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A 25-Kilodalton Fraction from *Mycobacterium tuberculosis* That Inhibits Hexose Monophosphate Shunt Activity, Lysozyme Release, and H_2O_2 Production: Reversal by Gamma Interferon

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This study examined the effects of a 25-kilodalton (kDa) glycolipoprotein derived from *Mycobacterium tuberculosis* on phagocyte functions associated with antimicrobial activity. The 25-kDa fraction inhibited the ability of both polymorphonuclear cells and cultured monocytes to release lysozyme and produce hydrogen peroxide. In addition, the glycolipoprotein was capable of reducing hexose monophosphate shunt activity and interfered with the ability of polymorphonuclear cells to reduce Nitro Blue Tetrazolium. Inhibition of these antimicrobial systems was optimal at a 50-μg/ml concentration of the 25-kDa fraction. Gamma interferon, but not alpha interferon, partially reversed the inhibitory effect of the mycobacterial component in all of the systems assessed. These studies indicate important mechanisms in the understanding of the pathogenesis of tuberculosis and suggest that gamma interferon may have a therapeutic role in mycobacterial diseases.

Macrophages are thought to be an essential and major component of the cell-mediated immune response to mycobacteria (6, 17, 18, 33). Efficient killing of mycobacteria by macrophages requires the production of oxygen metabolites, including superoxide anion (O_2^-), hydrogen peroxide (8, 11), and hydroxyl radical (27). The ability of macrophages to produce oxygen metabolites and kill mycobacteria depends upon their state of "priming" or "activation" (8, 27). Macrophages can be primed for an increased production of oxygen metabolites when they are exposed to lymphokines, especially gamma interferon (IFN-γ), which is the principal macrophage-activating factor (22, 25). IFN-γ derived from activated T cells or recombinant DNA techniques enhances macrophage antimicrobial activity against a wide variety of intracellular pathogens, such as *Toxoplasma gondii* (25), *Chlamydia psittaci* (22, 32), *Leishmania donovani* (21), and *Legionella pneumophila* (3).

Pathogenic mycobacteria, however, survive intracellular degradation by a variety of escape mechanisms. These include interference with macrophage activation (11), inhibition or neutralization of the production of oxygen metabolites (26, 29, 36), inhibition of phagosome-lysosome fusion (1), disruption of phagosomal membranes (23), and inhibition of degranulation (2, 7, 13).

We have previously demonstrated that sonic extracts from *Mycobacterium tuberculosis* inhibit the intracellular killing capacity of phagocytic cells (34). The active component of these extracts was shown to be a 25-kilodalton (kDa) glycolipoprotein which acted, presumably, by inhibiting phagosome-lysosome fusion. In view of the inhibitory effect of this fraction on bactericidal activity, the study reported here was undertaken to examine the effect of this fraction on a variety of other phagocytic functions associated with antimicrobial activity. Because of the stimulatory effect of IFN-γ on macrophage function, a further aspect of this study examined whether recombinant IFN-γ could in any way affect the inhibition caused by the 25-kDa fraction. The results indicate that IFN-γ reverses the effects induced by the mycobacterial fraction on phagocyte function and suggest that this lymphokine could have therapeutic potential in the treatment of tuberculosis.

MATERIALS AND METHODS

Establishment of monocyte monolayers. Peripheral blood monocytes were isolated from normal volunteers and maintained in culture by using a modification of the technique of Johnson et al. (12) as described previously (34). Briefly, 0.5 ml of mononuclear cells at a concentration of 3×10^6/ml was plated in 24-well Cel-Cult tissue culture plates (Sterilin Ltd., Feltham, England) and incubated for 30 min at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Nonadherent cells were removed, and the remaining adherent cells were cultured in 2.0 ml of Modular Medium (Highveld Biologicals, Johannesburg, South Africa) with 10% fresh autologous serum and 100 U of penicillin and streptomycin. Culture medium was replaced every 4 days by removing half of the supernatant fluid and replenishing with an equivalent amount of Modular Medium containing 10% fresh autologous serum. The growth of cells was examined with an inverted microscope, and cells were expanded when the monolayer had reached 75% confluency. The expansion of cells was performed by vigorous pipetting of cell cultures, followed by plating into equivalent wells with an equal volume of medium containing 10% autologous serum.

