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Evidence for Activation of a Respiratory Burst in the Interaction of Human Neutrophils with *Mycobacterium tuberculosis*

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We examined the capacity of human neutrophils to develop a respiratory burst, as monitored by superoxide release, in response to interaction with *Mycobacterium tuberculosis*. Serum-opsonized, heat-killed mycobacteria induced significant release of superoxide from neutrophils after 30 min of exposure, with a maximum release of 34 ± 1.7 nmol/30 min per 5 × 10^6 neutrophils occurring with a mycobacterium/neutrophil ratio of 40:1. Similar levels of superoxide release were induced by live mycobacteria. Neutrophil superoxide production was reduced significantly with exposure to unopsonized organisms or by substitution of heat-inactivated serum for opsonization. Mycobacterial components including culture filtrate, purified protein derivative, and the cell wall polysaccharide arabinogalactan failed to induce significant release of superoxide from neutrophils. Transmission electron microscopy demonstrated that more than 90% of the neutrophils had ingested heat-killed mycobacteria concomitant with the development of respiratory burst activity. These data suggest that the presumed failure of neutrophil killing of mycobacteria cannot be attributed to a lack of phagocytosis or respiratory burst activation.

New infections due to *Mycobacterium tuberculosis* number more than 20,000 per year in the United States (7). Worldwide, it is estimated that 20 million active infections occur at any one time (9). Despite this persistent case burden, a precise understanding of the pathogenesis of tuberculous infection has not been achieved. *M. tuberculosis* is recognized as a facultative intracellular pathogen which persists within macrophages after phagocytosis and produces granulomatous inflammation (18, 19, 31). As a result of these observations, it is often assumed that polymorphonuclear leukocytes (PMN) do not play a major role in host defense against *M. tuberculosis*, although specific data addressing this assertion are not available. We report that PMN exposed to *M. tuberculosis* in vitro undergo a respiratory burst during phagocytosis with release of superoxide (O_2^-) radicals, suggesting that PMN oxidative capacity is activated by contact with the mycobacteria for bactericidal activity.

PMN were isolated from heparinized venous blood of healthy adults with negative purified protein derivative (PPD) skin test results. Sequential Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) and 5% dextran sedimentation followed by hypotonic erythrocyte lysis resulted in a PMN suspension of >96% purity. Virulent *M. tuberculosis* H37Rv (TMC 102; kindly provided by the National Jewish Hospital, Denver, Colo.) was maintained in liquid Tween-albumin medium. The numbers of CFU in various inocula were determined by comparing the optical densities at 650 nm of suspension cultures with serial dilutions on 7H10 agar. Mycobacteria were either heat inactivated (68°C water bath, 2 h) or maintained live and washed three times in Hanks balanced salt solution (HBSS) (1,000 × *g*, 12 min) before opsonization. Opsonizing serum was pooled from healthy PPD skin test-negative adults and stored at −70°C until needed. The mycobacteria were incubated with serum in HBSS in a 1:4 (vol/vol) dilution (final serum concentration, 20%) for 30 min at 37°C, washed, and resuspended in HBSS at calculated CFU concentrations.

Superoxide release from PMN was assessed by the methods of Babior et al. (5) and was based on the reduction of ferricytochrome *c* (FCC) (horse heart type VI; Sigma Chemical Co., St. Louis, Mo.; final concentration, 100 μM) in the presence of O_2^-. PMN (5 × 10^6) were incubated in a shaking water bath at 37°C for 3 min with mycobacteria. After 30 min, FCC reduction was quenched by addition of 20 μl of superoxide dismutase (SOD) (bovine erythrocyte; Sigma Chemical Co; 2.5 mg/ml) to each tube followed by rapid centrifugation (17,500 × *g*, 2 min) and cooling to 4°C. Total FCC reduction was measured spectrophotometrically as the optical density at 550 nm as compared with that of an FCC/SOD blank. Superoxide production (in nanomoles) was calculated by using an extinction coefficient of 21 mM^{-1} cm^{-1} for FCC (21). All results were corrected for spontaneous, unstimulated FCC reduction in the presence of PMN alone, which was uniformly 4 to 9 nmol/30 min per 5 × 10^6 PMN.

