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The Effect of Exogenous Peroxidase on the Evolution of Murine Leprosy[1]

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Granuloma is the distinctive primary lesion of leprosy and tuberculosis. Mycobacteria-induced granulomas are classified as "reactive" or "non reactive," depending on the immunological status of the lesion. Reactive granulomas are made up of activated, macrophage-derived epithelioid cells, and large numbers of lymphocytes, mostly CD4+ Th1 cells. Fused epithelioid (giant or Langhans') cells are also often found in the reactive granulomas of tuberculosis and tuberculoid leprosy.

Non-reactive granulomas (the ones seen in lepromatous leprosy) consist of bacilli-laden macrophages intermixed with low numbers of CD4+ lymphocytes. The early studies on the lymphocytes found in the lesions of lepromatous leprosy, showed that they were predominantly CD8+ (suppressor) cells, with only a minor fraction of lymphocytes being CD4+ cells ([28, 44]). Now it is known that the predominant CD4+ T cell population in the lesions of lepromatous leprosy belong to the Th2 subpopulation ([27, 41]).

Immunological activation of macrophages enables them to kill intracellular parasites, including mycobacteria. This is the case with tuberculoid leprosy where granulomas are scarce in bacilli. In tuberculosis, the situation seems to be somehow different, because despite the activated status of macrophages, bacilli in the lesions are always present, albeit in variable stages of integrity. The excessive activity of cytotoxic cells and cytokines, and the release of hydrolytic enzymes from the killed parasitized macrophages are recognized as the cause of tissue damage in tuberculosis ([4, 5]). *Mycobacterium tuberculosis* resists the activated state of macrophages because it has developed diverse evasive mechanisms that assure its intracellular survival ([3, 7, 10, 14, 29, 43, 45]). This and other pathogenic mycobacteria, however, may eventually succumb to the deleterious effect of macrophages that have become activated through the mechanisms of cell-mediated immunity ([31, 35]).

Lepromatous leprosy, on the other hand, is a disease characterized by the gradual loss of the host's cell-mediated immune response to the antigens of *M. leprae.* In the absence of this form of immunity, macrophages do not become activated. Activation of macrophages involves a series of biochemical changes, some of which are related to these cells' ability to produce reactive oxygen and nitrogen intermediaries (ROI and RNI) in response to several stimuli. One of the most important oxygen intermediaries is hydrogen peroxide (H_2O_2) due to the fact that apart from being bactericidal in itself, it is a key participant in the myeloperoxidase (MPO)-halide microbicidal system, one of the strongest bactericidal mechanisms of phagocytic cells. The MPO-H_2O_2-halide system is of prime importance for the microbicidal activity of polymorph nuclear (PMN) neutrophilic leukocytes ([9, 15, 16]). These cells contain large amounts of MPO (at least 5% of their cellular mass) in their primary (azurophil) granules ([38]). During phagocytosis, these granules fuse with the endocytic vacuoles in which they discharge their contents to give rise to the phagolysosomes or digestive vacuoles. Within the acidic phagolysosomes the ingested microorganisms are first

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killed, then broken apart, and finally digested. Killing of microorganisms depends on both oxygen-dependent and oxygen-independent microbicidal mechanisms. Oxygen-dependent microbicidal mechanisms include the participation of free radicals (superoxide, hydroxyl ions, oxygen singlet, and hydrogen peroxide) and other substances such as MPO. Hydrogen peroxide, the MPO substrate, is transformed by the enzyme into unstable oxidant intermediaries, which avidly bind halides (Cl^-, I^- or Br-), transforming themselves into lethal halogenating bullets. The whole set of oxygen-derived intermediaries are responsible for the oxidative alteration or disruption and halogenation of target molecules on the ingested microorganism; oxidation and halogenation of key (vital) microbial molecules provoke irreversible changes that eventually lead to microbial death ([15, 37, 40]).

