INFECTION AND IMMUNITY, Sept. 1991, p. 2864–2869
0019-9567/91/092864-06$02.00/0

Vol. 59, No. 9

Effects of Activated Macrophages on *Mycobacterium leprae*

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Received 4 December 1990/Accepted 4 June 1991

Five alternative methods were used to explore in vitro the effects of normal and activated murine macrophages on the metabolic well-being of intracellular *Mycobacterium leprae*: fluorescein diacetate-ethidium bromide staining, ATP content, synthesis of phenolic glycolipid 1, and two techniques to quantitate oxidation of palmitic acid. In relatively short-term experiments (7 to 10 days), each of these procedures provided strong evidence that activated macrophages exerted a deleterious effect on the leprosy bacillus. These findings appear to confirm the contention that activated macrophages underlie host resistance to clinical leprosy and limitation of *M. leprae* growth in paucibacillary leprosy.

Clinical leprosy is characterized by a broad spectrum of host response, with great variability in clinical course of infection, histopathology, humoral antibody response, and cell-mediated immunity. As an obligate intracellular microorganism, *Mycobacterium leprae* is capable of prolific growth in macrophages (Mϕ) in vivo. However, little is known about the immune-mediated activation of Mϕ, and their ability to kill and digest *M. leprae* is an issue of central importance to understanding the spectrum of host resistance characteristic of leprosy (12). Unfortunately, the direct exploration of the interaction between *M. leprae* and the Mϕ has been stymied by the inability to rapidly quantitate the viability of the uncultivable leprosy bacillus.

The present study used a number of alternative assays to quantitate the relative viability and metabolism of *M. leprae* in vitro, and this report describes the detrimental effects of activated Mϕ on the leprosy bacillus.

MATERIALS AND METHODS

Mice. Athymic *nu/nu* mice (HSD-BR) were purchased from Harlan Sprague-Dawley, Inc., Indianapolis, Ind. BALB/c mice were locally bred. Swiss Webster retired breeders were purchased from Simonsen Laboratories, Gilroy, Calif.

Mϕ. Mϕ were obtained from the peritoneal cavities of unstimulated Swiss Webster or BALB/c mice as described previously (40). Briefly, peritoneal cells were harvested with cold heparinized (10 U/ml) Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), centrifuged, and resuspended in RPMI 1640 (GIBCO) supplemented with 100 U of penicillin per ml, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO), glutamine (GIBCO), and heat-inactivated 20% fetal calf serum (Sterile Systems, Inc., Logan, Utah). All buffer and culture media contained <0.1 ng of endotoxin per ml as assayed by the quantitative *Limulus* amoebocyte lysate assay (Whittaker MA Bioproducts, Walkersville, Md.). The cells were diluted in medium to contain 4×10^6 peritoneal cells per ml. For certain experiments, 5-ml aliquots were plated in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.), while for others, 0.5-ml aliquots were plated on 13-mm LUX plastic coverslips (Miles Laboratories, Naperville, Ill.) in wells of 24-well culture plates (Corning Glass Works). After a 2-h incubation at 37°C in an atmosphere of 5% CO_2, the nonadherent cells were washed off with Hanks balanced salt solution, the medium was replaced, and the Mϕ were infected with *M. leprae*.

***M. leprae*.** Viable leprosy bacilli were freshly harvested for each experiment from footpad tissues of athymic *nu/nu* mice infected 10 to 15 months previously (11). *M. leprae* organisms were suspended in RPMI 1640 and enumerated by the method of Shepard and McRae (35). The number of *M. leprae* per footpad averaged 1×10^{10} to 5×10^{10}.

Infection of Mϕ monolayers. Mϕ cultures in flasks were overlaid with a suspension of *M. leprae* in 5.0 ml of RPMI 1640–20% fetal calf serum adjusted to yield a bacterium/Mϕ ratio of 20:1. Mϕ monolayers on coverslips were infected with 0.5 ml of *M. leprae* suspension. After 2 h, noningested bacteria were removed by washing and the infected monolayers were reincubated.

