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Resistance to *Mycobacterium lepraemurium* is correlated with the capacity to generate macrophage activating factor(s) in response to mycobacterial antigens *in vitro*

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SUMMARY

The kinetics of cell-mediated immunity developed during the course of *Mycobacterium lepraemurium* infection were determined in resistant (C57BL) and susceptible (BALB/c) mice. Control of *M. lepraemurium* growth following footpad infection was T-cell dependent in C57BL mice as shown by the finding that T-cell deprived mice had enhanced bacterial counts in the footpad. In contrast, T-cell deprivation did not significantly alter the course of infection in BALB/c mice. However a T-cell dependent inflammatory response, resulting in an increase in size of the infected footpad, occurred in both strains, although it developed slightly later in BALB/c mice. Cells isolated from the lymph nodes, draining the infected foot-pads, were assayed for their proliferative responses to heat-killed *M. lepraemurium* (HK-MLM) antigens. Although lymph node cells from both mouse strains proliferated to HK-MLM early in the infection (1–2 weeks) both C57BL and BALB/c mice developed diminished *in vitro* proliferative reactivity within 4–6 weeks post-infection. Supernatants derived from cultures of lymph-node cells that had been stimulated with concanavalin A (Con A) or HK-MLM antigens, were assayed for the presence of macrophage-activating factor (MAF) activity using a tumour cytostasis assay and interferon (IFN) activity using a viral growth inhibition assay. Significantly higher levels of MAF and IFN were found in culture supernatants deprived from HK-MLM stimulated lymph-node cells from infected C57BL mice than from BALB/c mice during the first 8 weeks of infection. However, cells from infected mice of both strains produced similar amounts of both MAF and IFN in response to Con A.

INTRODUCTION

The development of cell-mediated immunity (CMI) involving macrophage activation and granuloma formation is believed to be of critical importance in determining the outcome of infections by obligate intracellular organisms (Hahn & Kaufmann, 1981).

Several studies have attempted to correlate various parameters of CMI in mice strains that are resistant or susceptible to subcutaneous *Myobacterium lepraemurium* infection. Both resistant (C57BL) and susceptible (BALB/c) mice develop cutaneous delayed type hypersensitivity reactions, which differ, however, in their kinetics (Alexander & Curtis, 1979). Depletion of T lymphocytes in resistant C57BL mice significantly exacerbates the infection (Adu, Curtis & Turk, 1983) while in contrast the course of infection was similar in nude athymic and heterozygous euthymic litter mates in strains susceptible to infection (Lefford, 1985). However, no correlation could be found between the proliferative capacity of cells from the draining lymph node and resistance to infection (Curtis, Adu & Turk, 1981). Susceptible BALB/c mice do appear to develop nylon wool non-adherent, T enriched cells capable of adoptively transferring resistance to infection, but these cells do not appear to be present until at least 6 weeks post-infection (Alexander, 1979).

The purpose of the present study was to monitor the development of T-cell immunity in resistant (C57BL) and susceptible (BALB/c) mice following *M. lepraemurium* infection. Initially this involved examining bacterial growth and the inflammatory response of the infected footpad in T-cell deprived mice. Levels of lymphocyte proliferation and release of macrophage activation factor(s) (MAF) and interferon (IFN) by draining lymph node cells in response to heat killed *M. lepraemurium* (HK-MLM) were followed at various times post-infection.

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MATERIALS AND METHODS

Animals

Female BALB/c and C57BL mice bred at the National Institute

for Medical Research were used in these experiments. Mice were 6–10 weeks of age at the time of infection.

Mycobacterium lepraemurium

The Douglas strain of MLM was maintained by serial passage of 10^9 bacilli intravenously in Parkes outbred mice. MLM were prepared for inoculation from infected livers as described previously (Brett, 1984) and counted by the method of Hart & Rees (1960). Since MLM cannot be readily grown *in vitro*, all organism counts refer to the number of intact organisms recovered and not to the number of viable organisms recovered.