Contrary to neutrophils, macrophages, the definitive cellular hosts for mycobacteria, lack granular MPO although they do contain small amounts of the enzyme both around the nucleus and also associated to the cell reticuloendoplasma ([38]). The amount of MPO in these cells represents, however, only a minor fraction of the MPO found in PMNs. *In vitro,* both *M. tuberculosis* and *M. leprae* are susceptible to the microbicidal effect of the MPO-H_2O_2-halide system ([1, 12, 17]), however, the deleterious effect of this system on *M. tuberculosis* has not been corroborated *in vivo.* In leprosy, several early pieces of evidence suggested a role for MPO in the pathogenesis of the disease. Gomez-Estrada, *et al.* ([11]), and Maslov and Juscenko ([23]), for instance, described the granulomas of lepromatous leprosy to be made of bacilli-free MPO-positive cells and bacilli-laden MPO-negative macrophages.

Similar observations were reported by McKeever, *et al.* ([26]), in the lepromatous lesions of *M. leprae*-infected armadillos, and by Rojas-Espinosa ([33]) in the lepromatous granulomas of murine leprosy. Mature granulomas of murine leprosy appeared to be made up of a core of highly parasitized, MPO-negative macrophages, surrounded by a multiple layer of MPO-positive, bacilli-free leukocytes (Fig. 1). These observations made us wonder on the fate of the intracellular bacilli in the case that

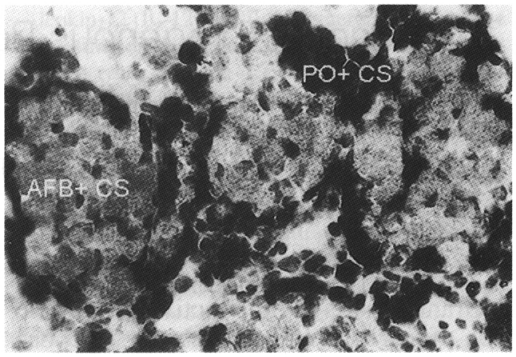

FIG. 1. Three coalescing-granulomas in the liver of a mouse bearing a three-month infection with *Mycobacterium lepraemurium.* The granulomas show a core of bacilli-laden macrophages lacking myeloperoxidase, surrounded by a layer of bacilli-free heterogeneous cell population, rich in myeloperoxidase. Acid-fast bacilli appear as a granular material in the core macrophages while peroxidase activity stains dark the peripheral cells (Peroxidase, Ziehl Neelsen and Harris' hematoxylin stains, 40×).

macrophages contained MPO. On this basis we infected mice with intact *Mycobacterium lepraemurium* or horseradish peroxidase-coated *M. lepraemurium,* and followed the progress of the infection under the two circumstances. In this study we used horseradish peroxidase (PO) first, because in a preliminary study (made only at 2.5 months of infection) we got similar results when using human neutrophil-extracted MPO or HRPO, secondly, because getting MPO from leukocytes is a less practical approach than just buying HRPO, and thirdly, because the enzymatic effect of HRPO and MPO on H_2O_2 cannot be distinguished from each other; they both promote the generation of oxygen-derived reactive intermediaries.

MATERIALS AND METHODS

Chemicals. Unless otherwise specified, chemicals and reagents were purchased from Sigma-Aldrich Chemical, St. Louis, Missouri, U.S.A.

Mice. Female, pathogen free, NIH mice were used in this study. Mice were infected with the Hawaiian strain of *M. lepraemurium* isolated from peritoneal lepromas freshly excised from mice bearing a six-month infection with the microorganism (see below).

Purification of *M. lepraemurium.* This was accomplished by combining the whole procedure of Prabhakaran ([30]) and the Percoll step of Draper's method ([6]), in that order. The procedure has been fully described in a previous communication ([34]).

Infection. Three groups of 16 mice were formed; one group was injected with 5×10^7 intact bacilli, by the intraperitoneal (i.p.) route; a second group was injected i.p. with 5×10^7 bacilli previously coated with horseradish peroxidase, and a third group was kept as a control, non-inoculated group.

Peroxidase coating of bacilli. Coating of *M. lepraemurium* with horseradish peroxidase (PO) was carried out according to Klebanoff and Shepard ([17]) by suspending 1×10^9 bacilli in 1.0 ml of phosphate buffer, pH 7.0, containing 500 units of PO type VI. The bacillary-PO mixture was then incubated at 37°C for 30 min. For injection, bacilli were collected by centrifugation (10,000 × g × 5 min), washed thrice with 1.0 ml of PBS, and then suspended in 10 ml of PBS (1×10^8 bacilli per ml). Control (PO-negative) bacterial suspensions were prepared as described, except that the washing was done immediately after the addition of PO to mycobacteria.

