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# Adoptive Transfer of BALB/c Mouse Splenocytes Reduces Lesion Severity and Induces Intestinal Pathophysiologic Changes in the *Mycobacterium avium* Subspecies *paratuberculosis* Beige/Scid Mouse Model

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**Successful immune reconstitution would enhance resistance of beige/scid mice to chronic infection with *Mycobacterium avium* subspecies *paratuberculosis,* but may cause damage to intestinal tissue. Therefore, we investigated the effect of adoptive transfer of BALB/c mouse splenocytes on lesion severity and intestinal physiology in beige/scid mice infected with *M. paratuberculosis*. Mice were inoculated intraperitoneally (i.p.) with *M. paratuberculosis*, and two weeks later were inoculated i.p. with viable spleen cells from immune-competent BALB/c mice. Mice were necropsied 12 weeks after infection when engraftment of lymphocytes, clinical disease, pathologic lesions, and intestinal electrophysiologic parameters were evaluated. Lymphocytes were rare in control beige/scid mice not inoculated with spleen cells. In contrast, high numbers of $CD4^+$, $CD8^+$, and $B220^+$ lymphocytes were detected in the spleen of all beige/scid mice (n = 24) inoculated with spleen cells, indicating that adoptive transfer resulted in successful engraftment of donor lymphocytes (immune reconstitution). Immune reconstitution of *M. paratuberculosis*-infected beige/scid mice significantly reduced the severity of clinical disease and pathologic lesions, and numbers of bacteria in the liver. However, intestinal electrophysiologic parameters studied in vitro indicated that intestinal tissues from reconstituted beige/scid mice had reduced short-circuit current responses (due to reduced ion secretion) following electrical, glucose, and forskolin stimulation. These abnormal responses suggested that neural or epithelial cells in the intestine were damaged. We conclude that successful immune reconstitution of beige/scid mice enhance their resistance to *M. paratuberculosis* infection, but may cause pathophysiologic changes associated with intestinal inflammation.**

Paratuberculosis is a chronic debilitating enteric disease of ruminants caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* and is of worldwide distribution (1, 2). The mechanisms that mediate protection or disease during infection with *M. paratuberculosis* are not well understood. However, since *M. paratuberculosis* is an intracellular pathogen, cell-mediated immune responses are expected to be critical in control of the infection (3). In this regard, in vitro evidence suggests that $CD4^+$, $CD8^+$, and γ/δ T cells mediate protective responses to *M. paratuberculosis* infection in cattle (4, 5). Immune responses to *M. paratuberculosis* have been implicated in the pathophysology of the chronic enteritis that accompanies paratuberculosis (2, 6). In clinical cases of ovine paratuberculosis, enhanced $T_h1$ immune responses recently have been demonstrated with intestinal lamina propria lymphocytes (7). Although the finding is still controversial, *M. paratuberculosis* has been implicated in some cases of Crohn's disease (1, 8, 9), an inflammatory bowel disease of humans believed to be a consequence of an exaggerated $T_h1$ immune response in the lamina propria in response to bacteria (10-13). Thus, there may be similarities in the immunologic mechanisms that mediate intestinal pathophysiology in paratuberculosis and inflammatory bowel disease (IBD) in humans.

Understanding the immunology and pathogenesis of paratuberculosis would be facilitated by the availability of a suitable laboratory animal model. We previously reported use of the severe combined immunodeficient beige (beige/scid) mouse as a model to study infections with *M. paratuberculosis* (14). The scid mutation in mice results in a loss of functional T and B lymphocytes (15-17). The beige mutation is principally a lysosomal defect (18). Doubly mutant *scid/beige/scid/beige* (beige/scid) mice lack functional lymphocytes and have decreased natural killer (NK) cell activity (19). Several investigators have used mice lacking components of the immune system to study resistance to pathogens. These include mice lacking α/β or γ/δ T cells (3), mice deficient in MHC class-I molecules (20), scid mice (21, 22), and beige/scid mice (14). By adoptively transferring the deficient immune components (immune reconstitution), it is possible to study the contribution of these components in the

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immunity or pathogenesis of infections. Immune reconstitution of scid mice with congenic splenic T cells was documented to protect against infection with *Leishmania major* (22) and *Pneumocystis carinii* (21). An alternative approach would be to deplete the desired immune components in clinically normal mice by using phenotype-specific antibodies. However, administration of anti-$CD4^+$ and anti-$CD8^+$ monoclonal antibodies did not significantly alter bacterial proliferation or pathologic changes in mice infected with *M. paratuberculosis* (23).

In our previous reports, beige/scid mice inoculated with *M. paratuberculosis* developed a clinical disease characterized by progressive weight loss, presence of bacteria and lesions in liver and intestine, and shedding of bacteria in feces within six months of infection (14, 24). In contrast, immune competent BALB/c mice similarly inoculated with *M. paratuberculosis* failed to develop obvious signs of clinical disease within 12 months, despite the presence of the mycobacteria in tissues (unpublished observation). These findings suggested that BALB/c mice are partially resistant to *M. paratuberculosis*, presumably by mounting immune responses that suppress the bacterial infection, consequently delaying the onset of clinical disease. Thus, we reasoned that if spleen cells from immune competent BALB/c mice were successfully engrafted in beige/scid mice, the reconstituted beige/scid mice would develop a degree of resistance similar to that observed in BALB/c mice. It was anticipated that if immune reconstitution with unseparated spleen cells modulated infection and disease, subsequent studies could be done using purified cells to define populations of effector cells involved.

*Mycobacterium paratuberculosis* has a propensity for the intestinal lamina propria of beige/scid mice (14), and the infection induces pathophysiologic changes in the small intestine (25). Aranda and co-workers (26) observed that adoptive transfer of spleen cells from normal (immune competent) mice into scid mice leads to infiltration of the intestinal lamina propria with donor lymphocytes. However, adoptive transfer of certain subsets of spleen cells ($CD45RB^{high}$ $CD4^+$, but not $CD45RB^{low}$ or $CD8^+$ T cells) tends to induce chronic intestinal inflammation (26-28). It is thought that immune cells and bacteria or bacterial products contribute to the development of intestinal pathologic changes (26).

