Immunology 1997 **90** 600–606

Evidence for a reduced chemokine response in the lungs of beige mice infected with *Mycobacterium avium*

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SUMMARY

The basis of the increased susceptibility of beige mice to *Mycobacterium avium* infections is still not clearly understood. In this study we examined the growth of three virulent strains of *M. avium* in beige mice and normal C57BL/6 controls. Depletion of natural killer (NK) cells by administration of anti-asialo GM1 antisera did not affect the growth of *M. avium* in any of the groups of animals. Similarly, interferon-γ (IFN-γ) gene-disrupted mice were more susceptible to infection than control mice but the growth of *M. avium* was not further affected by NK-cell depletion. In terms of effector immunity, beige mice showed enhanced expression of IFN-γ and tumour necrosis factor-α (TNF-α) when compared with wild-type C57BL/6 mice. In agreement with these results, I-A and interferon-inducible protein (IP-10) expression was also higher in beige mice than in wild-type animals, as was expression of the chemokines macrophage inflamatory protein-2 (MIP-2) and macrophage chemotactic protein (MCP-1) during latter stages of the infection. However, over the first few weeks of the infection, when the susceptibility of the beige mouse lung first becomes evident, MIP-1β and MIP-2 chemokine expression in the lungs was lower in beige mice than in wild-type animals. These data indicate, therefore, that the increased susceptibility of beige mice to *M. avium* infection in the lung is not due to lack of NK-cell activity, nor can it be explained in terms of the effector cytokine response. Instead, the lower early expression of the neutrophil chemoattractants MIP-1β and MIP-2 in the lungs of beige mice tends to suggest that the enhanced susceptibility of these mice to *M. avium* infection may be due in part to defective recruitment of neutrophils or other cells responsive to these specific chemokines.

INTRODUCTION

Disease due to members of the *Mycobacterium avium* complex continues to be the primary cause of disseminated bacterial infections in North American patients suffering from acquired immune deficiency syndrome (AIDS).[1,2] The disease is characterized by progressive infection of multiple organs of the body, in which the bacterial load can reach extremely high numbers.[3] This process is usually associated with very low circulating CD4$^+$ T-lymphocyte counts and contributes significantly to patient morbidity and mortality. A number of experimental mouse models have been used to mimic the progressive course of the disease seen in humans. The C57BL/6 inbred mouse strain allows the progressive growth of virulent strains of *M. avium*,[4] a susceptibility that is controlled at least partially by the *Bcg* gene cluster.[5–7] A mutant C57 strain, the C57BL/6 *bg*/*bg* or 'beige' mouse, is also highly susceptible. Dysfunction in the beige mouse is the equivalent of the Chediak–Higashi syndrome in man, and is associated with abnormal lysosomes in leucocytes and other cells in which enzymes such as serine proteases appear to be severely deficient.[8–13] As a consequence of this mutation, the animal has an impaired ability to kill invading micro-organisms.

The discovery that *M. avium* grows at an increased rate in the target organs of beige mutant mice compared to the wild-type strain, has led to the widespread use of this animal to meet the pressing need of identifying new drugs to treat *M. avium* infections in AIDS patients. In this regard the model has proven extremely useful, with important new data obtained regarding the activity of macrolides such as clarithromycin, and new rifamycins.[14–20]

It remains a matter of debate, however, as to whether the beige mouse is a model of *M. avium* infections in AIDS, as some workers have implied.[21–23] In fact, a tremendous paradox is the fact that both wild-type (C57BL/6) and the beige mutant strain are both apparently fully immunocompetent at the T-cell/monocyte axis,[24] and yet allow the mycobacteria to still grow and eventually kill the animal. Hence, we would suggest that in our enthusiasm to use the beige model to screen new drugs, we have lost sight of the fact that the pathogenesis of *M. avium*, both in mice and in humans, still remains essentially unexplained. The susceptibility of beige mice to *M. avium* infections has been attributed to defective natural killer (NK)

Received 17 October 1996; revised 15 December 1996; accepted 16 December 1996.