Mice were inoculated subcutaneously in the right hind footpad with 10^8 MLM organisms in 0·025 ml saline.

MLM was purified from infected livers and spleen using the method described previously (Brett, 1984) and the organisms were then killed by autoclaving for 15 min at 15 lb/ir^2 at 120°. These bacteria (HK-MLM) were then used as antigen in tissue culture experiments and were stored at 4° until use.

Adult thymectomy, irradiation and bone marrow reconstitution

Mice were thymectomized by suction at 5–6 weeks of age, using Avertin (Tribromoethyl alcohol, Winthrop) as the anaesthetic. Four weeks after thymectomy, mice were exposed to 800 rads whole body irradiation from a ^{60}Co source and reconstituted with 1×10^7 syngeneic bone marrow cells that had been treated with anti-Thy 1.2 (monoclonal IgM antibody, Olac Ltd, Oxon) plus rabbit complement (Buxted Rabbit Co., Buxted, Sussex). Mice were given tetracycline 1 week before and 4 weeks after irradiation to reduce the risk of infection. Eight weeks after irradiation the mice were infected subcutaneously with 10^8 MLM. Control mice (sham thymectomized) were treated in the same way, except that they had not been thymectomized.

Increase in size of infected foot

The size of the infected foot was measured with a screw gauge micrometer (Moore & Wright). The percentage increase in size of the foot was expressed by comparing the size of the uninfected left hindfoot with the size of the infected foot.

Assessment of resistance

At various times after MLM infection, mice were killed and right hindfeet removed and stored at −20°. Acid-fast bacilli were estimated in the feet by the method of Hart & Rees (1960), following homogenization in 2 ml of 0·1% bovine serum albumin in water.

Lymph node preparation

Mice were killed from 1–20 weeks after infection and the popliteal lymph node draining the infection site was removed aseptically and placed in culture medium. Cell suspensions were prepared by gently teasing the lymph node cells through a stainless steel sieve. Clumps were allowed to settle out and the lymph node cells were washed twice in tissue culture medium, then resuspended in RPMI-1640 medium supplemented with 5% fetal calf serum (Gibco-Biocult, Glasgow), 2 mM glutamine (Gibco-Biocult), 100 IU/ml penicillin (Glaxo, Middlesex), 100 μg/ml streptomycin (Evans, Middlesex), 5×10^{-5} M 2-mercaptoethanol and 10 mM HEPES. Lymph node cell viability, assessed by trypan blue exclusion, was greater than 90%.

Lymphocyte transformation test

Lymph node cells were cultured in round-bottomed microtitre plates (Nunc). Each well had 5×10^5 lymph node cells in 200 μl of complete medium as described above. Various concentrations of HK-MLM (μg/ml dry weight) were added as antigen to these cultures. Cultures were incubated at 37° in a humid atmosphere of 5% CO_2 in air for 3–4 days. Eighteen hours before harvesting 20 μl RPMI-1640 containing 1 μCi of tritiated thymidine ([^{3}H]TdR; spec.act. 2 Ci/mmol; Radiochemical Centre, Amersham, Bucks) were added to each well. Cultures were harvested onto glass fibre filters with a Skatron cell harvester.

Production of lymph node cell supernatants

Lymph node cells were cultured in 24-well Costar plates at a concentration of 5×10^6/ml with various doses of HK-MLM or concanavalin A (5 μg/ml), or left unstimulated. Cultures were incubated at 37° in humid atmosphere of 5% CO_2 in air for 48 hr. The contents of each well were harvested, centrifuged at 400 ***g*** for 10 min, and filtered through 0·22 μm millipore filters. Supernatants were aliquoted and stored at −20° until assayed.

Preparation of peritoneal exudate cells

Mice were injected i.p. with 3% thioglycollate and the peritoneal cells harvested 4 days later. The peritoneal cavities were washed out with 5 ml cold Dulbecco's phosphate buffered saline (Ca^{2+} and Mg^{2+} free). Peritoneal washouts from mice within one group were pooled, centrifuged at 400 ***g*** for 10 min, washed twice and resuspended in RPMI-1640 with 5% FCS and cell viability counts made. The peritoneal exudate cell populations in all cases contained $<1\%$ polymorphonuclear cells and $>90\%$ macrophage-like cells as assessed morphologically by crystal violet staining.

