INFECTION AND IMMUNITY, Sept. 1993, p. 3775–3784
0019-9567/93/093775-10$02.00/0
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Vol. 61, No. 9

Implication of Phagosome-Lysosome Fusion in Restriction of *Mycobacterium avium* Growth in Bone Marrow Macrophages from Genetically Resistant Mice

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Received 1 March 1993/Returned for modification 29 April 1993/Accepted 15 June 1993

The ability of the host to resist infection to a variety of intracellular pathogens, including mycobacteria, is strongly dependent upon the expression of the *Bcg* gene. Mouse strains which express the resistance phenotype (*Bcg^r^*) restrict bacterial growth, whereas susceptible strains (*Bcg^s^*) allow bacterial growth. Expression of the *Bcg* allele is known to influence the priming of host macrophages (Mφs) for bactericidal function. In the present work, bone marrow-derived Mφs from congenic BALB/c (*Bcg^s^*) and C.D2 (BALB/c.*Bcg^r^*) mice were infected with the virulent strain *Mycobacterium avium* TMC 724 to define the mechanism involved in growth restriction of *M. avium*. By combining CFU measurements and ultrastructural analyses, we show that growth of this bacterium is restricted in marrow Mφs from resistant mice. Using acid phosphatase as a lysosomal marker, we provide evidence that the hydrolytic activity of Mφs, as measured by the capacity of lysosomes to fuse with and transfer active hydrolytic enzymes to phagosomes in which *M. avium* resides, is an expression of the *Bcg* gene and that this phenomenon is a key antibacterial activity responsible for growth restriction of *M. avium*: (i) the percentage of phagosome-lysosome fusions was twice as high in *Bcg^r^* Mφs as in *Bcg^s^* Mφs, and (ii) the percentage of intact viable bacteria residing in acid phosphatase-negative phagosomes was twice as low in *Bcg^r^* Mφs as in their *Bcg^s^* counterparts. These differences are not due to a lower activity of the enzyme in *Bcg^r^* Mφs. The mechanism by which the *Bcg* gene exerts control over phagolysosomal fusion is discussed.

The ability of the host to resist infection with a variety of intracellular pathogens, including mycobacterium species, is strongly dependent upon the expression of a single gene, designated *Bcg* (11, 12, 19). Mouse strains which express the resistance phenotype (*Bcg^r^*) restrict bacterial growth, whereas susceptible strains (*Bcg^s^*) allow bacterial growth in their reticuloendothelial organs (1, 8, 11, 12, 19). The precise mechanism by which expression of the resistance allele inhibits intracellular multiplication of pathogens is unknown. However, expression of the *Bcg* alleles is known to influence the priming of host macrophages (Mφs) for bactericidal function (30). Several studies have been aimed at comparing functional and phenotypic parameters of activation in *Bcg^r^* and *Bcg^s^* Mφs populations. Following in vivo or in vitro challenge with *Mycobacterium bovis* BCG or *Mycobacterium smegmatis*, *Bcg^r^* Mφs have been shown to be superior producers of H_2O_2 and O_2^- compared with *Bcg^s^* Mφs (8, 10). However, it is unlikely that the superior degree of oxidative burst in the *Bcg^r^* Mφs is the mechanism of the enhanced antimicrobial activity of these Mφs, because the addition of inhibitors of the respiratory burst or scavengers of reactive oxygen intermediates does not alter the antimycobacterial activity of *Bcg^r^* Mφs (18).

Using immortalized Mφ lines isolated from the bone marrow of congenic mice bearing either the *Bcg^r^* (resistant, B10R line) or *Bcg^s^* (susceptible, B10S line) allele, Radzioch et al. (28) have shown that B10R Mφs are activated more efficiently for bactericidal function than their B10S counterparts when infected with mycobacteria and treated with gamma interferon. A critical difference lies in the ability of B10R Mφs to produce significant amounts of nitric oxide (NO_2), a major mediator of bactericidal activity (26). Furthermore, an examination of the surface expression of class II major histocompatibility complex (Ia) molecules by the cell lines has revealed a significantly higher expression by B10R cells (28). It had also been shown that peritoneal Mφs from *Bcg^r^* mice were significantly more efficient than their *Bcg^s^* counterparts in their antigen-presenting function for a variety of bacterial antigens (9). The lines of evidence support the concept of phenotypic expression of the *Bcg* gene in the regulation of Mφ activation.

In recent years, the clinical importance of *Mycobacterium avium* has been increasing because of the high incidence of infections, often lethal, by this pathogen in patients with AIDS (7, 34). It was shown in the mouse model of this infection that the resident peritoneal Mφs from *M. avium*-resistant *Bcg^r^* mice were more bacteriostatic compared with their *Bcg^s^* counterparts (1). It has been reported, however, that the amount of H_2O_2 and nitrites produced by peritoneal Mφs from *M. avium*-infected mice was similar in *Bcg^r^* and *Bcg^s^* congenic mice (1). This seems to indicate that the respiratory burst and the production of toxic nitrogen derivatives are not directly involved in growth restriction of this bacterium, at least in peritoneal Mφs. The survival strategies and the replication of *M. avium* have also been extensively studied in mouse bone marrow-derived Mφs. In this in vitro model (13) as well as in vivo (14), *M. avium* multiplies within the host phagosomes. It circumvents the hydrolytic activity of Mφs by strongly reducing fusion between the phagosomes that house the bacteria and the lysosomes containing the degradation enzymes (13). In addition, this bacterium is

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surrounded by an electron-transparent zone (16) that impedes the diffusion of lysosomal enzymes if phagosome-lysosome fusion happens to occur (13). These resistance strategies of *M. avium* were observed in marrow Mφs of the susceptible C57BL/6 mice.

The purpose of the present work was to determine whether growth of *M. avium* was restricted in marrow Mφs from resistant mice. More particularly, it was aimed at determining whether the hydrolytic activity of Mφs, as measured by the capacity of lysosomes to fuse with and transfer active hydrolytic enzymes to phagosomes housing *M. avium*, was an expression of the *Bcg* gene and whether this phenomenon was responsible for the restriction of bacterial growth in *Bcg*^{r} Mφs. Using bone marrow-derived Mφs from congenic BALB/c (*Bcg*^{s}) and C.D2 (BALB/c.*Bcg*^{r}) mice infected with *M. avium* TMC 724, we were able to show that (i) *Bcg*^{r} Mφs restrict growth of the virulent strain *M. avium* TMC 724, and (ii) the transfer of hydrolytic enzymes, via phagosome-lysosome fusion, is controlled by the *Bcg* gene and constitutes an important antibacterial activity in *Bcg*^{r} Mφs.