Exposure of PMN to opsonized, heat-killed *M. tuberculosis* resulted in significant release of O_2^- at 30 min that was dose dependent (Table 1). For comparison, the soluble stimulus phorbol myristate acetate (PMA) (10 ng/ml) released 59.3 ± 2.1 nmol of O_2^- from PMN in parallel assays (Table 1). Unopsonized, heat-killed mycobacteria also induced significant PMN O_2^- production at 30 min as compared with unstimulated controls. In comparison with opsonized mycobacteria, however, unopsonized, heat-killed mycobacteria and mycobacteria opsonized in heat-inactivated serum gave 41.1% ($P < 0.001$, paired *t* test) and 71.6% ($P < 0.001$, paired *t* test), respectively, of the O_2^- production of fully opsonized inocula (data not shown). The specificity of our assay for O_2^- release was confirmed by incubating the reaction mixtures in the presence of SOD. FCC reduction due to PMA was inhibited by 92.1% at 30 min in the presence of 50 μg of SOD per ml ($P < 0.005$, paired *t* test), while that due to opsonized, heat-killed mycobacteria was suppressed by 94.5% ($P < 0.001$, paired *t* test).

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TABLE 1. Net PMN superoxide production induced by opsonized, heat-killed *M. tuberculosis*

Stimulus	*n*	Net O_2^- production[a] (nmol/30 min per 5×10^6 PMN)	*P*[b]
Mycobacteria[c]			
5:1	4	9.9 ± 1.7	0.005
10:1	5	18.3 ± 1.8	0.001
20:1	9	24.8 ± 1.2	0.001
40:1	15	34.1 ± 1.7	0.001
PMA (10 ng/ml)	14	59.3 ± 2.1	0.001

[a] Net O_2^- is defined as the difference between total superoxide production and superoxide production by unstimulated control PMN. Ratios indicate proportions of opsonized mycobacteria to PMN (mean ± standard error).
[b] Paired *t* test, compared with unstimulated control PMN.
[c] Organism/PMN ratio.

We next sought to determine whether live mycobacteria also elicited O_2^- production. In three experiments, inocula containing 40 CFU of opsonized, live mycobacteria per PMN released 55.6 ± 4.1 nmol of O_2^-, a quantity similar to that produced by PMA (10 ng/ml) in simultaneous assays (Table 2). Unopsonized, live mycobacteria and live mycobacteria opsonized with heat-inactivated serum produced 51.7 and 64.7% of the response to opsonized, live mycobacteria, respectively (the same general pattern noted previously with heat-killed mycobacteria). In the presence of 50 μg of SOD, superoxide release due to opsonized, live mycobacteria also was suppressed by 93.0%.

Neither opsonized, heat-killed mycobacteria nor opsonized, live mycobacteria reduced FCC in significant amounts in the absence of PMN. In both cases, average apparent FCC reduction caused by 2×10^8 CFU/ml (equivalent to the inoculum used in a 40:1 assay) was less than 50% of the amount induced by 5×10^6 unstimulated control PMN. This apparent FCC reduction may, in part, have represented light scattering from small numbers of mycobacteria remaining in suspension after centrifugation, a problem arising from the high lipid content of mycobacterial cell walls. In addition, opsonized heat-killed or live mycobacteria did not suppress or scavenge the measured O_2^- release induced by PMA when PMN were incubated with both stimuli simultaneously (Table 3).

