LEISHMANIAL INFECTION:
IN BALB/C MICE AND C57BL/6 MICE

AN HONORS THESIS (ID 499)

BY

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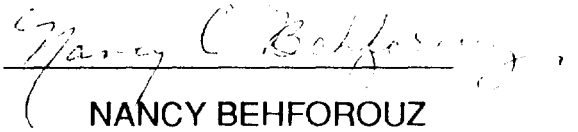

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INTRODUCTION

In this study, we compare and contrast various parameters of infection between Balb/c and C57BL/6 mice inoculated with Leishmania major. The Balb/c are susceptible to Leishmania major and eventually die from this infection. The C57BL/6 mice are resistant to serious disease, but do become briefly infected. We measured the progress of infection by examining spleen weight, lymph node weight, presence of L. major in the spleen, and the percentage of Ia positive macrophages in the peritoneal exudate at various times after infection in both strains of mice. From these experiments, we hope to better understand the parameters comprising an effective immune response against L. major, particularly the role of macrophage activation in both mouse strains. In previous experiments in our lab it has been determined that following infection, the Balb/c mice develop lymphoadenopathy, increased foot pad size with the eventual lose of the foot, metastasis of L. major to the spleen, and increased level of positive Ia peritoneal exudates.

In other experiments it has been shown that increased levels of Ia expression is related to an active immune response to certain intra-cellular parasites and autoimmune processes in genetically abnormal mice. For instance, Beller, Kiely, and Unanue demonstrated that A/St mice showed an increased number, from 7.1% to 69%, of Ia positive peritoneal macrophages when infected with Listeria monocytogenes. They also demonstrated that this increase in Ia was only induced when antigen specific T-cells were involved. (1) One way that T-cells are thought to induce Ia on the surface of macrophages is through the secretion of gamma interferon.(6)

MRL mice, a strain that becomes autoimmune, display an increase in their percentage of positive Ia peritoneal macrophages with the progression of autoimmunity. At 3 months the mice show no sign of autoimmunity. At this time 9% of their peritoneal macrophages are positive for Ia. At 4 months the mice start showing signs of autoimmunity and their percentage of Ia positive peritoneal macrophages increases to 35%. (4) It is thought that the increase in Ia expression may be directly related to the disease process occuring in these animals, possibly as a cause or result.

MATERIALS AND METHODS

Preparation for experiment one

Twenty two Balb/c male mice and 21 C57BL/6 male mice were injected with stationary phase, promastigote L. major diluted in saline to 2×10^7 ml. Each mouse was infected with 0.05 ml of 2×10^7 ml in the left foot pad to give a total inoculation of 1×10^6 protozoa/mouse. The L. major culture was grown in Medium 199(Sigma Chemical Company) plus 20% fetal calf serum(FCS).

Preparation of experiment two

Sixteen Balb/c male mice and 16 C57BL/6 male mice were injected with 0.05 ml of 2×10^7 promastigotes, as before. Five C57BL/6 mice were kept as uninfected controls.

Paraformaldehyde fixative

One percent paraformaldehyde was used to fix the adherent peritoneal cells after incubation. This was made fresh before use from 4% stock paraformaldehyde solution, diluted in a phosphate (PO_4) buffer solution each trial. The 4% solution was made every two weeks by dissolving 2 grams of paraformaldehyde in 47.5 ml of 0.1M PO_4 buffer (ph 7.3) solution. This was done by heating the solution to 70 degrees Celsius with continual stirring, then adding 2.5 ml of 0.001 M $CaCl_2$. The solution was cooled and filtered through a Buchner funnel with Whatman #1 filter paper. After filtering, another 5 ml of 0.1M PO_4 buffer solution was added. This 4% solution was then refrigerated and diluted on the day of the trial with additional PO_4 buffer.

Monoclonal Antibody

Two monoclonal antibodies recognizing different anti-Ia molecules were graciously provided by Dr. E. Unanue's laboratory and were used in order to detect Ia on the Balb/c macrophages (anti-Ia^d) and C57Bl/6 macrophages (anti-Ia^b). Previous experiments in our lab determined that the anti-Ia^d actually did detect the Ia of Balb/c mice specifically. Before use, the anti-ia^d was diluted on the day of each trial. Fifty microliters of anti-Ia^d was diluted on the day of each trial. Fifty microliters of anti-Ia^d was diluted in 450 microliters of rabbit serum diluent on the day of the trial. The rabbit serum diluent contains 10 mg/ml bovine serum albumin,

10% normal rabbit serum in RPMI 1640 Medium (Sigma Chemical Company).

Monoçlonal anti-Ia^b was used for the C57BL/6 mice and was also made fresh on the day of the trial. Fifty microliters was diluted in 450 microliters of rabbit serum. This antibody was also found to be highly specific for detecting Ia^b on the C57BL/6 macrophages, but did not react with Ia molecules (Ia^d) found on the Balb/c macrophages.

Fab'2 conjugate

An FITC (Fluorescein Isothiocyanate) conjugated antibody fragment (Fab'2) directed toward the Fc portion of the monoclonal anti-Ia was provided by Dr. E. Unanue's laboratory. This particular conjugate was only used at the beginning of the first experiment. The Fab'2 did not stain cells non-specifically. The conjugate was diluted in 198 ul of rabbit serum diluent to a concentration of approximately 86 ug/ml.

Later in our work, a Dichlorotriazinyl Amino Fluorescein (DTAF) conjugate was used to detect the presence of anti-Ia antibodies adhering to the macrophage surfaces. The conjugate was purchased from Pel Freeze Biologicals. Both conjugates had comparable staining capacity on macrophages from normal and infected animals. The DTAF was alliquoted into 20 ul quantities and stored in a freezer. The 20 ul quantities contained .66mg/ml of protein. The 20 ul quantities were diluted the day of the trial with 220 ul of rabbit serum, bringing the protein concentration to approximately .14mg/ml.

