Antigen-Coated Latex Particles as a Model System for Probing Monocyte Responses in Leprosy

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To study responses to Mycobacterium leprae antigens, we developed an in vitro model system in which latex particles coated with M. leprae sonic extract (MLSON) antigen were presented to monocytes. Uptake and oxidative response as measured by superoxide production to these antigens were investigated. Phagocytosis of MLSON-coated particles was greater than that of control particles in monocytes from both leprosy patients and controls from leprosy-endemic areas; uptake of MLSON-coated particles was higher in monocytes from lepromatous leprosy patients than in cells from tuberculoid leprosy patients and controls. In both patients and controls, uptake of latex particles coated with leprosy antigens triggered very little reduction of nitroblue tetrazolium although the cells were capable of mounting a respiratory burst. Antigen-coated latex particles can therefore be used as a tool to investigate monocyte responses to M. leprae and individual recombinant antigens.

The monocyte/macrophage is a central cell in the immune response to mycobacteria. In vitro macrophages have been shown to inhibit the growth of Mycobacterium bovis BCG (27) and Mycobacterium microti (30). On the other hand, macrophages have been implicated in the establishment of infection with Mycobacterium tuberculosis (6). In lepromatous leprosy, the macrophages contain higher numbers of bacteria than are found in cells from tuberculoid leprosy patients, possibly because of increased uptake of bacteria, decreased killing, or both. Using live Mycobacterium leprae, Mistry et al. (17) showed that monocytes from lepromatous patients acquired a greater bacterial load than do those from tuberculoid patients or normal controls. Monocytes or macrophages from lepromatous leprosy patients are able to kill other bacteria such as Listeria monocytogenes, Staphylococcus aureus, or Proteus vulgaris (5) and can be activated by gamma interferon to kill legionellae (13). There are two reports that blood-derived macrophages from lepromatous leprosy patients were unable to kill M. leprae, unlike those from tuberculoid patients or healthy controls, as assessed by lysis (1) or by a reduction in uptake of tritiated thymidine (21); other studies have failed to confirm these findings (4, 9).

There are a number of technical difficulties encountered when whole M. leprae is used in such studies. First, the small size of the bacteria and their tendency to clump makes counting by conventional microscopy difficult. Second, bacteria derived from lepromas contain a mixture of live and dead organisms, which may have different properties, while armadillo-derived material may have variable contamination with foreign proteins. In addition, the response to individual components of M. leprae needs to be analyzed. We have now developed a system in which mycobacterial antigens are coupled to latex particles and have used it to assess phagocytosis and oxidative response to M. leprae antigens by monocytes in leprosy.

MATERIALS AND METHODS

Patients and controls. Untreated leprosy patients, presenting at the Marie Adelaide Leprosy Centre, Karachi, Pakistan, and normal controls from leprosy-endemic areas, employees of the Aga Khan University, Karachi, Pakistan, were used. The leprosy patients were diagnosed clinically by bacterial counts on slit skin smears and histologically by using a 4-mm punch skin biopsy taken from the edge of an active lesion, according to the Ridley-Jopling classification (22). The majority of both patients and controls have not received BCG vaccination, but tuberculosis is highly endemic in Karachi.

Antigen coating of latex particles. A 5% suspension of carboxylated latex particles of 1.1-μm diameter (Seragen, Indianapolis, Ind.) was used. Aliquots (200 μl) of latex particles were washed three times with 0.1 M carbonate buffer (pH 9.6) and then three times with 0.02 M phosphate buffer (pH 4.5) by adding buffer to the particles and spinning the mixtures at 12,000 rpm. The particles were then resuspended in 500 μl of phosphate buffer, and 500 μl of 2% carbodiimide [1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride; Sigma Chemical Co., St. Louis, Mo.] was added drop by drop. The tubes were mixed at room temperature for 3 to 4 h. Unreacted carbodiimide was removed by washing the particles three times in 0.02 M phosphate buffer (pH 7.4). The antigen, M. leprae sonic extract (MLSON; batch CD 75), a soluble sonic extract of cobalt-irradiated armadillo-derived M. leprae, at concentrations of 50 to 200 μg/ml in a final volume of 1 ml was added, and the mixtures were incubated with mixing overnight at room temperature. Optimal coating was achieved with 200 μg of MLSON per ml. To block unreacted sites, 20 μl of ethanolamine (2-aminoethanol; Sigma) was added, and the particles were mixed for 1 h. After three washes in phosphate buffer (pH 7.4), any remaining sites were blocked by incubating the particles in 1 ml of 0.25% bovine serum albumin (BSA) in phosphate buffer. After a final three washes, the particles were stored as a 1% solution at 4°C in 0.02 M phosphate buffer (pH 7.4) with 8 mg of NaCl per ml, 10 mg of BSA per ml, and 0.02% sodium azide.