MATERIALS AND METHODS

Mice. BCG-susceptible BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, Maine). Congenic BCG-resistant BALB/c.*Bcg*^{r} (C.D2) mice were constructed by transfer of the *Bcg*^{r} allele of the DBA/2 strain into the BALB/c background by using the NX backcross system (27). The mice were bred in our facility (Faculté de Médecine Necker-Enfants Malades).

Cells and culture medium. Bone marrow Mφs were obtained by seeding 2 × 10^5 bone marrow cells from 8- to 13-week-old BALB/c or BALB/c.*Bcg*^{r} (C.D2) female mice per 35-mm-diameter tissue culture dish (Falcon; Becton Dickinson Labware, Meylan, France). These Mφs were used because once differentiated, after 7 to 10 days of culture, they can be maintained for at least 2 months as stationary cultures that retain appropriate Mφ characteristics (hydrolytic activity, phagocytosis, and endocytosis) (13). This is of utmost importance for long-term experiments with slowly growing mycobacteria.

The culture medium was Dulbecco modified Eagle medium with low glucose (1 g/liter) and high carbonate (3.7 g/liter) concentrations supplemented with 10% heat-inactivated fetal calf serum, 10% L-cell-conditioned medium (a source of CSF-1), and 2 mM L-glutamine. At 4 to 5 days after seeding, the adherent cells were rinsed twice with Hanks balanced salt solution containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and refed with fresh medium. Medium was then changed twice a week. No antibiotics were added.

Bacteria and growth medium. *M. avium* TMC 724 from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y., was kindly provided by Frank Collins. This strain yielded smooth, transparent colonies on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.). The bacteria were grown (without subculturing) in Middlebrook 7H9 broth (Difco) with Tween 80 added and stored in 1-ml tubes at −80°C until required. The frozen samples were quickly thawed, vortexed, and adjusted to the desired titer in cell culture medium.

Infection of Mφ monolayers. Mφ cultures (7 to 10 days old) were infected for 4 h at a mycobacterium/Mφ ratio of 1:10 or 1:1 for the kinetic studies of bacterial replication, 5:1 for acid phosphatase (AcPase) assays, or 10:1 for the electron microscopy studies (ultrastructure and AcPase cytochemistry). Cells were then washed in four changes of ice-cold phosphate-buffered saline (PBS) to eliminate noningested bacteria and refed with fresh medium devoid of antibiotics. The medium was renewed twice a week.

Evaluation of bacillary growth. (i) CFU counts. At selected intervals following infection, between 0 and 21 days, the medium was removed. Mφ monolayers were lysed with 0.9 ml of distilled water containing 0.05% sodium dodecyl sulfate. After 10 min of incubation at 37°C, 0.1 ml of 25% bovine serum albumin was added to the culture dishes. The number of viable bacteria per dish and in the medium removed from the dishes was then determined by plating 10-fold dilutions of the lysates (or supernatants) on Middlebrook 7H11 agar. Colonies were counted after incubation at 37°C for 21 days. For each time point, counts were made from three different culture dishes. During the first 14 days following infection, the number of viable bacteria in the supernatants was 1% or less of that found in the cell monolayers, thus ruling out complications due to continuous reinfection. For each time point, the number of Mφs per dish was determined by the method of Nakagawara and Nathan (25). Throughout the experiment, the number of Mφs per well remained stable at 10^6 cells ± 10%, and 98% or more of the cells were viable as determined by their capacity to internalize the pinocytotic marker horseradish peroxidase.

(ii) Morphological assessment. The number of bacteria per cell thin section was determined at selected intervals after infection (1, 7, 14, and 21 days) in 50 to 100 different cell profiles per time point. Three independent experiments were done. Data are from a typical experiment. We observed high reproducibility from one experiment to another (less than 10% and usually only 5% difference) for a given time point. Care was taken to avoid serial sections, and only those profiles exhibiting a nucleus were taken into consideration.

AcPase cytochemistry. Mφ monolayers were fixed for 1 h at 4°C (in the culture dishes) with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose. They were washed overnight with the same buffer, rinsed once with 0.1 M acetate buffer, pH 5.0, and incubated for 30 min at 37°C in prewarmed Gomori reaction medium (17). Mφs were rinsed twice with acetate buffer and once with cacodylate buffer. Control experiments performed in the presence of 10 mM NaF were negative. Cells were then prepared for electron microscopy as described below.

Subsequent processing for electron microscopy. Cells were fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were then scraped off the culture dishes with a rubber policeman, concentrated in agar, and treated for 1 h with 1% uranyl acetate in Veronal buffer at a final pH of 5.0. Samples were dehydrated in a graded series of acetone and embedded in Epon. Thin sections were stained with 2% uranyl acetate and lead citrate.

Assessment of intactness. In accord with our previous work (13), bacilli were considered to be intact (I) only if they had maintained their rod shape, if they were surrounded by their electron-transparent zone, if their cytoplasm had preserved its ultrastructural organization and electron density, and if no breaks in the cell wall or cytoplasmic membrane were observed. Otherwise, bacteria were considered to be damaged (D).

Assessment of fusion. The presence of electron-dense material within phagosomes after AcPase cytochemistry identified fusions with lysosomes (13). To statistically assess phagosome-lysosome fusions, we examined 200 to 1,000

different phagosomes per time point. Three independent experiments were performed. Data are from one typical experiment. We observed high reproducibility from one experiment to another (less than 10% difference) for a given time point. As for the assessment of bacillary growth, care was taken to avoid serial sections.

AcPase assays. To assay for AcPase activity, the medium was removed from the culture dishes and the cells were washed once with ice-cold PBS. Cells were lysed in 1 ml of 0.5% Triton X-100. After filtration through a 0.22-μm-pore-size membrane filter, AcPase activity was assayed by the method of Barrett and Heath (2) with *p*-nitrophenyl phosphate as a substrate. Controls lacking substrate or cell lysate were included. Enzyme activity is expressed as nanomoles of reaction product produced during 30 min at 37°C per milligram of protein by reference to standard curves prepared with a solution of *p*-nitrophenol (dissolved in Triton X-100 [0.5%]). All assays were done in duplicate from three different culture wells per time point.

Protein assay. The macrophage lysates were diluted fivefold in water to give a final concentration of Triton X-100 of 0.1%. The concentration of protein present in the lysates was estimated by the Bio-Rad reagent. Bovine serum albumin, fraction V, dissolved in 0.1% Triton X-100, was used as standard.

