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HYPER-Ia ANTIGEN EXPRESSION ON PERITONEAL MACROPHAGES CORRELATES WITH SEVERE *LEISHMANIA MAJOR* INFECTIONS IN MICE

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ABSTRACT: The role macrophages may play in the response of genetically susceptible and resistant strains of mice toward *Leishmania major* infections was studied. When infected subcutaneously, the highly susceptible Balb/c mice developed high levels of Ia^+ peritoneal macrophages which paralleled the development of severe, systemic disease. Whereas normal Balb/c mice had between 5-10% Ia^+ peritoneal macrophages, animals with severe leishmanial infections had 30-40%. The increased level of systemic macrophage activation in infected animals, which was detected as early as three weeks post infection, was not accompanied by an increase in total macrophage numbers in the peritoneal cavity. In contrast, the resistant C57B1/6 strain did not show increased levels of Ia^+ bearing macrophages following infection, even though mild, systemic infections did occur in these animals. Balb/c mice protected against serious, progressive infections by prophylactic treatment with cyclosporin A did not develop increased levels of Ia^+ peritoneal macrophages. *In vitro*, these phagocytes exhibited decreased phagocytic activity toward both *L. major* and *Candida albicans*, although their ability to destroy *L. major* did not seem to be impaired.

KEYWORDS: *Candida albicans,* cyclosporine, hyperactivation, Ia, *Leishmania major, Listeria monocytogenes,* macrophages, and mice.

INTRODUCTION

Depending on their genetic background, inbred mice vary markedly in their susceptibility to disease caused by *Leishmania major*. The Balb/c mouse strain is the most susceptible, with many other strains, including the C57B1/6 mice, showing considerable resistance (Mitchell, *et al.,* 1987). In general, the ability to mount an effective cell-mediated immune response appears to be responsible for recovery from infection, whereas antibodies seem to play little or no role in controlling the disease (Liew, *et al.,* 1984; Sadick, *et al.,* 1987). Although the reasons for the exquisite susceptibility of Balb/c mice toward this parasite are not clear, they do not include a failure to recognize or respond to infection. On the contrary, susceptible mice produce high antibody titers (Olobo, *et al.,* 1980; Sadick, *et al.,* 1987) and a rapid, hyper-expansion of activated $L3T4^+$ lymphocytes (Milon, *et al.,* 1986), which can transfer delayed-type hypersensitivi-

ty without establishing effective immunity (Dhaliwal, *et al.,* 1985). These cells have been shown to exacerbate disease when transferred to *L. major*-infected syngeneic mice (Titus, *et al.,* 1984). Several researchers (Milon, *et al.,* 1986; Modabber, *et al.,* 1987) have suggested that Balb/c mice are hyper-reactive rather than deficient in their responses to leishmanial infection. Their view is supported by the fact that various immunomodulatory or even immunosuppressive procedures, which affect a variety of cell types, render these mice more resistant to serious disease following infection. These procedures include prophylactic treatment with sublethal irradiation, anti-IgM antibody, anti-L3T4 antibodies, or, in our own laboratory, cyclosporin A (Howard, *et al.,* 1981; Sacks, *et al.,* 1984b; Titus, *et al.,* 1985; Behforouz, *et al.,* 1986). If given for only one week at the time of infection, cyclosporin A protects Balb/c mice from severe infections and renders them immune to reinfection (Behforouz, *et al.,* 1986).

Not only is there evidence of hyperactivity in the early stages of the infection in Balb/c mice, but there is also considerable symptomology and immunopathology related to an inappropriate immune response as the disease becomes progressively more severe. Lymphadenopathy, splenomegaly, hypergammaglobulinemia, and autoimmune antibodies characterize the late stages of the disease both in susceptible mice and in humans suffering from visceral leishmaniasis (Veres, *et al.,* 1977; Rezai, *et al.,* 1978; Djoko-Tamnou, *et al.,* 1981; Kharazmi, *et al.,* 1982; Behforouz, *et al.,* 1983).

The underlying reason for this excessive, inappropriate response to leishmanial infection is not clear but may be related to a faulty macrophage-T cell interaction. In recent years, the important immunoregulatory role of macrophages and other accessory cells has become increasingly clear (Unanue and Allen, 1987). A sensitive measure of the level of accessory cell activation and the ability to stimulate antigen-specific lymphocytes is the expression of the Class II MHC antigens, Ia, on the cell surface. Indeed, enhanced expression of Ia antigen on accessory cells has been associated not only with an active immune response to an acute infection in mice (Beller, *et al.,* 1980) but also to the immunopathology observed in several models of murine autoimmunity (Lu and Unanue, 1982; Monroe, *et al.,* 1985; Rosenberg and Kotzine, 1989) and to the dysregulation or dysfunction observed in AIDS (Buhl, *et al.,* 1993).

In order to determine the possible role of macrophage activation in the hyperactivity of Balb/c mice toward *L. major*, the level of Ia expression on peritoneal macrophages was determined in infected mice of both the Balb/c and the resistant C57B1/6 strains over time. Although C57B1/6 mice do develop mild systemic infections, including lymph node and splenic involvement, they maintain normal levels of Ia^+ peritoneal macrophages throughout the infection. Balb/c mice, on the other hand, develop an abnormally high level of Ia^+ peritoneal macrophages early after infection, and the number continues to increase slowly throughout the infectious process. However, Balb/c mice protected from serious disease by prophylactic treatment with cyclosporin A continuously maintain normal levels of Ia^+ peritoneal macrophages. Interestingly, the Ia^+ macrophages

retrieved from untreated, infected Balb/c animals with a heavy parasite burden did not harbor any *Leishmania* parasites but appeared to be functionally impaired with a generalized defect in phagocytic capacity.