Procedure for removal and staining of peritoneal exudate cells

For each trial, 2 infected Balb/c, 2 infected C57Bl/6 mice, 1 normal Balb/c, and on occasion a normal C57Bl/6 were sacrificed by asphiyxiation with CO_2 The peritoneal exudate cells (PEC's) were collected by lavage using 8 - 10 ml RPMI 1640 medium supplemented with 5% FCS. The cells were spun in a centrifuge at 2000 rpm for 15 minutes. They were then collected and raised in RPMI 1640 plus 5% FCS, counted, and adjusted to approximately 2 X 10^6/ml. Coverslips (Bellco. labs) were placed in a 24-well micro-titer plate (Falcon 3047 multiwell tissue culture plate) and 1 ml of the 2 X 10^6 /ml cell suspension was placed in each well (3 wells were set up for the normal mice 2 were stained with anti-Ia the other one was a control using PBS). The micro-titer plate was then spun for 5 minutes in the centifuge at 1000 rpm to aid in the adherence of the cells to the coverslips. The plate was then placed in an incubator for 2 hours at 37 degrees Celsius and 5% CO_2.

The staining procedure was carried out as described by Beller and Unanue with some amendment(1). The plate was removed from the incubator and each coverslip was washed with saline. The coverslips were then placed in clean wells with approximately 1 ml of 1% paraformaldehyde for 15 minutes at room temperature (the 1% paraformaldehyde did not affect detection of Ia and acted to kill and fix

the cells on the coverslips). (1) The coverslips were again washed with saline and then placed cell side down, within the depression of a hanging drop slide, containing 17-20 ul of either anti-Ia^b, anti-Ia^d, or PBS. The PBS was used as an control to detect non-specific staining. These slides were placed on ice for 30 minutes. After 30 minutes, the slides were washed gently but throughly with saline from a squeeze bottle and placed cell side down on a clean hanging drop slide containing 17-20 ul of anti Fab'2 conjugated with FITC or DTAF dye. The slides were placed on ice for 30 minutes and then the coverslips were washed again with saline. The coverslips were then placed separately in wells of a 24 well micro-titer plate. Each well contained 1ml of PBS. The cells were stored in the wells until they could be examined the next day under the Zeiss fluorescent microscope. These coverslips were taken from the wells and placed cell side down in 17-20 ul of PBS on a hanging drop slide and examined under 400X magnification.

Footpads

The infected foot pads of all mice were measured on the day of the trial with a Vernier caliper gauge by measuring the width from the top to the bottom of the footpad. This procedure was done to determine the rate of swelling of the infected foot and to establish normal levels using the normal mice.

Spleen culture

The spleens of the infected mice were removed sterilely after the PEC's were collected. The spleens were placed into a sterile petri dish with Hank's Balanced Salt Solution (HBSS) weighed and then ground with two frosted end glass slides. The spleen suspension was then transfered to a 15 ml centrifuge tube (Corning) and centrifuged at 7 for 8 minutes. After they were centrifuged, the supernantant was decanted and the spleen cells were vortexed and the resuspended in 10 ml of Medium 199 with 20% FCS and transferred into a 25 cm^2 culture bottle (Corning). The spleen cells were then incubated at 25 degrees Celsius. They were checked daily for the presence of Leishmania major by microscopic examination of a sample of the culture fluid. Negative cultures were reincubated for a period of three weeks before being discarded.

Lymph nodes

The hind leg popliteal lymph nodes of the infected mice were removed and weighed to determine the level of lymphoadenopathy. The popliteal lymph nodes that were removed were the regional draining lymph nodes of the infected foot.

RESULTS

Enlargement of the spleen

In both experimental trials the spleen size of the Balb/c enlarged through out the course of the experiment. The C57Bl/6 mice showed a slight increase at the beginning of the experiment one, but then their spleens returned to their normal sizes and remained normal in size throughout the rest of the experiment. (See Figure 1 and 2) On the graphs below the x axis represents the spleen weight in grams and the y axis represents how many days the mouse has been infected. Figure one represents the results of experiment one and figure two represents the results of experiment two.

