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Induction of Unresponsiveness to Gamma Interferon in Macrophages Infected with *Mycobacterium leprae*

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We have previously demonstrated that *Mycobacterium leprae*-burdened granuloma macrophages isolated from infected nude mice are refractory to activation by gamma interferon (IFN-γ). To explore further both the afferent and efferent functional capacity of *M. leprae*-infected macrophages, we examined the IFN-γ-mediated activation of resident mouse peritoneal macrophages infected in vitro with live or dead *M. leprae*. When IFN-γ was administered within 24 h of *M. leprae* infection, macrophages were fully activated. However, defective activation was evident at 3 to 5 days postinfection in macrophages that were heavily burdened with viable *M. leprae*. This defect was evident by four parameters of activation in which IFN-γ failed to stimulate the enhancement of microbicidal activity, cytotoxicity for tumor target cells, O_2^- production, and surface Ia antigen expression. The development of defective activation closely followed an increase in macrophage production of prostaglandin E_2. Defective activation of *M. leprae*-burdened macrophages was reversible by indomethacin, and a similar block in IFN-γ activation was observed in three of these four parameters in normal macrophages treated with exogenous prostaglandin E_2. Thus, infection of mouse macrophages with *M. leprae* appears to restrict IFN-γ-mediated activation at least in part by induction of inhibitory levels of prostaglandin E_2.

A prominent feature of lepromatous leprosy is the presence of macrophage-rich granulomas that harbor large numbers of intracellular *Mycobacterium leprae*. Previous studies have established that human peripheral blood monocytes from leprosy patients have a normal microbicidal capacity (8) and responsiveness to gamma interferon (IFN-γ) (16, 18, 27). However, this finding may not be true of tissue macrophages within the local lesion, of which little is known regarding functional capacity. We have recently demonstrated that macrophages isolated from lepromatous granulomas of *M. leprae*-infected nude mice are completely refractory to activation by IFN-γ (39, 40). This nonresponsive condition appears to be dependent on the heavy intracellular load of bacilli found in localized granulomas since peritoneal macrophages from these same mice are activated normally by IFN-γ.

Previous studies have demonstrated that uptake of live *M. leprae* may lead to a reduction of macrophage protein metabolism (34), reduced expression of surface receptors for Fc (3), and surface sialic acid residues (1). However, these studies have not addressed the consequence of *M. leprae* infection on the principal afferent and efferent functions of the macrophage in conferring cell-mediated resistance to intracellular microorganisms, i.e., antigen presentation and enhanced microbicidal capacity. In the present study, we have used normal mouse peritoneal macrophages to examine the development of defective macrophage activation as a consequence of in vitro infection with live versus dead *M. leprae*. The functional capacity of macrophages was evaluated by measuring an enhanced capacity for killing of the intracellular protozoan *Toxoplasma gondii*, enhanced cytostatic capacity for EL-4 tumor target cells, enhanced respiratory burst activity, and increased Ia receptor expression. These varied responses are functionally distinct in their mechanisms of action but share the common feature of being induced by IFN-γ in combination with low levels of endotoxin that together act as potent macrophage-activating signals (4, 20, 26, 29, 33). In addition, based on our previous observations that lepromatous granuloma macrophages produce elevated levels of prostaglandin E_2 (PGE_2) (40), we have examined the role of this inhibitory immunomodulator on macrophage activation.

MATERIALS AND METHODS

Cell culture. Resident peritoneal macrophages were collected from adult BALB/c or Swiss Webster mice and cultured on LUX cover slips (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) in 24-well plates at 2×10^6 cells per well or on Lab-Tek chamber slides (Miles) in complete medium as described previously (39, 40). Complete medium consisted of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1 mM glutamine (Irvine Scientific, Santa Ana, Calif.), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (GIBCO), and 100 U of penicillin (Sigma Chemical Co., St. Louis, Mo.) per ml containing 20% heat inactivated fetal bovine serum (FBS) (Sterile Systems, Inc., Logan, Utah). Tissue culture media and reagents used in this study contained ≤0.025 ng of endotoxin per ml as detected by the chromogenic *Limulus* amoebocyte assay (Whittaker MA Bioproducts, Walkersville, Md.). Indomethacin (Sigma) was prepared as a stock solution in 1.0 mg/ml in dimethyl sulfoxide and stored at −70°C. PGE_2 (10^{-3} M; Seragen, Inc., Boston, Mass.) and phorbol myristic acetate (PMA; 10 μg/ml; Sigma) were prepared as stock solutions in absolute ethanol and stored at −70°C.

