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Mycobacterium leprae Renders Schwann Cells and Mononuclear Phagocytes Susceptible or Resistant to Killer Cells

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Acquired resistance to *Mycobacterium leprae*, the etiologic agent of leprosy, crucially depends on cellular immune mechanisms. In addition to interleukin-mediated helper functions, killer mechanisms seem to be involved. This study addresses the question of how *M. leprae* renders mononuclear phagocytes and Schwann cells, its natural targets, susceptible or resistant to killer cells. Killer activities were stimulated in peripheral blood mononuclear cells from healthy individuals by incubation with mycobacteria plus interleukin-2. These cells lysed Schwann cells and mononuclear phagocytes which had been pulsed with dead *M. leprae*, while unpulsed targets remained virtually unaffected. Importantly, targets infected with viable *M. leprae* were not lysed; furthermore, infection with viable *M. leprae* as well as gamma interferon stimulation or heat shock caused resistance in otherwise susceptible targets which had been pulsed with dead *M. leprae*. Thus, *M. leprae* markedly influenced the effect of killer cells on Schwann cells and mononuclear phagocytes.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (15). This bacillus is an intracellular pathogen capable of replicating in the intracellular milieu of professional and nonprofessional phagocytes. Tissue macrophages and Schwann cells serve as major habitats for *M. leprae*, and because of the intracellular persistence of this pathogen, T lymphocytes are crucially required for resistance. According to current thinking, the activation of mononuclear phagocytes by T-cell-derived interleukins represents a major defense mechanism (17). Interleukins are primarily produced by CD4 T lymphocytes, which are considered particularly important for protection against leprosy. More recently, several lines of investigation have pointed to an additional role of cytolytic mechanisms. Killing of *M. leprae*-infected host cells can be achieved by specific cytolytic T cells of CD4 or CD8 phenotype and by nonspecific killer cells (1, 7).

Here, we show that pulsing of Schwann cells and mononuclear phagocytes with dead *M. leprae* renders them susceptible to attack by killer cells. In contrast, infection of these host cells with viable *M. leprae*, as well as stimulation with heat shock protein (HSP) inducers, reverses susceptibility to killing.

MATERIALS AND METHODS

Antigens and microorganisms. Dead *Mycobacterium tuberculosis* organisms (Difco Laboratories, Detroit, Mich.) and dead *M. leprae* organisms (kindly provided by P. Brennan, Colorado State University, Colo.) were suspended in phosphate-buffered saline (PBS) to give a final concentration of 1 mg/ml and stored at −20°C until use. Live *M. leprae* organisms (kindly supplied by J. Kazda, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany) were aliquoted and stored at −70°C before use.

Killer cells. Peripheral blood mononuclear cells from healthy donors were isolated over a Ficoll density gradient (ρ = 1.077 kg/liter). Cells were washed three times and cultured with 2 μg of dead *M. tuberculosis* per ml for 24 h. Cells were then stimulated twice a week with 200 U of recombinant interleukin-2 (IL-2) (kindly provided by Hoffmann-La Roche, Basel, Switzerland) per ml and used after 2 to 4 weeks of culture.

Schwann cell cultures. Human Schwann cells were obtained from cervical skin nerves of patients undergoing neck dissections. After careful removal of connective tissue, nerve fascicles were isolated, cut into small pieces, and incubated with equal volumes of 0.5% collagenase (Boehringer GmbH, Mannheim, Federal Republic of Germany) and 0.5% trypsin (Sigma, St. Louis, Mo.) for 3 to 4 h. Subsequently, DNase I (10 μg/ml) was added, and cells were dissociated by pipetting. After several washes with medium containing 10% fetal calf serum, cells were cultured in Iscove's modified Dulbecco's medium supplemented with 2% human serum, 2 μg of laminin per ml, and 10^{-5} M pituitary gland extract. Schwann cells were first grown nearly confluently in tissue culture flasks; after 24 to 48 h, they were washed with warm medium to remove nonadherent cells and then were plated onto round-bottom microtiter plates. Approximately 80% of the cells were Schwann cells, as determined by staining of the S100 antigen, an intracellular acidic protein specific for nervous tissue (24). This cell number was used for the calculation of effector-to-target cell ratios.