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were then incubated for 2 h at 37°C. The antigen-coated latex particles were washed five times in 0.02 M phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20 (Sigma) (PBS-Tween). The particles were then diluted in PBS containing 1% BSA and 0.05% Tween 20 (antibody diluent) and incubated in a high-titer pool of leprosy sera known to contain immunoglobulin G (IgG) anti-MLSON antibodies (12) diluted 1/100 for 1 h at 37°C and overnight at 4°C. They were washed five times in PBS-Tween, blocked with PBS containing 5% BSA, and washed again five times. The particles were then incubated for 2 h at 37°C with alkaline phosphatase-labelled goat anti-human IgG (Sigma). After a further five washes, color was developed by adding phosphatase substrate (Sigma), and the reaction was stopped by adding 100 μl of 3 M NaOH. The tubes were centrifuged, and the supernatants were read in an enzyme-linked immunosorbent assay (ELISA) reader at 410 nm.

(ii) Agglutination assay. A 0.1% suspension of antigen-coated latex particles was washed five times in PBS-Tween. The agglutination assay was performed in round-bottom microtiter plates containing 25 μl of antigen-coated latex, 25 μl of 0.2 M 2-amino-2-methyl-1-propanol-HCl (Eiken Chemical Co., Tokyo, Japan), and 25 μl of a serum known to contain a high titer of IgM antibodies to MLSON. A normal serum pool was used as a negative control. The test was read after overnight incubation at room temperature. The agglutination index was read according to standard criteria for agglutination, in which the extent of mat or button formation was assigned a value of 3 (total formation of mat), 2 (75 to 100% mat formation), 1 (50 to 75% mat formation), 0.5 (25 to 50% mat formation), or 0 (button formation). An agglutination index of 1 or greater is considered positive. The agglutination assay was performed on each batch of antigen-coated particles. To assess specificity of the agglutination assay, agglutination with five IgM antibody-positive sera from BL or LL leprosy patients in the presence and absence of soluble antigen was tested, using previously optimized concentrations of MLSON and an irrelevant Toxoplasma antigen supplied by M. Lunde, National Institutes of Health, Bethesda, Md.

Monocyte culture. Heparinized venous blood (20 IU of heparin per ml; Evans Medical Ltd., Horsham, United Kingdom) was diluted 1:1 with warmed RPMI 1640 tissue culture medium containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.2 mM L-glutamine, 25 U of penicillin per ml, and 25 μg of streptomycin per ml and separated on Histopaque 1077 (Sigma). After three washes, 10⁶ peripheral blood mononuclear cells in RPMI containing 20% autologous plasma (heat inactivated at 56°C for 30 min) were added to 13-mm-diameter coated Lux plastic coverslips (Flow Laboratories, Hertfordshire, United Kingdom), and monocytes were allowed to adhere for 1 h at 37°C in an atmosphere of 5% CO₂ in air. After the nonadherent cells were washed off with warm RPMI, fresh tissue culture medium was added and the cells were incubated overnight. In some experiments, 20% heat-inactivated human AB serum (Flow Laboratories) was used instead of autologous plasma. Before use, the adherent cells were again washed with warmed medium. The purity of the monocyte population was assessed by staining for nonspecific esterase (14); 80 to 90% of the adherent cells were positive (although nearly 100% of the cells reduced nitroblue tetrazolium [NBT] when stimulated with phorbol myristate acetate [PMA]).

