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Experimental Infection of Severe Combined Immunodeficient Beige Mice with *Mycobacterium paratuberculosis* of Bovine Origin

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Severe combined immunodeficient beige mice were inoculated orally and intraperitoneally with a bovine strain of *Mycobacterium paratuberculosis* to explore their potential as laboratory animal models in the study of paratuberculosis (Johne's disease). Control animals were similarly inoculated with heat-killed *M. paratuberculosis*. In the mice inoculated intraperitoneally, focal lesions and acid-fast bacilli were first detected in the livers (4 weeks postinfection) and later in the spleens and intestines of the test but not the control animals. No bacteria were seen in the hearts, kidneys, or lungs. At 12 weeks postinfection, all test mice had significant losses in body weight compared with those in controls ($P < 0.05$), a characteristic sign of bovine paratuberculosis. Tumor necrosis factor alpha was not detected in the serum. Histologic lesions were seen in the intestines, livers, and spleens of the animals in the orally inoculated test group after 26 weeks of infection. Our results suggest that the severe combined immunodeficient beige mouse may be a useful model for the investigation of paratuberculosis and cachexia and the evaluation of antimycobacterial drugs.

Paratuberculosis, also known as Johne's disease, is a chronic, infectious granulomatous enteritis of ruminants caused by *Mycobacterium paratuberculosis* (9). The ruminant disease has been recognized as a major problem in the United States and the rest of the world (5, 9, 19). It is believed that the disease causes heavy losses in the cattle industry, but the exact economic impact has not been adequately defined (9). Although best described in cattle, paratuberculosis is also a serious problem in sheep, goats (5, 9, 27), and wild ruminants (5, 9, 19, 31, 34). The recent isolation of *M. paratuberculosis*-like organisms from some patients with Crohn's disease (4, 8) has generated strong interest from human mycobacteriologists and suggested that paratuberculosis may be a human health hazard.

Infection early in life may lead to paratuberculosis after a long incubation period, usually several years. Emaciation and/or diarrhea are characteristic signs of the disease in cattle (5, 9, 19). Emaciation may be the only clinical sign in sheep and goats (5, 9, 27). Generally, the macroscopic and histologic lesions are confined to the intestines, associated lymph nodes, and occasionally the liver (7), but disseminated paratuberculosis infection has also been reported (18).

Previously, the cachexia seen in clinical paratuberculosis was attributed to a protein-loosing enteritis (for a review, see reference 9) or immunologic mechanisms involving competent lymphocytes (24, 25). It is now suggested that the cachexia associated with chronic infections can be a result of cachectin or tumor necrosis factor alpha (TNF-α) released from monocytic cells in chronic inflammations in mice (33). Whether cachectin contributes to cachexia in paratuberculosis needs to be proven.

A clear understanding of the immunology (2) and pathogenesis (17) of paratuberculosis is lacking in part because of the unavailability of a suitable laboratory animal model. To date, several have been tried, including immunocompetent mice, nude mice, hamsters, rabbits, rats, and voles (16, 20, 21, 26).

In this study, we investigated the potential of the severe combined immunodeficient beige (SCID bg) mouse as a model for the study of paratuberculosis. *scid/scid* mice lack functional T and B lymphocytes (6, 13, 14), while *scid/scid bg/bg* mice have, in addition, a decreased natural killer-cell activity (11). SCID mice have been used to study a number of infectious organisms, including listeria (12), rotaviruses (32), and human immunodeficiency virus type 1 (29), to which they are more susceptible than immunocompetent mice. Since SCID mice are immunocompromised, we reasoned that (i) they should be readily infected with relatively few organisms and rapidly develop disease and (ii) they may give an insight into the role, if any, played by immune mechanisms in the pathogenesis of cachexia in clinical paratuberculosis.

MATERIALS AND METHODS

Experimental animals. SCID bg mice of both sexes were provided by Anne Croy, Department of Biomedical Sciences, University of Guelph. The mice were housed in microisolator cages (Lab Products, Inc., Federalsburg, Md.) within a Horsfal isolation unit, fed sterile food and water, and handled as gnotobiotes.