Mononuclear phagocytes. Ficoll-separated peripheral blood mononuclear cells were cultured for 3 h in RPMI 1640 with 10% heat-inactivated human A^+ serum in 260-ml culture flasks. Adherent cells were gently washed with warm medium, removed with a cell scraper (Costar), and plated onto round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 10^4 cells per well in RPMI 1640 medium supplemented as described above. Approximately 90% of the cells became adherent, and this number was used for calculating effector-to-target cell ratios (22, 23).

Phenotype analysis. A phenotype analysis of the effector cell population used in this study is shown in Table 1. Nylon-wool-enriched peripheral blood mononuclear cells were incubated at a density of 2×10^5 cells for 30 min at 4°C

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TABLE 1. Phenotype analysis of effector cell population

T-cell marker	% Positive cells stimulated with[a]:	
	M. tuberculosis + IL-2	IL-2 only
CD56	71	34
CD3	56	ND
CD4	11	14
CD8	21	55
γ/δ	26	4
α/β	ND	79

[a] ND, Not determined.

with the following monoclonal antibodies (MAb; 50:1 dilution) in PBS containing 1% bovine serum albumin and 0.1% NaN_3: anti-CD3 (OKT3-phycoerythrin), anti-CD56 (Leu19 and Leu19-phycoerythrin), anti-CD4 (Leu3A-fluorescein isothiocyanate), and anti-CD8 (Leu2a-fluorescein isothiocyanate) (Becton Dickinson, Mississauga, Ontario, Canada); anti-T-cell receptor (TCR)-δ (TCR-δ 1-fluorescein isothiocyanate) (T Cell Science); and anti-TCR-α/β (BMA031, kindly provided by Behringwerke, Marburg, Federal Republic of Germany). Double staining was done sequentially, first with the indirectly and second with the directly labeled MAb. Cells were washed twice and analyzed on an Epics V flow cytometer (Coulter Electronics, Inc.).

Negative selection of killer cells. A total of 5×10^6 killer cells were incubated in a total volume of 50 μl of anti-CD56 MAb (50 μg/ml; Becton Dickinson) at 4°C for 30 min, washed twice with medium, and incubated with 500 μl of a 1:7-diluted rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) for 30 min at 37°C. Prior to the assay, cells were washed three times and viability was checked microscopically.

Killer assay. Human Schwann cells or mononuclear phagocytes were cultured for 4 days in 96-well round-bottom microtiter plates (Nunc) at a density of 10^4 cells per well in Iscove's modified Dulbecco's medium or RPMI 1640 medium supplemented as described above. Cells were infected with viable *M. leprae* organisms 48 h before the killer assay or pulsed with dead *M. leprae* 24 h prior to the assay. Cells were stimulated with gamma interferon (IFN-γ, 2,000 U/ml) for 2 days or heat shocked at 41°C for 20 min prior to killer assays. Schwann cells and mononuclear cells were labeled with 2.5 μCi of $Na^{51}CrO_4$ (Amersham Inc., Bucks, Great Britain) per ml for 15 and 6 h, respectively, and then washed three times with warm medium. Killer cells were added to target cells at the ratios indicated in Fig. 1 through 6 and 8. After 5 h, ^{51}Cr release was determined, and the percent lysis was calculated as follows: percent lysis = [(test cpm − spontaneous cpm)/(high cpm − spontaneous cpm)] × 100.