Fig. 1

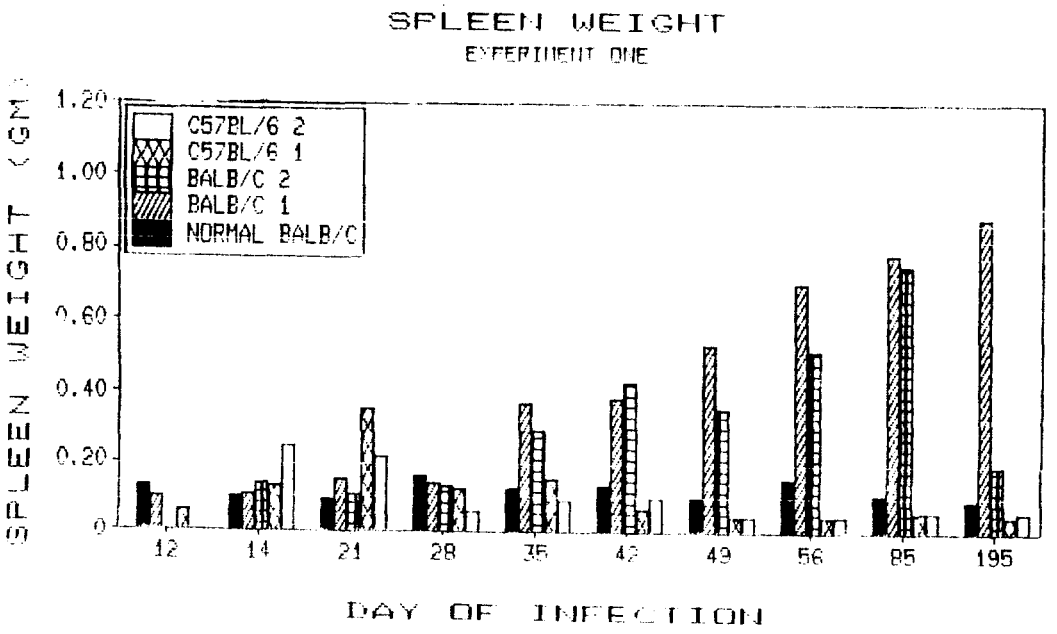
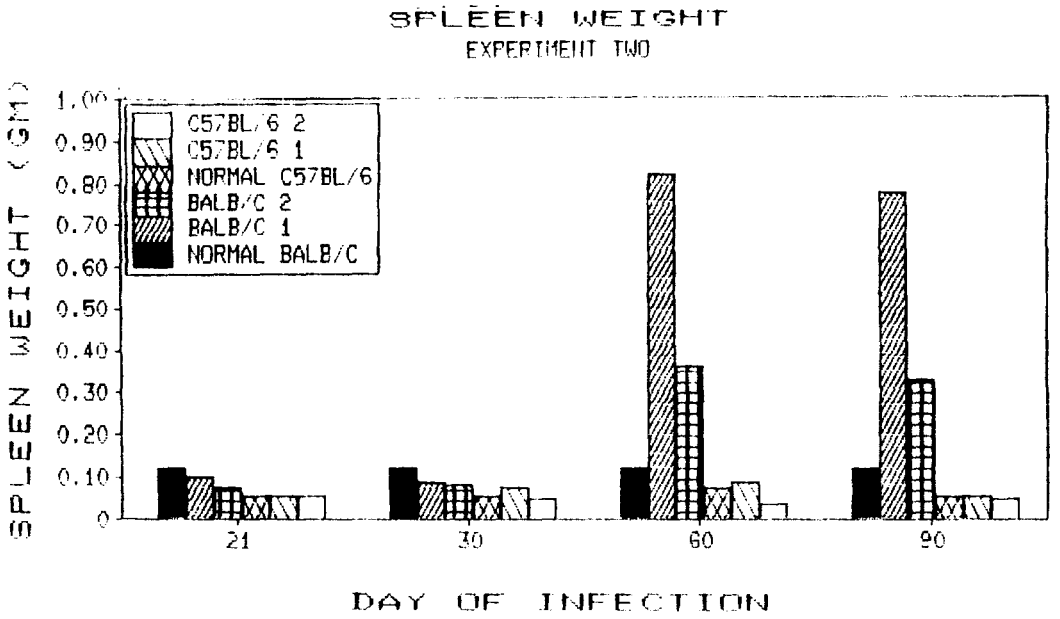


Fig. 2



Enlargement of the lymph nodes

The lymph nodes of both the Balb/c mice and the C57BL/6 mice became enlarged during the course of the infection. While the lymph nodes of the Balb/c mice grew progressively larger, the C57BL/c only slightly increased throughout the experiments. (See fig. 3 and 4)

Fig. 3

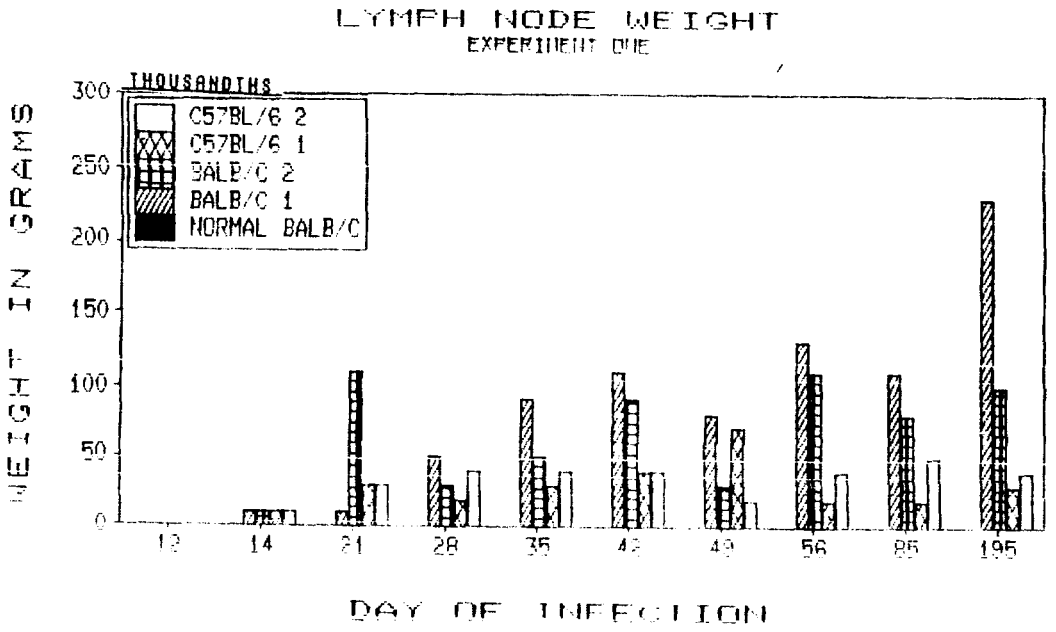
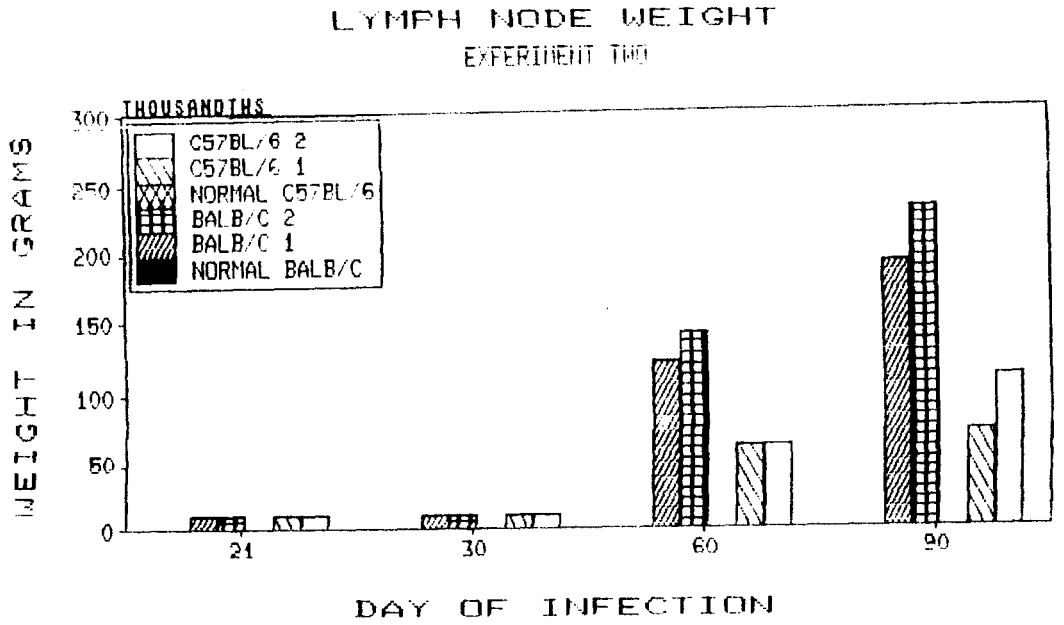