A kinetic analysis of superoxide production over time demonstrated rapid onset of O_2^- release for both PMA and

TABLE 2. Net PMN superoxide production induced by live *M. tuberculosis*[a]

Stimulus	Net O_2^- production (nmol/30 min per 5×10^6 PMN)	% of response due to opsonized live mycobacteria[b]
Opsonized mycobacteria	55.6 ± 4.1	
Unopsonized mycobacteria	28.8 ± 3.7	51.7 (<0.001)
Opsonized (heat-inactivated serum) mycobacteria	36.0 ± 6.1	64.7 (<0.02)
Opsonized mycobacteria + SOD (50 μg/ml)	3.9 ± 0.4	7.0 (<0.01)
PMA (10 ng/ml)	55.1 ± 4.5	

[a] Mycobacteria in appropriate sources of serum were incubated with PMN in a ratio of 40:1. Net O_2^- is as defined in Table 1, footnote *a* (mean ± standard error, $n = 3$).
[b] *P* values are given parenthetically (paired *t* test, compared with opsonized live mycobacteria).

TABLE 3. Effect of mycobacteria on PMA-elicited O_2^- production[a]

Mycobacteria	Net O_2^- production (nmol/30 min per 5×10^6 PMN)		
	PMA alone	Mycobacteria alone	PMA + mycobacteria
Opsonized, heat killed	54.7 ± 5.4	39.4 ± 2.3	51.5 ± 5.6
Opsonized, live	55.1 ± 4.5	55.6 ± 7.1	57.7 ± 4.6

[a] PMN were incubated with PMA (10 ng/ml) or mycobacteria alone at a ratio of 40:1 (mycobacteria to PMN). To some tubes, mycobacteria were added 15 s after incubation with PMA. Net O_2^- is as defined in Table 1, footnote *a* (mean ± standard error, $n = 3$).

opsonized mycobacteria (Fig. 1). Opsonized, heat-killed mycobacteria induced a maximum of 61.2 ± 2.3 nmol of cumulative O_2^- release at 30 min. The largest cumulative response to PMA (60.8 ± 2.7 nmol of O_2^-) occurred at15 min of incubation. A 40-CFU amount of unopsonized, heat-killed mycobacteria per PMN did not produce an apparent maximal response over the time period studied. However, the O_2^- production recorded for unopsonized mycobacteria at 60 min was no longer statistically distinguishable from the O_2^- release due to opsonized, heat-killed mycobacteria ($P < 0.5$, paired *t* test).

The mycobacterial surface polysaccharide arabinogalactan (AG; kindly provided by Thomas Daniel, Case Western Reserve University School of Medicine, Cleveland, Ohio), culture filtrate, and PPD skin test reagent were all examined for the capacity to stimulate PMN superoxide production. Dialyzed culture filtrates and PPD did not release significant O_2^- from PMN (final dilutions for both

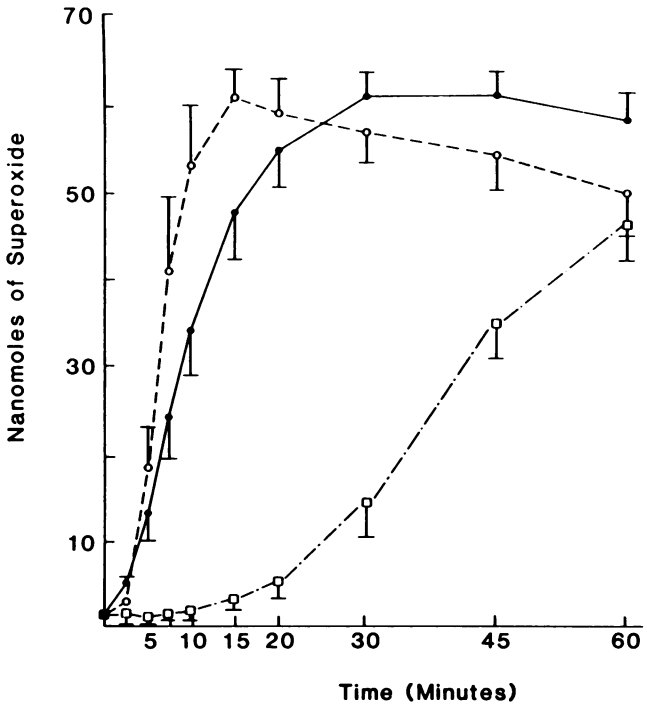

FIG. 1. Kinetic analysis of O_2^- release of neutrophils incubated with a 40:1 ratio of opsonized, heat-killed mycobacteria (●), unopsonized, heat-killed mycobacteria (□), or PMA (10 ng/ml) (○). Results are presented as the mean ± standard error (vertical bars) of four or five experiments and expressed as nanomoles per 30 min per 5×10^6 PMN.

