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Dissemination of Enteric *Mycobacterium avium* Infections in Mice Rendered Immunodeficient by Thymectomy and CD4 Depletion or by Prior Infection with Murine AIDS Retroviruses

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This study shows that infection of mice with the murine AIDS virus LP-BM5 or Du5H profoundly depressed the capacity of splenic T cells from these animals to respond to the T-cell mitogen phytohemagglutinin or concanavalin A or to alloantigens. Similar effects were also observed if mice were thymectomized and then infused with monoclonal anti-CD4 antibody (TxCD4$^-$ mice). When such mice were infected intravenously with *Mycobacterium avium*, growth of the infection was markedly exacerbated in the TxCD4$^-$ mice or in mice given murine AIDS virus 2 months earlier. In view of these data, we then investigated whether such treatments might cause dissemination of *M. avium* following enteric implantation of bacteria into the mouse cecum; this route was chosen in an attempt to model events in AIDS patients, in which the gut appears to be one of the major portals of *M. avium* infection. In this model, the entry and hematogenous dissemination of four clinical isolates of *M. avium* were monitored against time and found to be accelerated and enhanced in T-cell-deficient mice. In view of this finding, these novel approaches for enteric infection that use immunodeficient mice are presented as potential new models for the evaluation of immunotherapy and chemotherapy in a setting that bears some similarity to events believed to occur in AIDS patients.

Disease caused by *Mycobacterium avium* is the most common bacterial opportunistic infection associated with AIDS within the United States (5, 16–18, 22, 37). Upon autopsy, a disseminated form of *M. avium* infection is often seen, with very large numbers of acid-fast bacteria present in multiple tissues, including the spleen, liver, lymph nodes, and bone marrow. In addition, while a large infectious load is invariably seen in gut tissues, serious pulmonary involvement is much less common (18). This observation has led to the hypothesis that the gut, rather than the lungs, is the major portal of entry of *M. avium* infection (7, 37). Indeed, in this regard, a number of enteric pathogens are relatively common causes of infections in homosexual and bisexual men (1, 8, 9, 20, 25).

To date, however, the pathogenesis of *M. avium* infections in human immunodeficiency virus (HIV)-positive individuals is still not fully understood. In a recent report from this laboratory (21), it was shown that even HIV-negative homosexual men have substantially raised antibody levels to the glycopeptidolipid (serotyping) antigens of *M. avium*, suggesting that these individuals are more persistently exposed to this microorganism than are heterosexual control subjects. How increased colonization of such individuals might occur is unknown, but it may be exacerbated by other enteric pathogens as intimated above. Interestingly, some evidence suggests that administration of antigen via the lower gut may in itself depress local immune functions (31, 35). These factors, collectively, might permit some degree of colonization of *M. avium* within granulomas within the gut tissues, which might then break down and allow dissemination of the infection as systemic immunity is subsequently destroyed by HIV.

It is clear that substantial difficulties lie ahead in the treatment of *M. avium*, be it by chemotherapy, immunotherapy, or a combination of both approaches. Such initial testing is performed in an animal model, which to date has essentially consisted of the beige mouse model (12). This animal, which is a mutant of the C57BL/6 strain, has a defect in oxidative metabolism akin to Chediak-Higashi syndrome (32). Although T-cell and monocyte activity in such mice is essentially normal, these animals exhibit increased growth of *M. avium* in lung tissues following intravenous (i.v.) infection.

We have recently proposed an alternative model, the thymectomized CD4-depleted mouse (TxCD4$^-$) in which the growth of *M. avium* is enhanced (10). In the present study, we have compared this model with yet another new model in which mice are infected with murine retroviruses (murine AIDS [MAIDS] viruses [2, 13, 26, 36]) in an attempt to more closely mimic the immune depression and lymphadenopathy that occurs in human AIDS. In some experiments, in order to model the gut as a portal of infection in AIDS patients, immunocompromised mice were exposed to enteric infection with clinical isolates of *M. avium*. The results of this study reveal a direct association between T-cell deficiency and the degree of disseminated systemic disease arising from the enteric *M. avium* infection.