MATERIALS AND METHODS

Animals. The Balb/c and C57B1/6 mice used in this study were raised in our own breeding facilities from stock originally purchased from Harlan-Sprague Dawley, Inc. (Indianapolis, Indiana). Experiments were performed with animals of either sex, 8 to12 weeks in age.

Culture of *Leishmania major* and Infection of Mice. *Leishmania major* (Freidlin strain, NIH) was kindly provided by Dr. D. Sacks of NIH and was grown in medium 199 with 20% fetal bovine serum at 24° to 26° C. After 5 to 6 days, these promastigotes reached stationary phase and, as demonstrated by Sacks and Perkins (1984a), were highly infective for Balb/c mice. All mice were infected in the left hind foot pad with 1 x 10^6 stationary phase promastigotes of *L. major*. The size of the swelling or lesions which developed was estimated by measuring the diameter of the center of the foot (top to bottom) using vernier calipers. By 8 to 12 weeks after infection, most of the untreated Balb/c animals had lost their infected foot due to destructive necrosis.

Cyclosporin A Treatment. Cyclosporin A (CsA) was generously provided by Sandoz Ltd. (East Hanover, New Jersey) in the oral solution, Sandimmune®. For oral treatment, which was found to cause less toxicity in treated mice, the animals were given the undiluted oral solution (100 mg/ml) at 150 mg/kg twice daily for seven consecutive days beginning one day before infection with *L. major*. Depending on the weight of the mouse, between 0.3-0.5 ml of the Sandimmune solution was delivered orally using a 200 µl pipettor with a plastic tip. Infected control animals were given olive oil in the same manner.

Peritoneal Macrophage Collection. Peritoneal exudate cells (PEC) were collected aseptically by lavage from normal or infected mice using RPMI 1640 medium with 5% fetal bovine serum (FBS) and antibiotics. The cells were washed once, and their concentration in the above medium was adjusted to approximately 1-3 x 10^6 cells/ml. The cells were then cultured for 2 to 3 hr in 1 ml volumes in 24-well microtiter plates (Falcon 3047) containing a 9 x 9 glass coverslip (Bellco Biotechnology, Vineland, New Jersey). The microtiter plates were centrifuged for 5 minutes at 500 g to aid the adherence of the cells to the coverslips. Following a 2-hour incubation at 37° C in 5% CO_2, the coverslips with the PEC were removed by forceps and gently but exhaustively washed with saline from a squeeze bottle to remove any non-adherent cells. Cells collected in this way were found to be > 98% macrophages by staining for the presence of alpha-acetate-esterase (Manca, *et al.*, 1985), using a staining kit obtained from Sigma Chemical Company (St. Louis, Missouri).

Detection of Ia Antigens. Ia antigens on peritoneal macrophages of either Balb/c or C57B1/6 mice were detected by an indirect immunofluorescence assay using either the monoclonal antibody MK.D6 specific for Ia^d or the mon-

oclonal antibody 34-5-3 specific for Ia^b and an affinity purified fluorescein conjugated $F(ab')_2$ rabbit anti-mouse Ig (Pel-freeze Biologicals, Rogers, Arkansas). Both monoclonal antibodies were used as 1:10 dilutions of culture supernatants (diluent: medium plus 10% rabbit serum plus 10 mg/ml bovine serum albumin) kindly provided by Dr. E. Unanue (Washington University, St. Louis, Missouri). The conjugate was used at a final concentration of 85 µg/ml. The staining procedure used was essentially that of Beller, *et al.* (1980) with slight modification. Briefly, the washed coverslips coated with the adherent peritoneal cells were placed in clean microtiter wells with approximately 1 ml of freshly prepared 1% paraformaldehyde for 15 minutes at room temperature. Then, the coverslips were washed with saline and placed cell side down within the depression of a hanging drop slide containing 20 µl of either anti-Ia^b, anti-Ia^d, or diluent alone. These slides were placed on ice for 30 minutes, washed, and placed cell side down in the depression of a clean hanging drop slide containing 20 µl of the fluorescein conjugate on ice for another 30 minutes. The coverslips were washed again and placed singlely with the cell side up in the wells of microtiter plates containing 1 ml of phosphate buffered saline until the time of examination. The macrophages were examined at 400x with both bright light illumination and epifluorescent illumination using a Zeiss microscope after placing the coverslips cell side down in PBS in the depression of a hanging drop slide. In all experiments, duplicate coverslips were prepared for each experimental animal, and a total of at least 800 macrophages was counted on both coverslips as well as the number of brightly stained cells with distinct perimeters which were considered Ia positive. The specificity of the reaction of the anti-Ia and the fluorescein labeled $F(ab')_2$ anti-Ia was determined in the following manner. First, some of the resident peritoneal macrophages from normal C57B1/6 ($H\text{-}2^b$) were stained when anti-Ia^b antibodies were used but were not stained when anti-Ia^d antibodies were applied. Likewise, positive staining of macrophages from Balb/c animals ($H\text{-}2^d$) occurred only when anti-Ia^d but not anti-Ia^b antibodies were used. Second, the $F(ab')_2$ conjugate by itself did not stain the cells when diluent was used in place of anti-Ia antibody. Finally, Ia-positive macrophages represented only a small fraction of the adherent cells from normal animals, suggesting that the uptake of conjugate was a cell-specific reaction.

Spleen, PEC, and Lymph Node Involvement. The popliteal lymph nodes from the left leg and the spleens of infected mice were removed aseptically following collection of the PEC and were weighed. The spleens were placed in a pre-weighed sterile petri dish with HBSS, weighed, and then ground between two frosted-end glass slides. The resulting cell suspension was pelleted, resuspended in 10 ml of medium 199 plus 20% FBS for culture of *Leishmania*, and allowed to incubate at 25° C in a 25 cm^2 culture flask. Occasionally, suspensions of PEC collected from infected mice were also cultured and incubated in the same manner in 5 ml of the leishmanial culture medium. All cultures were checked daily for the presence of *L. major* promastigotes by a thorough microscopic

examination of a 20 µl ml sample. Negative cultures were discarded after 14 days.

