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Live but Not Heat-Killed Mycobacteria Cause Rapid Chemotaxis of Large Numbers of Eosinophils In Vivo and Are Ingested by the Attracted Granulocytes

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We studied leukocyte chemotaxis triggered by a local injection of mycobacteria (*Mycobacterium avium* and *M. smegmatis*) in BALB/c and C57BL/6 mice. Our experimental model consisted of the induction of a subcutaneous air pouch in the dorsal area of mice and inoculation 6 days later of 10^8 CFU of mycobacteria. Inflammatory exudates were harvested from the air pouch cavities 15, 30, and 45 min after the injection of the inocula. Injection of the microorganisms resulted in the migration of an elevated number of eosinophilic granulocytes into the inflammatory cavities. At 30 min after the inoculation of the mycobacteria, the air pouches contained between $(3.9 \pm 0.3) \times 10^5$ (*M. avium*) and $(3.3 \pm 0.3) \times 10^5$ (*M. smegmatis*) eosinophils, corresponding to more than one-third (41.4 to 38.3%) of the leukocytes present in the inflammatory cavities. Less than one-half of the eosinophils were attracted to the air pouches when the same number of heat-killed mycobacteria were inoculated [$(1.3 \pm 0.2) \times 10^5$ cells for *M. avium* and $(1.5 \pm 0.2) \times 10^5$ cells for *M. smegmatis*]. Injection of gram-negative bacteria (*Escherichia coli*), of latex beads, or of casein resulted in the attraction of inflammatory eosinophils in numbers that were comparable to those attracted by the heat-killed mycobacteria. Our data document the fact that live mycobacteria exert a rapid chemotactic effect on eosinophils. We therefore postulate that mycobacteria either contain or induce the production of an eosinophilotactic factor. Because this chemotactic effect occurs during the acute inflammatory response to mycobacteria, it cannot be due to the formation of immune complexes (a major infection-associated chemotactic factor for eosinophils). The attracted eosinophils had an important role in the local phagocytosis of mycobacteria, as indicated by our finding, derived from thin-section electron microscopy quantifications, that at 30 min after *M. avium* inoculation the inflammatory exudates contained $(2.2 \pm 0.5) \times 10^5$ mycobacterium-bearing eosinophils (corresponding to 57% of the total eosinophils), as compared with $(2.1 \pm 0.1) \times 10^5$ neutrophils and $(1.5 \pm 0.2) \times 10^5$ macrophages with ingested bacilli. We conclude that mycobacteria induce the attraction of eosinophils to inflammatory sites and that these granulocytes have the capacity to phagocytize these bacilli in situ.

Eosinophilic and neutrophilic granulocytes show different behaviors in inflammation and infection. Blood eosinophilia is a common finding in allergic states and in infections by helminthic parasites but is not a frequent consequence of bacterial infections (2, 6, 7, 11). High numbers of neutrophils, but few eosinophils, participate in the acute nonanaphylotactic inflammatory response, whereas eosinophils are abundant in areas of chronic inflammation (12, 21). These differences in behavior are derived, at least in part, from the distinct chemotactic sensitivities of the two types of granulocytes. In fact, the major chemotactic stimuli for eosinophils, i.e., anaphylotactic factors (11), antigen-antibody complexes (17), histamine (11, 24), and aggregated immunoglobulins (16), are not potent attractants for neutrophils.

Because human patients with mycobacterial infections frequently show eosinophilia (5, 23, 32), we decided to investigate whether an experimental acute inflammation caused by mycobacteria was associated with local eosinophilotaxis. Two mycobacterial species were tested: *Mycobacterium avium*, an opportunistic mycobacterial species that plagues AIDS patients (8, 13, 18) and elderly persons (22), and *M. smegmatis*, a fast-growing species that seldom causes disease in humans (33). We used the air pouch model of inflammation (25, 26) to determine the cell kinetics and leukocyte type present in the inflammatory exudates caused by mycobacterial inoculation. We report that the acute inflammatory response to the inoculated mycobacteria was associated with the migration of high numbers of eosinophils into the air pouch cavity and that significantly fewer eosinophils were obtained when heat-killed bacilli were injected. Light and electron microscopy analyses of the samples documented that inflammatory eosinophilic granulocytes had the ability to ingest significant numbers of mycobacteria.

