# CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ T Cell-subsets Can Confer Protection against *Leishmania m. mexicana* Infection

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*We studied the role of CD4$^+$, CD8$^+$, CD4$^-$ CD8$^-$ T cells and IgG anti-*Leishmania *after infection or vaccination in the CBA/ca mouse. Mice were either infected with L. m.* mexicana *promastigotes or vaccinated with parasite-membrane antigens incorporated into liposomes. Successfully vaccinated mice were used as cell-donors in adoptive transfer experiments. Naive, syngeneic recipients received highly-enriched CD4$^+$, CD8$^+$ or CD4$^-$ CD8$^-$ T cells from those two set of donors and challenged with live parasites. Our results showed that, both CD4$^+$ and CD8$^+$ T cells from infected or vaccinated donors conferred significant disease-resistance to naive recipients. In addition, adoptive transfer of CD4$^-$ CD8$^-$ T cells from vaccinated donors significantly delayed lesion growth in recipient mice. We concluded that vaccination of CBA mice correlates with the induction of protective CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ T cells and the synthesis of IgG anti-*Leishmania.

Key words: T cell-subpopulations - *Leishmania m. mexicana* - liposomes monoclonal antibodies

*Leishmania m. mexicana* is a protozoan parasite and is one of the causative organisms of new-world cutaneous leishmaniasis. The nature of the disease mechanisms and modes of protection have been the subject of intense research in humans and several mouse models (Blank et al. 1993, Holaday et al. 1993). Some such as the BALB/c and CBA/ca are considered susceptible to *L. m. mexicana* infection, since they develop non-curing infections, while others such as the C57BL/6 are resistant and only develop self-healing lesions after infection (Lezama 1991). Work has focussed on the role of T-cell subsets in these areas and histological studies have shown that susceptible mouse strains have a defect in the ability of T cells to migrate towards skin lesions (McElrath et al. 1987). Initial observations that CD4$^+$ T cells could transfer either resistance or exacerbation of the disease (Liew 1985, 1989) have evolved to the present view that IL-2, IFN-gama and TNF-beta producing murine CD4$^+$ lymphocytes (Th1 cells) confer resistance whereas, IL-4, IL-5, IL-6, IL-10 and IL-13 producing murine lymphocytes (Th2 cells) promote disease progression in mice (Scott 1989, Mosmann & Coffman 1989, Romagnani 1994) and perhaps in humans (Kemp et al. 1993,

Romagnani 1993). The role of CD8$^+$ T cells is less well defined although, it has been reported that *in vivo* depletion of these cells protects BALB/c mice from *L. major* infections (Titus et al. 1987). Cutaneous infection with *L. m. mexicana* would normally cause the development of potentially-growing lesions in the susceptible CBA/ca mouse. Intraperitoneal or subcutaneous vaccination with *Leishmania* surface antigens incorporated into liposomes can protect these mice from subsequent infection and this protective effect is transferred by T-cells (Russell & Alexander 1988). This work was designed to determine humoral and cellular mechanisms associated with successful vaccination and natural infection. We measured antibody levels and the ability of T-cell-subsets derived from infected and vaccinated donors to influence the course of subsequent infections in naive cell recipients. In this report we present evidence of the generation of protective CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ T cells after effective vaccination.

## MATERIALS AND METHODS

*Animals* - CBA/ca strains of mice between 8-12 weeks old, bred in the University of Strathclyde were used throughout this work. In any one experiment, mice were age and sex matched.

*Parasites* - *L. m. mexicana* (MNYC/BZ/62 /M379) was maintained by serial passage of amastigotes inoculated subcutaneously into the shaven rumps of CBA/ca mice. Stationary phase

promastigotes were produced by *in vitro* culture from amastigotes in RPMI-1640 (Gibco) supplemented with 10 % fetal calf serum (Gibco), penicillin G-potassium, (100 U/ml, Gifco) and streptomycin (100 μg/ml, Gibco), 2mM L-Glutamine (complete medium, pH=7.1).

*Antigen preparation and encapsulation into liposomes*-Membrane antigens were extracted from *L. m. mexicana* promastigotes following the procedure described by Russel and Weilhelm (1986). In brief, 2-5 X $10^{10}$ stationary phase promastigotes from culture flasks were washed three times with PBS, suspended in 40 ml of hypotonic buffer (10 mM Tris-HCl; 2 mM EDTA, pH=7.8; 0.050 mM of TLCK; 0.015 mM of leupeptin) and left in ice for 15 min. Parasites were then disrupted for 10-20 seconds at 500 psi in a cell-disruption chamber (Melsugen, West Germany), previously left to cool down at 0°C with a $CO_2$ steam. The suspension was examined under light microscopy to ensure for cell lysis and spun down at 10,000 X G for 30 min at 4°C. Supernatant (soluble antigen) was frozen at -20°C and the pellet was resuspended in 6 ml of PBS [0.050 mM TLCK, 0.015 mM leupeptide and 2% w/v octylglucoside, pH=7.1] left in ice for 15-20 min and spun down for 1 hr at 100,000 X G in a Beckman ultracentrifuge. The supernatant (membrane preparation) was frozen until use. Molecules contained in these fractions were polled and identified by SDS-PAGE followed by silver stain as previously described (Laemmli 1970, Wray et al. 1981).

Reconstitution of antigen into liposomes was a modification of the procedure of Russell and Alexander (1988) which is presented below: 1 ml suspension 32 mg (80%) soyabean lecithin, and 2 mg (5%) diacetyl phosphate were mixed with 2 ml of chloroform in a 500 ml conical flask and 6 mg (15%) of cholesterol were added. The mixture was dried with $N_2$ to produce a thin film and incubated for 1hr at 37°C. Parasite Ag in PBS/octylglucoside was then added to the lipid film and sonicated in an ice bath. A ratio of 2 μg of protein of parasite sample to a 1mg phospholipid-cholesterol mixture was mantained in all experiments. The detergent was removed by overnight dialysis against PBS at 4°C and the resulting vesicles washed with PBS, centrifuged for 1hr at 100,000 X G and stored at -20°C until use. The same procedure was applied for the preparation of empty liposomes, except that PBS without parasite antigen was used.