We hypothesized that immune reconstitution would enhance the resistance of beige/scid mice to *M. paratuberculosis* infection, but may induce pathophysiologic changes often associated with intestinal inflammation. These intestinal pathophysiologic changes include abnormalities in electrophysiologic transport parameters due to alterations ion secretions and suggest neuronal or epithelial cell damage. Thus, although reconstitution may enhance resistance to mycobacterial infection, it may induce tissue damage in the intestine. Therefore, the objective of the study reported here was to determine the effect of adoptive transfer of BALB/c mouse spleen cells on: clinical disease, pathologic changes, and bacteria; and intestinal electrophysiologic parameters associated with intestinal transport in a beige/scid mouse model of paratuberculosis.

## Materials and Methods

**Animal inoculation.** Animals were cared for and used as recommended by the Canadian Council on Animal Care in the "Guide to the Care and Use of Experimental Animals," and the experimental protocol was approved by the Animal Care Committee of the University of Guelph. The B6;CB17-*Lystbgj-Prkdcscid* (beige/scid) mice were bred by Dr. A. Croy (University of Guelph) from flora-defined (by use of Modified Schaedler Cocktail) CB-17-*Prkdcscid* males (provided by Dr. R. A. Philips, Toronto, Ontario, Canada) and C57BL/6J-*Lystbgj* females (Jackson Laboratory, Bar Harbor, Me.). Adult BALB/c mice were purchased from Charles River (St. Constant, Quebec, Canada). All mice were housed in microisolator cages (Lab Products Inc., Federalsburg, Md.) in the isolation facility. Beige/scid mice were maintained in a pathogen-free environment (temperature, 22°C; relative humidity, 45 to 55%), and were fed γ-irradiated mouse chow (Charles River) and autoclaved water. Sentinel CD1 mice (Charles River) were placed in the same environment and evaluated at regular intervals to monitor the colony for pathogen exposure. Sera from sentinel CD1 mice were consistently test negative for 17 common rodent pathogens (mouse level-II complete antibody profile; Microbiological Associates, Rockville, Md.). Mice were observed daily and weighed once a week.

A previously described bovine isolate of *M. paratuberculosis* (14) was grown as follows. A bacterial cell suspension was inoculated into Middlebrook 7H9 broth (DIFCO laboratories, Detroit, Mich.) supplemented with Middlebrook oleic acid albumin dextrose catalase (Becton Dickinson Microbiology systems, Cockeysville, Md.) and mycobactin J (Allied Labs Inc., Glenwood Springs, Colo.), and was incubated at 37°C for 4 weeks. An aliquot of the supernatant was cultured as described (14), and was found to contain $10^6$ colony-forming units (CFU)/ml. It was confirmed that the organism grown from the crude *M. paratuberculosis*-infected bovine intestinal mucosal homogenate contained the unique insertion sequence designated IS900 that is present in all bonafide strains of *M. avium* subspecies *paratuberculosis* (Dr. McFadden, University of Surrey, Guilford, Surrey, UK). Infection of mice was done by administration of a single intraperitoneal (i.p.) injection of $10^6$ CFU of *M. paratuberculosis* suspended in 1 ml of pyrogen-free saline.

Adoptive transfer of BALB/c mouse spleen cells to beige/scid mice (immune reconstitution) was performed by use of a single i.p. injection of the cell suspensions. Spleen cells were obtained from BALB/c mice, either inoculated with *M. paratuberculosis* 10 weeks prior to the cell transfer ("immune" spleen cells) or not inoculated with *M. paratuberculosis* (naïve spleen cells). Spleen cell suspensions were prepared as described (29). Briefly, mice were anesthetized, then were euthanized by cervical dislocation. The spleen was aseptically removed, placed in phosphate-buffered saline (PBS), then disrupted, using the flat rubber end of a syringe plunger. The resulting cell suspensions were pelleted by centrifugation, and red blood cells were lysed by addition of double-distilled water. The cell suspensions were resuspended in PBS. Cell viability was determined by use of trypan blue exclusion, and cells were counted in a hemocytometer. Two weeks after inoculation with *M. paratuberculosis*, approximately $3.0 \times 10^7$ viable spleen cells were administered to each beige/scid mouse.

Forty-eight (36 beige/scid and 12 BALB/c) mice, approximately six weeks old and of either sex, were assigned to eight groups (6 mice/group). Mice were inoculated with *M. paratuberculosis* or with spleen cells, and appropriate saline controls were included. Details of the infection and reconstitution status of the various groups are included in Tables 1 and 2.

**Delayed-type hypersensitivity reactions.** Attempts were made to determine delayed hypersensitivity skin reactions in the 20 BALB/c mice that were subsequently used as donors of the "immune" spleen cells. The footpad thickness of each hind foot of infected and uninfected BALB/c mice was measured, using callipers. Each left footpad was injected intradermally with 40 μl of johnin purified protein derivative (PPD; Agriculture Canada, Guelph, Ontario, Canada), whereas the right pads were injected with saline. Footpad thickness was measured at three, 24, 48, and 72 h after the injections. Increase in footpad thickness was not detected in BALB/c mice infected with *M. paratuberculosis*.

**Sample collection and evaluation of *M. paratuberculosis* infection.** Mice were observed for 12 weeks after infection (10 weeks after adoptive transfer), at which time mice were visually inspected and assigned a clinical score of 0 to 5; a score of 0 represented a clinically normal mouse, and a score of 5 indicated a very sick mouse. The clinical score assigned to each mouse was based on the severity of clinical signs of the experimentally induced disease (roughness of coat, poor body condition, and huddling) described in this model (14). Mice were killed, and a 20-cm segment of the distal portion of the small intestine was removed and placed in Kreb's buffer. A 1-cm segment (most distal) was fixed in formalin for histologic examination, and the adjacent 7-cm segment was processed for use in physiologic studies. Each carcass was then inspected and assigned a gross lesion score of 0 to 5; 0 for a normal carcass, and 5 for a carcass with the severest degree of gross abnormality, as indicated by enlarged spleen, liver lesions, absence of abdominal fat, and muscle wasting.