MATERIALS AND METHODS

Mice. Female C57BL/6J and C57BL/6J *bg/bg* (beige) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were housed under barrier conditions in our ABL-3 biohazard facility and used when 6 to 9 weeks of age.

Bacteria. A panel of clinical isolates of *M. avium* was kindly provided by Anna Tsang, National Jewish Center, Denver, Colo. In this study, three isolates from this panel were picked at random: strains 12–39 (serotype 1; smooth-

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transparent [SmT] colony morphology), 4–44 (serotype 4; SmT but with some rough colonies also present), and 2–8 (serotype 4; predominantly smooth domed [SmD]). The highly virulent strain 101 (serotype 1; SmT) was kindly provided by Lowell Young, Kuzell Institute, San Francisco, Calif. Strain 2–151 (serotype 2; cloned for SmT colonies) was kindly provided by John Belisle.

Viruses. Two murine leukemic MAIDS retroviruses were used in this study: LP-BM5 (clone G6), kindly provided by Janet Hartley, National Institutes of Health (15, 26, 36), and Du5H, kindly provided by Paul Jolicoeur, Clinical Research Institute of Montreal (2). Mice were infected intraperitoneally with approximately 10^4 PFU of virus (one injection of LP-BM5 and two injections 1 week apart of Du5H) and then used in experiments 8 weeks later, when T-cell depression and lymphadenopathy began to become evident.

Experimental infections. Some groups of mice were thymectomized at 4 weeks of age and then 1 week later given 250 μg of anti-CD4 monoclonal antibody (clone GK1.5) i.v. (10). For systemic infections, mice were injected i.v. with 10^4 viable bacteria suspended in 200 μl of sterile saline via a lateral tail vein. For enteric infections, mice were anesthetized and a small incision was made in the peritoneal wall. Mycobacteria (10^6 viable bacteria in 100 μl of saline) were then carefully injected directly into the lumen of the cecum, using a syringe fitted with a 30-gauge needle. The needle was withdrawn slowly to allow the gut muscle wall to contract around the puncture wound and hence prevent any leakage into the peritoneal fluid. The peritoneal wall was then closed with metal clips. Mice tolerated this procedure well, and regular inspection by veterinary staff indicated no evidence of subsequent peritonitis or other sequelae.

Numbers of viable bacteria in target organs were determined against time by plating serial dilutions of individual whole organ homogenates on nutrient 7H11 agar (Difco, Detroit, Mich.) and counting bacterial colony formation 10 to 20 days later after incubation at 37°C in humidified air.

Assays for T-cell enumeration and function. To enumerate $CD4^+$ cells, spleen cell suspensions were incubated with anti-J11d.2 monoclonal antibody plus complement to enrich for T cells (4) and were then washed and resuspended in phenol red- and biotin-deficient RPMI 1640 medium. Cells were stained with phycoerythrin-conjugated anti-CD3 (clone 145-2C11) and with fluorescein-conjugated anti-CD4 (clone RM-4-5). All antibodies were purchased from PharMingen, San Diego, Calif. After incubation for 20 to 30 min at 4°C, cells were washed and propidium iodide was added at a concentration of 2 μg/ml. Flow cytometric analysis was conducted, using a Coulter Epics II cytometer. Gating for viable lymphocytes was based upon light scatter and propidium iodide exclusion; 20,000 gated events were analyzed. In each case, pooled cells were analyzed; full statistical analysis of the counting procedures in this assay is described elsewhere (13).

To test overall T-cell responsiveness, spleen cell suspensions were pooled from groups of three mice and cultured in triplicate in the presence of the mitogens phytohemagglutinin PHA and concanavalin A, using a conventional technique as previously described (29). A mixed lymphocyte reaction was performed by using mitomycin-treated DBA/2 target cells as previously described (29).

Statistical analyses. Differences in gathered data were determined by Student's *t* test.