In Vitro Infection with *M. leprae*. *M. leprae* cells were obtained from log-phase growth in the nude mouse footpad (7) and were purified as described previously (12, 37). Briefly, infected footpads were minced in sterile Dubos

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albumin, pH 6.0, homogenized with a ground-glass homogenizer, and clarified by low-speed centrifugation at 100 × *g* for 10 min at 4°C to remove large tissue fragments and aggregates. The resulting suspension was pelleted at 2,700 × *g* for 60 min at 4°C, and the pellet was suspended in cold Dubos albumin. Live *M. leprae* suspensions were stored at 4°C and used within 48 h of harvest. Freshly harvested *M. leprae* cells were killed by treating for 12 h with 10% Formalin followed by five washings in Dubos albumin by centrifugation at 2,700 × *g* for 60 min. Control *M. leprae* cells were washed in parallel. *M. leprae* cells were counted by the method of Shepard and McRae (36) and diluted into complete medium for challenge of macrophage monolayers. Monolayers were allowed to phagocytose *M. leprae* until the mean number of intracellular bacilli reached levels of approximately 10 intracellular *M. leprae* per macrophage for examining effects of low burdens and approximately 50 intracellular *M. leprae* for examining the effects of high burdens of infection. Monolayers were then rinsed extensively in Hanks balanced salt solution (Whittaker) and recultured in RPMI–20% FBS. Challenge doses of live and Formalin-killed *M. leprae* were adjusted to obtain equal intracellular burdens in macrophages. Viability of macrophage monolayers was monitored by ethidium bromide-acridine orange staining as described previously (39).

PGE_2 production. To monitor production of PGE_2 by macrophage cultures, 1.0-ml supernatants were harvested at 1, 3, 5, and 7 days, clarified at 10,000 × *g* for 10 min at 4°C, and stored at −70°C. Control supernatants were harvested from cultures incubated with 1.0 μg of indomethacin per ml to inhibit prostaglandin synthesis. For analysis (40), 100-μl samples were incubated at 37°C, pH 10.0, for 24 h to convert PGE_2 to its stable end product bicyclo-PGE_2. Bicyclo-PGE_2 was quantitated for triplicate tubes per sample, using a radioimmunoassay kit (New England Nuclear Corp., Boston, Mass.).

PGE_2 treatment. The effect of PGE_2 on macrophage activation by IFN-γ was evaluated by exogenous treatment of normal macrophages with 10^{-8} M PGE_2. In parallel with experiments designed to assess the influence of *M. leprae* burden on macrophage responsiveness, normal macrophages were treated with 10^{-8} M PGE_2 by daily addition to their culture media for 3 consecutive days. In addition, to determine the time course effect of exogenous PGE_2 on macrophage response to IFN-γ, a single dose of 10^{-8} M PGE_2 was added to normal macrophages at intervals of 0, 1, 2, 3, and 4 days prior to activation with IFN-γ.

Macrophage activation. (i) Microbicidal capacity for toxoplasma. *T. gondii* RH cells were harvested from peritoneal cavities of 2-day-infected BALB/c mice in Hanks balanced salt solution containing 10 U of heparin per ml and purified by filtration through 3.0-μm polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) as described by Wilson et al. (44). Macrophage monolayers pretreated for 18 h with RPMI–20% FBS supplemented with 1.0 ng of endotoxin (*Escherichia coli* O111:B4; Sigma) per ml or with RPMI–20% FBS containing 200 U of IFN-γ plus 1.0 ng of endotoxin per ml were challenged with 5×10^5 freshly harvested *T. gondii* cells for 1 h, rinsed extensively, and returned to culture in RPMI–20% FBS. Microbicidal capacity was evaluated by counting the number of *T. gondii* cells per infected macrophage at 20 h postchallenge as described previously (39). Cell counts were made from 100 infected macrophages on triplicate cover slips from three to five separate experiments.

(ii) Cytostatic activity for tumor target cells. Cytostatic activity was quantitated as macrophage-mediated inhibition of thymidine uptake by EL-4 thymoma cells (21, 40). Macrophage monolayers pretreated for 6 h with 200 U of IFN-γ per ml or control monolayers were challenged with 10^5 EL-4 cells in 0.5 ml of RPMI–10% FBS supplemented with 1.0 ng of endotoxin and pulsed 18 h later with 5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear). At 24 h postchallenge, incorporation of [^{3}H]thymidine by EL-4 cells was quantitated in Aquasol-2 (New England Nuclear), using an LS 5801 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.) for triplicate samples from three to five separate experiments. Data are expressed as percentage of control, defined as the incorporation of [^{3}H]thymidine by EL-4 cells cocultured with normal macrophages.

(iii) O_2^- production. Production of O_2^- by stimulated macrophages was quantified by cytochrome *c* reduction, using a modification of the method described by Johnston et al. (17). Prior to O_2^- assays, macrophages were cultured for 48 h in either complete medium or medium supplemented with 200 U of IFN-γ per ml. Adherent macrophages were washed three times in phosphate-buffered saline and incubated for 90 min at 37°C in a reaction mixture consisting of 160 μM ferricytochrome *c* (Sigma) in phenol red-free Hanks balanced salt solution containing Ca^{2+} and Mg^{2+} (Whittaker) supplemented with 5% glucose. Stimuli consisted of 100 ng of PMA per ml added to the incubation media. Samples were corrected by subtraction of A_{500} readings for reaction mixtures incubated at 37°C without cells. Cytochrome *c* reduction was determined by measuring A_{500} and using the extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} for triplicate samples from three to five separate experiments.