MATERIALS AND METHODS

Animals. In each set of experiments, 25 groups (see Table 1 for a list of the experimental groups) of five to seven male BALB/c and C57BL/6 inbred mice were used. The animals were obtained from a local breeder (Instituto Gulbenkian de Ciência, Oeiras, Portugal) and used at 6 to 8 weeks of age.

Bacteria. *M. avium* (ATCC 25291) and *M. smegmatis* (strain 133001 from the Pasteur Institute, Paris, France) were cultured for 2 weeks or 3 days, respectively, at 37°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.)

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with 0.04% Tween 80 as described before (27). The mycobacteria were harvested from liquid cultures, washed in saline with 0.04% Tween 80, and diluted in the same vehicle to the appropriate concentrations before inoculation. *Escherichia coli* DH5 was cultured in nutrient broth (Difco) supplemented with 0.5% yeast extract (Difco) at 37°C, harvested, and washed in phosphate-buffered saline (PBS).

Air pouch formation. Ether-anesthetized mice were injected subcutaneously in the dorsal area with 5 ml of sterile air. The air injections were performed inside a laminar flow hood. A second subcutaneous injection of 3 ml of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air pouch model favors the formation of lining cells that increase the reactivity of the air pouch cavity (9, 14, 25). Microbial and other inflammatory stimuli were inoculated 24 h after the second air injection.

Experimental conditions. The air pouches of each group of animals were injected with the following inocula made in 1 ml of saline with 0.04% Tween 80: (i) 10^8 viable *M. avium* or *M. smegmatis* cells; (ii) 10^8 heat-killed *M. avium* or *M. smegmatis* cells (boiling of the mycobacterial aliquots for 2 h before injection); (iii) 10^8 viable *E. coli* cells; (iv) a latex bead suspension (1.23 μm in diameter; Sigma reference no. 7310) diluted 1:100; (v) 10% casein hydrolysate (Difco); and (vi) the vehicle alone (1 ml of saline with 0.04% Tween 80). All experiments were repeated at least four times, with similar results.

Inflammatory exudates. The exudates were recovered from the air pouches 15, 30, and 45 min after injection of the different inflammatory stimuli. Harvesting was done by injecting the air pouch cavities with 2 ml of PBS and recovering the lavage fluid. The number of cells collected from the air pouches was determined with an automatic cell counter. The cell suspension was spun down in a cytocentrifuge onto glass slides. The preparations were fixed with 10% Formol in ethanol and stained with erythrosine-eosine (1% [wt/vol] erythrosine B and 1% [wt/vol] eosine yellowish in distilled water). This staining method was adopted after preliminary assays revealed that it was more accurate for visualizing by light microscopy the acidophilic granules of mouse eosinophils than was staining with eosine alone (used as part of the Wright staining method). Leukocytes were counted by both light microscopy examination of the cytocentrifuge preparations and thin-section electron microscopy. *M. avium* and eosinophils were simultaneously visualized by electron microscopy and by treatment of the cytocentrifuge slides with Ziehl-Neelsen stain (without heating) and erythrosine-eosine stain.

Phagocytosis of mycobacteria. We used thin-section electron microscopy to quantify the number of phagocytes (eosinophils, neutrophils, and macrophages) with ingested mycobacteria in inflammatory exudates collected 30 min after the injection of the air pouches with 10^8 CFU of viable *M. avium*. This method was chosen because ultrastructural scrutiny of cells offers unambiguous identification of phagocytosis of mycobacteria (27). The number of *M. avium*-containing eosionophlis present in samples from the same exudates was also calculated by an independent method: light microscopy of cytocentrifuge preparations doubly stained with Ziehl-Neelsen stain (without heating) and erythrosine-eosine stain. The numerical values obtained by this second method were close to those obtained by thin-section electron microscopy scoring.