Activated Mϕ. Activated peritoneal Mϕ were obtained from mice chronically infected with the C-56 strain of *Toxoplasma gondii* (42) or from mice injected intraperitoneally with 700 μg of killed *Corynebacterium parvum* (Burroughs Wellcome, Inc., Research Triangle Park, N.C.) 7 to 14 days previously (16). The activation of normal Mϕ was achieved by treatment in vitro with murine recombinant gamma interferon (MuIFN-γ; Genentech, Inc., South San Francisco, Calif.) 18 h prior to infection or, in certain experiments, immediately after infection. MuIFN-γ was used at a concentration of 200 U/ml and was always supplemented with 2 ng of endotoxin per ml to provide the second molecular signal in the activation process (40). In certain experiments, supplemental fresh MuIFN-γ was added to the cells each day for 3 days.

Assays for anti-*M. leprae* effects of activated Mϕ. Several assays were used to quantify the effects of activated Mϕ on *M. leprae*.

(i) FDA-EB staining. A modification of the procedure described by Kvach and Veras (21) was used for *M. leprae*. At the termination of an experiment, the infected normal or activated Mϕ were scraped from the flasks and sonicated to release intracellular *M. leprae*. One-half milliliter of this suspension was washed two times in phosphate-buffered saline by centrifugation at 10,000 × *g* for 5 min and resuspended in 0.5 ml of phosphate-buffered saline. Triplicate smears of bacterial dilutions from each sample were prepared on microscope slides, air-dried, and coded for single blind examination. A 10-μl aliquot of fluorescein diacetate-

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ethidium bromide (FDA-EB; 40 μg of FDA per ml and 8 μg of EB per ml in phosphate-buffered saline) was overlaid on the smears, which were then covered with a 25-cm-diameter glass coverslip and incubated in the dark for 20 min. The smears were observed at ×900 under incident UV illumination, using a 390- to 400-nm excitation filter. A total of 200 bacteria were enumerated in each slide and graded as viable (green) or nonviable (red).

(ii) Quantitation of ATP content of *M. leprae*. ATP content of *M. leprae* was determined by a modification (11) of a procedure described previously for *M. leprae* by Kvach et al. (20). At the termination of the experiment (3 to 10 days postinfection), intracellular bacteria were released by sonication and centrifuged at 10,000 × *g* for 5 min, and bacterial ATP was quantitated by the luciferin-luciferase system described previously (11). Appropriate ATP standards (disodium ATP [Sigma] stored at −80°C) were used to construct a standard curve immediately following the measurement of bacterial ATP. ATP levels were normalized to picograms per 10^6 cells.

(iii) Biosynthesis of PGL-1 by intracellular *M. leprae*. Quantitation of the biosynthesis of phenolic glycolipid 1 (PGL-1) by *M. leprae* in Mϕ in vitro has been described previously (29, 30). In the present studies, flasks of activated and normal Mϕ infected with *M. leprae* were incubated for 72 h and then pulsed with 0.2 μCi of radiolabelled palmitic acid (PA) per ml ([U-^{14}C]PA; specific activity, 850 mCi/mmol; New England Nuclear, Berkley, Calif.) until the termination of the experiment 7 to 10 days later. Alternatively, tritium-labelled PA {[9,10-^{3}H(*N*)]PA; specific activity, 50 Ci/mmol; New England Nuclear} (<15 μCi/flask) was added on day 3 and the experiment was terminated 5 days later. Intracellular *M. leprae* was released from the Mϕ by using a lysing buffer, and the bacterial suspension was quantitated, autoclaved, and lyophilized before the lipids were extracted; PGL-1 synthesis was quantitated by thin-layer chromatography and liquid scintillation counting as described previously (30). The amount of [U-^{14}C]- or [^{3}H]PA incorporated into PGL-1 was then normalized as disintegrations per minute per 10^8 *M. leprae*.