Organism source and inoculum preparation. Frozen ileum from a cow experimentally infected with *M. paratuberculosis* was provided by Robert Duncan, Animal Diseases Research Institute, Nepean, Ontario, Canada.

The mucosa of the thawed tissue was scraped off with the edge of a clean glass microscope slide and homogenized in 0.75% hexadecylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo.) at a ratio of 1 g to 35 ml. The homogenate was left standing for 18 h at room temperature to eliminate normal gut flora. Coarse tissue debris was separated by centrifugation for 5 min at 120 × *g*. Bacteria in the supernatant were pelleted by centrifugation at 12,000 × *g* for 10 min

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and washed twice with sterile pyrogen-free 0.9% sodium chloride (Travenol Canada, Inc., Mississauga, Ontario, Canada). A sample was stained for acid-fast organisms and inoculated onto MacConkey and blood agar plates to assess the effectiveness of the decontamination process. Under continuous stirring, three-fourths of the suspension was dispensed in 2-ml aliquots and stored at −70°C until use. The remaining suspension was autoclaved and similarly dispensed and stored. One aliquot from each of the live and killed suspensions was cultured on Herrold's egg yolk medium (HEYM) with and without mycobactin J, incubated at 37°C, and checked weekly for growth. Before use, each aliquot was thawed and diluted 1:3 in pyrogen-free sterile saline. The number of colony forming units per milliliter was determined by plating serial dilutions of the suspension.

Inoculation procedures. All mice were weighed before inoculation.

(i) **Inoculation i.p.** A total of 84 mice (58 test mice and 26 controls) were used in three replicates. Each of the test mice was injected intraperitoneally (i.p.) with 1 ml of bacterial suspension containing 10^5 CFU while being restrained manually. Each of the control mice was injected with a similar dose of killed bacteria.

(ii) **Oral inoculation.** A total of 23 mice (18 test mice and 5 controls) were anesthetized by inhalation (metophane; Pitman-Moore Ltd., Mississauga, Ontario, Canada). Test mice were gavaged with 10^5 bacteria by using a blunt intragastric needle. Control mice were similarly dosed with killed bacteria.

All mice were observed daily for evidence of clinical abnormality with respect to attitude, activity, and fecal consistency and weighed weekly until 26 weeks postinoculation, when the experiment was terminated.

Postmortem procedure. (i) i.p. inoculated group. For the i.p. inoculated group, blood was collected under anesthesia from the orbital sinus by using heparinized capillary tubes (Allied Corp., Fisher Scientific, Pittsburg, Pa.) at weeks 2, 3, 4, 6, 8, 12, 16, 22, and 26 postinoculation from four to six test and two to four control mice. Plasma was separated from the blood, pooled by group and time interval, and stored at −20°C. These mice were then killed by cervical dislocation. One milliliter of pyrogen-free sterile saline was injected i.p. and aspirated through a needle after the abdominal cavity was opened. Two drops of this aspirate were stained for acid-fast organisms. The sternum was split to expose the thoracic organs. All organs were examined in situ, and any gross abnormalities were noted. The liver, spleen, intestines (terminal ileum and proximal colon), heart, lungs, and kidneys were excised. Half of each organ was fixed in 10% buffered formalin for histology. A small piece of liver (0.5 to 1.0 g) was removed aseptically for bacteriological culture. Samples of spleens and livers from selected animals were fixed in 2.5% glutaraldehyde for electron microscopy. The remainders of the organs were stored at −20°C for mycobacteriological culture.

(ii) **Orally inoculated group.** For the orally inoculated group, mice were similarly euthanized at weeks 8, 12, 16, 19, and 26 postinfection. All controls were killed at 26 weeks postinfection. Only samples for histology and bacteriology were taken.

