Involvement of Reactive Oxygen Intermediates in Tumor Necrosis Factor Alpha-Dependent Bacteriostasis of *Mycobacterium avium*

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Received 15 February 1996/Returned for modification 17 May 1996/Accepted 29 May 1996

We studied the involvement of reactive oxygen intermediates and reactive nitrogen intermediates in the bacteriostasis of two *Mycobacterium avium* strains differing in virulence by resident peritoneal macrophages. We found that both the highly virulent strain (25291) and the low-virulence strain (1983) of *M. avium* induced superoxide production but inhibited nitrite production in vitro. This inhibition was due to the production of superoxide, a nitric oxide scavenger. The stimulation of superoxide production was two- to fivefold higher in strain 1983-infected than in strain 25291-infected resident peritoneal macrophages and was independent of contaminating T cells or NK cells. Superoxide secretion was dependent on the tumor necrosis factor (TNF) produced endogenously by the macrophages. This was also true when macrophages were isolated from infected mice. Addition of TNF to the infected resident peritoneal macrophages caused only a slight, albeit significant, increase in superoxide production by strain 25291-infected macrophages. Incubation of resident peritoneal macrophages with different scavengers of reactive oxygen intermediates showed that strain 1983 was susceptible to hydrogen peroxide produced by resident peritoneal macrophages. Strain 25291 was shown to decrease superoxide secretion inside heavily infected bone marrow-derived macrophages. This strain was also shown to be a better trigger for production of reactive oxygen intermediates than strain 1983. In summary, strain 1983 induced high levels of TNF synthesis that acted in an autocrine fashion to stimulate production of reactive oxygen intermediates by macrophages leading to growth restriction mediated by hydrogen peroxide. The highly virulent strain 25291 induced low levels of TNF synthesis, and therefore little reactive oxygen intermediate production, and could also inhibit superoxide production by the infected macrophages.

Mycobacterium avium bacilli grow intracellularly inside macrophage phagosomes (20), circumventing the antimicrobial mechanisms of this phagocyte. However, macrophages can be activated to increase their microbiostatic and microbicidal capacity against *M. avium*. This activation may occur by T-cell-dependent (2, 28, 46, 47) as well as, possibly, T-cell-independent mechanisms, such as those involving NK cells (5, 18, 27, 42) or direct macrophage activation by microbial products (11, 26, 30, 41, 53). Among other mechanisms, there are two metabolic pathways involved in the microbicidal activity of macrophages: production of reactive oxygen intermediates (ROIs) and production of reactive nitrogen intermediates (RNIs). ROIs are microbicidal against several different microorganisms (25, 34–39, 44, 48). Their role in the antimycobacterial activity of mononuclear phagocytes is still not completely understood. Thus, *Mycobacterium microti* was shown to be susceptible to hydrogen peroxide produced by activated macrophages (52), but ROIs were found not to be involved in growth restriction of *Mycobacterium bovis* and *Mycobacterium tuberculosis* inside macrophages (13, 19). We have found that gamma interferon-induced growth restriction of virulent *M. avium* in bone marrow-derived macrophages was not dependent on ROI generation (3). However, low-virulence strains of *M. avium* and *Mycobacterium intracellulare* seem to trigger higher superoxide production by macrophages than highly virulent strains, suggesting that ROIs may act to restrict the growth of low-virulence strains (21, 49). A similar association between

virulence and susceptibility to macrophage-derived ROIs was found by Bermudez and Young (9).

In mouse macrophages, RNIs play an essential role in microbicidal activity against various microorganisms such as the protozoa *Leishmania* (24, 32), *Toxoplasma* (1, 31), *Trypanosoma* (22, 50, 51), and *Plasmodium* (40) species, the bacterium *Listeria monocytogenes* (6, 7, 10), and the fungi *Cryptococcus* (23) and *Candida* (12) species. RNIs also seem to be involved in the gamma interferon-induced growth restriction of *M. tuberculosis* in macrophages (13). However, conflicting data were reported in the literature concerning *M. avium*'s susceptibility to RNIs. Both our group (3) and Bermudez (7) showed that inhibition of nitric oxide synthesis had no effect on *M. avium* growth in activated human or murine macrophages. In a study comprising 72 strains of *M. avium*, Sato et al. reported that most of the strains were resistant to sodium nitrite in vitro (45). On the other hand, some *M. avium* strains were found to be susceptible to RNIs (14, 17).