Fig. 4



Percent of Ia positive macrophages

The percentage of Ia on the Balb/c mice began to increase early after infection and stayed above normal throughout the course of both experiments. The C57BL/6 mice never showed a significant increase in Ia level. In experiment 1 the marked increase in Ia in Balb/c mice did not appear until day 28 of infection whereas an indication of an increase in Ia in experiment two was first noted in mice sacrificed on day 21 of infection. A highly significant increase in Ia expression in the infected mice was not detected in these mice until day 60. (See Fig. 5 and 6)

Fig. 5

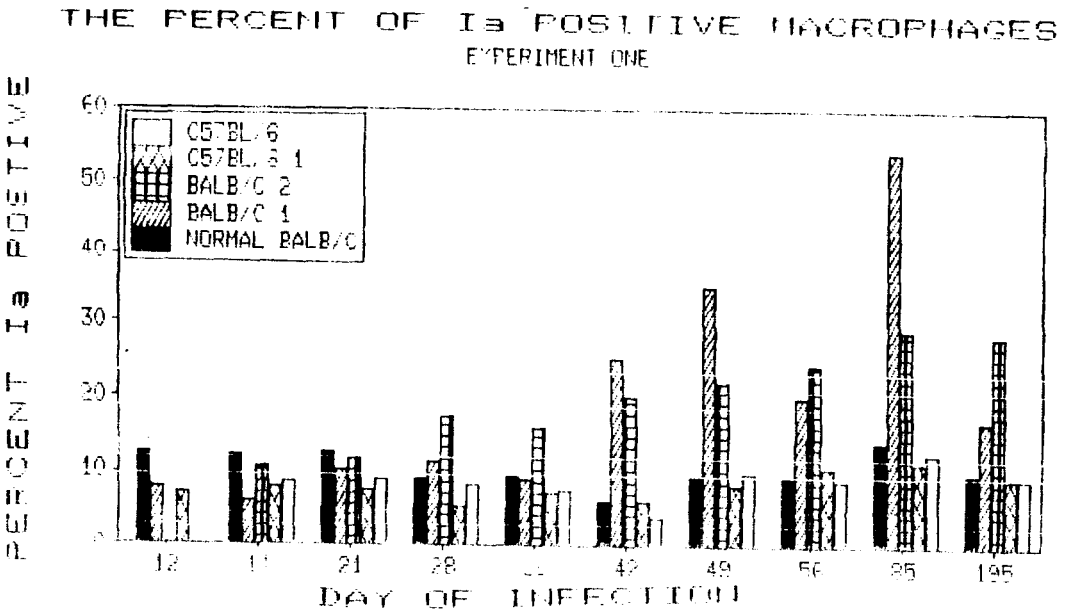
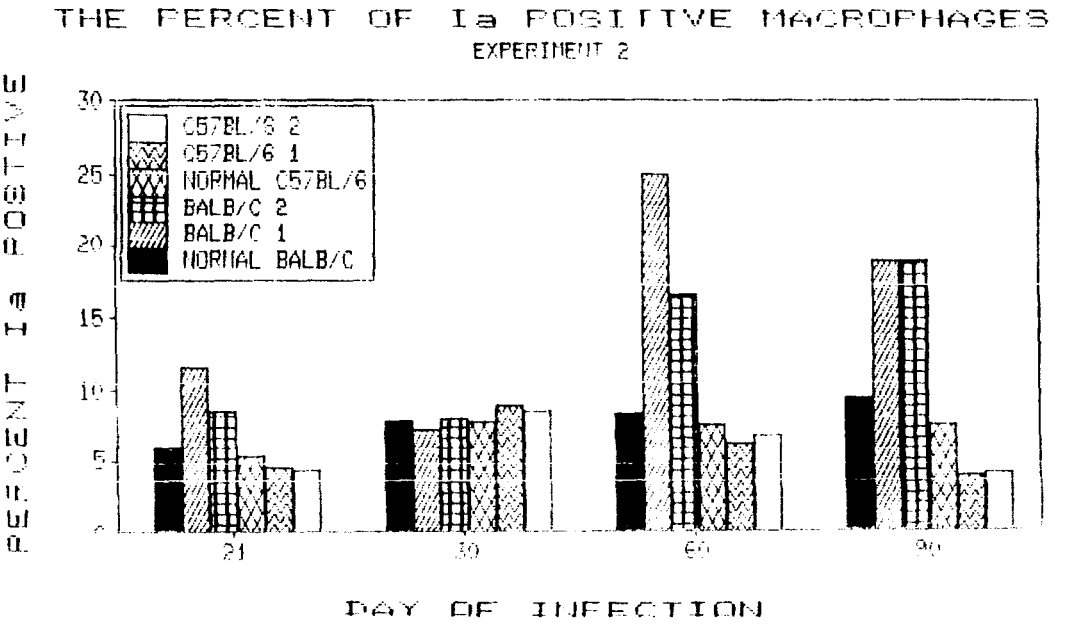

THE PERCENT OF Ia POSITIVE MACROPHAGES
EXPERIMENT ONE
PERCENT Ia POSITIVE
60
50
40
30
20
10
C57BL/6
C57BL/6 1
BALB/C 2
BALB/C 1
NORMAL BALB/C
12
21
28
42
49
56
95
195
DAY OF INFECTION

Fig. 6


THE PERCENT OF Ia POSITIVE MACROPHAGES
EXPERIMENT 2
PERCENT Ia POSITIVE
30
25
20
15
10
5
C57BL/6 2
C57BL/6 1
NORMAL C57BL/6
BALB/C 2
BALB/C 1
NORMAL BALB/C
21
30
60
90
DAY OF INFECTION

The line graphs of Figures 7,8,9, and 10 show the average of Ia positive macrophages in infected animals compared to the normal mice. The lined in region indicates the average of the normal mice including two standard deviations and the individual points represent each animal's percentage. The Balb/c mice showed an increase in the percentage of Ia positive peritoneal macrophages during the course of both experiments. The infected C57Bl/6 mice showed no marked increase in Ia percentage.

