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Autor: O'Daly C., J.A. / Cabrera, Z.
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Instituto Venezolano de Investigaciones Científicas (IVIC), Centro de Microbiología y Biología Celular, Caracas 1020-A, Venezuela

Immunization of hamsters with TLCK-killed parasites induces protection against *Leishmania* infection

J. A. O'Daly C., Z. Cabrera

Summary

Hamsters immunized with N-p-tosyl-L-lysine-chloromethyl ketone TLCK-treated *L. brasiliensis brasiliensis* (LB) from culture, infected with LB amastigotes presented: a gradual increase in T and B cell responsiveness to mitogens by lymph node lymphocytes, and an increased response to concanavalin A with no changes for dextran sulphate and pokeweed mitogen in splenocytes. Absence of parasites in lymph nodes after 6 weeks post-infection and a nodule 4 times smaller than that of infected control animals. The nodule was undetectable after 70 days of infection. Hamsters preimmunized with TLCK-treated *L. donovani* (LD) from culture did not show suppression of the blastogenic response to mitogens of spleen and lymph node cells after infection with LD amastigotes and survived for more than one year, whereas infected, unimmunized animals died five months after infection. Animals preimmunized with culture parasites (LB or LD) treated with phenyl-methyl-sulphonyl-fluoride (PMSF) and infected with LB or LD amastigotes did not show any protective effect.

Key words: leishmaniasis; vaccine; protease inhibitors; lymphocyte response; mitogens.

Introduction

Leishmania brasiliensis (LB) is the causative agent of mucocutaneous leishmaniasis in the New World. Infected humans present ulcers that heal spontaneously but protective immunity may not be complete since destructive

Correspondence: Dr. José A. O'Daly C., Instituto Venezolano de Investigaciones Científicas (IVIC), Centro de Microbiología y Biología Celular, Apdo. 1827, Caracas 1020-A, Venezuela

metastatic lesions have been described frequently in the mucosa of nasopharynx. *Leishmania donovani* (LD), *L. donovani chagasi* and *L. donovani infantum* are the pathogenic protozoa of visceral leishmaniasis (Kala-azar) (Lainson and Shaw, 1978). Excellent reviews have been published on the epidemiology, taxonomy, immunology, clinical and histological features of the different forms of leishmaniasis (Turk and Bryceson, 1971; Zuckerman, 1975; Preston and Dumonde, 1976).

Live parasites have been used to vaccinate humans against *L. tropica major* infection (Berberian, 1939; Koufman et al., 1978; Witztum et al., 1979), the only practical vaccination in use today.

Sonicated promastigotes of *L. brasiliensis panamensis* have been employed to vaccinate the African white-tailed rat (Beacham et al., 1982) but without satisfactory results. BALB/c mice immunized with gamma-irradiated live "non-infective" promastigotes exhibited protection against a fatal *L. major* infection (Howard et al., 1982). Radioattenuated *L. mexicana mexicana* amastigotes rendered CBA mice highly resistant to subsequent infection with *L. m. mexicana* (Alexander and Phillips, 1978). Formalin-killed LD promastigotes treated with glucan confer a significant degree of resistance against infection with viable LD promastigotes (Holbrook et al., 1981).

In this article we present evidence of significant protection against LD or LB amastigote infection by immunization of hamsters with dead in vitro cultured parasites, without adjuvants.

Materials and Methods

Animals. Outbred male hamsters weighing 125–145 g were used in all experiments. Purina Laboratory chow (Protinal, Valencia, Venezuela) and water were supplied ad libitum.The animals were housed in a temperature-controlled animal room in bonnet-covered plastic cages with six hamsters in each cage.

Parasites. For LB infection hamsters were injected subcutaneously at the base of the tail with 10^5 amastigotes obtained from the nodules of animals infected with the "Gladys Castillo" strain of *Leishmania brasiliensis brasiliensis.* This strain classified as LB following the criteria of Lainson and Shaw (1978), was obtained from a 9-year-old patient in Choroní, Aragua State, Venezuela, and was kindly supplied by Dr. Alexis Rodríguez, from the Instituto de Medicina Tropical, Universidad Central de Venezuela, and maintained by serial passages in hamsters. The patient had mucocutaneous lesions that healed after treatment. The nodular lesions of hamsters were dissected, placed in sterile 35×19 mm disposable Petri dishes and weighed at different times post-infection (p.i.). Subsequently RPMI-1640 culture medium was added and the nodule teased-homogenized with forceps, filtered through gauze and aliquots taken to measure parasite concentrations. Spleens and inguinal, axillary and brachial lymph nodes from LB-infected animals were also removed at different days p.i. and weighed in sterile 35×10 mm disposable Petri dishes.