In a previous work, we studied the ability of two *M. avium* strains, differing in virulence for mice, to induce tumor necrosis factor (TNF) secretion by resident peritoneal macrophages cultured in vitro. We found that the low-virulence strain (strain 1983) could induce secretion of higher levels of TNF than the highly virulent strain (strain ATCC 25291) and that neutralization of TNF, both in vitro and in vivo, led to enhanced growth of *M. avium* 1983 (43). To further extend these results, we studied the involvement of ROIs and RNIs in *M. avium* growth inside resident peritoneal macrophages, and the role of these intermediates in the TNF-dependent bacteriostatic activity of resident peritoneal macrophages against 1983 in vitro. We also studied the importance of the interaction of ROI and

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RNI metabolic pathways in the control of *M. avium* intracellular growth.

MATERIALS AND METHODS

Mice. Female BALB/c (*M. avium*-susceptible) mice were purchased from the Instituto Gulbenkian de Ciência (Oeiras, Portugal) and used at 8 to 12 weeks of age. Animals were kept under standard hygiene conditions, fed commercial chow, and given acidified drinking water ad libitum.

Reagents. Tween 80, saponin, phorbol myristate acetate (PMA), cytochrome *c*, nitroblue tetrazolium (NBT), latex beads (1.09 μ m in diameter), Percoll, *N*-(1-naphthyl)-ethylene-diamino dihydrochloride, and sulfanilamide were purchased from Sigma (St. Louis, Mo.). Middlebrook 7H9 and 7H10 media were obtained from Difco (Detroit, Mich.). Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle medium (DMEM), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and heat-inactivated fetal calf serum (FCS) were purchased from GIBCO (Paisley, United Kingdom). Recombinant mouse (rm) TNF- α was obtained from Genzyme (Cambridge, Mass.). Lyophilized rabbit complement was purchased from Serotec (Oxford, United Kingdom). *N*-Monomethyl L-arginine was obtained from Chem Biochem Research (Salt Lake City, Utah). All other reagents were purchased from Merck (Darmstadt, Germany).

Hybridomas and antibodies. The following hybridomas were used: 2.43, rat anti-Lyt 2.2/CD8 (ATCC TIB 210); GK 1.5, rat anti-L3T4/CD4 (ATCC TIB 207); MP6-XT22, monoclonal rat anti-mouse TNF- α (kindly provided by Paulo Vieira, DNAX Research Institute). All hybridomas were cultured in DMEM containing 10 mM HEPES buffer and 10% FCS until exponential growth, and supernatants of 2.43 and GK 1.5 were collected after culture centrifugation at $100 \times g$ for 10 min. Anti-TNF antibody was obtained from the ascitic fluid of HSD nude mice inoculated with the hybridoma MP6-XT22, after protein G-agarose affinity chromatography. GL 113 anti- β -galactosidase immunoglobulin G1 was kindly provided by P. Vieira (DNAX Research Institute) and used as an isotype control.

Mycobacteria. *M. avium* ATCC 25291 (highly virulent for mice) and 1983 (with low virulence for mice) were grown in Middlebrook 7H9 broth containing 0.04% Tween 80 at 37°C until the mid-log phase. The bacteria were collected by centrifugation and resuspended in a small volume of saline containing 0.04% Tween 80. The suspension was briefly sonicated (15 s at 50 W) with a Branson sonifier to disrupt bacterial clumps and then was diluted and frozen in aliquots at -70°C until use. The aliquots were thawed at 37°C and diluted to the desired concentration before inoculation.

Resident peritoneal macrophage culture. The peritoneal exudate was collected from untreated BALB/c mice by washing of the peritoneal cavity with 5 ml of HBSS. The cells were centrifuged at $100 \times g$ for 10 min, counted, and suspended at the concentration 3×10^6 cells per ml in DMEM containing 10 mM HEPES buffer and 10% FCS. One milliliter of the cell suspension was placed in each well of 24-well tissue culture plates, and the plates were incubated for 2 h at 37°C in a humidified atmosphere containing 7% CO₂. Nonadherent cells were removed by washing with warm HBSS, and the adherent cells were further incubated in DMEM.