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cells,[22,25] but recent data also points to a possible role of polymorphonuclear cells (PMN) in these infections.[26,27]

In the present study we show that the basis of susceptibility of beige mice to *M. avium* infections is not due to a defect at the level of production of cytokines such as interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α) which are known to play an important role in the acquired immune response to such infections. In addition, *in vivo* NK-cell depletion failed to enhance the susceptibility of normal mice to *M. avium* infections. Interestingly, in the lungs of beige mice, in which *M. avium* is able to grow at the highest rate, the early induction of mRNA for the neutrophil chemoattractants macrophage inflammatory protein-1β (MIP-1β) and MIP-2 was reduced compared to normal control animals, suggesting that a defective accumulation of neutrophils, or other effector cells responsive to the above chemokines, may underlie the increased susceptibility of the lungs of beige mice to *M. avium* infections.

MATERIALS AND METHODS

Experimental infections

C57BL/6 (wild-type) and C57BL/6 *bg/bg* (beige) mice were purchased from the Jackson Laboratory (Bar Harbour, ME). The wild-type BALB/c and IFN-γ gene-disrupted (GKO) were bred at the Laboratory Animal Resources facility at Colorado State University from stock kindly provided by Genentech (South San Francisco, CA). All mice were maintained under barrier conditions in an ABL3 facility. Food and water were given *ad libitum*. In all experiments age- and sex-matched animals were used.

Mice were infected intravenously with 10^7 colony-forming units (CFU) of *M. avium*. Strain 101 was a kind gift from Dr Lowell Young (Kuzell Institute, San Francisco, CA); it is a serotype 1 isolate from an AIDS patient with clinical disease and expresses a smooth transparent (SmT) colony type. Strain 2-151 is a serotype 2 isolated from a patient with pulmonary disease and was a kind gift from Dr Fred Crowle (Webb-Waring Institute, Denver, CO). The SmT colony type of 2-151 was isolated from plate cultures and frozen directly without growth in broth.[28] Strain TMC 724 is a serotype 2 isolate of veterinary origin and was originally obtained from the Trudeau Institute, Saranac Lake, NY.

At indicated times-points following inoculation, mice were killed by carbon dioxide inhalation. Target organs were harvested aseptically, and then individual whole organ homogenates were serially diluted in phosphate-buffered saline (PBS) and plated onto nutrient 7H11 agar medium. The number of bacterial colonies was counted after 1–2 weeks of incubation at 37° in humidified air. Blood samples were obtained by cardiac puncture.

In vivo *NK cell depletion*

In vivo depletion of NK cells was achieved by giving mice multiple intraperitoneal injections of 50 μl of anti-asialo-GM1 antiserum (Wako Chemicals, Richmond, VA), at the concentration recommended by the manufacturer to deplete NK-cell cytolytic activity. Preliminary experiments in our laboratories have shown that administration of this amount of the antibody reduced by over 95% the cytolytic activity of spleen cells towards the NK-sensitive YAC-1 target cell line, and reduced NK cells detectable by flow cytometry from 2·5–3·0% to undetectable levels. Antibody administration was 1 day before infection with *M. avium* and every 5 days throughout the course of the infection. A similar dose of normal rabbit serum (Sigma Chemical, St Louis, MO) was administered to control mice in the same way.

Semiquantitative (RT-PCR)

Liver and lung samples were taken from infected animals, homogenized in Ultraspec (Biotecx Laboratories Inc., Houston, TX) rapidly frozen and stored at $-70°$ until ready to be processed. Total mRNA was extracted and transcribed into cDNA by Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Grand Island, NY). Analysis of mRNA-specific cDNA sequences for cytokines and chemokines was performed by semiquantitative PCR.[29] Briefly, cDNA was diluted and amplified using *Taq* polymerase (Promega, Madison, WI) and specific primers. The PCR product was blotted, probed with specific internal probes and detected using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL). As a limiting number of cycles is used for each PCR, there is a relationship between the amount of signal and the relative amounts of cDNA specific for a particular product. The amount of readable RNA from each sample was compared using primers specific for the housekeeping gene hypoxanthine ribosyltransferase, HPRT. Samples which had similar HPRT signals were compared for the expression of cytokine-specific signals. The results were expressed as the fold increase in experimental signal over the control signal from uninfected animals.