For LD infection hamsters were inoculated intraperitoneally (i.p.) with 10^5 amastigote forms obtained from the spleens of animals infected with the "Chuao" strain of *L. donovani.* This strain was obtained from a 12-year-old patient in Chuao, Aragua State, Venezuela. The parasites had been maintained by serial passages in hamsters. Amastigotes were obtained from infected hamsters by homogenizing spleens in RPMI-1640 in a ground-glass homogenizer fitted with a teflon piston having a clearance of 0.012–0.017 mm, driven by a motor (Arthur Thomas Co. PA) set at maximum velocity

for 3–5 min at 4 °C in order to increase the yield of free amastigotes to a maximum. The crude homogenates were centrifuged at 50× *g* for 5 min and the supernatant containing the parasites was centrifuged again at 1000× *g* for 15 min at 4 °C. The pellets containing the parasites were resuspended in 5 ml of 5% (w/v) NH_4Cl for 5 min at 37 °C, centrifuged at 1000× *g* for 15 min and resuspended at the appropriate concentration for injection into hamsters (Murray et al., 1982).

Also to determine parasite load, we used the method previously described to quantify *L. enrietti* in tissues (Poulter, 1979). 0.2 ml aliquots from splenic cells were incubated in 10 ml of Eagle's MEM (GIBCO) containing 5% (v/v) fetal bovine serum in plastic disposable flasks (Falcon #3012). Parasite densities in cultures were determined using a hemocytometer.

Blastogenic assays. Experimental animals were exsanguinated by cardiac puncture under terminal ether anesthesia. Immediately after removal, each spleen was teased with forceps in RPMI-1640 culture medium, washed twice in the same medium and the cells adjusted to 2×10^6 cells/ml. Lymph node cells were obtained from the inguinal, axillary and brachial lymph nodes and processed as described for the spleens.

Triplicate cultures of 2×10^5 mononuclear splenic cells/well (Falcon #3040) in a volume of 100 µl of RPMI-1640 supplemented with 5% fetal bovine serum (FBS, GIBCO), penicillin (100 units/ml), streptomycin (100 µg/ml) and 25 mM HEPES (Sigma) were used for each assay. The ability of spleen cells to proliferate in response to mitogens was assessed by adding suboptimal, optimal or supraoptimal doses of Concanavalin A (Con A, 0.5, 1.0 or 2.5 µg/ml, Sigma), Dextran Sulphate (DS, 5, 10 or 25 µg/ml, Sigma), and Pokeweed Mitogen (PWM, 1.0, 2.0 or 4.0%, GIBCO), in 100 µl aliquots of RPMI-1640 – 5% FBS. Cells were incubated at 37 °C in 5% CO_2 for 48 h after which 0.2 µCi of ^{3}H-thymidine (2.5 Ci/mmol., New England Nuclear, Boston, Mass.), was added to each well and incubated for a further 18 h until the time of harvest.

Cultures were processed with a multiple channel automated cell harvester (M24, Brandell Gaitherburg, MD). After drying, the glass fiber discs (Reeve Angel grade 934, AH, Curtin) were placed in vials with Packard scintillation fluid and then counted. Results are expressed as % response calculated as $(E_1-C_1/E_2-C_2)\times100$. E_1 = cpm experimental cells + mitogens; C_1 = cpm experimental cells; E_2 = cpm normal cells + mitogens; C_2 = cpm normal cells. Each value represents the average ± standard deviation of three to four different experiments performed at different times each (one animal/time post-infection/experiment). For each experimental point on the curve a normal control (one uninfected animal/time p.i.) was set up from animals matched for weight and age. The idea behind the use of 3 concentrations of mitogens was to check any possible shift in the optimal concentration of the mitogens due to the effect of infection, nevertheless, only the values for the optimal concentrations will be reported (see Figs. 1 and 2) as no variation was observed. The P value in each figure corresponds to the probabilities for test versus control according to Student's t test at each day p.i.

Immunization of the animals. L. brasiliensis and *L. donovani* grown at 30 °C in a synthetic culture medium (O'Daly and Rodríguez, submitted for publication) supplemented with 5% FBS, were collected at the stationary phase of growth and washed three times with PBS, pH 7.5 by centrifugation at 900× *g* for 15 min at 4 °C. About 95% of the parasites were amastigotes as observed in May-Grünwald Giemsa stained preparations.