(iv) Ia receptors. The presence of Ia antigen on normal resident peritoneal macrophages from BALB/c mice was determined by indirect immunofluorescence labeling of cells cultured overnight in Lab-Tek slides (23a). Induction of Ia antigens was also examined after 48-h treatment with 200 U of IFN-γ or after similar culture in control RPMI–20% FBS. Primary antisera, consisting of mouse monoclonal anti-Ia^d (Becton Dickinson and Co., Mountain View, Calif.) or control NS1 myeloma supernatant (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), was diluted 1:20 in RPMI–1% FBS and incubated on live macrophages kept at 4°C for 30 min. Monolayers were rinsed three times in cold phosphate-buffered saline for 5 min each time and incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Dako Corp., Santa Barbara, Calif.) at 4°C for 30 min. After three additional 5-min washes in cold phosphate-buffered saline, the monolayers were mounted in glycerol–phosphate-buffered saline and examined with a Leitz Ortholux II epifluorescence microscope. The percentage of Ia-positive cells was quantified by examination of 10 fields from triplicate samples of 100 or more cells.

RESULTS

Production of PGE_2. To examine the unstimulated production of PGE_2 by normal and *M. leprae*-infected macrophages, supernatants were collected at intervals following in vitro cultivation. Due to the extremely short half-life of PGE_2 (13), production was monitored indirectly by quantifying the accumulation of the stable breakdown product bicyclo-PGE_2. Although this method does not allow precise determination of PGE_2 concentrations, the relative production during an extended interval is reflected by accumulation of bicyclo-PGE_2. Normal macrophages produced basal lev-

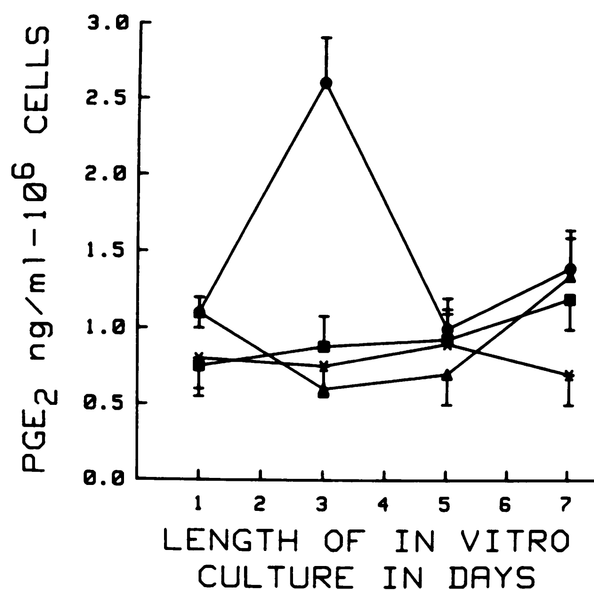

FIG. 1. Production of PGE_2 by macrophages during intervals of in vitro culture. Symbols: ▲, control monolayers; ●, live *M. leprae*-infected monolayers; *, live *M. leprae*-infected macrophages cultured in 1.0 μg of indomethacin per ml; ■, Formalin-killed *M. leprae*-infected monolayers. Values represent mean ± standard deviation for triplicate samples from a representative experiment.

els of bicyclo-PGE_2 of 0.5 ng/ml per 10^6 cells, which slowly increased to 1.2 ng/ml per 10^6 cells during 8 days in culture (Fig. 1). By comparison, RPMI–20% FBS used to culture macrophages contained 0.02 ng of bicyclo-PGE_2 per ml. Macrophages infected with viable *M. leprae* underwent a burst of PGE_2 production at day 2 to 3 after infection that reached levels of 2.6 ng/ml per 10^6 cells (approximately 10^{-8} M) (Fig. 1). The burst of PGE_2 production was not related to the initial phagocytosis of bacilli and returned to levels observed in noninfected cells by day 7 in culture. Treatment of *M. leprae*-infected macrophages with 1.0 μg of indomethacin per ml blocked the elevated production of PGE_2 (Fig. 1). Macrophages challenged with Formalin-killed *M. leprae* showed normal levels of bicyclo-PGE_2 production throughout this period of culture (Fig. 1).