Chemicals. Dulbecco modified Eagle medium, glutamine, PBS powder, and Hanks balanced salt solution were purchased from Seromed (Biochrom, KG, Berlin, Germany); bovine serum albumin (fraction V), β-glycerophosphate, glutaraldehyde, *p*-nitrophenyl phosphate, *p*-nitrophenol, Triton X-100, and SDS were from Sigma Chemical Co. (St. Louis, Mo.); protein assay reagent was from Bio-Rad Laboratories (Munich, Germany).

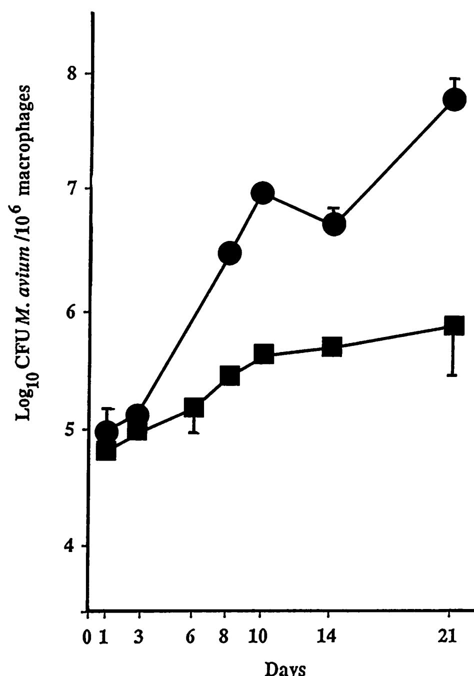

FIG. 1. Growth of *M. avium* in bone marrow-derived Mφs from susceptible BALB/c (●) and resistant congenic BALB/c.*Bcg*ʳ (■) mice. CFU counts were performed at different times after a 4-h infection with *M. avium*. An average of two different experiments is shown in this figure. Each value represents the mean ± standard error of the mean (three determinations per experiment).

RESULTS

Growth of *M. avium* within bone marrow-derived Mφs from BALB/c (*Bcg*ˢ) and BALB/c.*Bcg*ʳ mice. (i) CFU counts. Growth of *M. avium* in bone marrow-derived Mφs isolated from susceptible BALB/c and resistant C.D2 (BALB/c.*Bcg*ʳ) congenic mice was compared. Mφs were infected with *M. avium* at a ratio of 1:10 or 1:1 viable mycobacteria per Mφ and tested for mycobacterium content immediately after the 4-h infection and at different time points, ranging from 1 to 21 days after infection, by determination of CFU counts.

After infection of the monolayers, the number of Mφs remained stable at approximately 10^6 cells (±10%) per dish throughout the 21-day experimentation period. At the various intervals, 98% or more of the cells were viable as determined by their capacity to internalize the pinocytotic marker horseradish peroxidase. Identical growth patterns (i.e., same slope, with 1 log unit difference) were obtained whether cells had been infected with 1 bacterium per Mφ or 1 bacterium per 10 Mφs. Figure 1 depicts CFU counts after infection with 1 bacterium per 10 Mφs. During the first 3 days following infection, bacteria grew very slowly, with an identical replication index of 1.4 in both types of Mφs (Fig. 1; Table 1). In contrast, clear differences in growth were observed afterwards. (Fig. 1 and Table 1 show mean values calculated from two separate experiments.) Bacteria replicated rapidly in Mφs from the susceptible mice, while growth was restricted in Mφs from the resistant mice. Ten days after infection, the replication index was already more than 10-fold higher in *Bcg*ˢ Mφs than in *Bcg*ʳ ones. Twenty-one days after infection, the number of bacteria had increased by more than 600-fold in the susceptible Mφs and only 25-fold in resistant cells.

(ii) Quantitative ultrastructural analysis. In parallel experiments, *M. avium*-infected Mφs from *Bcg*ˢ and *Bcg*ʳ mice were processed for a quantitative ultrastructural evaluation of bacterial growth (Fig. 2, 3, and 4). For these experiments, cells were infected with 10 bacteria per Mφ. The important advantages of this type of analysis are that all bacteria can be individually counted, a more accurate evaluation of the amount of bacteria phagocytized by Mφs can be obtained, and finally, discrimination can be achieved between structurally intact (I), potentially live bacteria and damaged (D), presumably killed bacteria, as defined in our previous work (13). This allowed us to assess whether growth restriction

TABLE 1. Growth of *M. avium* in infected Mφs from *Bcg*ˢ and *Bcg*ʳ mice

Time (days) after infection	Replication index[a] of *M. avium*	
	*Bcg*ˢ Mφs	*Bcg*ʳ Mφs
1	1	1
3	1.4	1.4
6	ND[b]	2.1
8	37.1	4.4
10	112.1	5.9
14	67.6	8.4
21	660.4	24.1

[a] The replication index as defined by Stokes et al. (32) was calculated by dividing the viable bacteria count at the indicated times following infection by that at day 1.

[b] ND, not determined.

