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Immunological responses of *L. donovani* infection in mice and significance of T cell in resistance to experimental leishmaniasis

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SUMMARY

The parasite burdens of livers and spleens of several strains of mice following infection with *L. donovani* were determined for a period of over 120 days. The parasite loads of the spleens and livers were correlated with the development of immunity to reinfection, footpad sensitivity and antibody titres. The strains of mice studied could be divided into two groups—those highly susceptible and those relatively non-susceptible. The antibody in the susceptible strains appeared 12 days after infection and increased thereafter. The antibody in the less susceptible strains appeared some time later and remained at the same level throughout the test period. In strains CAFl, C57Bl/6 and BALB/c skin reactivity to Leishmania antigen developed at the time when the parasite burden had decreased from its peak. Skin reactivity never developed in DBA/1 mice, the most highly susceptible strain, and in A/j the least susceptible strain. The development of resistance to reinfection with *L. donovani* following i.v. and i.p. infection was observed in C57Bl mice. Upon cell transfer from these immune animals, it was found that protective immunity was mediated through the thymus-dependent lymphocytes. Serum from immune mice was not able to confer immunity to the recipient animals.

INTRODUCTION

Human visceral leishmaniasis is caused by *L. donovani* or *L. donovani infantum* (Bray, 1974). Patients with systemic leishmaniasis exhibit fever, hepatosplenomegaly, anaemia and reversal of the normal albumin:globulin ratio. Such patients have also been shown to possess a defect in cell-mediated immunity as detected by skin test (Manson-Bahr, 1961). Moreover, in the active stage of the disease the peripheral blood T/B cell ratio is reversed (Rezai *et al.*, 1978).

Although the course of infection with *L. donovani* in mice has been thoroughly investigated (Bradley & Kirkley, 1977; Bradley, 1977), our knowledge of immunological parameters in this disease has not significantly advanced in the last few years. Therefore, one of the objectives of this study was to investigate the immunological responses of different strains of mice at various times after infection with *L. donovani*. Indirect evidence has suggested that cell-mediated immunity plays an important role in the resistance of mice to *L. donovani* infection (Skov & Twohy, 1974). The most direct way of demonstrating a protective role for CMI mechanism would be to confer immunity to *L. donovani* infection on virgin recipients by transferring lymphocytes from immune mice. The results of such experiments are described in the present paper.

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MATERIALS AND METHODS

Animals. Female BALB/c, C57Bl/6, DAB/1, A/j, C3H, CAF1 (hybrid of BALB/c × A/j) and two congenic strains of C57Bl, B10 and B10A were obtained from Jackson Laboratories, Bar Harbor, Massachusetts. For all studies mice which were 6 weeks old, weighing 15–20 g, were used. These were maintained in the laboratory facilities of the Department of Pathobiology, University of Pennsylvania.

Parasite strain. hamsters infected with Sudan III stain of *L. donovani* were used as the source of amastigotes of *L. donovani*. An inoculum of 5×10^6 parasites suspended in 0·3 ml of sterile saline was used for infecting animals. The inoculum was prepared from spleens of infected hamsters. The spleens were excised aseptically and homogenized in a Ten-Broeck homogenizer with 10 ml of saline and the suspension was centrifuged at 600 r.p.m. for 6 min to remove tissue debris. The supernatant was centrifuged at 2,600–2,800 r.p.m. for 20 min. The sediment containing the parasites was resuspended in Tris-buffered ammonium chloride in order to lyse the red blood cells and spun for 20 min at 2,600 r.p.m. The deposit was washed twice with saline, counted using a haemocytometer and adjusted to the appropriate parasite concentration before injection.

Enumeration of parasite in tissues. The number of parasites was determined by the method suggested by Stauber (1955, 1958). A small piece of infected spleen or liver was first blotted on a paper towel to remove blood. Several smears were prepared from the tissues. The slides were air dried, fixed with absolute methanol and stained with Giemsa. The ratio of amastigotes to liver or spleen cell nuclei was determined by counting about 1,000 cells. If the parasites highly outnumbered the cell nuclei, only 500 cell nuclei were counted. The ratio of amastigote/cell nuclei was calculated and multiplied first by the weight of the organ in milligrams to give a measure of the parasite load in 'Leishman-Donovan' units (LDU) and then by 2×10^5 to give the approximate absolute number of parasites per organ, 'Leishman Donovans' (LDs).

Statisitical analysis. All data presented in Figs 1–4 are mean values of three mice. *P* values were determined by Student's *t*-test.

