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Demonstration of acquired resistance in *Bcg*^{r} inbred mouse strains infected with a low dose of BCG Montreal

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SUMMARY

The relationship between natural resistance to *Mycobacterium bovis* BCG, expressed by the *Bcg* gene, and the generation of acquired resistance to this infection in various selected inbred strains of mice was investigated. Consistent with previous findings, a low dose ($\sim 10^4$) of BCG Montreal grew progressively in the spleens of inbred mouse strains previously designated susceptible to BCG (*Bcg*^{s}), but grew poorly in resistant strains (*Bcg*^{r}). In contrast, however, little difference was observed in the growth of the organism in the liver or lungs of these mice, whereas furthermore, all animals behaved as *Bcg*^{s} when infected with the World Standard preparation of BCG, BCG Pasteur. Moreover, four strains tested (*Bcg*^{r}; A/J, C3H/HeJ, and *Bcg*^{s}; B10.A/J, BALB/c), all showed evidence of the generation of acquired resistance to a small inoculum of BCG Montreal, as demonstrated by their substantial protection against a subsequent intravenous challenge with virulent *M. tuberculosis*. These findings are interpreted as being inconsistent with the *Bcg* gene hypothesis and call into doubt the usage of the term *Bcg* as a gene designation.

Keywords *Bcg* gene natural resistance acquired resistance

INTRODUCTION

There are a number of established models in the literature which show that certain inbred strains of mice differ in their capacity to resist infection with intracellular bacterial or protozoal parasites. These models include, for example, resistance to infection with *Listeria monocytogenes* (Cheers & Mckenzie, 1978), *Salmonella typhimurium* (Plant & Glynn, 1974) and *Leishmania donovani* (Bradley & Kirkley, 1977). More recently, a series of reports have demonstrated that the resistance of certain inbred strains of mice to infection with BCG Montreal, an attenuated organism derived from *Mycobacterium bovis*, is under the control of a single, dominant, autosomal gene designated *Bcg* (Forget *et al.*, 1981; Gros, Skamene & Forget, 1981). Furthermore, linkage studies using recombinant inbred strains has led to the suggestion that the *Bcg* gene and the genes which control resistance to *S. typhimurium* (*Ity*) and *L. donovani* (*Lsh*) are identical or closely linked (Skamene *et al.*, 1982).

The ability of resistant strains of mice (*Bcg*^{r}) to control low dose infection with BCG Montreal has been ascribed to a natural resistance mechanism. This hypothesis followed from the demonstration of an apparent lack of evidence for acquired cell-mediated immunity within the spleens of infected mice (Pelletier *et al.*, 1982), leading in turn to the conclusion that control of the growth of the infectious organism resulted from natural, rather than acquired, resistance. The purpose of the present study was to test this hypothesis further by examining the resistance of *Bcg*^{r} and *Bcg*^{s} mice, following their inoculation with low doses of BCG Montreal, to a challenge infection

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with virulent *M. tuberculosis*. The results obtained, however, failed to support the hypothesis by showing that both *Bcg*^{r} and *Bcg*^{s} exhibited evidence of acquired resistance to *M. tuberculosis* following low dose BCG Montreal inoculation. Furthermore, a number of other observations were also incompatible with this hypothesis, and call into doubt the use of the term *Bcg* as a gene designation.

MATERIALS AND METHODS

Mice. Specific pathogen free female B6D2 (C57BL/6 × DBA/2) F_1 hybrids, C3H/He, DBA/2, BALB/c and C57BL/6 mice were obtained from the Trudeau Animal Breeding Facility, Saranac Lake, New York. In addition, specific pathogen free C57BL/6J, A/J, B10.A/J and C3H/HeJ female mice were obtained from Jackson Laboratory, Bar Harbor, Maine, USA. All mice weighed 20–22 g and were 6–7 weeks old at the start of the experiments. All animals were housed on sterile bedding, and maintained on sterile mouse chow and water throughout the experiments.

Experimental infections. Mycobacterium bovis (BCG Montreal), *M. bovis* BCG (BCG Pasteur) and *M. tuberculosis* Erdman were obtained from the Trudeau Mycobacterial Collection, Saranac Lake. The bacteria were grown in modified Sauton's medium as previously described (Collins, Wayne & Montalbine, 1974). Mice were infected via a lateral tail vein with dispersed inocula containing indicated numbers (colony forming units) of BCG suspended in 0·2 ml phosphate-buffered saline. Numbers of viable bacteria in the prepared inoculum was determined by plating serial dilutions on 7H10 agar (DIFCO, Detroit, Michigan, USA) as described below.