Electron microscopy. Inflammatory exudate cells were resuspended in 5% bovine serum albumin, and microbuffy coats were obtained by the method of Moura Nunes et al. (20). The buffy coats were fixed in 4% formaldehyde–1.25% glutaraldehyde–10 mM $CaCl_2$, washed in PBS, and postfixed in 1% OsO_4–10 mM $CaCl_2$ and then in 1% uranyl acetate as described before (28, 29). The specimens were dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed by electron microscopy (Siemens Elmiskop 1A or Zeiss EM9 microscope).

Statistical analysis. The numerical data were statistically compared with the Student *t* test.

RESULTS

We studied the leukocyte chemotaxis induced in vivo by *M. avium* and *M. smegmatis* by using a murine air pouch model of acute inflammation. Both BALB/c and C57BL/6 inbred mouse strains were used. This model allowed easy recovery and quantification of leukocytes from the inflammatory site. The leukocytes were harvested from the pouch 15, 30, and 45 min after the local inoculation of mycobacteria. The exudate was characterized by the total number of cells and leukocyte type. Before injection of the mycobacteria, the subcutaneous air pouch contained a moderate number of cells (2.4×10^5), with (1.8 ± 0.3) $\times 10^4$ eosinophilic granulocytes (7.3% of the total number of cells; see control values in Table 1).

Injection of the air pouch with 10^8 CFU of either mycobacterial species induced a striking increase in the number of leukocytes recovered from the inflammatory cavity. Eosinophilic granulocytes made up a large subpopulation of the leukocytes that were attracted to the air pouch by the mycobacteria (Table 1). These granulocytes were present in particularly high numbers 30 min after the injection of viable mycobacteria: (3.9 ± 0.3) $\times 10^5$ eosinophils were counted after *M. avium* injection and (3.3 ± 0.3) $\times 10^5$ eosinophils were counted after *M. smegmatis* injection, corresponding, at this time, to the largest subpopulation of phagocytes (38.3 to 41.4% of the total number of inflammatory leukocytes) (Fig. 1). The numbers of eosinophils attracted by the two mycobacterial species were not significantly different. Comparably high percentages of eosinophils were found in the air pouch 15 and 45 min after the mycobacterial injections. The peaks of eosinophil accumulation were different for *M. avium* and *M. smegmatis*. For *M. avium*, the number of eosinophils attracted to the air pouch increased from 15 to 30 min and decreased thereafter (Fig. 1). For *M. smegmatis*, the maximum number of attracted eosinophils was seen at 45 min for both viable [(3.5 ± 0.3) $\times 10^5$ eosinophils) and heat-killed [(1.7 ± 0.2) $\times 10^5$ eosinophils) bacilli and decreased at 60 min [(2.8 ± 0.4) $\times 10^5$ eosinophils attracted by viable bacilli; (1.2 ± 0.2) $\times 10^5$ eosinophils attracted by heat-killed bacilli; not depicted in Fig. 1]. In contrast with the kinetics for eosinophils, both neutrophils and mononuclear cells followed a different chronology of migration to the inflammatory cavity, since their numbers increased steadily during the period of acute inflammation that we studied, rather than peaking at 30 or 45 min, as observed for eosinophils (Table 1).

Significantly fewer eosinophils ($P < 0.01$) were attracted to the inflammatory pouches when inert particles (latex beads) of a size close to that of mycobacteria were injected. Comparable results were obtained after the injection of a solution of 10% casein hydrolysate (Fig. 1).