Fig. 7

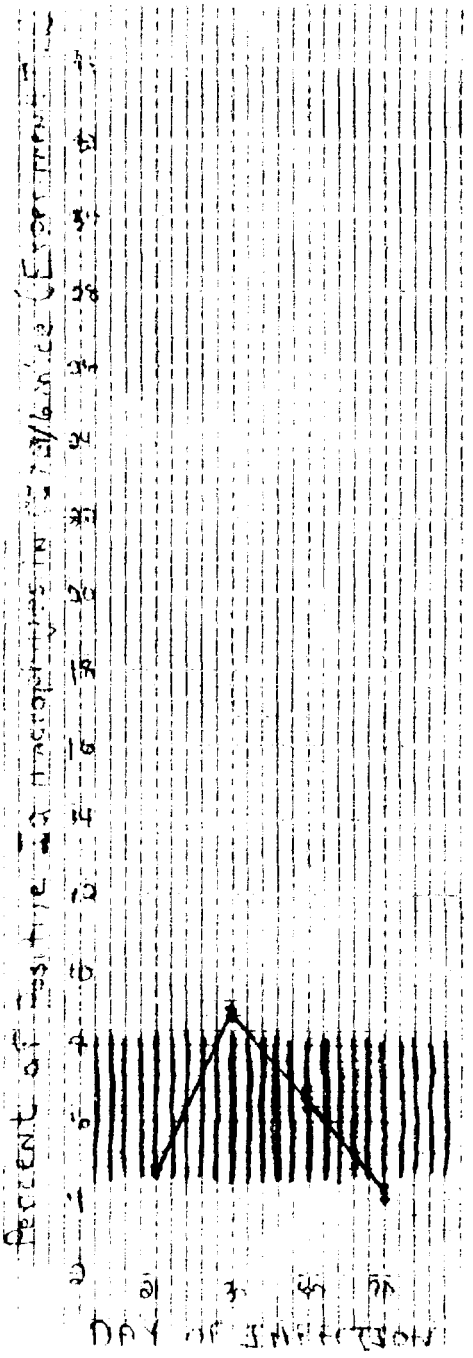


Fig. 8

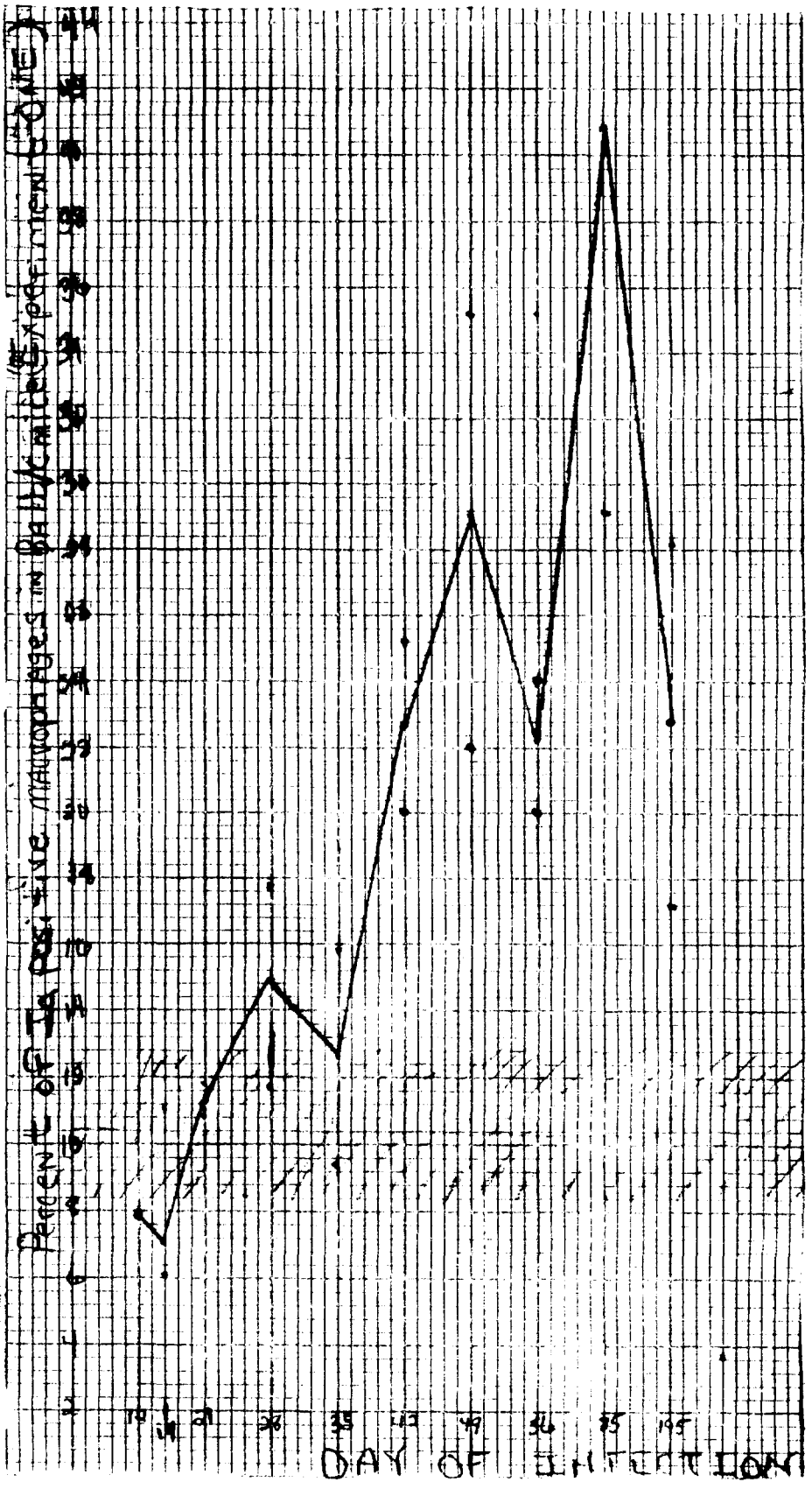