Antibody studies. All antibody titres were determined by the technique of immunofluorescence as described before (Behforouz, Rezai & Gettner, 1976) using a 1:20 dilution of fluorescein-isothiocyanate labelled anti-mouse serum obtained from the Capel Laboratory, Pennsylvania, USA.

Skin test. The skin test antigen was prepared from the promastigotes of *L. donovani* grown in Schneider's drosophila medium (GIBCO) to which 10% foetal calf serum was added. The organisms were washed, suspended in saline and sonicated until no intact protozoa were detected. The suspension was centrifuged and the protein content of the supernatant was measured by the Lowry technique and adjusted to 1,000 μg/ml. The footpads of infected mice were injected with 0·025 ml of the prepared antigen containing 25 μg protein. The antigen was also injected into footpads of normal mice as a control.

Preparation of enriched T and B lymphocytes. T- and B-enriched cells were prepared by the technique of Schwartz, Jackson & Paul (1975). Spleens were injected first with 3 ml of RPMI to loosen the tissue and then were cut into small pieces. A single cell suspension was prepared with a tissue mesh or coarse porosity tissue grinder in RPMI. The suspension was centrifuged in the cold for 10 min at 1,200 r.p.m. A nylon wool column was equilibrated first with saline and then with RPMI–HEPES buffer to which 5% foetal calf serum was added. The column containing RPMI was then incubated for 45 min at 37°C. Four millilitres of the prepared cell suspension was added to the column and incubated for 45 min. During this incubation period any evaporated medium was replaced every 15 min. The T cells were collected at room temperature by gently adding approximately 30 ml of the medium onto the column. The column was then washed with saline and the B cells were collected by rapidly and harshly adding 20–30 ml of medium onto the column.

Determination of surface immunoglobulins on lymphocytes. In order to stain surface immunoglobulins the following procedures were employed. A 1:15 dilution of conjugated antiserum was added to 2×10^6 lymphocytes in a small tube. The cell antiserum was mixed gently and then incubated for 30 min at 4°C on a rotator. The cells were then washed three times in phosphate-buffered saline containing 10% foetal calf serum. One drop of the cells was then mounted with one drop of 50/50 glycerine and phosphate-buffered saline and observed with a Zeiss fluorescent microscope.

Anti-brain-associated serum. Rabbit anti-mouse brain-associated thy I antiserum was used (Cederlane, Pennsylvania). The cytoxicity of anti-θ serum was tested against thymus and marrow suspensions according to the method of Golub (1971). A 1:15 dilution of the anti-mouse T cell serum killed all the thymus cells and approximately 25% of the BM cells *in vitro*.

RESULTS

Parasite burdens in various strains of mice

The mean number of parasites in the livers and spleens of infected mice was determined at days 12, 24, 36, 50, 75 and in some strains 130 days after infection. The results of these determinations for the liver are presented in Fig. 1. Among the mice strains studied, there were two distinct groups with regard to parasite burden of the liver: less susceptible and highly susceptible. The less susceptible group, in which the number of parasites did not increase considerably in the liver over the period studied included the A/j and C3H strains. The highest number of parasites in the livers of these strains was 1×10^7 at 36 days after infection. After this peak the parasite levels fell in both strains. The livers of these strains of mice were virtually parasite-free after about 75 days. In the highly susceptible groups which included DBA/1, C57Bl/6 and BALB/c strains, the parasite load increased considerably throughout the infection period. The peak parasite load in the livers of the C57Bl/6 and BALB/c strains observed by day 24 was $2{\cdot}4 \times 10^8$ and $3{\cdot}0 \times 10^8$ respectively. The number of amastigotes in the livers of these two strains fell gradually thereafter. The number of parasites in the livers of C57Bl/6 mice decreased much faster than in the BALB/c mice.

The DBA/1 mice appeared to be more highly susceptible to *L. donovani* than the other two susceptible strains. In this strain the parasite burden reached a higher peak and decreased more gradually than in the other two susceptible strains. There was a considerable parasite burden even after 130 days.

In the CAFl mice, which is a hybrid strain of BALB/c and A/j, the number of parasites in the liver reached a level somewhat higher than the less susceptible strains but lower than the highly susceptible ones which may indicate that genetic factors play a role in the susceptibility of these mice to *L. donovani* infection.