The spleen was removed, and cells were isolated for flow cytometric analysis. Two sections of the median lobe of the liver were removed; one section was frozen at -70°C until it was processed for bacterial culture, and the other was fixed in formalin. Two consecutive 5-μm sections were processed for histologic examination. One section was stained with hematoxylin and eosin (H&E), and the other was stained by use of the Ziehl-Neelsen technique for acid-fast (AF) bacteria, then both were examined by use of light microscopy. The microscopic lesions in the liver were scored blindly by a pathologist (JY) using two parameters: extent and severity of inflammation determined from examination of H&E-stained sections and scored on a scale of 0 to 5, 0 being normal without lesions and 5 having extensive coalescing multifocal lesions; and extent of the bacterial infection in AF-stained histologic sections scored on a scale of 0 to 3, 0 for no AF bacteria and 3 for many bacteria. Additionally, a semi-quantitative method was used to determine the numbers of viable bacteria (CFU) in the liver of individual mice. Previously frozen liver tissues were thawed, homogenized, and decontaminated with 0.75% hexadecylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo.) as described (14). Tissue suspensions were serially diluted to $10^{-2}$, $10^{-3}$, and $10^{-4}$ concentrations in saline, and 0.1 ml from each dilution from every mouse was cultured on four tubes of Herrold's egg yolk medium (HEYM) with mycobactin J, followed by incubation at 37°C for 12 weeks, when the colonies were counted (colony counts were corrected by weight of liver tissue).

**Preparation and identification of lymphocyte populations by use of flow cytometry.** We chose to use the spleen for the identification of engrafted lymphocytes because adoptively transferred (donor) cells originated from the organ, and the procedures for isolation and labelling of spleen cells for flow cytometry are well established. Spleen cells were isolated from individual mice, then were stained with the following IgG2b rat anti-mouse monoclonal antibodies: anti-L3/T4-FITC (anti-CD4; Cedarlane Laboratories, R. R. 2, Hornby, Ontario, Canada); anti-B220 (specific for a B-cell surface marker) and anti-ly-2-FITC (anti-CD8, provided by Dr. D. Snider, McMaster University, Hamilton, Ontario, Canada). Controls for non-specific labelling included isotype-matched irrelevant IgG2b-FITC and IgG2b-PE monoclonal antibodies (Cedarlane Laboratories). The final volume of the cell suspension was adjusted to 100 μl with 1% bovine serum albumin in PBS. The mixtures were vortexed briefly and incubated on ice for at least 30 min. They were then washed with 1 ml of PBS and centrifuged at 300 ×*g* for 5 min. The cell pellets were then resuspended in 1 ml of PBS. Flow cytometric analysis was performed by use of a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) and a Lysis II program.

**Physiologic studies in Ussing chambers.** Electrophysiologic parameters associated with intestinal transport were evaluated in Ussing chambers in vitro, using a described technique (30, 31). Briefly, a 7-cm segment of small intestinal tissue was immediately transferred to warm (37°C), oxygenated Kreb's solution containing 10 m*M* mannitol (pH 7.35). The intestinal section was then opened longitudinally at the mesenteric border and cut into four equal lengths. Each piece was rinsed with the buffer to dislodge any ingesta, then was mounted onto an Ussing chamber (WPI Instruments, Narco Scientific, Mississauga, Ontario, Canada), which included removable stimulating electrodes on opposite sides of the tissue. The mounted surface area of 0.6 $cm^2$ was exposed to Kreb's buffer, which on the serosal surface, contained 10 m*M* glucose (serosal buffer), and on the mucosal surface, contained 10 m*M* mannitol (mucosal buffer). The tissue was short-circuited at zero potential, using an automatic voltage clamp (WPI Instruments). The short-circuit current (Isc, in $\mu A/cm^2$) was recorded continuously. The potential difference (PD, in millivolts [mV]) was determined at 10-min intervals, and conductance (G) was calculated according to Ohm's law (millisiemens per square centimeter, $mS/cm^2$), as a measure of tissue viability. The mucosal and serosal buffer solutions were constantly aerated with 95% $O_2$/5% $Co_2$ and maintained at 37°C. Under these conditions, such intestinal tissue is known to remain viable for more than two hours.

After mounting, tissues were allowed to equilibrate for approximately 20 min, until a steady baseline Isc was observed, then baseline PD and G were recorded. After a further 15 min, the tissue received transmural electrical stimulation (TS, 10 mA, 10 Hz, 0.5 msec for 5 sec) to release neurotransmitters from submucosal nerves. The increase in Isc (Δ Isc) was recorded as the maximal change in current within 5 min. Tissues were also exposed to histamine ($10^{-3}$*M*; Sigma Chemical Co.) on the serosal surface and 10 m*M* glucose on the mucosal surface, and forskolin ($10^{-6}$*M*; Sigma Chemical Co.) was added simultaneously to the serosal and mucosal buffers. Transmural electrical stimulation, glucose, and forskolin usually will stimulate ion secretion (through different mechanisms), which would be reflected as Δ Isc.

**Statistical analysis.** Data obtained from flow cytometry, physiologic studies, and body weight were compared by use of analysis of variance. For the clinical, pathologic, and bacterial scores, a Kruskal-Wallis non-parametric test was used to compare differences between groups of mice. Statistical significance was set at $P < 0.05$.

## Results

Adoptive transfer of BALB/c splenocytes resulted in successful engraftment of lymphocytes in beige/scid mice. Significantly high numbers of $CD4^+$ T cells were detected in beige/scid mice injected with spleen cells and in BALB/c mice (Fig. 1a). In contrast, few $CD4^+$ T cells were detected in spleen cells of control and infected beige/scid mice that were not injected with spleen cells. Similarly, significantly high numbers of $CD8^+$ (Fig 1b) and $B220^+$ (data not shown) lymphocytes were detected in beige/scid mice that were injected with spleen cells, compared with uninjected beige/scid mice. Thus, injection of beige/scid mice with naïve or "immune" spleen cells from BALB/c mice resulted in significant reconstitution of lymphocyte populations.

Adoptive transfer of BALB/c splenocytes reduced the severity of clinical disease and lesions, and bacterial numbers in the liver of beige/scid mice infected with *M. paratuberculosis*: The effect of adoptive transfer of BALB/c spleen cells (immune reconstitution) on clinical disease, pathologic changes, and number of bacteria in beige/scid mice infected with *M. paratuberculosis* was evaluated by comparing reconstituted and unreconstituted beige/scid mice. Clinical disease in infected mice was evaluated during the experimental period by visual examination, monitoring of body weight, and assigning a clinical score just before necropsy.