For the immunization, parasites were incubated for 6 days at 30 °C in Eagle's MEM containing 0.01 M phenyl-methyl-sulfonyl-fluoride (PMSF, Sigma), or 150 µg/ml of N-p-tosyl-L-lysine-chloromethyl ketone, HCL (TLCK) in 2% (v/v) 2-propanol. No parasite survived these treatments as judged from examination of the cultures and/or infection in inoculated hamsters. Subsequently parasites were centrifuged at 900× *g* for 30 min at 4 °C and washed twice with PBS. One $\times10^8$ PMSF-treated or TLCK-treated parasites were inoculated i.p. in hamsters every week for 3 weeks. Seven days after the last dose, the animals were inoculated with 10^5 amastigotes obtained from nodules of LB-infected animals, or from spleens of LD-infected hamsters as described. Subsequently, infected unimmunized animals, immunized with PMSF- or TLCK-treated parasites but uninfected, immunized with PMSF-or TLCK-treated parasites and infected and one normal uninfected control were exsanguinated by cardiac puncture under ether anesthesia at 1 week intervals. Immediately, the spleens were

removed and teased with forceps in RPMI-1640 and the cells adjusted to 2×10^6 cells/ml. Lymph node cells were also obtained from the inguinal, axillary and brachial lymph nodes and processed as described for spleen. Blastogenesis assays were performed as described.