Experimental design. The course of BCG infection was monitored in the spleen, liver and lungs of inoculated mice by sacrificing groups of five animals 18 h and 7, 14, 28 and 42 days after inoculation. In the case of challenge experiments, various strains of mice were immunized with 1×10^4 BCG Montreal, and challenged intravenously 42 days later with 10^5 viable *M. tuberculosis*. The growth of the challenge organism was subsequently followed in the three target organs by sacrificing groups of five mice 18 h, 10 days, and 20 days after the *M. tuberculosis* challenge.

Enumeration of bacteria. Bacterial growth in target organs was followed against time by plating 10-fold serial dilutions of whole organ homogenates on Middlebrook 7H10 agar. Organs from five mice were each individually homogenized in ice cold sterile saline (0·85 g/100 ml NaCl); two samples were plated from each 10-fold dilution of the homogenate. Bacterial colonies were counted after 14–20 days incubation at 37°C in sealed plastic bags.

In experiments involving challenge infections with *M. tuberculosis*, organ homogenates were plated on 7H10 agar supplemented with 1 μg/ml 2-thiophene-carboxylic acid hydrazide (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) which selectively inhibits the growth of BCG.

Numbers of viable bacteria in a given organ was expressed as the geometric mean value of 10 determinations. Each presented result was representative of at least two experiments.

Statistical method. Comparisons were analysed by the Student's *t*-test for unpaired data.

RESULTS

Growth of BCG Pasteur in spleens of Bcg^r *and* Bcg^s *mouse strains*

In the initial series of experiments which led to the description of the *Bcg* gene (Forget *et al.*, 1981), it was found that expression of the *Bcg* gene became less distinctive as the infecting dose of BCG increased above $1{\cdot}55 \times 10^5$. In view of this knowledge, initial experiments in the present study were performed using doses of BCG ranging from 10-fold above this critical value (10^6) to doses 10-fold below (10^4). In these experiments groups of inbred strains of mice previously designated (Forget *et al.*, 1981) susceptible to BCG (*Bcg*^{s}; C57BL/6, BALB/c) or resistant to BCG (*Bcg*^{r}; C3H/He, DBA/2), and a hybrid (*Bcg*^{r}; B6D2 F_1) were infected with various doses of the World Standard strain of BCG (BCG Pasteur) and the course of the infection in the spleens of these animals subsequently followed with time.

The results obtained in these experiments (Fig. 1) show that the organism initially grew progressively in the spleens of all groups of mice regardless of the size of the infecting dose, and