RESULTS

All three strains of *M. avium* grow well in the target organs of both beige and wild-type animals

To examine the role of the beige mutation in controlling the growth of a variety of virulent *M. avium* strains, wild-type and beige mice were infected with 10^7 CFU of either 101, 2-151, or strain 724 and the course of infection was followed against time. As shown in Fig. 1, the highly virulent strain 724 was able to grow at similar rates in the spleen, liver and lung of both wild-type and beige mice (Fig. 1; right panel). Another virulent strain, 2-151, grew better in the spleen and lungs of the beige mice, but at a similar rate in the liver when compared to the wild-type controls (Fig. 1; middle panel). This was also seen in experiments using strain 101, which grew better in the spleens and lungs of beige mice (Fig. 1; left panel).

The degree of virulence of the three strains [724 > 2-151 > 101] was also reflected both in the degree of survival of the animals and in the degree of bacteraemia observed (Table 1). In each case, this was more pronounced in the beige mice than in the controls.

NK-cell depletion has no effect on the course of the *M. avium* infection in wild-type, beige, BALB/c or GKO mice

Because NK cells can both lyse target cells and secrete considerable quantities of IFN-γ, their role in anti-mycobacterial immunity could be due to either or both of

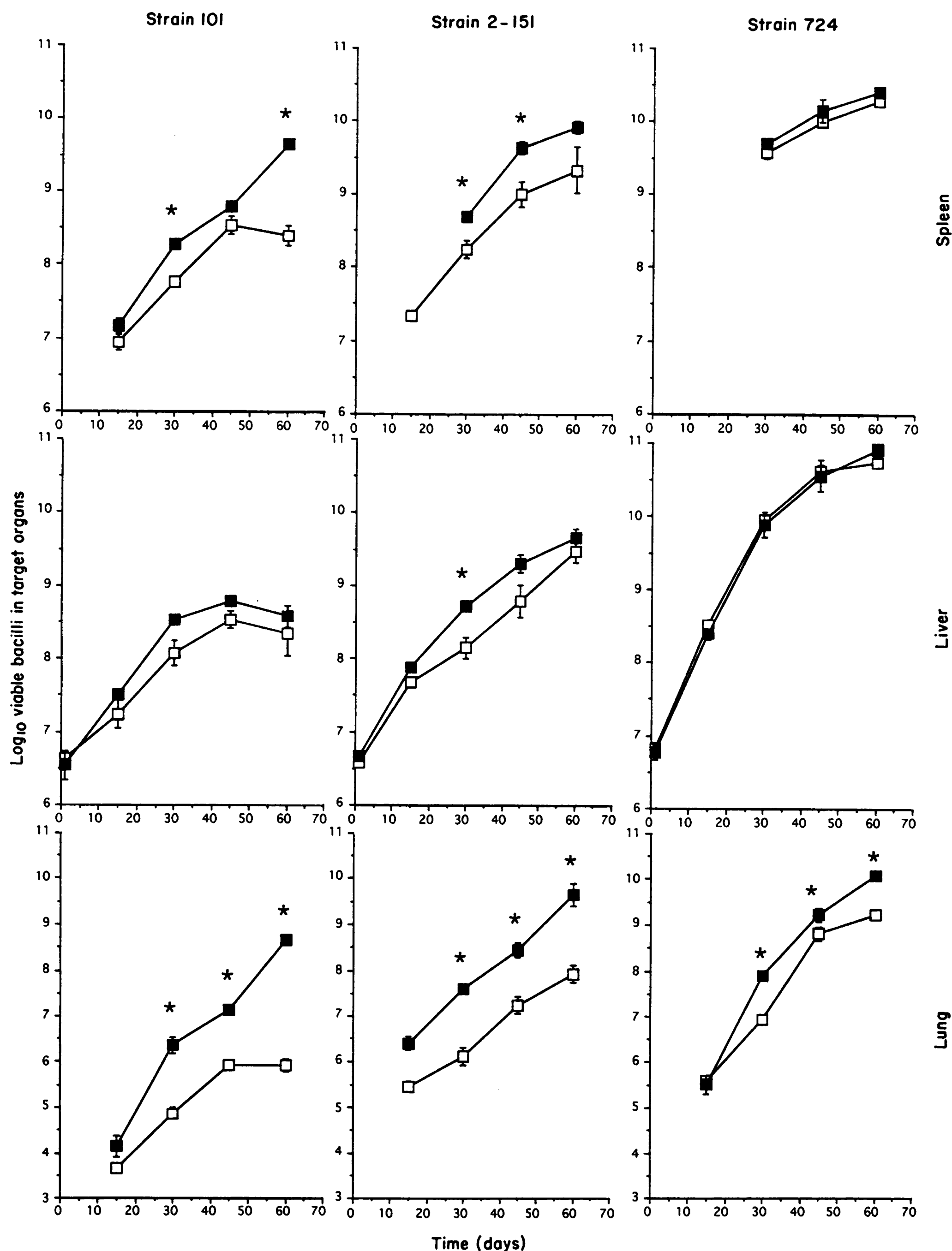

Figure 1. Growth of *M. avium* strains 101 (left panel), 2-151 (middle panel), or 724 (right panel) in the spleens, livers and lungs of C57BL/6 (open squares) and C57BL/6/*bg*/*bg* mice (closed squares). Each point represents the mean (±the standard error) numbers of viable bacilli in the target organs ($n=4$). The asterisk denotes values which were significantly increased in the beige mice (Student's paired *t*-test).