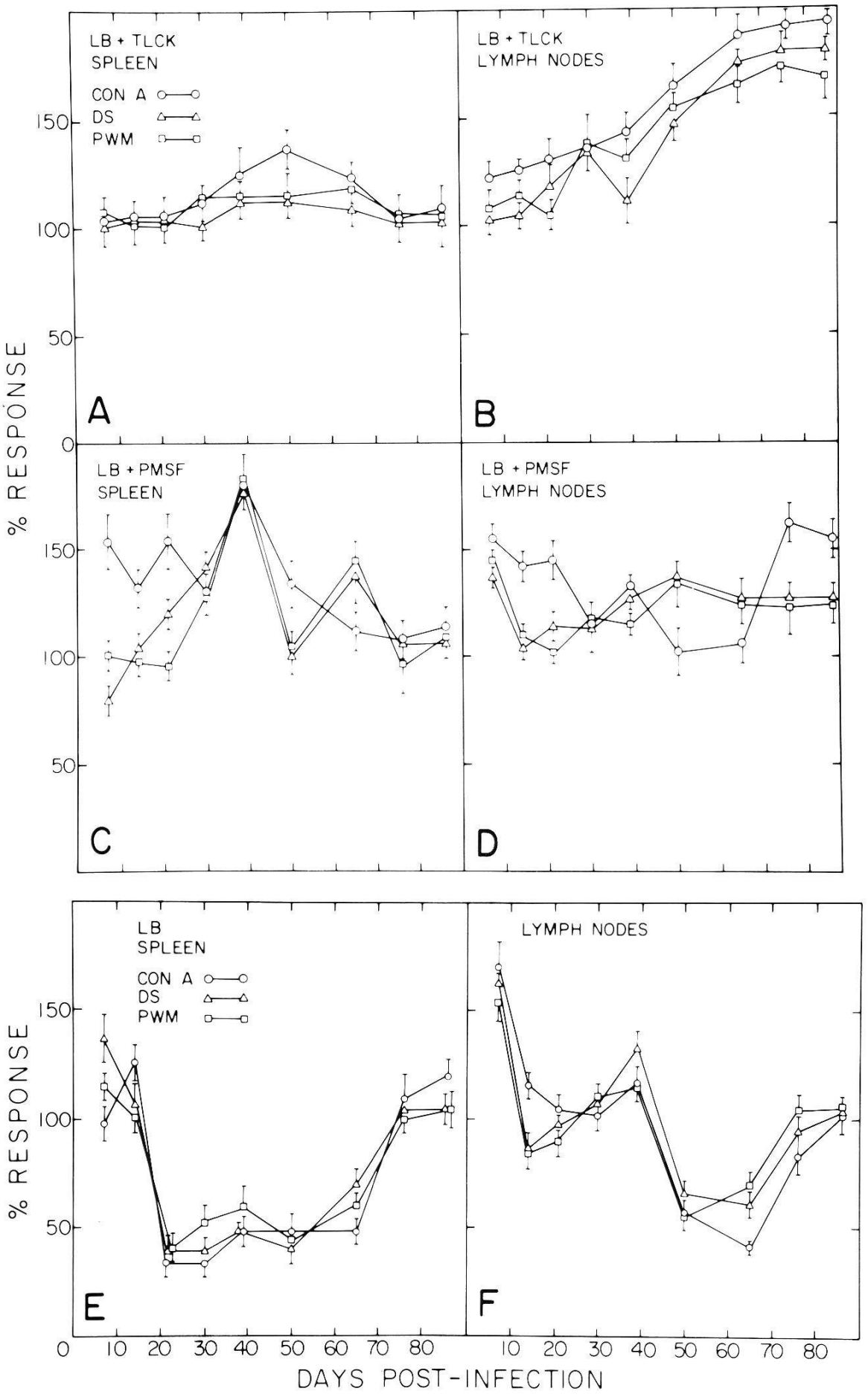
Results

When spleen cells from hamsters preimmunized with LB + TLCK and challenged with 1×10^5 amastigotes were tested for reactivity to mitogens, their lymphocytes showed an increased response to Con A between days 30 and 65 with normal values for DS and PWM (Fig. 1A), while lymph node lymphocytes showed a steady increase in their blastogenic response to all mitogens, reaching values of 160–200% after day 50 p.i. (Fig. 1B).

Spleen lymphocytes from hamsters which had been immunized with LB + PMSF and subsequently challenged with 1×10^5 LB amastigotes showed a significantly higher blastogenic response to T cell mitogens from the beginning of the infection, which returned to normal after 60 days of infection. The response to B cell mitogens increased steadily up to day 40 p.i., oscillating between 100–150% afterwards (Fig. 1C). By seven days, lymph node cells showed an increased response to all mitogens. After 14 and up to 42 days the B cell response was normal, increasing afterwards and remaining significantly high up to the end of the observation period. The response to Con A dropped to normal values by the 28th day and up to the 63rd day, increasing afterwards up to the end of the observation period (Fig. 1D).

Spleen cells from LB + TLCK preimmunized uninfected hamsters showed a normal response to all mitogens. A significant increase was observed in the cell response of lymph node cells reaching values up to 150% after 60 days post-immunization. Similarly, no suppression was observed when splenic or lymph node lymphocytes derived from LB + PMSF preimmunized uninfected animals were stimulated with B cell mitogens. In the case of Con A spleen cells from these animals showed a first cycle of increased responsiveness with a peak by day 21 (160%), a return to normal response from day 30–39 and a second cycle

Fig. 1. Blastogenic response to Con A (1 μg/ml), DS (10 μg/ml) and PWM (2%) exhibited by splenic and lymph node lymphocytes from hamsters preimmunized with TLCK- or PMSF-treated LB parasites and challenged with 10^5 LB amastigotes 7 days after the last immunization (A, B, C and D), and from infected non-immunized animals (E and F). Each experimental time point had its own control value (one non-immunized, non-infected animal) which was used to calculate the percent response of the different experimental groups on that particular day. Each value represents the average of 3–4 different experiments (one animal/time point post-infection/experiment) ± standard deviation. The P values for test versus control in B, after 50 days p.i. <.001 for all mitogens; in C from 25–45 days p.i. <.01 for all mitogens; in D after 75 days p.i. <.01 for Con A; in E at 21, 30, 39, 50 and 65 days p.i. <.01 for all mitogens, and in F at 7 days <.01 for all mitogens at 14 days <.05 for DS and PWM at 39 days p.i. <.05 for all mitogens and at 50 and 65 days <.01 for all mitogens. The cpm values for [^{3}H]-TdR uptake by lymphoid cells of non-immune, non-infected (control) animals were on the average (30 animals): spleen cells: Con A = 49,951±1,701; PWM = 16,792±1,482; DS = 5,554±742; lymph node cells: Con A = 53,147±1,224; PWM = 10,200±389; DS = 5,850±690.


LB + TLCK
SPLEEN
CON A
DS
PWM
LB + TLCK
LYMPH NODES
LB + PMSF
SPLEEN
LB + PMSF
LYMPH NODES
LB
SPLEEN
LYMPH NODES
A
B
C
D
E
F
% RESPONSE
DAYS POST-INFECTION
0
50
100
150
10
20
30
40
50
60
70
80

1

of increased responsiveness with a peak between 50 and 65 days (140%) that remained up to the end of the observation period. For lymph node cells a significant increase of the response to Con A was observed by day 60 (140%) with a return to normal afterwards (data not shown).

