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Growth of *Mycobacterium lepraemurium* in Nonstimulated and Stimulated Mouse Peritoneal-Derived and Bone Marrow-Derived Macrophages In Vitro

J. ALEXANDER* AND C. C. SMITH

National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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Mycobacterium lepraemurium cells were found to multiply in normal mouse peritoneal-derived and bone marrow-derived macrophages in vitro. Whereas activated peritoneal-derived macrophages demonstrated marked bacteriostasis for *M. lepraemurium*, significant bactericidal activity was exhibited by activated bone marrow-derived macrophages. However, only a small proportion of the bacteria were killed by activated bone marrow-derived macrophages with subsequent and enhanced bacterial growth. It is suggested that a rapid turnover of monocytes in active lesions is required to control mycobacterial infections in vivo. These results would suggest that careful consideration be given to the choice of the host cell in studies involving obligate intracellular parasites.

Effective immune responses against intracellular parasites are believed to be largely cell mediated (27). The carriers of this immunity are thought to be T lymphocytes (23). These cells, on encountering foreign antigen, are believed to release soluble products, lymphokines (15), which will not only induce an influx of mononuclear phagocytes into the region (7) but also increase the phagocytic and killing ability of surrounding macrophages (20).

Most in vitro studies on the interaction of intracellular organisms with their host cells have hitherto used peritoneal macrophages. Activated peritoneal macrophages have been shown to kill *Listeria monocytogenes* (25) and *Leishmania donovani* (22), but only to inhibit the growth of *Mycobacterium tuberculosis* (26) and *Toxoplasma gondii* (28), although antiserum-coated *T. gondii* (18) but not *M. tuberculosis* (2) are killed in these cells. Activated peritoneal macrophages from natural host animals were found to have no effect whatsoever on the growth of *Leishmania entrettii* or *Leishmania tropica* (3).

The mononuclear cells which accumulate at sites of inflammation are not, however, resident tissue macrophages such as those in the peritoneum but are derived from peripheral blood monocytes which, in turn, are derived from bone marrow precursors (29). Peripheral blood monocytes or their precursors, being relatively undifferentiated, would be expected to have more potential to respond to stimuli than peritoneal macrophages, and recent studies have cast doubt on the bone marrow origin of peritoneal macrophages (13, 29).

The study reported here was undertaken to compare the growth of *M. lepraemurium* in activated bone marrow-derived and peritoneal-derived macrophages. Macrophages were activated by culturing directly with lymphocytes or by incubation in mixed lymphocyte culture supernatants (MLC) or supernatants collected from lymphocytes incubated with *M. lepraemurium* antigen (LAC). The rate of destruction of dead (subjected to 100 Krads from a ^{60}Cobalt source) *M. lepraemurium* in these two cells types was also examined.

MATERIALS AND METHODS

Mice. Inbred BALB/c, C57Bl and CBA mice and outbred Parkes mice bred at the National Institute for Medical Research were used.

M. lepraemurium. The Douglas strain of *M. lepraemurium* was maintained by serial passage of 10^9 bacilli intravenously in Parkes outbred mice.

Preparation of *M. lepraemurium* for infecting cultures. *M. lepraemurium* cells were harvested from the livers of Parkes mice and partially purified by the method described by Draper (14). Acid-fast bacilli, stained by the Ziehl-Neelsen technique, were counted by the method of Hart and Rees (17).

Preparation of soluble *M. lepraemurium* antigen. Suspensions of purified bacteria in saline were disrupted by ultrasound. Cell walls and unbroken bacteria were removed by centrifugation (35,000 × *g* for 30 min), and protein estimation of the supernatant was carried out by the method of Lowry et al. (19).

Peritoneal-derived macrophage cultures. Macrophages were obtained from the peritoneal cavities of BALB/c or C57Bl mice. Animals killed by cervical dislocation were inoculated intraperitoneally with 3 ml of ice-cold NCTC 109 (Grand Island Biological Co. [GIBCO] Bio-Cult, Glasgow, Scotland) containing

heparin and penicillin at 5 and at 100 U/ml, respectively. Cells collected from 12 mice were pooled and counted in an Improved Neubauer haemacytometer and adjusted to 4×10^6 cells per ml. Volumes (1 ml each) were incubated in Leighton tubes for 2 h at 37°C, after which nonadherent cells were removed. The cultures were maintained at 37°C in Chang medium (11) containing 40% heat-inactivated horse serum (GIBCO Bio-Cult).