Macrophage activation. Normal mouse peritoneal macrophages and *M. leprae*-infected macrophages were tested for their capacity to respond to IFN-γ-mediated activation based on the induction of enhanced microbicidal activity for *T. gondii*. The effects of intracellular *M. leprae* burden and length of in vitro culture prior to administration of IGN-γ on macrophage activation are summarized in Table 1. Normal macrophages treated with IFN-γ and low levels of endotoxin restricted the growth of intracellular *T. gondii* compared with normal controls ($P \leq 0.001$). When IFN-γ stimulation was initiated 24 h following infection with high burdens of live *M. leprae*, activation responses were normal and resulted in inhibition of *T. gondii* similar to that seen in normal macrophages. However, when IFN-γ treatment was delayed until 5 days after in vitro infection, activation was inhibited in heavily burdened macrophages as evidenced by the unrestricted growth of *T. gondii*. At low doses of intracellular *M. leprae*, macrophage activation was normal at both 1 and 5 days after infection (Table 1). Infection with *M. leprae* did not affect viability or integrety of macrophage monolayers assessed by ethidium bromide-acridine orange staining.

Based on these initial findings, the activation potential of

TABLE 1. Activation of normal and *M. leprae*-infected macrophages by IFN-γ at intervals after in vitro culture

Macrophages	Mean no. of *T. gondii*/infected macrophage (± SD; n = 3)			
	Day 1		Day 5	
	Control	IFN-γ	Control	IFN-γ
Normal	4.5 ± 0.4	1.9 ± 0.2	4.9 ± 0.4	1.8 ± 0.3
M. leprae infected				
10:1[a]	4.8 ± 0.2	1.7 ± 0.5	4.7 ± 0.4	2.2 ± 0.3
50:1	5.1 ± 0.3	2.3 ± 0.3	4.9 ± 0.3	4.3 ± 0.3[b]

[a] Mean intracellular burden of *M. leprae* from viable suspensions.
[b] $P \leq 0.05$ versus 5-day IFN-γ-treated normal macrophages or IFN-γ-treated macrophages infected with *M. leprae* (10:1).

M. leprae-burdened macrophages cultured for 3 to 5 days in vitro prior to stimulation by IFN-γ was examined further by using both the induction of toxoplasmacidal activity and additional criteria of IFN-γ-mediated activation. The inclusion of these additional criteria was designed to test whether the inhibition of macrophage responsiveness was limited to expression of enhanced microbicidal activity or was a generalized failure to respond to IFN-γ. To assess the importance of *M. leprae* viability, macrophages were infected with either live or Formalin-killed *M. leprae*. These experiments also addressed the influence of PGE_2 on macrophage activation in normal macrophages treated for 3 days with exogenous PGE_2 at 10^8 M, a level that simulated the production of PGE_2 in *M. leprae*-burdened macrophages. Also, some cultures of *M. leprae*-burdened macrophages were treated with 1.0 μg of indomethacin per ml to suppress prostaglandin synthesis.

(i) Microbicidal activity. Normal macrophages treated with IFN-γ and low doses of endotoxin after 3 to 5 days of culture were activated as demonstrated by their capacity to restrict the intracellular growth of *T. gondii* (Fig. 2). However, macrophages treated with PGE_2 or macrophages infected in vitro with viable *M. leprae* showed significantly lower activation by IFN-γ as reflected by the increased growth of *T. gondii* ($P \leq 0.01$ versus normal macrophages treated with IFN-γ; Fig. 2). Decreased activation of IFN-γ-treated macrophages infected with viable *M. leprae* was reversed by coculture in 1.0 μg of indomethacin per ml (Fig. 2). Indomethacin treatment did not affect the toxoplasmacidal capacity of normal macrophages activated with IFN-γ (not shown). Macrophages infected with Formalin-killed *M. leprae* showed normal activation for restriction of *T. gondii* growth (Fig. 2). In the absence of IFN-γ treatment, *T. gondii* growth was not affected by indomethacin or PGE_2, nor did *M. leprae* infection of untreated macrophages affect the growth of *T. gondii* (Fig. 2).

(ii) Cytostatic activity. Normal macrophages cultured for 3 to 5 days and then treated with IFN-γ and low levels of endotoxin were activated to restrict the uptake of [^{3}H]thymidine by EL-4 tumor target cells ($P \leq 0.001$) (Fig. 3). In contrast, IFN-γ-mediated activation for cytostatic activity was significantly lower in macrophages that were treated in vitro with exogenous PGE_2 or in those infected with viable *M. leprae* ($P \leq 0.001$ versus normal macrophages treated with IFN-γ; Fig. 3). This defect in activation of macrophages infected with live *M. leprae* was reversed by coculture in 1.0 μg of indomethacin per ml and was not observed in macrophages infected in vitro with Formalin-killed *M. leprae* (Fig. 3). Indomethacin did not affect the cytostatic capacity of

