited macrophages with the factors for 6 days (see Materials and Methods); then IA expression was determined by RIA. IA expression by macrophages cultured in medium alone was 136 ± 40 ; in the presence of 10 μ g of R595 LPS per ml, it was 141 ± 87 .

generating Ia-inducing factors; and/or (iii) responsive to Ia-inducing factors. Because irradiated macrophages could respond in vitro to IFN- γ with increased Ia (data not shown), the effects of irradiation cannot be explained by a direct effect on the response of mature macrophages in vitro. Furthermore, since Ia induction in response to transfer of LPS-activated spleen cells was also radiation sensitive (Fig. 4) but the Ia-inducing capacity of the transferred spleen cells was not affected by irradiation (data not shown), it is unlikely that the irradiation-induced inhibition is due only to the lack of development of cells which generate Ia-inducing factors. Thus, the most likely target for the effects of irradiation is the myelopoietic system involved in the genesis of cells responsive to Ia-inducing factors or to the direct effects of LPS or to both.

An accessory lymphocytelike cell contributes to Ia induction in response to LPS. The first indication for such a cell was the secondary response to LPS that exhibited rapidity, memory, and specificity (Fig. 3 and Table 1). By analogy to other well-established anamnestic responses, this finding suggests that the secondary response involves a clonally expanded, LPS-reactive lymphocyte. Furthermore, the adoptive transfer with nonadherent (Thy-1⁺) spleen cells indicates that the LPS-reactive memory cell is a T lymphocyte.

The involvement of an accessory lymphocyte is also indicated by enhanced Ia expression following the transfer of LPS-activated spleen cells alone (prepared 5 days after LPS injection rather than 2 months as discussed above) (Fig. 4 and Table 3). The cell responsible for induction has been characterized as a Thy⁺, surface immunoglobulin-negative,

nonadherent cell (Table 3). The identification of the LPS-reactive cell as a typical T lymphocyte would be compatible with reports that LPS can affect T-lymphocyte activation (4, 14, 28). However, other observations appear to be inconsistent with this conclusion. We (31) and others (1) have observed Ia induction in athymic mice, indicating that the response can occur through a thymus-independent pathway. Therefore, the accessory cell(s) involved in the induction of Ia by LPS cannot be identified as a mature thymus-derived T lymphocyte in all circumstances. One candidate for the LPS-reactive cell in athymic nude mice is the natural killer cell. Natural killer cells have been shown to be heterogeneous in nature and capable not only of IFN- γ production (19), but, in some circumstances, of Thy-1 expression (21). Further studies to explore this issue are under way, utilizing additional lymphocyte and natural killer cell markers to characterize this cell(s) more definitively. In one experiment to date, intravenous treatment of mice with anti-asialo GM $_1$ antibody (to eliminate natural killer cells) reduced both natural killer cell activity and Ia induction in response to LPS (data not shown).

The mechanism of LPS-mediated Ia induction has been further defined by studies *in vitro*. Consistent with previous observations (13, 25), we found that LPS (i) did not induce macrophage Ia biosynthesis or expression *in vitro* (Fig. 6) and (ii) inhibited induction caused by lymphokine preparations and IFN- γ (Fig. 6).

LPS also slowed the decay of Ia expression usually observed upon culture of macrophages in the absence of inducing agents (Fig. 6). Since the expression of Ia by macrophages is generally a dynamic and transient event (2, 3, 27), a block in the conversion of Ia $^+$ to Ia $^-$ cells may cause an accumulation of Ia $^+$ macrophages at inflammatory sites.

We have found that LPS-elicited, but not resident, macrophages increased Ia in response to lymphokine *in vitro* (data not shown). Thus, like other eliciting agents (11), LPS appears to "prepare" macrophages to receive a second signal which subsequently induces Ia expression. This "priming" of macrophages may be critical before a response to an Ia-inducing factor produced by LPS-reactive accessory lymphocytes is possible. Whether this priming simply represents an elicitation of immature cells from the bone marrow that are responsive to IFN- γ or represents a change in cell function such as an increase in the number of IFN- γ receptors has not been addressed by the present study.

LPS was also involved in the production of Ia-inducing factors. Two conditions consistently resulted in Ia-inducing activity: (i) the culture of LPS-activated spleen cells in medium alone, and (ii) the addition of LPS to normal spleen cells after a 48-h preincubation *in vitro* (Table 4). The factor generated by the second condition was identified as IFN- γ by antiviral activity and pH 2 sensitivity; however, the factor elaborated under the first condition contained no detectable antiviral activity.

The most likely explanation for Ia induction without antiviral effects is that the antiviral assay is less sensitive than the Ia induction assay. The sensitivity of the antiviral assay is in the range of 2 to 5 U/ml, while <0.01 U of IFN- γ per ml can be detected in the Ia induction assay. As such, low concentrations of IFN- γ would appear to have no antiviral activity, yet still be capable of inducing Ia expression. It is also possible that both IFN- γ and another lymphokine(s) such as B-cell stimulatory factor-1/interleukin-4 (8), tumor necrosis factor- α (6), or others (10, 30) may play a role in Ia induction by LPS. However, a separate

FIG. 8. Regulation of macrophage Ia expression by LPS. A working model to account for the regulatory effects of LPS is shown. Boxed plus and minus signs indicate up or down regulation of the individual steps as indicated. See text for discussion of these events.

study using neutralizing antibodies to individual lymphokines will be required to address these issues properly.

The detection of antiviral effects without Ia-inducing activity (Table 4) is most likely due to the fact that LPS inhibits the Ia-inducing activity, but not the antiviral effects, of IFN- γ (Fig. 6). Thus, the presence of LPS in the factor preparations can mask the presence of IFN- γ when Ia induction is measured.

Our working hypothesis for the mechanism of LPS-mediated induction of macrophage Ia expression involves the events depicted in Fig. 8. An Ia-inducing factor elaborated by a lymphocytelike accessory cell(s) drives the production of Ia $^+$ macrophages from Ia $^-$ macrophages and bone marrow precursors. This pathway may be T-cell dependent or T-cell independent, as indicated by induction in athymic nude mice (31). Whether the stimulation of an accessory cell is direct through membrane-receptor interaction or indirect by the elaboration of other immunoregulatory molecules (5) is an issue which we did not address. LPS also contributes to increased Ia by priming macrophages to respond to Ia-inducing factors and by inhibiting the conversion of Ia $^+$ to Ia $^-$ macrophages. These effects may occur simultaneously and result in the accumulation of Ia-expressing macrophages at the site of an LPS-mediated inflammatory response. Our results may be interpreted in the context of the initial decrease and then subsequent increase in Ia after injection of LPS *i.p.* (32). The initial decrease may reflect the inhibitory effect of LPS on background levels of Ia expression regulated by a basal production of lymphokines. As the concentration of LPS decreases and Ia-inducing factor(s) increases, induction of Ia predominates. As such, Ia levels represent a critical balance between the inhibitory and stimulatory effects of LPS.

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