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Immune responsiveness in mice heavily infected with *Mycobacterium kansasii*

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Summary. Growth of *Mycobacterium kansasii* TMC 1203 in B6D2 F_1 hybrid mice was associated with increased splenic cellular proliferation, hyperplasia and the generation of non-specific antibacterial resistance. Both responses were dose dependent; the larger the inoculum, the more rapid and extensive the cellular response. However, such mice were still unable to reduce the mycobacterial load within the tissues, apparently because of their inherent resistance to inactivation by immunologically activated macrophages. On the other hand, mice infected with the non-persistent strain of *M. kansasii* 1214 exhibited only a transient increase in non-specific (anti-*listeria*) resistance which rapidly declined as the number of viable mycobacteria within the spleen fell below an arbitrary threshold level. Mice infected with either *M. kansasii* 1203 or 1214 could be immunized with sheep red blood cells (SRBCs), an unrelated T cell-dependent antigen. The humoral (PFC) response was not affected by the mycobacterial load within the spleen. However, the delayed footpad swelling reaction was severely depressed. The latter could be restored merely by increasing the size of the intravenous sensitizing inoculum 100-fold. The present study indicates that mice chronically infected with *M. kansasii* are not severely immunosuppressed (as had been inferred from earlier *in vitro* lymphoproliferation studies) but are fully capable of responding to appropriate *in vivo* stimuli.

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INTRODUCTION

Mycobacterium kansasii induces the formation of chronic lung infections both in experimentally infected mice (Collins, 1971) and in man (Wolinsky, 1979). However, not all strains of *M. kansasii* behave identically when introduced intravenously into normal mice (Collins & Cunningham, 1981). Both *M. kansasii* strains 1201 and 1203 multiply briefly in the spleen and lung, and this is followed by a prolonged plateau in which the growth curve persists unaltered, virtually for the remainder of the lifetime of the host (Collins, Morrison & Montalbine, 1978). On the other hand, both strains 1204 and 1214 are rapidly eliminated from the lungs and spleen and fail to establish persistent infections, even in very heavily infected mice (Cunningham & Collins, 1981). This striking difference in growth behaviour was ascribed to the development of a population of suppressor cells within the *M. kansasii* 1203-infected spleen which, somehow, inhibited the expression of antibacterial immunity by the anergic host, leading to the establishment of the chronic infection. This conclusion appeared consistent with the lack of suppressor cell activity in the *M. kansasii* 1214-infected spleen, as this inoculum was rapidly eliminated from the tissues (Cunningham & Collins, 1981). Implication of such an immunosuppressed state existing in these mice had been based solely on the result of *in vitro* lymphoproliferation assays following mitogenic or alloantigenic stimulation of spleen cells (Collins & Cunningham, 1981).

The present study examines the *in vivo* immune

responsiveness of mice that have been heavily infected with these two contrasting strains of *M. kansasii* (1203 and 1214). Contrary to previous studies, no evidence of immunosuppression was observed in terms of the *in vivo* responsiveness of such mice to a variety of antigenic stimuli, regardless of the growth behaviour of the mycobacterial population within the host tissues.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL-6 × DBA/2 (B6D2) F_1 hybrid mice were obtained from the Trudeau Animal Breeding Facility. Female mice, 6–8 weeks of age at the time of inoculation, were used throughout. They were fed sterilized commercial mouse chow and acidified water *ad libitum*.