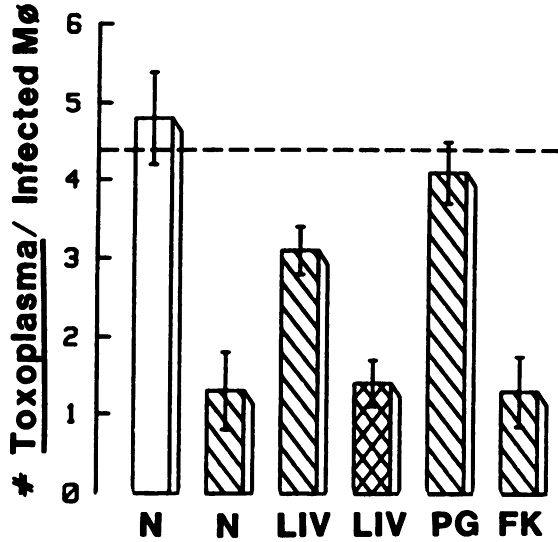

FIG. 2. Assessment of activation by IFN-γ in normal *M. leprae*-infected or PGE_2-treated macrophages after 3 to 5 days of culture as evaluated by toxoplasmacidal activity. N, Normal; LIV, live-*M. leprae* infected; FK, Formalin-killed-*M. leprae* infected; and PG, 10^{-8} M PGE_2 treated. The level of *Toxoplasma* replication in nonactivated macrophage monolayers from each of the experimental groups was equal to or greater than the value shown by the dotted line. Open bars denote control, diagonal bars denote IFN-γ treated, and hatched bars denote combination of indomethacin (1.0 μg/ml) and IFN-γ. Values represent mean ± standard error for three to five experiments.

normal macrophages activated with IFN-γ (not shown). In the absence of IFN-γ stimulation, EL-4 proliferation was not affected by coculture with PGE_2-treated macrophages, *M. leprae*-infected macrophages, or indomethacin-treated macrophages (Fig. 3).

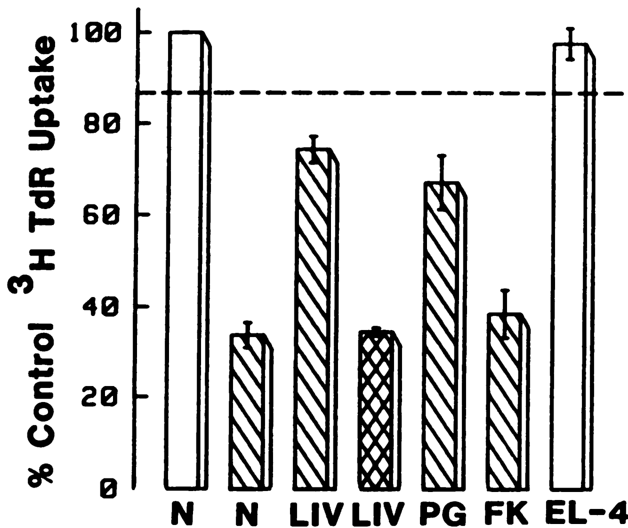

FIG. 3. Assessment of activation by IFN-γ in normal, *M. leprae*-infected, or PGE_2-treated macrophages after 3 to 4 days of culture as evaluated by inhibition of [^{3}H]thymidine uptake by EL-4 cells. Abbreviations and symbols as in the legend to Fig. 2. The level of [^{3}H]thymidine uptake by EL-4 cells in presence of nonactivated macrophage monolayers of each experimental group was greater than the value indicated by the dotted line. Values represent means ± standard error for three to five experiments.

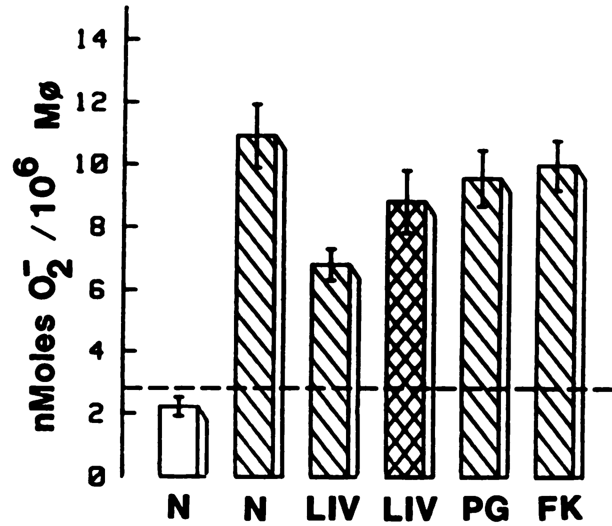

FIG. 4. Assessment of activation by IFN-γ in normal, *M. leprae*-infected, or PGE_2-treated macrophages as evaluated by an increased O_2^- production in response to PMA. Abbreviations and symbols as in the legend to Fig. 2. The level of O_2^- production by nonactivated macrophage monolayers treated with PMA from each experimental group was less than the value indicated by the dotted line. Values represent means ± standard error for three to five experiments.

