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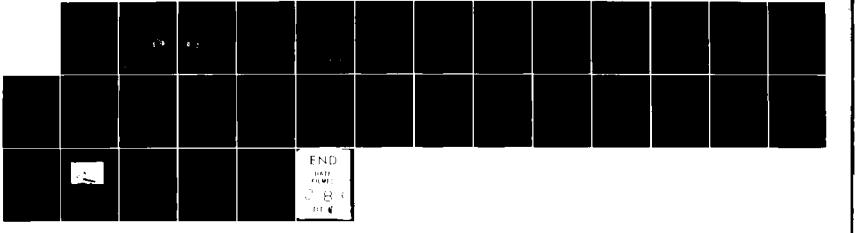

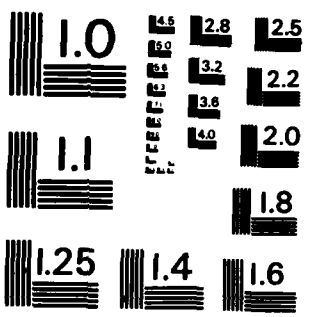

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Methods have been outlined for storage and reconstitution of various leishmania strains to be used as a vaccine. Investigations of cross immunity between L. tropica (Jericho) and L. braziliensis (panamensis) were made utilizing the African white tailed rat, Mystromys albacaudatus, model. It was established that a ulcerogenic dose L. tropica (Jericho) and L. braziliensis (panamensis) was $2x10^6$ promastigotes. Results indicated that L. tropica (Jericho) infected M. albacaudatus may develop some degree of protection from infection with not only the homologus strain but also against L. braziliensis (panamensis).

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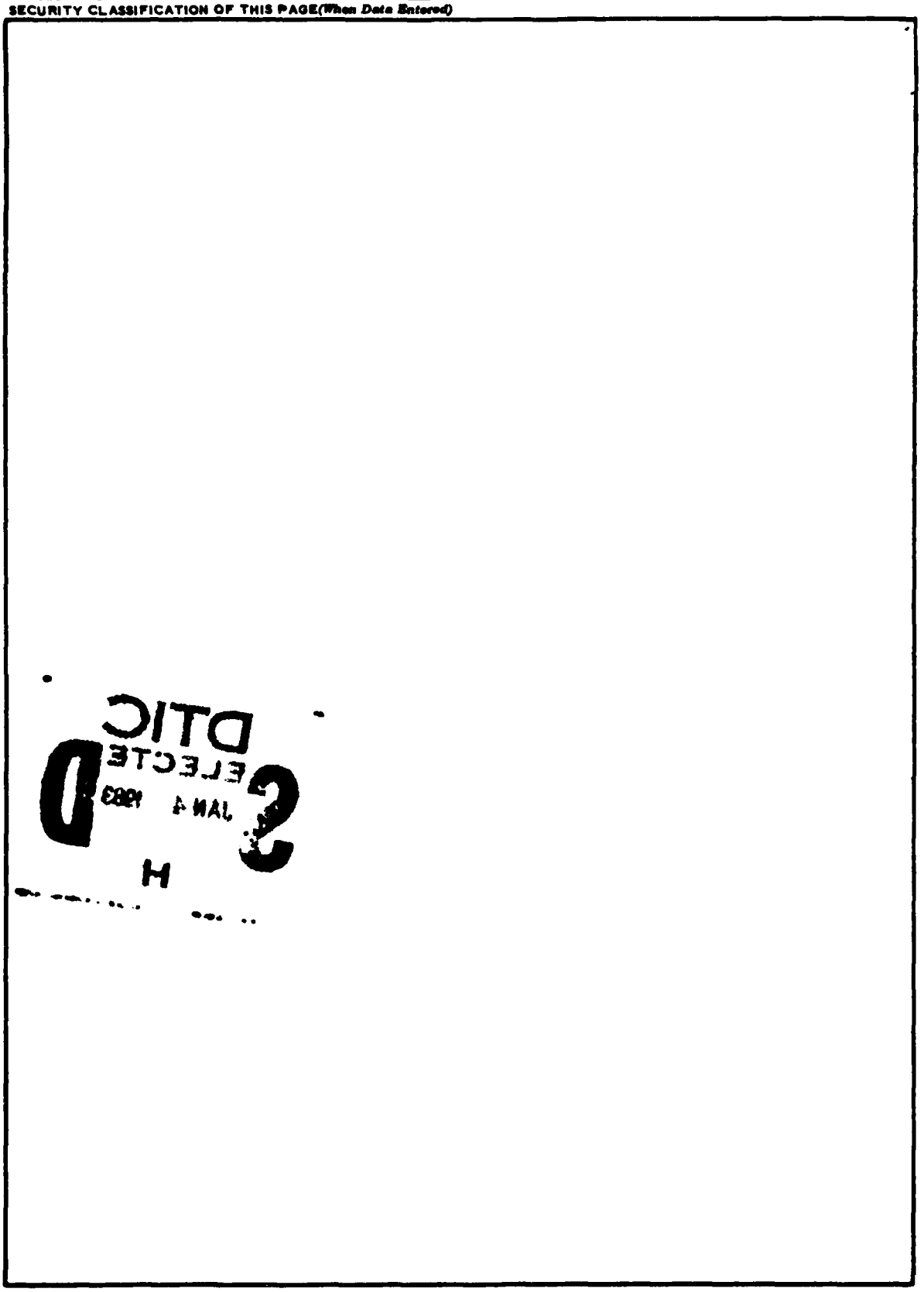

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"Investigations of Cross Immunity Between *Leishmania tropica* (Jericho) and *Leishmania braziliensis panamensis* in Experimentally Infected *Mystromys albacaudatus*"

Annual and Final Report

Bruce E. Beacham, M.D.