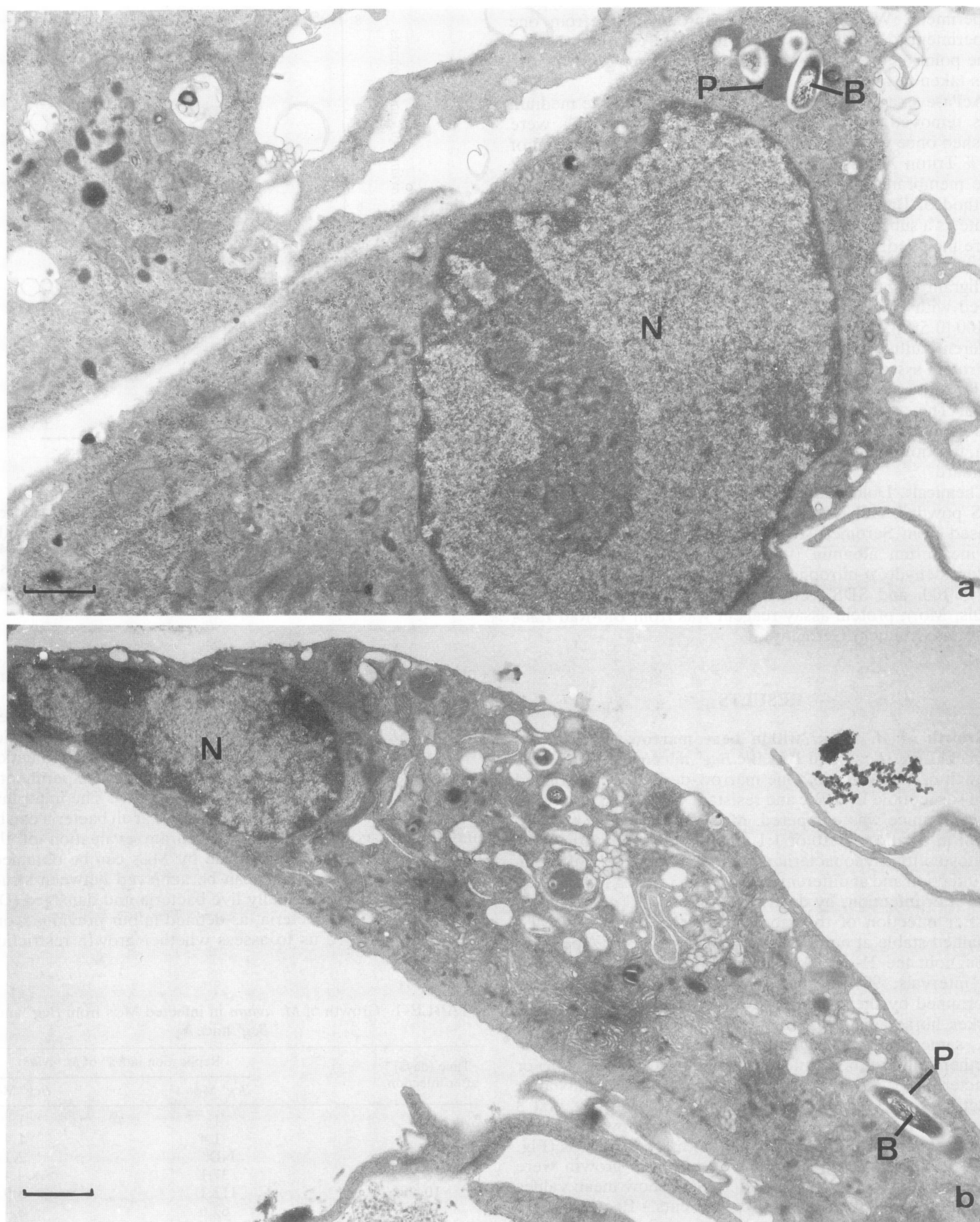

FIG. 2. Thin sections of Mϕs from resistant BALB/c.*Bcg*^{r} mice on day 1 (a) and day 14 (b) after infection with *M. avium*. P, phagosome; N, nucleus; B, bacterium. Bars, 1 μm.

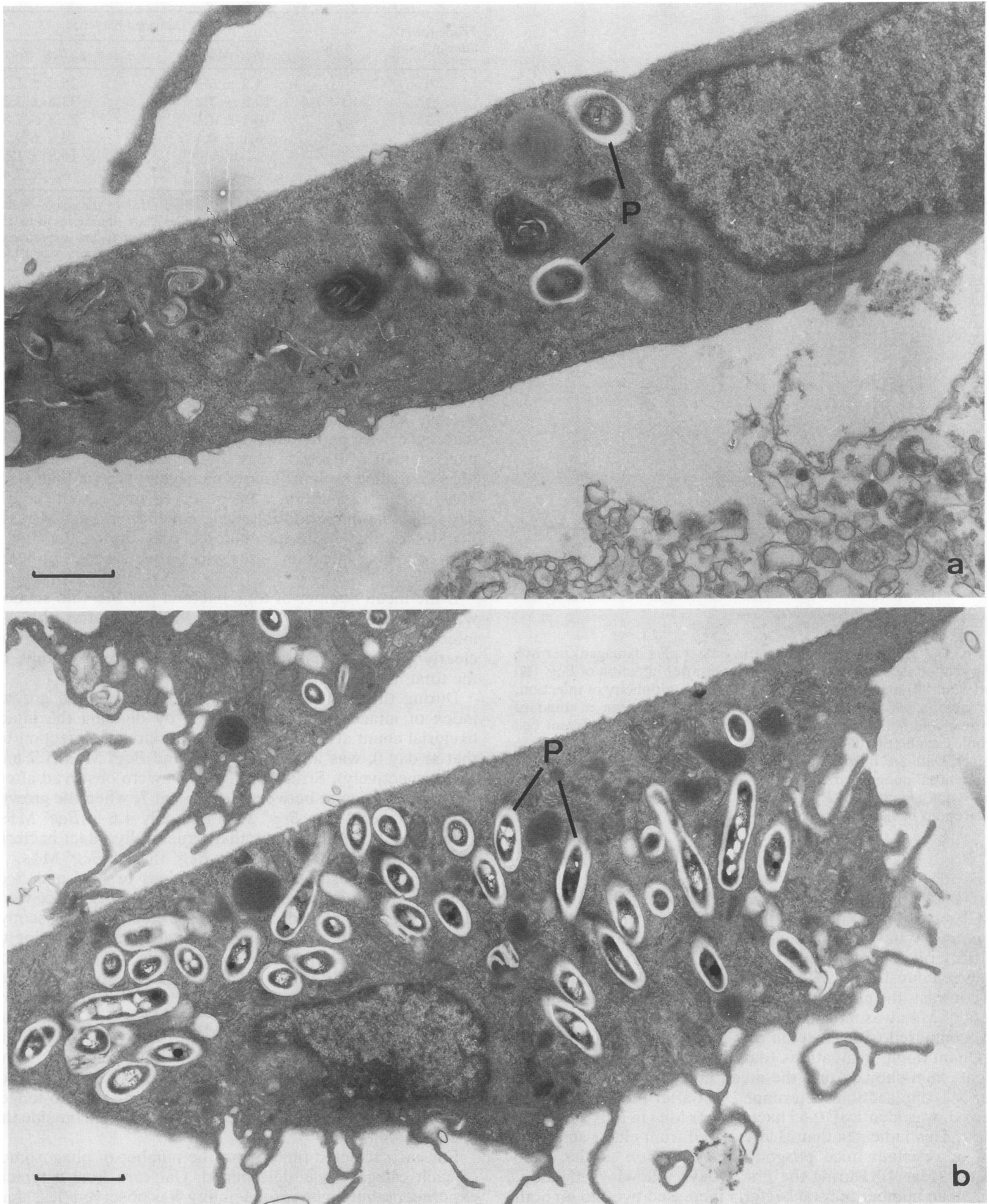

FIG. 3. Thin sections of Mφs from susceptible (*Bcg*^{s}) BALB/c mice on day 1 (a) and day 14 (b) after infection with *M. avium*. P, phagosome. Bars, 1 μm.