Fig. 9

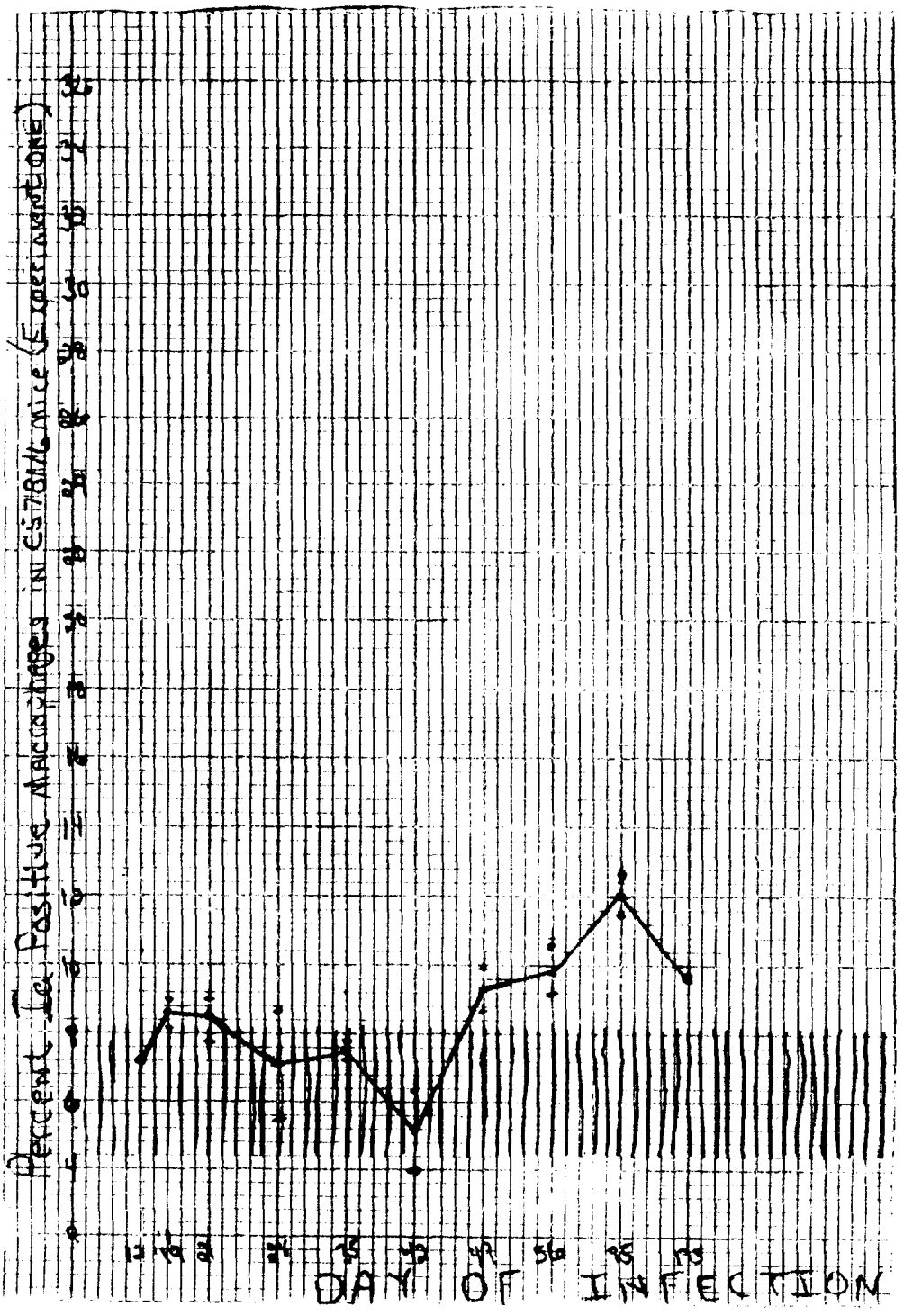
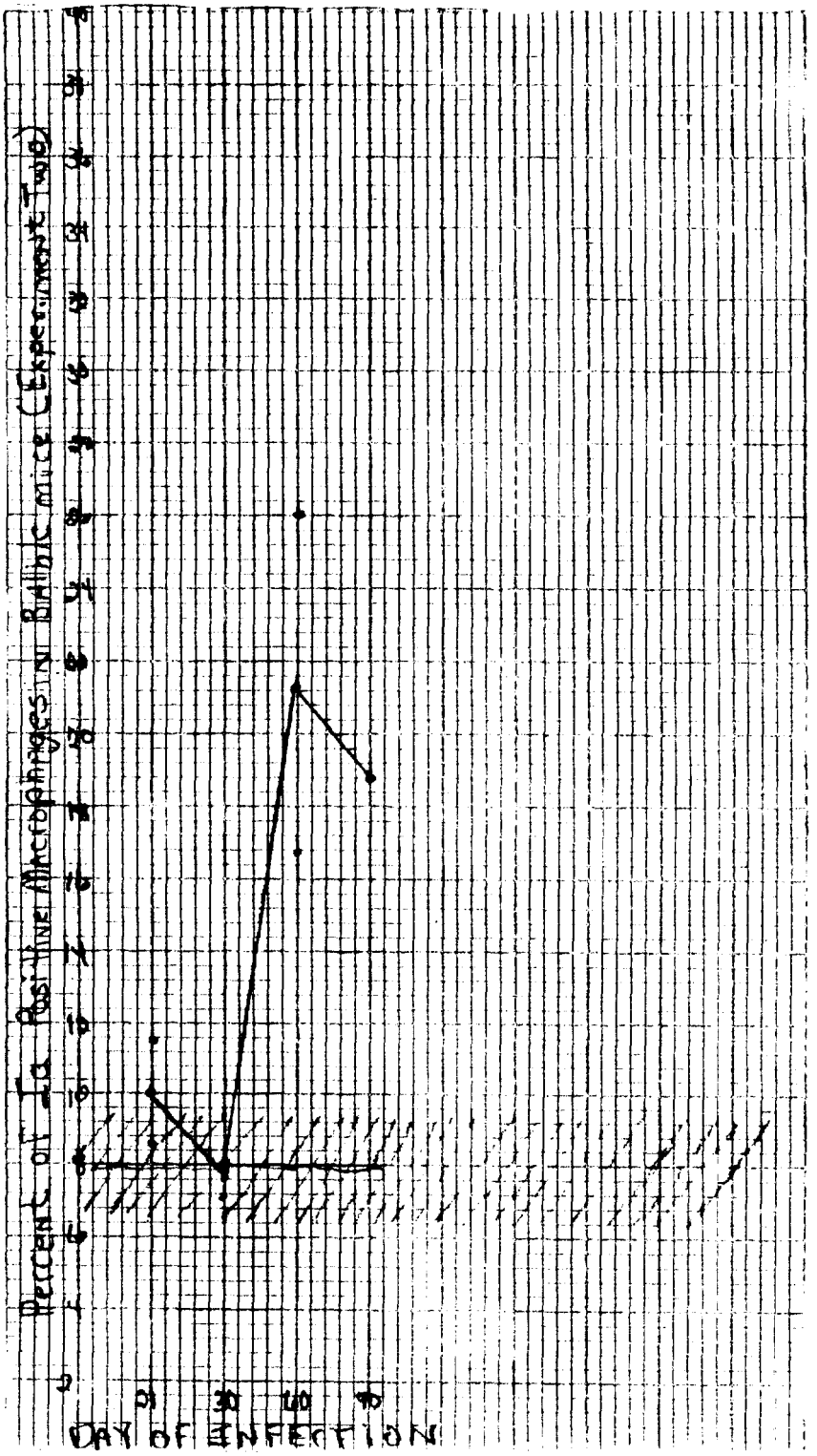