Histology. Two sequential 10-μm sections of each organ were stained by using the Ziehl-Neelsen technique and Ehrlich's hematoxylin and eosin. Selected sections were stained with Masson-Trichrome stain for the presence of collagen.

Determination of the liver index. A liver index, which was taken to represent the ability of organisms to proliferate within macrophages (15), was calculated by averaging the number of lesions in 47 light-microscopic fields of hematoxylin-and-eosin-stained sections magnified 25 times. In this study, a lesion was defined as any aggregate of inflammatory cells in the liver. A preliminary examination had shown that no such changes were apparent in the control sections. Orally infected mice were not included in this determination.

Electron microscopy. Approximately 1 mm^3 of fresh liver and spleen was trimmed and processed as described previously (30).

Culture technique. Organs pooled by group and time were thawed and then homogenized in saline. One milliliter of each suspension was cultured on each of three slants of HEYM, two with and one without mycobactin J, and onto MacConkey and blood agar plates and incubated at 37°C. The MacConkey and blood agar plates were checked daily for growth and discarded after 72 h. HEYM tubes were left in a slanted position until the surfaces were dry (usually 48 h). They were then sealed with tape and incubated at 37°C in an upright position. Slants were checked weekly for growth, and any contaminated slants were discarded. The appearance after at least 6 weeks of incubation of round, translucent colonies of acid-fast organisms on HEYM with mycobactin J was considered a positive indication of *M. paratuberculosis*.

TNF-α assay. A commercial multiple-antibody sandwich enzyme-linked immunosorbent assay (Genzyme Corp., Boston, Mass.), specific for murine TNF-α, was used to assay the plasma of test and control mice at 2, 4, 6, 8, 10, 12, 16, 22, 24, and 26 weeks postinfection. The manufacturer's instructions were followed.

Murine immunoglobulin assay. Although the SCID mice were randomly screened for murine immunoglobulins at the source, we examined pooled plasma samples from each group at different time intervals for mouse immunoglobulins by using radial immunodiffusion (Ouchterlony test).

Statistical analysis. Every week, the average weights of the mice in the treated and control groups were compared by the analysis-of-variance procedure in SAS (32a).

RESULTS

Clinical observations. One control mouse died of anaesthetic overdose at 11 weeks. Three test mice were found dead during weeks 19, 21, and 26 postinfection. Between weeks 10 and 14 postinfection, the test mice developed rough-hair coats and sluggish movement. By 16 weeks of infection, test animals appeared smaller in size than their uninfected controls (Fig. 1) and spent much time huddling. For the orally inoculated group, no deaths occurred and no clinical abnormalities were noticed.

Weight changes. The average weights of the control and test mice prior to inoculation were similar, but, 8 weeks after i.p. infection, the average weight of the test mice began to decrease (Fig. 2). A statistically significant difference in the mean weights between the two groups was determined 12 weeks postinfection and continued throughout the experimental period. At 15 weeks postinfection, the average weight of the test mice fell below the preinfection weight.

Postmortem changes. Gross evidence of infection was first detected in test mice at 6 weeks after i.p. inoculation. Livers from these mice had grayish focal lesions, giving the organ a mottled appearance. The spleens appeared two to three times larger than those of control mice. Over time, the liver lesions increased in number and size, imparting a pale color