Sampling. At two-week intervals, mice from each group were tail-bled (about 0.2 ml of blood) and their sera collected and individually tested for the presence of antibodies to protein- and lipid-antigens of *M. lepraemurium.* Antibodies were taken as an index of infection due to the fact that their level parallels the extent of the disease: the higher the level, the higher the extent.

Mycobacterial protein antigens. Protein antigens were prepared from purified *M. lepraemurium* by sonicating (15 1-min pulses at 80 watts) a heavy bacterial suspension in a cell disruptor (Heat Systems, ultrasonic, model W370, New York, New York, U.S.A.). The sonicate was dialysed against a borate buffer (0.392 g boric acid and 0.4768 g sodium tetraborate per liter of 0.85% sodium chloride, pH 8.4), centrifuged to eliminate cell-debris (10,000 × g × 15 min), 0.2-μm-Millipore-sterilized, divided into 0.5 ml aliquots, and kept frozen (–20°C) until used. Protein solutions were prepared to contain 20 μg per ml in the borate buffer.

Mycobacterial lipid antigens. Lipid antigens were extracted from purified bacilli following the method of Folch, *et al.* ([8]), as described elsewhere ([36]). After solvent evaporation, the waxy lipids were frozen until used. Lipid solutions were prepared by dissolving 1.0 mg of solid lipids in 100 μl of chloroform, then diluting the lipid solution to 50 ml with absolute ethanol (2 μg of lipids per 100 μl of solution).

Antibodies detection. Antibodies were detected by enzyme-linked immunosorbent assays (ELISAs), using Maxisorp plates (Nunc, Roskilde, Denmark) and 2.0 μg per well of the protein antigens in 100 μl of borate buffer, or 2.0 μg of the lipid antigens in 100 μl of absolute ethanol. Protein-containing plates were tightly sealed and incubated for 2 hr at 37°C and then overnight at 4°C. Lipid-containing plates were incubated unsealed for 24 hr at 37°C until solvent evaporation.

The ELISAs were standard procedures which involved a) coating of the wells with antigen, b) washing once with 150 μl of 0.01 M phosphate-0.15 M saline buffer, pH 7.0 (PBS), c) blocking of the wells with 150 μl of 2% skimmed milk in the borate buffer, d) addition of 100 μl of the test sera (diluted 1:50 in PBS), e) washing thrice with PBS, f) incubation for 20 min with 100 μl of a second (rabbit) peroxidated-antibody to mouse immunoglobulins (pretitered and used 1:1500), g) washing (4×) with PBS, h) incubation for 20 min with 100 μl of the chromogenic reagent (4.0 mg of *o*-phenylene diamine and 10 μl of 30% hydrogen peroxide in 10 ml of 0.025 M citrate 0.05 M phosphate buffer, pH 5.4), i) arresting the reaction with 20 μl of 3N sulfuric acid, and j) reading the absorbencies at 492 nm against control wells with no sera. Sera were tested in triplicate.

Histopathologic studies. Three animals of each group were sacrificed at one, two, or three months of infection. From these animals the liver and the spleen were excised and processed for cryostat sectioning. Six-micron thick sections were prepared and stained for acid-fast bacilli by the Ziehl-Neelsen method, and for general histology using the Harris' hematoxylin stain.

Statistical analysis. The results comparing peroxidase-coated MLM (PO-MLM) *versus* control MLM (Fig. 2) were analyzed by using a one way ANOVA, while the results comparing the level of antibodies in

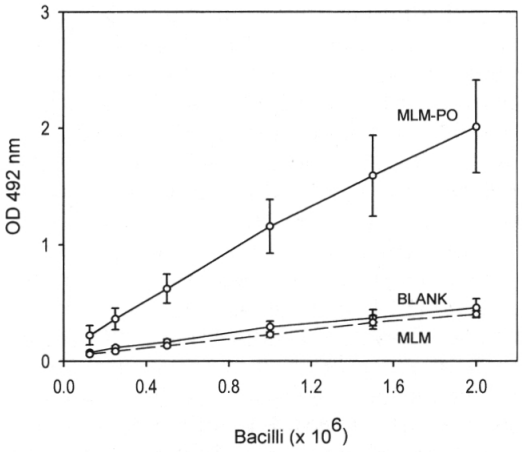

FIG. 2. Peroxidase activity of the PO-coated-(MLM-PO) and control-*Mycobacterium lepraemurium* (MLM) suspensions, as a function of the number of bacilli used in the assay. Curves differed from each other at the level of p = 0.001. When corrected by subtracting the blank curve, peroxidase activity in the control suspension became nonexistent.

the ML-infected *versus* the PO-MLM-infected mice (Figs. 3, 4) were analyzed by using a two way repetition ANOVA.

