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Host and bacterial factors control the *Mycobacterium avium*-induced chronic peritoneal granulocytosis in mice

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SUMMARY

Persistent peritoneal granulocytosis and elevated macrophage counts have been found in nine mouse strains from 8 to 90 days after infection with *Mycobacterium avium*. Peritoneal granulocytosis was higher in *M. avium*-resistant BALB/c. *Bcg*^{r} (C.D2) mice, compared with congenic *M. avium*-susceptible BALB/c (*Bcg*^{s}) animals. Although maximal granulocytosis values were not related to virulence of the inocula, the kinetics of the granulocytic response varied with the virulence of *M. avium*. Following infections by avirulent (rough) strains of *M. avium*, the peritoneal granulocytosis progressively declined in BALB/c and C3H/He mice. A similar decline in granulocyte number was observed in resistant C3H/He mice infected with virulent *M. avium* (smooth transparent strain). In both instances the decline in the peritoneal granulocytosis was associated with a progressive elimination of the inoculum. In the susceptible BALB/c mice, virulent *M. avium* strains induced progressive infection accompanied with a rapid decline in granulocyte number, whereas the infection with attenuated *M. avium*, which caused a chronic infection, induced persistent granulocytosis. The ability to recruit granulocytes following the intraperitoneal inoculation of a phlogistic substance (casein hydrolysate) was decreased in infected susceptible but not in infected resistant mice at 90 days of infection with virulent *M. avium*.

Keywords granulocytes granulocytosis mycobacterial infection *Mycobacterium avium*

INTRODUCTION

Mycobacteria are known to induce the accumulation of mononuclear cells at the foci of infection (Edwards & Kirkpatrick, 1986). These cells can be seen either organized in compact cellular clusterings, the granulomas, in tuberculosis or in tuberculoid leprosy, or in more loosely arranged cellular infiltrates as in lepromatous leprosy or miliary tuberculosis (Ridley, 1983). Granulocytes have been reported to be present only in the first few hours after bacterial inoculation in some experimental models of mycobacteriosis (Vorwald, 1932; Montgomery & Lemon, 1933; Bloch, 1948; Miler, 1955; Papadimitriou & Spector, 1972; Closs & Haugen, 1975). However, we have recently reported (Silva, Silva & Appelberg, 1989) that CD-1 mice inoculated intraperitoneally with several species of mycobacteria present a chronic peritoneal granulocytosis with a predominance of neutrophils in addition to the acute influx of these cells which takes place during the first 48 h post-inoculation. Such a persistent peritoneal granulocytosis was found to be regulated by the immune system (Appelberg & Silva, 1989). Moreover, we have shown that in the peritoneal exudate of mycobacteria-infected mice macrophages ingest neutrophils and that the anti-mycobacterial activity of macrophages *in vitro* is enhanced when these phagocytes have ingested granulocyte material (Silva *et al.*, 1989). The above results led us to propose (Silva *et al.*, 1989) that neutrophils would participate in the host defence against mycobacteria by enhancing the anti-microbial activity of macrophages after transferring some of their molecules into these phagocytes. Our interpretation is supported by data obtained by others, showing that neutrophils are unable to kill phagocytosed mycobacteria *in vitro* (Smith, Barr & Alexander, 1979; May & Spagnuolo, 1987) and that the transfer of neutrophilic material to macrophages increases their anti-microbial activity (Heifets, Imai & Goren, 1980; Lima & Kierszenbaum, 1985).

Here we extend our initial observations on the granulocytosis in mycobacterial infections by studying factors that influence the peritoneal granulocytic response in mice infected intraperitoneally with *M. avium*. Chronic granulocytosis was observed in all the nine mouse strains studied, and the magnitude of the granulocyte influx during *M. avium* infection varied with the strain used. Furthermore, the granulocytic response also varied with the virulence of the *M. avium* inoculum.