RESULTS

Lysis of K562 cells and of mononuclear phagocytes pulsed with dead *M. leprae* organisms. Peripheral blood mononuclear cells were activated with dead mycobacteria for 24 h, and then the cells were stimulated with IL-2 twice a week. After 2 to 4 weeks, cytolytic activities of these cells were assessed with different targets. Figure 1 shows strong killer activity against K562 cells. *M. leprae*-pulsed mononuclear phagocytes were killed, while unpulsed phagocytes remained virtually unaffected. Killing was apparently non-human leukocyte antigen restricted, since *M. leprae*-pulsed targets of different human leukocyte antigen type were lysed

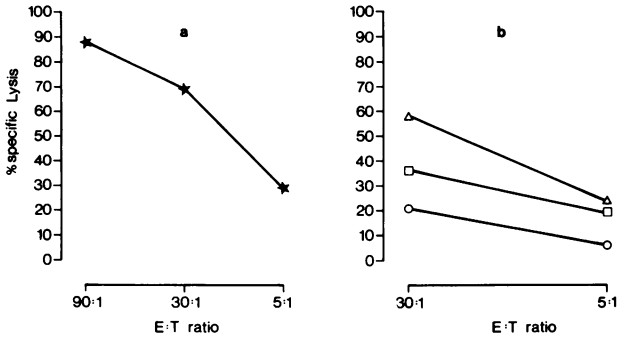

FIG. 1. Lysis of K562 cells and of *M. leprae*-pulsed mononuclear phagocytes. (a) K562 cells; (b) mononuclear phagocytes which were pulsed with 3 (△) or 10 (□) μg of dead *M. leprae* or were left unpulsed (○). Experiments were performed at least four times, with comparable results. Standard deviation, <25%. E, Effector cells; T, target cells.

by these effectors (data not shown). Similar though less striking activities were observed with killer cells which had been activated by IL-2 alone. Upon fluorescence-activated cell sorter analysis, the majority of effector cells expressed the CD56 marker (Table 1), and treatment of cells with anti-CD56 MAb plus complement abrogated killer activities (Fig. 2), indicating involvement of natural killer or lymphokine-activated killer cells. We conclude that mononuclear phagocytes pulsed with dead *M. leprae* become susceptible to killer cells, whereas unpulsed cells remain virtually unaffected.

Differential effects of dead and viable *M. leprae* on Schwann cell lysis. Schwann cells provide a major habitat for *M. leprae*. Hence, we wanted to assess whether *M. leprae* renders Schwann cells susceptible to killing as well. Peripheral blood leukocytes were activated as described above, and afterwards, their killer activities were assessed with Schwann cells which had been pulsed with dead *M. leprae* or infected with viable bacilli. Surprisingly, killer cells failed to efficiently lyse Schwann cells infected with viable *M. leprae*, whereas targets pulsed with dead *M. leprae* were lysed (Fig. 3). In principle, this observation could be explained by one of two alternatives. First, one could assume that killer cells failed to recognize infected target cells; second, it is possible

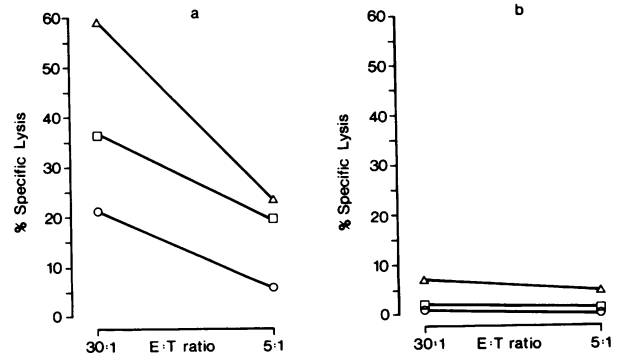

FIG. 2. Abrogation of killer activity by anti-CD56 MAb plus complement. Killer cells were left untreated (a) or were treated with anti-CD56 MAb plus complement (b). Mononuclear phagocytes were pulsed with 3 (△) or 10 (□) μg of dead *M. leprae* or were left unpulsed (○). Standard deviation, <15%. E, Effector cells; T, target cells.