Anti-Microbial Activity of Macrophages. PEC were collected aseptically (see above), washed, and their concentration was adjusted to 3 x 10^6/ml in medium plus 10% FBS. The cells were added in 400 µl volumes to the wells of eight-well Lab-Tek® culture slides. The cells were allowed to adhere overnight at 37° C in %5 CO_2 and then washed several times to remove non-adherent cells. Duplicate cultures of adherent macrophages were then infected with 300 µl of either 1 x 10^7/ml stationary phase *L. major* promastigotes in medium 199 with 20% FBS at 37° C in 5% CO_2 for two hours or 5 x 10^6/ml *Candida albicans* in RPMI medium plus 10% FBS for one hour. Following incubation, any non-ingested microbes were removed by extensive washing of the culture wells, and the cells were reincubated. After either two hours or several days in culture, the plastic wells were removed, and the slides were fixed in methanol, Giemsa stained, and examined microscopically for intracellular yeast or amastigotes. Both the percentage of infected macrophages and the number of parasites per macrophage were determined for each experimental group. A minimum of 400 macrophages were examined per culture, and the calculations were made as follows:

$$\frac{\text{number of macrophages bearing microbes}}{\text{number of total macrophages counted}} \times 100 = \text{Percent infected macrophages}$$

$$\frac{\text{number of intracellular microbes observed}}{\text{number of total infected macrophages}} \times 100 = \text{Mean number of microbes per infected microphage}$$

RESULTS

Hyper-Ia^d Expression in Infected Balb/c Mice. In preliminary experiments, peritoneal macrophages obtained from four Balb/c mice infected sixteen weeks previously with *Leishmania major* were found to express abnormally high levels of Ia^d in comparison to age-matched controls (i.e., 47 ± 11% vs 12 ± 4%). Although older mice generally have somewhat higher levels of Ia$^+$ peritoneal macrophages than younger mice (Beller, *et al.,* 1980), in repeated trials nearly half of the adherent cells obtained from the diseased animals consistently expressed Ia$^+$. The spleen weights of the diseased animals were nearly eight times heavier than normal with three times the monocytic cell number. Although the percentage of Ia-bearing macrophages detected in the spleens of the infected animals was high (> 50%), this value falls within the reported normal range (Cowing, *et al.,* 1978), and the percentage of Ia$^+$ adherent splenic cells from normal animals did not differ perceptibly from their percentage in the diseased animals.

In order to determine whether the activated state of the peritoneal macrophages was a late-developing feature of severe disease, Balb/c mice were infected

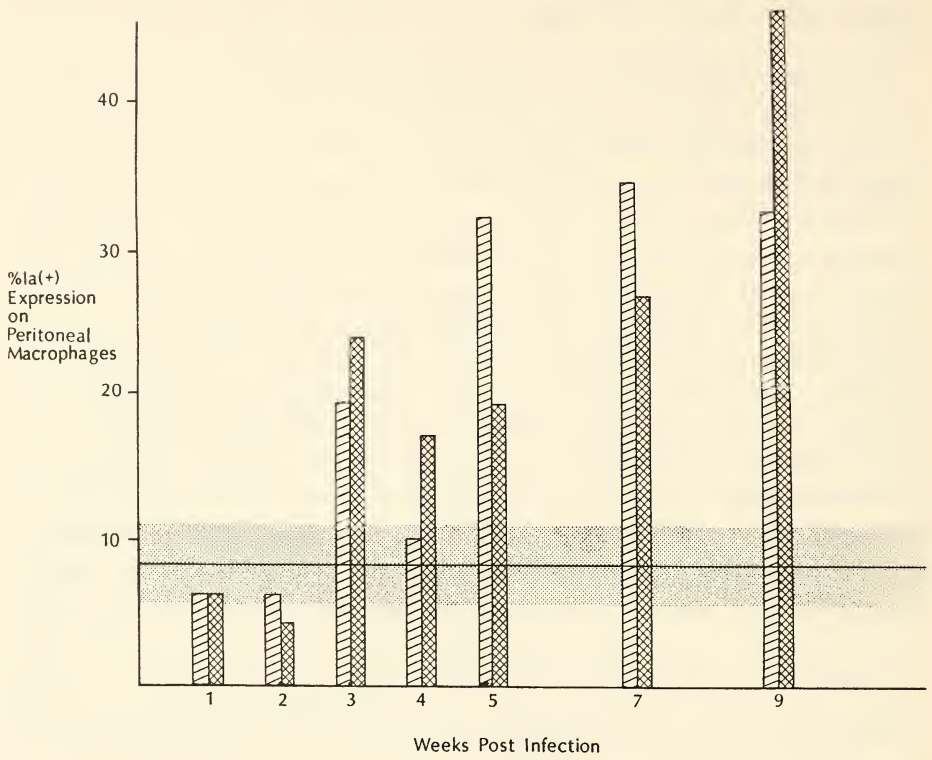

Figure 1. Balb/c mice infected with *Leishmania major* have increasing levels of Ia positive peritoneal macrophages. At various time intervals following infection with 1 x 10^6 promastigotes, two mice were sacrificed, and the percentage of Ia-bearing macrophages was determined for each (▨,▩). The mean ± standard deviation of eight normal, aged-matched controls is shown as the horizontal line within the shaded area.

with *L. major,* and the percentage of Ia^+ macrophages was determined at various time intervals following infection. Although the number of macrophages expressing Ia remained within the normal range of age matched controls for two weeks following infection, at three weeks post infection the individual mice began to show elevated levels of Ia expression (Figure 1). Whereas the level of Ia^+ cells was greater than 30% in the infected animals by week 9, the levels in normal, age-matched controls remained between 5% and 11%. At no time could *L. major* be recovered from the peritoneal macrophages of infected Balb/c animals under conditions permissive to outgrowth of *Leishmania* amastigotes, even in severely ill animals having a heavy parasite burden in the spleen (data not shown).