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MATERIALS AND METHODS

Bacteria

M. avium strain ATCC 25291 was grown for 2 weeks in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) plus 0·04% Tween 80 (Sigma, St Louis, MO). Bacterial inocula were prepared by briefly sonicating (15 sec with a Branson sonifier set at 50 W) the bacteria suspended in a small volume of saline–0·04% Tween 80 to disrupt bacterial clumps. The suspension was then diluted in the same vehicle to the desired concentration and kept frozen at −70°C until use. Then, it was quickly thawed at 37°C and $2{\cdot}5 \times 10^8$ cell-forming units (CFU) were inoculated intraperitoneally in 0·5 ml of vehicle. These bacterial preparations (referred to as the standard virulent inoculum) originated smooth-transparent (SmT) colonies when plated on Middlebrook 7H10 agar medium. Unless stated otherwise, this was the inoculum used.

In some experiments, two other inocula were used: (i) a rough colonial variant was isolated from extensively *in vitro*-passaged cultures of *M. avium* ATCC 25291 and inocula were prepared as described. This rough inoculum has been found to be avirulent for mice (manuscript in preparation); and (ii) inocula with decreased virulence (referred to as the attenuated inocula) were also prepared from extensively *in vitro*-passaged cultures with no isolation of particular colonial types (Appelberg *et al.*, 1989).

Mice

CD-1 and C57BL/6 mice were purchased from Instituto Gulbenkian de Ciência (Oeiras, Portugal); BALB/c, C3H/He, DBA/2, CBA/Ca, C57BL/10 were from Bantin & Kingman (Hull, UK) and DBA/1 from Harlan Olac (Bicester, UK). In some experiments, C3H/He and DBA/2 mice bred at our facilities were used. These mice were obtained from parents purchased from Bantin & Kingman. BALB/c.*Bcg*^{r} (C.D2) mice were bred at our facilities from parent mice generously given to us by Dr E. Skamene. The animals were kept under standard hygiene conditions, fed commercial chow and given acidified drinking water *ad libitum*. They were used at 6–10 weeks of age.

Evaluation of bacillary growth

The number of CFU of *M. avium* in the spleen and liver of infected mice was determined by serial diluting and plating the tissue homogenates onto 7H10 agar medium (Difco Laboratories). Results are expressed as the growth index which represents the difference between the $\log_{10}$ mean of CFU at day 90 of infection and the $\log_{10}$ mean of CFU at day 8 of infection. The CFU number in the peritoneal cavity was determined in the same way as with the tissue homogenates using an aliquot of the collected lavage fluid which was treated with 0·01% saponin (Sigma) to lyse the cells.

Study of the cellular response in the peritoneal exudates

At different periods of time after intraperitoneal infection, mice were killed by ether anaesthesia, and the peritoneal cavity was washed with 4 ml of phosphate-buffered saline (PBS). After massaging the abdomen gently, the peritoneal fluid was collected. Total cell counts were made using an electronic cell counter. Differential cell counts and determinations of the percentage of macrophages with recognizable ingested granulocytes were performed in cytospin preparations stained with the Wright's stain. Acid-fast bacilli were also visualized in the cytospin preparations of the peritoneal exudate after Ziehl–Neelsen staining.

Statistical analysis

The Student's *t*-test and the Mann–Whitney *U*-test were used.

RESULTS

The growth of *M. avium* ATCC 25291 (SmT) in different strains of mice has been described in detail elsewhere (Appelberg & Sarmento, 1990). Briefly, there was no net bacterial growth in the livers and spleens of CD-1, DBA/2, C3H/H3, CBA/Ca, and C-D2 mice. In contrast, C57BL/6, C57BL/10, BALB/c, and DBA/1 mice allowed an extensive systemic bacterial proliferation that resulted in the death of the animals. The peritoneal inoculum was initially cleared in both groups of mice during the initial 15–30 days. However, while resistant mice cleared the peritoneal bacterial load further, susceptible mice had a recrudescence of the peritoneal infection so that at 30 days they already had higher numbers of bacteria than did the resistant strains. When bone marrow cell cytospin preparations were examined, high loads of acid fast bacteria were found in cells from susceptible mice infected for 2 or 3 months with *M. avium* (not shown).