After the first 10 weeks of the experimental period (10 weeks after infection, eight weeks after reconstitution), visual observation indicated that three of six infected beige/scid mice that were not reconstituted (group 4) had obvious signs of clinical disease, but none of the infected beige/scid mice that were reconstituted with naïve (group 5) or "immune" cells (group 6) had obvious signs of clinical disease. At the end of the experimental period (12 weeks after infection, 10 weeks after reconstitution) when clinical scores were determined, all six (100%) mice of group 4 had developed clinical disease. In contrast, only three of six (50%) mice of groups 5 and 6 had evidence of clinical disease. The median clinical score for group-4 mice was 3.0, but was only 0.5 for reconstituted groups 5 and 6 (Table 1, column 5), and the clinical scores were significantly reduced in mice of group 6, compared with those of group 4. Infected BALB/c mice (group 7) did not develop clinical disease, confirming findings from a preliminary study (unpublished data). None of the uninfected BALB/c (group 8) or beige/scid mice (groups 1-3) developed clinical disease (Table 1, column 5).

Mean weekly body weight of group-4 mice did not change during the 12-week experimental period. In contrast, mean body weight increased in mice of all other groups (data not shown). However, mean body weight was not significantly different among the various groups due to a large variation in body weight of the beige/scid mice.

Gross lesions were seen at necropsy in all infected mice except two beige/scid mice infected and reconstituted with naïve cells (group 5), and one BALB/c mouse (group 7). When gross lesion scores were evaluated (Table 1, column 6), it became apparent that, compared with infected but not reconstituted beige/scid mice, reconstitution of *M. paratuberculosis*-infected beige/scid mice with either naïve or "immune" cells had significantly reduced gross lesions. The median gross lesion score for group 4 was 4.5, whereas that for groups 5 and 6 was only 1.0 and 1.5, respectively (Table 1, column 6). Additionally, gross lesion scores in reconstituted *M. paratuberculosis*-infected beige/scid mice were similar to those observed in infected BALB/c mice. Notice that mild gross lesions were seen in one of six beige/scid mice that were injected with "immune" cells only, but not with *M. paratuberculosis* (group 3). This was due to cotransfer of *M. paratuberculosis* along with spleen cells from previously infected BALB/c mice.

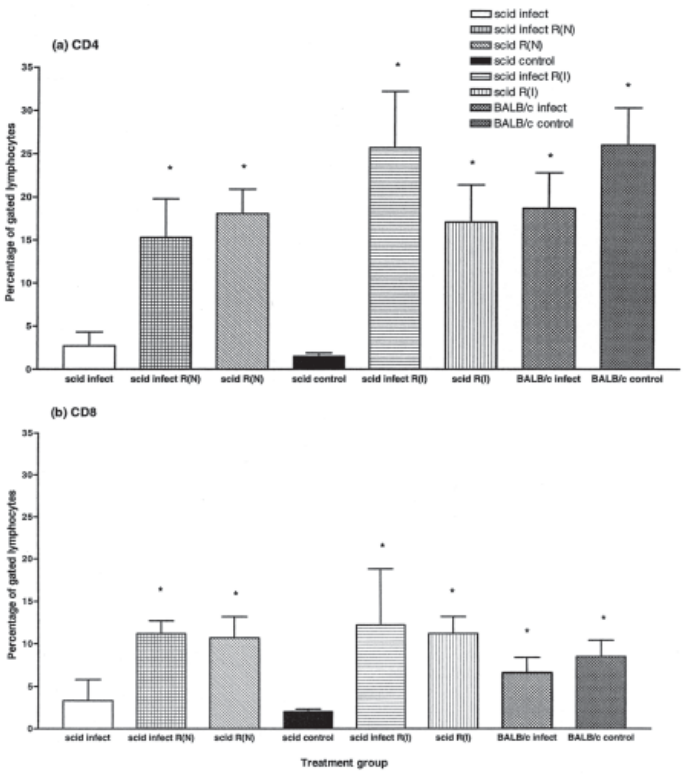


**Figure 1.** Adoptive transfer of BALB/c mouse spleen cells into beige/scid mice resulted in significant engraftment of T cells as indicated by the expression of (a) $CD4^+$ and (b) $CD8^+$ T cells as determined by use of flow cytometric analysis of splenocytes. Beige/scid mice were injected with naïve or immune BALB/c mouse splenocytes. Bars represent mean ± SD of percentage of gated lymphocytes from 4 to 6 mice/group analyzed separately. Groups: scid infect = beige/scid mice infected with *Mycobacterium paratuberculosis* (group 4); scid infect R(N) = beige/scid mice infected with *M. paratuberculosis* and reconstituted with spleen cells from naïve BALB/c (group 5); scid R(N) = beige/scid mice reconstituted with spleen cells from naïve BALB/c mice (group 2); scid control = beige/scid mice injected with saline (group 1); scid infected R(I) = beige/scid mice infected with *M. paratuberculosis* and reconstituted with spleen ("immune") cells from BALB/c mice previously infected with *M. paratuberculosis* (group 6); scid R(I) = beige/scid mice reconstituted with "immune" spleen cells (group 3); BALB/c infect = BALB/c mice infected with *M. paratuberculosis* (group 7); and BALB/c control = BALB/c mice injected with saline (group 8). *Significantly different from value for control beige/scid mice.

Histologic lesions were seen in H&E-stained liver sections of all mice infected with *M. paratuberculosis*, but not in control mice injected with saline. Histologic lesion scores (Table 1, column 7) indicated that reconstitution of *M. paratuberculosis*-infected beige/scid mice with either naïve (group 5) or "immune" cells (group 6) reduced severity of the histologic lesions, compared with those in infected, unreconstituted beige/scid mice (group 4) (Table 1, column 7), and severity of lesions was significantly reduced in group-6 mice. Additionally, AF bacteria were seen in Ziehl-Nielsen-stained liver sections of all infected mice (Table 2, column 5). Numbers of AF bacteria in liver were significantly reduced in mice of groups 5 and 6, compared with