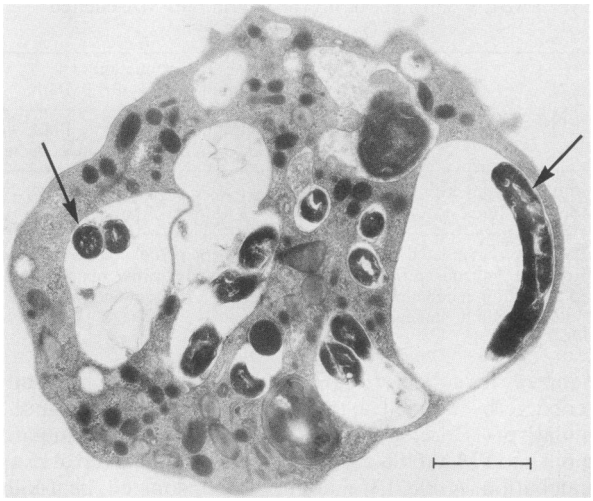

FIG. 2. Transmission electron micrograph of human PMN 30 min after exposure to heat-killed, opsonized mycobacteria. Cells were fixed in 2.5% phosphate-buffered glutaraldehyde and processed. Ultrathin sections were stained with uranyl acetate and lead citrate. Note numerous ingested mycobacteria in membrane-bound vacuolated phagosomes (arrows). ×12,700 (bar, 1 μm).

reagents of 1:5 and 1:20 [vol/vol] in HBSS; data not shown). AG ranging in concentrations from 10 ng/ml to 50 μg/ml also failed to induce O_2^- production. To determine whether AG or PPD presented as a particulate stimulus might induce O_2^- production, we coated latex beads with AG or PPD. At a bead/PMN ratio as great as 200:1, no significant difference in O_2^- release was detected between PPD-, AG-, and bovine serum albumin-coated beads (data not shown).

Examination of PMN after 30 min of incubation with heat-killed, opsonized mycobacteria revealed 96% viability by trypan blue exclusion. Both light microscopy (not shown) and transmission electron microscopy revealed that phagocytosis of the mycobacteria had occurred in more than 90% of PMN incubated with opsonized, heat-killed mycobacteria for 30 min (Fig. 2).

Facultative intracellular pathogens by definition evade PMN defenses in sufficient numbers to penetrate macrophages and persist within them. Several microbial strategies for intracellular survival have been described (11, 24, 27). *Salmonella typhi* organisms, for example, either suppress PMN superoxide release or fail to activate it fully (16), while *Brucella abortus* organisms inhibit PMN granule release (29). However, muted O_2^- release does not prevent neutrophils from ingesting and killing some of the virulent salmonella organisms they encounter (16), and brucella organisms succumb to nonoxidative killing by PMN granule fractions (28). The net contribution made by PMN to the host defense against these pathogens therefore remains speculative.

Whether PMN play a role at all in host defense against the intracellular pathogen *M. tuberculosis* has not been adequately investigated. Efforts to understand the pathogenesis of tuberculous infection have focused on the development of cell-mediated immunity, granuloma formation, and processing of the pathogen by monocytes or macrophages (18, 19, 31). Injection of virulent mycobacteria into tissues or the pleural space, however, produces an initial inflammatory response dominated by PMN; this response can persist for up to 48 h (6, 20, 23, 30). Light microscopic studies have demonstrated that PMN associate with and ingest mycobacteria when exposed to the organism in vitro and in vivo (1, 6, 20, 30). Antony and co-workers have recently shown that PMN release factors chemotactic for monocytes when stimulated by *Mycobacterium bovis* BCG (2), and animals rendered neutropenic in that study were significantly less able to mobilize monocytes to the pleural space than were nonneutropenic animals 72 h after intrapleural injection of BCG. These data indicate that PMN recognize mycobacteria and possibly initiate host cellular defenses against them, although details of the PMN-mycobacteria interaction are not known.