Assay for MAF production in vitro

MAF activity in lymph node cell supernatants was assessed by their ability to activate peritoneal macrophages to non-specifically inhibit the growth of EL4 tumour cells. Thioglycollate-elicited peritoneal macrophages from CBA mice were incubated in round-bottomed microtitre plates at 2×10^5 cells/well with 100 ng/ml lipopolysaccharide (LPS, Sigma) at 37° for 24 hr. Non-adherent cells were removed by 2–3 washes with complete medium and then incubated with lymph node cell supernatants (diluted 1/4) for 6 hr at 37° in 5% CO_2. Supernatants were then removed with 2–3 washes of medium and 5×10^3 EL4 cells were added to give an effector : target cell ratio of 40 : 1 and incubated for 18 hr at 37° with 5% CO_2. The cultures were pulsed with 1 μCi/well [^{3}H]TdR for 6 hr and then harvested onto glass fibre filters with the Skatron cell harvester. Control cultures containing peritoneal or EL4 cells only were also set up. Results were expressed as

$$\%\ \text{inhibition of EL4 growth} = 100 - \frac{\text{c.p.m. test group} - \text{PEC only}}{\text{c.p.m. EL4 only}} \times 100.$$

Interferon assay

Supernatants were assayed for IFN production by measuring the reduction of [^{3}H]uridine incorporation by Semliki Forest virus grown in L 929 fibroblasts (Taylor, Wraith & Askonas,

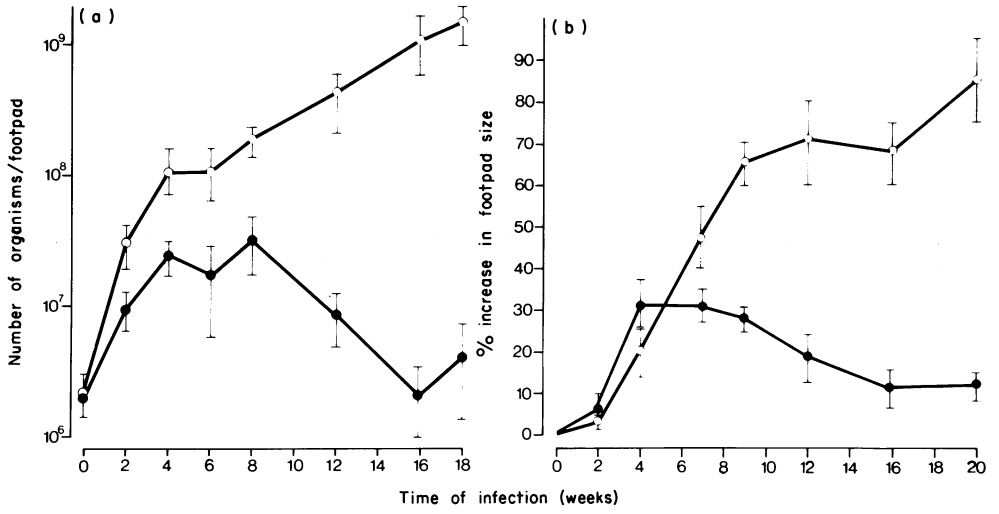

Figure 1. Course of *M. lepraemurium* infection following subcutaneous inoculation as monitored by (a); number of organisms/footpad and (b); percentage increase in footpad size in BALB/c (○——○) and C57BL (●——●) mice. Each point is the mean ± SE from 10 mice.

1985). The results were expressed as IFN units/ml by comparison with a known standard NIH IFN preparation of 700 U/ml.