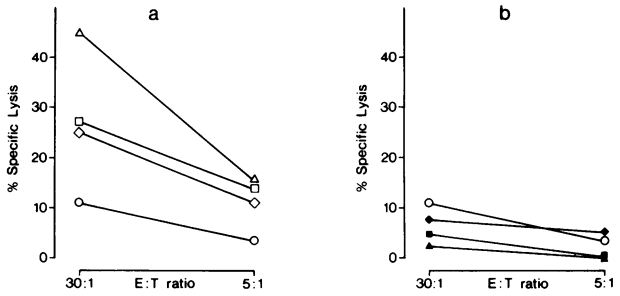

FIG. 3. Effects of dead and viable *M. leprae* on lysis of Schwann cells. (a) Schwann cells were pulsed with 3 (△), 10 (□), or 30 (◇) μg of dead *M. leprae* or were left untreated (○). (b) Schwann cells were infected with viable *M. leprae* at ratios of 30 (◆), 10 (■), or 5 (▲) bacteria per cell or were left untreated (○). Experiments were performed four times, with comparable results. Standard deviation, <25%. E, Effector cells; T, target cells.

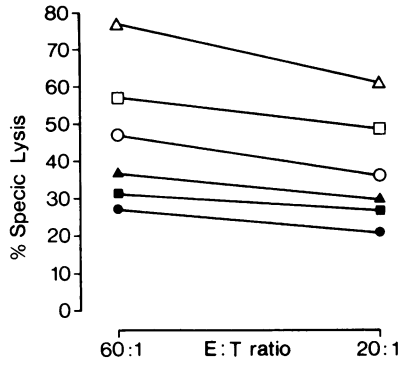

FIG. 5. Reduced killing of *M. leprae*-pulsed Schwann cells after infection with viable *M. leprae*. Schwann cells were pulsed with 3 (△) or 1 (□) μg of killed *M. leprae* or were left untreated (○). Alternatively, Schwann cells were infected with viable *M. leprae* at five bacteria per target cell alone (●) or 24 h before being pulsed with 3 (▲) or 1 (■) μg of dead *M. leprae*. Experiments were performed twice, with comparable results. Standard deviation, <20%. E, Effector cells; T, target cells.

that infected targets became resistant to killing. In the next experiment, we attempted to distinguish between these two alternatives.

Evidence that infection with viable *M. leprae* renders targets resistant to killing. Mononuclear phagocytes were infected with viable *M. leprae* and afterwards pulsed with dead leprosy bacilli. The data depicted in Fig. 4 show that infection of mononuclear phagocytes with viable *M. leprae* markedly reduced lysis of targets pulsed with dead bacilli. Similar data were obtained with Schwann cells (Fig. 5). In the particular experiment whose results are shown in Fig. 5, significant lysis was already observed with unpulsed Schwann cells, and it was increased by priming with dead *M. leprae*. Even in this case, infection with viable *M. leprae* reduced lysis by about 50%. These data suggest that infection with viable leprosy bacilli induces resistance to target cell lysis.

IFN-γ stimulation and heat shock induce resistance to killing. Evidence that prestimulation with IFN-γ or exposure of target cells to elevated temperature renders them resistant to subsequent killing by natural killer cells or by tumor necrosis factor has been presented by others (9, 11, 12, 25), and we wondered whether this also holds true for our system. Mononuclear phagocytes or Schwann cells were stimulated with IFN-γ for 48 h and pulsed with dead *M. leprae* 24 h before killer assays were performed. Prestimulation with IFN-γ induced marked resistance to lysis (Fig. 6 and 7). Because cells can protect themselves against harmful stimuli by increasing their intracellular HSP levels, it could be assumed that resistance to lysis after either infection with viable *M. leprae* or stimulation with IFN-γ was due at least in part to increased HSP synthesis. To further validate the possible role of HSP in resistance, *M. leprae*-pulsed cells were heat shocked immediately before the killer assay was performed. Although in this experiment lysis of untreated mononuclear phagocytes was already relatively high, it becomes clear from Fig. 8 that heat shock markedly reduced lysis. Thus, not only infection with viable *M. leprae* but also other stimuli, such as IFN-γ activation and heat shock, can induce resistance to killing. It is therefore tempting to speculate that resistance, at least in part, was imparted by the induction of HSPs (18, 28).

