HOST RESPONSE TO INFECTION

***Mycobacterium avium* infection in BALB/c and SCID mice**

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BALB/c and severe combined immunodeficient (SCID) mice were inoculated intraperitoneally with *Mycobacterium avium* and the numbers of cfu were monitored for 70 days in spleen, liver, lung, kidney, brain and peritoneum. While BALB/c mice formed typical granulomas and controlled bacterial growth in organs, a delay in development of lesions and a modest containment of infection were observed in SCID mice. In the spleen of BALB/c mice, in which bacterial growth was contained, macrophages (Mø) and natural killer (NK) cell numbers increased ≥ 4.2 times and T- and B-cell numbers increased ≥ 1.8 times after 42 days of infection; conversely, a low recruitment of mononuclear cells was observed in the spleen of SCID mice, where *M. avium* proliferated efficiently. Unlike visceral organs, a pronounced decrease in the number of cfu was observed in the peritoneum of BALB/c mice, concomitantly with a ≥ 31.7 -fold increase in Mø and NK cells and a ≥ 9.1 -fold increase in T and B cells. In the peritoneum of SCID mice only a bacteriostatic effect was observed despite a ≥ 56.7 -fold increase in Mø and NK cells and a ≥ 22.3 -fold increase in T and B cells. These results suggest that while an intact immune response can efficiently control *M. avium* infection in the spleen and peritoneum of BALB/c mice, cells of the innate immune system such as Mø and NK cells play a role in the containment of bacterial growth in the peritoneum, but not spleen, of SCID mice.

Introduction

Disseminating disease caused by *Mycobacterium avium* is the most common form of bacterial infection in HIV-infected patients. The disease is most often seen in patients with very low CD4⁺ T-cell counts and has been shown to contribute significantly to mortality [1].

Resistance to infection with bacterial pathogens in normal hosts with an intact immune system requires the co-ordinated interaction of both innate and acquired cellular immunity [2]. Macrophages (Mø) and natural killer (NK) cells operate in the former system while CD4⁺ and CD8⁺ T cells are the main components of the latter [3]. In immunocompetent

mice it has been shown that CD4⁺ but not CD8⁺ T cells [4, 5] play a major role in the control of *M. avium* infection, but other cell populations such as NK cells may be involved in early protection [2, 3, 6]. In the past, severe combined immunodeficient (SCID) mice, which are known to lack functional B and T cells [7], were used to study chronic infections with intracellular parasites [4, 8–11] to which they were more susceptible than immunocompetent mice. Such mice exhibit a cellular immune system operating without the modulation of B or T cells, that is, without acquired immunity, but retain normal function of NK cells and Mø [2].

In this study, both SCID and immunocompetent (BALB/c) mice were infected intraperitoneally with *M. avium* and bacterial growth and granuloma formation were examined at various times. In addition, a cytofluorometric examination of peritoneal and spleen cells was performed to investigate the pattern of inflammatory cells generated within compartments with different levels of anti-*M. avium* activity.

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Materials and methods

Mice

Specific pathogen-free BALB/c mice were obtained from Charles River (Calco, Lecco, Italy). CB-17 scid/scid (SCID) mice were purchased from Iffa Credo (Lyon, France). Male mice, aged 6–7 weeks, were used throughout the study. The animals were bred and maintained under barrier conditions, and fed sterilised chow and acidified water *ad libitum*.

Micro-organism and infection of mice

A clinical isolate of *M. avium* obtained from an AIDS patient (strain 485, type 21, transparent colonies [12]), was used throughout the study. Colonies grown on Middlebrook 7H10 Agar plates (Difco Laboratories) were suspended in phosphate-buffered saline (PBS) and sonicated briefly. A suspension adjusted to an optical density of 0.2 at 500 nm (corresponding to $c. 6 \times 10^8$ cfu/ml) was prepared. Mice were inoculated intraperitoneally with graded doses of mycobacteria (10^6 , 10^7 or 10^8 cfu/mouse, five mice/dose/time point) in 0.2 ml of PBS. At different time points mice were killed and 4 ml of sterile PBS were injected into the peritoneum. The fluid was withdrawn and added to an equal volume of Middlebrook 7H9 Broth (Difco). After a brief sonication (10 s, Soniprep 150; MSE, Crawley) the suspension was serially 10-fold diluted and plated on to 7H10 agar medium. Colonies were counted after incubation for 10–14 days at 37°C in a humidified CO₂ 5% atmosphere and the numbers of cfu/ml in recovered peritoneal fluid were determined. The organs collected under aseptic conditions were suspended in 7H9 medium, ground in homogenisers and briefly sonicated. The numbers of cfu/g of organ were determined as described above.