RESULTS

Course of infection

Following infection with 10^8 MLM s.c. there was a large drop in the number of organisms recovered from the infected footpads by 48 hr after inoculation, compared with the expected number of organisms in both BALB/c and C57BL strains (Fig. 1a). This was followed by an increase in bacterial numbers isolated from the footpads in both BALB/c and C57BL mice, until Week 4. In C57BL mice after Week 4 there was a plateau in bacterial numbers until Week 8, when there was a decline in the bacterial counts to about 10^8/footpad which was maintained until at least Week 35. In contrast BALB/c mice showed a progressive increase in bacterial numbers in the footpad. The rate of growth of the bacteria, however, appeared to slow down after 6–8 weeks.

The size of the infected footpad was monitored at various times during the infection (Fig. 1b). In C57BL mice there was a rapid increase in footpad size, reaching a maximum 4–6 weeks after infection, followed by a slow decrease; however, the feet were still significantly swollen by Week 20. BALB/c mice showed a slight lag when compared to C57BL and did not start to increase in size until Week 4. After this there was a rapid and progressive increase in size of the infected footpad which remained significantly more swollen than the C57BL until the end of the experiment.

Effect of adult thymectomy irradiation and bone marrow reconstitution on the course of MLM infection

In C57BL mice, adult thymectomy, irradiation and bone marrow reconstitution (TXBM) resulted in significantly higher numbers of organisms ($P > 0{\cdot}001$) in the infected footpad than in irradiation, bone marrow treated (STX) or in untreated C57BL mice (Table 1). In contrast similar numbers of organisms were harvested from TXBM and STX BALB/c mice ($P > 0{\cdot}05$) although both these groups had slightly higher levels than the untreated BALB/c mice.

Table 1. Organism counts in footpad 14 weeks after *M. lepraemurium*

Treatment		No. of mice	Acid-fast bacilli/footpad $\log_{10}$ mean (range)	
Untreated	C57BL	10	6·7 (6·47–7·04)	
STXBM	C57BL	7	6·81 (6·62–7·02)	$P < 0{\cdot}001$
TXBM	C57BL	9	8·02 (7·74–8·39)	
Untreated	BALB/c	8	8·54 (8·27–8·74)	
STXBM	BALB/c	7	9·0 (8·85–9·02)	$P > 0{\cdot}05$
TXBM	BALB/c	6	8·97 (8·69–9·32)	

The pattern of increase in footpad size in both C57BL and BALB/c mice was similar to that described above in both untreated and STX mice. However, in the TXBM-treated groups of both BALB/c and C57BL mice there was a significantly reduced increase in footpad size until the later stages of infection (Fig. 2a and b).

Proliferative response to HK-MLM antigen

The proliferative response of cells from the draining lymph node to HK-MLM antigen was assessed at various time points after s.c. infection with live MLM organisms. The incorporation of [^{3}H] TdR by the lymph node cells of BALB/c and C57BL mice to different doses of antigen ranging from 0·01 to 50 HK-MLM μg/ml (freeze dried wt) at 4 weeks after infection is shown in Fig. 3a. The results indicate that optimal levels of proliferation occurred at doses > 10–50 μg/ml. Higher levels of proliferation were consistently observed in BALB/c mice compared with C57BL mice. If however the lymph node cells were cultured in 24-well costar plates for 48 hr, non-adherent cells removed, washed, resuspended to give a cell concentration of 5×10^5/well, and then

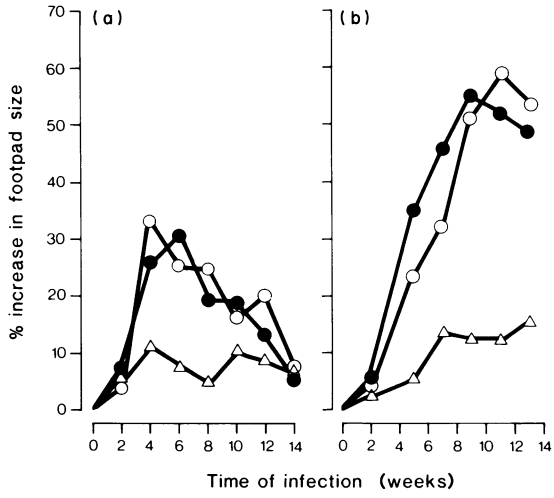

Figure 2. The percentage increase in the size of the infected footpad throughout the infection in (a) C57BL and (b) BALB/c mice that were (○) unmanipulated, (●) sham thymectomized or (△) thymectomized. Number of mice per group as indicated in Table 1.