Organisms

M. kansasii (TMC 1203 and 1214) and *Mycobacterium bovis* (BCG Pasteur, TMC 1011) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, NY. An acriflavine-resistant (A^R) variant of *Mycobacterium tuberculosis* Erdman (TMC 107), able to grow in the presence of 100 μg/ml acriflavin (J.T. Baker Chemical Co., Phillipsburg, NJ) per ml of Middlebrook 7H10 agar (Difco, Detroit, MI) was isolated as described elsewhere (Collins, Auclair & Mackaness, 1977). Such a concentration of acriflavine effectively inhibited the growth of the parent strain. The organisms were grown in 1 litre roller bottles containing modified Sauton's liquid medium (MSTA) which was incubated at 37° until the culture reached the late logarithmic growth phase (Collins, Wayne & Montalbine, 1974). The cultures were stored in 1 ml ampoules held at −70° until required. Challenge inocula were prepared by brief homogenization of the rapidly thawed suspension, followed by dilution in saline to the desired viable concentration. Viability of each inoculum was checked by plating suitable saline dilutions onto Middlebrook 7H10 agar. The plates were incubated at 37° in sealed plastic bags for 3 weeks before counting.

Listeria monocytogenes strain EGD (Mackaness, 1969) was grown at 37° in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) and harvested in the late logarithmic phase before being frozen at −70° in 1 ml aliquots. Inocula were prepared by appropriate dilution of the thawed suspension in sterile saline. The viability of the inoculum was checked by plating on Trypticase soy agar (TSA); this was incubated at 37° and the colonies were counted on the following day.

Determination of the in vivo *bacterial population*

The spleens and lungs were removed aseptically from five randomly selected mice and homogenized separately in cold saline (Collins, 1971). Viable bacteria were counted by plating 10-fold serial saline dilutions of the organ homogenates on Middlebrook 7H10 (for mycobacteria) or TSA (for *L. monocytogenes*). In rechallenge experiments, the Middlebrook 7H10 agar was supplemented with 100 μg/ml acriflavine to inhibit the growth of the primary *M. kansasii* 1203 or BCG in the doubly-infected animals (Collins *et al.*, 1977).

Spleen cell proliferation and hyperplasia

Mice were injected i.v. with 20 μCi tritiated thymidine ([^{3}H]TdR; 5 Ci/mmol; Radiochemical Centre, Amersham, U.K.). Thirty minutes later, the spleens were removed and single cell suspensions prepared. Hyperplasia was assessed by counting the number of nucleated cells per spleen after red blood cell lysis. Aliquots of the labelled cells were collected on a filter pad using a Mash II cell harvester (Microbiological Associates, Bethesda, MD). The radioactivity was determined with a Beckman liquid scintillation counter.

Resistance to secondary challenge

M. kansasii-infected mice were challenged i.v. with 5×10^4 CFU *L. monocytogenes* at intervals throughout the infection. Non-specific resistance was expressed in the form of a resistance index which was the $\log_{10}$ difference in the number of viable *Listeria* in the livers and spleens of the *M. kansasii*-infected mice 48 hr after challenge, compared with those for the age-matched non-*M. kansasii*-infected controls. Other *M. kansasii*-infected mice were challenged i.v. with *M. tuberculosis* Erdman A^R (5×10^5 CFU). In this case, the RI was based on the difference between the 14-day splenic counts for *M. tuberculosis* in the test and control groups of mice. All counts used in the calculation of these resistance indices were based on the mean of five mice/time point.

Resistance of M. kansasii *to activated macrophages*

Mice were infected i.v. with 10^8 CFU BCG and, 14 days post-inoculation, they were challenged i.v. with 10^6 CFU *M. kansasii* 1203. (This time had been determined to produce peak non-specific resistance to

a *Listeria* challenge). Growth of the *M. kansasii* 1203 was followed for 14 days.

Delayed footpad swelling and spleen plaque-forming cell (PFC) responses to sheep red blood cells (SRBCs)

Groups of *M. kansasii*-infected and age-matched uninfected control mice were injected via a lateral tail vein with 10^5–10^9 SRBCs (Colorado Serum Co., Denver, CO) suspended in 0·2 ml saline. Four days later, the mice were footpad tested with 1×10^8 SRBCs suspended in 20 μl saline (Miller, Mackaness & Lagrange, 1973). An equal volume of saline was injected into the contralateral footpad. Increases in footpad thickness were measured with a Schnelltaster dial gauge calipers after 3 hr and 24 hr (Collins, 1972).