Percent (%) Positive Attachments in ELISA to Virus (Experiment One)
DAY OF INFECTION

Fig. 10


Percent of i.p. Positive Macrophages in Balbic mice (Experiment Two)
DAY OF INFECTION

Footpad measurements

During the course of the experiment the Balb/c mices's footpads grew increasingly swollen and ulcerated until they eventually lost their foot. (See fig. 11 and 12) Foot loss is shown in the graph by the absense of bars. The C57BL/6 mice's footpads swelled minimumly in the beginning weeks of the infection then returned to their normal sizes.

Fig. 11

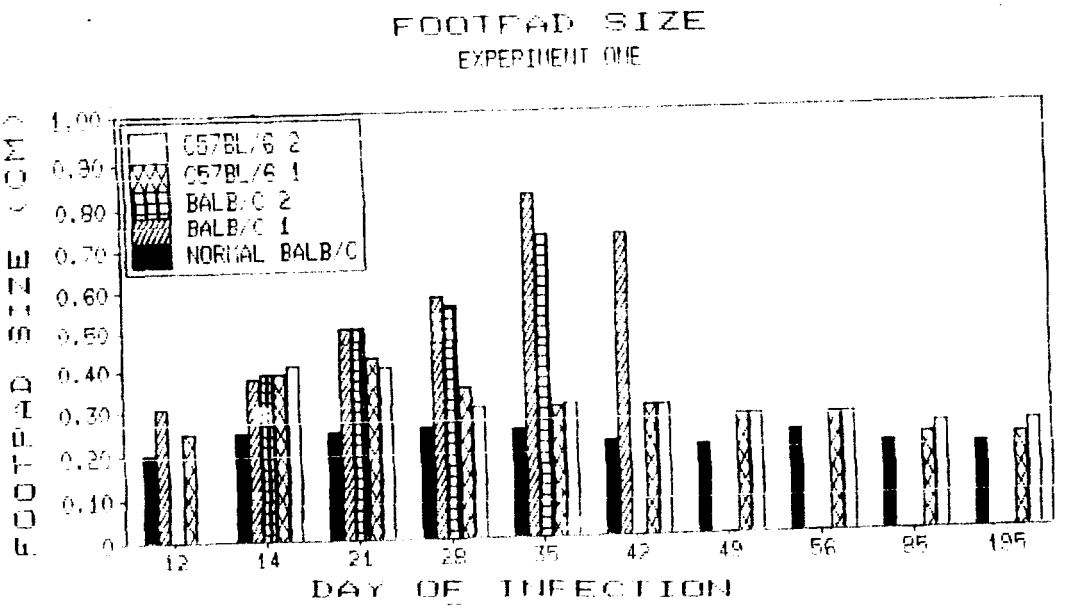
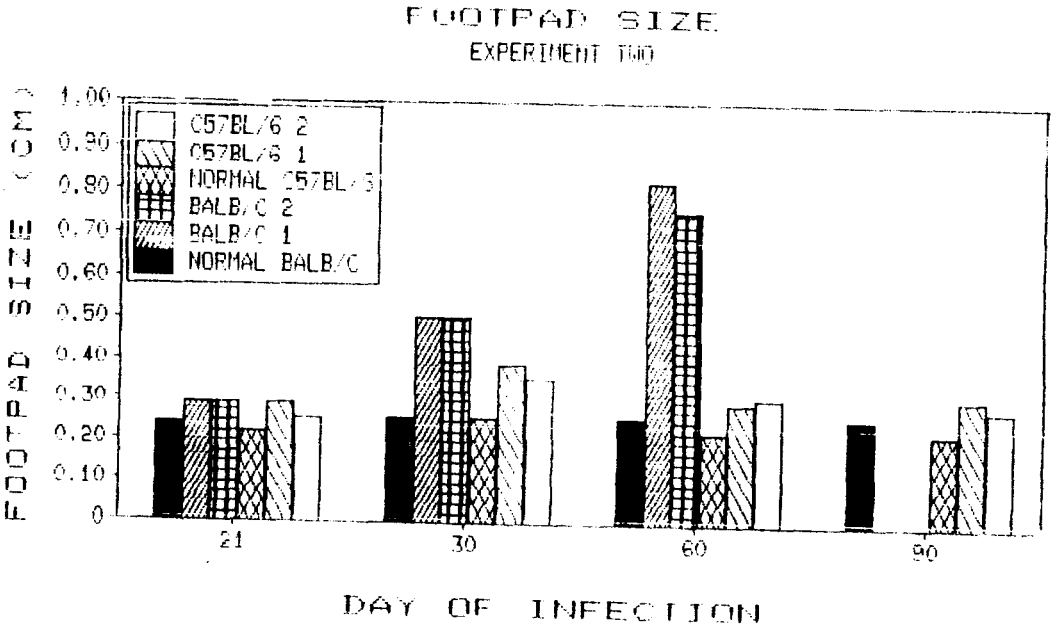