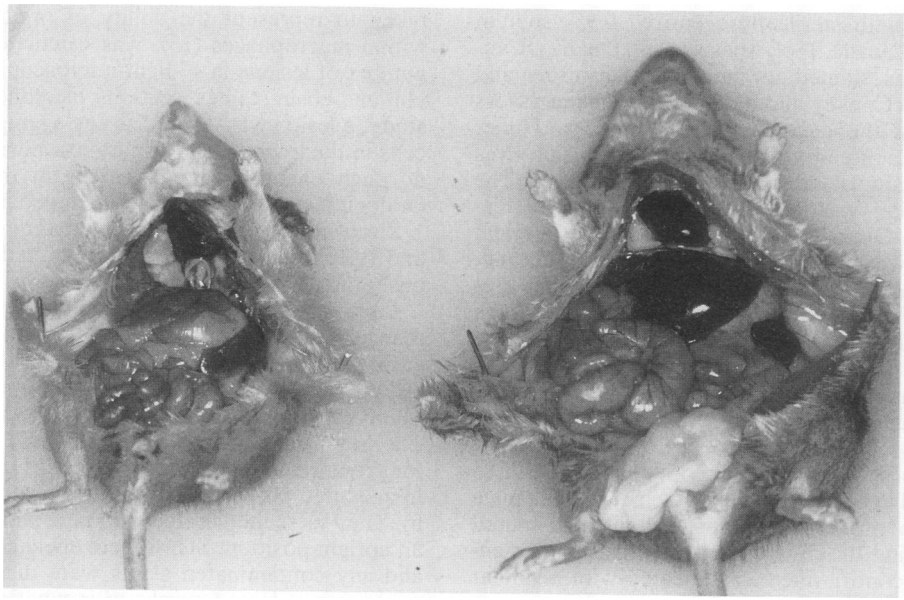

FIG. 1. SCID bg mice 26 weeks after intraperitoneal inoculation with live (left) and heat-killed (right) *M. paratuberculosis*. The test animal had lost weight and had a pale discolored liver, and its spleen was enlarged. The control animal was bigger, and fat was present in the abdomen at postmortem examination.

to the organ. From 16 weeks onwards, there was no fat in the abdomen (Fig. 1), the body tissues were dry, and there was generalized muscle wasting. The intestinal contents of the test mice were more mucoid than those of control mice. This characteristic was more pronounced in mice killed at 26 weeks.

No gross lesions were observed in the orally inoculated mice until 26 weeks after infection, when moderate splenomegaly was evident. Only one (1 of 18) mouse had a mottled liver and lacked abdominal fat. No gross abnormalities were seen in the control mice.

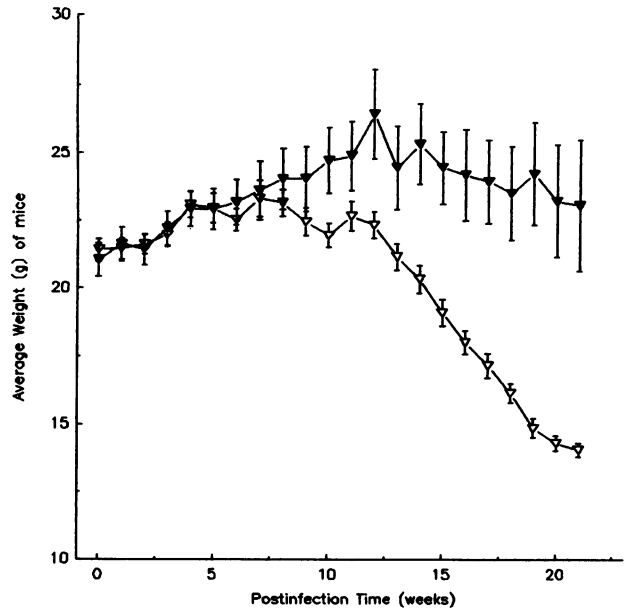

FIG. 2. Average weekly body weights (± standard error bars) of SCID bg mice inoculated intraperitoneally with live (▽) and heat-killed (▼) *M. paratuberculosis*. Starting at 12 weeks postinfection, there was a statistically significant difference in weights ($P < 0.001$) between the two groups.

Histology. Acid-fast bacilli (AFB) were seen in the cytoplasm of macrophages collected from the peritoneal cavity.

The extent of pathologic involvement of the organs progressed with time. The first lesions seen in the liver after 3 weeks of infection consisted of isolated aggregates of mononuclear and polymorphonuclear leukocytes (PMN). AFB were rarely seen at this time.