Parasite burden in the spleen

The results of the parasite burden of the spleen of various strains of mice are presented in Fig. 2. These results show that the parasite load of the spleen of various strains of mice parallels that in the

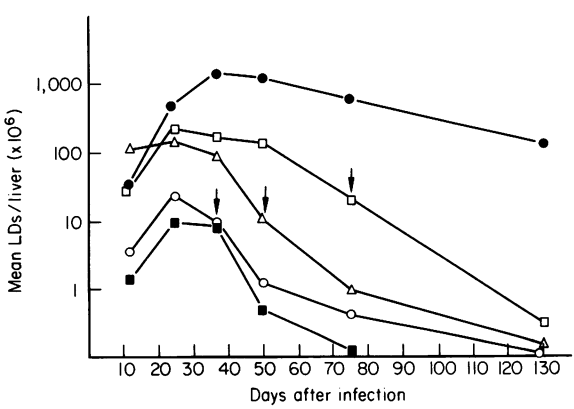

Fig. 1. Parasite burden in the liver of several strains of mice: C57Bl/6 (▵—▵), BALB/c (□—□), DBA/1 (●—●), A/j (■—■), CAF (○—○) (hybrid of BALB/c and A/j). The mice were infected intravenously with 5×10^6 *L. donovani* and counts were performed in the liver on groups of three mice. Development of delayed hypersensitivity (*arrows*)

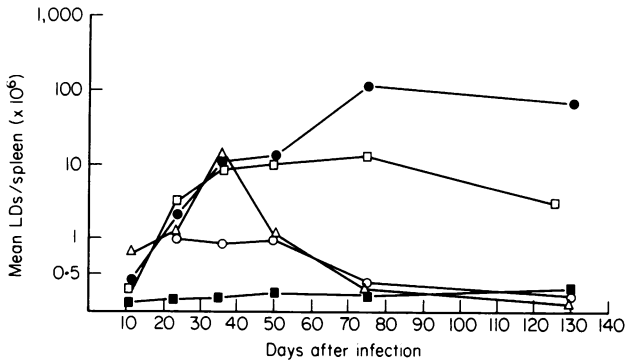

Fig. 2. Parasite burden in the spleen of several strains of mice: C57Bl (▵—▵), BALB/c (□—□), DBA/1 (●—●), A/j (■—■) and CAFl (○—○) (hybrid of BALB/c and A/j). The mice were infected intravenously with 5×10^6 *L. donovani* and counts were performed in the spleen on groups of three mice.

liver. The overall parasite burden in the spleen was, however, lower than in the liver and the parasite level reached a peak somewhat later than the peak in the liver. Parasite burden of the liver and spleen in C3H was the same as in A/j.

Susceptibility of mice to L. donovani infection

An attempt was made to investigate whether the susceptibility pattern of mice to *L. donovani* infection was H2-linked or not. For this purpose two congenic strains of C57Bl mice, B10 and B10A having H2B and H2a haplotype respectively, were infected with *L. donovani* and their parasite burden was determined after 12 days. It was observed that there was no difference in the susceptibility (parasite burden) of these two strains of mice to infection with *L. donovani* and thus one can probably conclude that susceptibility of mice to infection to this parasite is not H2-linked.

Development of resistance

Studies were performed to determine the development of resistance in a susceptible strain of infected mice whose parasite burden had fallen. For this purpose strain C57Bl/6 was used as the experimental animal. Four mice were first infected i.v. with 5×10^6 amastigotes and after 50 days these, along with four normal mice, were challenged with the same number of parasites (Table 1). The mean number of parasites in the liver of the four experimental mice determined 12 days after infection was approximately 1×10^7 whereas the parasite burden of the normal controls was 1×10^8. Experiments were also performed to determine if i.p. infection with amastigotes of *L. donovani* induces resistance to organisms introduced intravenously. The four C57Bl mice were first infected

Table 1. Acquired resistance to infection with *L. donovani* in C57Bl/6 mice

Primary infection	Parasite burden 12 days after challenge
i.v.	$1{\cdot}32 \times 10^7$
i.p.	$0{\cdot}91 \times 10^7$
No infection	$9{\cdot}2 \times 10^7$

Groups of mice that had been given 5×10^6 amastigote i.v. or i.p. 7 weeks earlier were challenged with 5×10^6 *L. donovani* amastigote i.v. For comparison, 5×10^6 *L. donovani* was injected i.v. into normal mice. Counts were performed 12 days later in the liver of a group of four mice.