Comparison of Macrophage Activation in Resistant and Susceptible Mice During Infection. Although C57Bl/6 mice developed infections with *L. major* as evidenced by the development of some foot pad swelling, significant lymphadenopathy, mild splenomegaly, and rapid metastasis of *L. major* to the

Table 1. Correlation of the expression of Ia on peritoneal macrophages with various disease parameters over time in Balb/c and C57Bl/6 mice infected with *L. major*[1].

Mouse Strains	Days Post Infection	Number of Animals	% Ia$^+$ Peritoneal Macrophages[2]	Foot Pad Size (cm)[2]	Lymph Node Weight (mg)[2]	Spleen Weight (g)[2]	*L. major* Positive Spleen Culture
Balb/c	0	6	8.2 ± 2.7	0.23 ± 0.01	1	0.12 ± 0.02	-
C57B1/6		4	6.9 ± 1.0	0.23 ± 0.02	1	0.07 ± 0.01	
Balb/c	7 - 14	7	6.8 ± 1.8	0.30 ± 0.05	7 ± 6	0.11 ± 0.02	4/7
C57B1/6		3	8.1 ± 0.7	0.35 ± 0.07	1	0.14 ± 0.07	2/3
Balb/c	21 - 30	12	13.1 ± 4.9*	0.43 ± 0.1	20 ± 10	0.11 ± 0.02	12/12
C57B1/6		8	7.2 ± 1.9	0.35 ± 0.07	28 ± 18	0.13 ± 0.09	8/8
Balb/c	35 - 42	6	20.2 ± 7.2*	0.67 ± 02	71 ± 26	0.28 ± 0.12	6/6
C57B1/6		4	6.4 ± 1.5	0.30 ± 0.01	37 ± 4	0.10 ± 0.03	4/4
Balb/c	49 - 60	6	27.2 ± 5.9**	NF[3]	98 ± 17	0.44 ± 015	6/6
C57B1/6		4	9.5 ± 0.7	0.28 ± 0	45 ± 18	0.05 ± 0.01	4/4
Balb/c	63 - 84	7	31.8 ± 12.7*	NF[3]	140 ± 48	0.55 ± 0.23	7/7
C57B1/6		6	7.5 ± 3.35	0.26 ± 0.02	55 ± 5	0.06 ± 0.02	5/6

[1] These results represent three experimental trials. The mice were infected and followed as described in the *Materials and Methods*.
[2] Mean ± Standard Deviation.
[3] No foot due to necrotic destruction.
* $p < 0.01$; Unpaired Student's t testing was used for analyzing the difference between the mean percentages of Ia positive cells in the two groups of mice.
** $p < 0.001$.

spleen, the percentage of peritoneal macrophages which expressed Ia^b never rose over normal levels throughout the infection period (Table 1). Interestingly, as late as 2 to 3 months post infection, when foot pad size and spleen weight had essentially returned to normal, 5/6 of the C57Bl/6 animals examined still harbored residual, viable *Leishmania* in the spleen, and the draining popliteal lymph nodes were still enlarged. Thus, resistance to and successful recovery from this parasite occurs in spite of a chronic, metastatic infection.

In Balb/c mice, the development of lymphadenopathy, splenomegaly, and foot pad swelling was nearly parallel to that in the C57Bl/6 mice for one month post infection. In fact, early in infection, average spleen and lymph node weights were somewhat larger in the C57Bl/6 mice than in the Balb/c mice. In contrast, the increase in Ia expression by peritoneal macrophages was again observed to appear as early as three weeks post infection in the susceptible mice, considerably before the appearance of splenomegaly, severe foot pad swelling, or ulceration. The average level of Ia$^+$ macrophages also appeared to increase as the infection progressed in the Balb/c mice. However, no consistent rise or fall in the total numbers of cells in the peritoneal cavities of either group of mice was noted during infection (data not shown).

Ia Expression and Disease Progression in Cyclosporin A Protected Balb/c Mice. In order to see whether or not hyper-Ia expression occurs during *L. major*

infections in susceptible Balb/c animals protected from serious disease, two groups of Balb/c animals were infected with *L. major*, and one group was prophylactically treated with CsA either subcutaneously or orally for seven days. Infected mice treated with CsA developed minimal foot pad swelling, minor spleen enlargement, and reduced lymphadenopathy when compared to the control mice (Figure 2). In addition, the percentage of Ia^+ peritoneal macrophages in these protected animals did not rise above normal levels throughout the ten-week post infection test period. Indeed, the pattern of disease symptoms and level of Ia on peritoneal macrophages observed in these CsA-treated animals was very similar to that observed in infected C57Bl/6 animals. In these studies, non-protected Balb/c animals once more showed a very early increase in the percentage of Ia^+ macrophages in response to *L. major* infection. As with the other infected groups, *L. major* could be cultured from the spleens of the drug-treated mice as early as one week and as late as three months post infection (data not shown).

As the above studies were continued for only ten weeks, several animals treated with the drug were maintained and compared to non-treated animals several months post infection. Although animals infected for three to four months without CsA treatment were severely diseased and had an average level of Ia-bearing macrophages of > 35%, the animals prophylactically protected with cyclosporin appeared essentially normal, and their level of Ia^+ peritoneal macrophages remained normal (data not shown.).