The lesions later organized into focal areas, and the numbers of AFB increased. A few AFB were also seen in the Von Kupffer cells of the liver. By 10 weeks, the size and number of lesions and the number of AFB had increased progressively. Many lesions became confluent, and cells appeared epithelioid. Giant cells were seen occasionally. PMN decreased in number and were absent from some lesions.

Lesions were seen in the spleen after 8 weeks of infection and consisted of AFB in cells in the periarteriolar sheath. No significant lesions or changes were observed in the intestines, kidneys, lungs, and heart.

Liver lesions containing only a few PMN coalesced after 12 weeks postinfection. Collagen was seen in some lesions. By 6 months, more than half of the organ demonstrated loss of normal architecture. The lesions varied in size and shape and were composed of mononuclear or epithelioid cells with occasional PMN (Fig. 3a). Most of the lesions were pink after Ziehl-Neelsen staining, indicating that most of the cells harbored AFB. Abundant collagen, indicating fibroblastic activity, was seen in the lesions.

In the spleen, the number of AFB increased, and mononuclear and epithelioid cells were apparent in both the red and white pulps. By 6 months, the red and white pulps were indistinguishable at low power, indicating that the organ had lost its normal architecture.

In the intestines of the i.p. infected mice, few AFB were seen at 3 months. The epithelia of a few villi were thickened