(iii) O_2^- production. Normal macrophages cultured for 3 to 5 days and then treated with IFN-γ demonstrated increased capacity to secrete O_2^- when stimulated with PMA ($P \leq 0.01$) (Fig. 4). However, IFN-γ-induced production of O_2^- was significantly inhibited in macrophages infected in vitro with viable *M. leprae* ($P \leq 0.05$ versus normal macrophages treated with IFN-γ) (Fig. 4). This defect was reversed when *M. leprae*-infected cells were cocultured in 1.0 μg of indomethacin per ml (Fig. 4). Macrophages treated in vitro with exogenous PGE_2 or infected with Formalin-killed *M. leprae* showed normal induction of O_2^- production when stimulated with IFN-γ (Fig. 4). Indomethacin treatment did not affect O_2^- production by normal macrophages activated with IFN-γ (not shown). In the absence of IFN-γ treatment, macrophage O_2^- production was not affected by indomethacin, PGE_2, or *M. leprae* infection (Fig. 4).

(iv) Ia antigen expression. Expression of Ia^d antigens by macrophages following in vitro culture is summarized in Table 2. Approximately 10% of normal peritoneal macrophages expressed Ia^d after 3 to 4 days in culture. In contrast, Ia^d expression was induced on >90% of normal macrophages treated in vitro with IFN-γ. Infection with live *M. leprae* blocked IFN-γ induction of Ia^d, a defect that was reversed by coculture in 1.0 μg of indomethacin per ml. Indomethacin alone did not affect Ia expression. Treatment of normal macrophages with exogenous PGE_2 in vitro also blocked IFN-γ induction of Ia^d. Macrophages infected with Formalin-killed *M. leprae* showed normal induction of Ia^d by IFN-γ. Control or IFN-γ-treated macrophages were not labeled by NS1 control sera (not shown).

(v) PGE_2 treatment. The inhibition of macrophage activation by a single dose of 10^{-8} M exogenous PGE_2 was evaluated by a decreased capacity for cytostatic activity against EL-4 target cells. A single exogenous PGE_2 treatment given 2 or 3 days prior to IFN-γ stimulation significantly reduced the capacity of macrophage monolayers to mount a cytostatic response ($P < 0.01$) (Fig. 5). However,

TABLE 2. Induction of Ia^d antigen expression by normal, *M. leprae*-infected, or PGE_2-treated macrophages stimulated with IFN-γ

Macrophages	% Positive[a]			
	Control		IFN-γ treated	
	− Indomethacin[b]	+ Indomethacin	− Indomethacin	+ Indomethacin
Normal	11.6	14.9	91.4	92.9
PGE_2 treated[c]	7.2	12.1	21.9	24.9
Live *M. leprae* infected	11.4	13.1	18.7	86.8
Formalin-killed *M. leprae* infected	12.3	11.1	80.7	90.5

[a] Mean of three determinations of 100 or more cells.
[b] 1.0 μg of indomethacin per ml.
[c] 10^{-6} M PGE_2.

this decreased activation was not seen in macrophages treated with PGE_2 simultaneously with IFN-γ. Moreover, the decreased activation potential was transient and was not seen in macrophages treated with PGE_2 4 or more days prior to IFN-γ activation.

DISCUSSION

The present report provides evidence for the development of defective IFN-γ-mediated activation in resident mouse peritoneal macrophages following in vitro infection with *M. leprae*. Unresponsiveness to IFN-γ was evident by the failure of IFN-γ to induce enhanced efferent (increased microbicidal capacity for *T. gondii* and cytotoxicity for tumor target cells) and afferent (induction of surface Ia antigen) effector function in *M. leprae*-infected macrophages. Defective macrophage response to IFN-γ was also evidenced by reduced oxidative metabolism (production of superoxide anion). A high intracellular burden of *M. leprae* was required, and only viable bacilli induced the defect. The