Bone marrow-derived macrophages. Femora from C57Bl or BALB/c mice were excised, and both ends were cut off to expose the marrow cavities. Marrow contents were washed out with 1 ml of ice-cold NCTC 109 containing heparin and penicillin at 5 and at 100 U/ml, respectively. Pooled bone marrow cells were incubated in 1-ml volumes overnight in 50 Leighton tubes at 37°C, in NCTC 109 containing 40% horse serum. Nonadherent cells were removed by washing, and cultures were subsequently maintained at 37°C in Chang medium containing 40% heat-inactivated horse serum.

Infection of macrophage cultures with *M. lepraemurium*. Cultures (7 days old) were infected with a freshly prepared suspension of *M. lepraemurium*. Each culture was infected by the addition of 10^8 *M. lepraemurium*, counted as acid-fast bacilli, in 0.1 ml of saline for 2 h at 37°C, after which unphagocytosed bacteria were removed by washing. Chang medium was then added, and it was changed every 10 days. Some experiments were undertaken to study the fate of killed *M. lepraemurium* in macrophages. Bacteria were subjected to 100 Krads from a ^{60}Co source and were then used to "infect" macrophage cultures as described above.

After infection, and at 10-day intervals, bacterial numbers were assessed by harvesting cultures in quadruplicate. The culture medium was removed and replaced with 1 ml of 0.1% (wt/vol) albumin in distilled water. Macrophages were disrupted by ultrasonic vibration (Luziesa 800 KHz ultrasonic generator and transducer), and the liberated bacteria were counted.

Lymphocyte culture supernatants. Spleens were dissected from uninfected mice or mice that had been infected with 10^7 *M. lepraemurium* in the footpad 8 weeks previously. The spleens were forced through a fine wire mesh into ice-cold NCTC 109 containing heparin and pencillin at 5 and at 100 U/ml, respectively. Large pieces of tissue were allowed to sediment, and the remaining supernatant was centrifuged at 250 $\times$ *g* for 10 min. Red blood cells were lysed with 0.83% ammonium chloride in tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.4 (9). Cells were washed in NCTC 109 and suspended in NCTC 109 containing 10% horse serum and 5 μg of soluble *M. lepraemurium* antigen per ml, the final concentration of lymphocytes being 2×10^6 to 3×10^6/ml. Viability, as measured by trypan blue exclusion, was >90%. After 48 h at 37°C, the cells were centrifuged, and the LAC supernatants were collected and stored at −20°C.

MLC supernatants were prepared from 8-week-old normal C57Bl and CBA mice. Spleen lymphocytes were harvested as described above, and cells of allogeneic strains were mixed in equal numbers and maintained in NCTC 109 with 10% horse serum at 37°C for 5 days before the supernatants were harvested. Control cultures contained 2×10^6 C57Bl or CBA lymphocytes per ml (autologous lymphocyte culture [ALC] supernatant).

Stimulation of macrophage cultures. Infected macrophage cultures were stimulated by adding 0.1 ml of MLC or LAC supernatant or by adding 2×10^5 lymphocytes from syngeneic *M. lepraemurium*-infected mice directly to cultures.

Experimental design. Normally, 48 bone marrow-derived and a similar number of peritoneal-derived macrophage cultures were used. Bacilli from four Leighton tube cultures of each macrophage type were harvested to provide a base line of *M. lepraemurium* uptake. The remaining cultures were divided into three groups: (A) 16 MLC, LAC, or sensitized lymphocyte-stimulated cultures; (B) 12 cultures containing appropriate control supernatants or normal lymphocytes; (C) 16 untreated cultures. At 10-day intervals, *M. lepraemurium* cells were harvested from four cultures of each group and counted while medium and supernatants or lymphocytes were replaced in the remaining cultures. At day 20, four group A cultures had their stimulating supernatants or lymphocytes removed, whereas four group C cultures had fresh MLC or LAC supernatants or lymphocytes added at this time.