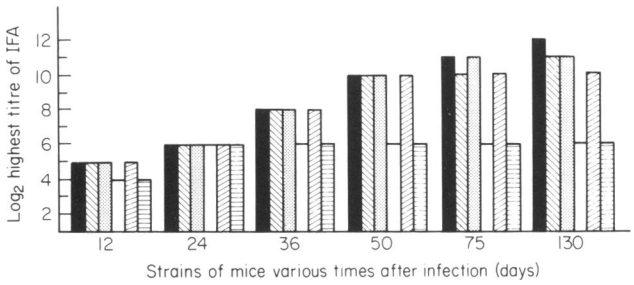

Fig. 3. Antibody response to *L. donovani* at various times after infection in several susceptible and less susceptible strains of mice: DBA/1 (■), C57BL ( ), BALB/c ( ), A/j (□), CAFL ( ) and C3H ( ).

i.p. with 5×10^6 parasites and 7 weeks later challenged i.v. with the same number of Leishmania. Twelve days later the number of parasites in the liver of the rechallenged mice was approximately ten-fold lower than the control groups, suggesting the development of resistance following intraperitoneal infection (Table 1).

Development of antibody

Results of antibody titration by indirect immunofluorescence are presented in Fig. 3. These results showed that antibody in the highly susceptible strains of mice (DBA/1, BALB/c and C57Bl/6) appeared at approximately 12 days after infection and increased to a high titre thereafter. The antibody titre in the less susceptible strains (C3H and A/j) first appeared about 24 days after infection and remained at the same titre throughout the test period. In the CAFl hybrid the antibody titre had a pattern somewhat similar to the highly susceptible strains.

Skin reactivity

The time of development of skin reactivity in the strains of mice infected with *L. donovani* is presented in Fig. 1. C57Bl/6 mice, one of the highly susceptible strains, developed skin reactivity approximately 50 days after infection, concomitant with the decrease of the parasite load from the peak and remained positive for the remainder of the test period. BALB/c, another susceptible strain, responded to the antigen 75 days after infection, again at the time when the parasite burden had decreased from the peak of the parasite load. DBA/1 mice, the most highly susceptible strain, never developed a positive skin test during the 130 days following infection. The parasite burden in the liver and spleen of this strain of mice was relatively high even 130 days after infection. The less susceptible strains also never developed skin reactivity to the antigen throughout the 130 days of the test period. The hybrid strain, CAFl, however, showed positive skin reaction 36 days after infection, again at the time when the parasite burden of the liver had decreased from the peak. These results indicate that, in general, skin reactivity to Leishmania antigen in mice may become positive only when the parasite load decreases to a certain level after the initial increase.

Adoptive transfer of cells and antibody

Attempts were made to investigate whether cells or antibody can passively transfer resistance to *L. donovani* infection in mice. For this purpose C57Bl/6 mice were infected i.v. with 5×10^6 amastigotes of *L. donovani*. After about 75 days, at the time when the parasite load of their livers had dropped considerably, their spleens were used as a source of immunocompetent cells. T- and B-enriched cell populations were obtaind by nylon wool separation (Schwartz *et al.*, 1975). Examination with labelled anti-immunoglobulin showed that the enriched T cell population was not contaminated with B cells as there was a complete absence of Ig-bearing lymphocytes in the preparation. However, since only 50% of the B cell preparation showed staining properties, one could assume that the B cell fraction was contaminated with other cell types, possible T cells. An attempt was made, therefore, to eliminate any residual T cells in the B cell preparation by treatment of the latter with anti-theta serum and complement. This treatment resulted in removal of the contaminated T cell from the B cell preparation as evaluated by immunofluorescence. Viable T- or B-enriched cells (1×10^8) or

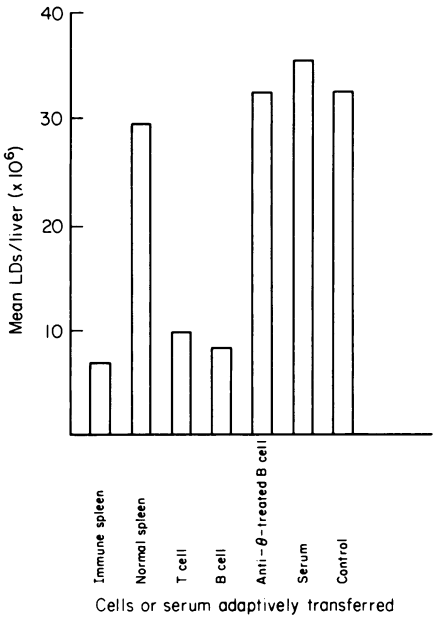

Fig. 4. Syngeneic adoptive transfer of immunity to *L. donovani* in C57Bl mice. Mice infected 75 days earlier with 5×10^6 promastigote were the source of the immune cells or serum. Whole viable spleen (2×10^5) or viable T or anti-θ-treated B cells (1×10^5) or 2 cc of immune serum were transferred to each mouse 24 hr earlier, then challenged with 5×10^6 *L. donovani* promastigote. Counts were performed in the liver on groups of four mice 12 days after challenge.