Phagocytic and Microbicidal Ability of Balb/c Mice Infected with *L. major*. Beginning as early as one month post infection, cultured macrophages obtained from the peritoneal cavity of *L. major*-infected mice were found to be consistently less active phagocytically toward *L. major* than normal PEC (Table 2). Compared to macrophages obtained from normal age-matched Balb/c animals, the percentage of cultured macrophages from infected animals bearing amastigotes following a 2-hour incubation with *L. major* was lower than in normal PEC cultures. This impairment was also observed in phagocytosis of the non-specific antigen, *Candida albicans* (Figure 3). The numbers of microbes (both *L. major* and *C. albicans*) phagocytosed per infected cell (rather than total cells) was also considerably lower throughout the infection period , indicating that all cells, even those capable of phagocytosis, had a generalized lower capacity for phagocytosis. Although the phagocytic potential of the macrophages from infected animals was less than normal, the leishmanicidal capacity *in vitro* appeared to be unaltered. In repeated trials, peritoneal macrophages from infected animals were found to be able to reduce their parasite burden at rates comparable to those of the normal macrophages. Figure 4 represents the results of one of these trials and demonstrates that, although the mean number of *L. major* taken up per macrophage was > 3 for the normal animals after 3 hours, the macrophages from infected animals had taken up < 1.5 parasites per cell. After four days incubation, however, cells from both the normal and diseased animals had reduced their parasite burden by at least 87%. Of course, the original para-

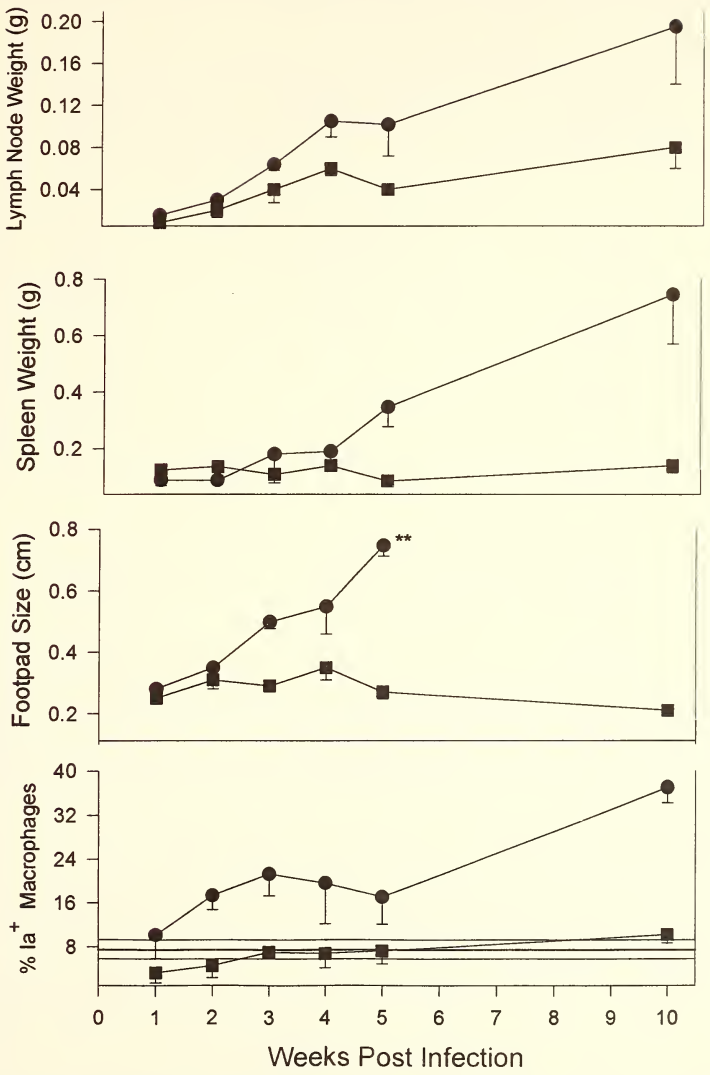

Figure 2. Infected Balb/c mice protected against severe disease by prophylactic treatment with CsA do not develop increased levels of Ia-bearing peritoneal macrophages. The figure reflects the combined results of three separate trials. In each trial, two groups of at least 12 mice each were infected with 1 x 10^6 *Leishmania major* in the left hind foot pad. One group received CsA for seven days as described in the *Materials and Methods* (■); the other infected group was not treated (•). At different intervals for a period of ten weeks, two to four mice in each group were sacrificed, and determinations of spleen and lymph node weight, foot pad size, and the percentage of Ia-bearing macrophages were made. Each point on the figure represents the mean ± standard deviation of the determinations for each time period. No foot pad measurements could be taken past this time point (**) because of necrotic destruction of the foot. The mean percent Ia^+ macrophages from 8 uninfected animals ± standard deviation is shown as the horizontal line with two surrounding lines. The normal values for Balb/c foot pad size and spleen weight are 0.25 ± 0.01 and 0.1 ± 0.01, respectively.

Table 2. Phagocytic capacity of peritoneal macrophages from infected Balb/c mice toward *L. major*[1].

Months Post Infection[2]	Source of Macrophages	% Macrophages Bearing *L. major*[3]	*L. major* /Infected Macrophage[4]
Experiment 1			
1	Normal	44 ± 7	2.1 ± 0.3
	Infected	18 ± 2	1.5 ± 0.3
2	Normal	50 ± 1	2.7 ± 0.7
	Infected	29 ± 8	2.4 ± 0.1
3	Normal	73 ± 2	3.7 ± 0.6
	Infected	51 ± 2	2.6 ± 0.6
4	Normal	43 ± 5 3	1 ± 0.2
	Infected	23 ± 4 1	8 ± 0.4
Experiment 2			
1	Normal	67 ± 6	2.6 ± 0.2
	Infected	45 ± 7	2.0 ± 0.1
3	Normal	59 ± 1	4.0 ± 0.9
	Infected	21 ± 13	2.3 ± 0.6

[1] PEC from each of 3 animals per group were individually adjusted to 1-2 x 10^6 cells/ml and allowed to adhere overnight in duplicate in Lab-Tek culture slides. The washed cells were infected with 1 x 10^7/ml *Leishmania major* for a period of 2 hours at 37° C in 5% CO_2 and were washed thoroughly to remove non-phagocytosed microbes. The slides were immediately air dried and stained with Geimsa before microscopic examination.