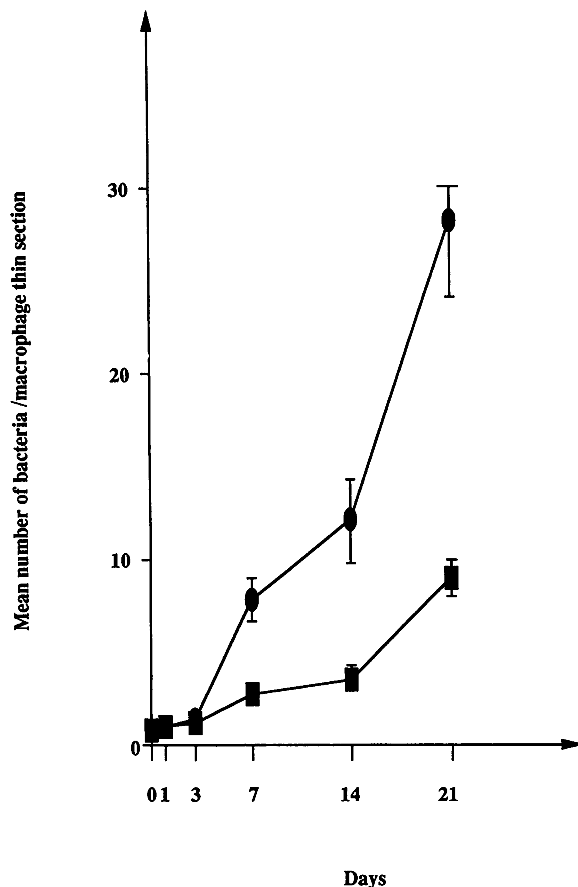

FIG. 4. Mean number of bacteria (intact plus damaged) per Mϕ thin section determined at different times after infection of *Bcg*[r] (■) and *Bcg*[s] (●) macrophages with *M. avium* (multiplicity of infection, 10 bacteria per Mϕ). Each value represents the mean ± standard error of the mean of five determinations on 10 to 30 different cell profiles each (therefore, 50 to 150 different cell profiles per time point). Data are from a typical experiment. Between days 0 and 3, differences among pairs (*Bcg*[s] versus *Bcg*[r]) for a given time point were not significant ($P > 0.5$, Student's t test). After day 3, differences became significant ($P < 0.05$).

was due to bacterial degradation and/or different rates of replication of intact bacteria in *Bcg*[r] and *Bcg*[s] Mϕs.

Observation of electron micrographs first showed that *M. avium* growth was restricted in *Bcg*[r] Mϕs, since the number of bacteria observed on thin sections increased only slightly between day 1 (Fig. 2a) and day 14 (Fig. 2b) after infection. In contrast, the number of bacteria observed on thin sections of *Bcg*[s] Mϕs was much higher after 14 days of infection (Fig. 3b) compared with those on day 1 (Fig. 3a).

Quantitative evaluations (data are from a typical experiment) then showed that the mean number of total bacteria per Mϕ thin section, determined just after the 4-h infection period, was identical (0.65 bacteria per Mϕ) in *Bcg*[s] and *Bcg*[r] Mϕs. This indicates that Mϕs isolated from either susceptible or resistant mice phagocytize *M. avium* to the same extent (Fig. 4). During the first 3 days following infection, the mean number of total bacteria increased twofold on both *Bcg*[s] and *Bcg*[r] Mϕs. No significant differences were observed (1.3 versus 1.2 bacteria per Mϕ, respectively). Afterwards, very significant differences were observed, since the mean number of total bacteria remained low in marrow Mϕs

TABLE 2. Damaged bacteria in infected Mϕs from *Bcg*[s] and *Bcg*[r] mice

Time (days) after infection	% Damaged bacteria[a]	
	Bcg[s] Mϕs	*Bcg*[r] Mϕs
0	4.2 ± 6.1	26.3 ± 12.1
1	20.2 ± 7.2	33.6 ± 7.2
3	10.1 ± 4.1	22.8 ± 7.8
7	6.1 ± 4.4	19.8 ± 9.2
14	0	16.8 ± 12.4
21	0	8.2 ± 7.4

[a] Each value represents the mean ± standard error of the mean of five determinations on 10 to 30 different cell profiles each (therefore 50 to 150 different cell profiles per time point). Data are from a typical experiment. According to Student's t test, differences among pairs (*Bcg*[s] versus *Bcg*[r]) for a given time point were significant ($P < 0.05$).

from the resistant mice, whereas it increased steadily in *Bcg*[s] Mϕs. Thus, the growth of *M. avium* is clearly restricted by *Bcg*[r] Mϕs but not by congenic *Bcg*[s] Mϕs.

At all time points, the percentage of damaged bacteria was at least twice as high in *Bcg*[r] as in *Bcg*[s] Mϕs (Table 2). This difference was particularly striking immediately following phagocytosis of bacteria (day 0 of reincubation) when *Bcg*[r] Mϕs contained six times more damaged bacteria than *Bcg*[s] Mϕs. Damaged bacteria were observed throughout the 21-day reincubation period following infection in *Bcg*[r] Mϕs; in *Bcg*[s] Mϕs, they were encountered only during the first 7 days following infection, after which time all bacteria were intact.

If the mean number of total bacteria per Mϕ thin section was identical in *Bcg*[s] and *Bcg*[r] Mϕs just after the 4-h infection period, the mean number of intact (I) bacteria was clearly different, representing 96 and 74%, respectively, of the total number of bacteria.

During the first 3 days following infection, the growth index of intact bacteria, calculated by dividing the intact bacterial count at the indicated times following infection by that at day 0, was identical in *Bcg*[s] and *Bcg*[r] Mϕs (1.7 and 1.8, respectively). Striking differences were observed afterwards, in particular between days 3 and 7, when the growth indexes were 11.3 in *Bcg*[s] Mϕs and only 4.6 in *Bcg*[r] Mϕs. From these data, it appears that structurally intact bacteria multiplied much more rapidly in *Bcg*[s] than in *Bcg*[r] Mϕs.

Pattern of phagosome-lysosome fusions in *Bcg*[s] and *Bcg*[r] Mϕs. *M. avium*-infected Mϕs from *Bcg*[r] and *Bcg*[s] mice were stained for AcPase and processed for electron microscopy. Phagosomes were divided into four categories according to the morphological appearance of bacteria, i.e., intact (I) or damaged (D), and to their content in AcPase reaction product (positive [+] or negative [−]). The four resulting categories, I^+, I^-, D^+, and D^-, are depicted in Fig. 5. The reaction product appeared as discrete patches located between the phagosome membrane and the electron-transparent zone (capsule) that surrounds bacteria. In severely damaged bacteria (Fig. 5c), the electron-transparent zone was no longer visible but the AcPase reaction product remained outside the bacterial wall.

For each selected time point, the number of phagosomes of each category was determined. Data are from a typical experiment, but high reproducibility was observed (less than 10% difference for a given time point) from one experiment to another. At all time points, except for day 1, the percentage of phagosomes displaying AcPase activity was much higher in *Bcg*[r] Mϕs than in their *Bcg*[s] counterparts (Fig. 6).