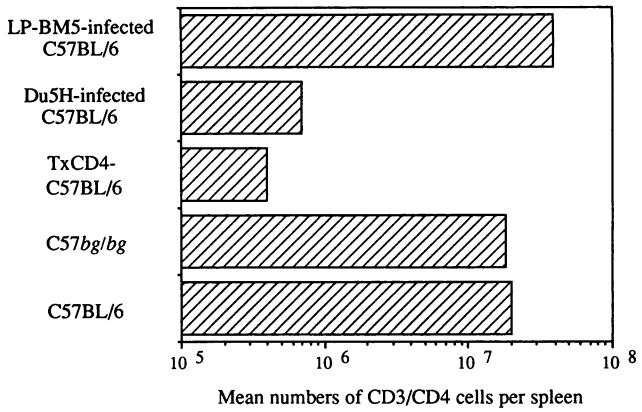

FIG. 1. Changes in numbers of T cells bearing the CD4 marker in various experimental groups, as determined by flow cytometry. Data were calculated from spleen cells pooled from three mice and are representative of two separate experiments.

RESULTS

Reduction in $CD4^+$ T-cell numbers and loss of responsiveness. The changes in numbers of cells expressing CD4 in the spleens of the various mouse models are shown in Fig. 1. While CD4 numbers in C57BL/6 controls and in beige mice were similar, monoclonal anti-CD4 depletion or infection with Du5H reduced the CD4 count in these mice by approximately 90%. In contrast, CD4 numbers in mice infected with LP-BM5 were actually increased approximately twofold, presumably as a result of observed severe lymphadenopathy, a hallmark of this viral infection (26). In all three of these latter models, however, T-cell responsiveness to mitogens or to alloantigens was severely reduced, being in most cases barely above background control responses (Fig. 2).

Association of T-cell deficiency with exacerbation of *M. avium* infection. Having established that MAIDS virus infection or direct depletion of CD4 cells substantially reduced overall T-cell responsiveness, we then determined whether such treatments might affect the course of a low-dose i.v. *M. avium* infection. To this end, indicated groups of mice were infected (10^4 i.v.) with a cloned SmT variant of *M. avium* 2–151. This particular isolate was chosen because of its previously established virulence in normal mice (30).

Infection with Du5H or anti-CD4 depletion markedly exacerbated the growth of the bacterial infection in the major target organs of these animals (Fig. 3). Significant increases were observed in the spleen, liver, and lungs of the $TxCD4^-$ mice ($P \leq 0.01$), and similar increases were seen in the liver and lungs of virus-infected mice. Similar data were obtained when LP-BM5 virus infection was used (not shown). In addition, the infection grew substantially better in the lungs of the beige mice, as previously reported (12), but was similar to that in controls in the other organs.

Dissemination of *M. avium* following enteric inoculation. Having determined the facets of the mouse models described above, we then sought to establish whether such animals could be useful in the construction of an enteric model of disease which might more closely model events believed to occur in AIDS patients. To test this possibility, we directly implanted *M. avium* 101 (as a "gold standard") and three other strains picked at random into the ceca of control and $TxCD4^-$ mice. In a pilot study, we earlier determined that dissemination was more pronounced with use of the LP-