**Table 1.** Median clinical, gross, and histologic lesion scores (range in parentheses) of mice after inoculation with *Mycobacterium paratuberculosis* and immune system reconstitution

| Group no. | Mouse strain | Infection status | Splenocyte reconstitution | Clinical score | Gross lesion score | Histologic lesion score |
|---|---|---|---|---|---|---|
| 1 | Beige/scid | - | - | 0 (0) | 0 (0) | 0 (0) |
| 2 | Beige/scid | - | Naïve cells | 0 (0) | 0 (0) | 0 (0) |
| 3 | Beige/scid | - | Immune cells | 0 (0) | 0 (0-1.0) | 1.0 (0-1.0) |
| 4 | Beige/scid | + | - | 3.0 (1.0-4.0) | 4.5 (3.0-5.0) | 3.0 (3.0-4.0) |
| 5 | Beige/scid | + | Naïve cells | 0.5 (0-1.5) | 1.0 (0-3.0) | 2.5 (1.0-3.0) |
| 6 | Beige/scid | + | Immune cells | 0.5 (0-1.0) | 1.5 (1.0-2.5) | 2.0 (1.0-2.0) |
| 7 | BALB/c | + | - | 0 (0) | 1.0 (0-2.0) | 1.5 (0.5-2.0) |
| 8 | BALB/c | - | - | 0 (0) | 0 (0) | 0 (0) |

**Table 2.** Median bacterial scores determined by use of liver histologic examination and *M. paratuberculosis* colony-forming units (CFU) isolated from the liver of mice following infection and immune system reconstitution

| Group no. | Mouse strain | Infection status | Splenocyte reconstitution | Bacterial scores (liver) | Bacteria isolation from liver (CFU) |
|---|---|---|---|---|---|
| 1 | Beige/scid | - | - | 0 (0) | 0 |
| 2 | Beige/scid | - | Naïve cells | 0 (0) | 0 |
| 3 | Beige/scid | - | Immune cells | 1.0 (0-1.0) | $3.3 \pm 1.1 \times 10^2$ |
| 4 | Beige/scid | + | - | 3.0 (3) | $1.0 \pm 0.0 \times 10^6$ |
| 5 | Beige/scid | + | Naïve cells | 1.5 (1-3) | $6.0 \pm 4.6 \times 10^5$ |
| 6 | Beige/scid | + | Immune cells | 1.5 (1.0-2.5) | $5.1 \pm 4.3 \times 10^4$ |
| 7 | BALB/c | + | - | 1.3 (0.5-2.0) | $2.8 \pm 3.9 \times 10^4$ |
| 8 | BALB/c | - | - | 0 (0) | 0 |

group-4 mice, as indicated by bacterial scores obtained on histologic examination of liver sections (Table 2, column 5). This was further confirmed by results of bacterial culture of liver tissue. More *M. paratuberculosis* organisms were isolated from the liver of group-4 mice, compared with mice of groups 5 and 6. Even lower numbers of bacteria were isolated from BALB/c mice. Some AF organisms were isolated from group-3 mice (Table 2, column 6). Thus, immune reconstitution reduced the severity of histologic lesions and numbers of bacteria in the liver. It was not possible to accurately count the exact number of bacterial CFU in group-4 mice even at the highest dilution due to the high number of colonies growing in the limited surface area on the HEYM slants. Therefore, any colonies over 100 CFU were recorded as 100 CFU, and thus, the SD value for group-4 mice was 0. Therefore, the reported CFU for group 4 (not in any other group) is an underestimate (notice that CFU counts in all the other groups were always < 100, and were accurately counted).

Representative histologic sections of the liver from beige/scid mice are shown in Fig. 2. Histologic lesions were not seen in liver sections from control group-1 mice (Fig. 2a, histologic score of 0). Extensive lesions were seen in liver sections from infected group-4 mice (Fig. 2b, histologic score of 4). These lesions consisted of multifocal, granulomatous or pyogranulomatous lesions randomly distributed throughout the hepatic parenchyma. Large numbers of AF bacteria were seen in phagocytes in sections stained for AF organisms. In contrast, only a reduced number of smaller lesions were seen in group-6 mice, clearly indicating that reconstitution reduced the severity of lesions in the liver (Fig. 2c, histologic score of 2). Livers from infected and reconstituted mice had evidence of lymphocytic infiltration, and small granulomas that contained a mixture of lymphocytes, macrophages, and few neutrophils. In most, but not all instances, this was associated with diminution in the number of bacteria within the macrophages and, hence, within the entire liver.

The effect of adoptive transfer (immune reconstitution) on morphology of the intestine was evaluated by microscopic examination of intestinal sections. Immune reconstitution of beige/scid mice with BALB/c spleen cells resulted in an obvious increase in lamina propria cellularity in the ileum, compared with that in control beige/scid mice. The lamina propria of reconstituted beige/scid mice contained mononuclear cells, plasma cells, and macrophages. Infected beige/scid mice that were not reconstituted with spleen cells had aggregates of macrophages which contained AF bacteria, and the lesions appeared as multifocal, small granuloma-like lesions in the upper half of villi containing neutrophils, but there was no substantial increase in lamina propria cellularity. In contrast, infected beige/scid mice reconstituted with naïve spleen cells had markedly increased infiltration with inflammatory cells in the lamina propria, consisting of mononuclear cells, plasma cells, and neutrophils. Acid-fast bacteria were seen within macrophages. Relevant inflammatory lesions or AF bacilli were not seen in intestinal sections from infected BALB/c mice.

Electrophysiologic responses of intestinal tissues to various stimuli were evaluated in vitro as an objective indicator of intestinal pathophysiology or injury that may arise from infection, reconstitution, or concurrent infection and reconstitution. Baseline electrical parameters were high in infected and reconstituted beige/scid mice, but were not significantly different from values in controls (data not shown).

When intestinal tissue from control beige/scid mice (group 1), and from BALB/c (groups 7 and 8) mice were subjected to TS, there was a transient large Δ Isc response (Fig. 3a), indicative of a net secretion of ions. However, when intestinal tissue from group 5 was subjected to the same stimuli, the Δ Isc in response to TS was significantly reduced, compared with that of control group-1 mice. Additionally, the Δ Isc in response to TS was reduced in intestinal tissue from mice of groups 2, 3, 4, and 6, but values were not significantly different from values for controls. Thus, reconstitution alone, or concurrent infection and reconstitution reduced capacity of intestinal tissue to respond to TS, presumably due to reduced ion secretion.