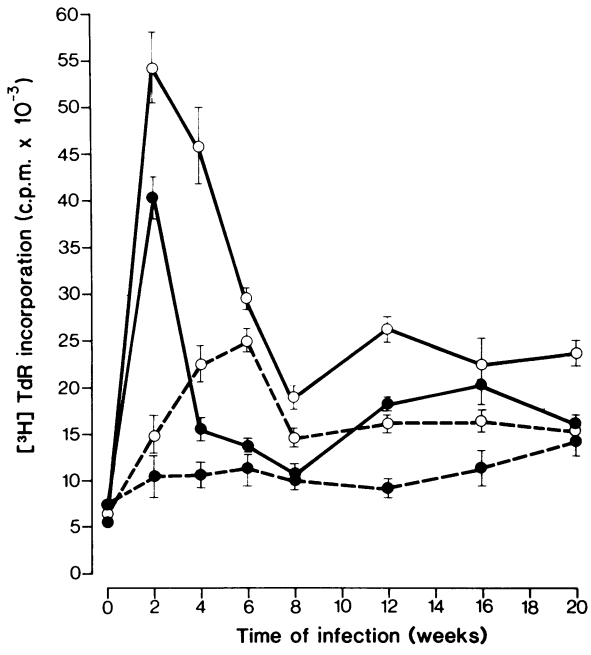

Figure 4. Lymphocyte transformation responses of cells from the draining popliteal lymph node of BALB/c (○) and C57BL (●) mice taken at different times following subcutaneous infection with *M. lepraemurium*. The cells were either cultured without antigen (– – –) or stimulated with HK-MLM 50 μg/ml (——).

pulsed with [^{3}H]TdR for 18 hr, similar proliferative responses were observed in both BALB/c and C57BL strains (Fig. 3b).

The proliferative responses of lymph node cells from BALB/c and C57BL in the standard lymphocyte transformation test at various time points after infection can be seen in Fig. 4. Maximum responsiveness to HK-MLM antigen occurred at 1–2 weeks post-infection, for both strains of mice. Thereafter, the proliferative responses of mice to MLM antigen declined, as the infection progressed. However, at all the time points studied, the response of BALB/c mice was consistently greater than that of C57BL mice. It was also observed that proliferation of unstimulated cultures from both infected BALB/c and C57BL mice were elevated, when compared with unstimulated cultures from normal mice. This may reflect antigen carryover, however it was observed that a high background was maintained by reconstituting purified T cells with fresh antigen presenting cells (manuscript in preparation).