Phagocytosis assay. A suspension of latex particles (3 x 10⁷ to 4 x 10⁷ per well) in RPMI containing 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories) was added to each monocyte monolayer and incubated for 2 h at 37°C. Non-phagocytosed beads were washed off with warm RPMI, and the cells were fixed with methanol and stained with 10% Giemsa solution. Phagocytosis was assessed in terms of the percentage of phagocytic cells or in terms of the number of particles ingested per phagocytic cell (1 to 5, 6 to 15, or >15 particles per cell). MLSON-coated latex particles were opsonized by incubation in a pool of high-titer antibody-positive lepromatous leprosy serum diluted 1/100 for 2 h at 37°C and then washed in Hanks balanced salt solution (HBSS). In some experiments, whole armadillo-derived cobalt-irradiated M. leprae was used at a concentration of 8 x 10⁶ per ml, the incubation time was increased to 4 h, and uptake was assessed microscopically after staining with modified Ziehl-Neelsen stain.

Superoxide anion production. The production of superoxide anions was measured by the reduction of NBT (Sigma). After overnight incubation, the adherent monocyte monolayers were washed with warm HBSS without phenol red (Flow Laboratories). A suspension of 1.2 x 10⁶ to 1.6 x 10⁶ latex particles in phenol-red free HBSS containing 10% FCS and 1 mg of NBT per ml was added to the cells (250 μl per well) and incubated for 1 h at 37°C. PMA (Sigma) at a final concentration of 1 μg/ml was used as a positive control. After incubation, the cells were washed with HBSS and counterstained with 2% methyl green for 40 min. A minimum of 200 phagocytic cells were counted on each coverslip, and the percentage of cells reducing NBT was expressed as a fraction of the total phagocytic cells. For PMA, the percentage of cells reducing NBT was expressed as a percentage of the total adherent cells.

RESULTS

Detection of antigen on latex particles. To assess antigen coating, an indirect ELISA method using a leprosy serum pool with high titers of IgG antibodies to MLSON was used, with a normal serum pool as a control. Antigen-coated latex bound antibodies in a highly specific manner; in addition, there was no nonspecific binding of the antibodies to the BSA-coated or control latex particles (Fig. 1). Increasing binding was detected at coating antigen concentrations of up to 200 μg/ml without a concomitant increase in nonspecific binding; therefore, this concentration was used in all subsequent experiments. The antigen coating assessed by an agglutination assay was highly reproducible in eight sequential batches, all serum titers being within 1 standard deviation of the mean (results not shown).

Another stringent measure of specificity of antibody binding to antigen bound on a solid matrix is competitive inhibition with soluble antigen. Figure 2 shows inhibition of the agglutination reaction by the specific antigen, MLSON, but not by an irrelevant soluble antigen preparation from Toxoplasma gondii with five separate lepromatous leprosy sera. These standardized latex particles were then used to probe monocyte function in leprosy patients.

Phagocytosis of antigen-coated latex by monocytes from leprosy patients and controls. Monocyte monolayers from both lepromatous and tuberculoid leprosy patients and from normal controls were tested for the ability to phagocytose MLSON-coated particles. In all three groups, the MLSON-
coated particles were phagocytosed to a greater extent than the control particles were (Table 1). The increase was seen not only in the total percentage of phagocytic cells but also in the number of particles phagocytosed per cell. In the lepromatous group, 9.41% ± 9.23% of the cells phagocytosed more than five MLSON-coated particles, while only 2.93% ± 4.08% of the cells phagocytosed more than five control particles (P = 0.004; Wilcoxon signed rank test). To ensure that the particles counted were actually phagocytosed and not merely attached to the membrane, the assays were carried out at 4°C and 37°C in three controls. In all cases, minimal binding of particles to the monocytes was observed at 4°C; at 37°C, particles could be seen in different planes of focus, indicating that the beads were phagocytosed rather than attached, although this has not been formally proved.