In one experiment, the peritoneal exudate cells were depleted of T cells. For that purpose, 50×10^6 peritoneal cells were incubated with 10 ml of culture supernatants of the hybridomas GK 1.5 and 2.43 (1:1), in the presence of rabbit complement (1:30 dilution) for 45 min, followed by two washes, before adhesion.

Bone marrow-derived macrophage culture. Bone marrow macrophages were obtained by culturing bone marrow cells for 10 days with L929 cell line-conditioned medium as previously described (3). The monolayers were inoculated as described below.

In vitro infection. After removal of the nonadherent cells, the macrophage-enriched monolayers were inoculated with 1×10^7 CFU of *M. avium* 25291 or *M. avium* 1983 per well; in one experiment, some wells were incubated with 2×10^6 or 2×10^7 CFU of *M. avium* 25291 per well. In another experiment, at the time of inoculation, macrophage cultures were incubated with each of several scavengers of ROIs: catalase (0.01 or 0.1 mg/ml), superoxide dismutase (SOD [150 or 1,500 U/ml]), histidine (0.3 or 3 mg/ml), and mannitol (1 or 10 mg/ml). The plates were centrifuged at $100 \times g$ for 10 min and incubated for 2 h under the conditions described for macrophage adherence. The extracellular bacteria were washed out, and O₂⁻ or NO₂⁻ production was immediately assessed (time zero); in some experiments, cells were lysed at time zero with 0.1% saponin, and serial dilutions were seeded onto Middlebrook 7H10 agar medium with OADC supplement for CFU counts. The infected cells were maintained in culture for 7 days, and the same assays were performed at 3 and/or 7 days. In one experiment, 5,000 neutralizing units of MP6-XT22 rat anti-mouse TNF- α monoclonal antibody, or the same protein content of isotype control monoclonal antibody, was added to a group of culture wells after removal of the nonphagocytosed bacteria. In another experiment, 500 U of recombinant mouse TNF- α per ml was added daily to the macrophage cultures.

In vivo infection. Mice were intraperitoneally (i.p.) infected with 2×10^8 CFU of *M. avium* 25291 or 1983 or infected with inoculum vehicle (saline with 0.04% Tween 80). Three hours after the infection, groups of mice were intravenously (i.v.) injected with 2 mg of MP6-XT22 anti-TNF monoclonal antibody. At day 7 after infection, the peritoneal exudate was collected and centrifuged over 55% isotonic Percoll. The cells in the interface (mononuclear cells) were collected,

counted, resuspended in DMEM, and plated in a 24-well tissue culture plate at a concentration of 3×10^6 cells per well. After a 2-h incubation at 37°C in a humidified atmosphere containing 7% CO₂, nonadherent cells were washed out and O₂⁻ production was assayed immediately.

Assay of O₂⁻ production. O₂⁻ production was assessed by the O₂⁻-dependent reduction of ferricytochrome *c*, as described previously (29). After the reaction time (90 min), the supernatants were collected and spectrophotometric measurements were made. The protein content of the cell monolayers was determined by the method of Lowry et al. (33), and results were expressed as nanomoles of O₂⁻ per milligram of protein per 90 min. In most assays, PMA was added as a triggering agent to the reaction mixture, at a concentration of 0.81 μ M. In a few experiments, *M. avium* bacilli were used as the triggering stimulus at the doses indicated in the figure legends.

Assay of NO₂⁻ production. Aliquots of the supernatants of the macrophage cultures, infected or not with *M. avium*, were collected, and the amount of nitrite present in these supernatants was quantified by a colorimetric assay with the Griess reagent as described elsewhere (16).