The PFC (humoral) response in mice injected with $5–10 \times 10^8$ SRBCs were compared in the *M. kansasii*-infected and uninfected control mice by removing the spleen 5 days after sensitization and determining the direct PFC count as described by Cunningham & Szenberg, 1968.

Statistical analysis

The differences between the group means for five determinations were evaluated by Student's *t*-test (Steel & Torrie, 1960). Differences were considered to be statistically significant at the 1% level.

RESULTS

Growth of *M. kansasii* in the spleens of B6D2 mice

Intravenous injection of groups of normal B6D2 mice with increasing numbers of viable *M. kansasii* 1203 produced the growth curves shown in Fig. 1. The number of viable *M. kansasii* within the spleen increased 5- to 10-fold over the first 7 days, after which the growth curves reached a stable plateau which lasted for at least 3 months. As the inoculum size was increased from 10^4 to 10^8 CFU, so the plateau phase appeared at a progressively earlier time. In contrast, the number of *M. kansasii* 1214 recovered from the spleen showed no early increase, but dropped in viability from the time of injection (Fig. 1). By day 30, more than 99% of the original inoculum had been killed and, although the rate of decline slowed somewhat after this time, the bacterial population within the spleen never produced a persistent plateau.

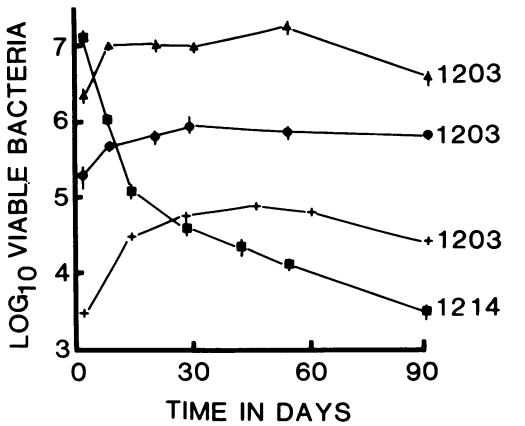

Figure 1. Viable *M. kansasii* present in the spleens of B6D2 mice at various times following intravenous inoculation with 10^4 CFU *M. kansasii* 1203 (+), 10^6 CFU 1203 (●), 10^7–10^8 CFU 1203 (▲), or 10^8 CFU 1214 (■). Each time point represents the mean ± SEM for five mice.

In vivo spleen cell proliferation and organ hyperplasia in response to the *M. kansasii* infection

The plateau phase of the growth curves for *M. kansasii* 1203 coincided with a sharp increase in cellular proliferation within the spleen, and this was shown by the rise in [^{3}H]TdR incorporation by cells within the

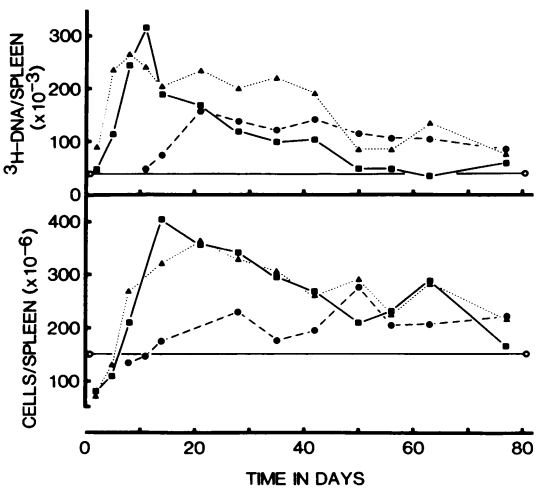

Figure 2. Cell proliferation and hyperplasia in the spleens of B6D2 mice at various times post-inoculation with 10^6 CFU *M. kansasii* 1203 (●), 10^7–10^8 CFU *M. kansasii* 1203 (■), or 10^8 CFU *M. kansasii* 1214 (▲). The values for uninfected control mice (○) are depicted as a mean for all the uninfected animals.