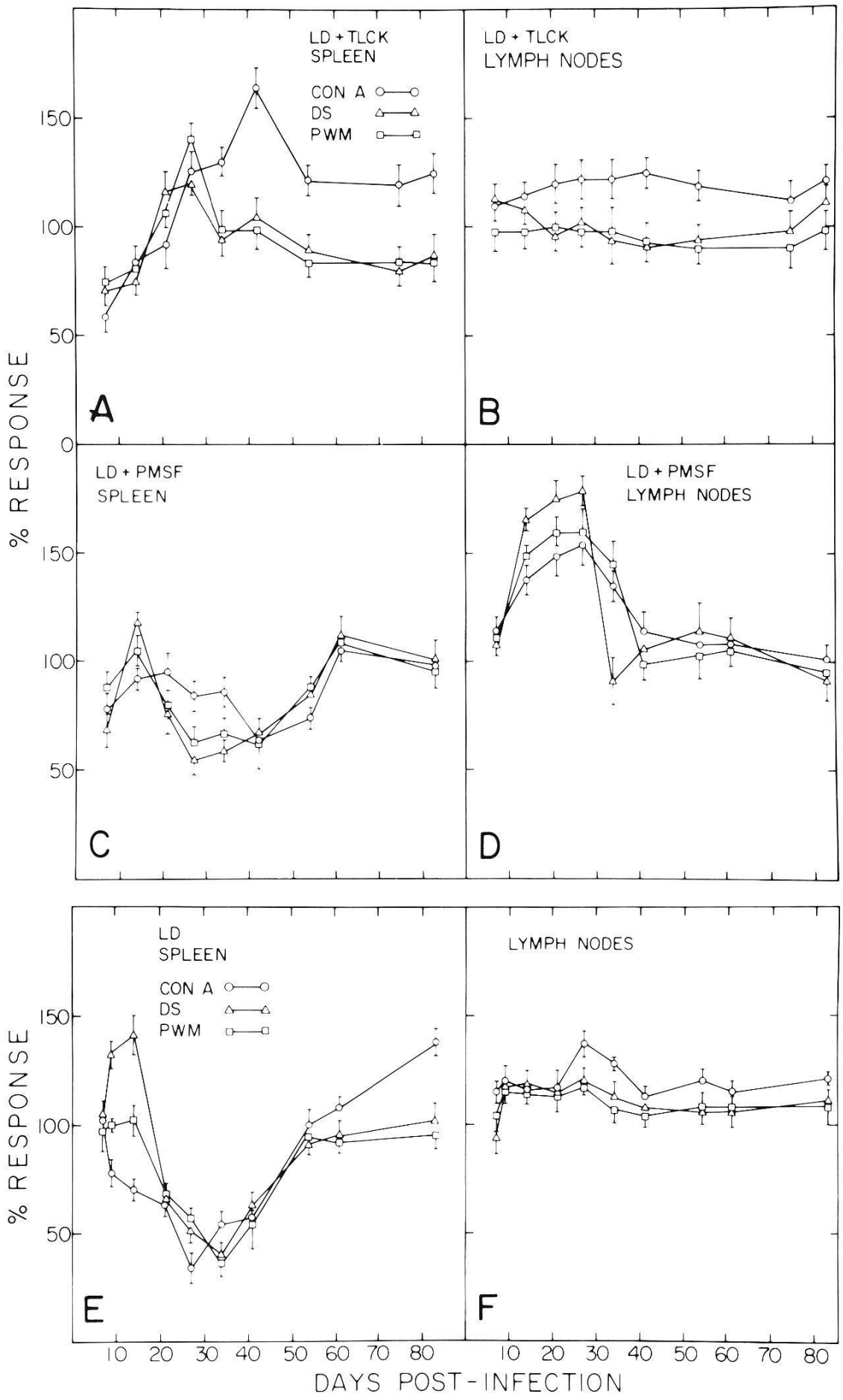
Hamsters infected with 1×10^5 LB amastigotes showed suppressed spleen cell responses to Con A, DS and PWM between 21 and 65 days p.i. with total recovery occurring between days 76 and 86 (Fig. 1E). At the beginning of the infection (7 days) there was a significant increase in the blastogenic response to DS and PWM. Lymph node cells of the same animals showed a significant increase in their blastogenic response to all mitogens (up to 160%) at day 7, and a decrease in the response (80%) to B cell mitogens by day 14. A second cycle of increased responsiveness to all mitogens occurred between days 25 and 40 with another period of significant suppression (70% or less) between days 45 and 70. By day 86 there was a total recovery of the response to all mitogens (Fig. 1F).