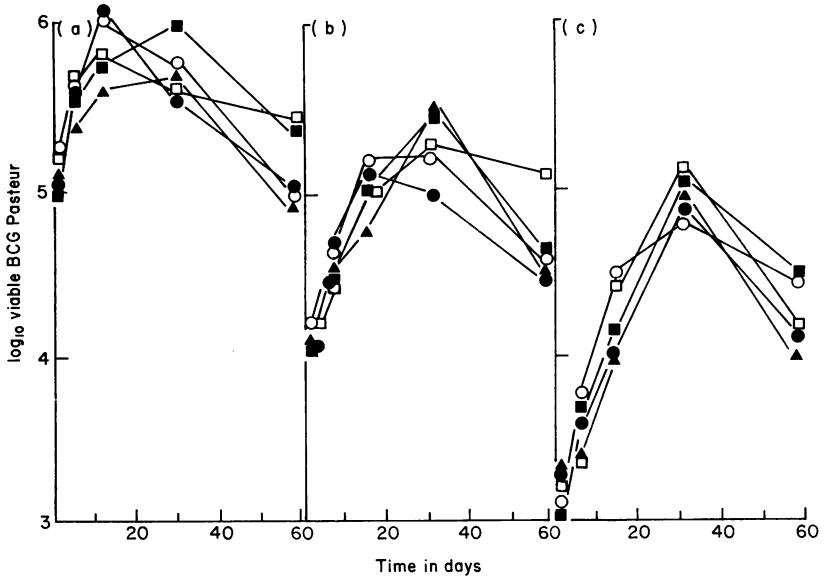

Fig. 1. Failure to find evidence of *Bcg* gene expression in the spleens of various inbred strains of mice inoculated with a range of doses of BCG Pasteur. Two strains of mice previously designated (see text) *Bcg*^{r} (● = C3/He and ○ = DBA/2), two designated *Bcg*^{s} (■ = C57BL/6 and □ = BALB/c) and a F_1 hybrid (▲ = B6D2, by definition *Bcg*^{r}) were inoculated intravenously with (a) $1{\cdot}8 \times 10^6$; (b) $1{\cdot}1 \times 10^5$ or (c) $1{\cdot}6 \times 10^4$ viable BCG. Data is expressed as the geometric mean of 10 determinations; for the sake of clarity standard error bars are omitted, in all cases these ranged from 0·05 to 0·22.

hence, following previous criteria (Forget *et al.*, 1981; Gros *et al.*, 1981) should be typed as *Bcg*^{s}. Furthermore, it was apparent that the cessation of progressive growth of the infection occurred at increasingly later time points as the size of the initial infectious inoculum decreased. For example, in mice given 10^6 BCG, the growth of the infection was curtailed in four out of five groups by day 14, whereas in mice given 10^5 BCG this occurred between day 14 and 28, and after day 28 in mice given 10^4 BCG. These observations are consistent with, and considerably expand, the original observations of Lefford (1970).

Comparison of the growth of BCG Pasteur and BCG Montreal infections in inbred mouse strains

Because of our failure, described in the previous section, to find evidence of *Bcg* gene expression in two previously designated *Bcg*^{r} inbred mouse strains, further experiments were performed using both the BCG Pasteur strain, and the strain of BCG (BCG Montreal) used in the initial reports (Forget *et al.*, 1981; Gros *et al.*, 1981). Selected inbred strains of mice (obtained from the same original source as these previous reports) were infected with 10^4 viable BCG (10-fold below the critical dose; Forget *et al.*, 1981) and the course of the infection followed in the spleen, liver and lungs over a period of 42 days.

The results obtained in these experiments (Fig. 2) were compatible with earlier reports (Gros *et al.*, 1981) in that they show that the BCG Montreal organism grew poorly in the spleens of resistant mouse strains (*Bcg*^{r}; C3H/HeJ, A/J) but progressively in susceptible strains (*Bcg*^{r}; B10.A/J, C57BL/6J); in contrast however, BCG Pasteur grew progressively in the spleens of all mouse strains. Moreover, despite a clear difference in growth in the spleens of BCG Montreal infected *Bcg*^{r} and *Bcg*^{s} mouse strains, little difference was observed in the growth of this organism in either the liver or the lungs. In these organs, furthermore, there was evidence that the growth of the infection was curtailed in both *Bcg*^{s} and *Bcg*^{r} mouse strains, an observation inconsistent with the finding elsewhere the *Bcg*^{r} mice do not express acquired immunity when infected with low doses ($< 1 \times 10^5$) of BCG Montreal (Pelletier *et al.*, 1982).