*ELISA assay for IgG determination* - An indirect non-competitive technique on solid phase was chosen and performed as described somewhere else (Ngo & Lenhoff 1981). In brief, *L. m. mexicana* membrane antigens were dried onto polyvinil microtiter plates previously covered with 1% BSA and 1:100 serum dilutions were added to microtiter plate wells in duplicate, including a pool of sera from chronically infected mice (positive control) and normal sera (negative control) and incubated at 37°C for 1.5hr and washed. Peroxidase conjugate (1:5000 diluted with 1% egg albumin in 0.02 M Tris, pH=7.4) was added and incubated for 1.5 hr at 37°C followed by washing with 0.02 M Tris-Tween (0.05%). Substrate solution (tetramethyl-benzidine in 0.1 M acetate-citrate buffer, pH=5.5) was added to each well and incubated in the dark at room temperature for 30 min.

Finally, the reaction was stopped by adding 50 μl of 2.5M of sulphuric acid and read in an ELISA reader (Titrek multiscan) at 450nm. Results are calculated by dividing the optical density of experimental samples by the optical density of negative controls.

*Vaccination protocol* - Vaccination procedures were conducted as described by Russell and Alexander (1988) with the following modifications: 8μg membrane preparations reconstituted into unilamelar liposomes were inoculated twice at two weeks interval, and two weeks after the last immunization, both controls and experimental CBA/ca mice were infected with $3 \times 10^6$ *L. m. mexicana* virulent promastigotes (some of the vaccinated mice were not infected and used for adoptive transfer experiments). Control groups included mice inoculated only with antigen/PBS and those inoculated with empty liposomes.

*Cytofluorography* - Cytofluorographic analysis of T cell subpopulations was performed as described by Mason et al. (1987). In brief, 5 X $10^5$ spleen cells were obtained from either vaccinated or infected mice two weeks after last vaccination (see vaccination protocol above) or two months after infection, spleen cells from normal mice were also analyzed by cytofluorography. Cells were dispensed into a microtitration well and mixed with a MoAb [anti Thy 1.1 (T cells marker) from Sera-Lab, anti-CD4 (L3T4) from Sera-Lab, anti-CD8 (Lyt-2) from Sera-Lab or anti-B220 (B cells marker) from The Department of Zoology, University of Edimburgh] diluted 1:500 (anti-Thy1.1, anti-CD4 and anti-CD8) or 1:10 (anti-B220), incubated for 45min at 4°C and washed with a solution of 1% BSA in PBS-Azide (0.01%). A second antibody (FITC-goat anti-rat IgG from Sigma) was added to the wells, incubated at 4°C and washed. Cells were pelleted and resuspended in PBS-formaldeyde (1%). Preparations were analyzed by one colour cytofluorography in a Becton Dickinson Cytofluorograph. Results are expressed as percentage of Thy $1.1^+$ $CD4^+$, $CD8^+$ or B-$220^+$ spleen cells.

*Preparation of T-cell subsets for adoptive transfer experiments* - Spleen cell suspensions were passed through nylon wool and T cells obtained as described by Julius et al. (1973). T cells were adjusted to $10^7$ cells/ml and treated with a

1:500 dilution of anti-CD4 (L3T4) and/or anti-CD8 (Lyt-2) MoAbs (Sera-Lab) for 60 min at 4°C. Following removal of excess antibody, cells were treated twice with 1:30 dilution of rabbit serum for 60min. Dead cells were removed by centrifugation (400 X g, 20 min, room temperature) using separation media (Lympho-prep from Sera-Lab). After thorough washing, viable cells were counted using the trypan blue test and adjusted to 3 X 10$^6$ for intraperitoneal transfer into naive recipients.

Efficiency of double or single depletions using anti-CD4 and/or anti-CD8 moAbs plus complement was monitored by cytofluorography in a Becton Dickinson instrument.

*Statistical analysis* - The unpair t test of Student was used in all statistical analysis.

## RESULTS

*Antigen preparation* - Parasite antigens were extracted using a non-ionic detergent (octylglucoside) and reconstituted into liposomes. Electrophoretic analysis (PAGE-SDS) of this material presented five strong and several weaker bands (Fig. 1). Antigens so prepared were encapsulated into liposomes and used to vaccinate CBA/ca mice.

## MOLECULAR WEIGHT MARKERS

205 ————— )

116 ————— )

6 6 ————— )
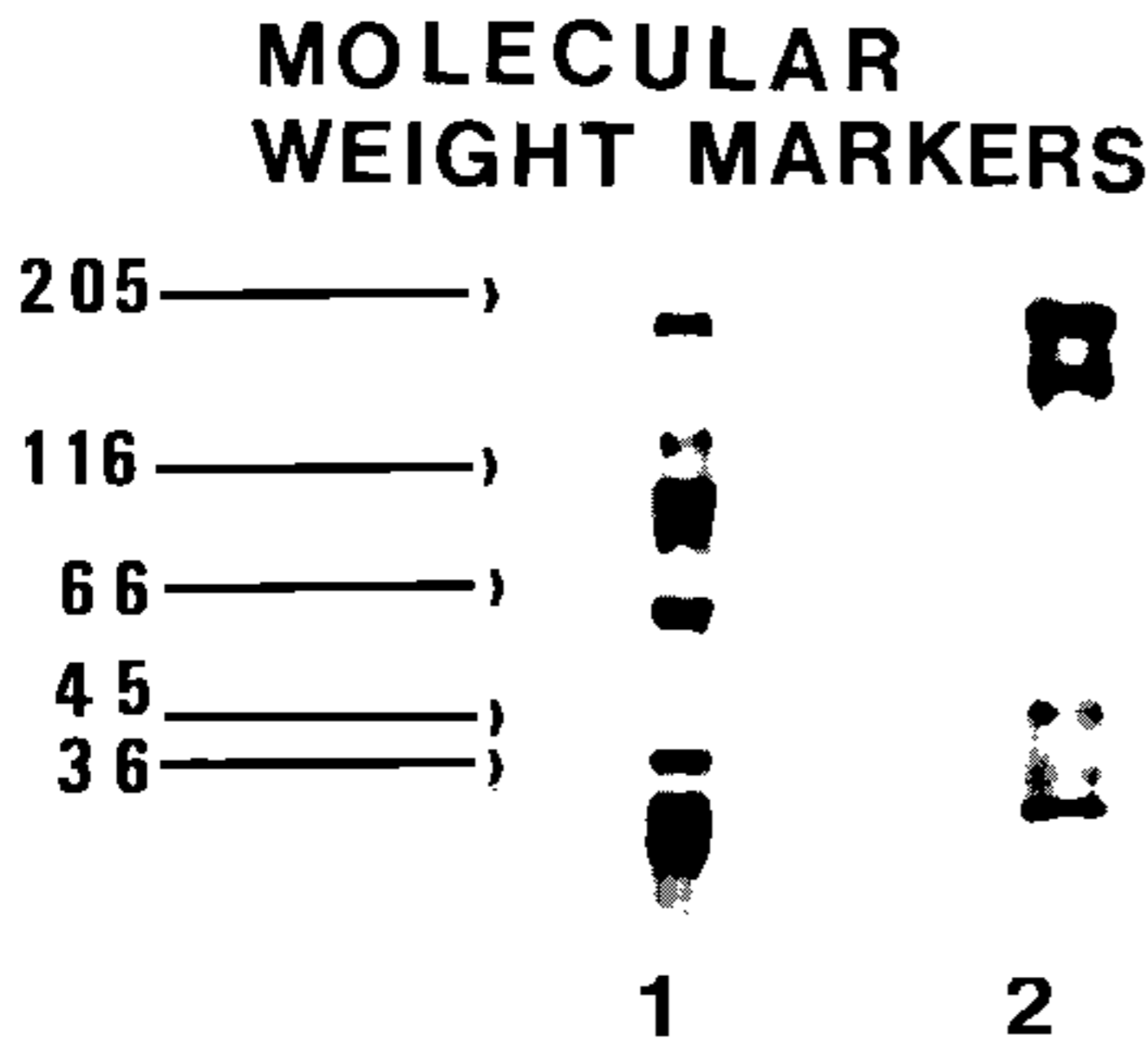
4 5 —————— )
3 6 ————— )