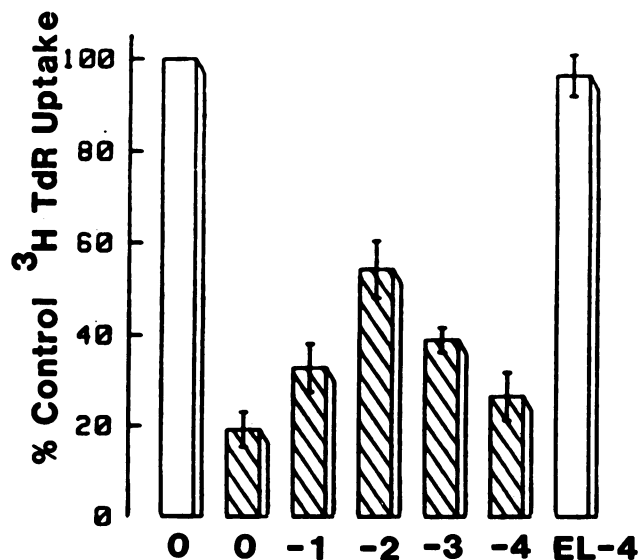

FIG. 5. Effect of a single treatment with 10^{-8} M PGE_2 on activation of macrophages by IFN-γ as evaluated by inhibition of [^{3}H]thymidine uptake by target EL-4 cells. Macrophage monolayers were pulsed with PGE_2 at intervals of 0 to 4 days prior to activation by IFN-γ. Open bars denote [^{3}H]thymidine uptake by EL-4 cells alone or cocultured with control macrophage monolayers, and hatched bars denote that by EL-4 cells treated with IFN-γ and endotoxin. Values represent means ± standard deviation (n = 3) for a representative experiment.

defective responses to IFN-γ were not observed in recently infected macrophages (24 h or less) but was consistently seen when macrophages harbored leprosy bacilli for 3 to 5 days before stimulation with IFN-γ. While we have not directly examined the effects of varying concentrations of IFN-γ or endotoxin in this report, these studies were conducted with doses of IFN-γ and endotoxin well above the threshold necessary to induce activation in normal macrophages (39, 40).

The development of defective activation was closely correlated with elevated PGE_2 production by live *M. leprae*-infected macrophages that peaked at 48 to 72 h postinfection at a level of approximately 10^{-8} M. However, when *M. leprae*-infected macrophages were cocultured with 1.0 μg of indomethacin per ml, prostaglandin production was reduced to normal levels and IFN-γ-mediated activation was restored to normal. It was previously shown that PGE_2 does not inhibit IFN-γ activation for enhanced tumoricidal activity when these two modulators were added simultaneously (43). However, in the present report, when macrophages were pulsed with repeated doses of exogenous PGE_2 for intervals of 3 to 4 days prior to IFN-γ activation, their capacity to become activated was severely restricted. The defect in activation produced by repeated PGE_2 treatments was evident as a reduction in microbicidal and tumoricidal capacity and as inhibition of IFN-γ-induced Ia expression. These findings are consistent with previous reports that PGE_2 inhibits induction of macrophage Ia expression by lymphokines (42) and demonstrate that prolonged exposure to PGE_2 inhibits the expression of macrophage effector functions normally enhanced by IFN-γ. Moreover, a single dose of exogenous PGE_2 significantly decreased macrophage activation when given 2 to 3 days prior to, but not after, being given simultaneously with IFN-γ treatment. This finding correlates with our observation that the burst of PGE_2 production induced by infection with live *M. leprae* was followed 1 or 2 days later by reduced responsiveness to IFN-γ.

Collectively, the present findings demonstrate a role for induction of PGE_2 production leading to decreased capacity for activation as a major consequence of *M. leprae* infection in mouse peritoneal macrophages. A similar role for heightened production of PGE_2 by macrophages leading to in vivo immune suppression has recently been demonstrated for *M. intracellulare* infection in mice (9). In addition to the generalized block in macrophage activation induced by live *M. leprae* (39, 40; present report), *Leishmania donovani* (31), *Mycobacterium microti* (19), and *Mycobacterium kansasii* (Mshana et al., in press) appear also to restrict macrophage responsiveness to lymphokine activation as measured by induction of surface Ia antigens. Thus, inhibition of macro-

phage activation may be a general consequence of chronic intracellular microorganisms that reside in localized macrophage granulomas.

In the present study, elevated levels of PGE_2 induced by infection with live *M. leprae* were correlated with reduced macrophage activation as evaluated by toxoplasmicidal and tumoristatic activity and by expression of Ia antigens. However, although PMA-triggered respiratory burst activity by IFN-γ-stimulated macrophages was reduced in *M. leprae*-infected macrophages, their unresponsiveness could not be induced by addition of exogenous PGE_2. While elevated respiratory burst activity has been correlated with the enhanced cytotoxic and microbicidal state of activated macrophages, this correlation is not universal (6, 17, 41). The disassociation observed between the role of PGE_2 in unresponsiveness to IFN-γ enhancement of efferent and afferent induction of respiratory function and the failure of PGE_2 to block IFN-γ burst activity may reflect a different underlying mechanism in the induction of these responses by IFN-γ. Regardless, the uptake of *M. leprae* by macrophages is a very poor activator of the respiratory burst (15), suggesting that even slight inhibition of the respiratory burst may be important for intracellular survival.

