Mycobacterium leprae Surface Components Intervene in the Early Phagosome-Lysosome Fusion Inhibition Event

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Received 8 June 1987/Accepted 19 August 1987

Bone marrow-derived cultured macrophages were infected with Mycobacterium leprae. The bacteria were either used as freshly isolated organisms or incubated with M. leprae antiserum (1:5) for 30 min prior to phagocytosis. Immediately after inoculation (1 to 4 h) and at 1 to 8 days later, macrophages were stained for acid phosphatase activity to assess fusions between phagosomes and lysosomes. Inhibition of fusions was essentially apparent as an early event, which was partially reversed by antiserum treatment of the bacteria, suggesting a role for M. leprae immunogenic surface components in this early phenomenon. Later incubation times (1 to 8 days) did not show any considerable difference between antiserum-treated and nontreated bacteria. The formation of an electron-transparent zone around phagocytized bacteria and its role in phagosome-lysosome fusion was investigated, and a direct relationship could not be established.

All pathogenic mycobacteria are capable of intracellular growth inside the phagosomes of macrophages; however, the mechanisms involved in their intracellular survival differ greatly from one pathogenic species to another (5). It is known that Mycobacterium tuberculosis mainly escapes host defense mechanisms by phagosome-lysosome fusion inhibition (1, 2, 10), whereas Mycobacterium avium survives by both fusion inhibition and electron-transparent zone (ETZ) formation around phagocytized bacteria, the latter lessening the diffusion of lysosomal enzymes towards the bacterial surface (7, 8). The mechanism by which Mycobacterium leprae resists destruction remains poorly understood. Although it has been stated that M. leprae is capable of survival within the phagolysosomes (5) or of escaping the phagosomal environment (12), the phagosome-lysosome fusion inhibition was only recently quantified in the resident peritoneal macrophage system (19). The latter work by Sibley et al. (19) emphasized that viable M. leprae hindered phagosome-lysosome fusion in infected resident peritoneal macrophages; however, contrary to the earlier observations of Armstrong and d’Arcy Hart (1) with M. tuberculosis, these authors showed that antibody coating of the bacteria prior to phagocytosis did not revert the phagosome-lysosome fusion inhibition.

Independently of the research by Sibley et al. (19), we have been actively engaged in elucidating the role of mycobacterial surface components in their intracellular survival (7, 8, 17). While Sibley’s group was engaged in M. leprae interactions with resident peritoneal macrophages, utilizing Thoria-Sol labeling of secondary lysosomes to assess phagosome-lysosome fusions, we were simultaneously investigating M. leprae-mouse bone marrow-derived macrophage interactions, using acid phosphatase (AcPase) cytochemistry to assess phagosome-lysosome fusions (7). The AcPase method has an efficacy rate of 80% positive fusions in the case of macrophages loaded with nonpathogenic bacteria (7).

In a previous study (17), we defined the intracellular survival of M. leprae within bone marrow macrophages and assigned a protective role to the ETZ which surrounded the phagocytized bacteria. In the present investigation, we demonstrate that M. leprae surface components intervene in the early phagosome-lysosome fusion inhibition, and, contrary to the report by Sibley et al. (19), we hereby show that, in our experimental model, specific antiserum coating of M. leprae prior to phagocytosis partially reverts the early phagosome-lysosome fusion inhibition. In addition, the use of protein A-gold immunocytochemistry (16) permitted us to follow the formation of ETZ and to investigate whether a relationship between ETZ formation and phagosome-lysosome fusion inhibition could be established.

MATERIALS AND METHODS

Cells and culture medium. Bone marrow macrophages were obtained by seeding 2 × 105 bone marrow cells, isolated from 6- to 8-week-old C57BL/6 female mice, per 35-mm tissue culture dish (Falcon; Becton Dickinson Labware, Oxnard, Calif.). The culture medium consisted of Dulbecco modified Eagle medium with low glucose (1 g/liter) and high carbonate (3.7 g/liter) concentrations and enriched with 10% fetal calf serum heat inactivated for 40 min at 56°C, 10% L-cell-conditioned medium, and 2 mM L-glutamine. At 4 to 5 days after seeding, the attached cells were rinsed with Hanks balanced salt solution and refed with fresh medium.