The injection of the same numbers of heat-killed *M. avium* or *M. smegmatis* cells into the air pouches attracted signif-

TABLE 1. Numbers of cells present in air pouches of BALB/c mice after injection of various inocula[a]

Inoculum	Time (min)	No. (10^4)[b] of the following cells:		
		Mononuclear	Neutrophils	Eosinophils
Control		16.5 ± 2.2	6.1 ± 1.3	1.8 ± 0.3
Viable *M. avium*	15	20.1 ± 1.8	17.2 ± 1.5	16.0 ± 2.0
	30	29.2 ± 4.8	26.0 ± 1.3	39.0 ± 3.2
	45	29.3 ± 1.8	34.2 ± 1.6	30.0 ± 2.8
Heat-killed *M. avium*	15	23.0 ± 1.5	16.0 ± 2.1	8.2 ± 1.0
	30	25.1 ± 3.9	21.3 ± 3.4	13.5 ± 2.0
	45	33.4 ± 2.9	28.6 ± 4.2	10.8 ± 2.0
Viable *M. smegmatis*	15	19.0 ± 1.5	15.1 ± 1.1	14.0 ± 1.4
	30	25.1 ± 2.0	28.2 ± 1.5	33.1 ± 3.0
	45	26.1 ± 2.3	33.3 ± 1.0	35.2 ± 2.8
Heat-killed *M. smegmatis*	15	21.4 ± 3.8	16.7 ± 1.1	8.2 ± 1.7
	30	23.6 ± 3.6	22.8 ± 2.4	15.0 ± 1.6
	45	25.3 ± 3.5	25.0 ± 1.6	17.6 ± 2.0
E. coli	15	12.6 ± 1.3	7.8 ± 1.1	6.9 ± 1.3
	30	20.4 ± 1.5	18.5 ± 1.3	16.1 ± 1.5
	45	23.0 ± 1.6	20.1 ± 1.5	16.0 ± 1.2
Latex beads	15	22.8 ± 2.1	10.1 ± 2.0	4.0 ± 0.4
	30	24.2 ± 3.5	18.0 ± 3.6	8.9 ± 0.2
	45	32.0 ± 1.9	22.1 ± 2.8	6.5 ± 0.4
Casein	15	23.4 ± 3.2	7.1 ± 2.2	2.1 ± 0.3
	30	28.9 ± 2.3	9.0 ± 1.9	6.1 ± 0.2
	45	31.0 ± 2.4	14.0 ± 1.8	4.5 ± 0.3
Saline	15	21.4 ± 2.8	8.0 ± 1.1	2.0 ± 0.4
	30	32.0 ± 3.0	11.2 ± 1.8	3.6 ± 0.2
	45	31.6 ± 4.0	14.0 ± 2.3	3.0 ± 0.4

[a] Air pouches were injected with the following inocula: 10^8 viable or heat-killed *M. avium* and *M. smegmatis* cells; 10^8 viable *E. coli*; and noninfectious phlogistic agents (latex beads and 10% casein hydrolysate). The number of leukocytes present in nontreated air pouches is shown in the top row (control). The number of leukocytes attracted by the vehicle alone are shown in the last three rows (saline). Statistically significant differences ($P < 0.01$) were found in the numbers of attracted eosinophils between the following experimental groups: viable mycobacteria (*M. avium* or *M. smegmatis*) versus latex beads, casein, or saline; viable mycobacteria versus heat-killed mycobacteria; and viable mycobacteria versus viable *E. coli*.

[b] Values are reported as means ± standard deviations of the numbers of cells in groups of five to seven mice.