Cell viability was assessed by trypan blue exclusion, and cell cultures were usually maintained for 3 months or longer. More than 95% of these cells reacted with OKM1 monoclonal antibody (Ortho Diagnostics, Inc., Raritan, N.J.) and were actively phagocytic. Monocytes that were in culture for 6 weeks or longer were used in the present studies.

Isolation of PMN leukocytes. Polymorphonuclear (PMN) cells were obtained from normal volunteers after centrifugation of heparinized peripheral blood on Hypaque-Ficoll density gradients. Cell pellets so obtained were suspended in 38 ml of phosphate-buffered saline (PBS) and mixed with 12 ml of 3% gelatin (Difco Laboratories, Detroit, Mich.) in PBS. The cell-gelatin suspension was incubated at 37°C for approximately 30 min, and the leukocyte-rich supernatant

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was removed, centrifuged, and washed three times in PBS. Residual erythrocytes were lysed with 0.83% ammonium chloride at 4°C by the method of Boyle (5). The remaining PMN cells were washed, counted, and suspended in PBS.

Mycobacterial preparation. *M. tuberculosis* organisms were obtained by scraping colonies off Lowenstein-Jensen slopes. Organisms were washed, heat killed, homogenized, counted, and made up to a concentration of 10^6/ml in PBS, pH 7.2. Mycobacterial extracts were obtained by sonication in an MSE 50-W ultrasonic disintegrator as described previously (35). The sonicated material was centrifuged at 2,000 × *g* for 10 min at 4°C, the supernatant was collected, and the amount of protein was determined with a protein estimation kit (Bio-Rad Laboratories, Richmond, Calif.). Fractionation of this material was performed on precalibrated Sephacryl S-200 columns (Pharmacia, Uppsala, Sweden), as previously described (34). Fractions were collected in 2.0-ml aliquots, and protein elution profiles were plotted by measuring the amount of protein in each fraction. Of the 11 major peaks obtained from this fractionation procedure, previous studies (34) demonstrated that the 25-kDa fraction consistently inhibited the intracellular killing ability of phagocytic cells. The fraction was also shown to demonstrate maximal inhibitory activity when it was in the form of an intact carbohydrate-lipid-protein complex. The important components of the molecule appear to be both carbohydrate and lipid. The molecule also appears to be heat stable at 56°C for 1 h and is unaffected by repeated freeze-thawing (unpublished observations). This fraction, henceforth called a 25-kDa fraction, was used at a concentration of 50 μg/ml in all studies unless stated otherwise.

Preparation of *Candida albicans* and *Saccharomyces cerevisiae* (bakers' yeast). *C. albicans* was grown overnight in dextrose-saline, washed three times in PBS, and killed by heating at 80°C for 1 h. Commercially available bakers' yeast was prepared by suspending a small pellet in physiological saline and heat killed as described above. Both organisms were then washed with PBS, suspended to a concentration of 6×10^7/ml in PBS, and stored at −20°C until used.

Lysozyme production and assay. Reactions were initiated by incubating 1.0 ml of PMN cells or cultured monocytes at a concentration of 10×10^6/ml with 200 μl of bakers' yeast particles in the presence of 200 μl of fresh human serum in tubes containing 100 μl of various concentrations of the mycobacterial fraction, interferons, or RPMI 1640. Tubes were incubated for various periods of time, after which they were centrifuged and the supernatants were collected and stored at −20°C until used.

Lysozyme was assayed by the method of Gordon et al. (9) by measuring the initial rate of lysis of a suspension of *Micrococcus lysodeikticus* with the aid of a PYE Unicam PU 8800 spectrophotometer fitted with an automatic recorder. Standards of lysozyme-rich materials were diluted in RPMI 1640 containing 5% fetal calf serum. Reaction conditions were as follows. A 1.0-ml volume of sample was mixed at room temperature with 2.0 ml of a suspension of *M. lysodeikticus* at a concentration of 0.25 mg/ml in 0.067 M phosphate buffer, pH 6.3. The rate of decrease in turbidity was measured at 540 nm, with a ratio switch position of 0.5 and with the full recording scale equal to 0.3 optical density unit. The initial reaction was linear for at least 1 min and directly proportional to egg lysozyme concentrations in the range of 0.2 to 2.0 μg/ml.