January 1981

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TABLE ON CONTENTS

List of Tables

ABSTRACT

Methods have been outlined for storage and reconstitution of various leishmania strains to be used as a vaccine. Investigations of cross immunity between L. tropica (Jericho) and L. braziliensis panamensis were made utilizing Mystromys albacaudatus, the African white tailed rat as an animal model. Data derived from our first year of study is included in Tables I-III and indicates how optimal infectivity relating to dose and strain as well as immunogenicity was established.

Thirty matched control animals were vaccinated with vehicle and challenged with $2x10^6$ live L. braziliensis panamensis promastigotes. Ulcers developed in 26 animals in a mean time of 29 days and were a mean size of 0.82cm at 8 weeks post inoculation. Twenty animals healed in a mean time of 140 days while six hadn't healed after 12 months and four animals never ulcerated.

Forty-one animals received two inoculations of $2x10^6$ live L. tropica (Jericho) promastigotes. Twenty-one animals developed ulcers in a mean time of 30 days with a mean size of 0.33cm. Mean healing time of ulcers was 95 days. Some degree of protection was imparted to animals that received live vaccination of L. tropica (Jericho) when challenged with L. braziliensis panamensis.

I. INTRODUCTION

The purpose of this report is to bring to attention the results of investigations dealing with possible cross immunity between L. tropica and L. braziliensis panamensis in an animal model. At this point in time, it would appear that there is some evidence that cross immunity does exist utilizing M. albacaudatus as an animal model.

If suitable experimental animals are successfully vacinated with promastigotes of L. tropica (jericho) solid immunity will develop to challenge L. braziliensis or L. braziliensis panamensis ? Part of this hypothesis is supported by the work of Lainson and Bray (1966) as mentioned above and part by the rich history of the use of related species of parasites or species with reduced virulence to prevent disease.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care, as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council (DHEW Publication No. 78-23, Revised 1978).

II. BACKGROUND

The use of related species of parasites or species with reduced virulence is a well established form of prevention of disease in man. This method of immunization in parasitic disease to date has been limited to scattered reports of success of zooprophylaxis occurring with malaria, Babesiosis and Trypanosomiasis (Nelson, 1974). These reports demonstrate amelioration or prevention of disease by exposure to heterologous infections of animal origin.

Leishmania investigators, for a considerable length of time, have addressed the antigenic relationships of different species and strains of leishmania -- in particular, relationships existing between new and old-world disease forms (Adler, 1964). Adler and Gunders (1964) demonstrated that patients recovered from typical oriental sores were immune to subsequent challenge with Leishmania mexicana. Thus, prior infection with recovery from a nonmetastasizing cutaneous leishmaniasis might provide immunity to other forms of new-world leishmaniasis in man. This hypothesis was confirmed in animals in 1966 by Lainson and Bray who demonstrated that Rhesus monkeys recovered from L. mexicana infection were refractory to challenge with L. braziliensis but were easily infected by L. panamensis. In 1966 Lainson and Shaw reported a human volunteer immune to L. mexicana infection but completely susceptible to Panamanian cutaneous leishmaniasis. They concluded that L. mexicana and the causative agent of Panamanian cutaneous leishmaniasis were antigenically distinct, thus ruling out the use of L. mexicana as a vaccination source for Panamanian cutaneous leishmanial disease.

The above work was reported over ten years ago, but unfortunately no further progress has been made in the development of an effective human va-cine against new-world leishmanial disease. This hiatus can perhaps be explained by: (1) The difficulty encountered in evaluating immunity in humans, and (2) the lack of a suitable animal model which could be adequately immunized without significant metastatic leishmanial disease.

Recent developments suggest that the above two obstacles may be overcome. First, Naggan, et al (1970), reported on the successful vaccination of a small group of young adults in Israel with a new strain of leishmania isolated from humans residing in the Jericho region of Israel. Effective immunity could be produced in approximately four to six weeks after healing

of the initial cutaneous ulcer with significant reduction in the attack rate of cutaneous old-world leishmaniasis in military personnel stationed in an endemic area (Naggan, et al, 1972). More recently, Koufman, et al (1978), reported a gradual decline in the rates of takes of inoculations utilizing the same strain of L. tropica as used by Naggan in 1968. In 1968 Naggan reported an 85.7 percent take. In 1975 this rate was reduced to 21.3 percent take. The authors felt that L. tropica tends to lose its virulence after prolonged storage and multiple passages. They demonstrated that using a new strain, isolated just a few months before the vaccination trial was performed, resulted in a greater than 60 percent positive take rate. This loss of virulence secondary to long storage and in multiple passages has been reported in numerous other parasitic strains (Gunders, et al, 1972; Manson-Bahr, 1964; Heyneman, 1971). Adler and Zuckerman (1948) were able to infect volunteers with an L. tropica strain maintained for 22 years although the incubation period of eight months was unusually long. It is also not known whether this phenomenon is very critical in cryogenically stored leishmania strains.

In addition to the above statements, it should also be noted that no significant complications were reported in the vaccinations of approximately 1,200 soldiers with L. tropica (jericho). It also should be noted that Naggan's results indicate that immunity which was thought to only be acquired after the healing process has commenced may be at least partially acquired as early as three to six weeks after inoculation.

Second, an ideal animal model for the study of cutaneous leishmaniasis has been found (Hendricks, 1977). Mystromys albacaudatus is easily infected with conventional ulcer-producing doses of two million promastigotes of L. tropica. These ulcers self-cure in approximately three months and there has been no evidence of metastatic spread of the leishmanial disease. Furthermore, this animal has an average life span of four to five years making it ideal for relatively long-term evaluation of the immunologic status of the immunized and nonimmunized animals.

Because of these two relatively recent developments it would appear that ideal conditions exist to obtain more specific information concerning the cross immunity between old- and new-world leishmaniasis.

The approaches to immunological prophylaxis in protozoal infections can be divided into passive and active immunization:

Passive immunization in protozal disease has centered around experience with Plasmodium falciparum malaria in man (Cohen and Sadun, 1976). The antibody is directed against the merozaites and prevents the reinvasion of the red blood cell by blocking the attachment of the parasite to the erythrocyte membrane. However, these antibodies are vatiant-specific and substantial problems were encountered in the development of a vaccination program against malaria (Brown, 1976).