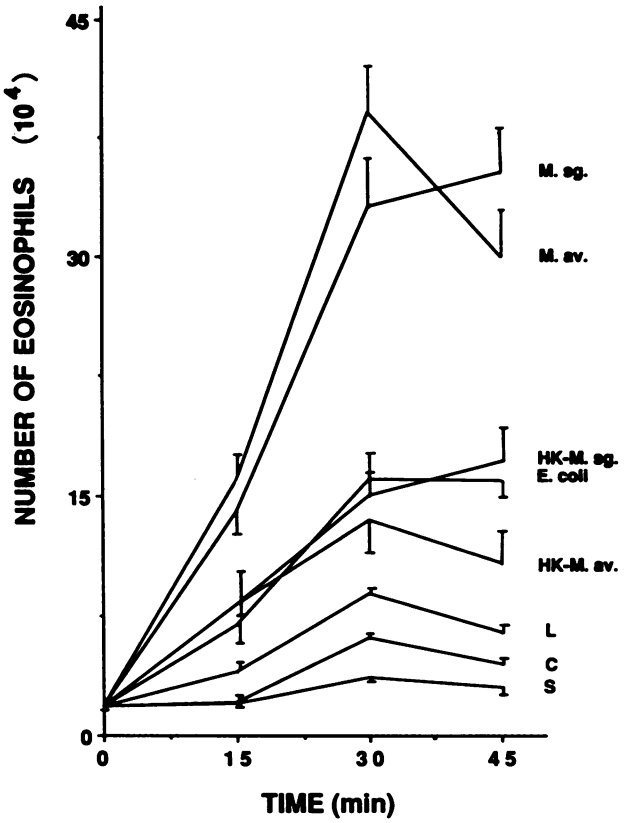

FIG. 1. Kinetics (15, 30, and 45 min) for the numbers of eosinophils attracted to air pouch cavities of BALB/c mice by 10^8 cells of viable and heat-killed (HK) *M. avium* (M. av.) and *M. smegmatis* (M. sg.), of 10^8 viable cells of the gram-negative bacterium *E. coli* (E. coli), and of several phlogistic agents (C, 10% casein hydrolysate; L, 1.23-μm latex beads). The effect of the vehicle (saline, S) alone is also shown. Viable mycobacteria attracted significantly higher numbers of eosinophils to inflammatory cavities than did the same number of heat-killed mycobacteria or viable *E. coli*. The eosinophilotactic action of the mycobacteria was also significantly higher than was that of inert particles (latex beads) or casein.

icantly fewer eosinophils to the inflammatory cavities than did viable mycobacteria (Fig. 1). The difference was statistically significant ($P < 0.01$). Dead bacilli also resulted in the presence of fewer neutrophils in the inflammatory cavities than did viable bacilli. In contrast, the number of mononuclear cells attracted by the dead bacilli was not significantly different from that attracted by viable *M. avium* or *M. smegmatis* (Table 1). We also compared the chemotactic effect of the mycobacteria (gram-positive bacilli) with that of viable *E. coli*, a gram-negative bacterium. *E. coli* cells attracted eosinophils in numbers comparable to those attracted by latex beads or heat-killed mycobacteria and, therefore, were significantly less eosinophilotactic than were live mycobacteria.

We used thin-section electron microscopy to determine the number of eosinophils with ingested mycobacteria at 30 min after the injection of 10^8 CFU of *M. avium* into the air pouches of BALB/c mice. This quantitative evaluation revealed that $(2.2 \pm 0.5) \times 10^5$ eosinophils contained phagocytosed *M. avium* bacilli (Fig. 2). This number represents a significant complement (57%) of the total number of inflammatory eosinophils present in these exudates. Quantification of *M. avium*-containing eosinophils in the same samples was also done by light microscopy after double staining of cytocentrifuge slides of the inflammatory exudates with Ziehl-Neelsen stain (to visualize the mycobacteria) and erythrosine-eosine stain (to identify eosinophils). Scoring by this method indicated that the number of *M. avium*-containing eosinophils $[(2.4 \pm 0.2) \times 10^5$ cells] was not significantly different from that obtained in the electron microscopy examination of the same samples. We also found that, at 30 min, the number of eosinophils with phagocytosed *M. avium* was comparable to that of mycobacterium-containing neutrophils $[(2.1 \pm 0.1) \times 10^5$ cells] and higher than that of *M. avium*-containing macrophages $[(1.5 \pm 0.2) \times 10^5$ cells] detected at this time.

In thin-section electron microscopy preparations, the *M. avium*-containing eosinophils showed the characteristic crystal-bearing, football-shaped granules of these granulocytes. Some of the eosinophil granules showed signs of decondensation of their matrices (arrowheads in Fig. 2).