1          2

Fig. 1: polyacrylamide gel electrophoresis of isolated promastigote membrane antigens (for details see materials and methods). Silver stained 10% polyacrylamide gel membrane antigens extracted with 1% octylglucoside (Track 2) and molecular weight markers (Track 1).

*Vaccination* - To determine effectiveness of vaccination, a group of five CBA/ca mice were intraperitoneally vaccinated twice at two weeks interval using membrane antigens (8 μg of protein/mouse) reconstituted into liposomes. Together with a group of nine non-immunized control mice, vaccinated animals were exposed to a homologous challenge (i.e. mice vaccinated with *L. m. mexicana* antigens and infected with the

same parasite) of 3 X 10$^6$ stationary phase *L. m. mexicana* promastigotes and lesion growth was recorded for 16 weeks. Vaccinated mice proved to be completely protected developing no lesions at the site of inoculation, whereas in the control group eight out of a total of nine mice developed non-curing lesions and differences between last lesion measurements of controls and vaccinated animals were highly significant (p<0.0001; Fig. 2). Groups of mice inoculated either with empty liposomes or antigen alone showed a slight delay in lesion growth but, all of them developed non-curing lesions (data not shown).
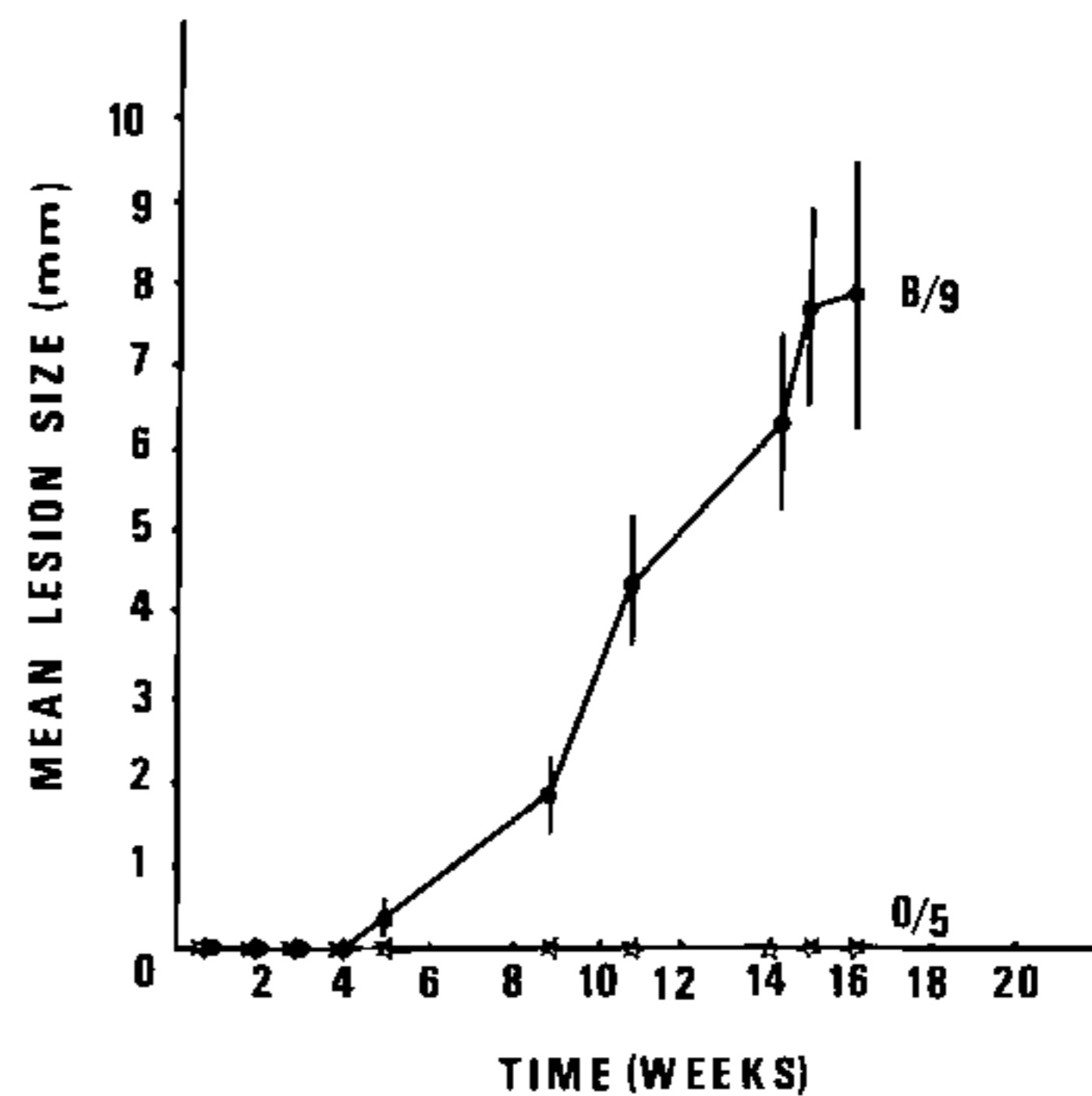