Active immunization has been investigated in protozoal disease by four methods. (1) The first method, perhaps least acceptable in humans, is

the use of standardized doses of normal infective stages with the development of disease which is terminated by an appropriate antiparasitic drug. (2) The second method, most practical at present, is the use of related species with reduced virulence. (3) The third method is the use of artifically attenuated infective stages. (4) The fourth method is the use of in vitro organisms from which specific antigens may be isolated and used to immunize.

The most desirable approach to the development of a vaccine for humans would be the use of attenuated human strains of leishmania which are antigenically related to L. braziliensis and have reduced virulence. In the event that a solid cross immunity between L. braziliensis and leishmania strains with reduced virulence can be developed utilizing a rodent model, further work utilizing primates and eventually humans could proceed. It would also be appropriate to investigate the immunologic status of one animal model in a more extensive manner utilizing *in vitro* and *in vivo* measures of both humoral and cell-mediated immunity.

Cell-mediated immunity and macrophage function significantly influence the degree, course and final outcome of leishmanial infection. Participation of cell-mediated immunity is well documented in various leishmanial animal models, including the guinea pig and mouse (Blewett, et al, 1971; Turk and Bryceson, 1971; Lemma and Yau, 1973; Preston, et al, 1972; Skov and Twohy, 1974a and 1974b). The degree of effectiveness of cell-mediated immunity may determine the clinical manifestations of the leishmanial disease (Turk and Bryceson, 1971). Disseminated cutaneous leishmaniasis most closely correlates with the lack of effective cell-mediated immunity and the recidivens type of leishmaniasis is characterized by healed disease with only a very few parasitized histiocytes. The role of the macrophage in acquired immunity in leishmanial infection has not been clearly defined. There is also good evidence that the macrophage is not the sole controller of parasite burden in chronically infected animals and most likely acts in conjunction with antibody response to the organism (Herman and Farrell, 1977).

The development of a positive delayed skin test can be correlated with the in vitro production of lymphokines and monokines in the development of blast transformation (Blewett, et al, 1971). It would be useful to establish a correlation between time of vaccination and time of adequate immunity as detected by in vitro cell-mediated measurements such as described above. It was previously thought that immunity would not develop until several weeks or months after the initial ulcer of cutaneous leishmaniasis had healed (Senekji and Beattie, 1941; Berberian, 1944). However, observations made in Naggan's study (1970) and again in follow-up studies reported by Koufman in 1978 revealed the development of at least partial immunity in soldiers before the beginning of the healing phase of the ulcer. If there is a correlation between the measurement of cell-mediated immunity and refractiveness to infection with cutaneous leishmaniasis, a longer than necessary waiting period prior to entering an endemic area would be obviated.

Additionally, by recording and correlating cell-mediated immune responses in vaccinated diseased animals exposed to various cutaneous leishmanial species, a scale could be constructed which might serve as a guideline

to the prognosis of existing disease or detection of factors associated with the breakdown in immunity. Since adequate immunization is essential to the development of a successful vaccine, several other avenues of immunization might be mentioned. It has been suggested that the use of amastigotes, the disease producing entity in humans, might be more antigenic than the usual promastigote form (personal communication). The last avenue open at this time would be the utilization of irradiated killed promastigotes of L. braziliensis. Precedence of this exists in malaria with the radiation of attenuated sporozoites (Nussenzweig, Vanderberg and Most, 1969) as well as parasitized erythrocytes (Wellde and Sadun, 1967) in an attempt to develop vaccines and has met with little success because of resistance secondary to strain variation (Brown, 1976). Other discouraging results using this approach were reported by Lemma and Cole (1974) who were unable to induce immunity against L. enriettei in guinea pigs utilizing irradiated promastigotes of an homologous strain.

Finally, since most of this hypothesis relies on the use of closely antigenically related species, how does one determine what parasite is causing disease when challenge may produce a lesion? In the event this problem arises, there now exists a reliable sensitive rapid means of identification of various strains of leishmania by radiorespirometry reported by Decker, et al (1977). In their preliminary study they were able to consistently differentiate between Leishmania donovani, Leishmania tropica and Leishmania braziliensis.

III. APPROACH TO THE PROBLEM

We have already determined that $2x10^6$ promastigotes of L. tropica (Jericho strain) injected intrademally, or even subcutaneously, in a properly shaved region over the back of Mystromys albacaudatus will produce an ulcer in approximately 30 days. This ulcer has been observed to self-heal in approximately two to three months, at which time the animals are reported to be refractory to challenge with homologous strains of L. tropica (Jericho) (personal experience and personal communication). However, as mentioned in the background section, we have observed that as many as 25 percent of the initially inoculated animals developed ulcers when challenged with homologous strains. It should also be noted that these 25 percent developed the smallest primary lesions after the first innoculation.

In order to maintain the ulcers produced during vaccination, the area surrounding the ulcer must be depilated by shaving with a #40 shaving head, followed by a 30-second massage using a cream depliatory at weekly intervals.

In order to produce the vaccine which was utilized, it was necessary to reconstitute cryogenically stored leishmania obtained from Dr. Larry Hendricks of the Walter Reed Army Institute of Research. The promastigotes were reconstituted as per the method of Hendricks, et al (1978), and various concentrations established after five to six days of growth in 30 percent fetal calf serum in Schneider's insect media revised.

Our hypothesis was tested in vivo since this is the most direct and practical method . We also utilized various sized groups of animals to (1) establish the infective dose (50) for the L. tropica (Jericho) vaccine and L. braziliensis inoculant, (2) determine the approximate length of time needed for homologous immunity to develop after initial immunization with L. tropica (Jericho), and (3) define the immunogenicity of a variety of dosages and schedules of vaccinations of L. tropica (Jericho) promastigotes when challenged with L. panamensis and L. braziliensis.