Hydrogen peroxide assay. H_2O_2 production by monocytes and PMN cells was measured by a microtechnique with 96-well, flat-bottomed microdilution plates (Sterilin), essentially as described by Paton and Ferrante (30). To each well was added 70 μl of PMN cells or monocytes, 20 μl of horseradish peroxidase (250 μg/ml; type IV, Sigma Chemical Co., St. Louis, Mo.), 70 μl of phenol red (500 μg/ml), and 40 μl of bakers' yeast. All solutions were made up in Hanks balanced salt solution. Test or control systems contained 20 μl of mycobaterial factor or Hanks balanced salt solution, respectively. The plates were incubated at 37°C for 60 min, after which 25 μl of a 2 N NaOH solution was added to stop the reaction. The A_{620} was measured in a Titertek Multiscan MC spectrophotometer. The assay was standardized with freshly diluted H_2O_2 (BDH, Poole, England) and was linear over the range of 0 to 60 μM.

HMPS activity. Hexose monophosphate shunt (HMPS) activity was assessed by the production of $^{14}CO_2$ from [1-^{14}C]glucose (New England Nuclear Corp., Boston, Mass.) by the method of Wood et al. (37). Cells were suspended to a final concentration of 2×10^7/ml in 0.15 M glucose-free PBS. The test apparatus was a 10-ml glass scintillation vial (Packard Instrument Co., Inc., Randburg, South Africa), which served as the outer chamber, stoppered with a tightly fitting perforated metal lid. Inside this chamber was placed a 1.0-ml autoanalyzer cup, which served as the inner chamber. The reaction mixture, consisting of 700 μl of [1-^{14}C]glucose (0.06 Ci), 100 μl of *C. albicans* organisms, 100 μl of fresh serum, and 100 μl of mycobacterial factor, IFNs (100 U/ml), or PBS, was placed in the outer chamber and 600 μl of a solution of 1 M KOH was placed in the inner chamber. The outer chamber was stoppered and allowed to stand in a 37°C water bath for 10 min. The reaction was initiated by the introduction of 200 μl of cell suspension into the outer chamber by injection through the rubber-lined cap with a long-needle syringe. The reaction was terminated by the addition of 2.0 ml of 2 N HCl after 60 min. The chambers were allowed to stand at room temperature for a further 60 min to permit the release of $^{14}CO_2$ and its absorption by the KOH. Samples of 500 μl of the inner chamber mixture were then transferred to scintillation vials containing 10 ml of acidified Instagel (Packard) and counted in an SMM liquid scintillation counter for 10 min each.

Controls consisted of the reaction mixture devoid of the cell suspension, and the counts so obtained were substracted from the corresponding experimental values. All tests were performed in triplicate and expressed as corrected (minus background) counts per minute.

NBT reduction. The Nitro Blue Tetrazolium (NBT) reagent (grade III, Sigma) was prepared by dissolving 1.0 mg of NBT in 1.0 ml of PBS. The solution was then centrifuged at 3,000 × *g* for 10 min, and the supernatant was collected and stored in the dark at 4°C.

Complement was activated by adding 5 mg of *Escherichia coli* endotoxin (O127:B8, Difco) to 1.0 ml of fresh human serum. The mixture was incubated at 37°C for 45 min, after which 200 μl of this endotoxin-activated serum was incubated with 200 μl of cell suspension in the presence of the 25-kDa fraction, 100 U of IFNs per ml, or both. After an incubation period of 30 min at 37°C, 400 μl of NBT solution was added and the cell suspension was allowed to incubate for 15 min at 37°C, followed by a further 15 min at room temperature. Smears of each sample were then made, allowed to air dry, fixed in methanol for 1 min, and stained with well-filtered hematoxylin. Smears were examined under oil immersion, and 100 phagocytes were counted. Cells with a large bluish precipitate were counted as positive.