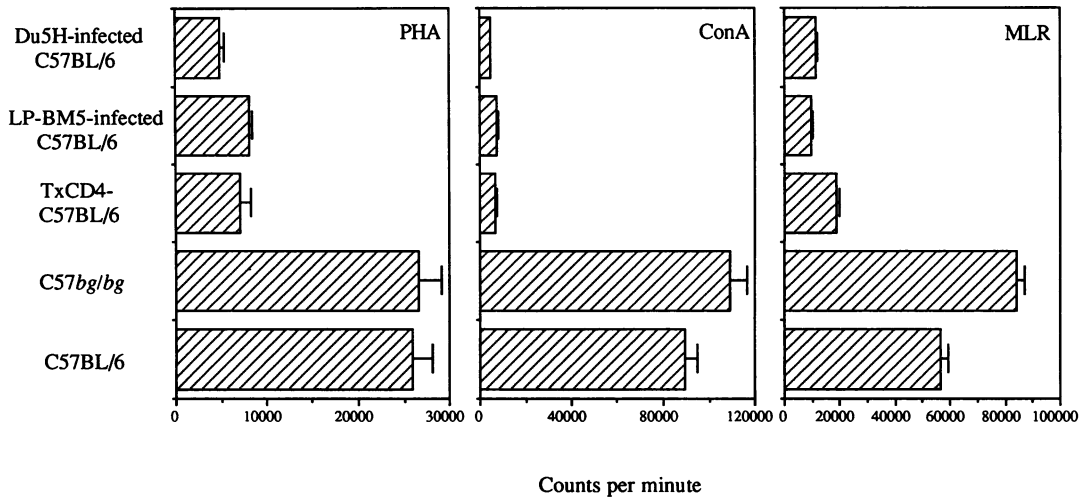

FIG. 2. Evidence that viral infection, or thymectomy and anti-CD4 treatment ($TxCD4^-$), decreased the T-cell response of mice to the T-cell mitogen phytohemagglutinin (PHA; 2.5 μg/ml) or concanavalin A (ConA; 1.25 μg/ml) or to $H\text{-}2^d$ alloantigens in a mixed lymphocyte reaction (MLR). Data are expressed as mean values ± standard errors of the means (n = 3 mice). Background responses of untreated C57BL/6 mice were 3,073 ± 206 (phytohemagglutinin), 3,340 ± 132 (concanavalin A), and 7,920 ± 745 (mixed lymphocyte reaction).

BM5 MAIDS virus, and hence this virus was used in this series of experiments.

Representative results from two experiments are shown in Fig. 4 to 6. In three of four cases, dissemination from the gut to the spleen was observed in all three models. Only strain 2–8 (SmD) failed to escape from the gut of control mice (or, if it did so, was then destroyed). However, all four strains of bacteria were detected in the spleens of virus-infected or $TxCD4^-$ mice (Fig. 4).

Similar patterns of disseminating disease were observed in the lungs, although overall bacterial numbers were relatively low, and their appearance was delayed or absent in normal controls. In the case of strain 2–8, bacteria were observed only in the lungs of mice infected with LP-BM5 virus (Fig. 5).

The bacterial counts in the bone marrow of a representative experiment are shown in Fig. 6. In three of four infections, dissemination of bacteria to the bone marrow was observed in immunodeficient mice; in contrast, no appearance of bacteria was observed in healthy controls. Moreover, no bacteremia was ever observed in control mice, whereas small numbers (1 to 3 log units) of bacteria were seen in mice infected with virus and then with strain 101 or 4-44 (data not shown). In these latter experiments, substantial standard error values were observed (0.3 to 0.6 log unit), indicating substantial variation between animals.

DISCUSSION

The results of this study show that infection of mice with the MAIDS virus Du5H or LP-BM5 or direct depletion of CD4 lymphocytes by i.v. administration of antibody adversely affects T-cell responsiveness in these mice. It was further shown that this T-cell deficiency extended to acquired immunity to infection with *M. avium*, in that systemic infection with this organism was exacerbated, as was dissemination of an enteric implant of these bacteria. Given the similarity of this latter experimental system to events that

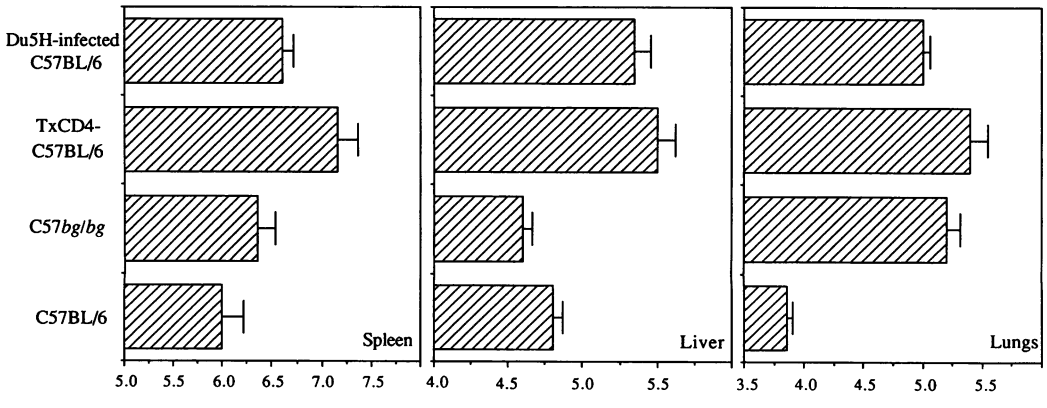

FIG. 3. Evidence that the growth of *M. avium* is increased in virus-infected or $TxCD4^-$ mice. Mice were infected i.v. with 10^4 *M. avium* 2-151 (SmT), and bacterial numbers were assayed 60 days later. Data shown are representative of two experiments and are expressed as means ± standard errors of the means (n = 4 mice).