IV. RESULTS WITH DISCUSSION OF RESULTS

In Table I, we have established an optimal ulcerogenic dose of L. tropica (Jericho) newly isolated strain and L. braziliensis (panamensis) to be $2x10^6$ promastigotes. Additionally, L. tropica (Jericho) old strain (two years old) needed a higher number of promastigores to effectively produce a lesion. This most likely represents a storage phenomenon which has been described by many investigators. The results also indicate a clear difference between L. tropica (Jericho) and L. braziliensis panamensis in duration of the infection -- L. braziliensis panamensis demonstrating a duration of anywhere from five months or longer compared with one to three months with L. tropica (Jericho).

As a sham control 10 Mystromys albacaudatus were inoculated with media without subsequent ulceration. These same 30 animals were then inoculated with $2x10^6$ L. braziliensis panamensis after which nine developed ulcers.

Table II presents the initial results of the first inoculation of Mystromys albacaudatus with old and newly isolated strains of L. tropica (Jericho). Most of these animals were males because of previous poor results in successfully inoculating females in some preliminary studies. A total of 79 animals have been inoculated with L. tropica (Jericho) utilizing the old strain in 14 and the new strain in 65. The incubation period depended upon varied dosages from an average of 14 days in the case of the highest dose of L. tropica (Jericho old strain) to an average of 30 days with .2cc L. tropica (Jericho new strain) with lesions ranging from 5mm to 1.5cm respectively. In general, the new strain seemed to need a slightly longer incubation period, have a longer healing time and result in a larger lesion.

Table III perhaps presents the most exciting results concerning cross immunity. Preliminary results indicated that L. tropica (Jericho new strain) infected Mystromys albacaudatus may impart immunity against infection with not only the homologous strain but also against L. braziliensis panamensis. These results, if confirmed with greater numbers, should suggest that a future, more medically significant, experiment would be the use of L. braziliensis braziliensis as the challenge agent.

As a result of the above encouraging preliminary data a larger more conclusive study of in vivo investigations of cross immunity between Leishmania tropica (Jericho) and Leishmania braziliensis panamensis in experimentally infected Mystromys albacaudatus was performed. Seventy-six animals were infected utilizing $2x10^6$ live L. tropica (Jericho) promastigotes suspended in Schneider's Drosophila Media Revised with 30 percent fetal calf serum (DMR30). Each animal had its left hind quarter shaved followed by topical application of a depilatory cream. The inoculation sites were shaven and depilated weekly for a period of three months. Ulcers developed in a mean time of 27.8 days and ranged from 0.2cm to 2.5cm in size with a mean of 0.63cm. Lesions healed, in a mean time of 80.4 days, with 15 animals failing t eal during an observation period of nine months. Ten of these animals wi non-healing ulcers died over the observation period secondary to a necrot g gram negative bacterial pneumonia without evidence of visceral leishma sis. Five animals with non-healing ulcers survived and remained culture pos ve for leishmania for greater than nine months. These animals were observed idn't receive a second inoculation with L. tropica (Jericho).

Table IV presents data derived from the above animals that were challenged with L. braziliensis panamensis after one or two inoculations with L. tropica (Jericho) and those control animals inoculated with DMR30 alone.

Ten animals received one inoculation of live $2x10^6$ L. tropica (Jericho) promastigotes, ulcerated and healed; were challenged 2 months after healing with $2x10^6$ L. braziliensis panamensis promastigotes injected into the shaved right hind quarter. Six of these ten animals (60 percent) developed ulcers in a mean time of 36.7 days and were a mean size of 0.48cm when measured at eight weeks post-inoculation. All ulcers healed in a mean time of 101 days.

Eighty-one animals received a second inoculation of live $2x10^6$ L. tropica (Jericho) promastigotes near the healed region on the left-hind quarter in the same manner as the first inoculation. Five of these animals developed a second ulceration in a mean time of 27.8 days with a mean size of 0.47cm. Four of these five animals healed in a mean time of 45 days and one failed to heal after a 90 day observation period. These 51 animals were then inoculated intradermally in the shaved and depilated right hind quarter two months after the second L. tropica (Jericho) inoculation had been given or had healed. Ten animals died during the post challenge period secondary to a necrotizing pneumonitis of unknown etiology. All autopsied animals had no signs of systemic leishmania disease.

Forty-one animals survived the challenge with L. braziliensis panamensis. Twenty-one animals developed ulcers in a mean time of 29.6 days with a mean size of 0.33cm. These ulcers healed in a mean time of 95 days. There was no significant difference in the group of animals that ulcerated after the second inoculation of L. tropica (Jericho) in terms of ulceration or healing time or size of ulcer when compared to those animals that didn't ulcerate after the second inoculation.

A matched group of 30 control animals were inoculated with DMR30 on two occasions, three months apart into the shaved left hind quarter. The animals and inoculation sites were maintained in the same manner as was described for the experimental animals. No lesions occurred in the control group following the above inoculations. All control animals were inoculated with $2x10^6$ live L. braziliensis panamensis promastigotes intradermally into the shaved right hind quarter. Ulcers developed in 26 (88 percent) animals in a mean time of 28.7 days. The mean size of these ulcers was 0.82cm when measured at 8 weeks post inoculation. Twenty animals with ulcers healed in a mean time of 140 days while six animals had not healed after twelve months of observation. Four animals remained refractory to challenge with L. braziliensis panamensis.

All scarred areas were cultured for leishmania two months after healing and were negative. Twenty animals remained refractory to challenge with L. braziliensis panamensis three months after their challenge. Autopsies of ten animals in the experimental group inoculated twice with L. tropica (Jericho) and challenged with L. braziliensis panamensis which developed ulcers revealed no signs of systemic involvement with leishmania.