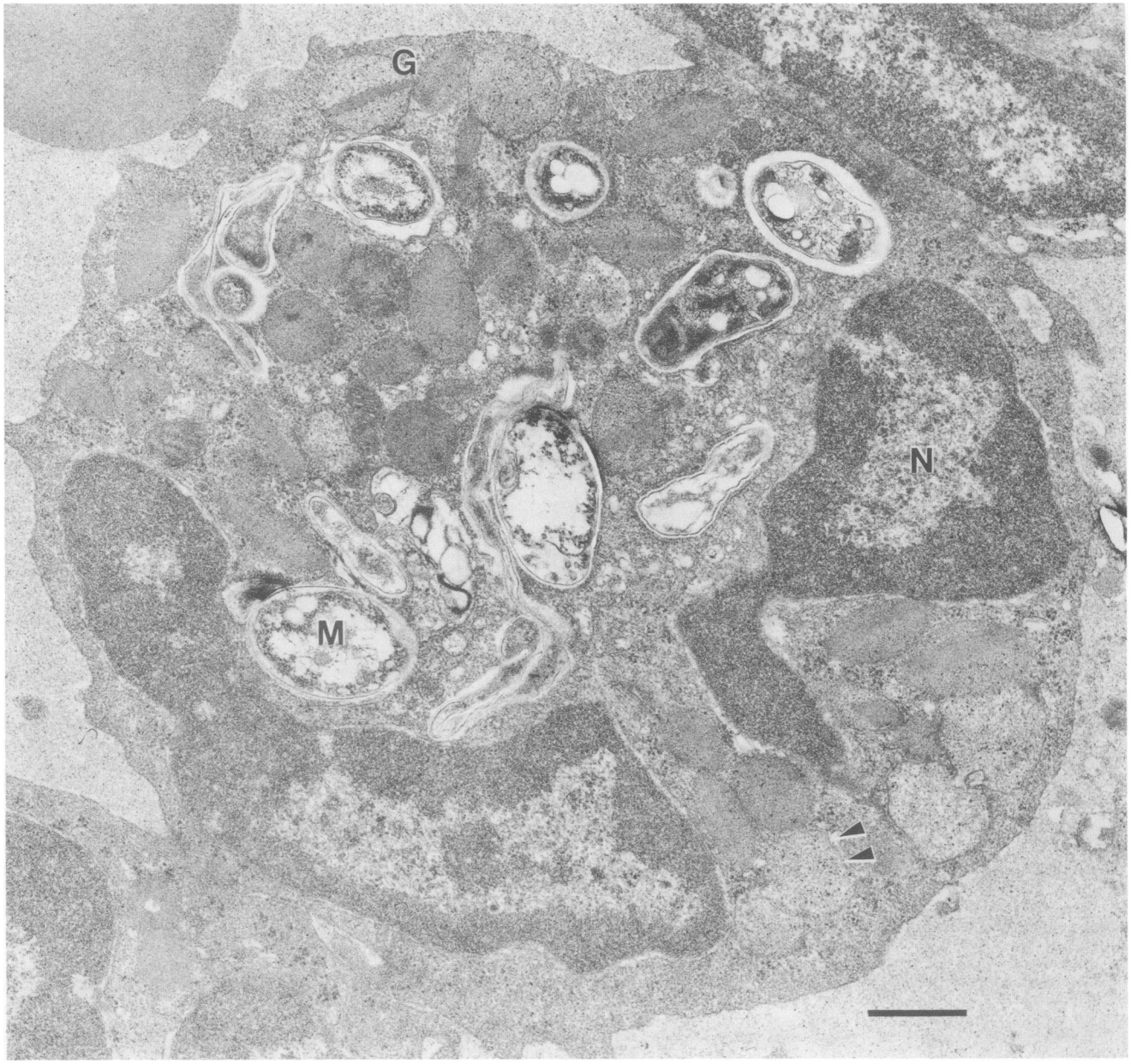

FIG. 2. Thin-section electron micrograph of mycobacterium-containing eosinophil recovered from the air pouch of a BALB/c mouse injected 30 min before with 10^8 CFU of *M. avium*. The granulocyte shows the characteristic crystal-bearing granules (G) of eosinophils, some of which show signs of decondensation (arrowheads). N, nucleus; M, mycobacteria. Magnification, ×25,600. Bar, 0.6 μm.