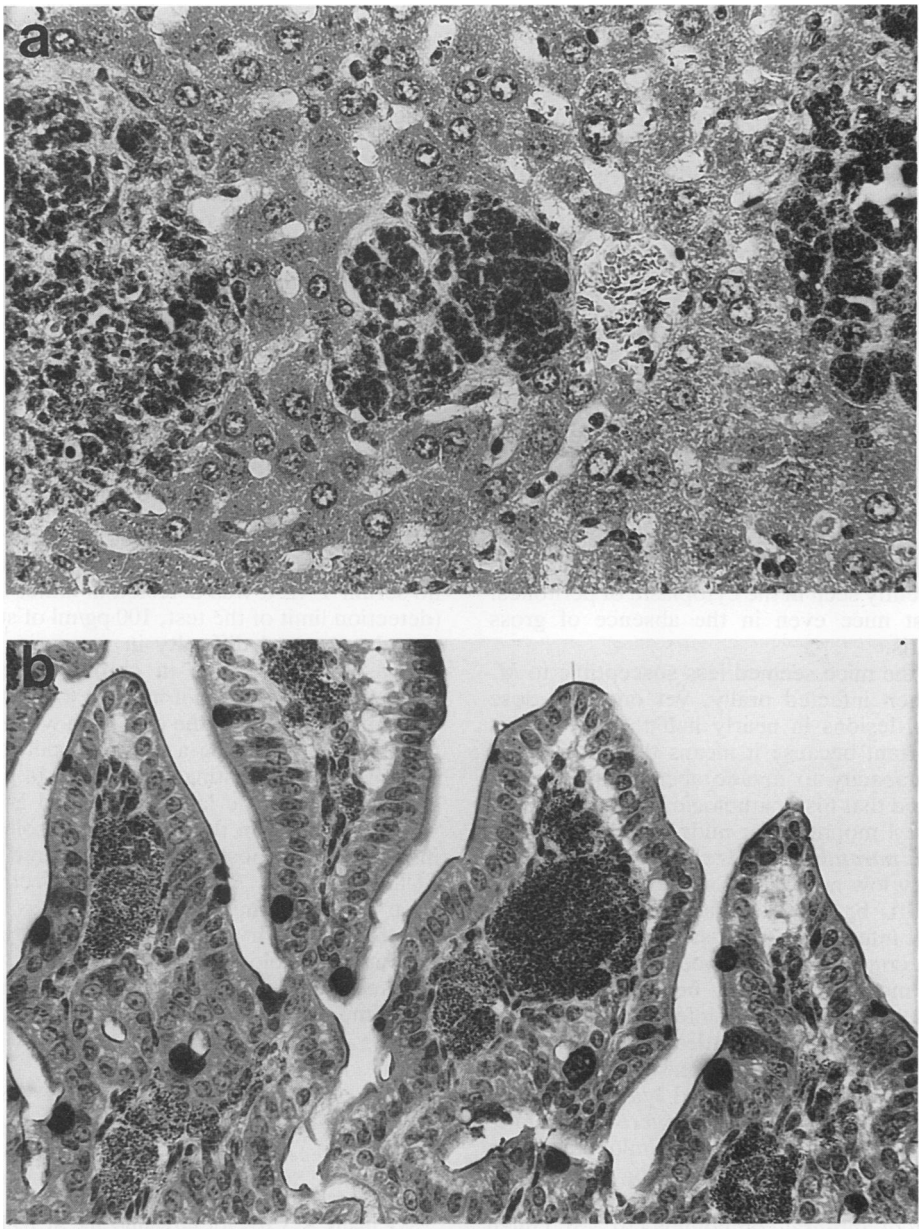

FIG. 3. Liver (a) and small intestinal villi (b) of SCID bg mouse after 26 weeks of infection with *M. paratuberculosis*. Darkly stained areas represent many AFB located in the intracytoplasmic compartment of mononuclear cells (Ziehl-Nielsen stain; magnification, ×25).

and hyperplastic. Later, foci of PMN were seen in the tips and bases of crypts and lamina propria of the villi. There were also multifocal aggregates of mononuclear cells with abundant cytoplasm. AFB were seen in the submucosa, associated with many mononuclear cells and a few PMN. By 26 weeks of infection, the epithelia had become more thickened and the number of AFB had increased markedly (Fig. 3b).

At weeks 2, 3, 4, and 6, the liver index values (± standard deviations) were 0.102 ± 0.305, 0.571 ± 0.790, 3.346 ± 3.950, and 24.195 ± 6.025, respectively. Beyond 6 weeks, the foci became confluent and/or too numerous to count.

At 26 weeks postinfection, four of nine orally inoculated test mice had lesions in the liver, spleen, and intestines similar to those seen in the i.p. test mice at this time point. However, in contrast to SCID bg mice infected by the i.p. route, all of which showed generalized muscle wasting and depletion of abdominal fat, only one of nine mice infected by the oral route showed these changes.

Electron microscopy. Electron micrographs (not shown) revealed mycobacterial organisms in the cytoplasm of macrophages.

TNF-α assay. Despite the high sensitivity of the assay (100 pg/ml), TNF-α was not detected in the sera of either test or control mice (results not shown).

Murine immunoglobulins. None of the serum samples were positive for immunoglobulins (results not shown).

Bacteriology. There was no growth from the inoculum or organs (regardless of group) inoculated on MacConkey and blood agar plates, indicating that hexadecylpyridinium chlo-

ride decontamination was effective. Characteristic colonies of *M. paratuberculosis* were seen on HEYM slants with mycobactin J from the livers, spleens, and intestines of i.p. inoculated test mice after 8 weeks of incubation. There was no growth from slants without mycobactin. Growth also occurred in organs of four orally inoculated test mice that also had histologic changes. There was no growth from any of the tissues in control mice irrespective of the presence of the mycobactin in the culture medium.

DISCUSSION

Although we did not do a comparative study, there is circumstantial evidence in the literature that large numbers of *M. paratuberculosis* organisms are required to establish an i.p. infection in conventional mice. Madge (21) injected 10^8 to 10^9 organisms i.p. into immunocompetent mice and repeated the injections 4 weeks later, while Frelier et al. (15) used 1.1×10^9 organisms to establish infections in conventional mice. We injected only 1×10^5 to 2×10^5 CFU as a single dose i.p., and yet all test mice developed evidence of progressive infection, weight loss, and emaciation.

AFB were consistently seen in the cytoplasm of peritoneal macrophages of test mice even in the absence of gross evidence of peritonitis.