Luminal exposure of intestinal tissue to glucose resulted in a transient large Δ Isc response in control beige/scid mice (Fig. 3b). The Δ Isc in response to glucose was significantly reduced in mice of groups 2 and 5. Additionally, this Δ Isc in response to glucose

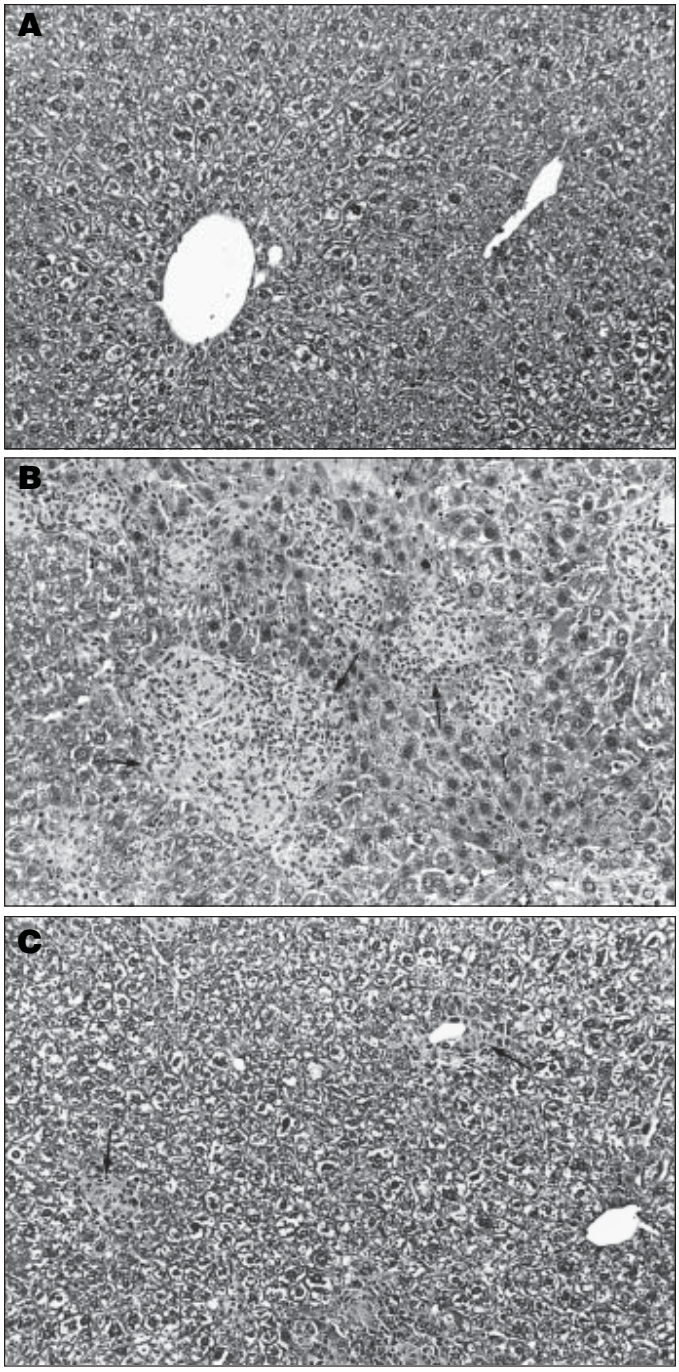


**Figure 2.** Adoptive transfer of BALB/c spleen cells into beige/scid mice infected with *M. paratuberculosis* reduced the severity of histologic lesions in the liver. Representative histologic liver sections from; (a) control beige/scid mice injected with saline (histologic score of 0), (b) beige/scid mice infected with M. paratuberculosis ([histologic score of 4), and (c) beige/scid mice infected with *M. paratuberculosis* and two weeks later injected with "immune" BALB/c spleen cells (histologic score of 2). Arrows point to areas of inflammation. H&E stain; magnification, 26×).

was reduced in mice of groups 3, 4, and 6, but the reduction was not significant. Thus, reconstitution with naïve cells significantly reduced the capacity of intestinal tissue to respond to glucose stimulation, whereas the reduced responses associated with reconstitution with "immune" cells were not significant.

Intestinal tissue from control beige/scid and BALB/c mice responded well to forskolin stimulation (Fig. 3c). As with TS and glucose stimulation, responses to forskolin were significantly reduced in group-5 mice, compared with group-1 control mice. Similarly, responses to forskolin were significantly reduced in mice of groups 2 and 3. Mice of groups 4 and 6 had reduced Δ Isc responses to forskolin, but these responses were not significantly different from responses of controls. Thus reconstitution with either naïve or "immune" cells significantly reduced responses to forskolin

Together, these data indicate that reconstitution alone (especially with naïve cells), was associated with significantly reduced (abnormal) electrophysiologic responses of intestinal tissues to stimulation with two of the three stimuli.

Although infection alone reduced the responses, this reduction was not significant. However, concurrent infection and reconstitution with naïve cells consistently resulted in significant reduction of intestinal responses, and these responses were the poorest to all three stimuli.

## Discussion

Results of this study indicate that adoptive transfer of immune competent BALB/c mouse spleen cells into beige/scid mice resulted in successful engraftment of significant numbers of lymphocytes (immune reconstitution), reduced lesion severity of *M. paratuberculosis* infection, and induced abnormal electrophysiologic responses indicative of pathophysiologic changes in the intestine.

Immune reconstitution reduced the severity of clinical disease in beige/scid mice infected with *M. paratuberculosis*. Furthermore, immune reconstitution reduced the severity of gross and microscopic lesions, and numbers of *M. paratuberculosis* organisms in the liver. These observations suggest that immune reconstitution of beige/scid mice with immune competent BALB/c mouse spleen cells reduced the severity of *M. paratuberculosis* infection in beige/scid mice by suppressing bacterial growth in the liver (and probably other organs outside of the intestine), and subsequently slowing the development of clinical and pathologic disease. Our results are consistent with those from other investigators which indicated that reconstitution of scid mice with BALB/c splenic T cells can protect against infection with *Leishmania major* (22) and *Pneumocystis carinii* (21). The partially protective effects of the adoptive transfer likely reflects reconstitution of the immune system in beige/scid mice, with the donor cells from BALB/c contributing to the development of immunity against *M. paratuberculosis* infection in the beige/scid mice. Further experiments are required to determine the nature of *M. paratuberculosis*-specific immune responses. This can be achieved by conducting in vitro immune function assays (e.g., lymphocyte proliferative responses and interferon [IFN]-γ production) to detect cell-mediated immune responses in infected beige/scid mice.