The numbers of granulocytes in the undisturbed peritoneal cavity varied from 1 to 5×10^5 among the different mouse strains and were mainly eosinophils. The initial influx of neutrophils which takes place in the first 48 h following the intraperitoneal inoculation of mycobacteria has already been reported (Silva *et al.*, 1989) and was not analysed here. All mouse strains studied showed a persistent granulocytosis (mostly neutrophils) in the peritoneal cavity after the inoculation of $2{\cdot}5 \times 10^8$ CFU of *M. avium* (Fig. 1). The number of granulocytes accumulating in the peritoneal cavity of infected mice varied among the different mouse strains. During the first month of infection, granulocyte numbers were significantly higher among resistant mice as compared to susceptible mice ($P < 0{\cdot}05$ at 15 days of infection) when the values of each group of four strains (resistant *versus* susceptible) were pooled (Fig. 1). When the geometric means of granulocytes at 15 days of infection of each individual strain were compared, significant differences were found between CD-1 mice and all susceptible strains and between DBA/2 and all susceptible strains but DBA/1. CBA/Ca only differed significantly from DBA/1, and C3H/He did not differ from any susceptible strain. Significant differences were also found between strains in the same group (resistant or susceptible). Thus, CD-1 and DBA/2 differed from the other resistant strains and between themselves, DBA/1 differed from all susceptible strains but C57BL/6, and C57BL/6 significantly differed from BALB/c and C57BL/10. After the first month of infection, the granulocyte number decreased more rapidly in the susceptible strains of mice (Fig. 1). When mice from individual strains were compared for the geometric mean of granulocytes present in the peritoneal cavity at day 90 of infection, CD-1, DBA/2 and C3H/He strains showed significantly higher granulocyte number than all susceptible strains and did not show significant differences between themselves. Granulocyte number in CBA/Ca mice was not significantly different from any susceptible strain and was significantly lower than that of CD-1 and DBA/2 mice. No significant differences were found between susceptible strains.

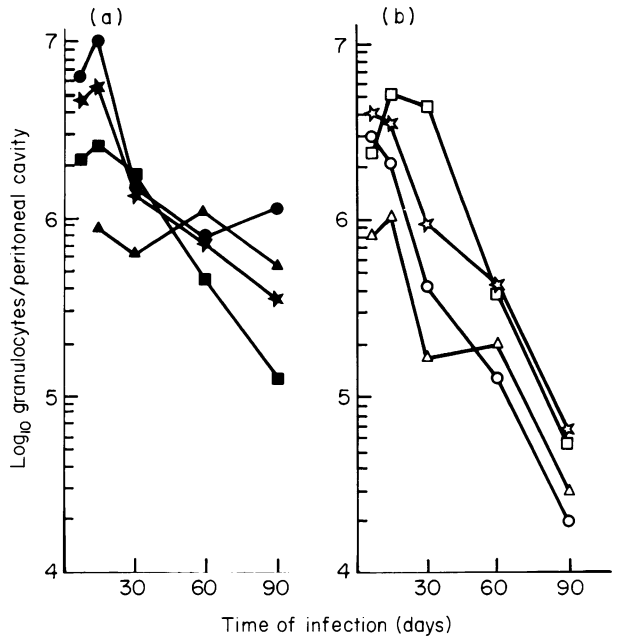

Fig. 1. Total number of granulocytes per peritoneal cavity of *M. avium*-resistant (a) and susceptible (b) mice intraperitoneally infected with $2{\cdot}5 \times 10^8$ CFU of *M. avium* (SmT variant). Each time-point represents the mean of the granulocyte number for four to eight mice of the C57BL/6 (open circles), C57BL/10 (open squares), BALB/c (open stars), DBA/1 (open triangles), DBA/2 (closed triangles), CD-1 (closed circles), CBA/Ca (closed squares), and C3H/He (closed stars) strains. The s.d. of the geometric mean varied from 2 to 12% of the value of the mean.