Bacteria. M. leprae was freshly isolated from an experimentally infected armadillo. Infected tissues were manually ground, using mortar and pestle in the presence of sterilized sand. To facilitate grinding, Spizizen’s salt solution (20) was added in small quantities to obtain at the end of grinding not more than twice the volume of original tissues. The mixture was centrifuged at 4°C and 800 × g to remove the larger fragments, which were treated similarly two to three times to further extract the bacteria. The pooled supernatants were then centrifuged at 800 × g and 3,000 × g successively to remove the sand plus tissue debris and the larger bacterial clumps, respectively. The bacilli from the 3,000 × g supernatant were recovered by centrifugation at 10,000 × g and 4°C and washed three times with 250 volumes of Spizizen’s salt solution to remove the tissue debris. The bacteria were then treated with 4% (vol/vol) H2SO4 for 10 min at room temperature, neutralized with NaOH, and then washed three times with 250 volumes of the salt solution. The quality of the bacteria at this step was controlled by Ziehl-Neelsen

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staining and electron microscopy, and the absence of any microbiological contamination was verified by plating the bacterial suspension on the usual growth medium.

**Antibody-coating experiments.** Specific rabbit antiserum against total *M. leprae* antigens prepared and characterized as reported earlier (13) was a kind gift from F. Papa, Unité de la Tuberculose et des Mycobactériés, Institut Pasteur. The bacteria were washed and suspended in phosphate-buffered saline at an optical density of 0.3 (630 nm), as measured with a Coleman Junior II spectrophotometer, and were then incubated for 30 min at 20°C with *M. leprae* antiserum (1:5). A parallel control was performed by incubating the bacteria with nonimmune rabbit serum (1:5). In both cases, the bacteria were washed twice with phosphate-buffered saline and either used immediately for macrophage infection or postincubated with protein A-gold as described below.

**Infection of macrophage monolayers.** The 10-day-old macrophages were overlaid with *M. leprae* suspensions (antibody coated or nonimmune serum coated) to give a bacteria/macrophage ratio of 50 to 100:1. Macrophages were infected for 1 to 4 h for assessing the early phagosome-lysosome fusion events. For late events (1 to 8 days), macrophages were infected for 4 h, washed twice with phosphate-buffered saline and coated with fresh medium. The medium was renewed every 5 days.

**AcPase cytochemistry.** Macrophage monolayers were fixed for 1 h at 4°C (in the culture dishes) with 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose. They were washed overnight with the same buffer, rinsed once with 0.1 M acetate buffer (pH 5.0), and incubated for 30 min at 37°C in prewarmed Gomori reaction medium (9). Macrophages were rinsed twice with acetate buffer and once with cacodylate buffer and prepared for electron microscopy as follows. Cells were fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were then scraped off the culture dishes with a rubber policeman, concentrated in agar, and treated for 1 h with 1% uranyl acetate in Veronal buffer ( Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.) at a final pH of 5.0. Samples were dehydrated in a graded series of acetone and embedded in Epon. Thin sections were stained with 2% uranyl acetate and lead citrate. Control experiments performed in the presence of 10 mM NaF were negative.

**Assessment of fusion.** The presence of electron-dense material within phagosomes after AcPase cytochemistry identified fusions with lysosomes. To statistically assess the phagosome-lysosome fusion, about 50 macrophages were examined per sample, corresponding to 200 to 400 phagosomes in each experiment. The results were calculated as the mean of three different experiments. To avoid any statistical error, we carefully avoided serial sections, and only profiles exhibiting a nucleus were examined.