organ. This could also be correlated with an increased number of nucleated cells within the infected spleen (Fig. 2). Mice infected with 10^7–10^8 CFU of either *M. kansasii* 1203 or 1214 both showed increased [^{3}H]TdR incorporation, peaking between days 7 and 10 of the infection. As the infection progressed, this increased level of [^{3}H]TdR incorporation (and the number of nucleated cells) both declined, returning to near control levels by 60–90 days. When the mice were infected with only 10^5–10^6 CFU *M. kansasii*, the [^{3}H]DNA levels increased modestly and did not peak until 3 weeks into the infection, after which they declined again slowly. This was reflected by marginal increases in the number of nucleated cells found within the spleen (Fig. 2).

Non-specific resistance to challenge with *L. monocytogenes*

Acquired resistance to intracellular parasites such as *L. monocytogenes* and *M. tuberculosis* can be correlated with the generation of a T cell-mediated response within the infected spleen (North, 1973). At this time, it is not known whether *M. kansasii* induces an analogous T cell-mediated immune response. If such a response does occur, the activated macrophages within the spleen should express an enhanced antibacterial response against a *Listeria* challenge (Mackaness, 1964). To determine the level of antibacterial resistance within the *M. kansasii*-infected spleen, mice were superinfected with a lethal dose of *L. monocytogenes* (10^4 CFU in 0·2 ml saline, injected i.v.).

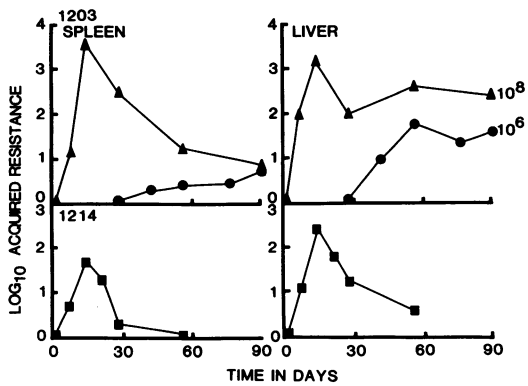

Figure 3. Acquired resistance to *L. monocytogenes* in the spleens and livers of B6D2 mice at various times post-inoculation with 10^6 CFU *M. kansasii* 1203 (●), 10^8 CFU *M. kansasii* 1203 (▲), or 10^7–10^8 CFU *M. kansasii* 1214 (■).

Enhanced killing of the secondary challenge inoculum could first be detected by day 7 (peaking on day 14). Macrophage activation occurred in mice inoculated with a large dose (10^7–10^8 CFU) of either *M. kansasii* 1203 or 1214 (Fig. 3). Later, this level of non-specific resistance declined again to non-significant levels in the *M. kansasii* 1214-infected mice, whereas it remained significantly elevated throughout the experiment in the 1203 mice. Even after 90 days, this resistance index was high enough to be significant ($P<0·01$). In mice receiving the lower dose of *M. kansasii* 1203, *Listeria* inactivation occurred later and was generally of a lower magnitude than that seen in the more heavily infected animals. The resistance indices for the liver peaked somewhat earlier (about day 60 post-inoculation).

Resistance to a virulent tuberculosis challenge

Some mice infected with *M. kansasii* were later challenged with virulent *M. tuberculosis* A^R. This type of challenge organism is highly resistant to normal tissue macrophages and, thus, should provide a better indicator of the presence of antituberculous activity in the *M. kansasii*-activated macrophages. The resistance index curve shown in Fig. 4 for the *M. tuberculosis*