RESULTS

All experiments were carried out by using cultured macrophages from BALB/c and C57Bl mice. Because results obtained from both mouse strains were reproducible, representative experiments involving only BALB/c mice are reported.

Effect of MLC supernatants on the growth of *M. lepraemurium* in peritoneal- and bone marrow-derived macrophages. The growth of *M. lepraemurium* in peritoneal-derived macrophages incubated in MLC supernatants was markedly inhibited compared with *M. lepraemurium* grown in ALC-treated and untreated cultures (Fig. 1A). Removal of MLC supernatant from group A cultures at day 20 resulted in renewed *M. lepraemurium* growth, whereas treatment of group C cultures at day 20 with MLC supernatant significantly inhibited further bacillary multiplication.

In bone marrow-derived macrophages (Fig. 1B), a significant reduction in *M. lepraemurium* was recorded in group A cultures over the first 20 days of infection but, thereafter, greatly enhanced mycobacterial multiplication was noted, whether MLC supernatant was maintained in cultures or not. Those group C cultures incubated in MLC supernatant from day 20 were found to have fewer recoverable *M. lepraemurium* at day 30 than at day 20. Group B and untreated group C cultures demonstrated *M. lepraemurium* growth for the duration of the experiment.

Effect of sensitized lymphocyte overlays

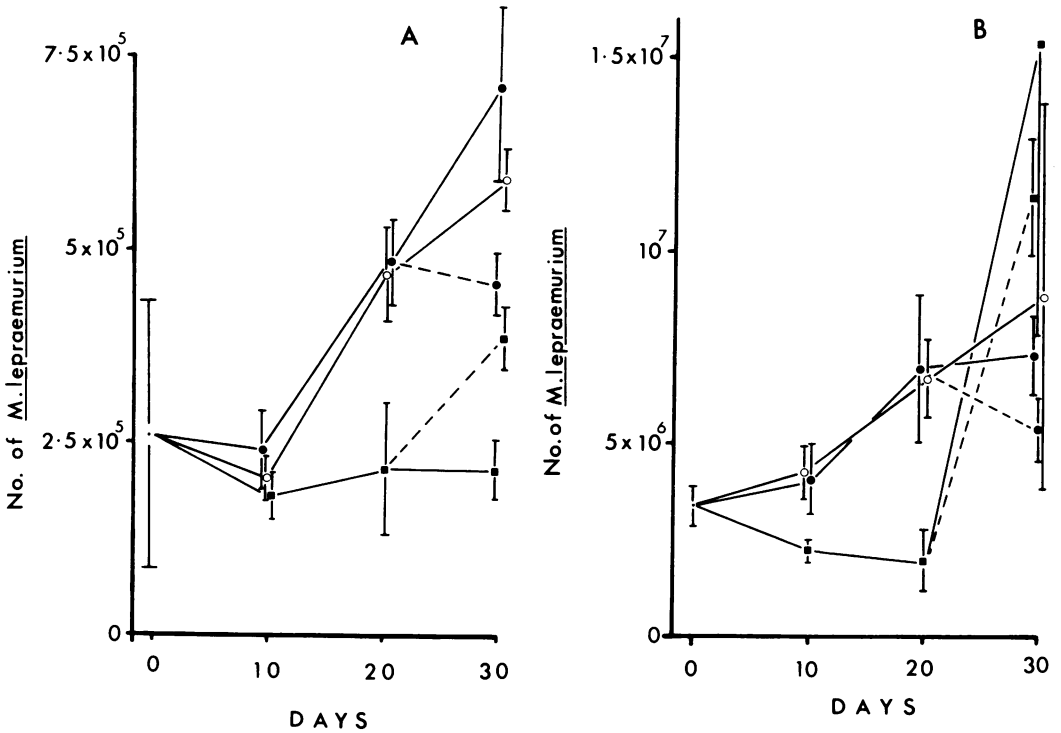

FIG. 1. *Growth of M. lepraemurium in MLC supernatant-stimulated, peritoneal-derived (A) and bone marrow-derived (B) macrophages. Each value represents the mean of four cultures ± standard deviation. Symbols: ●——●, untreated macrophage cultures; ■——■, MLC supernatant-cultured macrophages; ○——○, ALC supernatant-cultured macrophages; ●- - -●, untreated macrophage cultures treated with MLC supernatant at day 20; ■- - -■, cultures washed free of MLC supernatant at day 20.*