(iv) Radiorespirometric assay for *M. leprae* viability. Normal Mϕ and Mϕ activated in vitro with MuIFN-γ and endotoxin or in vivo by treatment with *C. parvum* were infected for 2 h with viable *M. leprae* freshly harvested from the footpads of *nu/nu* mice. Coverslips were washed to remove extracellular bacteria, and after 24 h of culture, coverslips containing the infected Mϕ monolayers were transferred to wells containing 0.4 ml of 0.1 N NaOH. After 5 min of incubation at room temperature, 0.3 ml of the bacterial suspension released from the Mϕ was transferred to the appropriate vessel for quantitation of PA oxidation by either the Buddemeyer (4, 8) or the BACTEC 460 system as described recently for *M. leprae* by Franzblau (9).

(a) Buddemeyer system. Bacterial suspensions (0.3 ml) were transferred to 6-ml, screw-cap vials containing 4 ml of 7H12 medium, pH 5.8, and 1 μCi of [1-^{14}C]PA (5.7 mCi/mmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) per ml. The use of medium adjusted to pH 5.8 allowed for the direct addition of 0.3 ml of 0.1 N NaOH without prior neutralization of the lysate. Evolution of [$^{14}CO_2$] was measured as described previously (8). The vials, with caps loosened, were placed in wide-mouthed scintillation vials containing a hollow cylinder of Whatman no. 1 filter paper which had been dipped previously in concentrated liquid scintillation counting solution {Liquiflor PPO-POPOP [2,5-diphenyloxazole–1,4-bis(5-phenyloxazolyl)benzene] toluene concentrate},

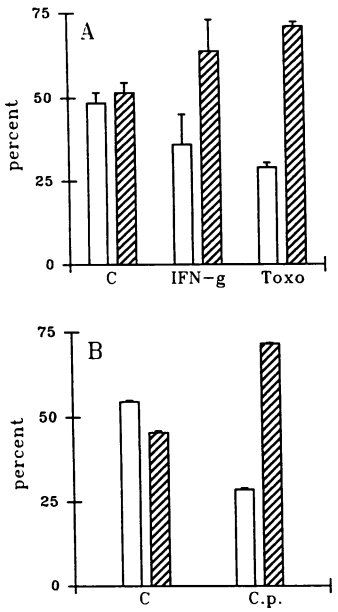

FIG. 1. FDA-EB staining characteristics of *M. leprae* released after 10 (A) or 5 (B) days of culture in normal control Mϕ (C) or Mϕ activated in vitro with MuIFN-γ (IFN-g) or in vivo by chronic *T. gondii* infection (Toxo) or treatment with heat-killed *C. parvum* (C.p.). A total of 200 bacteria per sample were counted and graded as viable (green; open bars) or nonviable (red; hatched bars). Values represent means ± standard deviations of triplicate samples.

dried, and charged with 100 μl of 2 N NaOH. The caps of the scintillation vials were firmly tightened, and the assembly was incubated at 33°C. Cumulative $^{14}CO_2$ production was measured at 24-h intervals in a Beckman model LS-5801 liquid scintillation counter.

(b) BACTEC 460 system. Bacterial suspensions (0.3 ml) from lysed Mϕ were dispensed into 20-ml BACTEC stoppered serum vials containing 4 ml of 7H12 medium, pH 5.8, and 1 μCi of [1-^{14}C]PA per ml. The vials were flushed with 2.5% O_2–10% CO_2–balance N_2. This low-oxygen gassing mixture was shown to be optimal for metabolic maintenance of *M. leprae* (13). The vials were incubated at 33°C for 1 week, and evolved $^{14}CO_2$ was measured in growth index units (100 growth index units = 0.025 μCi) in a BACTEC 460 instrument.

Statistical analysis. Student's *t* test was used to test the significance of experimental results.