IFN treatment. In experiments in which the effects of IFN on phagocyte function were examined, PMN cells or mono-

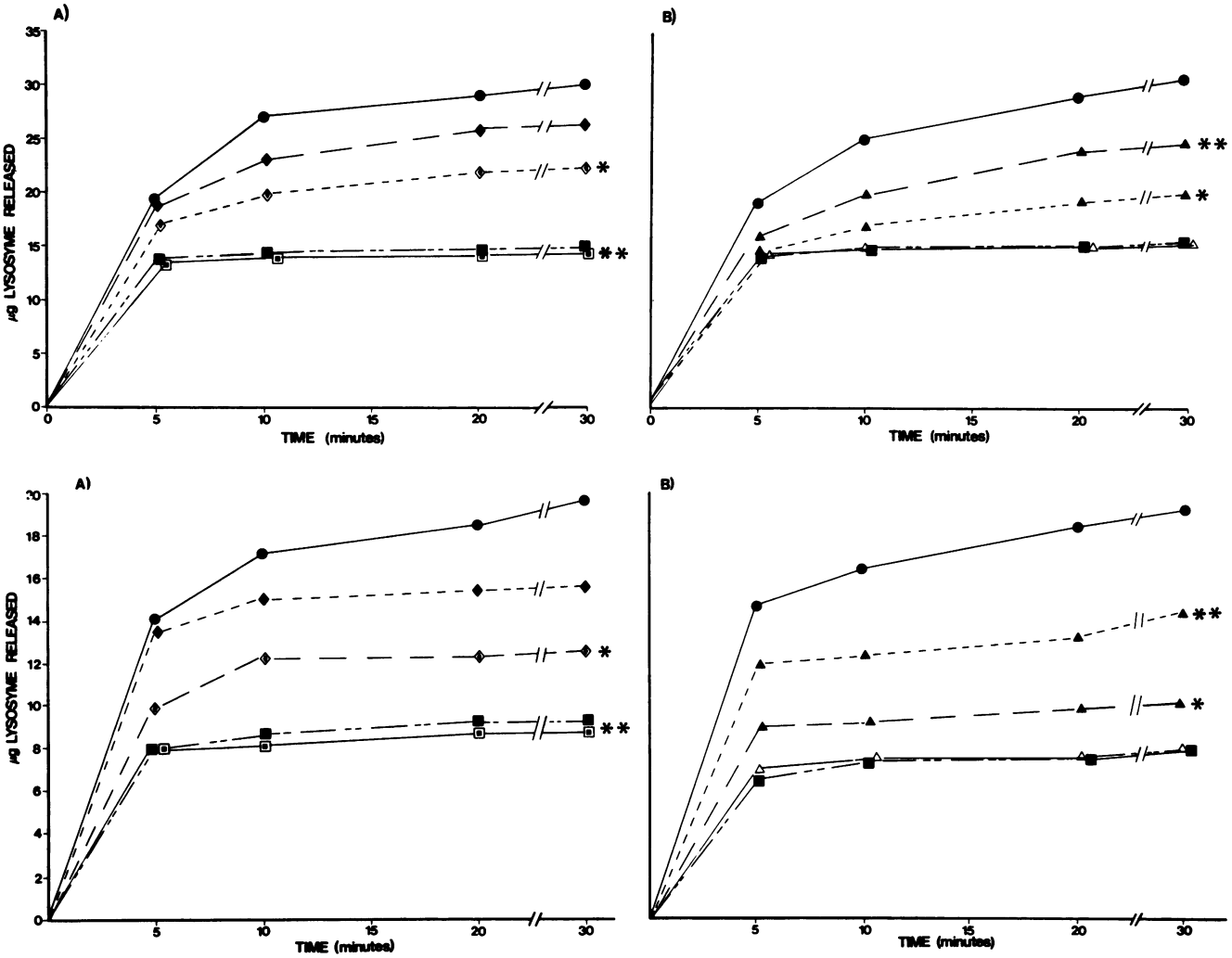

FIG. 1. Lysozyme release by PMN cells (top) and activated cultured monocytes (bottom) in the presence of medium (●) or increasing concentrations of the 25-kDa fraction (◆, 10 μg/ml; ◈, 25 μg/ml; ■, 50 μg/ml; ▣, 100 μg/ml) (A) and in the presence of 50 μg of the 25-kDa fraction per ml alone (■) or with increasing concentrations of IFN-γ (△, 50 U/ml; ◬, 100 U/ml; ▲, 200 U/ml) (B). *, $P < 0.01$; **, $P < 0.001$.