Comparison of uptake between patients and controls from endemic areas showed higher phagocytosis of MLSON-coated particles by monocytes from lepromatous patients compared with monocytes from tuberculoid patients or from controls. Interestingly, a similar trend was observed with the uncoated control particles and with whole M. leprae (Table 1). Some of this uptake may have been due to some opsonization of the particles by cross-reacting antibodies in the FCS. However, since the conditions used were the same for all three study groups, this could not explain the greater uptake in lepromatous patients. Moreover, when MLSON-coated particles opsonized with lepromatous serum were used, phagocytosis was increased and was still significantly higher in the lepromatous patients (61.2% ± 14.0% of phagocytic cells) than in the controls (46.2% ± 15.0% of phagocytic cells) (P > 0.01; Mann-Whitney test). Thus, any opsonization by the FCS was minimal, and there must be additional functional differences between the monocytes in lepromatous patients and controls.

Superoxide anion production by monocytes in response to phagocytosis. Monocytes from leprosy patients and controls were able to trigger a respiratory burst in response to PMA, with a mean response rate of 95% of the cells reducing NBT in all groups. The system was also able to detect NBT reduction around BSA-coated latex. In comparison with PMA- and BSA-coated particles, MLSON-coated latex resulted in a lower rate of NBT reduction (Fig. 3). The spontaneous reduction of NBT by monocytes after overnight incubation was extremely low, with <5% of the monocytes being positive in a 1-h incubation period.

The low respiratory burst response to MLSON-coated latex was also seen in the larger donor panel; in most donors, the response to MLSON-coated particles was not higher than the response to control particles, and with the exception of two normal controls, the percentage of cells reducing NBT was below 10% in all donors (Fig. 4). Thus, the MLSON-coated particles appeared to be phagocytosed via a receptor whose modulation does not induce a respiratory burst.

DISCUSSION

For studies of host interactions with M. leprae, the model closest to the in vivo situation is one using whole bacteria. Since M. leprae cannot be grown in culture, such studies use...
organisms obtained from infected armadillo tissue (liver or spleen) or extracted from human lepromas. The different sources and methods of isolation, however, are a source of variation; as many of the M. leprae antigens recognized to date are heat shock proteins produced by the organism in response to stress (33, 34), one might expect variation in their expression in different tissues. Differences in viability of live organisms would also be expected. All of these factors make it difficult to relate results from different studies using whole organisms. Furthermore, whole bacteria do not allow analysis of the host interaction at the level of individual components, which is important in order to identify antigens important for virulence.

To study the interaction of leprosy antigens with monocytes and macrophages, it is necessary to have a system in which functional capabilities, including activation and subsequent cellular events, can be quantitated. Some authors have used electron microscopy to assess phagocytosis of M. leprae (25) or labeled similar organisms with fluorescent dye to make quantitation by light microscopy easier (8). Antigen-coated latex particles have been used in other systems (10, 19) and have a number of advantages over the use of whole bacteria. The particle size chosen here, 1.1-μm diameter, makes the refractile round particles easier to quantitate by light microscopy than the slender M. leprae (3.0 by 0.3 μm), which have a tendency to clump and therefore cannot be counted accurately. Induction of a respiratory burst in response to phagocytosed particles can also be easily observed. The covalent linking of antigens to latex particles by the carbodiimide method proved stable, and recognition of the antigens by antibodies in two assay systems proved that the proteins were present on the particles in an antigenic form. Finally, the system can be adapted to investigate the responses to individual protein antigens, such as the M. leprae recombinant 18-kDa antigen (28), which was successfully coupled to latex by the same technique (results not shown).

Phagocytosis of antigen-coated beads was greater in lepromatous leprosy patients than in those with tuberculoid disease or normal controls. Lepromatous leprosy patients

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FIG. 3. Oxidative responses to phagocytosed latex particles and PMA. The oxidative responses to MLSON-coated (open bars) and BSA-coated (hatched bars) latex particles and to PMA (solid bars) were compared in monocytes from three controls. The results, expressed as the percentage of phagocytic cells reducing NBT or of total monocytes reducing NBT for the PMA responses, represent the means and standard errors of triplicate wells.