Histopathological studies

A histopathological study was performed on spleen, liver, lung, kidney and brain tissues. Portions of each organ collected at 21 and 56 days after infection were fixed in neutral formalin 10% for 1 week and embedded in paraffin. Sections of organs (5 µm thick) were stained either with haematoxylin and eosin or by the Ziehl–Neelsen acid-fast method.

Flow cytometry

Cells were stained with fluorescein isothiocyanate or phycoerythrin-conjugated anti-CD4 (RM4-5, IgG2a), anti-CD8 (53-6.7, IgG2a), anti-B cells (B220, RA3-6B2, IgG2a), anti-macrophage (Mø) Ly-6C (AL-21, IgM), anti-natural killer (NK) cells (5E6, IgG2a) monoclonal antibodies (MAbs; Pharmingen, San Diego, CA, USA). Cells incubated with MAbs ($1 \mu\text{g}/10^6$ cells) on ice for 30 min were washed three times with PBS containing fetal calf serum 1% and sodium azide 0.2% and treated with a lysing solution (Becton

Dickinson Immunocytocchemical Systems, San Jose, CA, USA). The cells were resuspended in 0.5 ml of FACS flow solution and analysed in a FACScan apparatus (Becton Dickinson) with Lysis II software. Data were reported as means and SEM and compared by Student's *t* test.

Results

Pathogenicity of *M. avium* for BALB/c and SCID mice

When inoculated with 10^8 cfu, BALB/c mice resisted the infection, none was killed by this dose within 70 days. In contrast, this inoculum was lethal for SCID mice, which all died between 49 and 70 days. A dose of 10^7 cfu/mouse did not cause any deaths in either strain; this dose was then chosen to study chronic infection during a 70-day period (Fig. 1). After 24 h, *c.* 15 and 30% of the inoculum was still present in the peritoneum of BALB/c and SCID mice, respectively, and *c.* 1–2% of the micro-organisms were found in liver, spleen, lung and, at a lower level, in kidney and brain.

In BALB/c mice, a substantial containment of infection was seen in lung and kidney, while in liver and spleen a plateau in cfu number was preceded in the first 21 days by an increase in the number of cfu of 1.5 and $2 \log_{10}$, respectively. In brain, but even more in peritoneum, a remarkable reduction in cfu was seen, followed by a late regrowth at 8–9 weeks. In SCID mice, a constant increase in the number of cfu was seen, reaching a maximum in the kidney ($4 \log_{10}$); viable counts increased $>3 \log_{10}$ in spleen and lung and $>2 \log_{10}$ in liver. In brain, after a $1 \log_{10}$ increase in the first week, the number of cfu remained constant up to 70 days; in peritoneum, the infection was contained from the onset and no relevant variation in the number of cfu was observed with time.

Development and morphology of granulomas

In spleen of BALB/c mice, granulomas formed by aggregates of epithelioid Mø mostly located in and around the marginal zone at the periphery of lymph follicles were observed on day 21 (Fig. 2a). On day 56, these granulomas were still present as small accumulations of epithelioid cells inside the follicles (Fig. 2b) and occasionally contained intracellular mycobacteria. On day 21, splenic granulomas larger than those observed in BALB/c mice were found in SCID mice (Fig. 2c); these lesions appeared to be increased on day 56 (Fig. 2d) and contained a high number of intracellular mycobacteria.