NBT reduction. In some experiments, macrophage adhesion was performed over glass coverslips for NBT reduction assays. At the infection times studied, cells were incubated with a 0.2% solution of NBT in phosphate-buffered saline (PBS) for 30 min. After this time, the coverslips in each well were removed and washed in warm PBS. The cells were then fixed in a mixture of formal-ethanol (1:1), stained for acid-fast bacilli, and counterstained in a 2% solution of malachite green. The coverslip was attached to a slide with Entellan (Merck), and the percentage of formazan-positive (superoxide-producing) and formazan-negative (superoxide-nonproducing) cells was determined.

Statistical analysis. The Student *t* test was used to compare pairs of data.

RESULTS

Superoxide and nitrite production during resident peritoneal macrophage infection with *M. avium* 25291 or 1983. Resident peritoneal macrophages were infected in vitro with *M. avium* 25291 or 1983, as described in Materials and Methods, or left uninfected. At time zero (immediately after washing off the extracellular bacteria), on days 3 and 7 after infection (or culture), the amounts of superoxide (Fig. 1A) and nitrite (Fig. 1B) produced were evaluated. Results were compared with the values obtained with uninfected cells. *M. avium*-infected macrophages produced significantly larger amounts of PMA-triggered superoxide than uninfected macrophages 3 and 7 days after infection, and the highest values were obtained for strain 1983-infected macrophages 7 days after infection (Fig. 1A). In contrast, *M. avium*-infected resident peritoneal macrophages produced lower levels of nitrite than uninfected macrophages at all times studied (Fig. 1B). No significant differences were found between strain 25291- and strain 1983-infected macrophages with regard to nitrite secretion. The addition of 1,500 U of SOD to macrophages infected with either of the two strains led to a six- to eightfold increase in nitrite levels (not shown) showing that superoxide was scavenging nitric oxide. The enhancement in nitrite secretion was abrogated by *N*-monomethyl L-arginine, showing that it was being produced through the nitric oxide synthase pathway (not shown).

Effect of T-cell depletion on superoxide production by resident peritoneal macrophages in vitro. To test whether T cells present in the macrophage monolayer could be involved in the stimulation of superoxide production observed in infected resident peritoneal macrophages, the peritoneal cells were treated, prior to adhesion, with anti-CD4 and anti-CD8 antibodies in the presence of complement. This treatment eliminated the T-cell population, as determined by fluorescence-activated cell sorter analysis (results not shown). Superoxide production was assayed at day 7 after 25291 or 1983 infection in macrophages left untreated, treated with the antibodies and complement, and treated with complement alone. No differences between the different treatments were observed (data not shown). Furthermore, no NK cell activity could be detected in the peritoneal washouts as determined by a YAC-1 cytotoxicity assay (data not shown), suggesting that NK cell-mediated macrophage activation was not occurring. Previous

FIG. 1. Kinetics of superoxide (A) and nitrite (B) production by resident peritoneal macrophages infected in vitro with *M. avium* 25291 or 1983. Macrophages were infected for 2 h with 10^7 viable mycobacteria, washed, and stimulated with PMA either immediately (time zero) or 3 or 7 days later (A). Supernatants from similar cultures were examined for nitrite content at the same time points. The results are from a typical experiment, and each value represents the mean of three determinations $\pm$ standard deviation. Statistically significant differences between infected and uninfected macrophages are labeled with asterisks (*, $P < 0.05$; **, $P < 0.01$). ND, not detected.

experiments with NK cell-depleting antibodies have shown no differences in the macrophage activation induced by infection with these two *M. avium* strains (43).

Effect of anti-TNF antibody or rmTNF addition on superoxide production by resident peritoneal macrophages. We tested whether TNF produced by macrophages in culture was responsible for the observed stimulation of superoxide production. Both uninfected and *M. avium*-infected macrophage cultures were exposed to the MP6-XT22 anti-TNF monoclonal antibody or an isotype control, immediately after washing the extracellular mycobacteria and throughout the infection period. Anti-TNF significantly inhibited superoxide production by 25291- and 1983-infected macrophages at day 7 after infection, compared with the isotype control monoclonal antibody-treated infected macrophages (Fig. 2A). This was most marked in the macrophage cultures infected with strain 1983 which showed the highest levels of superoxide secretion in the control cultures and a reduction to levels comparable to those found in strain 25291-infected macrophages after TNF neutralization.