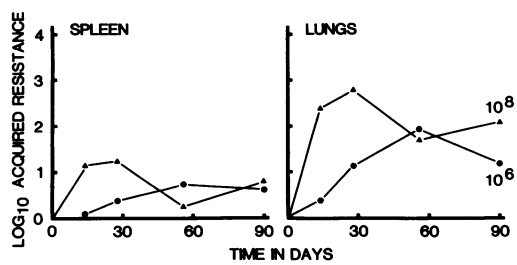

Figure 4. Acquired resistance to *M. tuberculosis* in the spleens and lungs of B6D2 mice at various times post-inoculation with 10^6 (●) or 10^8 CFU (▲) *M. kansasii* 1203.

challenge followed a similar course to that observed earlier for *L. monocytogenes* Fig. 3). Mice inoculated with 10^7–10^8 CFU of *M. kansasii* 1203 reduced the growth of the virulent tubercle bacilli within the spleen 10-fold or more 14 days into the primary (*M. kansasii*) infection. Even when smaller numbers of *M. kansasii* were used, increased antituberculous resistance was expressed by day 56 (Fig. 4).

Table 1. Immune response to sheep red blood cells in mice infected with *M. kansasii* 1203

Days post infection	Inoculum	Direct PFC/spleen*	Direct PFC/10^6 spleen cells	Average increase in footpad thickness†
0	Nil	64,500 ± 12,000	795 ± 140	12·2 ± 0·7
14	Nil	64,000 ± 3090	780 ± 120	11·0 ± 0·8
	10^6	57,680 ± 11,000	575 ± 130	14·6 ± 0·8
	10^8	59,600 ± 15,400	926 ± 170	1·8 ± 0·2‡
28	Nil	98,000 ± 12,150	754 ± 130	13·8 ± 0·5
	10^6	193,600 ± 21,000‡	1498 ± 330	14·6 ± 1·3
	10^8	224,750 ± 6316‡	1196 ± 235	2·0 ± 0·3‡
56	Nil	42,320 ± 3777	555 ± 131	14·6 ± 0·7
	10^6	74,000 ± 15,750	536 ± 128	12·6 ± 1·1
	10^8	117,600 ± 13,750‡	818 ± 303	5·0 ± 0·9‡
90	Nil	53,800 ± 5300	875 ± 111	12·4 ± 1·3
	10^6	119,000 ± 33,860	1117 ± 370	13·8 ± 1·3
	10^8	119,800 ± 30,442‡	840 ± 180	1·8 ± 0·7‡

* Mean ± SEM for five determinations.
† Mean of Schnelltaster units (10 units = 1 mm) ± SEM for five mice.
‡ $P < 0·01$ versus normal control.

Humoral and cellular responses to SRBCs in the *M. kansasii*-infected mice

Intravenous injection with 5×10^8 SRBCs induces a primarily humoral (PFC) response in normal mice but, as the sensitizing inoculum is reduced, so an increasing cellular response develops with little or no antibody formation (Lagrange, Mackaness & Miller, 1974). In the present study, the antibody response to an inoculum of 5×10^8 produced a similar PFC response per 10^6 spleen cells, regardless of whether the mice had been infected with 10^6 or 10^8 CFU *M. kansasii* 1203 (Table 1). However, when the response was calculated on a per spleen basis, the PFC values were found to be substantially increased in the 10^8 *M. Kansasii*-infected mice, reflecting the hyperplasia (splenomegaly) observed in these animals (Fig. 2).

Mice inoculated 14 days earlier with either 10^8 *M. kansasii* 1203 or 1214 developed little or no footpad swelling following their sensitization with 10^5 SRBCs (Table 1). In the *M. kansasii* 1214 infected mice, much of this responsiveness returned by day 28 (data not shown); this was in sharp contrast to the mice infected with 10^8 CFU *M. kansasii* 1203 which remained unresponsive to the footpad SRBC for the remainder of the experiment. Mice inoculated with smaller inocula of *M. kansasii* 1203 (10^5–10^6 CFU) showed no such depression in their SRBC–delayed footpad swelling responsiveness.