Granulomas were evident in liver of BALB/c mice, on day 21 (Fig. 2e) and decreased in size on day 56 (Fig. 2f). In SCID mice, on day 21, liver lesions appeared as focal accumulations of myelomonocytic cells

Fig. 1. Time course of *M. avium* infection in (a) spleen, (b) liver, (c) lung, (d) kidney, (e) brain and (f) peritoneum of SCID (○) and BALB/c (●) mice inoculated with 1×10^7 cfu intraperitoneally. Results represent the mean and SD cfu/g of organ or cfu/ml of peritoneal lavage in each group at each time point.

lacking the lymphocytic cuff seen in BALB/c mice (Fig. 2g); their size had increased on day 56 (Fig. 2h). At the latter time, isolated Kupffer cells heavily filled with mycobacteria but not surrounded by inflammatory infiltrates were frequently seen in SCID but not BALB/c mice.

In lung, epithelioid granulomas with few mycobacteria were occasionally observed in BALB/c mice on days 21 (Fig. 3a) and 56. At the latter time point, rare aggregates of heavily infected Mø resembling small granulomas were observed in the lung of SCID mice; in some cases, accumulation of infected inflammatory cells within the adjacent bronchial lumen was seen

(Fig. 3b). No lesions were evident in kidney and brain of either mouse strain.

Cytofluorometric analysis of BALB/c and SCID mice cells

To investigate the nature of immune cells generated during infection, flow cytometry analysis of spleen (Table 1) and peritoneal cells (Table 2) was performed on day 42. Cells of uninfected mice (controls) were examined at the same time.

BALB/c mice spleen controls (130×10^6 total cells) contained 45% B cells, 24% Mø, 13% CD4⁺ T cells,

Fig. 2. Haematoxylin and eosin-stained sections of *M. avium*-induced lesions in spleen (a–d) and liver (e–h) of BALB/c and SCID mice inoculated with 1×10^7 cfu intraperitoneally. Bar = 35 μ m. a, BALB/c mice, 21 days, spleen; b, BALB/c mice, 56 days, spleen; c, SCID mice, 21 days, spleen; d, SCID mice, 56 days, spleen; e, BALB/c mice, 21 days, liver; f, BALB/c mice, 56 days, liver; g, SCID mice, 21 days, liver; h, SCID mice, 56 days, liver.

10% CD8⁺ T cells and 3% NK cells. Following infection, the total spleen cell number was raised 2-fold, with a ≥ 4 -fold increase in the absolute number of M ϕ (47% of the total) and NK cells and a 2-, 3-

and 2-fold increase in the absolute numbers of CD4⁺, CD8⁺ and B cells, respectively; some cells showed a double positivity, as shown by total marker percentages being higher than 100%.

Fig. 3. Haematoxylin and eosin-stained sections of *M. avium*-induced lesions in lung of mice inoculated with 1×10^7 cfu intraperitoneally. Bar = 35 μ m. **a**, BALB/c mice, 21 days after infection; **b**, SCID mice, 56 days after infection.

In spleens of uninfected SCID mice (35×10^6 cells/spleen), 38% of the cells were not stained by any of the MAbs used and most of the stained cells were M ϕ and NK cells (37% and 12% of the total, respectively). *M. avium* infection induced a two-fold increase in the total cell number with relatively low changes in the proportion of single cell populations.

In the peritoneal lavages of BALB/c mice, 55% of the cells were not stained by the MAbs used and a relevant proportion of the stained cells (26% of the total) were B cells. After infection, the total cell

number increased 6.4-fold and the absolute numbers of M ϕ and NK cells (38% and 16% of the total, respectively) increased ≥ 32 -fold and those of CD4 $^+$, CD8 $^+$ and B cells increased 12-, 14- and 9-fold, respectively.

A large increase in the total cell number (11.3-fold) and in the number of the single cell lineages was also induced by *M. avium* infection in the peritoneal cavity of SCID mice, with a ≥ 56.7 -fold increase in the absolute numbers of M ϕ and NK cells and a 22-, 55- and 41-fold increase in those of CD4 $^+$, CD8 $^+$ and B cells, respectively.