these properties. To approach this question, the beige mouse was used as a lysis-deficient model, and IFN-γ gene-disrupted mice (GKO) as an interferon-deficient model. Each group of mice, and appropriate controls, was depleted of NK cells as described above and then challenged with *M. avium* and the effect of the depletion on bacterial growth was then determined. The administration of the anti-NK-cell antibody had no effect on the growth of mycobacteria in any of the groups of mice (Fig. 2). As expected,[30,31] the GKO mice were less able to control infection than control mice.

The kinetics of emergence and loss of tissue cytokine and chemokine production during the course of the infection

The expression of acquired immunity in infected tissues to mycobacterial disease involves the efficient accumulation of

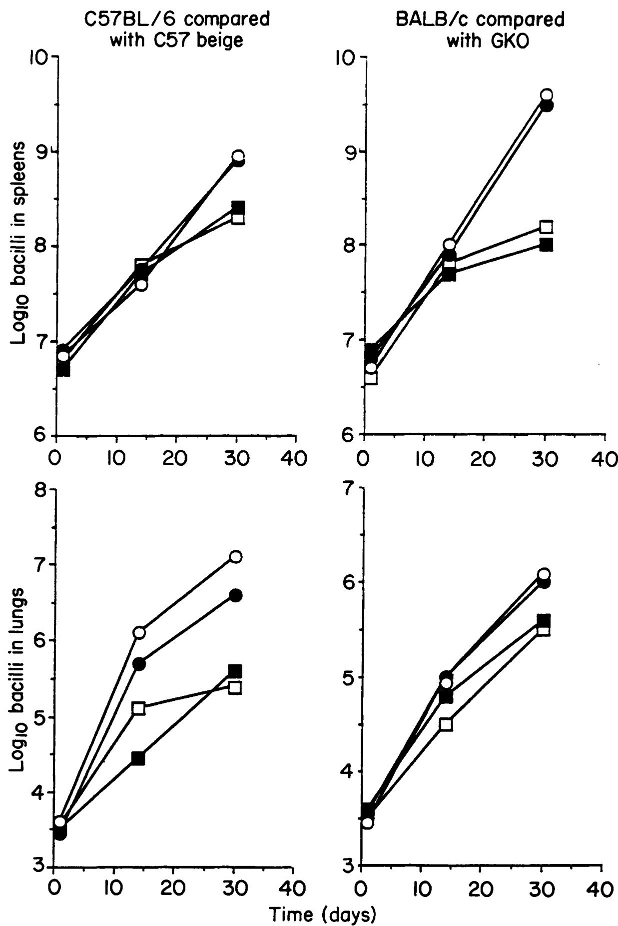

Figure 2. Evidence that NK-cell depletion had no effect on the course *M. avium* infection in C57BL/6, C57BL/6/*bg*/*bg*, BALB/c and IFN-γ gene-disrupted (GKO) BALB/c mice. Mycobacterial growth was measured in the spleens (upper panel) and lungs (lower panel) of control (squares) or mutant (circles) mice infected with 10^7 CFU *M. avium* strain 2-151, and treated with either anti-asialo GM1 serum (closed symbols) or normal rabbit serum (open symbols). Each point represents the mean numbers of viable bacilli ($n=4$). In each case there was no significant difference between mice receiving the anti-asialo GM1 or normal serum as determined by the Student's paired t-test analysis.