DISCUSSION

We investigated here the chemotactic effect of mycobacteria (*M. avium* and *M. smegmatis*) for leukocytes during acute inflammation. We used a modification of the air pouch model of inflammation adapted to mice to obtain an easy and reliable recovery of cells from the inflammatory site (9, 25). Our protocol involved the injection of 6-day-old air pouches with 10^8 CFU of *M. avium* and harvesting of the exudates 15, 30, and 45 min later. High numbers of inflammatory eosinophils were collected from the air pouches after the mycobacterial injection. In these samples, the eosinophils made up more than one-third of the total number of inflammatory leukocytes present in the air pouches. Viable mycobacteria attracted significantly higher numbers of eosinophils than did equal numbers of heat-killed mycobacteria or of the gram-negative bacterium *E. coli*. Phlogistic agents (e.g., casein) or inert particles (latex beads) of a size approximating that of the microorganisms also induced significantly less eosinophilotaxis than did viable mycobacteria.

Mycobacteria are among the infectious agents associated with eosinophilia in humans (5, 23, 32). Our experimental data are consistent with this view, although we have not addressed here mycobacterium-induced chronic eosinophilia. In addition, we have also found a significant difference between live and heat-killed mycobacteria in their abilities to attract eosinophils to inflammatory sites. Inter-

estingly, it was recently reported that live mycobacteria also attract more neutrophilic granulocytes to the peritoneal cavity of mice than do heat-killed mycobacteria (30). Together, the two sets of data indicate that mycobacteria may produce or induce chemotactic agents for both types of granulocytes. This idea is of particular importance because of recent evidence of an important role for granulocytes in host defense against mycobacterial infections (1, 3, 10, 15, 30). Interestingly, the kinetics of attraction of eosinophils to the acute inflammatory site studied here are distinct from the kinetics of attraction of neutrophils or mononuclear cells: the number of eosinophils in the air pouches reached its highest level earlier than did that of neutrophils or mononuclear cells.

The eosinophilia that accompanies mycobacteriosis is commonly seen as a result of chronic recruitment and retention of eosinophils, a phenomenon that is interpreted as the consequence of the formation of a large number of immune complexes produced as a response to the infection (5, 19, 21). This view is consistent with the well-established induction of polyclonal B cell activation by mycobacteria leading to high antibody levels in host serum and to elevated concentrations of circulating antigen-antibody complexes (4, 31). The immune complexes would be the major eosinophilotactic factor associated with mycobacterial infection (19, 21).

We report here that the eosinophilotaxis induced by mycobacteria at the site of inflammation is a rapid event. This result establishes that, at least in our model, mycobacteria can also induce eosinophilotaxis by nonimmune mechanisms. Clearly, the formation of immune complexes occurs only well after the short period (45 min) of the acute response to infection that we investigated here. Because we found that viable *M. avium* and *M. smegmatis* attracted significantly higher numbers of eosinophils than did dead bacilli, we propose that mycobacteria may contain a heat-labile factor or secrete a component that exerts, directly or indirectly, the eosinophilotactic effect.

The attraction of eosinophils to the inflammatory cavity by viable *M. avium* was followed by an important participation of the eosinophils in the phagocytosis of the mycobacteria, detected and quantified by thin-section electron microscopy. In fact, we found that at 30 min after *M. avium* injection there were more mycobacterium-containing eosinophils than neutrophils or macrophages with ingested bacilli. This result indicates that when extracellular mycobacteria are available, eosinophilic granulocytes will participate in the scavenging of the microorganisms in infected tissues. Taken together, our results suggest that eosinophils may have a significant role in the natural history of mycobacterial infections.

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