Discussion

M. avium infection was more severe in SCID than in BALB/c mice, although less progressive and lethal than that reported in SCID mice infected with BCG or *M. tuberculosis* [9, 10]. This is in keeping with the knowledge that *M. avium* can cause disseminated infections in severely immunocompromised individuals such as AIDS patients and focal diseases limited to lungs in normal hosts [1]. After intraperitoneal inoculation of 10^8 cfu, SCID mice died between days 49 and 70 while they survived >70 days with an inoculum of 10^7 cfu; these results confirm and extend previous observations [4, 11] and show that even in mice devoid of functional T and B cells a partial containment of *M. avium* infection can be obtained. It is difficult to correlate the results of mortality in mice with human infections; indeed, various mycobacterial loads are found at the post-mortem examination of *M. avium*-infected AIDS patients [13, 14], but it should also be noted that in these patients the primary cause of death is not always the *M. avium* infection.

Bacterial counts were contained in BALB/c mice, after a period of rapid increase in the number of cfu, and a clear decreasing trend was observed in peritoneum and brain. In SCID mice, control of bacterial multiplication was seen in the peritoneum and brain, but not in other organs. The end of the first

Table 1. Cytofluorometric analysis of spleen cells in uninfected and *M. avium*-infected BALB/c and SCID mice on day 42

Group	Mean number of cells $\times 10^6$ * (%)					
	Total cells	CD4 $^+$ T cells	CD8 $^+$ T cells	M ϕ	B cells	NK cells
Uninfected BALB/c	130	16.2 (12.5 SEM 4.8)	12.7 (9.8 SEM 0.7)	31.5 (24.2 SEM 4.7)	58.1 (44.7 SEM 2.9)	4.3 (3.3 SEM 0.1)
Infected BALB/c	280	28.8 (10.3 SEM 2.5)	36.4 (13 SEM 2.1)	132.4 (47.3 SEM 0.3) [†]	106.7 (38.1 SEM 1.5)	19.3 (6.9 SEM 1.3) [†]
Uninfected SCID	35	1.4 (4 SEM 1.2)	0.6 (1.6 SEM 0.5)	13.1 (37.4 SEM 1.7)	2.3 (6.6 SEM 0.9)	4.3 (12.3 SEM 0.8)
Infected SCID	71	2.1 (3 SEM 0.6)	0.6 (0.9 SEM 0.4)	30 (42.2 SEM 3.1)	5.8 (8.2 SEM 1.7)	6.8 (9.6 SEM 1.2)

*Data shown are representative of two experiments (n = 3 mice per group in each experiment).

[†]p < 0.05, compared with percentage in uninfected mice, Student's *t* test.

Table 2. Cytofluorometric analysis of peritoneal cells in uninfected and *M. avium*-infected BALB/c and SCID mice on day 42

Group	Mean number of cells $\times 10^4$ * (%)					
	Total cells	CD4 ⁺ T cells	CD8 ⁺ T cells	M ϕ	B cells	NK cells
Uninfected BALB/c	250	17.5 (7 SEM 1)	6.2 (2.5 SEM 1.5)	15 (6 SEM 0.8)	65 (26 SEM 1.4)	8.2 (3.3 SEM 0.3)
Infected BALB/c	1,600	208 (13 SEM 1.3) [†]	88 (5.5 SEM 1.5)	608 (38 SEM 1.4) ^{††}	592 (37 SEM 1.5) [†]	260 (16.2 SEM 1.8) [†]
Uninfected SCID	30	0.7 (2.3 SEM 0.2)	0.2 (0.7 SEM 0.2)	0.3 (1.1 SEM 0.1)	0.3 (1 SEM 0.2)	0.15 (0.5 SEM 0.1)
Infected SCID	340	15.6 (4.6 SEM 0.4) [†]	10.9 (3.2 SEM 0.9) [†]	41.1 (12.1 SEM 1) ^{††}	12.2 (3.6 SEM 0.6) [†]	8.5 (2.5 SEM 0.1) ^{††}

*Data shown are representative of two experiments (n = 3 mice/group in each experiment).