TABLE 1

Initial inoculation of *Mystromys albacaudatus* with *L. tropica* (jericho) and *L. braziliensis* for determination of optimal dose

Number of Animals	Species and Strain	Innoculum Size	Number Infected	Incubation Period	Duration
10	*L. tropica* (old Jericho)	$.5x10^6$ pros	3	5 weeks	4 weeks
10	*L. tropica* (old Jericho)	$2x10^6$ pros	5	4 weeks	4 weeks
10	*L. tropica* (old Jericho)	$2x10^7$ pros	7	3 weeks	4 weeks
10	*L. tropica* (new Jericho)	$2x10^6$	9	3 weeks	12 weeks
10	*L. braziliensis panamensis*	$2x10^6$	8	2 weeks	21 weeks
10	*L. braziliensis panamensis*	$2x10^8$	4	4 weeks	21 weeks

TABLE II

Initial inoculation of Myatromya albacaudatus with
L. tropica (Jericho old & new) and *L. braziliensis panamensis*

Date of Innocu-Lation	Number of Animals	Species and strain	Innoculum Size	Number Infected	Incuba-tion Peroid	Duration	Size of Lesion
2/79	10 ♂	*L. tropica* (old Jericho)	$2x10^7$ pros (.1ml)	9	20 days	4 wks	.5cm
2/79	4 ♂	*L. tropica* (old Jericho)	$2x10^8$ pros (.1ml)	4	9 days	16 wks	.5cm
2/79	2 ♂	*L. braziliensis panamensis*	$2x10^8$ pros (.1ml)	1	14 days	6 mos	1.0cm
3/79	20 ♂	*L. tropica* (new Jericho)	$2x10^6$ pros (.1ml)	20	25 days	10-24 wks	1.5cm
4/79	25 ♂	*L. tropica* (new Jericho)	$2x10^6$ pros (.2ml)	25	42 days	2-4 mos	1.5cm
8/79	11 ♂	*L. tropica* (new Jericho)	$2x10^6$ pros	Pending----------			
8/79	9 ♀	*L. tropica* (new Jericho)	$2x10^6$ pros	Pending----------			

TABLE III

Mystromys albacadatus inoculated with *L. tropica* (Jericho) and subsequently challenged with $2x10^6$ *L. braziliensis panamensis* and an homologous strain of $2x10^6$ promastigotes

Originally Infecting Type and strain	# of Infected Animals	Challenge Type & Strain	# Animals Infected With Challenge
L. tropica (old Jericho)	5	*L. tropica* (new Jericho)	1
L. tropica (new Jericho)	5	*L. tropica* (new Jericho)	0
L. tropica (old Jericho)	10	*L. braziliensis panamensis*	0
L. tropica (new Jericho)	2	*L. braziliensis panamensis*	1

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TABLE IV

Experimental and control Mystromys albacaudatus vaccinated with $2x10^6$ L. tropica (Jericho) promastigotes and Drosophila media revised with 30 percent fetal calf serum (DMR30), respectively, and subsequently challenged with $2x10^6$ L. braziliensis panamensis promastigotes.

Animals vaccinated with $2x10^6$ live L. tropica (Jericho) promastigotes once and challenged with $2x10^6$ L. braziliensis panamensis.

No. of Animals	No. of Animals with Ulcers	Time to Ulcerate (days)	Mean Ulcer Size (cm) at 8 wks	Healing Time (days)
10	6	37	0.48	101

Animals vaccinated with $2x10^6$ live L. tropica (Jericho) promastigotes twice and challenged with $2x10^6$ L. braziliensis panamensis promastigotes.

No. of Animals	No. of Animals with Ulcers	Time to Ulcerate (days)	Mean Ulcer Size (cm) at 8 wks	Healing Time (days)
41	21	29.6	.33	95

Control animals vaccinated with DMR30 twice and challenged with $2x10^6$ L. braziliensis panamensis promastigotes.

No. of Animals	No. of Animals with Ulcers	Time to Ulcerate (days)	Mean Ulcer Size (cm) at 8 wks	Healing Time (days)
30	26	28.7	.82	140

V. CONCLUSIONS

Some degree of protection was imparted to animals that received live vaccinations of L. tropica (Jericho) when challenged with L. braziliensis panamensis. This protection was demonstrated by refractoriness to challenge and by shortening healing time and decreasing ulcer size. There was no evidence of systemic Leishmanial involvement but some animals did develop non-healing ulcers secondary to bacterial infection and other unknown causes. Some inoculated animals did die from a necrotizing pneumonitis which may indicate an alteration of their immune status. Variations in response to the live attenuated vaccine could have been attributed to our methods in quantitation and delivery of live promastigotes.

VI. RECOMMENDATIONS

These encouraging in vivo results indicate a need to investigate the efficacy of a live attenuated vaccine but also the efficacy of a killed vaccine. In regards to the latter, the use of a specific antigen may prove to be highly protective and might encourage the use of passive immunization if a monoclonal antibody to such an antigen could be developed.

VII. FINAL SUMMARY REPORT

This report represents the final results and conclusions of the in vivo investigations of cross immunity between Leishmania tropica (Jericho) and Leishmania braziliensis panamensis in experimentally infected Mystromys albacaudatus. Seventy-six animals were infected utilizing $2x10^6$ live L. tropica (Jericho) promastigotes suspended in Schneider's Drosophila media revised, which contained 30 percent fetal calf serum. Each animal had its left hind quarter shaved followed by topical application of a depilatory cream. The inoculation sites were shaven and depilated weekly for a period of three months. Ulcers developed in a mean time of 27.8 days and ranged from 0.2cm to 2.5cm in size with a mean of 0.63cm. Lesions healed, in a mean time of 80.4 days, with 15 animals failing to heal during an observation period of nine months. Ten of these animals with non-healing ulcers died over the observation period secondary to a necrotizing gram negative bacterial pneumonia without evidence of visceral leishmaniasis. Five animals with non-healing ulcers survived and remained culture positive for leishmania for greater than nine months. These animals were observed but didn't receive a second inoculation with L. tropica (Jericho).

Fifty-one animals received a second inoculation of live $2x10^6$ L. tropica (Jericho) promastigotes near the healed region on the left hind quarter in the same manner as the first inoculation. Five of these animals developed a second ulceration in a mean time of 27.8 days with a mean size of 0.42cm. Four of these five animals healed in a mean time of 45 days and one failed to heal after a 90 day observation period.