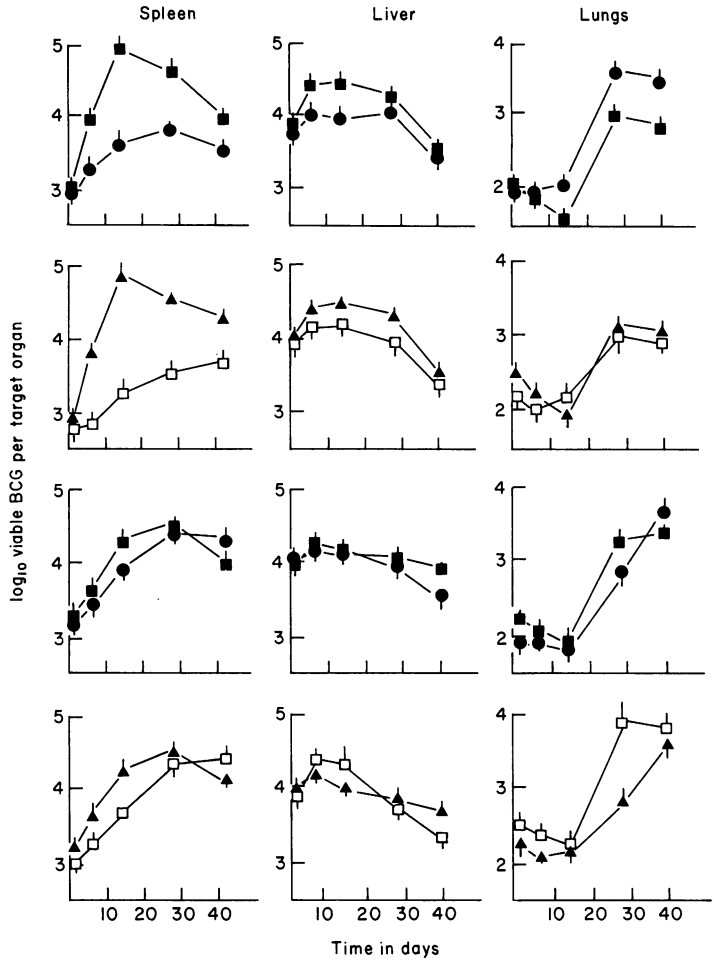

Fig. 2. *Bcg* gene activity can be inferred from the growth of BCG Montreal in the spleens, but not in the livers or lungs, of inbred strains of mice. Mice were intravenously inoculated with $1{\cdot}2 \times 10^4$ viable BCG Montreal (top two panels) or with $1{\cdot}1 \times 10^4$ viable BCG Pasteur (bottom two panels). Two strains of mice previously designated Bcg^r (● = C3H/HeJ and □ = A/J) and two Bcg^s strains (■ = C57BL/6J and ▲ = B10.A/J) were tested. Statistical differences in the growth of BCG Montreal in the spleens of Bcg^r and Bcg^s first became apparent on day 7 of the infection ($P < 0{\cdot}01$); in the case of infection in the liver, day 14 values in Bcg^r mice were significantly lower ($P < 0{\cdot}05$) but in both cases completely overlapped in terms of their 95% confidence limits (mean ± 2 s.d.). Data expressed as mean ± s.e. ($n = 10$).

Demonstration of acquired immunity in Bcg^r *mice infected with a low dose of BCG Montreal*

In order to test the hypothesis (Pelletier *et al.*, 1982) that Bcg^r mice inoculated with a low dose ($< 10^5$) of BCG Montreal did not subsequently generate acquired immunity to immunizing infection, inoculated mice and groups of normal controls were challenged intravenously with *M. tuberculosis*, and the ability of immunized and non-immunized mice to resist this tuberculous challenge subsequently compared in the three target organs (Fig. 3). The results obtained show that both Bcg^r and Bcg^s strains of mice were highly resistant to subsequent *M. tuberculosis* challenge following their inoculation with 10^4 BCG Montreal and that this resistance was strongly expressed in all three target organs monitored. This finding is consistent with the hypothesis, therefore, the Bcg^r inbred strains of mice generate acquired immunity when immunized with 10^4 BCG Montreal.