Fig. 2: effect of intraperitoneal vaccination using membrane antigens reconstituted into liposomes on lesion growth in the shaven rump of CBA/ca mice. One experimental group of five mice (stars) were intraperitoneally vaccinated with membrane antigens reconstituted into liposomes (8 μg of protein/mouse). A control group (circles) consisted of 9 mice subcutaneously infected with 3x10$^6$ promastigotes of *Leishmania m. mexicana* at the same time as experimental group. Data are expressed as the mean ± SEM (N=8-9) and the number of mice which developed lesions is indicated after each line (i.e No. of mice with lesions/total No. of mice included in the experiment). Results shown represent a replicate of three separate experiments.

*Anti-Leishmania IgG levels* - The IgG response in vaccinated CBA/ca mice, measured two weeks after the last immunization or infection with virulent parasites showed a significant increase as compared to control mice inoculated only with PBS (p<0.05; Fig. 3). An additional control consisted of mice inoculated with empty liposomes which did not induce the production of IgG anti-*Leishmania* (data not shown).

*Cytofluorographic analysis of lymphocyte-subpopulations* - Effectiveness of double and sin-
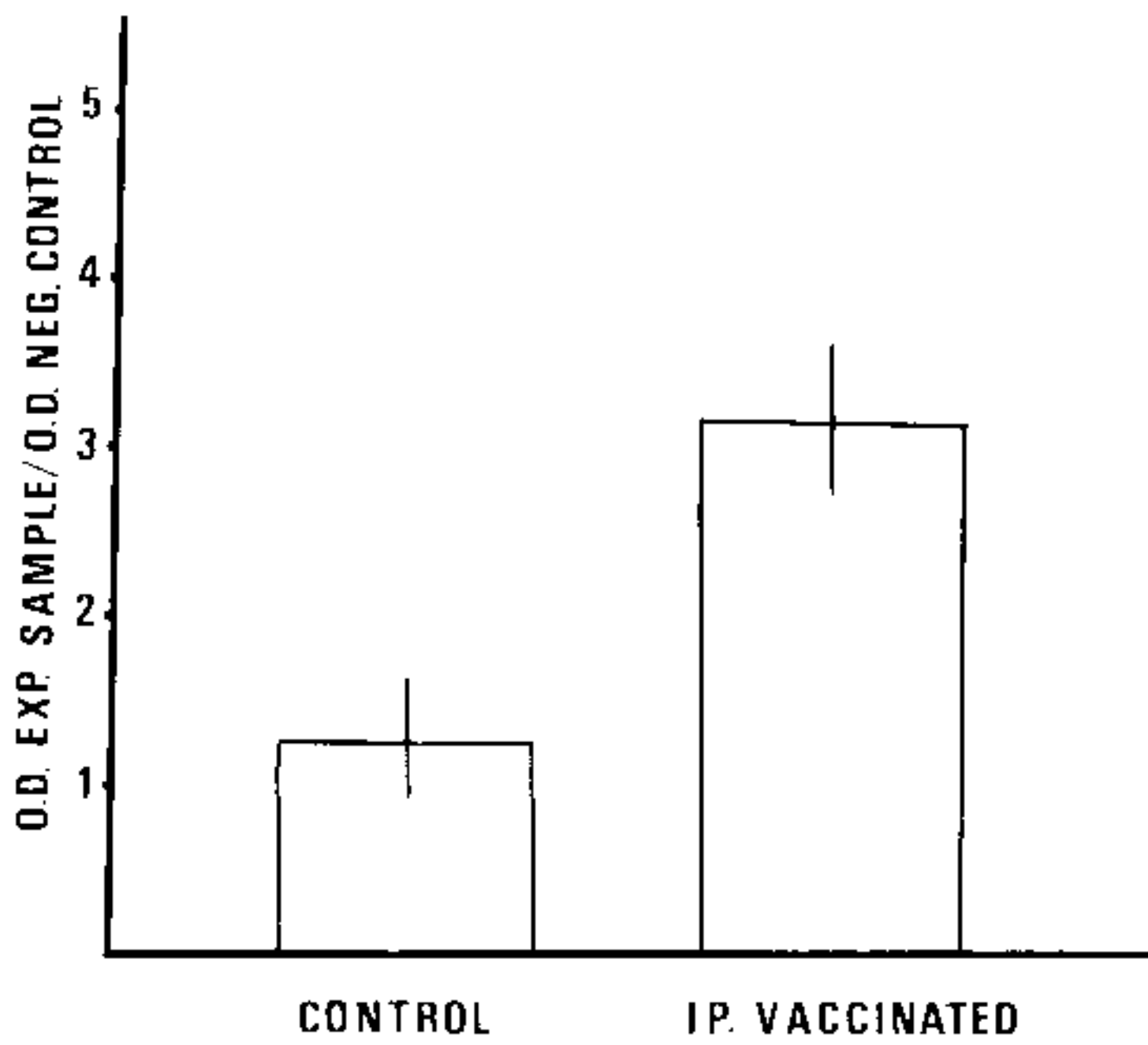
Fig. 3: antibody responses measured in mice (CBA/ca) after vaccination with *Leishmania m. mexicana* membrane antigens reconstituted into liposomes. Mice were vaccinated intraperitoneally twice at two weeks interval (8 μg of membrane proteins/mouse). A control group consisted of mice inoculated only with PBS. Data are expressed as the mean ± SEM (N=3). These results represent a replicate of three separate experiments.