Splenic lymphocytes from animals immunized with LD + TLCK and challenged with LD showed a blastogenic response lower than normal for the first two weeks p.i. increasing steadily up to the 30th day p.i. It was equal or lower than normal for B cell mitogens after 35 days p.i. (Fig. 2A). A significant increase in the response to Con A was exhibited by lymphocytes 28 days after infection with a peak (165%) by day 42 and stayed elevated for the rest of the infection. Lymph node lymphocytes of the same animals showed a significant increase in the response (up to 120%) to Con A after 2–3 weeks of infection with normal values for DS and PWM at all times (Fig. 2B).

Splenic lymphocytes from animals immunized with PMSF-treated LD and infected with 1×10^5 LD amastigotes gave less than 80% of the control response at the time when suppression had also been found in infected unimmunized animals (Fig. 2C). Lymph node lymphocytes from the same hamsters showed a significant increase in the blastogenic response for all mitogens (up to 180%) at days 12, 21 and 27 p.i. (Fig. 2D) and remained within normal values during the rest of the observation period.

The responsiveness of splenic lymphocytes from animals immunized with LD + PMSF, but uninfected, showed a significant increase in the blastogenic

Fig.2. Blastogenic response to Con A (1 μg/ml), DS (10 μg/ml) and PWM (2%) exhibited by splenic and lymph node lymphocytes from hamsters preimmunized with TLCK- or PMSF-treated LD parasites and challenged with 10^5 LD amastigotes seven days after the last immunization (A, B, C and D), and from infected non-immunized animals (E and F). Each experimental time point had its own control value (one non-immunized, non-infected animal) which was used to calculate the percent response of the different experimental groups on that particular day. Each value represents the average of 3–4 different experiments (one animal/time point post-infection/experiment) ± standard deviation. The P values for test versus control in A after 30 days p.i. <.01 for Con A, after 50 days p.i. <.02; in C, at 27 and 34 days p.i. <.02 for PWM and DS, at 41 days p.i. <.02 for all mitogens; in D, after 12,21 and 27 days p.i. <.01 for all mitogens; in E, at 21–45 days p.i. <.01 for all mitogens, and in F, at 25–35 days p.i. <.01 for Con A. The cpm values or [^{3}H]-TdR uptake by lymphoid cells of non-immune, non-infected (control) animals were very similar to those reported in Fig. 1.


LD + TLCK
SPLEEN
CON A
DS
PWM
LD + TLCK
LYMPH NODES
LD + PMSF
SPLEEN
LD + PMSF
LYMPH NODES
LD
SPLEEN
LYMPH NODES
% RESPONSE
% RESPONSE
DAYS POST-INFECTION
A
B
C
D
E
F
0
50
100
150
10 20 30 40 50 60 70 80

2

response to all mitogens up to 150% from days 14–28 post-immunization dropping to normal values (100%) by day 42 and increasing by day 63 up to 160%. Lymph node lymphocytes showed a steady increase in the blastogenic response to all mitogens (up to 170%) from days 14–28 dropping to 130% and staying within that range for the rest of the observation period. Spleen cells from animals preimmunized with TLCK-treated LD and uninfected showed a normal blastogenic response to B cell mitogens during the whole period. There was an increase for Con A responsiveness by day 28 with a maximum of 145% by day 42, dropping to 130% and staying in that range up to the end of the observation period. Lymph node cells from the same animals showed a blastogenic response of $\simeq$120% of controls for all mitogens at all times (data not shown).

When responsiveness of splenic lymphocytes from LD-infected hamsters was measured, no change was observed on day 7 p.i. (Fig. 2E). However, between days 9 and 41 a significant decrease in responsiveness to Con A was observed with the lowest value at day 27 p.i. A gradual recovery of responsiveness followed until day 54 with a significant increase from day 61 to 83. A similar decrease in responsiveness to PWM of splenic lymphocytes was observed later, between days 21 and 41 p.i., with a peak by day 34. Interestingly, when the blastogenic response to DS was measured, a significant increase over controls was observed between days 9 and 14 followed by a decrease response between days 21 and 41, as observed with PWM and Con A. A return to normal responsiveness to PWM and DS was observed between days 54 and 83. In contrast, lymph node lymphocytes showed a slight stimulation by day 10 to all mitogens with a tendency to return to normal values by day 40 with the exception of Con A that showed stimulation throughout the whole study (Fig. 2F).