[2] Balb/c animals were infected with *L. major* as described in the *Materials and Methods*. The normal animals were age-matched Balb/c mice.

[3] The percent of stained macrophages bearing intracellular *L. major* was determined as described in the *Materials and Methods*. The results are shown as the mean percent infection of cells from 3 animals per time point. The PECs from infected animals displayed at least a 2-fold higher level of Ia^+ cells at all time points over those from normal animals.

[4] The number of *L. major* per infected macrophages was determined as described in the *Materials and Methods*. The results are shown as the mean number of amastigotes per infected cell ± SD per time point.

site burden/cell for the cells of the infected host was smaller, and the cultures were incubated for several days outside the potentially inhibitory surroundings of the diseased animal.

DISCUSSION

The data reported here demonstrate that Balb/c mice infected with *Leishmania major*, which are destined to develop severe, overwhelming infections, also develop abnormally high levels of Ia positive peritoneal macrophages early in the infection. This apparent systemic activation of macrophages distant from the original lesion continues for the remainder of the infection and tends to increase over time. In contrast, genetically resistant mice do not develop increased levels of Ia-bearing macrophages at any time in response to the parasite even though they develop mild systemic infections. Interestingly, the percentage of

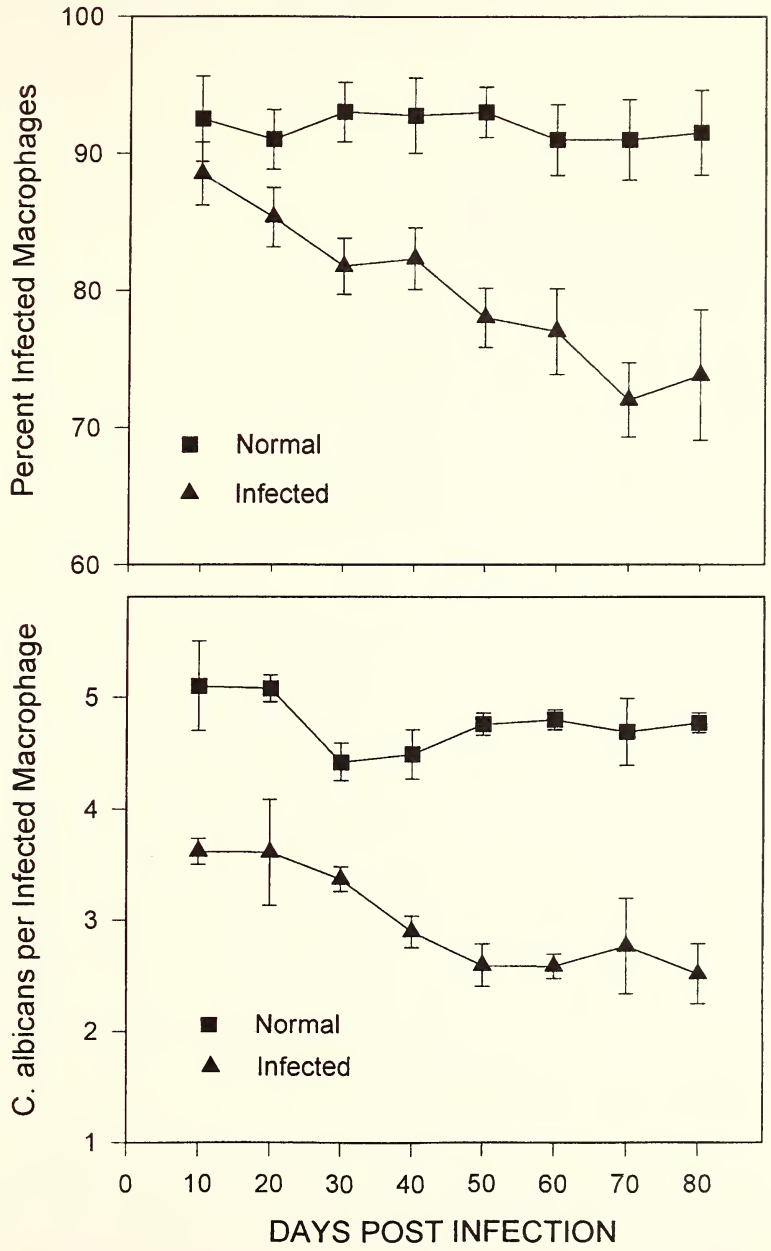

Figure 3. Peritoneal macrophages from *L. major*-infected animals exhibit a general defect in phagocytosis. Adherent PEC from each of three *L. major*-infected or normal age-matched Balb/c animals were cultured, infected, and stained individually in duplicate as described in Table 2 except that the washed cells were exposed to 5 x 10^6/ml *Candida albicans* rather than *L. major* for a period of 2 hours. The percentage of stained macrophages bearing intracellular *C. albicans* was determined as described in the *Materials and Methods*. The results are shown either as the mean percent infection of cells from three animals (A) or the mean number of *C. albicans* per infected macrophage (B) per time point ± standard deviation.