TABLE

Effect of infection and vaccination on the frequency of spleenlymphocytes in CBA/ca mice

| Group of donor mice | % of lymphocytes with phenotype | | | | |
|---|---|---|---|---|---|
| | Thy-1 | B-220 | L3T4 (CD4) | Lyt-2 (CD8) | Ratio CD4/ CD8 |
| Normal | 25 | 31 | 18 | 9 | 2 |
| Infected | 24 | 32 | 18 | 8 | 2.2 |
| Vaccinated | 23 | 31 | 18 | 6 | 2.9 |

Cytofluorographic analysis of T cell subpopulations using whole spleen cells. A two step assay was performed using anti-L3T4 (CD4) or anti-Lyt-2 (CD8) as first antibody and fluorochrome linked anti-rat IgG as second antibody. Results presented here represent one of three replicate experiments.

gle depletions using anti-CD4 and/or anti-CD8 MoAbs plus complement was determined by cytofluorography and no more than 10% of contaminating cells were present in any one of the adoptive transfer experiments performed during this study. Our results showed that the proportion of CD4$^+$ and CD8$^+$ T cells present in the double depleted T cells from infected or vaccinated donors used for adoptive transfer experiments was four (CD4$^+$) and five (CD8$^+$) percent and four (CD4$^+$) and six (CD8$^+$) percent respectively. Further cytofluorographic analysis of murine T cell-subpopulations and whole T and B cells was performed to assess the frequency of these cells in the spleen of CBA/ca mice. The analysis of whole spleen cells revealed that the percentage of Thy-1$^+$, CD4$^+$, CD8$^+$ and B-220$^+$ cells did not change appreciably among normal, infected and vaccinated mice (Table).

*Effect of transferred T cell-subsets on lesion growth in recipient mice* - In order to find out the functional role of T cell-subpopulations from donor mice stimulated in a different way (infected or vaccinated mice) adoptive transfer experiments were performed. Highly viable (>95%) nylon wool purified donor cells were treated *in vitro* with monoclonal antibodies (either anti-CD4 and/or anti-CD8) plus complement and immediately transferred into naive recipients. Together with control mice (i.e. mice inoculated only with medium, but not cells), recipient mice were infected with 3x10$^6$ *L. m. mexicana* pro-