RESULTS

Peroxidase coating of bacilli. Success in the coating of bacilli was assessed by measuring the PO activity in both the PO-coated and the PO-control bacterial suspensions. Variable amounts of bacilli (from 0.1 to 2 × 10^6 microorganisms) were suspended in a fixed volume (100 µl) of PBS and incubated with 100 µl of the substrate-chromogen mixture (H_2O_2 and OPD). Then, the enzyme reaction was allowed to proceed for 20 min before stopping the reaction with sulfuric acid (see the ELISA assay). A PO solution, identical to the one used to prepare the PO-coated and PO-control bacteria was similarly processed ("washed" and diluted) and used to correct the optical density-readings. Fig. 2 shows the successful coating of the bacilli that were incubated with PO for 30 min at 37°C. The instantaneous contact of bacilli with peroxidase in the control suspension produced no detectable labeling. Curves prepared with the suspensions of PO-MLM and control MLM, differed from each other at the level of p <0.001.

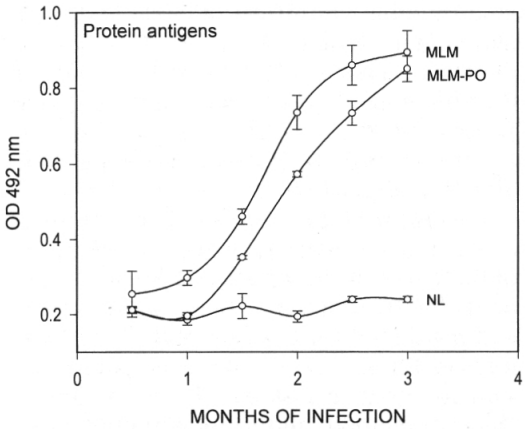

FIG. 3. Antibodies to protein antigens of *M. lepraemurium* in the serum of mice infected with PO-coated *M. lepraemurium* (MLM-PO) or control *M. lepraemurium* (MLM). In the experiment illustrated, the curves differed from each other at the level of p = 0.03; in other experiments the differences were even more significant (p = 0.05–0.001).

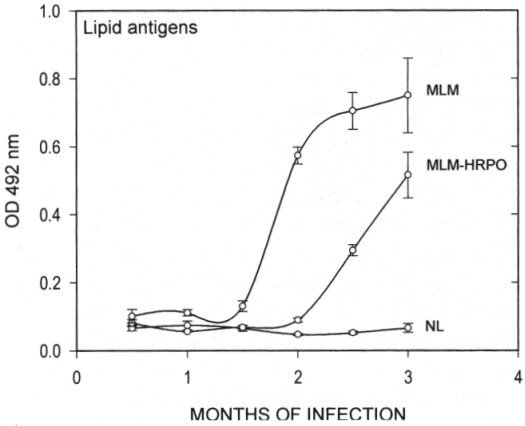

FIG. 4. Antibodies to lipid antigens of *M. lepraemurium* in the serum of mice infected with PO-coated *M. lepraemurium* (MLM-HRPO) or control *M. lepraemurium* (MLM). The two groups differed from each other at the level of p = 0.001.