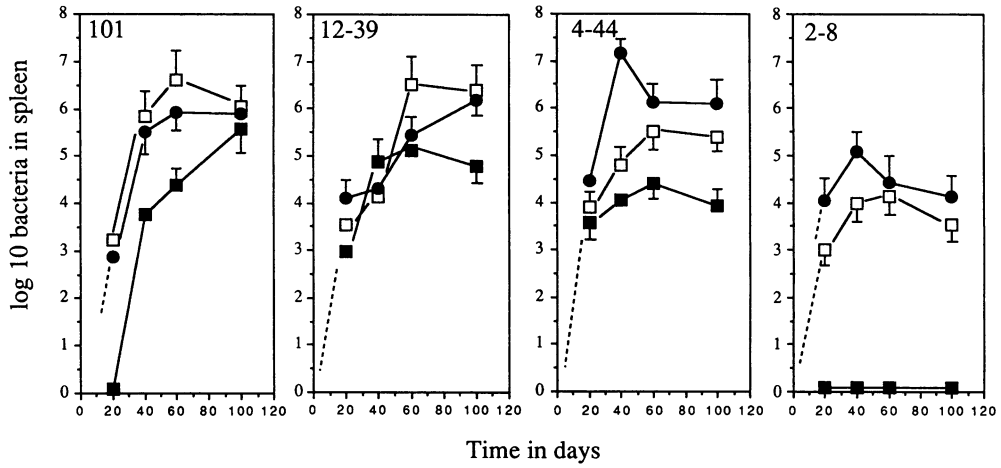

FIG. 4. Dissemination of *M. avium* 101, 12-39, 4-44, and 2-8 to the spleen following enteric inoculation of normal (■), TxCD4$^-$ (□), or LP-BM5-infected (●) mice. Data are expressed as mean numbers of bacteria per target organ (n = 4 mice). Bars indicating standard errors of the means are shown only when values were greater than ±0.2.

may occur in AIDS patients infected in the gut with *M. avium*, we present this system as a potential new experimental model both for evaluation of the immunotherapy and chemotherapy of disseminating *M. avium* disease and for determination of its pathogenesis. The key term in this statement is experimental model, for we concede that it is unlikely that patients are exposed to a bolus of *M. avium* in the manner used in these experiments. In this regard, however, the etiology of *M. avium* infection in AIDS patients remains unknown; there is often major gut involvement, leading to the hypothesis that these tissues are the major portal of infection (37), but recent data suggest that the pulmonary route of infection may also be important (19, 27).

In terms of enteric infection, we believe it unlikely that *M. avium* bacilli free in suspension could survive the acidic pH of the stomach, but it is possible that viable bacilli might escape into the intestinal system if embedded in food particles. Some entry of a few bacilli into the gut tissue or the draining lymph nodes might then occur, and this might be increased if there is also a secondary infection causing local inflammation (or, in the model presented above, the possible inflammatory effects of cecal implantation), resulting in the attraction of host macrophages which could then engulf the mycobacteria. As a result, small numbers of bacilli might then be contained by the formation of granulomas, a mechanism that would be lacking in the immunocompromised host. It is equally possible that dissemination of latent disease might be triggered from such granulomas following the onset of HIV-induced immunodeficiency, akin to that now observed in HIV-positive individuals with active (possibly recrudescent) tuberculosis (33).