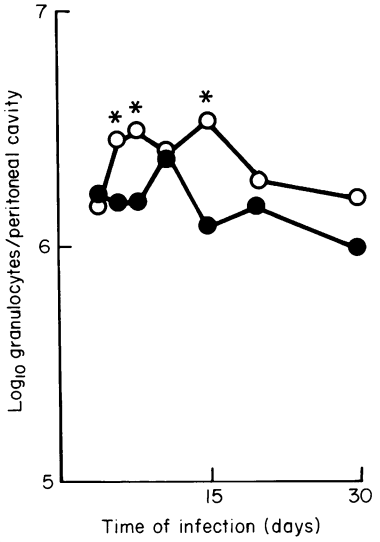

Fig. 3. Number of granulocytes per peritoneal cavity of BALB/c (closed symbols) and C.D2 (open symbols) mice (four animals per group) infected with $2{\cdot}5 \times 10^8$ CFU of *M. avium* (SmT variant). * Significant differences ($P < 0{\cdot}05$, Mann–Whitney *U*-test).

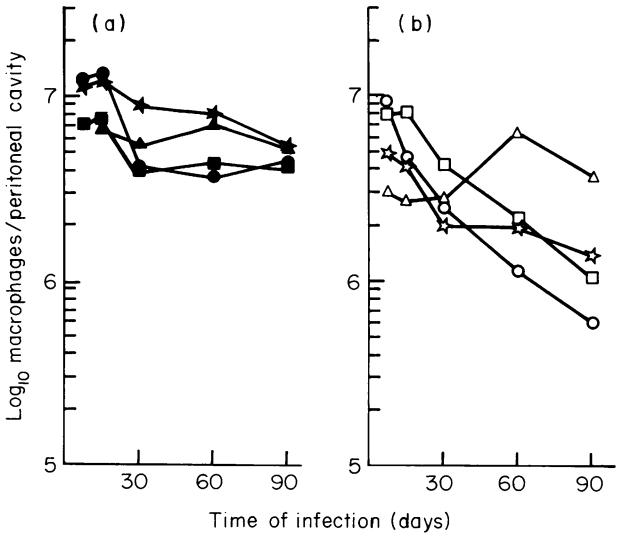

Fig. 2. Total number of macrophages per peritoneal cavity of the same mice as in Fig. 1. (a) Resistant; (b) susceptible.

Macrophages also accumulated in the peritoneal cavity of *M. avium*-infected mice with maximal numbers peaking at 8–15 days post-inoculation (Fig. 2). After the first month of infection, the number of macrophages remained constant in the resistant strains, and declined progressively in the susceptible mice—with the exception of DBA/1 mice, who showed a late peak of macrophage accumulation at 60 days post-inoculation. The decrease in macrophage number in susceptible mice paralleled that observed with granulocytes.

Since the presence of the Bcg^r allele in BALB/c mice renders them resistant to *M. avium* infection (Appelberg & Sarmento, 1990), we analysed the peritoneal response to *M. avium* intraperitoneal infection in BALB/c as compared with C.D2 mice. The latter animals were able to induce a higher granulocyte influx than the former (Fig. 3). This was confirmed in other independent experiments not shown here.

The granulocytic response was studied in BALB/c (*M. avium*-susceptible) and C3H/He (*M. avium*-resistant) mice, comparing bacterial inocula of different virulences. The growth pattern of these different inocula in the peritoneal cavity of the two mouse strains is shown in Fig. 4 (a, b). The magnitude of systemic proliferation was studied in the liver and spleen by performing viable counts at 8, 15, 30, 60, and 90 days post-inoculation. Since growth or elimination of the micro-organism was roughly linear with time of infection, data were converted into growth indices as described in Materials and Methods and are shown in Table 1. Rough variants were eliminated from both BALB/c and C3H/He mice (Fig. 4a, b; Table 1). The virulent *M. avium* (forming SmT colonies) proliferated extensively in BALB/c mice but not in C3H/He animals. In the latter strain, bacteria were progressively cleared from the peritoneal cavity (Fig. 4b) and there was no net bacterial growth in the liver and spleen of these mice (Table 1). An attenuated inoculum of *M. avium* had limited virulence for BALB/c mice, growing in the peritoneal cavity after the first month of infection (Fig. 4a) but proliferating only slightly in the liver and spleen of these animals (Table 1).