Antibodies to protein antigens. The sera from both *M. lepraemurium*-infected groups showed antibodies to protein mycobacterial antigens, but these antibodies appeared at an earlier stage in the group of animals infected with control bacilli than in the group of mice infected with PO-bacilli. Also, the antibody levels were always significantly higher in the group of mice infected with control bacilli than in the group infected with peroxidated-*M. lepraemurium*. Normal, uninfected mice contained low levels of antibodies to mycobacterial proteins that did not significantly

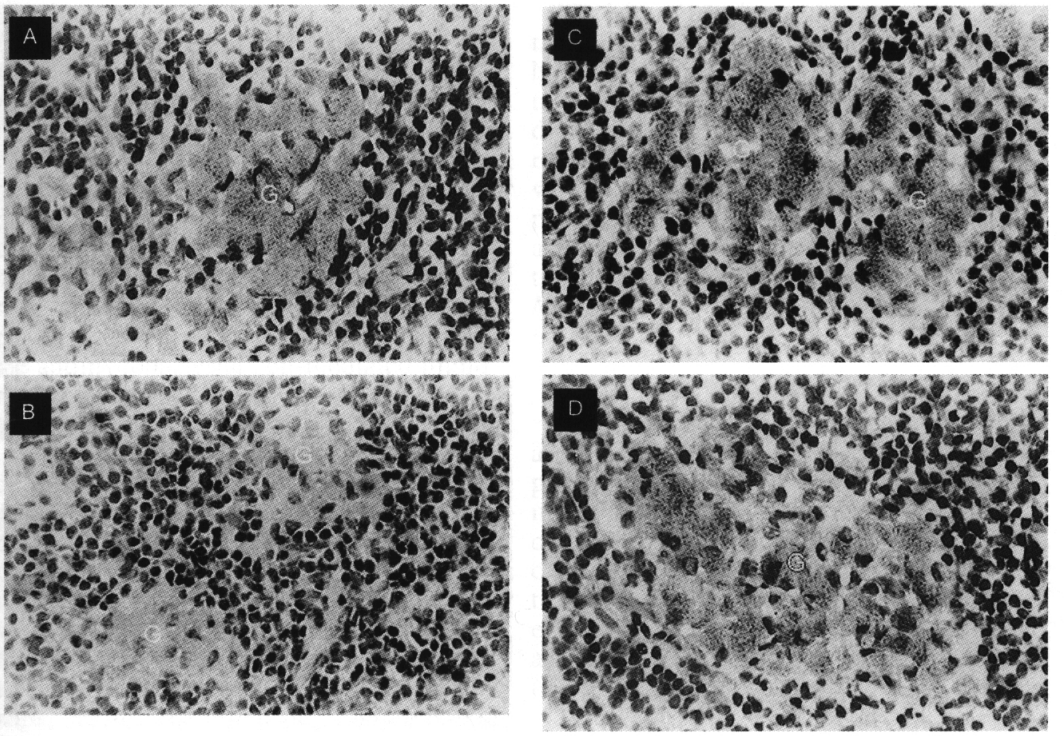

FIG. 5. Spleen sections of mice bearing a two-month infection with MLM (**A**) or MLM-PO (**B**), or a three-month infection with MLM (**C**) or MLM-PO (**D**). Although some differences are observed between **A** and **B** (larger and somehow more bacilliferous granulomas in the first case), a similar degree of infection is observed between **C** and **D** (large granulomas containing bacilli-laden macrophages) (Ziehl-Neelsen and Harris' hematoxylin stains, 400×).

change during the period of the study. Fig. 3 shows a representative result from one out of two independent experiments performed under similar conditions; the statistical analysis, comparing the behavior of the two experimental groups, indicated a difference that was significant at the level of $p < 0.039$.

Antibodies to lipid antigens. As with mycobacterial protein antigens, mice inoculated with control, non-peroxidated-*M. lepraemurium,* developed higher levels of antibodies to the lipid mycobacterial antigens than mice inoculated with peroxidated-*M. lepraemurium.* Fig. 4 illustrates a representative result from one out of two similar experiments. The antibody-concentration curves of the two experimental groups differed from each other at the level of $p < 0.001$. Again, uninfected mice developed only negligible amounts of anti-mycobacterial-lipid antibodies.