Table 1. Survival and level of bacteraemia in C57Bl/6 and C57Bl/6/*bg*/*bg* mice infected with 10^7 CFU of *M. avium* strains 101, 2–151 and 724. Per cent survival was calculated from a total of 20 (day 30) or 12 (day 60) infected animals for each *M. avium* strain studied. Viable mycobacteria were also quantified in the blood of four animals at day 60 of infection and the number of mycobacteria in the blood is represented as mean ± standard deviation

M. avium strain	Mouse strain	Survival (%) D30	Survival (%) D60	Log_{10}CFU/200 μl blood D60
101	C57Bl/6	100	100	1·90 ± 0·07
	C57Bl/6/*bg*/*bg*	100	75	3·39 ± 0·12
2–151	C57Bl/6	95	92	2·86 ± 0·29
	C57Bl/6/*bg*/*bg*	90	58	3·89 ± 0·27
724	C57Bl/6	80	66	4·79 ± 0·17
	C57Bl/6/*bg*/*bg*	75	58	5·54 ± 0·14

leucocytes to the site, mediated by chemoattractant chemokines,[32] followed by activation of these cells by IFN-γ. The expression of such molecules was determined in terms of the early differences between beige mice and controls in the lungs, and at later time-points in the liver given the inter-strain differences observed.

It was found (Fig. 3) that during the early stages of the infection in the lungs, macrophages from both beige and control mice expressed similar low levels of TNF-α and interleukin-12 (IL-12)p40 chain. Expression of the chemokine interferon-inducible protein (IP-10) was raised in beige mice lungs, whereas in contrast the expression of macrophage inflammatory chemokines MIP-2 and MIP-1β were substantially lower in the beige mice by the end of the first month of infection (Fig. 3). As acquired immunity was gradually generated, however, this trend disappeared and most signals subsequently observed in the liver were either similar or much higher in the beige mice (Fig. 4). These included mRNA for TNF-α and IFN-γ, which are known to be important in the host response to *M. avium*.[30]

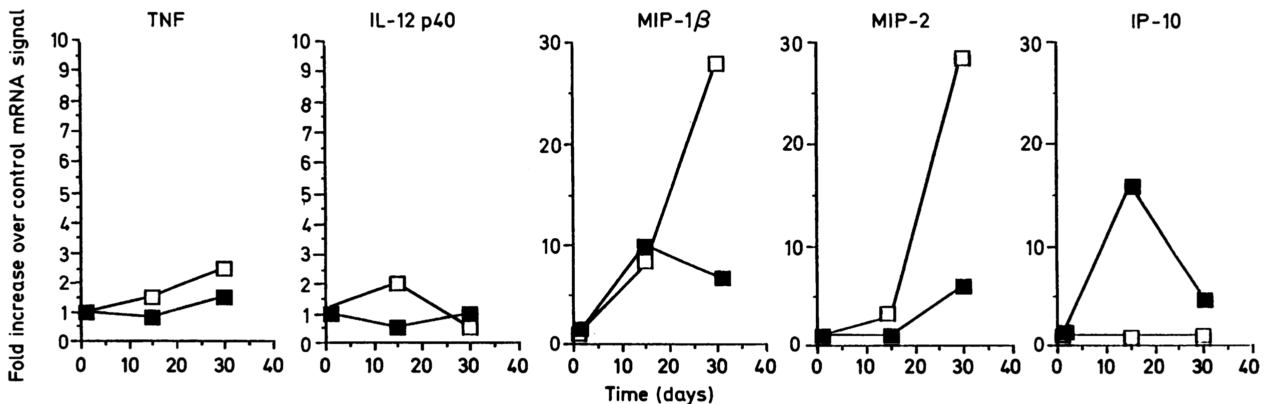

Figure 3. RT–PCR semiquantitative analysis of cytokine and chemokine gene expression in the lungs of C57BL/6 (open symbols) and C57BL/6/*bg*/*bg* mice (closed symbols) infected with *M. avium* strain 101. Similar data were seen for strain 2-151 (not shown). Data are presented as fold increase in experimental signal over the signal from control uninfected animals. Each point represents the mean signal derived from four test animals.