In contrast to the findings that heavily burdened macrophages are refractory to further activation by IFN-γ (39, 40; present report), macrophages treated with immune lymphokines, including IFN-γ, prior to encountering *M. leprae* attain an activated state and significantly inhibit the intracellular metabolism and survival of *M. leprae* (37; N. Ramasesh, S. Franzblau, and J. Krahenbuhl, manuscript in preparation). In the present study, the development of defective activation was not simply a consequence of phagocytosis or early events following the ingestion of live bacilli but was delayed 72 to 96 h. Collectively, these findings underscore the importance of the timing of macrophage uptake of viable *M. leprae* in relationship with the capacity for IFN-γ-mediated activation. Thus, the development of defective activation in infected macrophages observed in the present report appears to be dependent on the accumulative influence of an intracellular burden of live *M. leprae*. Whether IFN-γ activation is blocked by a threshold dose of an actively synthesized constituent of live leprosy bacilli is under investigation. In recent preliminary studies (38), we have reported that lipoarabinomannan, an abundant, highly immunogenic component of the mycobacterial cell wall, is a potent inhibitor of IFN-γ-mediated activation of mouse macrophages by mechanisms that do not appear to involve production of PGE_2.

In *M. leprae*-infected nude mice, defective macrophage activation is confined to heavily burdened macrophages isolated from granulomas of the footpad and lymph nodes, whereas peritoneal macrophages from these same mice exhibit normal IFN-γ responsiveness (39, 40). Although the lepromatous macrophages isolated from granulomas in the nude mouse produce high levels of PGE_2, the failure of indomethacin treatment to restore IFN-γ activation of these lepromatous macrophages (40) suggests that nonprostanoid mechanisms of inhibition are also involved in the restriction of their activation. While the underlying basis of this defect is not known, the present report emphasizes the importance of heightened PGE_2 production by *M. leprae*-infected macrophages in leading to compromised responsiveness to activating signals. Thus, newly arrived bone-marrow-derived monocytes likely encounter conditions in the localized lepromatous granuloma that rapidly restrict their responsiveness to IFN-γ.

We have recently examined the content of PGE_2 in supernatants produced by in vitro cultivation of skin biopsies from the lesions of leprosy patients that were initially collected for studies of local modulation of monocyte leucotactic responses (5). Preliminary analyses (Sibley and Krahenbuhl, unpublished results) indicate that supernatants from unstimulated biopsies from both borderline and lepromatous leprosy patients contain elevated levels of PGE_2. Elevated levels of PGE_2 have been shown to dramatically influence a number of T-cell and macrophage functions, including inhibition of T-cell proliferation (10), suppression of Ia antigen expression by macrophages treated with lymphokines (42), and inhibition of macrophage tumoricidal capacity (43). Although the local cellular source of the elevated PGE_2 production in lesions of leprosy patients is not known, the abundance of macrophages in these lesions and the induction of PGE_2 production by human monocytes exposed to *M. leprae* in vitro (32) implicate the *M. leprae*-infected macrophage. Support for this hypothesis is provided by studies that use a mouse model of experimental leprosy which show elevated spontaneous release of PGE_2 in vitro by *M. leprae*-infected macrophages (present study) and in vivo by *M. leprae*-burdened macrophages from local lepromatous granulomas (39, 40). Studies on the production of arachidonic acid metabolites by stimulated human monocytes indicate that thromboxane rather than PGE_2 is the predominate cyclooxygenase metabolite (30). Thus, while PGE_2 may be an important local mediator in the response of mouse macrophages to *M. leprae*, the role of PGE_2, thromboxane, and other arachidonic acid metabolites in modulating human macrophage responses in leprosy will require further study.

With the exception of their inability to cope with the leprosy bacillus, macrophages from lepromatous patients are functionally normal as evidenced by the in vitro microbicidal capacity of peripheral blood monocytes (8) and the observation that these patients are not susceptible to the facultative and obligate intracellular pathogens characteristically seen in the immunocompromised host. Although monocytes from lepromatous patients appear to produce lower levels of the oxidative metabolite H_2O_2, this function is restored following in vivo (27) or in vitro (18) administration of IFN-γ. Moreover, enhanced microbicidal capacity for *Legionella pneumophila* is induced in vitro by IFN-γ in monocyte-derived macrophages from lepromatous patients (16). Thus, while peripheral blood monocytes of leprosy patients appear to be functionally normal in their microbicidal capacity and their responsiveness to IFN-γ (8, 16, 18, 27), their arrival in the granulomatous lesion may lead to exposure to conditions which inhibit their capacity to respond to macrophage-activating signals. Previous studies of cell-mediated responses of leprosy patients have emphasized the importance of T-cell anergy that results in lowered production of interleukin-2 and IFN-γ (14, 16, 28) and fewer interleukin-2^+ lymphocytes (23) and the role of suppressor T cells (22, 24, 25) as a mechanism underlying this specific anergy. In this context, it is noteworthy that PGE_2 has been shown to preferentially encourage T suppressor rather than helper cell proliferation (11). Collectively, our studies of the interaction of *M. leprae* with mouse macrophages (39, 40; present report) emphasize that defects in cell-mediated immunity in lepromatous leprosy likely extend beyond the level of the T cell to include localized restriction of macrophage afferent and efferent functions influenced by lymphokines.

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