The respective percentage of phagosomes containing ultrastructurally intact (I) and degraded (D) bacteria was calculated as follows: I bacteria were those maintaining rod-shape, electron opacity, and normal cytoplasmic organization with unbroken cytoplasmic membranes and cell walls, whereas D bacteria lacked at least one of these criteria.

**Protein A-gold labeling of antibody-coated bacteria.** Antibody-coated bacteria were incubated with a protein A-gold suspension (5 nm) in a 1:1 ratio. The protein A-gold suspension was prepared as described earlier (16) and used in excess.

After 30 min of incubation at 20°C, bacteria were washed twice with phosphate-buffered saline and either used immediately for macrophage infection or processed for electron microscopy. Macrophage infection was performed as described above, and cells were fixed for electron microscopy at 1 to 24 h.

**Fixation and embedding for morphological studies.** Bacteria were fixed overnight at 4°C with a mixture of 1.25% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde in cacodylate buffer (pH 7.2; 0.1 M) containing 5 mM CaCl₂ and MgCl₂. Bacilli were then washed for 4 h at 4°C with same buffer and successively postfixed overnight at 20°C with 1% (wt/vol) OsO₄ in cacodylate buffer, followed by uranyl acetate (1%, wt/vol, in Veronal buffer) for 1 h at 20°C. Dehydration was done in a graded acetone series, followed by embedding in Epon 812. Thin sections mounted on Formvar-coated grids were stained with 2% (wt/vol) uranyl acetate and lead citrate and observed under a Siemens 101 microscope.

Ruthenium red staining of the bacteria was performed as reported earlier (14) by adding 0.05% (wt/vol) ruthenium red during fixation and dehydration steps.

A similar fixation and embedding procedure was used for infected macrophages, except that cacodylate buffer also contained 0.1 M sucrose and osmium postfixation was performed only for 1 h.

**Chemicals.** Protein A, β-glycerophosphate, and glutaraldehyde grade I were from Sigma Chemical Co., St. Louis, Mo. Paraformaldehyde was from Serva, Heidelberg, Federal Republic of Germany. Fetal calf serum was from GIBCO Europe, Paris, France. Dulbecco modified Eagle medium and glutamine were from Biopro, Mulhouse, France.

**RESULTS**

**Quantitative assessment of phagosome-lysosome fusion during *M. leprae* infection.** As viable counts of *M. leprae* cannot be obtained, the ultrastructural intactness and the ATP content of these bacilli prior to phagocytosis are a good indication of their probable viability. Observed under the electron microscope (Fig. 1A), our *M. leprae* preparation was devoid of host tissue debris and also preserved the typical polysaccharide outer layer (14). Moreover, our method of *M. leprae* isolation yields bacilli which contained about 400 pg of ATP per 10⁶ organisms (4), quite similar to that obtained by Sibley et al. (19).

Under the electron microscope, D and I bacteria could be distinguished from each other, as reported in Materials and Methods. As phagosomes contained clumps of essentially heterogeneous *M. leprae* (three to six bacilli), for each phagosome the numbers of D and I bacilli as well as the ones surrounded by electron-dense AcPase deposit were tabulated. Figure 1B and C shows AcPase cytochemistry in *M. leprae*-infected macrophages at 4 and 24 h, respectively.

Our results concerning the phagosome-lysosome fusion during *M. leprae* infection are presented in Table 1. Phagosome-lysosome fusion inhibition is evident in particular in the early steps of infection by *M. leprae*, as the positive phagosome-lysosome fusion index was only about 25%. This early fusion inhibition was independent of the morphological stage of the bacteria. Starting from day 1, the positive fusion index increased to about 60% and remained constant for up to 3 days. However, it decreased to about 40% at day 8 of infection.