Since the heavily-infected spleen was known to contain large numbers of activated macrophages (Fig. 3), it was possible that this lack of delayed footpad reactivity (Table 1) represented an increased destruction of the small (10^5) sensitizing dose of SRBCs reaching the spleen. This, in effect, reduced the antigenic stimulus to subsensitizing levels. By increasing the size of the intravenous sensitizing inoculum to 10^7 or 10^8 SRBCs, the amount of sensitin reaching the T cells in the spleen was increased and this restored the footpad reactivity. The dose-response curves shown in Fig. 5 indicate that when the 14-day infected mice (the time of maximum macrophage activation in Fig. 3) were primed with a normally optimal sensitizing dose (10^5 SRBCs), little or no 24 hr footpad swelling was observed in either the *M. kansasii* 1203- 1214-infected mice (Fig. 5). On the other hand, a substantial level of footpad reactivity developed in these same mice when the sensitizing inoculum of SRBCs was increased 100-fold, to a level which was supra-optimal for normal control mice.

Resistance of *M. kansasii* to killing by immunologically activated macrophages

Despite the fact that the splenic macrophages from *M. kansasii*-infected mice exhibited substantial listericidal and tuberculocidal activity, the primary *M. kansasii*

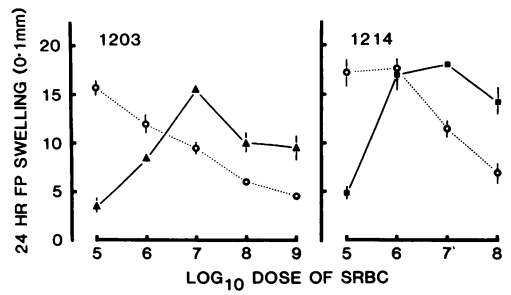

Figure 5. Effect of increasing the sensitizing dosage of SRBCs on the DTH response in normal mice (○) and mice inoculated 14 days earlier with either 10^7–10^8 CFU *M. kansasii* 1203 (▲) or 1214 (■). Each data point represents the mean ± SEM of five mice.

1203 population within the spleen did not decline with time (cf. Figs 1 and 4). One explanation for this apparent paradox might be an unusually high level of innate resistance to intracellular killing by *M. kansasii* (Orme & Collins, 1983). To examine this possibility, mice were inoculated with 10^8 CFU BCG and then superinfected with 10^6 CFU *M. kansasii* 1203 at the time of peak antilistericidal activity within the spleen (day 14). It can be seen in Fig. 6 that the BCG infection prevented the early growth of the *M. kansasii* inoculum within the spleen, but then there was no further significant drop (< 0·05 log) in viable counts for the *M. kansasii* population over the subsequent 28-day period of the experiment.

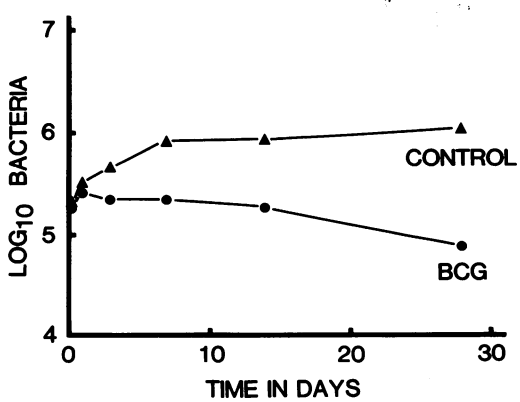

Figure 6. Resistance of *M. kansasii* to killing by BCG-activated macrophages. Viable numbers of *M. kansasii* 1203 in the spleens of uninfected control (▲) and BCG-activated (●) mice.