The current report confirms with transmission electron microscopy that the majority of human neutrophils exposed in vitro to heat-killed, opsonized mycobacteria ingest the bacilli into complete phagosomes by 30 min. As demonstrated in previous reports, this uptake of mycobacteria is not toxic to PMN (20). The present investigation documents for the first time, however, that PMN phagocytosis of heat-killed and live mycobacteria results in a dose-dependent initiation of the respiratory burst. Inocula of heat-killed mycobacteria clearly induce superoxide production by PMN, and substitution of live organisms for heat-killed organisms does not blunt this response. Abrogation of these responses by SOD confirmed specificity for O_2^- release.

These findings suggest that mycobacterial persistence at sites of infection cannot be attributed to a failure to trigger PMN oxidative metabolism. Either the quantity of superoxide liberated is insufficient for killing or other bacterial defense strategies are operative to prevent destruction of the mycobacteria (15). Although we considered the possibility that mycobacteria inactivate O_2^- with their own SOD (17), our data indicate that neither heat-killed nor live mycobacteria suppress the O_2^- release generated by PMN incubated with PMA.

That unopsonized mycobacteria were able to induce O_2^- release from PMN is also noteworthy. One possible explanation for this finding is that PMN were reacting to mycobacterial surface components without the intervention of serum opsonins. The mycobacterial cell wall polysaccharide AG, a fairly uniform branched carbohydrate found covalently linked to the peptidoglycan layer of all mycobacteria (22), was unable to stimulate PMN O_2^- production in this study. Similar results were seen with mycobacterial culture filtrates and PPD.

The ultimate fate of mycobacteria within PMN is unclear. In macrophages from nonimmune animals, virulent mycobacteria are known to inhibit fusion of lysosomes with phagosomes containing the bacilli, a phenomenon attributed in part to the activity of mycobacterial sulfatides (3, 4, 14). Mycobacteria can also rupture macrophage phagosomes (25). The current investigation demonstrates that the presumed failure of PMN killing of *M. tuberculosis* occurs distal to the point of phagocytosis and respiratory burst activation induced by this pathogen.

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LITERATURE CITED

1. **Allgower, M., and H. Bloch.** 1949. The effect of tubercle bacilli on the migration of phagocytes in vitro. Am. Rev. Tuberc. **59**:562–566.
2. **Antony, V. B., S. A. Sahn, A. C. Antony, and J. E. Repine.** 1985.