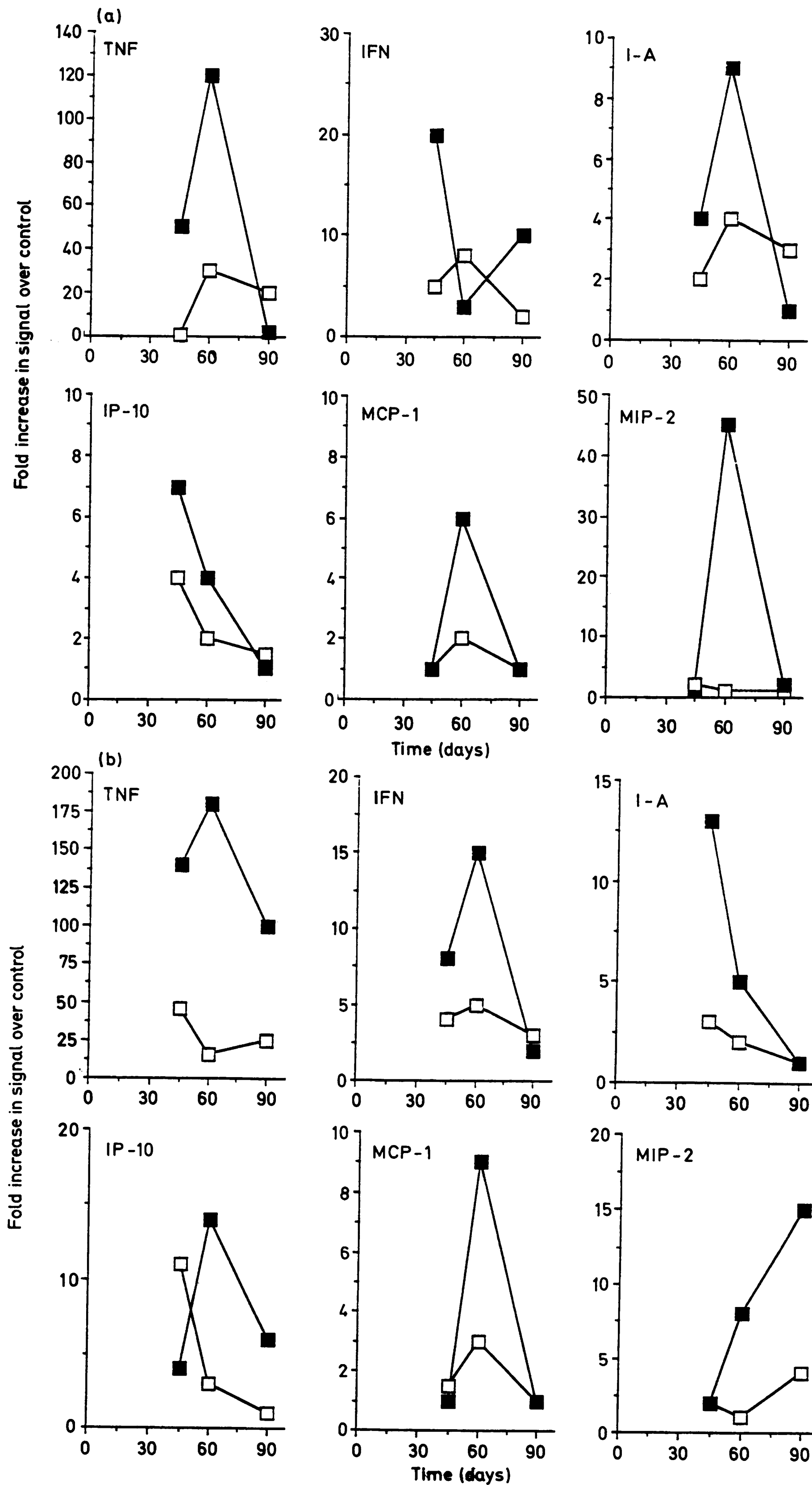

Figure 4. RT–PCR semiquantitative analysis of cytokine and chemokine gene expression in the livers of C57BL/6 (open symbols) and C57BL/6/*bg*/*bg* mice (closed symbols) infected with *M. avium* strains 101 (a) or 2-151 (b). Data are presented as fold increase in experimental signal over the signal from control uninfected animals. Each point represents the mean signal derived from at least three animals.