Histopathological changes. The histopathologic study was limited to the search for granulomas and acid-fast bacilli in the spleen and liver in the two experimental groups of mice. No well-defined alterations were observed in either group of animals at the end of the first month of infection, but significant differences were observed at the end of the second month. Although the observed changes were not exclusive to either group of mice, those animals infected with *M. lepraemurium* showed a predominance of better-defined granulomas and more bacilli than those mice inoculated with PO-*M. lepraemurium.* Nonetheless, at the time this experiment was suspended (three months), the animals in both experimental groups were no longer distinguishable from each other, neither on the basis of their antibody levels to protein antigens nor on the basis of their pathological alterations. The histological study of the liver of *M. lepraemurium*- or PO-*M. lepraemurium*-infected mice, as to the extent of the granuloma fraction and the number of bacilli, showed a similar degree of involvement These results are depicted in Fig. 5

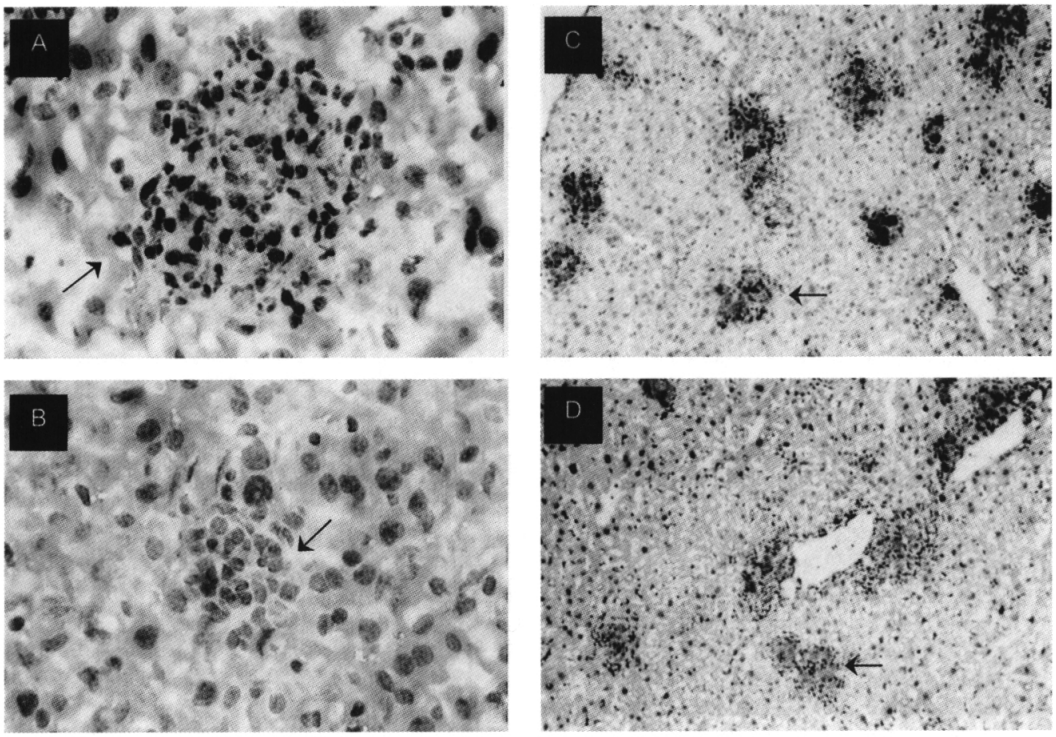

FIG. 6. Liver sections of mice bearing a two-month infection with MLM (**A**) or MLM-PO (**B**), and a three-month infection with MLM (**C**) or MLM-PO (**D**). Although some differences are observed between **A** and **B** (larger and somehow more bacilliferous granulomas in the first case), a similar degree of infection is observed between **C** and **D** (large granulomas containing bacilli-laden macrophages) (Ziehl-Neelsen and Harris' hematoxylin stains, **A** and **B** at 100×, **C** and **D** at 25×).

(and/or Fig. 6). Only one of the largest lesions found in the examined tissue-sections were photographed.

DISCUSSION

We interpret the results presented above, in the following manner. On intraperitoneal administration of *M. lepraemurium,* bacilli are picked up by the resident cell population of neutrophils and macrophages. Neutrophils, which contain MPO, might theoretically be able to ingest and kill mycobacteria, even if the bacteria were administered free of peroxidase ([2, 13]). However, due to the fact that PMN leukocytes are terminal, i.e., no longer dividing cells, these cells do not survive the phagocytic act and die, and in doing so, release the formerly ingested bacteria and much of their granular contents, including MPO. These materials, including intact and surviving bacteria, are then picked up by macrophages, within which the microorganisms eventually lodge. In the presence of minute quantities of proinflammatory cytokines, young macrophages become activated, divide at least once, and change their metabolism and morphology, acquiring first the characteristics of epithelioid cells and then the characteristics of giant, multinucleated, cells (Dannenberg, *et al.,* 1975). Macrophages will also take the bacilli to other sites, such as the skin, liver, spleen and bone marrow, allowing dissemination of the disease.