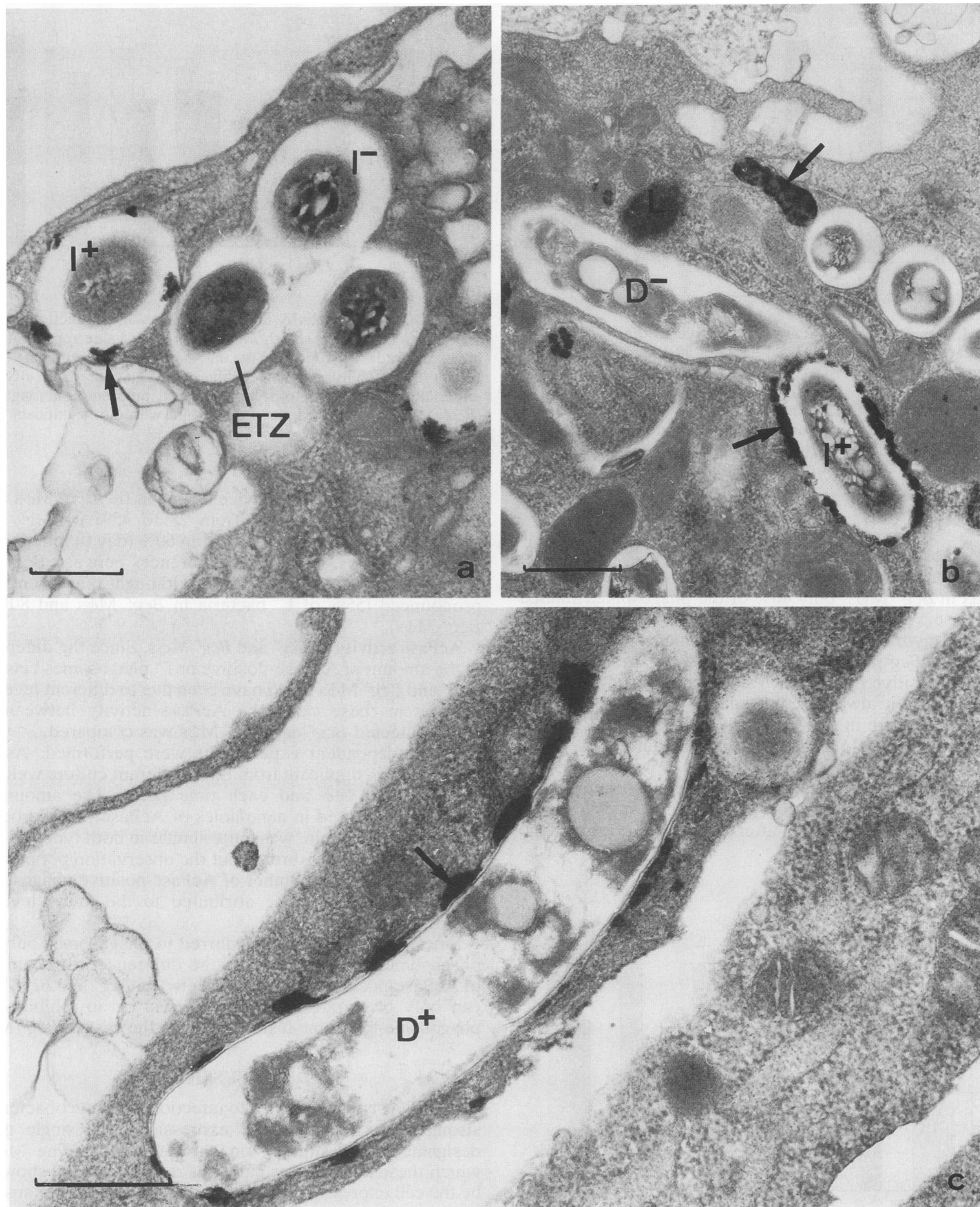

FIG. 5. Enlarged views of Mϕ thin sections showing the different types of phagosomes. (a) Phagosomes with intact bacteria (I) with (+) or without (−) AcPase reaction product; (b) AcPase-negative (−) phagosome with a damaged (D) bacterium; (c) an AcPase-positive (+) phagosome with a damaged (D) bacterium. This latter bacterium has lost the electron-transparent zone (ETZ) that surrounds intact bacteria. L, lysosome. Bars, 0.5 μm.

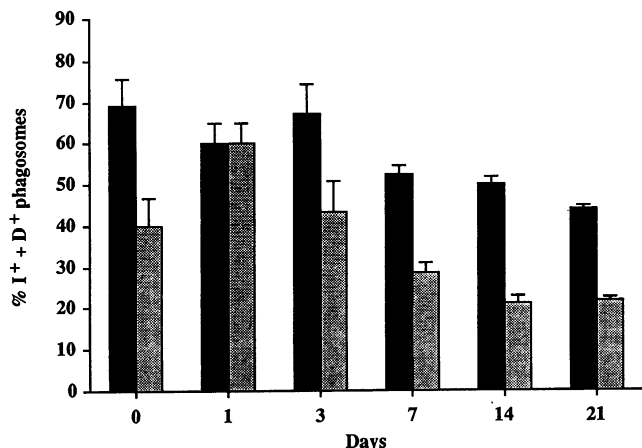

FIG. 6. Percentage of AcPase-positive phagosomes containing either intact (I^+) or damaged (D^+) bacteria at different times after infection of *Bcg*^{r} (■) and *Bcg*^{s} (▨) Mϕs with *M. avium*. Each value represents the mean ± standard error of the mean of five determinations on 40 to 200 different phagosomes each (therefore, 200 to 1,000 phagosomes per time point). Data are from a typical experiment. According to Student's *t* test, differences among pairs (*Bcg*^{r} versus *Bcg*^{s}) for a given time point, except for day 1, were significant ($P < 0.05$).

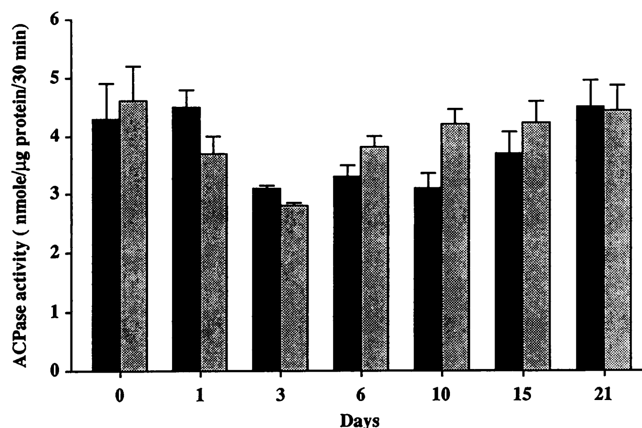

FIG. 8. AcPase activity at different times after infection of *Bcg*^{r} (■) and *Bcg*^{s} (▨) Mϕs with *M. avium*. Each value represents the mean ± standard error of the mean. Assays were in duplicate (from three different culture wells per time point). Data are from a typical experiment. According to Student's *t* test, differences among pairs (*Bcg*^{r} versus *Bcg*^{s}) for a given time point were not significant ($P >$ 0.5).