To extend the results described above, we assessed the effect of adding 50 or 500 U of rmTNF per day on superoxide production by resident peritoneal macrophage cultures infected or not infected with *M. avium*. TNF treatment significantly increased superoxide production by uninfected macrophages in a

FIG. 2. Superoxide production by resident peritoneal macrophages infected or not infected with *M. avium* for 7 days and treated with an anti-TNF- α monoclonal antibody (A) or different doses of recombinant mouse TNF- α (B). Macrophages were triggered with PMA, and superoxide was released and then detected as described before. IgG, immunoglobulin G. Results are presented as described in the legend to Fig. 1.

dose-dependent way (Fig. 2B). However, addition of 500 U/day caused only a slight, although significant, increase in 25291-infected macrophages, and neither dose of TNF added had any effect on 1983-infected macrophages (Fig. 2B).

Superoxide production by peritoneal macrophages isolated from *M. avium*-infected mice treated or not treated with anti-TNF. We studied superoxide production by peritoneal macrophages isolated from *M. avium*-infected mice or mice injected with the inoculum vehicle at day 7 after i.p. inoculation. The MP6-XT22 anti-TNF monoclonal antibody was injected i.v. 3 h after i.p. infection into a group of mice (2 mg per mouse). Although there were no differences observed in superoxide production between macrophages from 25291- and 1983-infected mice, macrophages from anti-TNF-treated mice showed decreased superoxide production compared with that of macrophages from untreated, infected mice (Fig. 3).

Effects of ROI scavengers on *M. avium* growth in resident peritoneal macrophages in vitro. The results presented here as well as those already published (43) show that strain 1983 induces a higher secretion of TNF- α responsible for a higher secretion of ROIs in infected macrophages than that in strain 25291, leading to the restriction of growth of strain 1983. It was also shown that TNF neutralization promoted the proliferation of strain 1983 in in vitro-cultured macrophages and in vivo in BALB/c mice (43). These data prompted us to assess the role of ROI in growth restriction of strain 1983 in this pathway of autocrine or paracrine macrophage activation. We thus studied the effect of the addition of catalase or SOD (scavengers of hydrogen peroxide and superoxide anion, respectively) or his-

FIG. 3. Superoxide production by macrophage-enriched peritoneal exudate cells from BALB/c mice i.p. infected for 7 days with 2×10^8 CFU of *M. avium* 25291 or strain 1983. Half of the animals were treated i.v. 3 h prior to the infection with 2 mg of anti-TNF- α monoclonal antibody. Control animals received the inoculum vehicle (0.5 ml of saline with 0.04% Tween 80). Macrophages were triggered with PMA, and superoxide secretion was studied as described before. Results are presented as described in the legend to Fig. 1.

tidine or mannitol (scavengers of the hydroxyl radical) on the growth of *M. avium* 25291 and 1983 inside resident peritoneal macrophages. All scavengers were added to the macrophage cultures at the same time as the infecting mycobacteria, so they could be internalized with the bacilli. Catalase caused a statistically significant increase in the growth of strain 1983 (Fig. 4). Boiled catalase did not have this effect (results not shown).

FIG. 4. Effects of four scavengers of ROIs on *M. avium* 1983 growth in macrophages infected in vitro for 7 days. Results are presented as the log increase in CFU from day zero (immediately after washing out of nonphagocytosed bacteria) to day 7. These results are representative of three different experiments. Statistically significant differences between controls and scavenger-treated macrophages are labeled with asterisks (*, $P = 0.012$; **, $P = 0.000$).

TABLE 1. Ability of two different doses of *M. avium* 1983 or 25291 to trigger superoxide secretion by uninfected or infected macrophages

Priming agent	Amt of superoxide secretion (nmol of O ₂ ⁻ /mg of protein/90 min) triggered by ^a :			
	1983		25291	
	10 ⁶ CFU	10 ⁷ CFU	10 ⁶ CFU	10 ⁷ CFU
None	0	0	0	24.4 ± 18.7
1983	270 ± 48.4	259 ± 25.9	ND ^b	ND
25291	ND	ND	113 ± 31.4	166 ± 2.62

^a Infected macrophages were resident peritoneal macrophages primed with strain 1983 or strain 25291 and cultured in vitro for 7 days. Uninfected macrophages were also cultured in vitro for 7 days.