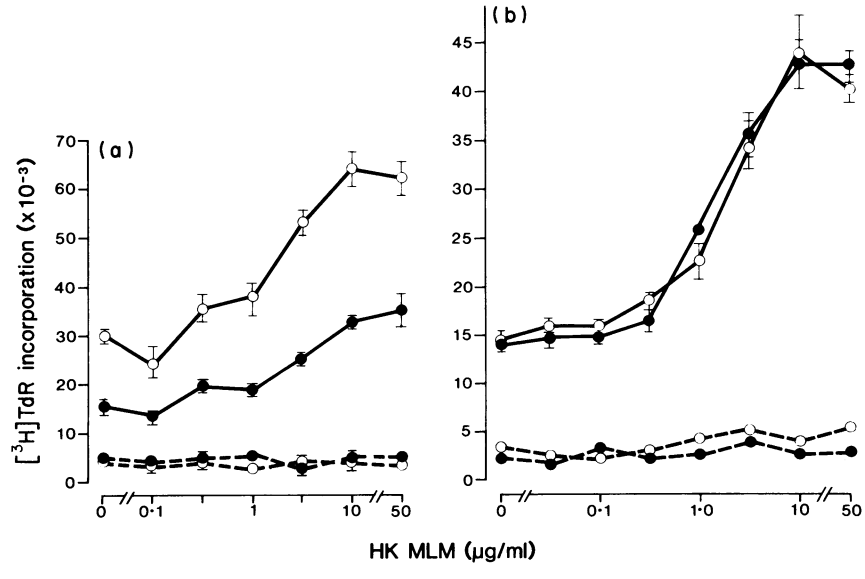

Figure 3. Lymphocyte proliferative responses of cells from the draining popliteal lymph node of uninfected (– – –), and 4 week infected (——), C57BL (●) and BALB/c (○) mice to various doses of HK-MLM, using unseparated lymph node cells (a) or lymph node cells depleted of adherent cells for the last 18 hr of experiment (b).

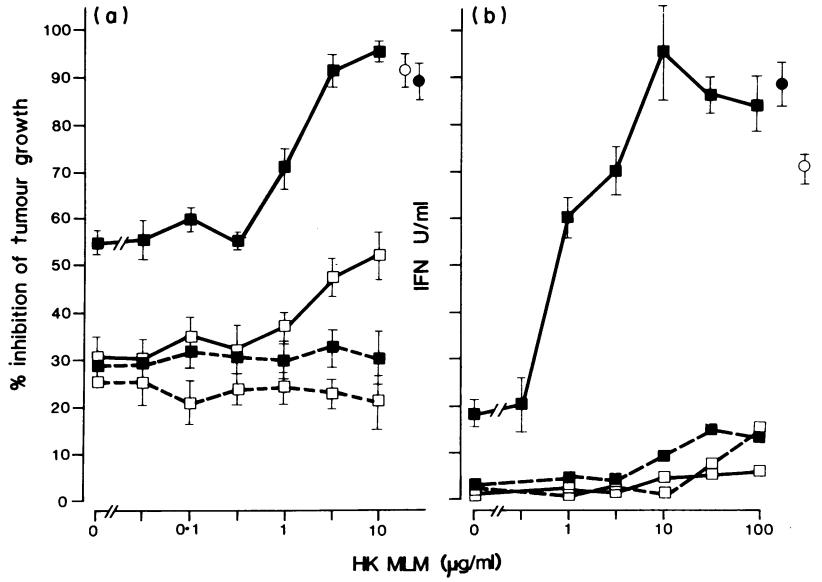

Figure 5. Dose response of MAF [a] or IFN (b) production to KH-MLM antigens (μg/ml) by draining popliteal lymph node cells from uninfected (- - -) or *M. lepraemurium* infected (——) BALB/c mice (□) or C57BL (■) mice. Response of infected BALB/c (○) mice or C57BL (●) mice to Con A (5 μg/ml) is also shown.

Macrophage activation factor(s) induced by HK-MLM antigen

The ability of lymph node cells from uninfected and 5 week infected mice to release MAF into the surrounding medium upon stimulation with a range of doses of HK-MLM was assessed. The supernatants were incubated with macrophages that were then tested for their ability to inhibit EL4 tumour growth in the cytostasis assay (Fig. 5a). The results indicated that lymph node cells from the infected C57BL mice released significantly more MAF on stimulation with specific antigen than BALB/c mice. However, lymph node cells from both strains of infected mice released similar amounts of MAF with an optimal dose of Con A.

Interferon release

Supernatants were also monitored for their levels of interferon in a standard viral assay that measures α β and γ IFN (Fig. 5b). Lymph node cells from uninfected BALB/c and C57BL mice released small but detectable levels of interferon in response to increasing doses of HK-MLM. Lymph node cells from infected C57BL mice released significant amounts of interferon in response to increasing doses of HK-MLM. In contrast BALB/c mice, did not release any detectable interferon in response to HK-MLM. Lymph node cells from both strains of infected mice released similar amounts of IFN with an optimal dose of Con A. In other experiments some interferon was detected in supernatants from infected BALB/c mice in response to HK-MLM although this was invariably at significantly lower levels than from infected C57BL mice.