**Effect of antiserum coating on phagosome-lysosome fusion.** Figure 2A shows antiserum coating of *M. leprae* by the protein A-gold method. All bacteria, irrespective of their
FIG. 1. Electron microscopy. (A) Thin section of purified *M. leprae* suspension stained by the ruthenium red method. The total cell surface in all bacteria is covered by an ruthenium red-positive deposit. (B) Phagocytized *M. leprae* observed after 4 h of infection in bone marrow macrophages. AcPase cytochemistry shows the presence of lysosomal enzymes as electron-dense deposits (arrows) around both I and D bacilli. (C) AcPase cytochemistry of macrophages 24 h after ingestion of *M. leprae*. AcPase deposits are visible around bacilli (arrows). I, Intact bacteria; D, damaged bacteria; L, lysosomes. Bar, 0.2 μm.
TABLE 1. Phagosome-lysosome fusion in macrophages infected by M. leprae

<table>
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<tr>
<th>Time</th>
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<th>Postinfection</th>
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<td></td>
<td>I</td>
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<td>2 h</td>
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<td>59.0</td>
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<tr>
<td>3 days</td>
<td>58.2</td>
<td>59.1</td>
</tr>
<tr>
<td>8 days</td>
<td>43.1</td>
<td>41.2</td>
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* Control values correspond to M. leprae treated with nonimmune serum, whereas the test values (antiserum) correspond to M. leprae treated with specific antiserum. The percentage represents the number of AcPase-positive phagosomes containing I or D bacteria compared with the total number of I- or D-containing phagosomes of phag-lys fusion. For T bacilli, on the contrary, the percentage is tabulated irrespective of the morphological state of the bacteria. The data represent means of two or three different experiments.

**DISCUSSION**

It is well established that host protection against mycobacterial diseases, including leprosy, and recovery are mainly dependent on cell-mediated immunity, in which T-lymphocyte–macrophage interactions predominate (15). As far as humoral immunity to mycobacterial infections is concerned, no protective role has yet been assigned to antibodies, and their presence is not always relevant to the morphological stage of preservation, were found to be covered by specific antiserum. The fate of antiserum-coated bacilli in the macrophages after infection was verified by electron microscopy. A typical observation is represented in Fig. 2B, showing that most of the phagocytized bacilli were still covered by the antiserum at 4 h of infection, which is important concerning the early phagosome-lysosome fusion inhibition. The initial gold labeling of the M. leprae surface is located at the periphery of the ETZ (Fig. 2B), an observation similar to earlier results of Tereletsky and Barrow (21) with antiserum-coated M. intracellulare.

AcPase cytochemistry of the antiserum-coated M. leprae in macrophages is illustrated in Fig. 3, which shows the abundance of the electron-dense AcPase deposit around phagocytized bacteria at 4 h and 3 days of infection.

Table 1 shows a quantitative assessment of the phagosome-lysosome fusion by antiserum-coated M. leprae. Compared with the control (Table 1), a partial reversal of the early fusion inhibition event is demonstrated, as the positive fusion index was about 50% with antiserum-coated bacilli versus about 25% observed with control experiments. No significant variation for D and I bacilli could be observed. At later steps of infection (1 to 8 days), this reversal of phagosome-lysosome inhibition was not evident.

**Relationship between ETZ formation and phagosome-lysosome fusion.** As phagosome-lysosome fusion inhibition was an early event, we chose the 1-h infection point to determine whether a relationship exists between the bacteria capable of forming ETZ within 1 h of phagocytosis and the essentially early phagosome-lysosome fusion inhibition event. No such relationship could be established.

**FIG. 2.** Same bacterial suspension as Fig. 1A, observed after (A) antiserum-coating and subsequent revelation by the protein A-gold method and (B) 4 h of phagocytosis by mouse bone marrow macrophages. (A) All bacilli were effectively covered by antiserum (arrows). (B) Gold particles (arrows) are visible around the periphery of bacterial ETZ. Bar, 0.2 μm.
stage of infection or to diagnosis (18). Nonetheless, as early as 1975, Armstrong and d’Arcy Hart (1) emphasized that exposure of M. tuberculosis to specific antiserum prior to phagocytosis reverted the usual nonfusion pattern of phagosome-lysosome interactions. This observation has since been limited to M. tuberculosis.