^b ND, not done.

Neither SOD nor mannitol addition affected bacterial growth, and histidine had only a slight bacteriostatic effect at 3 mg/ml. None of the scavengers used affected the growth of strain 25291 (not shown).

Triggering ability of both *M. avium* strains for superoxide production by resident peritoneal macrophages. The resistance of *M. avium* 25291 to ROI could depend on its inability to trigger superoxide secretion rather than or in addition to its deficient priming effect on ROI metabolism of infected macrophages. To study the capacity of strains 1983 and 25291 to trigger a respiratory burst response on the macrophages, resident peritoneal macrophages were infected or not infected with either *M. avium* strain and cultured for 7 days. At this time point, the mycobacteria were added to the culture wells to trigger the oxidative response. As shown in Table 1, when strain 1983 was used as a trigger for macrophages infected with *M. avium* 1983, there was a higher level of secretion of superoxide than when strain 25291 was used as a trigger for macrophages infected with *M. avium* 25291 ($P < 0.05$ at the two inoculum doses tested). However, when the priming for ROI secretion was the same for both triggers (2 days with 500 U of rmTNF per ml), strain 25291 was shown to be a better trigger than strain 1983 (Fig. 5).

Growth of *M. avium* 25291 on superoxide-producing and nonproducing bone marrow-derived macrophages. In addition to being a poor inducer of TNF and therefore leading to a minimal priming for ROI secretion, strain 25291 appeared to make macrophages insensitive to TNF-mediated priming for superoxide release (Fig. 2B). To test whether infection by *M. avium* 25291 affected the superoxide-producing capacity of the macrophages, we incubated strain 25291-infected bone marrow-derived macrophages with an NBT solution at time zero, 3 and 7 days after infection and counted the number of acid-fast bacilli in formazan-positive (superoxide-producing) and formazan-negative (superoxide-nonproducing) macrophages (Fig. 6). We found that at day 7 after infection, the macrophages containing larger numbers of 25291 were formazan negative. To extend these latter results, we inoculated in vitro-cultured bone marrow-derived macrophages with strain 25291 at three different inoculum concentrations for 24 h to test what effects increasing numbers of inoculated bacteria had on the capacity of infected macrophages to produce superoxide anion; the results (determined by reduction of NBT added to the macrophages 24 h after infection) were as follows. The ratios of formazan-positive to formazan-negative infected macrophages were 3.10, 0.54, and 0.31 at inocula of 2×10^6 , 1×10^7 , and 2×10^7 CFU per well, respectively. These ratios were determined by visual inspection of 1,000 cells in three replica

FIG. 5. Ability of *M. avium* 25291 or 1983 to trigger the respiratory burst in resident peritoneal macrophages cultured in vitro for 2 days in the presence of 500 U of TNF- α per ml. The results shown represent the mean of three replicate wells $\pm$ standard deviation. Statistically significant differences are labeled with an asterisk ($P < 0.05$).

slides. There was a dose-dependent decrease in the percentage of formazan-positive cells, which suggested an inhibition of macrophage superoxide production by large numbers of 25291 bacilli.

DISCUSSION

In a previous work, we found that a low-virulence strain of *M. avium* (1983) stimulated TNF production by resident peritoneal macrophages cultured in vitro, which in an autocrine way increased the bacteriostatic capacity of these cells for that *M. avium* strain (43). That was not found with another strain of *M. avium* with a high level of virulence for mice. Although RNIs and ROIs are known to participate in the growth restriction of several microorganisms, little is known about their involvement in the bacteriostasis of macrophages against low-virulence *M. avium* strains. For this reason and since TNF- α is involved in the priming of both ROI and RNI production, we investigated whether these effector mechanisms of the macrophages participated in the TNF-dependent bacteriostasis against strain 1983 in vitro and compared the results obtained with that strain with the ones obtained with the highly virulent strain 25291 to analyze the basis of the virulence of the latter strain.