The change in nodular lesion weight as an index of infection is shown in Fig. 3. Nodules in infected unimmunized animals appeared 2 weeks after infection, reached a maximum size and began to ulcerate 7 weeks after infection, followed by a decrease in size but persistence of the nodule in the chronic period of infection. Animals preimmunized with PMSF-treated LB and infected showed a delayed nodular lesion, 5 weeks after infection, this lesion continued growing and became ulcerated 11 weeks after infection. Hamsters preimmunized with TLCK-treated LB and infected showed a nodule that appeared 4 weeks after infection, reaching a maximum size 4 times smaller than the one of unimmunized infected animals. The lesion ulcerated at 7 weeks p.i. and thereafter decreased, disappearing after 10 weeks of infection.

The presence of parasites in lymph node cultures is an additional parameter to follow the course of infection. In unimmunized LB infected or in LB + PMSF preimmunized and infected animals parasites could be detected in lymph node cell cultures after the 6th week of infection up to the end of the observation period (13 weeks), while in LB + TLCK preimmunized and infected animals parasites were seen at the 6th week of infection only. Cultures taken from the 7th up to 13th week p.i. failed to reveal parasites.

Parasites appeared in spleen at 34 days p.i. in LD-infected unimmunized hamsters and in animals preimmunized with PMSF-treated LD and then challenged with LD. Parasites were detected in spleens of hamsters preimmunized with TLCK-treated LD and then challenged with LD at 45 days p.i. and up to 63 days p.i. Their numbers were significantly lower than in the spleens of unimmunized infected animals. Infected and preimmunized animals with LD + PMSF presented a parasite density in the spleens similar to unimmunized infected animals. Infected animals, and preimmunized with PMSF-treated LD and infected, did not survive more than 5 months, while preimmunized with LD + TLCK and infected, survived more than a year.

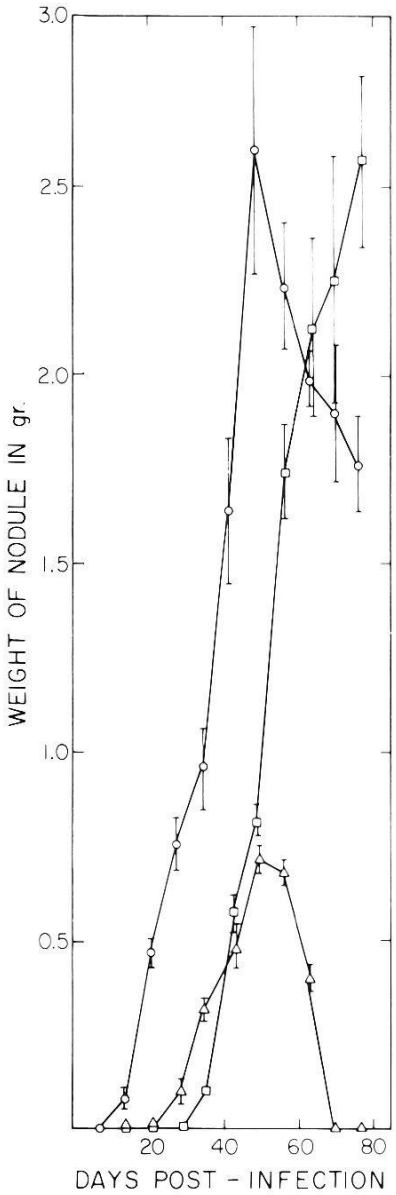


Fig. 3. Weight of nodules from hamsters infected with *L. brasiliensis* with and without previous immunization with PMSF- or TLCK-treated parasites. Each point is the average of 5 animals. The weight of the nodule is presented in unimmunized, infected hamster (○——○); animals preimmunized with TLCK-treated LB (△——△) or PMSF-treated LB (□——□) parasites from culture. All animals were challenged with 10^5 LB amastigotes.

Discussion

The evolution of infection in hamsters with mucocutaneous or visceral leishmaniasis has been studied in comparison with the effect of preimmunization with culture parasites treated in several ways. Transient hyporeactivity to T and B cell mitogens in spleen cells of LD-infected animals and in spleen and lymph node cells of LB-infected hamsters was observed. In both infections animals recovered their lymphocyte responsiveness to mitogens in the chronic period of the disease.