mastigotes one day later and lesion growth was weekly recorded for 6-9 weeks. Results from these experiments are shown in Fig. 4. Fig. 4A shows that, unfractionated T cells from infected donors did not have any significant effect on lesion growth, as compared to control mice. There was, however, an inhibition when either CD4 and CD8 depleted T cells were inoculated into syngeneic recipients. Lesion size measurements of control and transferred mice were significantly different only for mice reconstituted with CD8 depleted T cells both, five and six weeks post-infection (p<0.05) and only three out of a total of six mice per group (i. e. groups of recipient mice inoculated with CD4 or CD8 depleted T cells respectively) developed palpable lesions. Fig. 4B shows that adoptive transfer of unfractionated T cells from vaccinated CBA/ca syngeneic donor mice induced a delay in lesion growth (up to five weeks after infection), but this effect was much more dramatic when either CD4 or CD8 depleted T cells were transferred into naive recipients. Lesion measurements between control and mice transferred with CD8 depleted T cells were significantly different six (p<0.005) and seven (p<0.05) weeks after infection, whereas those differences between controls and mice transferred with CD4 depleted T cells were significant at six (p<0.05) and seven (p<0.05) weeks post-infection. Four out of a total of six recipient mice developed lesions when inoculated with CD4 depleted T cells and only two did it when reconstituted with CD8 depleted T cells. Results shown in Fig.5A indicate that double depleted T cells (i.e. Nylon wool purified spleen cells treated with anti-CD4 and anti-CD8 MoAb plus complement) from infected donors produce a transient arrest on
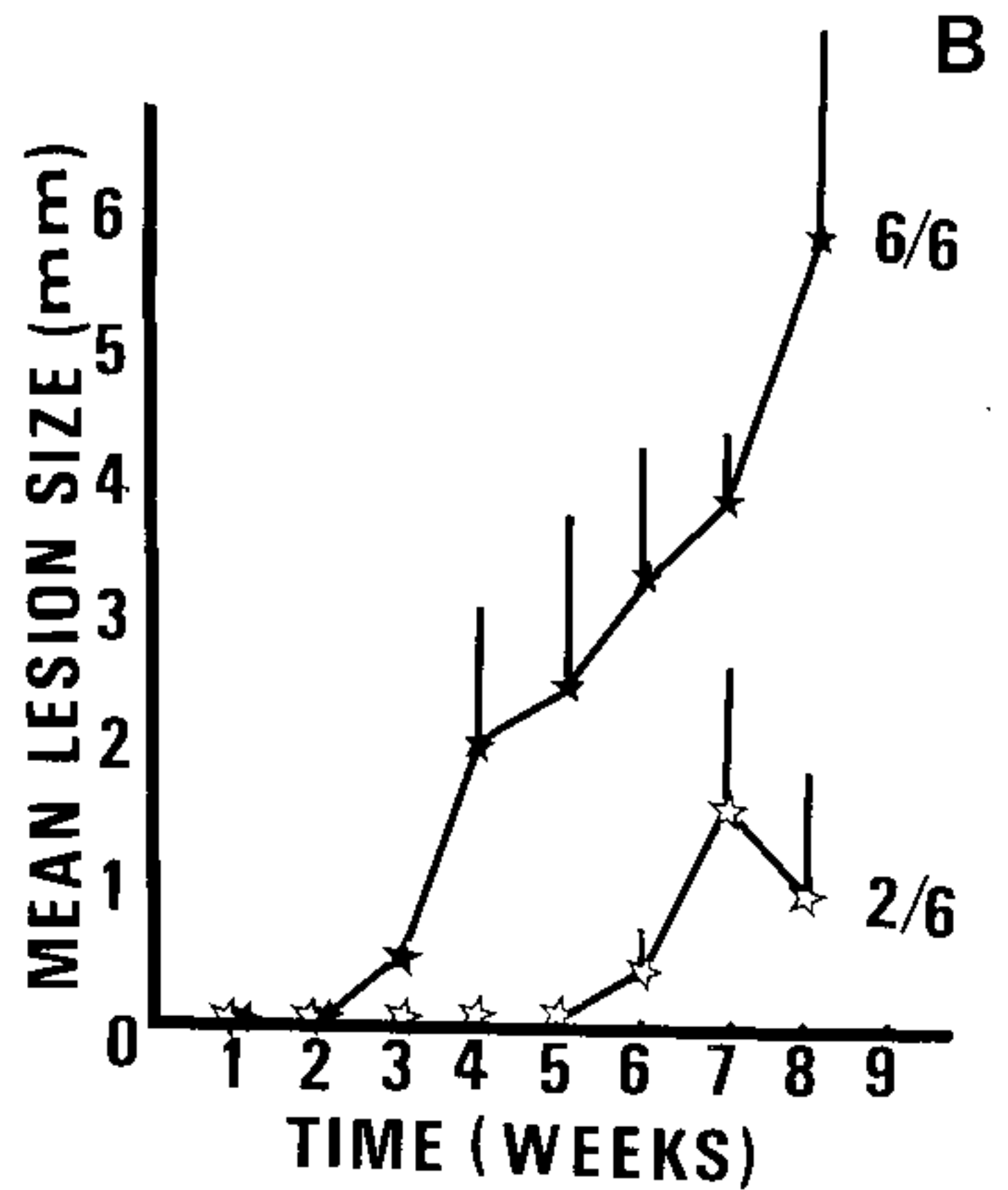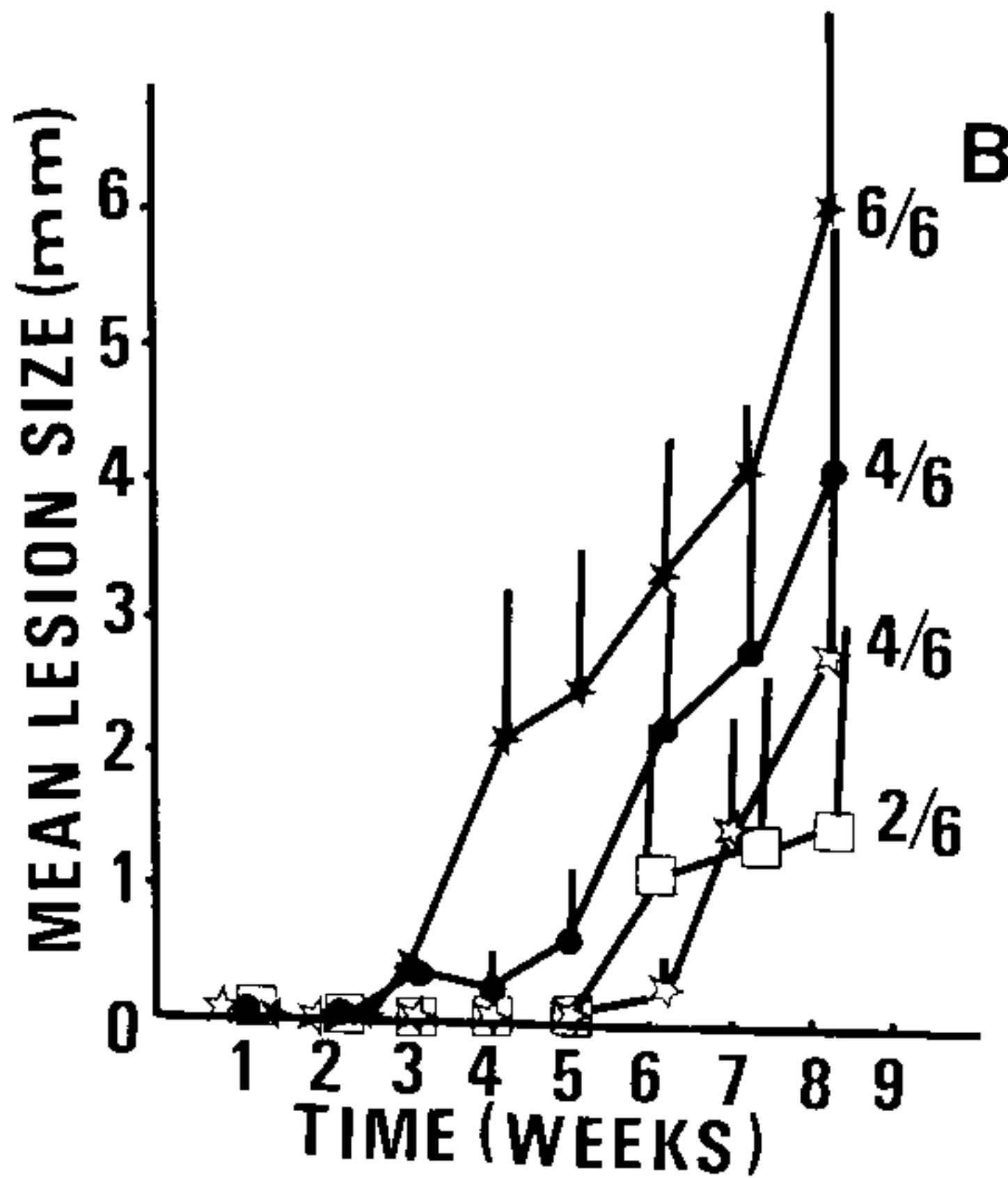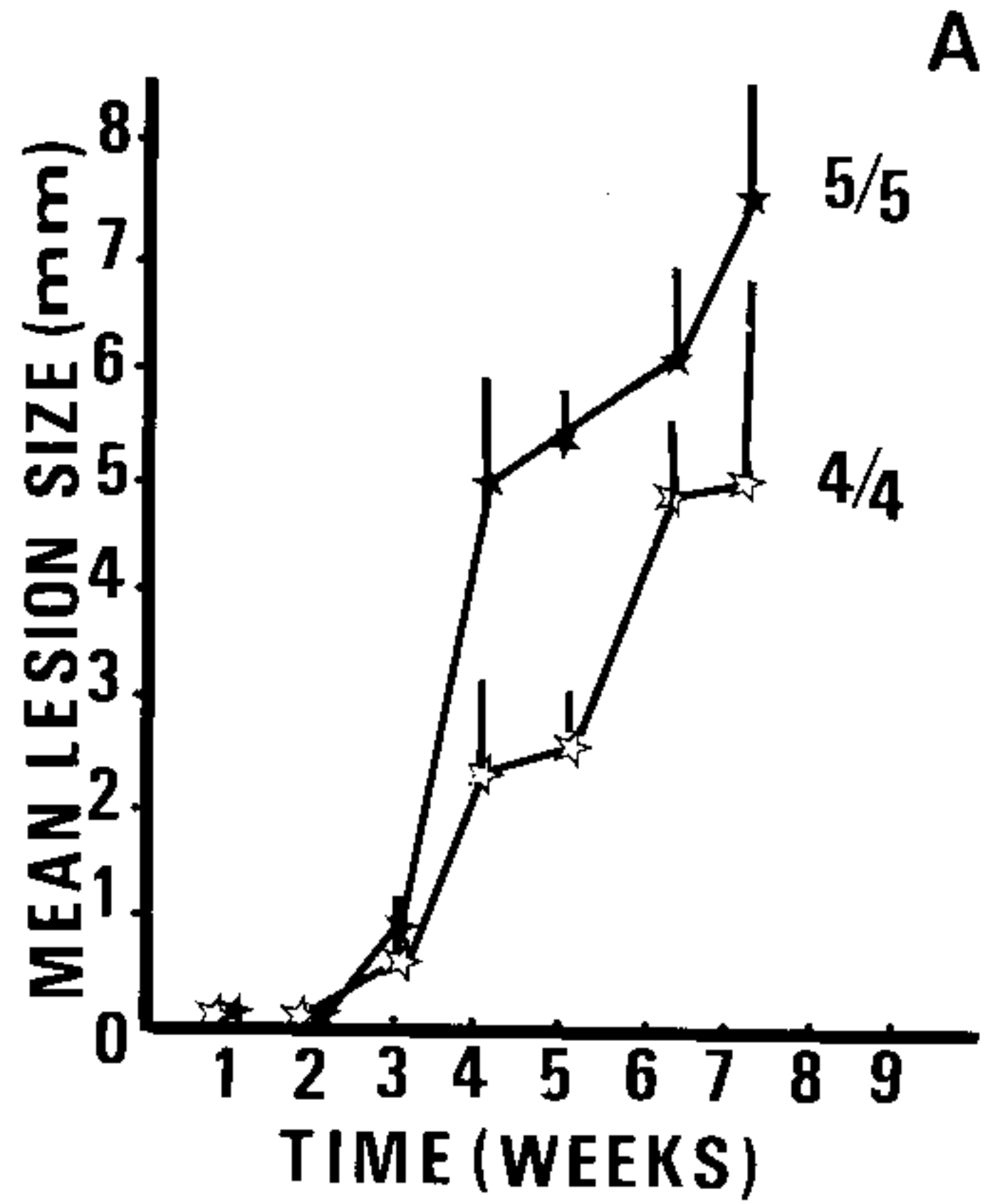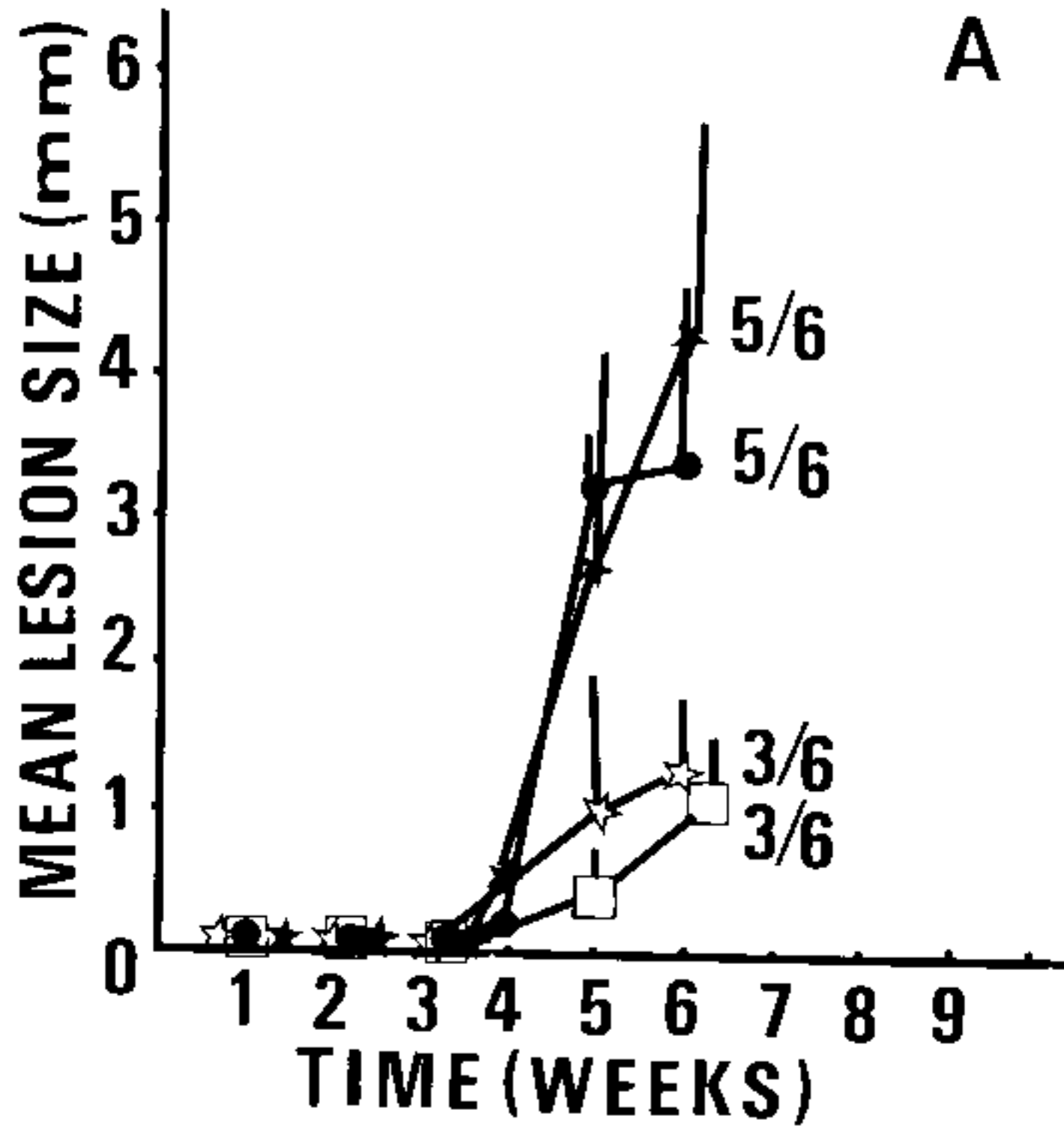
Fig. 4: adoptive transfer of T cell-subpopulations from infected or vaccinated donors into naive recipients. Groups of mice were i.p. inoculated with $3 \times 10^6$ viable (> 95%) nylon wool purified cells from infected (4A) or vaccinated (4B) donors alone (closed circles) or treated *in vitro* with anti-CD4 (open stars) or anti-CD8 (open squares) MoAbs and complement. Control groups (closed stars) were all inoculated only with RPMI-1640 (no cells were transferred). One day later all mice were infected with $3 \times 10^6$ promastigotes of *Leishmania m. mexicana*. Data are expressed as mean ± SEM (N=6). The number of mice that developed lesions is indicated after each line (i. e. No. of mice with lesions at the end of the experiment/total No. of mice included in the group). Results shown represent a replicate of three separate experiments.