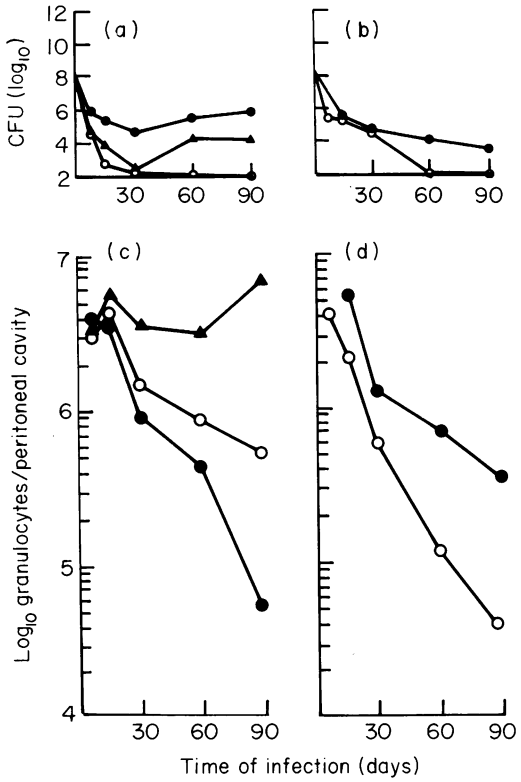

Fig. 4. CFU counts in the peritoneal cavity of BALB/c (a) and C3H/He (b) mice infected with $2{\cdot}5 \times 10^8$ CFU of virulent inocula (closed circles), non-virulent rough variant (open circles) and attenuated inocula (triangles); and number of peritoneal granulocytes induced by such infections in the BALB/c (c) and C3H/He (d) animals (four animals per group).

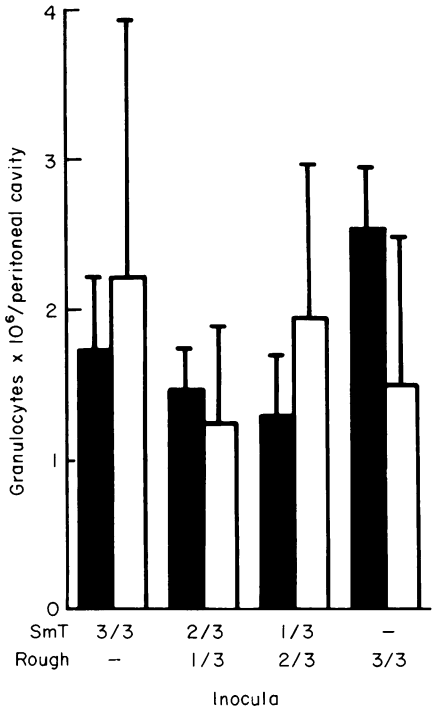

Fig. 5. Number of granulocytes per peritoneal cavity of BALB/c (solid columns) and C3H/He (open columns) mice (four animals per group) infected with $2{\cdot}0 \times 10^8$ CFU of *M. avium* with the indicated proportions of the virulent (SmT) and the avirulent (rough) strains of the bacteria.

Table 1. Growth index in BALB/c and C3H/He mice infected by different preparations of *M. avium*

Bacterial preparation	C3H/He		BALB/c	
	liver	spleen	liver	spleen
Virulent	0·07	0·97	4·11	4·06
Avirulent	−2·82	−1·77	−2·84	−0·15
Attenuated	ND	ND	0·30	1·03

The growth index was calculated by subtracting the log_{10} CFU in day 8 infected organs to the log_{10} CFU in day 90 infected organs. Negative values thus indicate bacterial elimination.