It has been reported that vaccination of the African white-tailed rat with sonicated promastigotes and then infected with *L. b. panamensis* induced partial protection. Of the treated animals, 43.75% developed ulcers at the site of challenge and 56.2% remained free from ulcers 7 months after challenge (Beacham et al., 1982).

Immuniziation with gamma-irradiated promastigotes reduced mortality to less than 33%, with 30–78% of infected BALB/c mice developing minimal primary lesions or none at all. This protective immunization abrogated the suppression of DTH in response to *L. tropica* infection (Howard et al., 1982).

Previously, we have described several proteases in *Trypanosoma* and *Leishmania* extracts that destroyed parasite antigens in a short period of time (O'Daly et al., 1983). Thus, in this work we explore the effect of protease inhibitors on parasite antigens and its potential in vaccination.

The TLCK + LB preimmunized and infected hamster was protected as judged by: 1. normal B cell blastogenic responses with a transitory increase in T cell response by splenic lymphocytes; 2. a significant stimulation of the response of lymph node lymphocytes to all mitogens, above the values obtained with the TLCK-treated LB preimmunized uninfected animals; 3. a brief appearance of parasites in lymph nodes only at the 6th week p.i.; 4. a nodular lesion 4 times smaller than infected controls, disappearing 70 days after infection; 5. absence of suppression of the mitogenic response in spleen and lymph node cells after infection (Figs. 1A, 1B and 3).

On the other hand the preimmunization with PMSF-treated LB produced a significant increase in the response to Con A in both spleen and lymph node lymphocytes with no changes for DS and PWM responses. Once those animals were infected, a displacement of the peak response for Con A was observed for both types of lymphocytes with a significant increase of the response of B cells (Figs. 1C and 1D). Nevertheless, even though the nodular lesion appeared with a delay, its evolution was similar to the control. Also, parasites were present in lymph nodes from the 6th week onward. It should be stressed that the preimmunization with either TLCK or PMSF treated LB parasites induces a general state of hyper-reactivity both unspecific (to mitogens for the immunized, uninfected animals) and specific (to the parasites in the challenge for the immunized infected animals) compared to normal hamsters, but that only the TLCK treatment seems to be producing additionally to this activation of the immune

system the preservation of some immunoprotective antigens, which does not seem to be the case for the PMSF treatment, as evidenced by the lack of parasite elimination and nodular lesion healing for the latter treatment, even in the presence of a hyperstimulated reactivity to mitogens.

It is of interest that these TLCK-treated LB hamsters were the only animals that showed a continuous increase, since the beginning of infection, of the blastogenic response to mitogens by lymph node cells (Fig. 1B), which suggests a key role for these cells in the control of LB amastigote proliferation. In the PMSF-treated LB animals it is clear that once amastigotes start appearing in lymph nodes, a drop in the Con A response with a leveling off in the response of B cell mitogens occurred (Fig. 1D) suggesting that, at this moment, parasites would overcome the immunological response and proliferate. Since the primary lesion in LB is cutaneous, it is not surprising that the draining lymphocytes are playing a major role in the control of the disease.

Once more, the effectiveness of TLCK-treatment is evidenced in LD infection, since no significant suppression of the blastogenic response to mitogens for both spleen and lymph node lymphocytes was observed (Figs. 2A and 2B), while the treatment of LD parasites with PMSF used as immunogen, even though produced hyperstimulation of the blastogenic response after vaccination, did not avoid the appearance of suppression of the spleen cell response of the immunized and infected hamsters to mitogens, which was similar to the infected control (Figs. 2C and 2E). In the same animals the response of lymph node lymphocytes was not affected as compared with the vaccinated uninfected controls (Fig. 2D), which together with the fact that LD infection does not affect the blastogenic response of lymph node lymphocytes to mitogens (Fig. 2F) stresses the importance of splenocytes in the control of LD infection.

The finding of a T and B cell response equal or above the normal control in most of the experimental conditions suggests that no variation occurred in the relative proportion of T and B lymphocytes. The variation in the macrophage number was not evaluated and is a parameter to be considered for future experiments, since preliminary data in our laboratory suggest that macrophages play a major role in the suppression induced by LB infection, while the suppression in LD infection seems to be mediated by non-adherent cells.

Formalin-treated, sonicated, heat killed and glutaraldehyde-treated parasites have also been used (data not shown) but none of these procedures induced the same protection as TLCK treatment, which should provide a useful basis for the study of those parasite antigens involved in the induction of immunity.

Acknowledgments

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