In *Bcg*^{r} Mϕs, 70% of the phagosomes displayed hydrolytic enzymes immediately following the 4-h infection period, whereas in *Bcg*^{s} Mϕs, only 40% of the phagosomes stained for AcPase. This indicates that at this early time point, transfer of hydrolytic enzymes is 75% higher in *Bcg*^{r} Mϕs than in their *Bcg*^{s} counterparts. After day 3, the percentage of AcPase-positive phagosomes slowly decreased to 50% in *Bcg*^{r} Mϕs, but it always remained 75% (1.5-fold) to 100% (2-fold) higher than in *Bcg*^{s} Mϕs.

The percentage of phagosomes that contained intact bacteria but no AcPase reaction product (I^-) was determined next (Fig. 7). This is most probably the only category of bacteria capable of multiplying within the host cell. Up to day 14 (and except for day 1) after infection, the percentage of such phagosomes was twice as low in the *Bcg*^{r} Mϕs as in the *Bcg*^{s} ones, increasing from 28 to 45% in *Bcg*^{r} Mϕs between day 0 and day 14 and from 60% (day 0) to 80% (day 14) in *Bcg*^{s} Mϕs. At day 21, differences between *Bcg*^{r} and *Bcg*^{s} Mϕs were still observed, although they were less pronounced (55% of I^- bacteria in *Bcg*^{r} Mϕs and 80% in *Bcg*^{s} Mϕs).

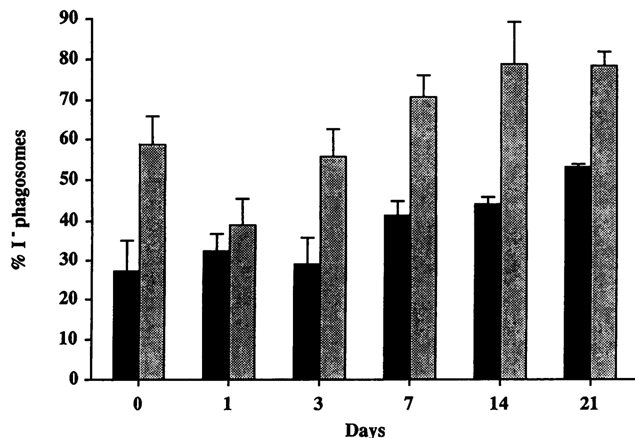

FIG. 7. Percentage of AcPase-negative phagosomes with intact bacteria (I^-) at different times after infection of *Bcg*^{r} (■) and *Bcg*^{s} (▨) Mϕs with *M. avium*. Quantitations were made as for Fig. 6 on the same cell profiles, with the same statistical analysis. Differences were significant, as described in the legend to Fig. 6.

AcPase activity in *Bcg*^{s} and *Bcg*^{r} Mϕs. Since the difference in the amount of AcPase-positive or I^- phagosomes between *Bcg*^{r} and *Bcg*^{s} Mϕs could have been due to different levels of enzyme in these cells, the AcPase activity between *M. avium*-infected *Bcg*^{s} and *Bcg*^{r} Mϕs was compared.

Two independent experiments were performed. Assays were made in duplicate from three different culture wells for each type of Mϕ and each time point. The amount of enzyme, expressed in nanomoles of AcPase per microgram of protein per 30 min, was quite similar in both types of Mϕs and remained stable throughout the observation period (Fig. 8). Thus, the lower number of AcPase-positive phagosomes in *Bcg*^{s} Mϕs cannot be attributed to the lower level of enzyme in these cells.

Since AcPase can be transferred to phagosomes only via phagosome-lysosome fusions, the difference in the amount of AcPase-positive phagosomes between *Bcg*^{r} and *Bcg*^{s} Mϕs can only be explained by a decrease or an inhibition of phagosome-lysosome fusion events in the case of *Bcg*^{s} Mϕs.

DISCUSSION

Innate resistance of mice to infection with mycobacteria is strongly dependent on the expression of a single gene, designated *Bcg*, situated on chromosome 1. The Mϕ in which these intracellular parasites reside has been shown to be the cell expressing the phenotype of resistance or susceptibility to infection at the cellular level (5). Using CFU measurement methods or [^{3}H]uracil uptake assays, different authors have shown that the proliferative activity of mycobacteria was significantly reduced in Mϕs from *Bcg*^{r} mice. These studies had been performed with either splenic or resident peritoneal Mϕs from *Bcg*^{r} and *Bcg*^{s} mice infected with BCG (11, 12, 19, 30), *M. smegmatis* (8), or *M. avium* (1,

18, 32) or with immortalized Mϕ cell lines, from congenic resistant or susceptible mice, and infected with *Bcg* or *M. smegmatis* (28).

In the present work, we have combined CFU measurements and ultrastructural analyses to compare the growth of *M. avium* in primary cultures of bone marrow-derived Mϕs from resistant (C.D2) and susceptible (BALB/c) congenic mice. The combination of these two methods clearly shows that, in this cell model, growth of the virulent strain of *M. avium* TMC 724 is restricted in *Bcg*^{r} Mϕs, whereas these bacteria multiply within the marrow Mϕs from the susceptible congenic mice. CFU measurements showed that the number of bacteria increased by more than 600-fold in the susceptible Mϕs and by only 25-fold in the resistant cells during a 21-day experimentation period following infection. However, in all experiments, the number of viable bacilli determined by CFU measurements consistently decreased between days 10 and 14 after infection, and bacteria replicated more slowly afterwards, although light microscope observations seemed to indicate a steady increase in the number of bacteria. One likely explanation for the abnormally low counts is that at the late time points, bacteria form clumps upon cell lysis. As a result, each CFU would correspond to several bacteria instead of a single bacterium. Although *M. avium* does not usually form clumps when grown in culture medium, contrary to how other mycobacteria act, the bacilli do indeed seem to be more difficult to disperse after cell lysis at the late time points. It is possible that during bacterial replication within Mϕs the bacterial cell wall components reorganize in such a way that bacteria stick together after cell lysis. Such cell wall modifications had been suggested by previous work (13).