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FIG. 4. Oxidative responses to phagocytosis of MLSON-coated (solid bars) and control (hatched bars) latex in monocytes from leprosy patients and controls. Results are expressed as the percentage of phagocytic cells (PC) reducing NBT. Means and standard errors of triplicate wells are shown for each donor. The mean response rate of monocytes in all groups when tested with PMA as a positive control was 95%.
have been shown to have 10- to 100-fold-higher concentrations of antibodies than tuberculoid patients do (11, 16). Although the assays were performed in tissue culture medium containing FCS, passive absorption of antibodies to the monocyte Fc receptors may have occurred during the overnight incubation in medium containing autologous plasma. The absorbed antibodies would increase binding and phagocytosis of antigen-coated particles. It is also possible that the number of monocyte receptors is increased on monocytes in lepromatous leprosy patients, although viable M. leprae has been shown to be downregulated Fc receptors in lepromatous leprosy (17). A number of studies have also noted increased uptake of M. leprae by cells from lepromatous patients (15, 17), although comparable uptake of Corynebacterium pseudotropicalis by monocytes from leprosy patients and from controls has been observed (7).

Enhancement of phagocytosis by antibody, however, cannot explain increased uptake of MLSON-coated particles compared with uncoated control particles by monocytes from tuberculoid leprosy patients and controls. This enhancement may be due to interactions between components of MLSON and receptors or molecules on the monocytes. The monocyte membrane contains a number of other receptors through which ligand binding and phagocytosis can occur. For example, Pneumocystis carinii is phagocyted via the macrophage mannose receptor (8), Leishmania parasites are phagocyted by gp63 binding to the CR3 receptor (23), and the yeast Histoplasma capsulatum is phagocyted via C3, LFA-1, and the p190,95 complex (2). Recent studies demonstrated that M. leprae can be phagocyted by the CR1 and CR3 receptors, even in the absence of C3 in the serum used for the assays and that M. leprae can activate the alternative complement cascade, leading to phenolic glycolipid 1-mediated fixation of C3, with the resultant deposition of C3 on the bacterial surface (24, 25). The complement receptors CR1 and CR3 have also been found to mediate ingestion of M. tuberculosis (20) and Mycobacterium avium (29). In our study, all sera used were heat inactivated prior to use. However, it is possible that complement components released by the monocytes were able to mediate the phagocytosis of the antigen-coated latex.

The observation that phagocytosis of the antigen-coated latex triggered a minimal respiratory burst would support the hypothesis that the particles were interacting with complement receptors, as it has been shown that binding to this receptor does not initiate a respiratory burst, and would argue against opsonization of the particles by cross-reactive antibodies in the FCS, leading to uptake via Fc receptors (31, 32). The response to PMA was normal, as shown earlier by Sharp and Bannerjee (26). In fact, the BSA-coated latex particles triggered more NBT reduction than did the particles coated with the MLSON antigens; this result also proves that the system was capable of detecting NBT reduction around phagocytosed particles. The specific lack of an oxidative response to M. leprae agrees with an earlier study which showed a poor oxidative response to either live or irradiated M. leprae (12). Other studies have reported either similar production of hydrogen peroxide and superoxide anion by monocytes from leprosy patients and controls in response to M. leprae (26) or weak superoxide production in cells from lepromatous leprosy patients compared with normal controls (15). These differences may reflect variations in the sensitivities of the methods used or patient variation. It is also possible that phenolic glycolipid, some of which is present in the MLSON antigen used in our study, is scavenging the oxygen radicals (3, 18).

We have described a system in which the macrophage response to various antigen presented on latex beads can be analyzed. Phagocytosis of the particles may allow antigen processing and presentation in a manner more similar to when whole bacteria are used than when soluble antigens are used. Other applications of the antigen-coated latex particles include analysis of cytokine release in response to the particles. We are also using this system to compare the effect of opsonization with antibodies from tuberculoid or lepromatous leprosy patients on phagocytosis and the respiratory burst.

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REFERENCES