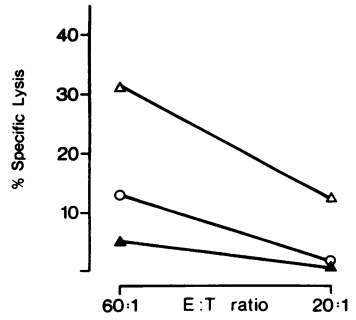

FIG. 4. Reduced killing of *M. leprae*-pulsed mononuclear phagocytes after infection with viable *M. leprae*. Mononuclear phagocytes were pulsed with 3 μg of killed *M. leprae* (△) or were left untreated (○). Alternatively, mononuclear phagocytes were infected with viable *M. leprae* at five bacteria per target cell 24 h before being primed with 3 μg of killed *M. leprae* (▲). Experiments were performed three times, with comparable results. Standard deviation, <15%. E, Effector cells; T, target cells.

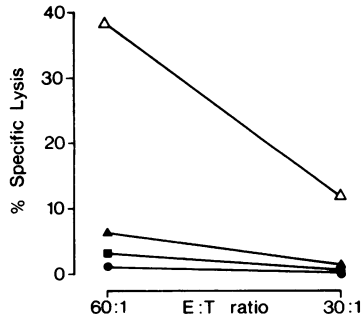

FIG. 6. Resistance to lysis of IFN-γ-stimulated mononuclear phagocytes. Mononuclear phagocytes were stimulated with IFN-γ (2,000 U) and pulsed with 3 (▲) or 1 (■) μg of dead *M. leprae* or were left unpulsed (●). As a control, cells were pulsed only with 3 (△) μg of dead *M. leprae*. Experiments were performed twice, with comparable results. Standard deviation, <20%. E, Effector cells; T, target cells.

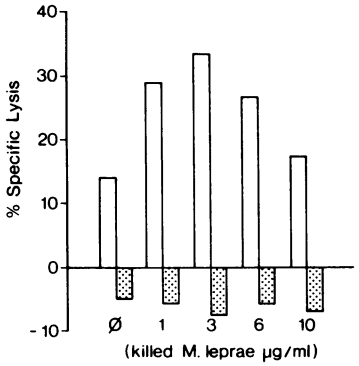

FIG. 7. Resistance to lysis of IFN-γ-stimulated Schwann cells. Targets were pulsed with the amount of dead *M. leprae* indicated. Experiments were performed twice, with comparable results. Standard deviation, <15%. Symbols: ☐, unstimulated Schwann cells; ▩, IFN-γ (500 U)-stimulated Schwann cells. Effector-to-target ratio, 40:1.

DISCUSSION

Although it is generally assumed that macrophage activation by interleukins represents a major mechanism of acquired resistance to leprosy, more recent studies have provided evidence suggesting that in addition, lysis of infected host cells occurs in leprosy (1, 7, 16, 27). Besides specific cytolytic T cells of CD8 and CD4 phenotypes, nonspecific killer cells have been identified. Although these in vitro experiments as well as recent in situ studies by Kaplan and coworkers (13, 14) suggest an in vivo role of target cell killing in leprosy, its biological relevance is not fully understood. On the one hand, target cell lysis could contribute to pathogenesis, and Schwann cell destruction may be taken as an example of pathogenic sequelae of such a mechanism. On the other hand, target cell lysis may contribute to protection by releasing bacteria from host cells, which are insufficiently equipped for intracellular killing, facilitating bacterial engulfment by more efficient effector cells. The present study shows that components of *M. leprae* render Schwann cells and mononuclear phagocytes susceptible to attack by killer cells, whereas infection with viable *M. leprae* renders them resistant. Furthermore, our study provides evidence for a role of HSP in resistance to this type of killing.

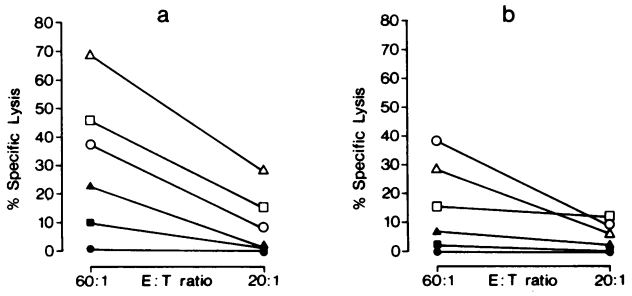

FIG. 8. Resistance to killing induced in mononuclear phagocytes by heat shock. (a) Priming with dead *M. leprae*. Targets were pulsed with 3 (△, ▲) or 10 (☐, ■) µg of dead *M. leprae* or were left unpulsed (○, ●). (b) Infection with viable *M. leprae*. Targets were infected with viable *M. leprae* at 5 (△, ▲) or 100 (☐, ■) bacilli per target or were left untreated (○, ●). Experiments were performed three times, with comparable results. Standard deviation, <20%. E, Effector cells; T, target cells. Closed symbols, Heat-shocked cells; open symbols, unshocked cells.