Reconstitution with "immune" cells (from BALB/c mice infected with *M. paratuberculosis*) did not result in significantly better protection, compared with results of reconstitution with naïve cells, as we had anticipated. This comparison may have been confounded by the co-transfer of viable *M. paratuberculosis* organisms along with the donor "immune" cells, which may have exposed these mice to a higher number of organisms. Further investigation is needed to explore the effect of reconstitution with "immune" cells on *M. paratuberculosis* infection in beige/scid

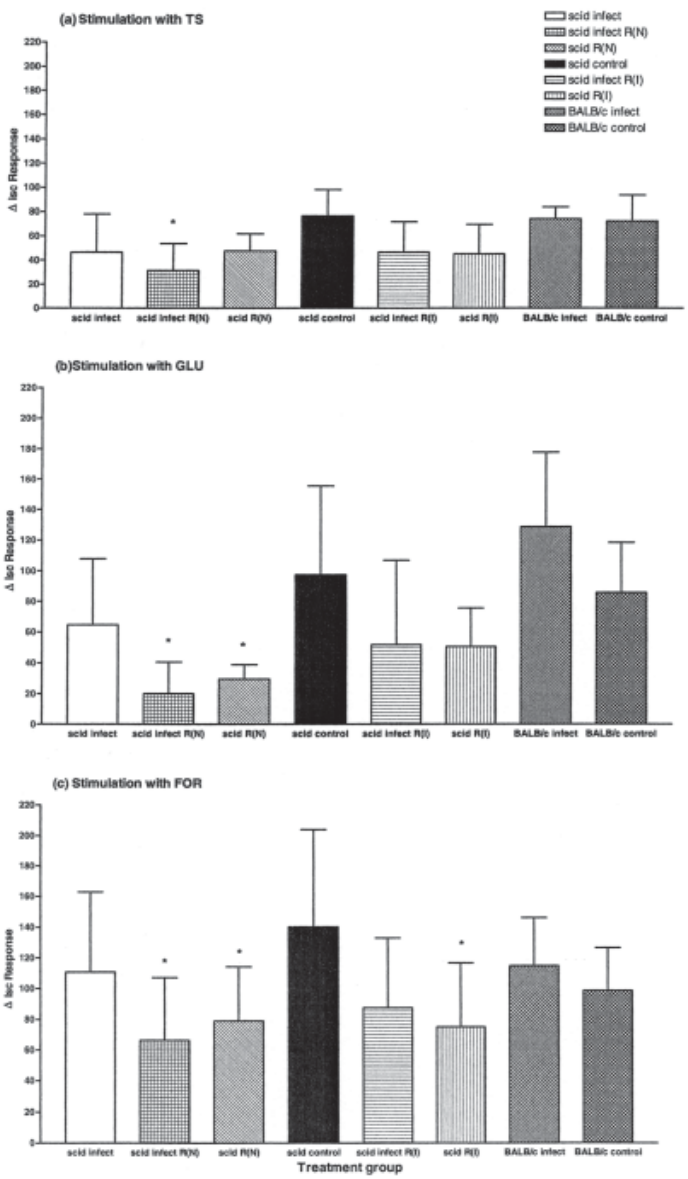


**Figure 3.** Adoptive transfer of BALB/c spleen cells into beige/scid mice was associated with reduced (abnormal) intestinal electrophysiologic responses. Mice were inoculated with *M. paratuberculosis*, then injected with the spleen cells. Mice were killed 12 weeks after inoculation (10 weeks after adoptive transfer). Small intestinal tissues were mounted on Ussing chambers in vitro and the change in short circuit current (Δ Isc) in response to electrical stimulation (TS), glucose (GLU), and forskolin (FOR) was measured. Adoptive transfer (a) or infection (b) reduced intestinal responses to stimuli. Adoptive transfer into infected scid.bg mice further reduced these responses (c). *See* Fig. 1 for key. *Significantly different from value for control beige/scid mice.

mice. Such experiments can be performed by immunizing BALB/c mice with non-pathogenic mycobacteria or include a step to deplete macrophages from donor spleen cells (which contain mycobacteria) prior to the adoptive transfer. Our failure to document a delayed hypersensitivity skin reaction in *M. paratuberculosis*-infected BALB/c mice, and that of others (32), does not necessarily mean that cell-mediated immunity had not developed in these mice (33). In vitro immune assays could be useful for documenting the presence (or absence) of immunity in BALB/c mice.

Despite the beneficial effects observed clinically and in liver tissue, immune reconstitution of beige/scid mice was associated with pathophysiologic changes (injury) to the intestinal tissue, as indicated by abnormal electrophysiologic (reduced Isc) responses of this tissue following stimulation. The abnormal electrophysiologic responses are presumably due to altered ion secretion after injury to intestinal tissue, and are often associated with intestinal pathophysiology and inflammation. Electrical stimulation of the intestine induces mucosal nerves to release endogenous neurotransmitters, which stimulate secretion of $Cl^-$ by intestinal epithelium (34), consequently increasing Δ Isc. The Δ Isc response to transmural electrical stimulation is reduced during intestinal inflammation, and the poor response can be due to abnormality of mucosal nerves (31). However, from results of our study, it remains to be determined whether the poor response is due to neuronal or epithelial abnormality, or both.

The transport of glucose is carrier mediated and is associated with the absorption of $Na^+$. Thus a relative decrease in the Isc response to glucose stimulation would suggest a defect in the carrier system.

Responses to another ion secretion stimulator, forskolin, did not differ between control and infected mice. The stimulatory activity of forskolin is mediated directly via activation of cAMP, a secondary messenger responsible for ion secretion. The fact that responses to forskolin were reduced suggests that reconstitution affected the cAMP system.

The intestinal pathophysiologic changes observed after immune reconstitution alone (in the absence of *M. paratuberculosis* infection) confirm our earlier finding from a preliminary experiment (unpublished data). This observation bares some similarities with animal models of IBD. Spleen cells from normal mice adoptively transferred into scid mice migrate to the epithelium and lamina propria of the intestine (26). Scid mice reconstituted with normal mouse spleen $CD45RB^{high}$ $CD4^+$ T cells, but not with $CD45RB^{low}$ or $CD8^+$ T cells or unseparated splenocytes develop chronic intestinal inflammation similar to that associated with IBD (26-28). It has been hypothesized that reconstitution with cells alone may not be sufficient for disease development, and that presence of bacteria or bacterial products, such as in enteric flora, may be required to generate pathogenic T cells (26). Evidence is accumulating to suggest that the presence of bacteria (or bacterial products) appears to be the stimulus for immune-mediated intestinal inflammation (11, 16, 35). Furthermore, scid mice infected with *Helicobacter hepaticus*, then reconstituted with $CD45RB^{high}$ $CD4^+$ T cells, developed more severe intestinal inflammation than did controls or mice with either factor alone (27). We speculate that, in our study, reconstitution of the immune system in beige/scid mice generated immune-competent cells, which in turn, generated immune responses against bacteria or bacterial products from enteric flora, as suggested by others (26). It is possible that immune responses directed against *M. paratuberculosis* may have contributed to the more pathophysiologic changes observed in concurrently infected and reconstituted beige/scid mice.