cyte cultures were incubated with recombinant IFN-γ (Amersham Corp., Bucks, England) or recombinant IFN-α (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Johannesburg, South Africa) in the presence or absence of the 25-kDa mycobacterial fraction. IFNs were added to cultures for 1 h, and experiments were initiated without any further washing of cells.

RESULTS

Lysozyme production in vitro. The production of lysozyme by PMN cells and cultured monocytes is shown in Fig. 1A (mean of three experiments). When lysozyme production was assessed in the presence of several concentrations of the 25-kDa fraction, a dose-related reduction was observed. Maximal effects were seen when 50 μg of the glycolipoprotein per ml was used in these assays. Higher concentrations could not reduce lysozyme production any further. When IFN-γ was included in cultures containing the 25-kDa fraction, lysozyme production was partially restored (Fig. 1B), with maximal activity being observed at an IFN-γ concentration of 200 U/ml. IFN-α had no effect on lysozyme release (results not shown).

Hydrogen peroxide production. The effect of the 25-kDa fraction on the ability of phagocytes to produce the oxygen-derived reactive species H_2O_2 is shown in Fig. 2. At a variety of concentrations, the 25-kDa fraction significantly reduced H_2O_2 production in PMN cells and macrophages (Fig. 2B). Although IFN-γ on its own had no effect on H_2O_2 production, it was capable of partially restoring the inhibition produced by the 25-kDa fraction (Fig. 2D). This effect was dose dependent and maximal at 200 U/ml (results not shown). IFN-α had no such restorative activity (Fig. 2C).

HMPS activity. Phagocytic cells incubated with *C. albicans* in the presence of fresh autologous serum demonstrated a burst of HMPS activity (Table 1). However, in the presence of the 25-kDa fraction, there was a significant decrease in HMPS activity. This reduction in HMPS activity due to the 25-kDa fraction was partially, although significantly, restored by IFN-γ. No restorative ability was demonstrated by IFN-α. IFN-γ on its own had no effect on HMPS activity.

NBT reduction. When NBT reduction induced by endotoxin-activated serum was measured in the presence of the 25-kDa fraction, a significant decrease in the number of cells