and LAC supernatants on the growth of *M. lepraemurium* in cultured peritoneal and bone marrow-derived macrophages. LAC supernatants and overlays of sensitized lymphocytes were found to produce prolonged inhibition of *M. lepraemurium* growth in peritoneal macrophages (Fig. 2A). Control supernatants and normal lymphocytes, although producing inhibition of *M. lepraemurium* growth compared with untreated cultures, were significantly less efficient in stimulating peritoneal macrophage inhibition of *M. lepraemurium* growth than cultures with sensitized lymphocytes or their supernatants. Results were not recorded after day 20, owing to a shortage of healthy cultures.

In bone marrow-derived macrophages, LAC supernatants and lymphocytes, whether from infected or noninfected donor mice, produced a short-term but significant drop in *M. lepraemurium* numbers (Fig. 2B). After day 20, marked bacillary multiplication became evident in these cultures. In untreated group C cultures, growth continued unhindered over the course of the experiment. If these group C cultures were overlaid with lymphocytes or incubated in LAC supernatant from day 20, a significant fall in recoverable *M. lepraemurium* was recorded at day 30.

Degradation of γ-irradiated (100 Krads) *M. lepraemurium* in stimulated and nonstimulated bone marrow- and peritoneal-derived macrophages. MLC supernatant stimulation of bone marrow-derived macrophages significantly increased the rate of degradation of killed *M. lepraemurium* compared with nonstimulated cells over a 20-day period (Fig. 3). Over a similar period of time, MLC supernatant-stimulated, bone marrow-derived macrophages demonstrated greater powers of degradation of killed *M. lepraemurium* than similarly stimulated peritoneal-derived macrophages (Fig. 4).

DISCUSSION

Although *M. lepraemurium* grows equally well in untreated, in vitro-cultured, peritoneal-derived and bone marrow-derived macrophages, its fate in each of these cell types after stimulation appears to be quite different. Whereas peritoneal macrophages have the capability to limit bacterial growth for extended periods, bone marrow-derived macrophages can significantly re-