Activation of killer cells by mycobacterial products has been described before. Thus, as early as 1967, stimulation of killer activities in peripheral blood cells of healthy individuals was shown by Holm and Perlmann (10). The presence in mice of antigen-specific T cells with reactivity to mycobacteria which specifically lyse *M. leprae*-pulsed macrophages and Schwann cells has been established (7, 27, 28). More recently, mycobacterium-specific cytolytic CD4 T cells and natural killer cells have been activated from peripheral blood cells of leprosy patients and healthy individuals (1, 24, 25).

In the present study, a pulse with dead *M. leprae* rendered mononuclear phagocytes and Schwann cells susceptible to killing, whereas infection with viable *M. leprae* induced resistance in both targets. Either mycobacterial components themselves provided a ligand for recognition by killer cells, or they induced such a ligand in their targets. Because concomitant treatment of target cells with nonviable and viable *M. leprae* resulted in resistance to killing, we assume that infection with viable *M. leprae* actively caused resistance and that resistance cannot be explained by a lack of ligands. One possible mechanism responsible for resistance is the induction of HSP (4, 6, 8, 18, 29). This assumption is supported by our finding that resistance to killing was caused not only by viable *M. leprae* but also by IFN-γ stimulation and by heat shock. At sublethal doses, stress stimuli render cells resistant to subsequent, otherwise lethal insults. Although formal proof is still lacking, it is therefore tempting to speculate that resistance of *M. leprae*-infected host cells was due at least in part to induction of HSP synthesis. Increased resistance after either IFN-γ stimulation or heat shock to killing by tumor necrosis factor or by lymphokine-activated killer cells has been described by others (11, 12, 25, 26).

It has been suggested that natural killer cells discriminate among host cells according to the level of major histocompatibility complex class I expression (for a review, see reference 20). Although we cannot formally exclude influences of differential class I expression, we consider them less relevant in the case of *M. leprae*-confronted Schwann cells since we failed to observe significant differences in major histocompatibility complex class I expression by untreated and *M. leprae*-treated Schwann cells (data not shown).

Several in vitro studies have pointed to a role for killer cells in the host response to different intracellular bacteria, including not only tubercle and leprosy bacilli but also *Legionella pneumophila*, *Shigella flexneri*, atypical mycobacteria, and typhus-group rickettsiae (2, 3, 5, 19). In these studies, viable bacteria induced macrophage lysis by killer cells, whereas in our experiments, susceptibility to killing was achieved only with dead *M. leprae*.

What could be the reason for the differential effects on target lysis of viable and dead *M. leprae*? It is known that viable *M. leprae* are able to invade from the endosomal into the cytoplasmic compartment, whereas dead *M. leprae* remain in the endosomal compartment (21). Perhaps resistance to killing is induced only after translocation of bacteria from phagosomes into the cytoplasmic compartment, depending on the viability of the organism.

The findings described here shed another light on the complexity of the host-parasite relationship in leprosy since they indicate that depending on the viability of the bacillus, susceptibility or resistance to killing can be induced. At the moment, the value of differential susceptibility to killing for the host is hard to assess, and in fact, differential suscepti-

bility to killing may be beneficial as well as detrimental. Thus, it may be favorable for the host if Schwann cells harboring few viable *M. leprae* in their cytoplasmic compartments become resistant to killing. Also, the elimination of tissue macrophages packed with mycobacterial detritus could be beneficial. On the other hand, lysis of macrophages actively involved in the elimination of *M. leprae* could reflect a more harmful aspect of this relationship.

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