Bacillus Calmette-Guerin-stimulated neutrophils release chemotaxins for monocytes in rabbit pleural spaces and in vitro. J. Clin. Invest. **76**:1514–1521.
3. **Armstrong, J. A., and P. D. 'A. Hart.** 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134**:713–739.
4. **Armstrong, J. A., and P. D. 'A. Hart.** 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. J. Exp. Med. **142**:1–16.
5. **Babior, B. M., R. S. Kipnes, and J. T. Curnutte.** 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52**:741–744.
6. **Bloch, H.** 1948. The relationship between phagocytic cells and human tubercle bacilli. Am. Rev. Tuberc. **58**:662–670.
7. **Centers for Disease Control.** 1985. Tuberculosis—United States, 1984. Morbid. Mortal. Weekly Rep. **34**:299-307.
8. **Chick, T. W., S. E. Goldblum, N. D. Smith, C. Butler, B. J. Skipper, J. A. Winklestein, L. C. Cork, and W. P. Reed.** 1984. Pneumococcal-induced pulmonary leukostasis and hemodynamic changes: role of complement and granulocytes. J. Lab. Clin. Med. **103**:180–192.
9. **Daniel, T. M.** 1984. Tuberculosis, p. 787–797. *In* K. S. Warren and A. A. F. Mahmoud (ed.), Tropical and geographic medicine. McGraw-Hill, New York.
10. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42**:84–113.
11. **Densen, P., and G. L. Mandell.** 1980. Phagocyte strategy vs. microbial tactics. Rev. Infect. Dis. **2**:817–838.
12. **Fearon, D. T., S. Ruddy, P. H. Schur, and W. R. McCabe.** 1975. Activation of the properdin pathway in patients with gram-negative bacteremia. N. Engl. J. Med. **292**:937–940.
13. **Fleck, L.** 1956. Recent investigations on leukergy. Tex. Rep. Biol. Med. **14**:424–431.
14. **Goren, M. B., P. D. 'A. Hart, M. R. Young, and J. A. Armstrong.** 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **73**:2510–2514.
15. **Jackett, P. S., V. R. Aber, and D. B. Lowrie.** 1978. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104**:37–45.
16. **Kossack, R. E., R. L. Guerrant, P. Densen, J. Schadelin, and G. L. Mandell.** 1981. Diminished neutrophil oxidative metabolism after phagocytosis of virulent *Salmonella typhi*. Infect. Immun. **31**:674–678.
17. **Kusunose, E., K. Ichihara, Y. Noda, and M. Kusunose.** 1976. Superoxide dismutase from *Mycobacterium tuberculosis*. J. Biochem. **80**:1343–1352.
18. **Lagrange, P. H.** 1984. Cell-mediated diminunity and delayed-type hypersensitivity, p. 681–720. *In* G. P. Kubica and L. G. Wayne (ed.), The Mycobacteria: a sourcebook, part B. Marcel Dekker, Inc., New York.
19. **Lowrie, D. B.** 1983. Mononuclear phagocyte-*Mycobacterium* interaction, p. 235–275. *In* C. Ratledge and J. Stanford (ed.), The biology of the mycobacteria, vol. 2. Academic Press, Inc. (London), Ltd., London.
20. **Martin, S. P., C. H. Pierce, G. Middlebrook, and R. J. Dubos.** 1950. The effect of tubercle bacilli on the polymorphonuclear leukocytes of normal animals. J. Exp. Med. **91**:381–391.
21. **Massey, V.** 1959. The microestimation of succinate and the extinction coefficient of cytochrome *c*. Biochem. Biophys. Acta **34**:255–256.
22. **Misaki, A., N. Seto, and I. Azuma.** 1974. Structure and immunological properties of D-arabino-D-galactans isolated from *Mycobacterium* species. J. Biochem. **76**:15–23.
23. **Montgomery, L. G., and W. S. Lemon.** 1933. The cellular reaction of the pleura to infection with *Mycobacterium tuberculosis*. J. Thorac. Surg. **2**:429–438.
24. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49**:298–337.
25. **Myrvik, Q. N., E. S. Leake, and M. J. Wright.** 1984. Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **129**:322–328.
26. **Nelson, D. S.** 1963. Immune adherence. Adv. Immunol. **3**: 131–180.
27. **Quie, P. G.** 1983. Perturbation of the normal mechanisms of intraleukocytic killing of bacteria. J. Infect. Dis. **148**:189–193.
28. **Riley, L. K., and D. C. Robertson.** 1984. Brucellacidal activity of human and bovine polymorphonuclear leukocyte granule extracts against smooth and rough strains of *Brucella abortus*. Infect. Immun. **46**:231–236.
29. **Riley, L. K., and D. C. Robertson.** 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect. Immun. **46**:224–230.
30. **Vorwald, A. J.** 1932. The early cellular reactions in the lungs of rabbits injected intravenously with human tubercle bacilli. Am. Rev. Tuberc. **25**:74–88.
31. **Youmans, G. P.** 1979. Tuberculosis, p. 285–301. W. B. Saunders Co., Philadelphia.