ND, not done.

The granulocytic response to these different inocula varied (Fig. 4c, d). Maximal accumulation of granulocytes did not significantly differ between the two mouse strains or between the different inocula. In another experiment, mice were inoculated with either the virulent (SmT variant) or the avirulent (rough variant) *M. avium* strains or with different proportions of both strains and the number of granulocytes present after 15 days of intraperitoneal infection counted (Fig. 5). Again, no significant differences were found between the two mouse strains or between the different inocula.

The decline in granulocyte number after the first month of infection varied, however, according to the mouse strain and *M. avium* inoculum. The elimination of the avirulent *M. avium* from the peritoneal cavity of both mouse strains was accompanied by a substantial decline in granulocytosis (Fig. 4c, d). In the resistent C3H/He mice, the virulent *M. avium* inoculum was eliminated from the peritoneal cavity and granulocyte number also decreased. However, in susceptible BALB/c mice there was a recrudescence of the peritoneal infection by the virulent *M. avium* and the decrease in granulocytes was more rapid than with the resistant C3H/He mice. To test whether this was due to an inhibitory effect of the systemic infection on the production of granulocytes in the susceptible mouse strain, two experiments were performed.

First, the kinetics of the granulocytic response of an attenuated preparation of *M. avium* were studied. This inoculum did not proliferate extensively in BALB/c mice (Table 1) but caused a persistent peritoneal infection (Fig. 4a). As shown in Fig. 4c, the peritoneal granulocytosis remained high during the 3 months of the infection. When two mice were killed after 9 months of infection by this attenuated *M. avium*, they exhibited extensive systemic bacterial proliferation and the number of peritoneal granulocytes had decreased drastically (not shown).

In the second set of experiments, resistant CD-1 and susceptible C57BL/6 mice were infected with virulent *M. avium*

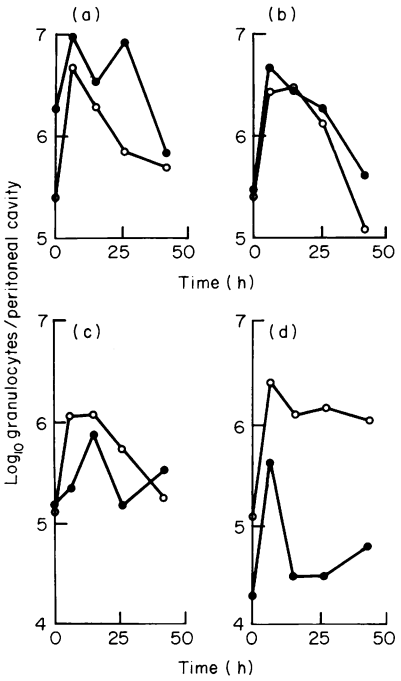

Fig. 6. Number of granulocytes per peritoneal cavity of CD-1 (a, b) and C57BL/6 (c, d) mice (four animals per time point) inoculated intraperitoneally with 1 ml of 10% casein hydrolysate. Uninfected mice (open symbols) were compared with mice infected with $2{\cdot}5 \times 10^8$ CFU of virulent *M. avium* (closed symbols) for 45 days (a), 90 days (b, d) or 30 days (c).

and challenged intraperitoneally at two times of infection with a phlogistic substance (10% casein hydrolysate) to examine the ability of infected mice to recruit granulocytes. Whereas resistant mice had similar peritoneal granulocytic responses before or after 45 or 90 days of infection by *M. avium* (Fig. 6a, b), susceptible mice had a decreased response to casein hydrolysate, showing a granulocytic response after 90 days of infection by *M. avium* that was significantly ($P < 0{\cdot}05$) lower than that observed in normal mice (Fig. 6d). The granulocytic response of 30-day-infected mice was already lower than normal (Fig. 6c) but differences were not statistically significant.