The *in vivo* ingestion of the bacteria by macrophages may trigger in these cells mechanisms leading to production of microbicidal metabolites, including hydrogen peroxide ([15, 40]). In the presence of exogenous peroxidase (the one coating the bacillus), *M. lepraemurium* might become damaged by the effect of the MPO-$H2O_2$-Cl^- system ([35]). Hydrogen peroxide might be produced as a result of the macrophage's normal metabolism or its production might be co-induced via T-cell-derived cytokines ([39]). However, the toxic effect of the oxidative-halogenating system does not prevent the progress of

the disease, because of one (or more) of the following possibilities: a) due to its thick envelope of lipids and carbohydrates, *M. lepraemurium* highly resists the deleterious effect of the oxidative-halogenating system (survivors will then take over to continue the infection), b) the exogenous peroxidase dilutes itself because of the replication of the bacillus, c) when still viable, *M. lepraemurium* releases outer components and gets rid of the attached peroxidase, or d) macrophages do not produce any more H_2O_2 when the antimycobacterial cell-mediated immunity has vanished ([35]); the bacilli will then grow freely.

Some macrophages, but perhaps not the ones highly parasitized, will still be able to process mycobacteria and to present mycobacterial antigens to the reactive Th2 cell population. These cells will then cooperate with *M. lepraemurium*-reactive B cells to stimulate the synthesis of antibodies. In addition, parasitized macrophages, when destroyed by the overload of bacilli and, perhaps, also by the effect of some cytotoxic CD8+ cells (which in lepromatous leprosy predominate over the CD4+ cell population) ([42]), will release intact and damaged bacilli, as well as soluble and particulate antigens. Some of these antigens, namely carbohydrates and lipids, will directly stimulate B cells to produce antibodies. This is why the levels of anti-mycobacterial antibodies can be taken as an index of the extent of the disease. The lower levels of antibodies to the mycobacterial protein antigens and the clearly diminished levels of antibodies to the lipid antigens in the animals infected with PO-coated *M. lepraemurium,* suggest a certain protective role of the peroxidase-hydrogen peroxide-halide system in murine leprosy.

Similar results to the ones presented here, but related to other microorganisms, have also been reported. *In vitro* pretreatment of phagocytic cells with recombinant MPO ([19, 20, 21]) or *in vivo* administration to animals of exogenous, purified or recombinant MPO, or horseradish PO (HRPO) ([24, 25]), increases their microbicidal activity against several microorganisms, including *E. coli, Candida albicans* and *M. leprae,* and depleting phagocytes of MPO with hydrogen peroxide, decreases their microbicidal activity ([25]).

Based on the above presented information, and being aware that the MPO-H_2O_2-halide system is not the sole microbicidal mechanism of macrophages ([18]) [acid hydrolases ([32]) and nitric oxide also play a role (Rojas-Espinosa, *et al.,* submitted)], we conclude that if these cells contained peroxidase they would be able to kill, or at least, limit the intracellular proliferation of pathogenic mycobacteria such as *M. tuberculosis, M. leprae,* and *M. lepraemurium.*

SUMMARY

Mycobacterium lepraemurium (MLM) is a successful parasite of murine macrophages; *in vitro,* this microorganism infects macrophages without triggering these cells' ability to produce either the reactive oxygen intermediaries (ROI) or the reactive nitrogen intermediaries (RNI), and ends up lodging within these cells, that, in addition, do not contain myeloperoxidase (MPO).

In this study, we analyzed the effect of exogenous peroxidase on the evolution of murine leprosy. Bacilli were intraperitoneally injected, either alone (MLM) or precoated with horseradish peroxidase (MLM-PO), into two different groups of mice. At two-week intervals, the groups were blood-sampled to measure the levels of antibodies to protein- or lipid-MLM antigens. The extent of the disease was also assessed by looking at the histopathologic changes that occurred both in the liver and the spleen of the infected animals.