MAF release during the course of MLM infection

MAF production, by draining lymph node cells of C57BL and BALB/c mice, at various times after s.c. infection with MLM,

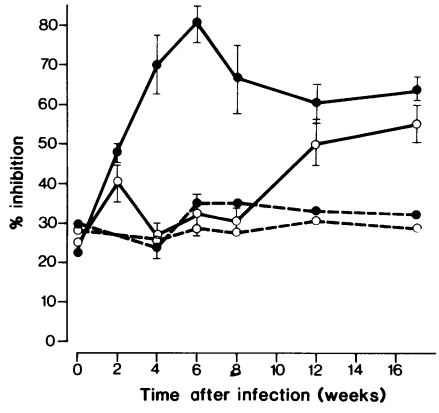

Figure 6. Kinetics of MAF production by draining popliteal lymph node cells from BALB/c (○) or C57BL (●) mice, taken at different time points after subcutaneous *M. lepraemurium* infection that were either unstimulated (- - -) or stimulated (——) with 50 μg/ml HK-MLM.

was monitored using the cytostasis assay (Fig. 6). Fairly high levels of MAF were produced by lymph node cells from C57BL mice stimulated with MLM during the early stages of infection i.e. Weeks 2–8, after which the levels declined slightly. In contrast only low levels of MAF were detected upon antigen stimulation of draining lymph node cells from BALB/c mice in the first 8 weeks of infection. At later times after the infection of BALB/c mice higher levels of MAF activity could be detected.

DISCUSSION

C57BL mice were able to control the growth of *M. lepraemurium*, 4–6 weeks after subcutaneous infection. Control of bac-

terial growth was T-cell dependent, as shown by the exacerbated infection in TXBM mice, confirming the findings of Adu *et al.* (1983). In contrast there was a progressive increase in bacterial counts in BALB/c mice that were not significantly different in TXBM mice, supporting observations in nude mice with susceptible background genes (Lefford, 1985). The mechanisms underlying susceptibility in BALB/c mice to *M. lepraemurium* infection appear to be different from those involved in susceptibility of BALB/c mice to s.c. infection with *Leishmania tropica* (Howard, Hale & Liew, 1980, 1981). In the latter infection both adult TXBM or sub-lethal whole body irradiation (550 rads) increased the resistance of BALB/c mice. However, neither TXBM nor 550 rads (unpublished observation) increased resistance of BALB/c mice to *M. lepraemurium*, which more closely resembles the pattern seen with *Leishmania mexicana* (Alexander & Kaye, 1985).

A T-cell dependent inflammatory response to *M. lepraemurium*, as monitored by increase in the size of the infected footpad, was found to occur in both resistant and susceptible mouse strains, confirming previous observations (Adu *et al.*, 1983; Lefford, 1985). This suggests that there is a T-cell dependent immune response to *M. lepraemurium* antigens in BALB/c mice that attracts various cell types into the foci of infection. However, this response does not appear to be sufficient to control bacterial growth. This contrasts with the findings with *Listeria monocytogenes*, where the influx of inflammatory cells is able to restrict growth to a certain extent (Czuyprynski, Hensen & Campbell, 1985). It is probable that the freshly recruited macrophages in the inflammatory response may exacerbate the infection by providing a good supply of host cells. It has been demonstrated, for example, that the growth rate of MLM is faster in bone marrow than in the liver or spleen (Brain & Krensien, 1976).

The pattern of the antigen specific proliferative response to HK-MLM antigen by draining lymph node cells was similar to that described by Curtis *et al.* (1981) using a cell free extract of *M. lepraemurium*. The lymphocyte transformation test contains a variety of interacting cell types, and although the proliferative response is T-cell dependent (manuscript in preparation), once T cells are activated they may induce other cell types, such as B cells, to divide and these would then contribute to the total proliferation measured. In the present study it was also observed that the maximum antigen-specific proliferative response to HK-MLM antigen was masked in cultures containing lymph node cells from infected C57BL mice but not BALB/c mice, by the development of inhibitory adherent cells. Further studies are therefore required using limiting dilution analysis with T-cell enriched populations to compare the frequency of antigen-specific T cells in resistant and susceptible strains, before firm conclusions can be drawn about the relative levels of T-cell activation between the strains.