Fig. 12



Increased numbers of Leishmania major

As the infection progressed , the L. major was detected in the spleens of both strains in progressively shorter times. (See tables 1 and 2) At the beginning of the experiment both types of mice had L. major in their spleens. At the conclusion of the experiment most of the C57BL/6 mice no longer had the protozoa in their spleens, showing that they could effectively rid themselves of this parasite.

Table 1 (exp. 1)

DAY OF INFECTION C57BL/6	BALB/C	DAY POSITIVE
12		6, ND
	12	13, ND
14		4, ND
	14	11, 5
21		3, 3
	21	3, POS
28		5, 5
	28	5, 5
35		4, 5
	35	4, 4
42		4, 4
	42	4, 4
49		3, ND
	49	3, 1
85		8, 5
	85	3,3
95		NEG.
	95	1, 1

Table 2 (exp. 2)

DAY OF INFECTION C57BL/6	BALB/C	DAY POSITIVE
21		22, ND
	21	7, 6
30		13, 13
	30	13, 13
60		POS
	60	POS
90		4, ND
	90	4, ND
134		7, NEG

DISCUSSION

The results of this investigation indicate that the immune response to infection with L. major in two inbred strains of mice, Balb/ c and C57Bl/6 is characterized by significant differences which may be correlated to the different susceptibilities of the two mouse strains to the parasite. Following infection, the resistant C57BL/6 mice developed some footpad swelling which disappeared after several weeks, and some local lymph node enlargement. There was no splenic enlargement although the L. major did metastasize to the spleen as early as day 12 and was detectable in the spleen for up to 4 months. In addition, there was no detectable increase in the expression of Ia on adherant peritoneal exudate cells, the macrophages, during the course of infection. In contrast, the highly susceptible Balb/c mouse strain developed footpad swelling and lesions and eventually loss of the infected foot accompanied by increasing local lymph node enlargement. The size of the spleens of these animals increased dramatically during the course of the infection indicating generalized lymphoproliferation. Within a few weeks the peritoneal macrophages of the infected Balb/c mice began to show increased expression of Ia, a molecular marker of macrophage activation. As early as day 28 following infection in experiment one the macrophages of the Balb/c mice appear to become activated. In experiment two the Balb/c mice's macrophages seem to be activated by day 60. This longer time period before activation in experiment two is probably because the whole infection process seemed to be slower. One week after this activation,

day 35 in experiment one, and simultaneously in experiment two the spleen weight and lymph node weight of the Balb/c mice increase drastically, indicating that the activation of the macrophages may occur before marked splenomegaly and lymphoproliferation. This macrophage activation only oocurs in the Balb/c mice as shown in both experiments. The fact that no macrophage activation nor increase in spleen weight occurs in C57BL/6 mice suggests that increased macrophage activation may be correlated with strain susceptibility to L. major.

The increase of Ia on the macrophage is thought to be the result of either an increased uptake of antigen or stimulation by T- cells or perhaps a combination of both. What exactly causes the increased Ia level on peritoneal macrophages is not clear. It is thought that the way T-cells induce macrophages to display Ia is by secreting gamma interferon and other lymphokines (6). As the resistant C57BL/6 strains have normal levels of Ia throughout the infection, this increased level of Ia expression in Balb/c mice is thought to be either the result or of the cause of a non-protective immune response.

The increased number of activated macrophages in the Balb/c mice may be due to faulty procesing of antigen by the macrophage , or some T-cell abnormality. This T-cell abnormality may cause the T-cell to secrete

an Ia inducing substance that causes these macrophages to become activated which then further activates the abnormal T-cell. Indeed, an Ia inducing factor is found to induce Ia in the autoimmune MRL-lpr mice (4).

Experiments ar now being conducted in which Balb/c mice are given Cyclosporine. When the mice are given this immunosuppressant, they become resistant to serious disease, but not to infection, if the drug is given a few days before the parasite is injected. It has been shown in other experiments that Cyclosporine depresses Class two major histocompatibility antigens (3 and 4). We suspect that this drug will prevent the increase of Ia positive macrophages in the Balb/c mice.

CONCLUSIONS

In general the infected Balb/c mice showed a dramatic increase in footpad size, spleen weight, lymph node weight, and the percent of Ia positive macrophages in their peritoneal exudate. The C57BL/6 , on the othe r hand, showed a slight increase in spleen weight, footpad size, lymph node weight during the beginning of the infection with an eventual return to normal levels and no increase in their percent of Ia positive peritoneal exudate macrophages. More experiments like these and experiments using Cyclosporine will elucidate the failure of the Balb/c mice to develop protective immunity against L. major.

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