A matched group of 30 control animals were inoculated with Schneider's Drosophila media with 30 percent fetal calf serum on two occasions, three months apart into the shaved left hind quarter. The animals and inoculation sites

were maintained in the same manner as was described for the experimental animals. No lesions occurred in the control group following the above inoculations. All control animals were inoculated with $2x10^6$ live L. braziliensis panamensis promastigotes intradermally into the shaved right hind quarter. Ulcers developed in 26 (88 percent) animals in a mean time of 28.7 days. The mean size of these ulcers was 0.82cm when measured at eight weeks post inoculation. Twenty animals with ulcers healed in a mean time of 140 days while six animals had not healed after twelve months of observation. Four animals remained refractory to challenge with L. braziliensis.

Ten animals received one inoculation of live $2x10^6$ L. tropica (Jericho) promastigotes, ulcerated and healed; were challenged two months after healing with $2x10^6$ L. braziliensis panamensis injected into the shaved right hind quarter. Six of these ten animals (60 percent) developed ulcers in a mean time of 36.7 days and were a mean size of 0.48cm when measured at eight weeks post-inoculation. All ulcers healed in a mean time of 101 days.

Fifty-one animals that received two inoculations of $2x10^6$ live L. tropica (Jericho) promastigotes were inoculated intradermally in the shaved and depilated right hind quarter two months after the second L. tropica (Jericho) inoculation had been given or had healed. Ten animals died during the post challenge period secondary to a necrotizing pneumonitis of unknown etiology. All autopsied animals had no signs of systemic Leishmanial disease.

Forty-one animals survived the challenge with L. braziliensis panamensis. Twenty-one animals developed ulcers in a mean time of 29.6 days with a mean size of 0.33cm. These ulcers healed in a mean time of 95 days. There was no significant difference in the group of animals that ulcerated after the second inoculation of L. tropica (Jericho) in terms of ulceration or healing time or size of ulcer when compared to those animals that didn't ulcerate after the second inoculation.

All scarred areas were cultured for Leishmania two months after healing and were negative. Twenty animals remained refractory to challenge with L. braziliensis panamensis 3 months after their challenge. Autopsies of ten animals in the experimental group inoculated twice with L. tropica (Jericho) and challenged with L. braziliensis panamensis which developed ulcers revealed no signs of systemic involvement with Leishmania.

CONCLUSIONS

1. Some degree of protection was imparted to animals that received live vaccinations of L. tropica (Jericho) when challenged with L. braziliensis panamensis.

2. Protection was demonstrated by refractoriness to challenge and by shortening the healing time and decreasing the ulcer size.

3. The live vaccine, L. tropica (Jericho), didn't cause systemic disease, however, some animals developed non-healing ulcers secondary to bacterial infection and other unknown causes.

4. The live vaccine is slightly less protective than a killed homologous vaccine. (As demonstrated by a previous study submitted to you for approval in February 1981).

5. Variation in response to the live attenuated vaccine could have been attributed to our methods in quantitation and delivery of live promastigotes.

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PUBLICATIONS RESULTING FROM RESEARCH

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VACCINATION OF THE AFRICAN WHITE-TAILED RAT, *MYSTROMYS ALBACAUDATUS*, WITH SONICATED *LEISHMANIA BRAZILIENSIS PANAMENSIS* PROMASTIGOTES*

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Abstract. The usefulness of sonicated *Leishmania braziliensis panamensis* promastigotes for vaccination was evaluated in *Mystromys albacaudatus*, the African white-tailed rat. Thirty-two animals received three intradermal injections of 2×10^6 sonicated promastigotes derived from *L. braziliensis panamensis* at weekly intervals. One month after completion of the immunization schedule, the experimental group of animals was challenged in vivo with 2×10^6 live *L. braziliensis panamensis* promastogotes. At the same time, a matched group of 40 control animals was similarly challenged. Within 2 months, 35 of the 40 animals (87.5%) the control group developed ulcers, while only 14 of 32 previously vaccinated animals (43.7%) developed ulcers at the site of challenge within this same time period. The remaining 18 vaccinated animals (56.2%) remain free from ulcers 7 months after challenge. When lymphoctes from the spleens of vaccinated and control animals were challenged in vitro with antigen derived from sonicates of varying numbers of promastigotes, only cells from immunized animals responded vigorously to the antigenic challenge, a response which was not enhanced by the addition of immune antiserum to the reaction.

The induction of protective immunity utilizing homologous killed vaccines would be a significant step in the control of human cutaneous leishmaniasis. However, control of parasitic infections by prior immunization (vaccination) has not always met with success.[1–3] For example, Lemma and Cole were unable to induce immunity to *Leishmania enriettii* in guinea pigs using irradiated promastigotes of a homologous strain.[4] Recently, however, Mayrink et al. reported successful vaccination against cutaneous leishmaniasis in man by intramuscular injection of killed promastigotes of *Leishmania braziliensis*.[5] They pointed out, however, that their encouraging results were only preliminary.

Although results from animal studies cannot be directly extrapolated to human disease, the use of animal models in evaluating the efficacy of homologous, killed vaccines against cutaneous Leishmaniasis can be very informative. There are now several experimental animal models for studying cutaneous leishmaniasis, one of which has been developed in the African white-tailed rat, *Mystromys albacaudatus*.[6,7] We used this model to determine whether protection against *L. braziliensis panamensis* infection could be induced by vaccination with killed promastigotes of a homologous strain.

METHODS

In vivo studies

Previous work with the *M. albacaudatus* model has established that 2×10^6 live promastigotes of *L. braziliensis panamensis*, when inoculated intradermally into an area of skin kept free of hair, infected approximately 90% of the animals (Beacham and Ramito, unpublished data). Ulcers developed in 3 to 4 weeks and lasted approximately 3 to 4 months before healing (Fig. 1).