RESULTS

Effects of activated Mϕ on viability staining of *M. leprae*. Figure 1A compares the FDA-EB staining characteristics of *M. leprae* released from normal or activated Mϕ after 10 days in culture. As shown, approximately 50% of the bacteria released from normal Mϕ stained green, but the percentage of viable bacteria from the Mϕ activated in vivo by *T. gondii* infection or in vitro by treatment with MuIFN-γ was lower (25 to 30%). Interestingly, while 15 to 20% of the bacteria graded as green in the control group were solidly stained, many of the bacteria graded as green in the activated Mϕ groups were highly beaded. Similar observations were made in our studies with *C. parvum*-activated Mϕ after 5 days in culture (Fig. 1B). Not shown are observations made at the earlier time points of 24 and 48 h after infection when

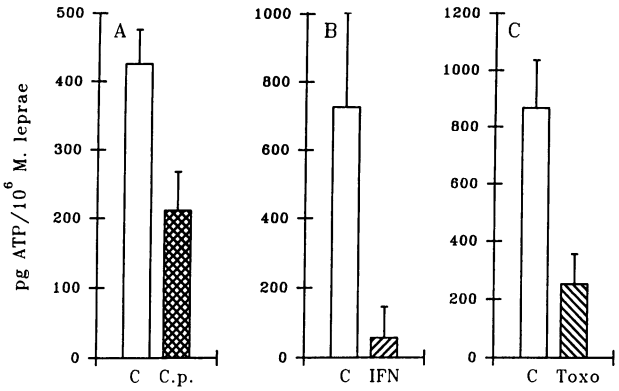

FIG. 2. Quantitation of ATP content of *M. leprae* released after 3 (A), 8 (B), or 10 (C) days of culture in normal control Mϕ (C) or Mϕ activated in vitro with MuIFN-γ (IFN) or in vivo by treatment with heat-killed *C. parvum* (C.p.) or chronic *T. gondii* infection (Toxo). Values represent means ± standard deviations of triplicate samples.

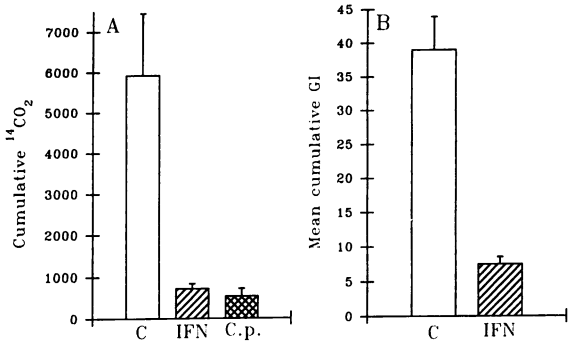

FIG. 4. Oxidation of [^{14}C]PA by *M. leprae* released after 24 h of culture from normal control Mϕ (C) or Mϕ activated in vitro with MuIFN-γ (IFN) or in vivo by treatment with *C. parvum* (C.p.). Bacilli were transferred to either the Buddemeyer (A) or BACTEC 460 (B) system and monitored for production of $^{14}CO_2$. Values represent means ± standard deviations of triplicate samples. Each of these experiments is representative of four similar studies.

no difference in the FDA-EB staining quality could be discerned in bacteria released from normal or activated Mϕ.

ATP content. Figure 2 shows the effects of *C. parvum*, MuIFN-γ, and *T. gondii*-activated Mϕ on the ATP content of *M. leprae* at 3, 8, or 10 days, respectively, after infection. In each experiment, there was a significant ($P < 0.5$) depression of ATP content in bacilli released from the activated group.