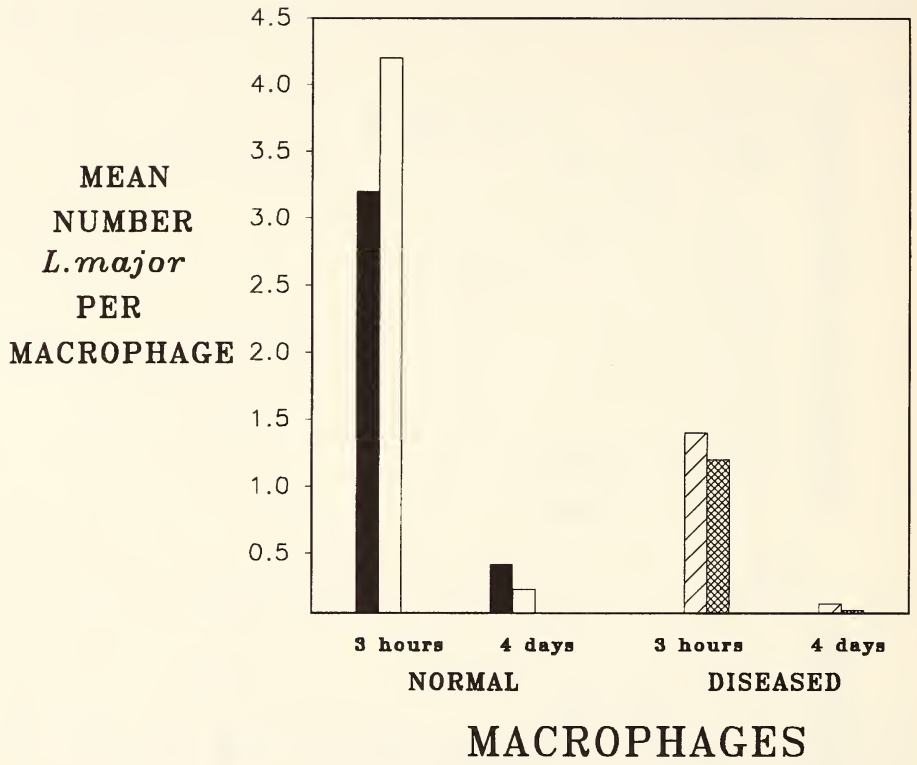

Figure 4. Peritoneal macrophages from *L. major*-infected animals do not appear to have impaired leishmanicidal capacity upon *in vitro* cultivation. Adherent PEC from each of two animals infected three months previously (▨,▩) and two normal animals (■,□) were cultured individually in quadruplicate as described in Table 2 and infected and incubated with 1 x 10^7/ml *L. major* for a period of three hours. After a thorough washing, duplicate slides for each animal were immediately stained and examined microscopically, while two others were reincubated for a period of four days at 37° C in 5% CO_2 before staining and examination. The mean number of *L. major* amastigotes per macrophage was determined as described in the *Materials and Methods*.

MHC class II bearing cells did not perceptibly increase in the spleens of the infected Balb/c animals, even though considerable splenic and lymph node enlargement did occur during the infection. The rise in Ia expression in infected Balb/c animals does not appear to be an irrelevant nor trivial difference between the two strains of mice, since Balb/c mice protected from serious disease by cyclosporin treatment also fail to develop increased levels of Ia-bearing macrophages at any time following infection. Thus, there appears to be a direct correlation between progressive disease and systemic hyper-Ia expression.

The systemic increase in Ia expression in mice has been observed in a number of disease states, including *Listeria monocytogenes* infection (Beller, *et al.*, 1980), autoimmune disease (Lu and Unanue, 1982; Rosenberg and Kotzin,

1989), and tumor-bearing animals (Evans, *et al.,* 1986). In all cases, the increased level of Ia-bearing macrophages was associated with an ongoing, active, immune response, and, in the case of infection and autoimmune models, the hyper-Ia expression was associated with severe disease. Recently, Buhl, *et al.* (1993) reported that class II MHC molecules are expressed more frequently and heavily on alveolar macrophages from asymptomatic HIV-infected individuals; they suggested that this upregulation was further evidence of the immune derangement, dysfunction, and deficiency observed in the pre-AIDS condition and in AIDS itself (Edelman and Zolla-Pazner, 1989). The development of abnormally high levels of Ia expression on peritoneal macrophages in mice with severe leishmanial infections may also indicate a role for these activated macrophages in the pathogenesis of murine leishmaniasis. Although the reason(s) for the failure of certain strains of mice to inhibit leishmanial infections is (are) still unclear, the failure may be related to an inappropriate stimulation of certain helper T lymphocyte subpopulations, which may lead to an exacerbated, rather than controlled infection (Titus, *et al.,* 1984; Louis, *et al.,* 1987). The preferential induction of certain subpopulations of T cells may be related either causally or secondarily to the presence of systemic, hyperactivated macrophages.

The results obtained in this study show that the level of macrophage activation in the peritoneum of an infected susceptible animal is abnormally high and becomes so relatively early in the infection before severe symptoms are evident. Whether or not this abnormality is central to the immune defect in these animals or is a consequence of stimulation by an activated T cell population is unclear. In murine leishmaniasis, the Th2 subpopulation of $L3T4^+$ cells appears to be preferentially stimulated in susceptible animals, whereas the Th1 helper subset is found to expand in resistant animals (Heinzel, *et al.,* 1989). The Th2 subset appears to uniquely secrete IL-4, IL-5, whereas the Th1 subset secretes IL-2 and γ-IFN (Mosmann, *et al.,* 1986). As γ-IFN is thought to be the central lymphokine responsible for activating macrophages to kill *Leishmania* amastigotes, the presence of a Th2 T cell response leading to IL-4 rather than γ-IFN production would be counterproductive for the murine host. Indeed, investigators have shown that there is a reciprocal expression of interferon-γ or IL-4 during resolution or progression of murine leishmaniasis (Heinzel, *et al.,* 1989). Perhaps, the hyper-Ia antigen expression noted in this study of infected Balb/c animals is induced and maintained by the expanded Th2 clone which produces an inappropriate balance of critical lymphokines, including an excessive amount of IL-4. This lymphokine has been shown to be a potent inducer of MHC Class II expression of lymphoid cells, including macrophages (Stuart, *et al.,* 1988; Crawford, *et al.,* 1987; and TeVelde, *et al.,* 1988).