The parasite, designated WR-209, was originally isolated on blood agar from a human ulcer in Panama in 1974 and presumed to be *L. b. panamensis* based on geographic location, clinical

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In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as promulgated by the Institute of Laboratory Animal Resources, National Academy of Science, National Research Council.

manifestation, chemotherapeutic response and growth characteristics in culture. The isolate was inoculated intradermally into hamsters, reisolated, frozen and the stabilate was retained in liquid nigrogen until used. The inoculum of promastigotes was prepared by cultivation in Schneider's Drosophila medium, Revised (GIBCO, Grand Island Biological Co.) and 30% V/V fetal bovine sera, according to the method described by Hendricks et al.[8] Hereafter, this media will be referred to as Schneider's DMR 30. The number of organisms was adjusted to a final concentration equivalent to 2×10^6 leishmanial promastigotes with the aid of hemocytometer and microscope after appropriate dilutions. Promastigotes in suspensions were killed by sonication using a sonic dismembrater (Artek Co., Farmingdale, NY) at a setting greater than 90 decibels for 3 minutes while being cooled in an ice bath. Sonicates were quick-frozen to −70°C and reconstituted at room temperature prior to injection. The sites of inoculation were shaven and depilated weekly for a period of 2 months after completion of the final vaccination.

In the present studies, 15 8-week-old male and female white-tailed rats received 2×10^6 sonicated promastigotes of *L. b. panamensis* (Walter Reed Strain #209) suspended in 0.2 cc Schneider's DMR 30 in the right rump region by an intradermal route weekly for 3 consecutive weeks. It was important to keep the skin in the region of inoculation free of hair, since regrowth impeded the development of ulceration (unpublished observation). Thus, these skin regions were prepared by shaving with an electric animal shaver, followed by topical application of a depilatory cream. The cream was massaged onto the skin for 30 seconds and then washed with a slow stream of lukewarm tap water.

A matched control group of 20 animals was maintained in an identical manner as the vaccinated group, but received three weekly intradermal injections of 0.2 cc Schneider's DMR 30. Following these injections, these animals were also shaven and depilated weekly for a period of 2 months.

One month after completion of the final vaccination, 15 animals were challenged with 2×10^6 live *L. b. panamensis* promastigotes (WR-209) suspended in 0.2 cc of Schneider's DMR 30 by intradermal injection into a shaven left rump. The challenge sites were shaven and depilated weekly as described previously for a period of 3 months. The matched group of 20 control animals was also inoculated with 2×10^6 live *L. b. panamensis* and maintained in an identical manner as the vaccinated group.

In view of the initial sample size, the first in vivo experiment was repeated several months after its completion. The number of control animals in the repeat experiment was identical (20), but the vaccinated group consisted of 17 animals. All methods utilized during the repeat study were identical to the first in vivo experiment.

In vitro studies

Six vaccinated and three control animals in three different sets of experiments, were sacrificed one month after they had received their initial series of inoculations with sonicated promastigotes or 0.2 ml DMR 30 respectively. Spleens of these animals were obtained as the source of lymphocytes and monocytes for in vitro analysis of the immune status of the vaccinated and control animals. Cell suspensions were obtained by forcing the spleen through a sterile stainless steel screen into media containing RPMI 1640 (Grand Island Biological Co., Grand Island, NY), Hepes buffer (1 mm final concentration; Grand Island Biological Co., Grand Island, NY), glutamine (1 mm final concentration; Grand Island Biological Co., Grand Island, NY) gentamycin (50 mg/ml; Sigma Chemical Co., St. Louis, MO) and 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). Large aggregates of tissue were removed by filtering the cell suspensions through a 12-cc syringe which had been packed with sterile gauze. Cell numbers were determined with a Coulter electronic cell counter (Coulter Electronics, Hialeah, FL). Splenic lymphocytes and monocytes were separated by incubating whole cell suspensions on plastic dishes for 90 min and then collecting the non-adherent cells (lymphocytes). Monocytes were then separately collected by scraping adherent cells from the plastic dishes, using a rubber policeperson. Cell suspensions were adjusted to concentrations of 3×10^6 cells/ml and tested at serial 1:3 dilutions. One hundred μl of the appropriate cell suspension were then mixed with 100 μl of sonicated antigen at varying concentrations (see results), or media alone, in triplicate wells of microtiter plates, and incubated at 37°D in a CO_2 incubator. After five days, 20 μl of triated-thymidine (^{3}H-TdR) were added to each well. Eighteen hours later, the radio-labeled cell

mixtures were harvested using a Skatron A.S. automatic harvester (Flow Laboratories, Rockville, MD) and uptake of ^{3}H-TdR was determined in a liquid scintillation counter (Beckman LS250, Beckman Instruments, Palo Alto, CA). Blastogenic responses were evaluated by: 1) determining the increase in counts-per-minute of ^{3}H-TdR in stimulated cultures, compared to untreated controls; and by 2) a Blast Transformation Index, determined by dividing the mean number of counts in given experimental treatment by the mean of the controls for a given experiment.

RESULTS

In vivo studies

Data derived from both in vivo studies appear in Tables 1 and 2.

At 24 hour post-injection (PI), with 2×10^6 live *L. b. panamensis* promastigotes, small papules (<2 mm in diameter) appeared at the site of injection but were undetectable 72 hours PI. Six weeks PI, 35 or 40 control animals (87.5%) developed ulcers (mean 8.9 mm in diameter) on the left rump region (Fig. 1), while only 14 of 32 vaccinated animals (43.7%) developed slightly smaller ulcers (mean 5.0 mm in diameter) over the left rump region in the same time period. Twelve weeks PI, the ulcers of the control animals measured a mean diameter of 17.7 mm while the ulcers of the vaccinated animals measured a mean diameter of 8.1 mm. The difference in sizes of ulcers in the control and vaccinated groups both at 6 and 12 weeks, were significant ($P < 0.05$).