Although the above conclusion is largely conjecture, a recent report from this laboratory (21) has shown that HIV-negative nonhomosexual individuals have low but detectable antibody levels to the unique glycopeptidolipid antigens of *M. avium*, suggesting low-level exposure to this organism, whereas these antibody levels are substantially raised both in HIV-positive and HIV-negative homosexual men. There are no concrete data to explain these increased levels, but we have speculated (28) that they may arise from colonization by *M. avium* occurring in concert with other inflammatory infections in the gut. As pointed out above,

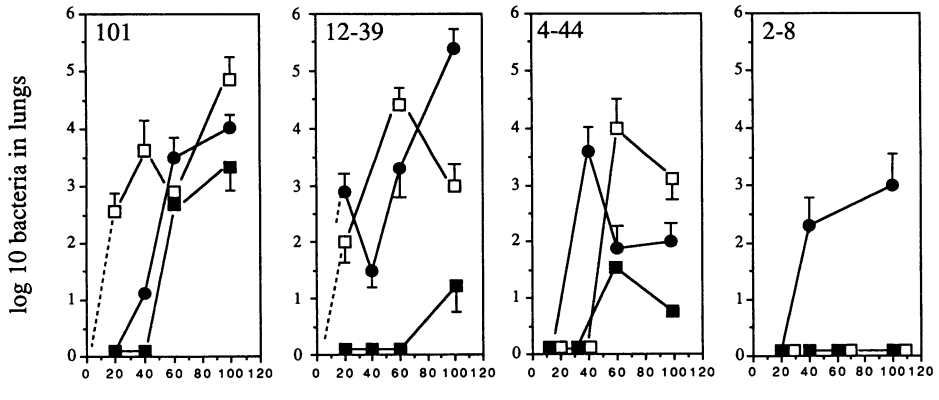

FIG. 5. Dissemination of *M. avium* 101, 12-39, 4-44, and 2-8 to the lungs following enteric inoculation of normal (■), TxCD4$^-$ (□), or LP-BM5-infected (●) mice. Data are expressed as mean numbers of bacteria per target organ (n = 4 mice). Bars indicating standard errors of the means are shown only when values were greater than ±0.2.

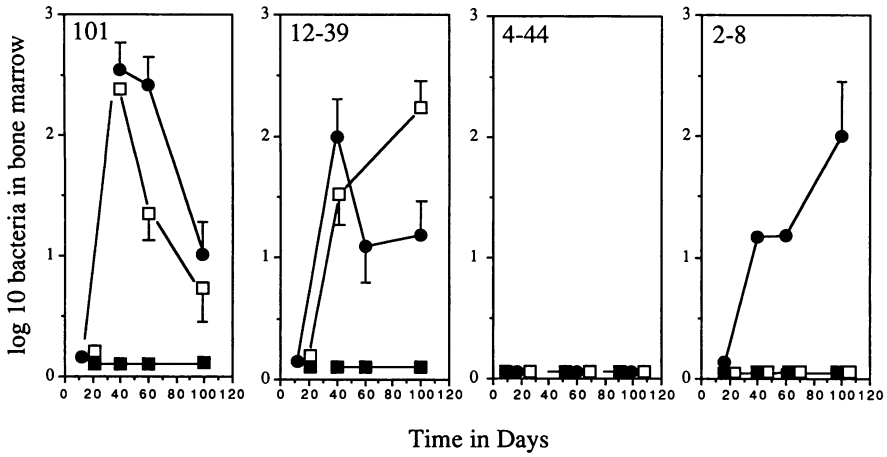

FIG. 6. Dissemination of *M. avium* 101, 12-39, 4-44, and 2-8 to the bone marrow following enteric inoculation of normal (■), TxCD4$^-$ (□), or LP-BM5-infected (●) mice. Data are expressed as mean numbers of bacteria per target organ (n = 4 mice). Bars indicating standard errors of the means are shown only when values were greater than ±0.2.

various protozoa and other gut pathogens were seen in increased incidence in individuals with homosexual lifestyles long before the advent of the HIV epidemic (1, 8, 9).

In the enteric infection mouse model presented above, it was clear from extrapolation of the early time points that only very small numbers of bacteria (approximately 0.01% of the implanted inoculum) were escaping from the gut into the blood. This dissemination, plus the subsequent growth of the infection in target organs, was both accelerated and exacerbated in animals that were rendered T-cell deficient by MAIDS virus infection or by direct depletion of CD4 T cells. These data thus allow us to hypothesize that a direct association exists between the degree of entry and dissemination of enteric *M. avium* infection and the lack of functional CD4$^+$ T cells.