viable spleen cells (2×10^8) were transferred into each of four syngeneic mice i.p. The sera from eight immune mice were pooled and transferred into four other syngeneic animals. Each mouse received approximately 2 cc of serum i.p. After 24 hr all recipient mice were infected with 5×10^6 amastigotes i.v. and the parasite burdens of their livers were determined after 12 days. The results of passive transfer experiments which are reported as the mean parasite burden of eight mice from two experiments are presented in Fig. 4. These results showed that the animals which received either immune spleen or enriched T cells had approximately a four-fold lower parasite burden in their livers than those of the control mice indicating that immune T and spleen cells passively transferred resistance to the recipient syngeneic animals. The mice which had receive normal spleen, anti-θ-treated B cells, or immune serum had parasite burdens similar to those of the normal controls.

DISCUSSION

Variation in susceptibility of mouse strains to a variety of bacterial, viral and protozoan infections has been well known for many years (Webster, 1933; Plant & Glynn, 1976; Preston & Dumonde, 1975; Bradley, 1977). Our studies, using various inbred strains of mice, confirm Bradley's findings that the susceptibility of mice to *L. donovani* infection is genetically controlled. Although little variation in susceptibility exists within inbred strains, a relatively large variation was observed between mouse strains. Our results strongly implicate the animal's immune system as being germane to the generation of specific host defences over and above those which may naturally exist. Acquired immunity developed as a result of prior i.v. or i.p. inoculation of *L. donovani* approximately 50 days after infection as evidenced by resistance to reinfection.

There are a number of infectious diseases, notably leishmaniasis, which exhibit a wide spectrum of clinical forms. In leishmaniasis these may range from the lupoid cutaneous type to the fulminating systemic infection (Turk & Bryceson, 1971). There seems to be a definite correlation between the differing clinical forms of leishmaniasis and the state of the cell-mediated reactivity towards the Leishmania antigen. In the lupoid form both the highly positive Montenegro reaction and the

hypersensitive granuloma indicate a strong cell-mediated immune response. Moreover, only very rare or no *L. tropica* can be found in the tissue (Ardehali *et al.*, 1979). At the other end of the spectrum, there is systemic leishmaniasis which is noted for its lack of cell-mediated immunity to Leishmania antigen. The reticuloendothelial organs of such patients are loaded with *L. donovani*. Kala-azar patients are not totally tolerant to *L. donovani*, however, because their serum contains a high level of antibody. In mice, as in humans, anti-Leishmania antibody as detected by indirect immunofluorescence is produced at an early stage of the infection. A rapid increase of antibody concentration in the serum was observed in the more susceptible strains, though anti-Leishmania antibody titres remained at very low level throughout the infection in the more resistant strains.

Since strains of mice differ in their parasite burden after infection with *L. donovani* it was predicted that differences in the development of cell-mediated immune responses in these strains of mice would also occur. In this study the footpad skin test was used as the parameter of the cell-mediated immune response towards *L. donovani* antigen. In those which developed a positive test, it was observed that delayed skin reactivity developed approximately 5 weeks or more after infection depending upon the strain of mice. This study indicates that antigen concentration in internal organs may play some role in the development of skin reactivity. The pattern of the development of skin reactivity among various strains of mice indicated that positive skin reaction appeared when the parasite concentration in the liver and spleen decreased to a certain level. Positive skin reaction did not develop in A/j mice, the less susceptible strain whose parasite burden did not increase considerably, or in the DBA/1 strain whose parasite level remained at a high level even at 130 days.

Although *L. donovani* induces both humoral as well as cell-mediated immune responses, a number of observations have suggested that cell-mediated immune responses play an important role in the protection of mice against *L. donovani* infection (Skov & Twohy, 1974). Indeed, an attempt to transfer immunity with spleen cells and T cells from mice whose parasite burden was reduced considerably from the peak was successful. After infection of mice with *L. donovani*, animals which had previously received immune spleen cells and T cells had a parasite burden in their liver four times less than the control animals. Mouse serum containing a high titre of anti-Leishmania antibody, however, was not able to confer immunity to the recipient animals.

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