PGL-1 synthesis. Figure 3 shows the effects of activated Mϕ on PGL-1 synthesis by *M. leprae*. In the representative experiments depicted in Fig. 3A and B, [^{14}C]- and [^{3}H]PA, respectively, were used to label PGL-1. While *M. leprae* in normal Mϕ labelled with [U-^{14}C]PA incorporated over 15,000 dpm in the extractable PGL-1, there was negligible incorporation into *M. leprae* in the activated groups (Fig. 3A). These findings were confirmed and extended in the experiments depicted in Fig. 3B, in which the effects of Mϕ activated by *C. parvum* treatment in vivo are compared with those of Mϕ activated by addition of MuIFN-γ to the cultures after they were infected with *M. leprae*. The results

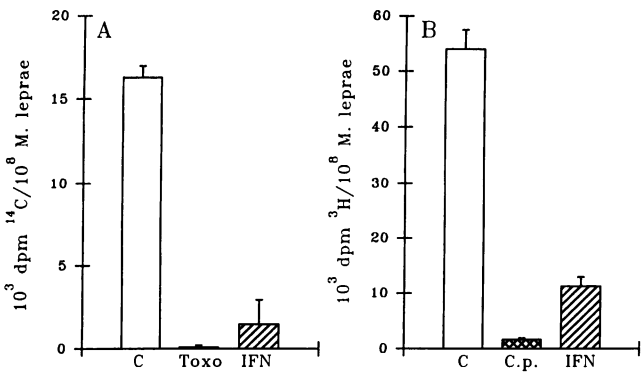

FIG. 3. Synthesis of PGL-1 by *M. leprae* in normal control Mϕ (C) or Mϕ activated in vitro with MuIFN-γ (IFN) or in vivo by treatment with *C. parvum* (C.p.) or chronic infection with *T. gondii* (Toxo). Values represent means ± standard deviations of triplicate samples. (A) [U-^{14}C]PA was used (7-day incubation period); (B) [^{3}H]PA was used (5-day incubation period). Data are representative of four (A) and three (B) experiments.

of both types of study clearly show a significant ($P < 0.01$) inhibition of PGL-1 synthesis by *M. leprae* in activated Mϕ.

Radiorespirometry. Figure 4 shows the effects of activated Mϕ on the metabolic ability of *M. leprae* to oxidize [^{14}C]PA to $^{14}CO_2$. After 24-h culture in Mϕ activated either in vitro with MuIFN-γ or in vivo with *C. parvum*, the metabolism of *M. leprae* was markedly and significantly ($P < 0.01$) diminished as measured in both the Buddemeyer (Fig. 4A) and BACTEC 460 (Fig. 4B) systems.

DISCUSSION

In the past, Mϕ effector function in leprosy has been addressed indirectly by exploring mononuclear phagocytes from patients' blood, with contradictory results. There is some evidence that Mϕ from lepromatous leprosy (LL) patients are deficient in their ability to digest *M. leprae* (2, 26). However, well-controlled studies (6, 32, 43) failed to find any correlation between lepromin activity and the ability of an individual's Mϕ to kill or destroy *M. leprae*. Sharp and Banerjee (33) tried to identify a defect in Mϕ function in leprosy patients but found that the oxidative metabolism of patient Mϕ was normal or above normal. In contrast, Nathan et al. (23) reported that monocytes from LL patients were deficient in hydrogen peroxide production and presumably microbicidal function as well. However, other studies of the innate microbicidal properties of LL patients' mononuclear phagocytes for other intracellular pathogens (6) failed to show a defect. Moreover, the anergy in LL is *M. leprae* specific; LL patients are not prone to infection with the opportunistic pathogens that plague patients with immunodeficiency diseases.

A fundamental problem in directly addressing the role of the Mϕ in resistance to *M. leprae* is the inability to quantitate routinely the viability of the uncultivable leprosy bacillus. *M. leprae* can obviously survive and replicate in Mϕ in vivo, but confirming that they remain viable in vitro is difficult. To monitor the viability of the noncultivable leprosy bacillus, researchers have relied on a highly subjective evaluation (34) of acid-fast staining patterns of the bacilli (morphological index) or determination of the viability of *M. leprae* by titration of growth in the mouse footpad, an expensive method requiring 1 year or more to complete (45). The accuracy of the morphological index procedure as a