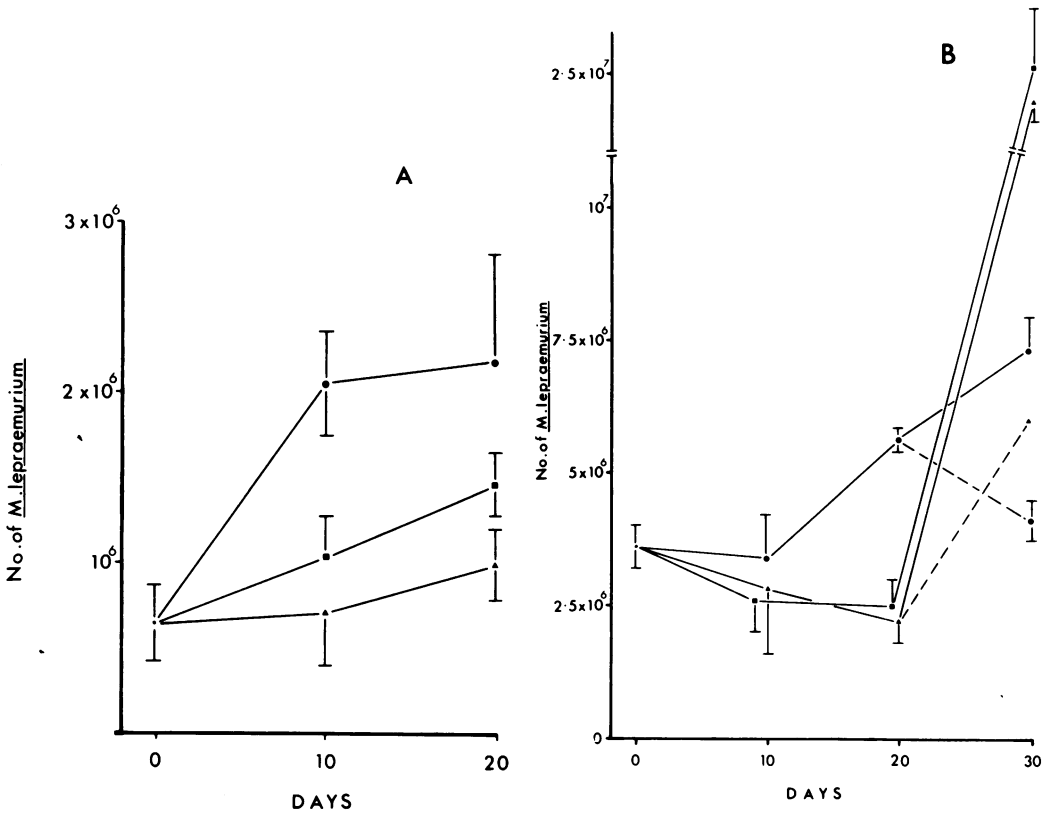

FIG. 2. *Growth of M. lepraemurium in peritoneal-derived (A) and bone marrow-derived (B) macrophage cultures overlaid with lymphocytes and 5 μg of M. lepraemurium sonically isolated soluble antigen. Each value represents the mean of four cultures ± standard deviation. Symbols: ●——●, untreated macrophage cultures; ■——■, normal lymphocyte overlay; ▲——▲, sensitized lymphocyte overlay; ●- - -●, untreated cultures overlaid with sensitized lymphocytes on day 20; ▲- - -▲, cultures washed free of sensitized lymphocytes at day 20 (only one culture available in this group).*

duce bacillus numbers for a short period. *M. lepraemurium* is highly resistant to degradation, as demonstrated by the relatively slow disappearance of killed bacilli from macrophage cultures. Degradation is markedly accelerated, however, in activated bone marrow-derived macrophages.

The information provided by these results would certainly imply that bone marrow-derived macrophages are functionally different from those resident in the peritoneum. Whereas resident tissue macrophages, as found in the peritoneum, have already differentiated in vivo, macrophages from the bone marrow still have the potential to differentiate in vitro. The relative immaturity of bone marrow-derived macrophages is indicated by their rapid division and their acquisition, after 3 to 5 days, of Fc and C3 plasma membrane receptors (R. Vasquez and J. Alexander, unpublished data). Although Daems et al. (13) and Volkman (29) have suggested that resident peritoneal macrophages may not be bone marrow derived, this has been contested by other workers (8). Because bone marrow-derived macrophages are found to have granules containing peroxidase even after 5 weeks in culture (J. Alexander, unpublished data), and peritoneal-derived macrophages never possess such organelles, the relationship between these two macrophage types must remain somewhat obscure.

Peritoneal macrophages were found to inhibit *M. lepraemurium* growth to a greater or lesser extent, depending on the mode of stimulation; overlaying peritoneal macrophage cultures with normal lymphocytes inhibited *M. lepraemurium* growth significantly compared with untreated controls or ALC supernatants but less than MLC supernatants or lymphocytes from *M. lepraemurium*-infected mice. The ability of activated peritoneal macrophages to inhibit mycobacterial growth has been reported previously in experi-

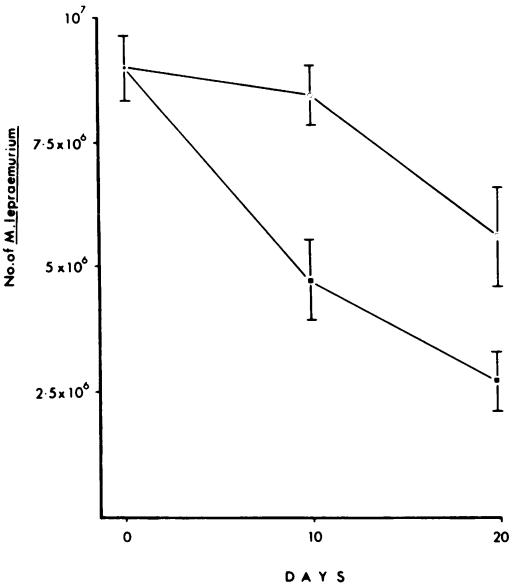

FIG. 3. *Rate of disappearance of killed M. lepraemurium (100 Krads; ^{60}Co) in MLC supernatant-stimulated (■——■) and unstimulated (○——○), bone marrow-derived macrophages. Each value represents the mean of four cultures ± standard deviation.*