Not surprisingly, the mice seemed less susceptible to *M. paratuberculosis* when infected orally, yet one oral dose resulted in intestinal lesions in nearly half the mice. This observation is important because it means that *M. paratuberculosis* has the capacity to invade aberrant hosts. Recently, it was reported that histopathologic lesions and AFB were rare in the first 4 months after nude mice were orally infected with 10^{10} *M. paratuberculosis* organisms (16). Considering the relatively low number of organisms we used, it would seem that SCID bg mice are more susceptible than nude mice. In the i.p. infected group, histopathologic lesions and AFB were seen consistently at 4 weeks after infection. Thus, the SCID bg mouse model may be a useful tool for laboratory diagnosis of mycobacterial infections.

The lesions of paratuberculosis in cattle and nude mice are granulomatous and dominated by macrophages, giant cells (Langerhan's type), epithelioid cells, and lymphocytes (9, 16), whereas granulocytes are rare. There were no lymphocytes, giant cells were rare, and few granulocytes were involved in the lesions induced in the SCID bg mice.

SCID mice have shown some promise as a means of studying the efficacy of antiviral compounds (10, 23). Given the rapidity with which the *M. paratuberculosis* infection is established, the SCID mouse, if further manipulated, may serve as an appropriate model for testing the efficacy of antimycobacterial agents. This model could be further evaluated and may be useful for isolation of other slow-growing mycobacteria. In the three replicates of this work, all test mice invariably lost weight after 12 weeks of infection. Emaciation, usually with diarrhea, is a characteristic sign in clinical Johne's disease in cattle (5, 9), while emaciation is often the only sign exhibited by sheep and goats (5, 27). Previously, the emaciation was attributed to a protein-loosing enteritis (for a review, see reference 9). In this study, fecal consistency did not change and histological evidence of intestinal infection did not appear until 12 weeks postinfection, by which time a significant weight loss had already occurred.

Merkal et al. (24, 25) suggested that an interaction between an antigen and competent lymphocytes may liberate pyrogens and cytotoxins which mediate febrile responses, emaciation, and anemia. Up to 10% of SCID mice may secrete detectable immunoglobulin and are thus tested for this (28). At present, simple in vivo tests to assess the functional status of endogenous T lymphocytes in SCID mice are not available. In separate experiments, we have injected Johnin purified protein derivative into footpads of SCID bg mice infected with *M. paratuberculosis*, but no hypersensitivity reaction was provoked (unpublished observations). Our findings suggest that competent lymphocytes may not be necessary in the pathogenesis of cachexia in *M. paratuberculosis* infections in SCID bg mice. Although these results cannot be directly applied to ruminants, they raise important questions regarding the role of an intact immune system in the cachexia of paratuberculosis.

Cytokines released as a result of immunologic mechanisms may play a role in the muscle atrophy and emaciation associated with paratuberculosis (9). There has been some speculation that TNF-α may be the cause of the emaciation in *M. paratuberculosis* infections. SCID mice are capable of producing various cytokines that are important in disease, including cachectin and gamma interferon, albeit in a T-lymphocyte-independent mechanism (1). In this study, however, no serum TNF-α was detected in either test or control mice (detection limit of the test, 100 pg/ml of serum). Others have already reported difficulty in measuring the production of serum TNF-α in vivo in chronic illnesses (3, 16). This perhaps reflects the brief half-life (6 min) of this cytokine in circulation (3). Given the ease with which SCID bg mice are infected, they may be a useful model for the study of the pathogenesis of wasting that is seen in paratuberculosis.

Since SCID mice lack both T and B lymphocytes, they would be useful in the study of the role of natural (innate) immunity in the absence of the acquired type. In one study, SCID mice were found to be as effective as conventional mice in combating *Candida albicans* (22). While orally infected laboratory animals are appropriate for the study of the pathophysiology of paratuberculosis, the SCID bg mouse infected i.p. may be more appropriate for the broad study of the immunopathogenesis and chemotherapy of paratuberculosis.

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