measure of viability remains controversial (5, 14, 15, 19, 24) and would have proven to be of little value in our relatively short-term in vitro studies. In unpublished preliminary studies, we used the footpad technique to evaluate the effects of activated Mϕ on *M. leprae* and observed approximately a 10-fold difference in the minimum number of bacilli required to establish infection. Groups of 10 mice each were used to titrate 10-fold dilutions of *M. leprae* released from single cultures of infected normal or activated (*C. parvum*) Mϕ. After 11 months, *M. leprae* growth occurred in all recipients of 5×10^3 and 5×10^2 organisms. Titration of 5×10^1 bacilli from normal or activated Mϕ yielded positive growth in 9 of 10 and 2 of 10 mice, respectively. When the titrated inoculum was 5×10^0, growth was seen in 8 of 10 recipients of bacilli from normal Mϕ but only 1 of 10 mice became infected when injected with *M. leprae* from activated Mϕ. Almost 1 year was required to complete these expensive, labor-intensive studies, yet the number of variables explored was limited to a single *M. leprae*-infected normal Mϕ monolayer and a single *M. leprae*-infected activated Mϕ monolayer.

In other studies, we used the mouse footpad technique and showed that mice with potent populations of activated Mϕ or mice treated locally with immunopotentiators were markedly resistant to footpad infection with *M. leprae* (16, 17). Others have used the uptake of radiolabelled markers by *M. leprae* in Mϕ as a means of quantitating metabolism and studying the effects of antileprosy drugs in vitro (22, 28), but we were unable to obtain satisfactory results with these procedures (unpublished results). More recently, we studied, in vitro, the fusion of Thoriasol-labelled secondary lysosomes with phagosomes containing live *M. leprae* in infected mouse Mϕ (36). Activated Mϕ clearly had a higher percentage of fused vacuoles than did normal Mϕ.

In the present study, we evaluated five alternative techniques and examined the in vitro effects of activated Mϕ on *M. leprae*. Our approach to this goal has a marked advantage over previous attempts to study Mϕ and *M. leprae* in vitro. Only freshly harvested bacilli, obtained from the infected athymic mouse footpad, were used in each experiment. This resource provides a reliable source of large numbers of leprosy bacilli, which has allowed us to refine harvest and purification procedures (11, 36) that yield highly viable organisms relatively free of host tissues.

We are least confident of our results obtained with the FDA-EB stain. Our results suggest that activated Mϕ have a deleterious effect on the enzyme activity and membrane integrity of *M. leprae*. However, a cautionary note must be emphasized in interpretation of these FDA-EB data. As described originally (21), FDA-EB staining patterns accurately measured the viability of cultivable mycobacteria (*M. smegmatis* and *M. phlei*), but the relationship between staining and viability in *M. leprae* is more difficult to ascertain. Host tissue components appeared to complicate FDA-EB staining of *M. leprae*, and there was no correlation between FDA-EB viability estimates and those obtained by the morphological index (19). Moreover, Katoch et al. (14) described no correlation between the effects of chemotherapy on FDA-EB staining and bacillary ATP content. In addition, whereas repeated freeze-thawing of armadillo-derived *M. leprae* had little effect on viability as judged by FDA-EB staining (23), this treatment markedly reduced viability as judged by growth in the mouse footpad (27) and ATP content (9a).

The quantitation of the intracellular ATP content of *M. leprae* represents a more objective measure of viability, and this technique has been used as an indicator of the metabolic status of *M. leprae* harvested from armadillos (20) and from human biopsies after drug treatment (14). ATP content has also been used as a rapid in vitro metabolic screen for antileprosy compounds (11) and to ascertain the suitability of potential in vitro culture media (10). Our own findings that *M. leprae* released from activated Mϕ possess far less bacterial ATP than bacteria from control Mϕ suggest loss of viability.