DISCUSSION

The increased susceptibility of the beige mouse to growth in the lungs by members of the *M. avium* complex, plus its tendency to develop bacteraemia after intravenous infection, has led to the widespread use of this mutant strain in evaluating new chemotherapies for the treatment of disseminated *M. avium* in AIDS patients. In addition, however, it has become regarded by some laboratories as a 'model of AIDS', despite the fact that it is not apparently immunocompromised.[24] In fact, the ability of the more virulent strains of *M. avium* to grow progressively in both beige and normal C57BL/6 mice still remains unexplained.

The results of this study show differences in the course of such infections, even between strains selected for high virulence. Thus, strain 724 grew at a similar rate in all three organs in both beige and control mice. In contrast, in mice infected with strains 2-151 or 101, the effect of the beige mutation was evident early in the lungs of the beige mice, and later in the spleen, but was not evident in the liver. These data tend to suggest therefore that certain strains of *M. avium* are of sufficient virulence to mask any increased susceptibility encoded by the beige mutation.

The results also show that bacteraemia is not just limited to beige mice, but also occurs, albeit at a lower level, in normal mice infected with these virulent strains. These observations are therefore contrary to the observations of others[22] who have emphasized this parameter in beige mice as a model of the bacteraemia seen in AIDS patients, and have stated that bacteraemia is a late event in immunocompetent mice occurring only after 19–20 weeks of infection.[22]

The major findings of the current study, however, are the demonstrations that the increased susceptibility of beige mice did not appear to be due to a deficiency in NK-cell activity, or could be attributed to a failure to make key cytokines associated with acquired immunity. In this regard, depletion of NK activity in normal mice did not increase the bacterial load in any target organ.

The higher susceptibility exhibited by the GKO mice to *M. avium* infection in the major target organs compared to control mice agrees with the reported evidence that IFN-γ is important to the control of mycobacterial infections.[30–33] Hence, together, the failure further to exacerbate the growth of the infection in either beige mice or GKO mice by NK-cell depletion, rules out the role of this cell in either lysis or IFN-γ secretion effector mechanisms.

In turn, TNF-α has also been shown to be protective in *M. avium* infections both in *in vitro* and *in vivo* models.[30,34–37] In view of the importance of TNF-α and IFN-γ in resistance to *M. avium*, it was possible that the enhanced susceptibility of beige animals to *M. avium* might be related to the defective production of these molecules. However the results of this study clearly demonstrated higher amounts of both TNF-α and IFN-γ mRNA in the livers of infected beige mice as compared to controls. In addition, IP-10 and I-A expression, which are directly influenced by local IFN-γ production, were also higher.

Given its nature, lysosomal abnormalities resulting from dysfunctional homotypic fusion due to mutations in a gene, *Lyst*, that controls cell signal response coupling,[9] the beige mutation can affect various cell types including monocytes, neutrophils and eosinophils. Additional mechanisms may also be involved, and it was therefore interesting to note that two neutrophil attractant chemokines, MIP-1β and MIP-2, were poorly expressed in the lungs of beige mice early during the infection, but later recovered during the phase of acquired immunity.

This observation is interesting in light of previous work that suggests a role for neutrophils in immunity to *M. avium*. For instance, recombinant IFN-γ given intraperitoneally to mice infected with *M. bovis* or *M. avium* has been shown to induce an enhanced recruitment of neutrophils,[26] and the administration of PMN cells to beige mice infected with *M. avium* leads to a decrease in bacterial loads in the liver and spleen[27] thus suggesting a protective role of these cells against mycobacterial infections. Given this evidence, it is reasonable to hypothesize that a defective recruitment and function of neutrophils, or other cells responsive to these chemokines, may be at least partially responsible for the increased early growth of the infection seen in the lungs of the beige mice.

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