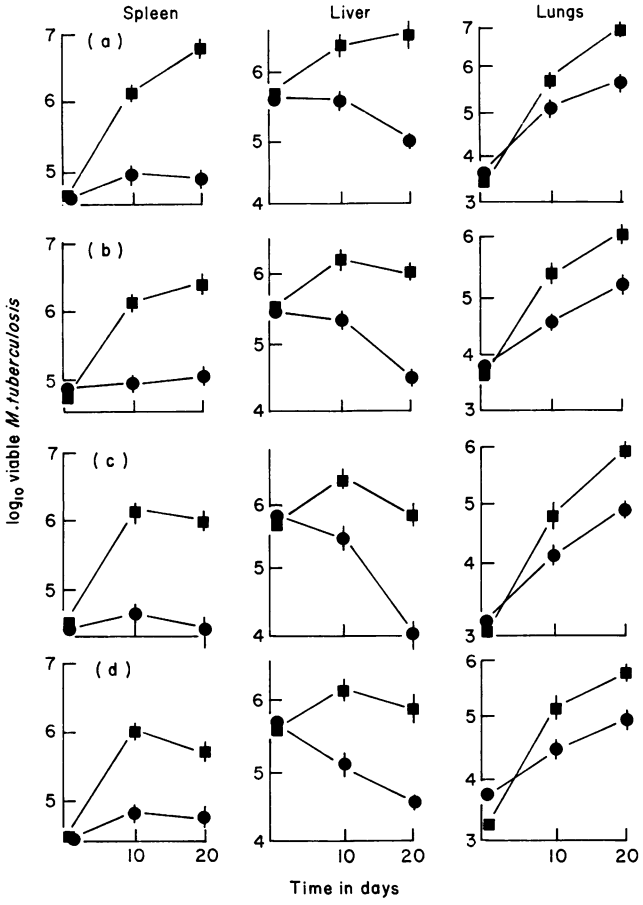

Fig. 3. Demonstration of acquired resistance to *M. tuberculosis* in both *Bcg*^{s} and *Bcg*^{r} inbred strains of mice inoculated with BCG Montreal. Six weeks after intravenous inoculation with $1{\cdot}3 \times 10^4$ BCG Montreal, inoculated mice (●——●) plus normal controls (■——■) were challenged intravenously with $4{\cdot}2 \times 10^5$ *M. tuberculosis* Erdman. Strains tested were C3H/HeJ (a; *Bcg*^{r}), BALB/c (b; *Bcg*^{s}), B10.A/J (c; *Bcg*^{s}) and A/J (d; *Bcg*^{r}). All animals exhibited significant anti-tuberculous resistance compared to unimmunized controls by day 10 of the *M. tuberculosis* challenge infection ($P < 0{\cdot}01$). Data expressed as mean ± s.e. ($n = 10$).

DISCUSSION

This paper shows that despite the apparent inability of BCG Montreal to grow progressively in the spleens of inbred strains of mice previously designated (Forget *et al.*, 1981; Gros *et al.*, 1981) as expressing the resistant allele of the *Bcg* gene (*Bcg*^{r}), such animals nevertheless responded to low dose infection with this organism by the generation of an acquired immune response, as evidenced by the capacity of such mice to express substantial acquired resistance to a subsequent challenge with virulent *M. tuberculosis*. The findings of the present study are therefore incompatible with previous findings (Pelletier *et al.*, 1982) which proposed the hypothesis that because of the failure to find evidence of demonstrable acquired immunity in *Bcg*^{r} mice following inoculation with 10^4 BCG Montreal, the inability of the organism to grow progressively in the spleen was due to a mechanism of natural rather than acquired resistance, under the control of a single, dominant gene designated *Bcg*.

The present study also raised the question of the practicality of using the designation *Bcg* as a gene description in view of the failure of these experiments to demonstrate any differences in the response of a number of inbred mouse strains to the much more commonly used BCG preparation, BCG Pasteur. The finding that there was no difference between Bcg^r and Bcg^s mice in terms of the increase in numbers of viable BCG Pasteur organisms in the spleen following low dose inoculation, leads us to speculate whether the phenomenon of 'BCG-resistant' mice is limited to BCG Montreal infection alone.

The finding that BCG Montreal was able to grow in the liver and lungs of Bcg^r mice is also incompatible with the hypothesis of natural resistance expressed by a *Bcg* gene. It would be surprising, in the light of present knowledge, to find that *Bcg* gene activity was expressed by resident macrophages in the spleen, but not by such cells in the liver or lungs, despite the central role of both resident and circulating mononuclear phagocytes in the primary defense to mycobacterial infections. The role played by these cells in mechanisms of genetic resistance to infection is clearly a subject of fundamental importance and in this regard it is singularly important to distinguish between native resistance and acquired resistance to intracellular bacteria in the infected host. Clearly, a bacteriostatic or bactericidal mechanism possessed by host phagocytic cells would constitute a purely native resistance mechanism. On the other hand, however, in the case of intracellular bacterial parasites the intracellular environment must provide an appropriate milieu to allow the organism to proliferate in an unrestricted manner; one explanation to account for the apparent inability of BCG Montreal to grow in the spleens of C3H/HeJ and A/J mice might be the absence of an intrinsic physiological requirement within spleen macrophages which is required for the growth of this particular organism, rather than an expression of innate natural resistance. Whatever the reason however, interpretation of mechanisms which are ascribed to natural or native resistance is only possible following a clear demonstration of the absence of acquired immunological resistance to the infecting agent. It was apparent in the present study that the decline in numbers of the BCG organism in the livers of Bcg^r mice, and the cessation of progressive proliferation in the lungs, was consistent with the interpretation that these animals had generated an acquired cell-mediated immune response to BCG Montreal. Subsequent challenge of these animals with *M. tuberculosis* Erdman confirmed this interpretation, by showing that these mice possessed the capacity to express substantial acquired resistance to this organism. Such findings are therefore consistent with the hypothesis that mice designated Bcg^r are able to generate acquired immunity, (presumably in other host lymphoid tissues because of the lack of growth of the organism in the spleen) when inoculated with small doses of BCG Montreal. Circulating T lymphocytes which were then acquired as a result of this immunizing infection presumably resulted in the ability of the host to express anti-tuberculous resistance in all target organs, follwing subsequent challenge with *M. tuberculosis*.