T-cell dependent macrophage activation plays a central role in control of replication of various intracellular organisms such as *Leishmania donovani* (Hockyemer *et al.*, 1984), *L. tropica* (Titus, Kelso & Louis, 1984), *Trypanosoma cruzi* (Nogueira & Cohn, 1978), *M. microti* (Walker & Lowrie, 1981) and also *M. lepraemurium* (Alexander & Smith, 1978). Several studies have shown that purified or recombinant γ IFN is extremely potent in activating macrophages for enhanced killing of intracellular organisms (Nathan *et al.*, 1984) and tumour cytostasis or cytotoxicity (Kildahl-Andersen & Nissen-Meyer, 1985). It is clear, however, that other mediators may be involved in macrophage activation. For example supernatants from *M. tuberculosis* specific T-cell clones contained a lymphokine that activated macrophages to release H_2O_2, but had only low levels of γ IFN (Andrew *et al.*, 1984). Several studies have reported that the amount of macrophage activation factor/IFN released after antigenic stimulation of cells from infected mice, correlated with susceptibility to infection, for example with *L. donovani* (Murray, Masur & Keithly, 1982), *L. tropica* (Sadick *et al.*, 1986), *T. cruzi* (Nogueira *et al.*, 1981), *Listeria monocytogenes* (Buchmeier & Schreiber, 1985) and human leprosy (Nogueira *et al.*, 1983).

In the present study it was found that resistant C57BL mice released significantly higher levels of MAF and/or IFN than susceptible mice, especially at the earlier stages of infection. It has also been observed that *in vivo* challenge via peritoneum with HK-MLM and thioglycollate resulted in significantly higher levels of macrophage activation in infected C57BL mice compared with infected BALB/c mice (unpublished observations). In other experimental systems such as BCG sensitized mice challenged with PPD (Huygen & Palfliet, 1983), *Trypanosoma brucei* infection (De Gee, Sonnenfield & Mansfeld, 1985), pokeweed mitogen stimulation (Virelizier, 1982), and *L. tropica* infection (Sadick *et al.*, 1986), C57BL mice also released significantly higher amounts of IFN than BALB/c mice. Further studies are, however, required to assess whether γ IFN and/or other macrophage activating factors, which may be present in the supernatants from MLM stimulated lymph node cells from C57BL, are capable of activating macrophages to control MLM growth, both *in vivo* and *in vitro*. This would then answer more directly whether the lower amounts of MAF/IFN produced by BALB/c mice, contributes to their relatively susceptibility to *M. lepraemurium* infection. The use of recombinant γ IFN should allow the role of this lymphokine in *M. lepraemurium* infections to be more precisely defined. Further studies could then be carried out to assess whether the lower levels of IFN produced by BALB/c mice in response to MLM are due to the presence of regulatory cells, general inability to produce appropriate levels of IFN or, alternatively, to a parasite-induced perturbation in immune function.

Release of MAF in response to stimulation with HK-MLM, as well as macrophage activation following *in vivo* challenge with HK-MLM, were detected at the later stages of infection in BALB/c mice, although these were at lower levels than in C57BL mice. This supports previous findings that while C57BL were invariably better at controlling primary and challenge infections than BALB/c mice, BALB/c mice did acquire some protective immunity towards challenge at the later stages of infection (Preston, 1982).

In conclusion, it appears that susceptible BALB/c mice develop sensitized antigen-specific T cells in response to s.c. infection with *M. lepraemurium*. While these T cells are able to proliferative and mediate a local inflammatory response at the site of infection, they do not control bacterial growth. This may be due to failure of BALB/c mice to release large amounts of MAF/IFN in response to antigen, at the early stages of infection.

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