The ultrastructural analyses allowed us to gain better insight into the restriction of growth of *M. avium* in *Bcg*^{r} Mϕs, because with this method all bacteria can be visualized and discrimination can be achieved between structurally intact, presumably live, bacteria able to multiply within the cells, and damaged killed bacteria no longer able to replicate. One can therefore determine whether restriction of growth is because bacilli are killed or whether their proliferative abilities are impaired in *Bcg*^{r} Mϕs.

The electron microscope approach first showed that *M. avium* was phagocytized to the same extent by both *Bcg*^{r} and *Bcg*^{s} Mϕs, contrary to what had been observed for the phagocytosis of *M. smegmatis* by immortalized Mϕ cell lines (28) of B10R and B10S mice. Several receptors are known to be implicated in the phagocytosis of *M. avium* (3, 6). At present, we do not know whether *M. avium* enters into *Bcg*^{r} and *Bcg*^{s} marrow Mϕs via the same receptors or not and whether this has any influence on its replication pattern, which is totally different in these two Mϕ populations.

In thin sections, we consistently observed a steady increase in the number of bacteria in the Mϕs from *Bcg*^{s} mice throughout the 21 days of experimentation, including the 10- to 14-day postinfection interval. This observation showed that there was no arrest of bacterial growth during this latter interval and further suggested that the apparent decrease in CFU counts was probably secondary to bacterial clumping. In *Bcg*^{r} Mϕs, the total number of bacteria increased very slowly during the first 14 days following infection. Between day 14 and 21 this increase was more important, suggesting that restriction of growth became less efficient. This could be due to the loss of bactericidal properties because of lengthy cell culturing.

The quantitative analysis of the amount of intact and damaged bacilli found in thin sections during the 21-day postinfection interval showed that restriction of growth of *Bcg*^{r} Mϕ had two causes. First, the rate of replication of viable bacteria was lower in *Bcg*^{r} Mϕs than in the *Bcg*^{s} counterparts, especially during the 3- to 7-day postinfection interval, when the growth index of intact bacteria was 2.5-fold lower in *Bcg*^{r} than in *Bcg*^{s} Mϕs. The second, and certainly most important, cause was the extent of bacterial degradation: *Bcg*^{r} Mϕs displayed at least twice as many damaged bacilli as *Bcg*^{s} Mϕs. This was observed at a very early stage of infection immediately following the 4-h infection period. In addition, damaged bacilli were encountered throughout the 21-day experimentation interval in *Bcg*^{r} Mϕs but only until day 7 in *Bcg*^{s} Mϕs, after which time all bacteria were intact.

The question that arises is which antimicrobial activity of Mϕs is responsible for the degradation of *M. avium* in *Bcg*^{r} Mϕs and hence for the restriction of bacterial growth. We have shown previously that *M. avium* (13), as well as other intracellular pathogens such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Legionella pneumophila*, *Toxoplasma gondii*, and *Salmonella typhimurium* (4, 15, 20, 21, 23), survives and multiplies within Mϕs by inhibiting phagosome fusion with lysosomes, thereby preventing exposure to toxic lysosomal contents. Using the lysosomal enzyme AcPase as a marker, we show here that the percentage of phagosome-lysosome fusions is twice as high in *Bcg*^{r} as in *Bcg*^{s} Mϕs and that the percentage of intact viable bacteria residing in AcPase-negative phagosomes in about twice as low in *Bcg*^{r} Mϕs as in their *Bcg*^{s} counterparts. These differences are not due to a lower activity of the enzyme in *Bcg*^{r} Mϕs, as previously suggested by Stokes and Collins (31). From the present work it can therefore be concluded that the pattern of phagosome-lysosome fusions is under the control of the *Bcg* gene and that it constitutes a key antibacterial activity responsible for the restriction of *M. avium* growth in *Bcg*^{r} Mϕs in the first hours following infection and throughout a 21-day experimentation period as well. It cannot be excluded that bacterial damage could be induced prior to phagosome-lysosome fusion events because of the production of toxic oxygen metabolites or nitrogen derivatives that could render bacteria more susceptible to the lysosomal lytic machinery. In the case of *M. avium*-infected peritoneal Mϕs from BALB/c and C.D2 mice, it has been shown, however, that H_2O_2 production was as high or even higher among the susceptible strain as in Mϕs from the mouse strain resistant to *M. avium* (1). In addition, the secretion of reactive nitrogen intermediates was not associated with any pattern of resistance or susceptibility to infection with *M. avium* (1), contrary to what had been observed in BCG- or *M. smegmatis*-infected Mϕs (28). For these reasons, we favor the idea that degradation of *M. avium* is induced solely by phagosome-lysosome fusions events.

The mechanism by which the *Bcg* gene exerts control over phagosome-lysosome fusion is a matter of speculation, for the moment. Results of all the studies dealing with the phenotypic expression of the *Bcg* gene are congruous with the hypothesis that the gene product regulates a discrete, yet unidentified, step in the cascade of Mϕ priming for activation. The phenotypic differences between the *Bcg*^{s} and *Bcg*^{r} Mϕs are extremely pleiotropic (downregulation of membrane 5′ nucleotidase, upregulation of Ia mRNA, upregulation of antigen presentation, upregulation of nitric oxide synthase, and enhanced bactericidal activity), and thus they can be viewed as a consequence of the heightened state of Mϕ activation controlled by the *Bcg*^{r} allele. The simplest, unitarian explanation of the findings presented in this paper would, therefore, be that an enhanced phagolysosomal fusion in the *Bcg*^{r} Mϕ is yet another manifestation of the

pleiotropic effects of the *Bcg*^{r} allele that appear as a result of genetically controlled upregulation of Mϕ activation. In support of this thesis are several lines of evidence suggesting that Mϕ activation does indeed result in the enhancement of phagolysosomal fusion (22, 24, 29). An alternate hypothesis, namely, that the *Bcg* gene product itself controls a biochemical process which leads to upregulation of phagolysosomal fusion, is plausible but less likely in view of the known pleiotropy of the phenotypic manifestations of this gene. The recent cloning of a *Bcg* gene candidate (33) will stimulate structure-function studies that will address this issue directly.

ACKNOWLEDGMENTS

We thank Patrick Berche for helpful advice, Luis Barrera and Alex Apt for critical reading of the manuscript, and Nathalie Laurent for excellent technical assistance.

This work received financial support from INSERM (CJF contract 90-04) and from the Ministère Français des Affaires Etrangères and the Ministère Québécois de l'Enseignement Supérieur et de la Science (Projets Conjoints de Coopération Franco-Québécoise en Recherche Médicale 14-90 and 14-91).

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