The above findings would, at first sight, appear to be inconsistent with the previous findings of Pelletier *et al.* (1982), who found that Bcg^r mice showed no evidence of a secondary response in the spleen following reinfection with a challenge inoculum of BCG Montreal, and no protection against a subsequent challenge with *L. monocytogenes*. However, we feel that the approach used by Pelletier *et al.* to test for the absence or presence of acquired immunity in Bcg^r mice is open to criticism on the following grounds. The A/J strain of mouse, it can be argued, is 'resistant' to BCG Montreal in the sense that low dose inocula ($\sim 10^4$) grow poorly or not at all in the spleens of these animals. To test for the presence or absence of acquired immunity Pelletier *et al.* (1982) rechallenged infected A/J mice with $2{\cdot}5 \times 10^4$ BCG Montreal and subsequently found no difference in the numbers of BCG in the spleens of the infected and control groups of A/J mice. However, this type of resistance assay has no valid basis unless the challenge organism is either rapidly or demonstrably killed in the reinfected animal (which in the present system would by necessity require the use of a drug resistant BCG organism; Orme & Collins, 1983a) or alternatively grows sufficiently in the control group to the extent that the resistance of immune animals to the infection can be measured; since the BCG Montreal challenge inoculum size used was of the magnitude that does not grow progressively in the spleens of A/J mice, then it is difficult to follow how this approach could be used to determine the presence or absence of acquired immunity in this strain of mice.

In this regard, although no direct formal proof is presently available, there is considerable

circumstantial evidence which suggests that the emergence of acquired protective cell-mediated immunity is temporally associated with the active metabolic phase of progressive growing mycobacteria (Orme & Collins, 1983b) and hence, if correct, then such a hypothesis would predict that protective (effector) T cell activity would not be expressed in the spleens of A/J mice infected with very low doses of BCG Montreal. It would therefore follow, that the presence of acquired immunity in these animals could only be tested if an heterologous, but cross-reacting, organism capable of progressive growth in the spleens of *Brg*^{r} mice was used as the challenge infection. To achieve this, the present study has used a virulent strain of *M. tuberculosis* as a challenge infection, which subsequently confirmed the presence of acquired immunity in both *Bcg*^{r} strains of mice tested.

The findings of the present study, furthermore, illustrate the need to consider the response of the whole animal to such infections. The findings of others (Forget *et al.*, 1981; Gros *et al.*, 1981), leading to the designation of *Bcg*^{r} and *Bcg*^{s} mouse strains based on the growth of BCG Montreal in the spleen are clearly correct and reproduced in the present study. However, the growth of the organism in other target organs resulting in the emergence of acquired immunity are not compatible with these resistance gene designations. A further illustration of this need to consider other organs has been provided elsewhere (Ho & Cheers, 1982) in which studies directed towards the basis of genetic control to intracellular bacteria have shown that certain inbred strains of mice differed significantly in their capacity to resolve *Brucella abortus* infections in the spleen whereas, in contrast, the clearance of bacteria from the liver was relatively efficient in all strains tested; such findings provide a clear analogy to the results of the present study.

Although single gene control has been suggested for a number of intracellular bacterial parasites (Skamene *et al.*, 1982), it is probably more likely that a number of genes play a role in the control of such infections; it is clear that a similar conclusion is emerging with regard to present knowledge of the control of *S. typhimurium* infections (Hormaeche, 1979). In the present case of infection with small doses of BCG Montreal however, the results of this study did not permit eludication of the activities of the genes expressing natural resistance to BCG in view of the overriding presence of acquired resistance to this organism. Furthermore, the observation that BCG Montreal was able to grow in organs other than the spleen of *Bcg*^{r} mice is inconsistent with the possibility of control by a single resistance gene, and hence suggests that other parameters, as yet undefined, play a role in this phenomenon.

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