This hypothesis is strongly supported by the findings of Hamilton and colleagues (14), who found that immunodeficiency, resulting in this case from thymic aplasia in nude mice, caused marked exacerbation of intestinal *M. paratuberculosis* infection in comparison with euthymic controls. In this study, substantial enteritis and fecal shedding of bacteria were observed, whereas in our study, only minimal evidence of enteritis was seen (with the exception of intestinal thickening due to lymphoproliferation in virus-infected gut-associated lymphoid tissues). These differences may reflect a better adaptation to gut infection by *M. paratuberculosis* than by *M. avium*. Despite this difference, these reports, taken in concert, clearly indicate that the gut may act as a potential reservoir of active mycobacterial disease in immunocompromised mice.

Moreover, if one accepts the contention that the inoculum size used in these experiments may be several magnitudes higher than events in reality, it is reasonable to speculate that invasion of the gut in normal individuals by *M. avium* may actually be a rare event, occurring with increased frequency only if local surveillance mechanisms are less effective as a result of immune deficiency or are perturbed by local inflammation caused by other gut infections. Indeed, the clinical evidence (6) that indicates that *M. avium* is not present to any significant degree in stool samples from normal individuals would tend to support the idea that the numbers of viable bacilli passing through the lower gut are usually relatively small.

These experiments also provide the first indications that the observed virulence of a given clinical isolate of *M. avium* may not be a primary factor in the pathogenesis of the disease in severely immunocompromised individuals. Various in vitro measures, such as growth in human monocytes or in murine macrophages, suggest that there is substantial variation between isolates (11, 23, 34). In our hands, using a bone marrow-derived macrophage culture system (11), we have determined that strains 101 and 12-39 grow progressively in these cells, whereas strains 4-44 and 2-8 at best persist with no increase in numbers. However, in the present study, all four strains disseminated and grew in MAIDS virus-infected mice.

A further interesting observation concerned the colonial morphology of the strains tested. It is well established that isolates that exist primarily in the SmT colonial type are usually the most virulent, whereas SmD forms are invariably avirulent, both in vivo and in vitro (11, 24, 34), and thus the progressive growth of the SmT strains 101 and 12-39 was not surprising. On the other hand, strain 2-8, which is of the SmD type, also grew and disseminated in the immunodeficient mouse models. This latter finding may therefore suggest that the observed inability of an SmD isolate to grow in macrophages from normal individuals in vitro cannot necessarily always be extrapolated to predict events in severely immunocompromised individuals.

Both the TxCD4$^-$ model and the MAIDS virus model are technically easy to generate and thus may prove useful in future evaluations of new strategies of immunotherapy and chemotherapy of *M. avium* disease. Perhaps most importantly, to our mind, is the factor that both models induce profound T-cell deficiency but not complete T-cell depletion. In this regard, they model the reduced but not totally absent numbers of CD4 cells seen in AIDS patients and hence differ from the *scid* mouse, which possesses no lymphocytes. We would suggest, moreover, that the new models may prove equal to or better than the existing and popular beige mouse model (12) for testing experimental therapies. We do not regard this latter model as particularly useful because although it possesses a defect in oxidative metabolism, the production of superoxide against *M. avium* may be unimportant (3). Also, while the beige mouse is clearly more susceptible to infection in the lungs, this susceptibility is not usually

reflected in other organs and is clearly not a result of T-cell deficiency.

These criticisms may not be trivial. A possible major avenue of future therapy of *M. avium* infections in AIDS may be cytokine administration. To date, however, evaluation of cytokine therapy has essentially been limited to normal mice or the beige mouse. Thus, in both cases, conclusions regarding the efficacy of a given cytokine have been reached on the basis of experiments in which large quantities of these materials have been administered to mice in which T-cell-mediated acquired immunity is presumably already in the process of generation. While positive effects of therapy have then been regarded as adjunctive, it is quite possible that the regulatory effects of high concentrations of a single cytokine that may effect both T-cell and macrophage activation and subsequent behavior may not always occur similarly in immunodeficient animals in which the T-cell axis is dysfunctional.

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