In vitro metabolism of *M. leprae* was assessed by two methods. The first method examined an anabolic pathway and measured the incorporation of radiolabelled PA into the biochemically unique and immunologically specific product of *M. leprae*, PGL-1. In our previous studies (29, 30), we had demonstrated that only viable *M. leprae* are capable of synthesizing PGL-1, while no biosynthesis of PGL-1 could be detected in noninfected Mϕ, Mϕ infected with formalin-killed *M. leprae*, or Mϕ containing live bacteria and treated with the microbicidal drug rifampin or other antimicrobial compounds (30). The advantage of this assay lies in the biochemically and immunologically unique identity that PGL-1 has for *M. leprae* (3, 13). Clearly, the metabolism of *M. leprae* is markedly curtailed when the organisms reside in activated Mϕ. Both [^{14}C]- and [^{3}H]PA can be used in this assay, [^{3}H]PA being less expensive, routinely providing higher counts, and requiring only 5 days of incorporation as opposed to the 7 days needed when [^{14}C]PA is used. The disadvantages of the PGL-1 assay relate to its requirement for flasks of Mϕ monolayers. This requirement for very large numbers of Mϕ and the tedious PGL-1 extraction procedure limit the number of variables that can be explored in an experiment.

The second method evaluated *M. leprae* metabolism by measuring bacterial oxidation of [^{14}C]PA to $^{14}CO_2$. Because individual monolayers on coverslips are used, a number of variables can be explored with a single experiment. Although this catabolic pathway is not species specific, its detection is rapid, sensitive, and relatively easy for determining the viability of pure cultures of *M. leprae* (8, 9). We exploited these observations to study the effects of activated Mϕ on the leprosy bacillus. The results obtained with both the BACTEC 460 (9) and Buddemeyer (8) methods convincingly suggest that activated Mϕ cause a profound loss in viability of *M. leprae*. Since *M. leprae* organisms were released from the Mϕ after only 24 h, these techniques are clearly able to detect a rapid harmful effect of activated Mϕ on the leprosy bacillus.

The apparent deleterious ex vivo effects of *C. parvum*- and *T. gondii*-activated mouse Mϕ on *M. leprae* are consistent with the nonspecific microbicidal function of the activated Mϕ against a broad spectrum of phylogenetically diverse intracellular pathogens (18). In contrast to these results with human Mϕ, a number of previous reports have demonstrated a role for the enhanced microbicidal capacity of the activated mouse Mϕ in in vitro interaction with various pathogenic mycobacteria, including *M. tuberculosis* (25, 31), *M. bovis* BCG (7, 31, 41), *M. microti* (44), and *M. avium* (1).

The experimental conditions used in the present studies were designed to explore the ability of the activated Mϕ to cope with relatively few bacilli (i.e., <10/Mϕ), a situation more relevant to host resistance to infection or the tuberculoid form of leprosy. These conditions do not approach those we used previously to obtain Mϕ so heavily burdened with *M. leprae* that they were refractory to activation by MuIFN-γ (37–40).

Rather than study the inability of the Mϕ to cope with *M.*

leprae, as in human LL or experimental infection of the *nu/nu* mouse, we chose here to explore in vitro the role the Mϕ may play in resistance to establishment of *M. leprae* infection or the role the Mϕ may play in the events culminating in formation of the epithelioid cell granuloma and elimination of local bacilli in indeterminate or tuberculoid leprosy. On the basis of five different in vitro methods used to measure the metabolism of *M. leprae*, we conclude that, in contrast to normal Mϕ, activated murine Mϕ have a marked deleterious effect on the leprosy bacillus. Whether this effect is microbicidal or microbistatic is an important question and is currently under investigation. Moreover, our adaptation of radiorespirometry methods to the present study demonstrates that the number of Mϕ required per sample can be scaled down to allow us to use human peripheral blood-derived mononuclear phagocytes and determine whether the effects of the differences in response to IFN-γ among species described above for other mycobacteria hold as well for *M. leprae*.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Julie Loesch, J. P. Pasqua, Ken White, and Anita Biswas and the clerical help of Penne Cason.

This work was supported by Public Health Service grant AI-22442 from the National Institutes of Health.

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