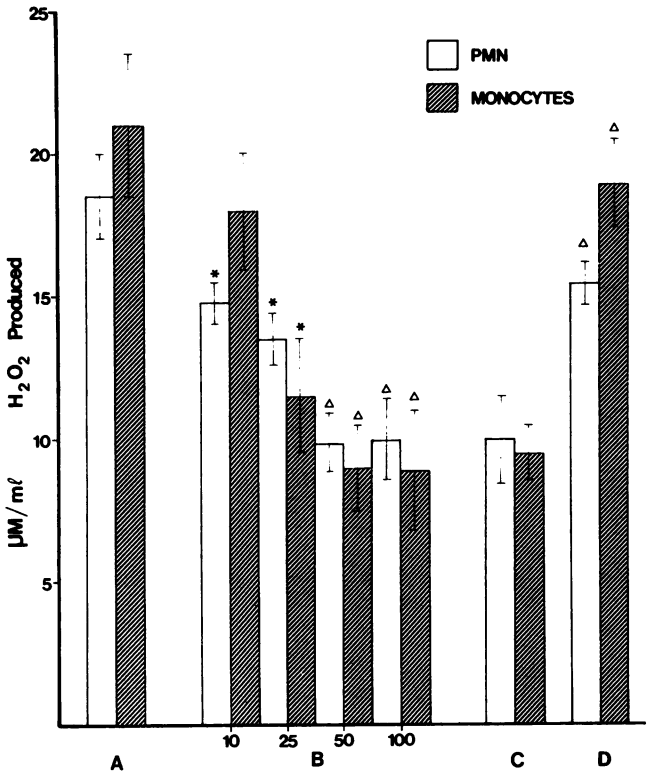

FIG. 2. H_2O_2 production by PMN cells (□) and monocytes (▨) incubated with medium (A); various doses (micrograms per milliliter) of the 25-kDa fraction (B); 50 μg of the 25-kDa fraction per ml with IFN-α (100 U/ml) (C); and 50 μg of the 25-kDa fraction per ml with IFN-γ (100 U/ml) (D). Mean ± standard deviation of three experiments. *, $P < 0.01$; △, $P < 0.001$.

with positive staining was observed. When IFN-γ was incubated together with the 25-kDa fraction, NBT reduction was partially restored (Table 2). No such restorative effect was observed when IFN-α was used in these assays (Table 2).

DISCUSSION

We have previously shown that *M. tuberculosis* extracts, and in particular the 25-kDa fraction derived from such extracts, markedly inhibited the intracellular killing ability of phagocytic cells (34). This was due, presumably, to an inhibition of phagosome-lysosome fusion. To further investigate the mechanism(s) responsible for this depressed microbicidal activity, in the present study we undertook to examine several additional parameters known to be operative in phagocyte bactericidal activity.

TABLE 1. Effect of the 25-kDa fraction and IFN on phagocyte HMPS activity

Activated cells incubated with:	Mean cpm ± SD[a]	
	PMN cells	Cultured monocytes
Medium	24,792 ± 2,098	14,715 ± 554
IFN-γ	24,121 ± 3,764	15,540 ± 664
25-kDa fraction	10,074 ± 1,160[b]	7,186 ± 1,920[b]
25-kDa fraction + IFN-γ	16,167 ± 1,652	12,727 ± 994
25-kDa fraction + IFN-α	11,070 ± 1,480	6,064 ± 1,489

[a] Three experiments.
[b] $P < 0.001$.

TABLE 2. Effect of the 25-kDa mycobacterial fraction on PMN NBT reduction

Cell system	No. of cells demonstrating NBT reduction[a]
PMN (resting)	9 ± 1
PMN + EAS[b]	58 ± 7
PMN + EAS + 25-kDa fraction	26 ± 5[c]
PMN + EAS + IFN-γ	61 ± 6
PMN + EAS + 25-kDa fraction + IFN-γ	40 ± 3[d]
PMN + EAS + 25-kDa fraction + IFN-α	29 ± 4

[a] Mean ± standard deviation of three experiments.
[b] EAS, Endotoxin-activated serum.
[c] $P < 0.001$.
[d] $P < 0.01$.

An important antimicrobial mechanism of both PMN cells and monocytes is the generation of the oxygen-derived reactive species, including H_2O_2 and superoxide anion, and the release of lysosomal enzymes. It has been demonstrated recently that both *Mycobacterium leprae* (14) and *M. tuberculosis* (16) are susceptible to peroxidase-mediated H_2O_2 killing in vitro. Despite this susceptibility, however, mycobacteria are still not effectively eliminated by competent phagocytes. To examine the possibility that mycobacterial survival may result from a failure or reduced capacity of phagocytes to generate H_2O_2, we examined the effects of the 25-kDa mycobacterial fraction on H_2O_2 production by activated phagocytes. Our studies indicate that the 25-kDa mycobacterial fraction inhibits the production of H_2O_2 by activated PMN cells and cultured monocytes. Inhibition of H_2O_2 by this fraction was dose dependent, with maximal effects observed at a concentration of 50 μg/ml. This was not due to a direct cytotoxic effect on leukocytes since cell viability after incubation with the 25-kDa fraction for 2 h was consistently greater than 95% (results not shown). In addition, our previous experiments (34) also demonstrate that the phagocytic ability of these cells is unaltered by the 25-kDa mycobacterial fraction.