Several investigators (Preston, *et al.,* 1978; Colle, *et al.,* 1983; Modabber, 1987) have suggested that an underlying reason for the susceptibility of Balb/c animals is hyper-reactivity toward the parasite, which leads to a generalized inflammatory response characterized initially by an inappropriate immune reactivity to *Leishmania* as well as other antigens. The fact that many types of pro-

phylactic immunosuppressive treatments, which decrease the activity or numbers of B cells, T cells, and even macrophages, induce some level of resistance in susceptible animals offers support for this view (Howard, *et al.,* 1981; Sacks, *et al.,* 1984b; Titus, *et al.,* 1985; Behforouz, *et al.,* 1986). Indeed, in many respects, the late stages of leishmanial disease in susceptible animals parallels that seen in MRL/1pr autoimmune mice. MRL/1pr mice develop spontaneous autoimmune disease with the production of a constellation of autoantibodies, hypergammaglobulinemia, increased helper cell activity, and massive spleen and lymph node enlargement. These mice also display an increased, systemic expression of Ia^+ on their peritoneal macrophages (Lu and Unanue, 1982). Hypergammaglobulinemia, splenomegaly, lymphadenopathy, and autoantibodies characterize systemic leishmaniasis in mouse and man (Djoko-Tamnou, *et al.,* 1981; Kharazmi, *et al.,* 1982). Lu and Unanue (1982) have suggested that lymphokine secreting T cells in MRL/1pr mice activate a large number of macrophages to express Ia which, in turn, may enhance T cell activities thus establishing a cycle of macrophage-T cell interactions that aggravate the hyperactive autoimmune responses and lymphoproliferation. A very similar situation may also occur in systemic leishmaniasis with an initial hyper-reactivity to antigen expanding into a destructive autoimmune response. The fact that the levels of Ia^+ macrophages in the peritoneum become elevated during infection only in susceptible animals may help us understand the etiology of the autoimmune component of leishmanial disease. Our finding that this population of heightened Ia-bearing cells was also severely compromised in their phagocytic ability toward both *L. major* and *C. albicans* may also be related to the profound failure to control the systemic spread and proliferation of the parasite in susceptible animals. Although these cells, shortly after removal from the host, were less capable phagocytically, their microbicidal capability after several days in culture appeared normal. Perhaps these cells regain normal cidal function following removal from the environment of the infected host and several days of *in vitro* culture. *In vivo*, the macrophage dysfunction may also extend to leishmaniacidal activity, particularly as the internal milieu of the host may include inhibitory lymphokines such as IL-4, which have been shown to impair phagocytic activity (Lehn, *et al.,* 1989). Panosian and Wyler (1983) reported that peritoneal macrophages from the genetically resistant C57Bl/6 mice infected with *L. major* are phagocytically less active toward *L. major* but not latex beads or opsonized SRBC during the first month of infection. This defect resolved as the animals recovered from their infections. The abnormally low phagocytic activity observed in the present study in susceptible Balb/c animals appears to be significantly different; i.e., there is a generalized reduction in phagocytosis, and there is no recovery. This defect in peritoneal macrophage activity concurrent with excessive Ia expression may reflect the systemic, non-protective hyperactivation observed in these animals.

The ability of cyclosporin A to prophylactically protect susceptible animals from severe infection is somewhat surprising in that CsA has been shown to inhibit production of both IL-2 and γ-IFN; it would seem that this drug would

exacerbate rather than promote healing of the infection. In order to protect animals, however, cyclosporin A is given only briefly at the beginning of the infection. If given later, following appearance of the lesion, CsA does indeed exacerbate the disease (Behforouz, *et al.,* 1986). The ability of a brief, intense treatment with cyclosporin A to promote a protective immune response only at a very early stage in infection would seem to indicate that the susceptibility of Balb/c mice to *L. major* results primarily from a flawed induction phase of the immune response. Thus, prophylactic CsA treatment may suppress irreversibly the hyperactive or unbalanced immune response initially induced by first exposure to leishmanial antigens. This effect might be accomplished by the selective suppression of the Th2 helper subset which appears to inappropriately expand in these animals or by an overall suppression of all T helper activity early in the infection. One of the critical consequences of this T cell suppression might be the suppression or modulation of MHC class II antigens, namely Ia, on the surface of the antigen-presenting cells. Indeed, CsA has been shown to block the induction of MHC class I and II antigens in experimental GVHD (Autenreid and Halloran, 1985) and to inhibit increased expression of MHC products in MRL/1pr mice (Halloran, *et al.,* 1988). Just as in the leishmanial disease studied here, CsA treatment in both GVHD and MRL/1pr leads to decreased disease symptoms and immunopathology (Van Bekkum, *et al.,* 1980; Mountz, *et al.,* 1987). The authors also attempted to alter the outcome of infections by treating susceptible mice with intraperitoneal injections of an anti-Ia^d monoclonal antibody (D3137) shown in another investigation (Kurlander and Jones, 1987) to increase resistance toward *Listeria monocytogenes*. No protection was observed, however, when the anti-Ia antibody was given to mice at various times within the first three weeks of infection (data not shown). Indeed, the antibody treatments appeared to have an exacerbating effect when given within the first week of infection. Perhaps the route, dose, and timing of the antibody treatments were inadequate in light of the generalized lymphoproliferative response occurring in the animals.

Evidence collected in this study indicates that early CsA treatment somehow permanently prevents both the development of serious disease and hyper-Ia expression in infected susceptible mice. Indeed, the two phenomenon seem inextricably linked. Whether or not the inhibition of Ia expression by CsA is indeed the cause, or simply a result, of enhanced resistance in the Balb/c mice is unclear. There does appear, however, to be a correlation between an early, sustained level of peritoneal macrophage Ia expression accompanied with a generalized defect in phagocytic activity and the development of severe, progressive murine leishmaniasis.

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