For M. leprae, in addition to a recent study that used Thoria-Sol labeling (19), we hereby demonstrate with the AcPase probe that M. leprae inhibited phagosome-lysosome fusions in bone marrow macrophages. This fusion inhibition was essentially an early event (1 to 4 h) and diminished during late postinfection (1 to 8 days). These results are similar to those we observed for M. avium (7), except that with M. leprae the early fusion inhibition was independent of the morphological state of the bacteria: D bacteria were as effective in phagosome-lysosome fusion inhibition as I bacteria. Consequently, our report differed from the recent work of Sibley et al. (19) on two accounts. First, D bacilli, which actually represented nonviable bacteria, were as efficient as I bacilli in inhibiting phagosome-lysosome fusions. Second, this inhibition was observed during the first hours of phagocytosis (1 to 4 h) and not in later steps (1 to 8 days), probably corresponding to the “plateau” period in Sibley’s data. As both I and D bacteria impaired 1- to 4-h phagosome-lysosome fusions, it can be assumed that in our model system the early fusion inhibition was independent of bacterial viability.

The second part of this study evaluates the influence of specific antiserum coating of M. leprae prior to phagocytosis on phagosome-lysosome fusion and ETZ formation. Our results showed that antiserum coating of M. leprae partially reversed the early phagosome-lysosome fusion inhibition. As antiserum was used to coat the bacterial surfaces only prior to their engulfment, surface components are implicated in this phenomenon and are probably immunogenic. The paper by Sibley et al. (19), on the other hand, does not demonstrate a significant reversal of phagosome-lysosome fusion inhibition when the bacilli are coated with a specific antiserum. Once again (Table 1), this reversal is only evident in the early steps of infection, not in the later steps (1 to 8 days). Another benefit of the antiserum-coated M. leprae and their subsequent revelation by protein A-gold labeling was the observation that, once phagocytized, these bacilli were still covered by the specific antiserum, as observed earlier for M. avium (21). Although the saturation of cell surface antigens caused the reversal of phagosome-lysosome fusion inhibition, it did not impair ETZ formation. Consequently, it appeared that distinct bacterial components in M. leprae determined phagosome-lysosome fusion inhibition and ETZ formation. It was earlier postulated that mycobacterial cell wall components such as mycoside C might be implicated in ETZ formation and subsequent bacterial protection against host defense mechanisms (6). This study, moreover, suggests that ETZ formation in phagocytized M. leprae is not due to a specific recognition of bacterial surface antigens by host macrophages, as ETZ was formed even if M. leprae were antiserum coated prior to phagocytosis.

Despite the limitations of the in vitro macrophage-bacteria interaction model used in this study, our findings are promising as an aid to understanding host-M. leprae interactions. This investigation suggests not only the role of M. leprae surface components in the phagosome-lysosome fusion inhibition, but also that specific antibodies might contribute to at least partial host protection against leprosy by facilitating higher phagosome-lysosome fusion in compromised hosts.

The mechanism(s) involved in the phagosome-lysosome fusion inhibition remains to be established for M. leprae and may be due to one or more of the following: diminished...
activity or change of enzyme profiles in lysosomal bodies (11); impairment of membrane fusions between different vacuolar compartments (7); altered intracytoplasmic movement of lysosomes (3). We are presently continuing with macrophage-M. leprae interactions to elucidate this mechanism(s) and to identify the mycobacterial components involved.

ACKNOWLEDGMENTS

We thank H. L. David and A. Ryter for constructive criticism of our work and F. Papa for the kind gift of M. leprae antiserum. The excellent technical help of R. Daty and S. Cadou is gratefully acknowledged. We also thank C. Pham for typing this manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (contract 0620) and the Centre National de la Recherche Scientifique (UA 04-1148).

LITERATURE CITED