Both *M. avium* strains were found to prime for ROI secretion by macrophages in the absence of T cells and NK cells or their products. Strain 1983 stimulated the production of two to fivefold more superoxide anion than strain 25291, depending on the experiments. On the contrary, neither strain stimulated nitrite production; in fact, infected macrophages showed

FIG. 6. Acid-fast bacilli (AFB) counts in formazan-positive and formazan-negative bone marrow-derived macrophages. Macrophages were infected with 10^7 CFU of *M. avium* 25291 and cultured for 7 days. Each value represents the mean number of AFB counts per macrophage (at least 400 cells were counted) in three slides. Data were statistically compared between formazan-positive and -negative cells, and significant results are labeled with an asterisk ($P < 0.05$).

a decrease in nitrite secretion compared with uninfected macrophages. The increase in nitrite production observed with the addition of SOD to the macrophage cultures showed that the superoxide produced by the *M. avium*-infected cells was acting as a scavenger of nitric oxide. These results as well as previous ones (3) led us not to further test the role of RNIs.

The TNF synthesis induced by strain 1983 in macrophages (43) was found here to prime for superoxide production in an autocrine or paracrine way. On the other hand, strain 25291, a poor inducer of TNF synthesis by macrophages (43), induced low levels of priming for superoxide secretion. We therefore showed the existence of a TNF-dependent mechanism for the priming of macrophages for superoxide production. This mechanism was also observed ex vivo in peritoneal macrophages of BALB/c mice infected i.p. with either one of the mycobacterial strains. In this situation, we did not find any significant difference in macrophage superoxide production between strain 25291- and strain 1983-infected mice, probably because other factors, e.g., serum factors and NK cell products, are present and may regulate ROI production in vivo.

TNF addition to strain 1983-infected resident peritoneal macrophage cultures did not further stimulate superoxide production—probably because these cells were already fully activated and could not respond to further stimuli. Although strain 25291 is not a good priming agent for TNF secretion by resident peritoneal macrophages (43), strain 25291-infected macrophages were also low responders to exogenously added TNF with respect to stimulation of superoxide production. In addition, we were unable to detect superoxide production inside macrophages heavily infected with strain 25291 when the NBT

assay was used. Thus, it is likely that the virulent strain may downregulate the capacity of these phagocytes to synthesize ROIs or that the large numbers of these bacilli found inside them may be eliminating the superoxide being produced. We have reported augmented ROI production by peritoneal macrophages of BALB/c mice infected with strain 25291 at day 30 of infection (4). In this situation, the mycobacteria were proliferating extensively despite the ability of the macrophages to produce ROIs. This apparent contradiction may be explained by the results presented here that show that even when there is an overall priming of the macrophage population, the bacilli could be inhibiting the ROI produced in the infected peritoneal macrophages themselves, a minority among the macrophages recruited to the infectious foci. Synthesis of other mediators (e.g., interleukin 6) by strain 25291-infected macrophages could decrease expression of membrane TNF receptors and hence render these phagocytes low responders to TNF stimulation (8, 15).

The association of the induction of TNF secretion with both the induction of bacteriostasis and the priming for ROI release led us to directly test the possible role of ROIs in bacteriostasis. Addition of catalase to *M. avium* 1983-infected macrophages showed that this strain was susceptible to hydrogen peroxide produced by the phagocyte. The other scavengers were not effective in that regard, an observation that was similar to the one reported for the *Leishmania* model (34). Finally, our results do not support the notion that virulence is associated with a lower ability to trigger (as opposed to priming) ROI secretion.

In conclusion, a low-virulence strain of *M. avium* (1983) induced TNF production by macrophages, which in turn acted as a good priming agent for macrophage ROI production. This mycobacterium was susceptible to the hydrogen peroxide produced by macrophages and therefore had its growth restricted. Although this is a mechanism by which endogenously produced TNF caused bacteriostasis of strain 1983, other mechanisms are not excluded, namely when macrophages are activated by gamma interferon (3). These results also suggest that at least one of the bases of the virulence of other *M. avium* strains such as strain 25291 might be the avoidance of induction of TNF production and possible downregulation of the ability of infected macrophages to respond to TNF produced in the cellular environment.

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Editor: S. H. E. Kaufmann