We found that the animals injected with MLM-PO developed a disease that evolved at a slower pace than the disease that occurred in the animals injected with intact MLM. The difference between groups, both in terms of antibody levels and histological changes, was clearly evident at the intermediate stages of the disease (2 to 2.5 months), but was not so obvious at the more advanced stage of 3 months. Several possibilities to explain how the PO-coated bacilli might have regained their infectiousness are discussed. Lowering the infective dose of MLM and MLM-PO from 5×10^7 bacilli to 5×10^6 bacilli would, probably, have resulted in a different outcome of the disease: more extended in the MLM-group than in the MLM-PO group.

RESUMEN

Mycobacterium lepraemurium (MLM) es un parásito exitoso de los macrófagos murinos; *in vitro,* este microorganismo infecta a los macrófagos sin despertar en ellos la producción, ni de radicales libres del oxígeno (ROI) ni de radicales libres del nitrógeno (RNI), y termina instalándose en estas células, que, además, no contienen mieloperoxidasa (MPO).

En este estudio, analizamos el efecto de la peroxidasa exógena sobre la evolución de la lepra murina. Los bacilos fueron administrados por la vía intraperitoneal, solos (MLM) o recubiertos con peroxidasa de rábano (MLM-PO), en dos grupos diferentes de animales. Después, a intervalos de 2 semanas, los grupos fueron muestreados para medir los niveles de anticuerpos contra antígenos proteicos o lipídicos de MLM. También se registró el grado de enfermedad midiendo los cambios histopatológicos ocurridos en el hígado y el bazo de los animales infectados.

Encontramos que los animales inyectados con MLM-PO desarrollaron una enfermedad que evolucionó más lentamente que la enfermedad ocurrida en los animales infectados con MLM intacto. La diferencia entre los grupos, tanto en términos de niveles de anticuerpos como de cambios histopatológicos, fue claramente evidente en las etapas intermedias de la enfermedad (2 a 2.5 meses), pero no fue tan obvia al tiempo más avanzado de 3 meses. Se discuten varias posibilidades para explicar como es que los bacilos recubiertos con PO pudieron haber recuperado su infecciosidad. Una disminución de la dosis infectante de MLM y MLM-PO de 5×10^7 bacilos a 5×10^6 bacilos podría, probablemente, haber resultado en un diferente grado de efermedad: más extenso en el grupo MLM que en el grupo MLM-PO.

RÉSUMÉ

Mycobacterium lepraemurium (MLM) est un parasite montrant un bon succès adaptatif vis à vis du macrophage de la souris ; *in vitro,* ce micro-organisme infecte les macrophages sans déclencher leur capacité à produire des radicaux libres intermédiaires réactifs provenant de l'oxygène (ROI) ou de l'azote (RNI), et finissent par résider dans ces cellules qui, en plus, ne contiennent pas de myélopéroxydase (MPO).

Dans cette étude, nous avons analysé l'effet de l'ajout de péroxydase exogène sur l'évolution de la lèpre murine. Des bacilles furent injectés par voie intrapéritonéale, soit seuls (MLM) ou bien recouverts par de la péroxydase de raifort (MLM-PO), dans deux groupes de souris. Toute les deux semaines, les souris des 2 groupes furent soumises à des prises de sang afin de mesurer les niveaux d'anticorps dirigés contre les antigènes protéiques ou lipidiques spécifiques de MLM. La sévérité de la maladie fut également évaluée en examinant les modifications histopathologiques apparaissant dans le foie et la rate de ces animaux.

Les animaux injectés avec MLM-PO ont développé une maladie d'évolution plus lente que celle induite par l'injection de MLM intactes. La différence entre les groupes, à la fois en terme de niveaux d'anticorps et de modifications histologiques, était clairement visible aux stades intermédiaires de la maladie (entre 2 et 2,5 mois), puis diminuait au stade plus avancé de 3 mois. Les multiples possibilités pour expliquer comment les bacilles recouverts de PO pourraient avoir récupéré leur potentiel infectieux sont discutées. Une diminution de la dose infectieuse de MLM et MLM-PO, comme par exemple de 5×10^7 à 5×10^6 bacilles, auraient probablement changé l'issue de la maladie : plus étendue dans le groupe MLM que dans le groupe MLM-PO.

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