In further experiments on the antimicrobial activity of phagocytes, we examined the reduction of NBT within cells or measured phagocyte HMPS activity. In addition to our findings of reduced H_2O_2 production, we demonstrated a depression of both NBT reduction and HMPS activity. These inhibitory effects of the 25-kDa mycobacterial fraction on the oxygen-dependent respiratory burst ranged between 51 and 65%. Furthermore, depression was evident at concentrations of 10 μg/ml, reaching a peak inhibitory dose of 50 μg/ml. In all of these assays, however, this concentration did not affect resting or basal levels (results not shown). These findings are in discord with those reported by May and Spagnuolo (19), who demonstrated that neither heat-killed nor live mycobacteria suppress the O_2^- release generated by PMN cells incubated with phorbol myristate acetate. Other studies (8) have indicated that mycobacterial sulfatide inhibits the priming of monocytes, resulting in a decline in the release of O_2^-. Furthermore, mycobacteria have also been implicated in the inactivation of O_2^- by producing their own superoxide dismutase (16). Even though the possibility does exist that our 25-kDa mycobacterial fraction may be demonstrating superoxide dismutase-like activity or contain the enzyme, the fact that other intracellular killing mechanisms are also interfered with makes this unlikely. The inhibition of

phagocyte function by bacterial fractions is not restricted to mycobacteria, as evidenced by reports on similar effects with pneumolysin derived from *Streptococcus pneumoniae* (24) or by reports on *Salmonella typhi* organisms demonstrating a suppression of PMN superoxide release or incomplete superoxide activation (15). It has also been suggested that *Listeria monocytogenes* may produce catalase to resist oxidative antibacterial agents (4).

High concentrations of lysozyme have been demonstrated in phagocytes (9). As lysosome is found in both the azurophil and specific granules of phagocytic cells, it was of interest to note the effects of the 25-kDa fraction on lysozyme release.

Our studies indicate that in addition to depressing the respiratory burst, the 25-kDa fraction significantly reduced lysozyme release in cells given a phagocytic stimulus. Inhibition was shown to be dose dependent and could be observed 5 min after cell activation. The observation that this fraction depressed lysozyme levels in activated leukocyte supernatants is in keeping with the findings of Gordon et al. (9) and Ridley et al. (31), who showed that lysozyme synthesis ceases in macrophages which have ingested *M. leprae*. It is possible that the organisms degraded by macrophages could release a fraction similar to that described herein and that this could inhibit the release of lysozyme or could neutralize any formed lysozyme.

Considerable data have now accumulated indicating that IFN-γ activates a variety of monocyte-marcophage functions, including the activation of antimicrobial activities (10, 28). Although the mechanism by which the latter occurs is unknown, we have demonstrated recently that IFN-γ, but not IFN-α, promotes phagosome-lysosome fusion and that it reverses the inhibitory effect of the 25-kDa mycobacterial fraction on leukocyte intracellular killing ability (34). IFN-γ has been demonstrated to enhance the respiratory burst and to induce the production of H_2O_2 in response to stimuli (20, 25).

Other workers, however, have indicated that phagocyte activation by IFN-γ depended on the agent utilized for cell activation (33). It was therefore of interest to examine the effects of these IFNs on other monocyte functions inhibited by the 25-kDa fraction. It was not surprising, therefore, that IFN-γ, but not IFN-α, reversed the inhibitory effects of the mycobacterial factor on H_2O_2 production, NBT reduction, HMPS activity, and lysozyme release. In some experiments, cultures were pretreated with the 25-kDa fraction, followed by IFN-γ treatment. In these experiments, IFN-γ was not able to reverse the inhibition caused by the mycobacterial fraction (results not shown). Our findings thus confirm that IFN-γ is a potent primer of these intracellular killing mechanisms as IFN-γ had no stimulatory effect on its own but was capable of partially restoring the functions inhibited by the 25-kDa fraction.

The results reported in this study may have a number of important clinical and therapeutic implications in the understanding of the pathogenesis of mycobacterial disease. It is possible that mycobacteria ingested by phagocytes could, upon degradation, release the 25-kDa fraction, which inhibits further microbicidal abilities of the phagocyte. Newly ingested mycobacteria would therefore resist destruction and might accumulate intracellularly, as seen in tuberculosis and leprosy. Reversal of this inhibition by IFN-γ may have clinical relevance in the treatment of these diseases.

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