DISCUSSION

Earlier studies indicated that spleen cells from mice heavily infected with *M. kansasii* 1203 were unresponsive to polyclonal T cell mitogens, as well as to allo-antigenic stimulation *in vitro* (Cunningham & Collins, 1981). Such mice were presumed to be severely immunosuppressed as a result of suppressor cell activity within the spleen. However, the present data indicate that substantial cellular reactivity could still be expressed within the spleen against both *L. monocytogenes* and *M. tuberculosis* challenges at a time when maximum depression of *in vitro* responsiveness (days 20–30 of the *M. kansasii* infection) had earlier been reported. The appearance of substantial cellular resistance within the heavily-infected spleen could be associated with a phase of intensive cellular proliferation throughout the lymphoreticular system. Thus, far from being severely immunosuppressed and unresponsive, the *M. kansasii*-infected animal was actually in a highly activated state. Such animals were able to respond fully to appropriate doses of SRBCs *in vivo*, and the conclusion seems to be inescapable that their cellular defences were not, in fact, severely immunosuppressed. The failure of *M. kansasii* 1203 spleen cells to produce anti-SRBC antibodies when exposed *in vitro* to SRBCs in culture (Cunningham & Collins, 1981) was not reflected by a corresponding drop in the PFC responses seen *in vivo* (Table 1). This finding is in accordance with earlier data obtained in anergic *Mycobacterium avium*-infected mice (Watson & Collins, 1981). Similarly, the apparent suppression of footpad reactivity seen in the conventionally sensitized animal was, at first sight, compatible with those earlier findings, but the dose-response curves shown in Fig. 5 indicate that much of the unresponsiveness was an experimental artefact due to the activated state of the host defenses within the spleens of these mice. The size of the initial mycobacterial population within the spleen had to remain above some arbitrary threshold level in order to induce a sufficient level of non-specific resistance to destroy the red cell antigens before they could sensitize the T cell population. Sensitization of the mice with 10^5 SRBCs was possible only when the *M. kansasii* population declined below 10^4–10^5 CFU/spleen. In the *M. kansasii* 1214-infected mice, this required a period of about 4 weeks. Such an interpretation has recently been confirmed by a lack of sensitized T cells in an adoptive transfer system using *M. avium*-infected mice (Orme & Collins, 1984).

Mice infected with an intracellular parasite are able

to control the *in vivo* growth of the challenge population by a T cell-mediated immune response beginning within the infected spleen (North, 1974). *M. kansasii* 1203 was an intracellular pathogen which induced the production of a substantial population of activated macrophages within the spleen (the rate of development and magnitude of this response was directly related to the initial size of the infecting inoculum). Such findings are in general agreement with those reported by Lefford (1970) in BCG-vaccinated mice. However, the *M. kansasii*-induced response was dependent on the ability of the infecting strain to persist within the reticuloendothelial organs of the host for an extended period of time. This is also consistent with the effect of being very short-lived in mice infected with large numbers of *M. kansasii* 1214, dropping back to normal levels as the number of viable mycobacteria declined (Fig. 3).

This model is subject to the curious paradox that the cellular defenses in both groups of heavily-infected mice were in a highly activated state, and yet they were unable to completely eliminate the *M. kansasii* from the tissues within a reasonable time period (Collins and Cunningham, 1981). In particular, despite the presence of large numbers of activated macrophages within the heavily-infected spleen, there was little or no apparent decrease in the number of viable *M. kansasii* 1203 with time. The only indication of an immune response on the part of the host defenses was a limitation to further growth by the mycobacterial population in the spleen 10–14 days into the infection. The inability of the host defences to eliminate the *M. kansasii* 1203 population, even after activation by live BCG vaccine (Fig. 6), is in agreement with similar findings by Orme and Collins (1983) studying a number of other atypical mycobacteria species. The viable *M. kansasii* 1203 within the spleen (and the lungs, not shown) were clearly resistant to inactivation by the BCG-activated mcrophages; it is presumably this ability which is responsible for the persistence of these oganisms within chronic lung tubercules in man (Wolinsky, 1979). However, the reason for this extraordinary resistance to the activated bactericidal mechanisms of the macrophage within the developing granuloma is presently unclear and deserves further study.

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