Fig. 5: adoptive transfer of T cell-subpopulations from infected or vaccinated donors into naive recipients. Groups of mice were i.p. inoculated with $3 \times 10^6$ viable Nylon wool purified cells from infected (5A) or vaccinated (5B) donors and treated *in vitro* with anti-CD4 and anti-CD8 MoAbs and complement (open stars). Control mice (closed stars) were all inoculated with RPMI-1640 only. One day later all mice were infected with $3 \times 10^6$ promastigotes of *Leishmania m. mexicana*. Data are expressed as mean ± SEM (N=5-6), the number of mice that developed lesions is indicated after each line (i. e. No. of mice that presented lesions at the end of the experiment/total No. of mice included in the group). Results shown represent a replicate of three separate experiments.

lesion growth five weeks after infection (p<0.01) but, all animals (controls and reconstituted mice) developed lesions by the fifth week post-infection, whereas lesions on mice transferred with double depleted T cells from vaccinated donors were clearly arrested soon after infection with *L. m. mexicana* promastigotes and onwards. Lesion size measurements of controls and mice transferred with double depleted T cells from vaccinated donors were significantly different at five (p<0.05), six (p<0.001) and nine (p<0.01) weeks after infection and only two out of a total of six mice developed lesions at the end of the experiment (Fig. 5B).

## DISCUSSION

Our results showed that, intraperitoneal vaccination of CBA/ca mice with promastigote-derived membrane antigens in liposomes fully protects them against a normally non-curing infection and this effect correlates with induction of protective T cell-subpopulations and an increase in specific IgG levels. Liposomes have proved to be an efficient adjuvant acceptable for intradermal use in humans which, act enhancing both humoral and cellular responses (Russell & Alexander 1988, Kahl et al. 1989). Recently, it has been shown that liposome-preparations loaded with *L. major* soluble antigen presented a preferential entrapment with GP-63 and LPG and lead to protection against a homologous challenge with low IL-3 production (Kahl et al. 1990). Russell and Alexander (1988), have reported that CBA/ca mice can be fully protected against infection by i.p. or s.c. immunization using liposomes containing GP-63 and/or LPG. In this report we present evidence showing that resistance to murine leishmaniasis can be transferred to naive recipients with CD4+ T cells from infected mice and with CD4+, CD8+ and CD4- CD8- T cells from vaccinated donors. Both CD4+ and CD8+ T cells might be involved in an effector mechanism relying on IFN-gamma they produce (Kaufmann 1988). The participation of CD8+ T cells in protection during *Leishmania* infection has been controversial. Liew (1985, 1989) has long supported the contention that, if they do have a role in protection against *Leishmania* infection it is secondary to CD4+ T cells, while Titus (1987), has reported that *in vivo* elimination of CD4+ and CD8+ cells ended in exacerbation to *L. major* infection and Kaye (1987), reported that a substantial number of CD8+ T cells are activated during *L. donovani* infection. In addition, McElrath et al. (1987) has reported that, 40% of the T cells infiltrating the lesion site in resistant mice are CD8+ cells. Further evidence supporting the importance of CD8+ T cells comes out from experiments performed by Hill et al. (1989), who suggested that *in vivo* elimination of CD4+ "suppressor" T cells releases CD8+ T cells that mediate healing to *L. major* infection. Recently, Farrell et al. (1989)

discovered that *in vivo* depletion of CD8+ T cells effectively prevented the induction of resistance that can be observed following i.v. immunization with killed promastigotes. More recently, Smith et al. (1991) showed that CD8+ T cells induce macrophage leishmanicidal activity by the release of IFN-gamma. In addition, CD4+ T cell clones have been developed which protect BALB/c mice against an otherwise fatal disease (Müller & Louis 1989). However, Müller et al. (1989), have also produced CD4+ T cell clones which transfer disease exacerbation. This dichotomy can be fully explained accepting that disease progression is controled by two (or more) different T cells that could be distinguished by the lymphokine pattern they produce (Romagnani et al. 1993) and could be stimulated with different parasite antigens (Scott et al. 1988, Scott, 1989, Lezama et al. 1992, Kemp et al. 1993). This information suggests yet again, that different immunological pathways are followed during natural resistance of CBA/ca mice to *L. major* infection and experimentally-induced protection against *L. m. mexicana* challenge (Alexander & Kaye 1985). Previous reports have indicated that the ratio CD4/CD8 T cells in the draining lymph nodes of CBA mice resistant to *L. major* infection presented equal proportion of CD4+ and CD8+ cells (Milon et al. 1986). Our experiments showed that vaccinated CBA/ca mice contained a slightly higher proportion of CD8+ T cells. Our experiments also showed that, adoptive transfer of CD4- CD8- T cells from vaccinated mice strongly delayed the growth of *L. m. mexicana* in syngeneic recipients. Although, the role of such cells in leishmaniasis has not been previously established, it is of interest that cells expressing the gamma-delta T cell-receptor have been found infiltrating lesions produced by *L. aethiopica* or *L. braziliensis* in humans (Modlin et al. 1989, Uyemura et al. 1992). Lymphocyte-lines expressing the gamma-delta T-cell receptor have been prepared from leprosy skin lesions which proliferate *in vitro* specifically to mycobacterial antigens (Mehra & Modlin 1990). It has been demonstrated that, murine splenic double negative T cells expressing the alpha-beta T cell receptor produce IL-4 and it was especulated that they would induce the differentiation of CD4+ Th0 cells into Th2 phenotype (Zlotnik et al. 1992). Furthermore, it has recently been found that, intraepithelial (IEP) double negative T cells expressing the gamma-delta TCR can restore antibody responses in mice that had been orally tolerized with antigen (Fujihashi et al. 1992) and more recently Rust and coworkers (1992), showed that the number of these cells is notably increased in patiens with coeliac disease. In addition, double negative T cells are present in the spleen of adult mice and express cytotoxic capabilities (Skinner et al. 1992). Although in our experiments we did not formally demonstrated that

protective double depleted T cells expressed the gamma-delta T cell-receptor, it could be of interest the isolation of double negative lymphocyte lines from *Leishmania* skin lesions and their full functional characterization would allow a better understanding of the whole cellular mechanisms associated with the development of chronic leishmaniasis.

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