*Mycobacterium paratuberculosis* infection alone reduced intestinal responses in beige/scid mice, but these were not significantly different from those of controls. Infection alone would most likely have caused appreciable pathophysiologic changes had the experimental period been extended. In a separate study,

we found that beige/scid mice infected for 32 weeks with as few as $10^2$ CFU of *M. paratuberculosis* develop substantial intestinal pathophysiologic changes in the absence of immune reconstitution (25). Interestingly, concurrent infection and reconstitution of beige/scid mice with naïve cells (but not with "immune" cells) consistently reduced intestinal responses significantly to all stimuli, and always induced the poorest responses. This trend suggests a potential interaction between donor cells (from reconstitution) and *M. paratuberculosis* resulting in more extensive pathophysiologic changes. However, since responses of intestinal tissues from concurrently infected and reconstituted beige/scid mice were not significantly different from those of reconstituted (but not infected) mice, further investigation is required to formerly prove a specific interaction between donor cells and *M. paratuberculosis*.

The results of the study reported here also raised some interesting questions. It is not clear why reconstitution with "immune" cells was not as effective as with naïve cells in inducing pathophysiologic changes in intestines. This could be due to a number of factors including differences in the ability of naïve and "immune" cells to migrate and expand in the intestine, or to differences in cytokine activities or profiles obtained from these lymphocyte populations (26). It is also not clear why pathophysiologic changes were not detected in intestinal tissues of infected BALB/c mice. This may be due to several reasons, including the composition of the lymphocyte population in the intestine or the nature of the intestinal epithelial barrier. The BALB/c mice have an intact immune system that may prevent the entry of antigens or enteric flora from getting into the lamina propria, or may have an environment capable of controlling potentially harmful immune responses in the lamina propria (11). In contrast, the immune system of beige/scid mice may have permitted the entry and persistence of antigen from enteric flora. Reconstitution of these mice probably led to homing and expansion of populations of lymphocytes that generated cell-mediated responses to antigens in the lamina propria which, if not appropriately controlled, may result in intestinal injury. The BALB/c mice have an intact immune system and presumably prevented establishment of infection in the intestine (AF bacteria were not seen in intestinal tissue from BALB/c mice). More work is needed to determine whether establishment of *M. paratuberculosis* infection in the intestine of BALB/c mice could cause any pathophysiologic changes. It is speculated that up to $10^3$ to $10^4$ CFU of mycobacteria may be necessary to initiate a focus of inflammation (6).

Intestinal injury is usually associated with a $T_h1$ response to bacterial antigen in the lamina propria (11, 12). Scid mice have the ability to mount $T_h1$ responses on reconstitution with spleen cells and subsequent infection with *L. major* (22). The $T_h1$ immune response (indicated by high IFN-γ and interleukin 2 activities) has been documented, using lamina propria lymphocytes from sheep affected with clinical paratuberculosis (7). Further work is required to evaluate the local intestinal cytokine responses to determine whether a $T_h1$ response is the cause of the observed intestinal pathophysiologic changes in the beige/scid mouse model.

It has been reported that immune responses to infection can vary substantially in different organs in the same animal; infection can resolve in one organ, whereas the pathogen can persist in another organ (36). This could be due to the presence of different chemokine or cytokine profiles in each organ, or organ-specific growth patterns of the pathogen, both of which could have profound effects on organ pathologic changes (36). For example, *L. donovani* growth in the liver results in a granulomatous response with no appreciable impact on liver function, whereas persistent infection of the spleen results in substantial structural damage (36).

We observed substantial reconstitution with $CD4^+$ and $CD8^+$ T cells after adoptive transfer of BALB/c spleen cells. Cell-mediated immune responses are largely mediated by $CD4^+$ and $CD8^+$ T cells, and are required for resistance to intracellular bacterial infections (37-39). Reagents were not available to detect γ/δ T cells, which recognize mycobacterial antigens, and can mount $T_h1$ responses when stimulated with intracellular pathogens in vitro (38). However, other investigators have reported that γ/δ T-cells were apparently not essential for protecting mice against primary infection with the bacillus of Calmette-Guerin (40), although these cells may have an immunoregulatory function (38), suggested by in vitro data for *M. paratuberculosis* (4, 5). Despite the requirement for T cells in protection against intracellular bacterial infections, there are no data to suggest a role for B cells in protection against *Salmonella typhimurium* infection (41). Although evidence from studies of other mycobacterial infections suggest that $CD4^+$ and $CD8^+$ T lymphocytes contributed to the protective effects observed in our study, adoptive transfer experiments using purified cell populations are required to confirm their specific roles.

In the study reported here and on the basis of results of flow cytometry, lymphocytes were extremely rare in all the beige/scid mice not injected with BALB/c spleen cells, indicating that beige/scid mice did not become "leaky." Substantial numbers of lymphocytes in scid mice older than one year have been reported by use of flow cytometry, a phenomena referred to as "leakiness" (42, 43). "Leakiness" is apparently less likely in beige/scid mice (44). The scid defect involves the lymphocyte lineage, and is not apparently associated with abnormalities of other cells, such as macrophages, involved in the generation of immune responses (45). Beige/scid mice, on the other hand, have approximately 50% less NK-cell activity in addition to the scid defect, and appear to have lymphokine activated killer-like effector cells and LAK cell precursors (19). Beige/scid mice may have some NK cells and may produce cytokines, such as IFN-γ, on activation (44). Despite these differences, *M. paratuberculosis* infections were similar in scid and beige/scid mice (unpublished observations); others found beige and normal mice to be equally susceptible to *M. tuberculosis* infections (20).

In conclusion, results of this study indicate that immune reconstitution by adoptive transfer of BALB/c spleen cells into *M. paratuberculosis*-infected beige/scid mice reduced the severity of clinical disease and pathologic lesions in the liver. However, immune reconstitution was associated with pathophysiologic changes in the intestine.

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