DISCUSSION

In a previous work we have studied the peritoneal inflammatory cell response to infection by several mycobacterial species in CD-1 mice (Silva *et al.*, 1989). We showed that in addition to the already described acute accumulation of neutrophils that takes place during the first 48 h post-inoculation (Vorwald, 1932; Montgomery & Lemon, 1933; Bloch, 1948; Miler, 1955; Papadimitriou & Spector, 1972; Closs & Haugen, 1975), a persistent peritoneal granulocytosis occurred which lasted for different periods of time according to the mycobacterial infection. We studied here the peritoneal granulocytic response to *M. avium*, using additional strains of mice and different bacterial inocula of distinct virulence.

Four mouse strains, C57BL/6, C57BL/10, DBA/1 and BALB/c, known to be susceptible to *M. avium* infection, and four strains known to be resistant to this mycobacterium, CD-1, DBA/2, CBA/Ca and C3H/He (Orme, Stokes & Collins, 1986; Stokes, Orme & Collins, 1986; Appelberg & Sarmento, 1990) were studied. They all presented a chronic granulocytic response to the intraperitoneal inoculation of *M. avium* but there was considerable variation in the numbers of granulocytes (mostly neutrophils) present in the peritoneal cavities among the different mouse strains studied. This variation in granulocyte number among strains of mice suggests that genetic factors are involved in the regulation of peritoneal granulocytosis. The granulocyte numbers tended to be higher in the resistant strains. The late decrease in granulocyte number was more marked in the susceptible strains. In these strains the decrease may be related to bone marrow failure due to infiltration by parasitized macrophages, as seen in cytospin preparations of bone marrow cell suspensions. This interpretation is further strengthened by the fact that the number of macrophages also decreased in a similar fashion, and that the inflammatory responses to the intraperitoneal inoculation of casein were reduced in the susceptible C57BL/6 but not in the resistant CD-1 mice infected by $2{\cdot}5 \times 10^8$ CFU of *M. avium*. The late decrease in granulocyte numbers that occurs in resistant strains may be related to the removal of the bacteria from the peritoneal cavity and thus the removal of the stimulus for continued influx of granulocytes.

Although the genetic control of the granulocytic response is most probably polygenic, we found that *Bcg*-encoded functions influence the *M. avium*-induced peritoneal granulocytosis, as shown by the experiments comparing BALB/c and C.D2 mice. This is concordant with the results obtained with the seven inbred strains studied in the first experiment.

In addition to host factors, bacterial factors also influenced the peritoneal granulocytosis. Thus, the granulocytic response varied with the virulence of the bacterial inoculum of *M. avium*. Although maximal accumulation of granulocytes did not seem to be associated with virulence of the *M. avium* inocula, the decline in granulocyte number observed after the first month of infection was different according to the bacterial preparation studied. Two factors seemed to control the persistence or decline of the granulocytic response: the elimination of the peritoneal inoculum (rough preparations in both mouse strains and SmT preparations in resistant mice) was associated with decreased granulocyte number; the extensive systemic proliferation of SmT bacteria in susceptible mice was associated with drastic decrease in peritoneal granulocytosis despite the presence of high numbers of bacteria in the peritoneal cavity. We hypothesize that such systemic infections hinders myelopoiesis and thus blocks the ability to mount peritoneal granulocytosis. This hypothesis was supported by the findings that such heavily infected mice had decreased peritoneal response to secondary challenge with a phlogistic substance and that a less virulent inoculum that persisted in the peritoneal cavity but did not proliferate extensively systemically was a good stimulus for persistently high peritoneal granulocytosis.

Persistent peritoneal granulocytosis was evidenced in all mouse strains studied including those that were resistant and those that were susceptible to *M. avium* infection. Both host genetic factors and bacterial virulence properties seemed to be involved in the regulation of the magnitude of the granulocytic response. We further propose that sustained granulocytic responses depend on the persistence of bacteria in the peritoneal cavity and on an adequate supply of granulocytes by a functional myelopoietic system.

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