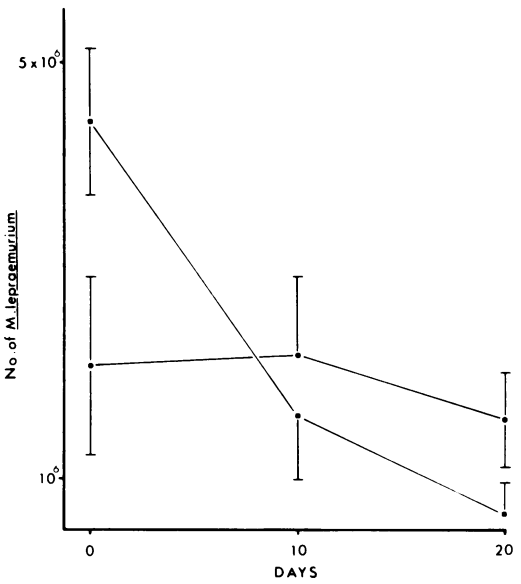

FIG. 4. *Rate of disappearance of killed M. lepraemurium (100 Krads; ^{60}Co) in MLC supernatant-stimulated peritoneal-derived (●——●) and bone marrow-derived (■——■) macrophages. Each value represents the mean of 4 cultures ± standard deviation.*

ments involving *M. tuberculosis* (10, 24, 26, 28) and *M. lepraemurium* (U. Sengupta, unpublished data).

Activation of bone marrow-derived macrophages appears to be something of an "all or nothing" response with "normal," as well as sensitized lymphocytes and LAC and MLC supernatants, but not ALC supernatants, inducing a short-term reduction in *M. lepraemurium*. This is the first time, to our knowledge, that bactericidal potential, as opposed to merely bacteriostatic potential, has been demonstrated against mycobacteria by macrophages. Although Godal et al. (16) studied the growth of *M. lepraemurium* in MLC supernatant-activated rabbit peripheral blood monocyte-derived macrophages and demonstrated marked inhibition of mycobacterial growth, they did not observe any bactericidal activity. Because these workers maintained infected cultures for only 10 days, and degradation of killed *M. lepraemurium* is a slow process, further observations may have revealed a significant reduction in mycobacterial numbers.

Bactericidal activity is not maintained in our cultures after day 20, and renewed supergrowth of *M. lepraemurium* is noted thereafter. A similar pattern of short-term killing followed by growth, although not supergrowth, has been described for *L. monocytogenes* in activated human peripheral monocyte-derived macrophages (4). This work suggests that renewed growth of *L. monocytogenes* could be due to in vitro exhaustion of the cellular bactericidal mechanisms, or perhaps the existence of a susceptible subpopulation of macrophages (5), or even a resistant bacterial subpopulation. Published evidence would suggest that macrophages could have limited killing ability in vivo, yet still promote bacterial clearance. Thus, Ando and Dannenburg (1) have demonstrated that blood monocyte-derived macrophages rapidly enter and die in tuberculosis lesions and that the quicker the turnover the more effective the killing of *M. tuberculosis*. Similarly, a rapid turnover of macrophages in *M. lepraemurium* lesions in mice is associated with the disappearance of acid-fast bacilli (12). Blanden et al. (6) have recently suggested that cytotoxic lymphocytes could lyse macrophages infected with mycobacteria in the same way that they lyse virus-infected macrophages. Not only could such a mechanism be easily tested, but its occurrence would promote macrophage turnover and lead to increased mycobacterial clearance. Increasing the lymphocyte concentration in culture during these experiments tended to result in macrophage lysis.

The superiority of bone marrow-derived macrophages over peritoneal-derived macrophages under certain experimental conditions has also

been demonstrated by Meerpohl et al. (21). These workers have shown that bone marrow-derived macrophages are more cytotoxic for tumor cells than are peritoneal-derived macrophages. What these and our results indicate is that it is extremely important when using in vitro techniques to determine which cells are actively involved in vivo and to utilize these in subsequent in vitro experiments.

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