[†]p < 0.05, ^{††}p < 0.01, compared with percentage in uninfected mice, Student's *t* test.

phase of rapid bacterial multiplication (day 21) coincided in spleen and liver with the formation of granulomas with a typical structure in BALB/c mice and with a less compact aspect in SCID mice, which lasted throughout the following phase of chronic infection. Similar granulomas have been observed in spleen and liver of BCG-infected mice [9] and in liver of *M. avium*-infected SCID mice [11], where they were shown to be ineffective in containing the infection. While containment of the bacterial growth in the lung of BALB/c mice was associated with the formation of epithelioid granulomas, in SCID mice a tendency not to form granulomas even at late stages of infection was associated with a faster rate of bacterial growth in this organ, in keeping with that previously observed in BCG-infected SCID mice [9].

The comparison of mononuclear cells from spleens of uninfected or infected BALB/c mice and SCID mice showed that, in the first mouse strain, not only a significant increase in the M ϕ number could be observed (c. four times that seen in uninfected mice and half of the total cell number), but also a significant rise in NK cells. In uninfected SCID mice spleens – where the total cell number was about one-quarter of that observed in BALB/c mice – a lower cellular response was found, in good accordance with the poor containment of bacterial growth in this organ. A close relationship between cellularity and resistance was also more evident in the peritoneum, where a massive increase in M ϕ and NK cells, and also in T and B cells, was associated with a rapid clearance of bacterial inoculum in infected BALB/c mice. In the peritoneum of uninfected SCID mice, not only was a paucity in T and B cells, in comparison with uninfected immunocompetent mice, found, but also a reduction in the number of M ϕ and NK cells, suggesting that the lack of functional T and B cells and of their products can markedly influence the recruitment and the functions of cells of the innate immune system in this region. However, the large increase in total mononuclear cells (including not only M ϕ and NK cells but also CD4⁺ and CD8⁺ T cells and B cells) following infection allowed the contain-

ment of the bacterial growth at the initial inoculum level, despite a total cell number which was only 21% of that found in infected BALB/c mice.

While CD4⁺ T cells are known to be involved in defence against *M. avium* in mice [4], CD8⁺ T cells are not known to play a direct role in the containment of murine infection [4, 5]. Thus, the increase in CD8⁺ T cells in peritoneum of both mouse strains could be explained by a general activation occurring in this compartment following peritoneal infection. A similar explanation can be given for the increase in B cells, whose role in protection is possibly not associated with production of antibodies (known to correlate with susceptibility to *M. avium* infection [15]), but more likely with their activity as antigen presenting cells [16]. Contemporary increases in NK cells and M ϕ in the peritoneum of both mouse strains are consistent with previous observations in which NK cells participated in *M. avium* resistance in mice through secretion of M ϕ -activating cytokines [11].

Therefore, a relevant decrease or containment of the number of cfu of *M. avium* at peritoneum level is not unexpected, because, unlike other non-tuberculous mycobacteria (*M. fortuitum*, *M. chelonae*, *M. goodii*) which have been implicated in human peritonitis [17, 18], to our knowledge, only one case of *M. avium* peritonitis in an AIDS patient [19] and four cases of peritonitis during continuous ambulatory peritoneal dialysis or liver disease [17, 20–22], have been described. Moreover, tuberculous peritonitis has been reported as the rarest form of extrapulmonary tuberculosis [23]. Rapid peritoneal clearance of micro-organisms in the peritoneum of immunocompetent hosts can be related both to intraperitoneal circulation [24] and acquisition of an activation state induced by cytokines secreted by the M ϕ themselves or mesothelium [25]. Relevant *M. avium* clearance by the peritoneal cavity has been reported in immunocompetent Bcg^s and Bcg^r mice, with the peritoneum of Bcg^r mice being much more effective than that of Bcg^s mice [26]. The observation of mycobacteriostatic activity in the peritoneum of SCID mice suggests that

cells of the innate immune system such as Mø and NK cells are critical for effective inhibition of *M. avium* growth in this compartment.

Overall, these observations suggest the occurrence of a relevant response of serosal peritoneal surfaces to *M. avium* infection. This has also been observed in man for other body cavities such as the pleural space, which is known to react to *M. tuberculosis* by producing more T-helper 1 cytokines than blood [27], thus allowing patients with tuberculous pleuritis to mount an effective immune response to the infection. Taken together, the experimental evidence presented above and clinical observations in man can be used to facilitate further cellular or humoral studies of local host defences developed in the peritoneum against *M. avium*.

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