The control animals exhibited a mean incubation time (measured time from injection until ulceration) of 32.4 days while the incubation time for the vaccinated animals was a mean of 43.3 days. The control animals' mean duration of ulceration (measured time from ulceration to healing) was 108.6 days compared to 86.5 days for the vaccinated animals. Leishmania organisms were readily cultured from lesions on both control and vaccinated groups by aspiration and cultivation according to the method described by Hendricks and Wright.[9] Eighteen of 32 vaccinated animals (56.2%) exhibited complete resistance to challenge and were free of lesions 6 months after reexposure ($\lambda^2 = 10.50; P < .005$). One month post challenge with live *L. b. panamensis*, the resistant animals were cultured for leishmania in the same manner as described above and were found to be negative.

TABLE 1

In vivo results of inoculation of control and vaccinated Mystromys albacaudatus *with* 2×10^6 *sonicated* Leishmania braziliensis panamensis *promastigotes*

Groups	No. animals	No. infected	% infected	% resistant
Vaccinated I	15	6	40.0	60.0
Vaccinated II	17	8	47.0	53.0
Vaccinated I and II	32	14	43.7	56.3
Control I	20	18	90.0	10.0
Control II	20	17	85.0	15.0
Control I and II	40	35	87.5	12.5
Vaccinated but not challenged	6	—	—	—

In vitro studies

Lymphocytes and monocytes obtained from the spleens of three control animals, and from six animals which had been vaccinated as described, were cultured in triplicate wells of microtiter plates with antigen derived from sonicated suspensions of *L. braziliensis panamensis* promastigotes at concentrations ranging from 1×10^8 organisms to 3×10^4 organisms per ml.

When tested by themselves, purified adherent cells (monocytes) did not proliferate or take up ^{3}H-

TABLE 2

In vivo results of incubation period duration and size of ulceration following inoculation of control and vaccinated M. albacaudatus *with* 2×10^6 *sonicated* L. b. panamensis *promastigotes*

Groups	Mean incubation time (days)	Mean ulcer size at 6 weeks (mm)	Mean ulcer size at 12 weeks (mm)	Mean duration of ulceration (days)
Vaccinated I	40 ± 1.90	5.23 ± 0.19	8.2 ± 0.46	80 ± 6.23
Vaccinated II	47 ± 2.67	4.79 ± 0.29	8.0 ± 0.35	88 ± 4.87
Vaccinated I and II	44 ± 4.26	4.98 ± 0.33	8.09 ± 0.40	84.57 ± 6.68
Control I	32 ± 3.24	8.39 ± 0.32	17.96 ± 0.53	108 ± 6.83
Control II	32.71 ± 3.18	9.4 ± 0.42	17.4 ± 1.01	110 ± 5.06
Control I and II	32.34 ± 3.18	8.88 ± 0.63	17.69 ± 0.84	108.97 ± 6.04

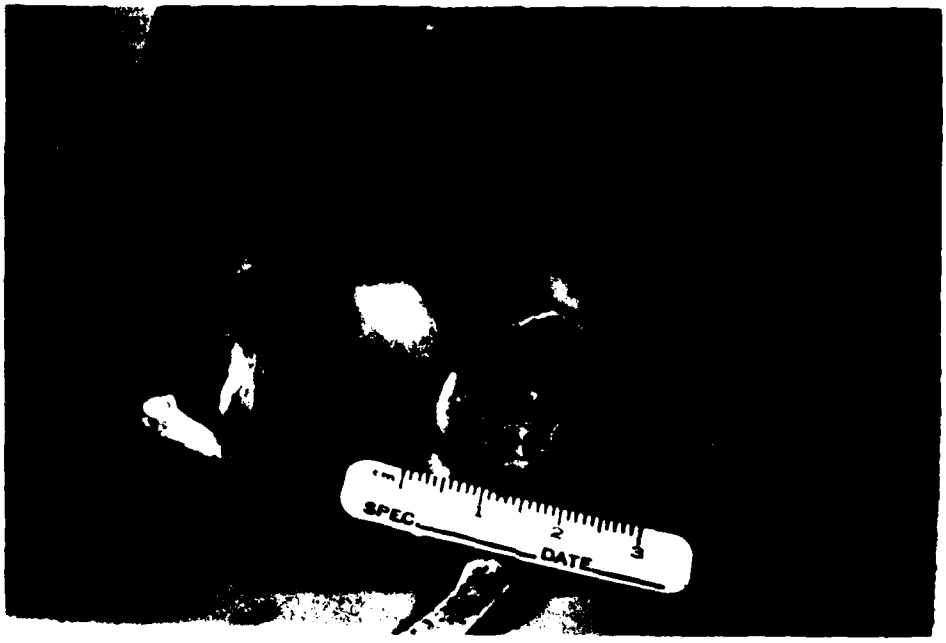

FIGURE 1. *Eroded, punched-out ulcer* over rump region of African white-tailed rat, *Mystromys albacaudatus*, 2 months after intradermal inoculation of 2×10^6 live promastigotes of *L. braziliensis panamensis* (Walter Reed Strain #209).

TdR in response to incubation with sonicated antigen. Further, the proliferative responses of splenic lymphocytes (nonadherent cells) were not abrogated, and were in fact slightly enhanced, when most adherent cells were removed by incubation on plastic prior to testing of the lymphocytes.

Following 5 days of incubation with antigen derived from sonicates of various concentrates of promastigotes, lymphocytes from vaccinated animals showed significantly higher proliferative responses to the antigen than did the non-immunized controls, as measured by uptake of ^{3}H-TdR, and shown for a representative experiment in Table 1. As shown, lymphocytes responded to antigen derived from 2×10^7 promastigotes, or less, with marked proliferation, whereas, in the presence of antigen derived from very high concentrations of organisms (1×10^8 or more promastigotes), the lymphocyte response was markedly suppressed compared to unstimulated controls. In three other experiments (not shown), results were similar, except that there were variations in baseline uptake by controls (two experiments had backgrounds of 10,000 and 16,000 counts), and peak stimulation in two experiments occurred when cells were incubated with from 4 to 8×10^5 organisms, in contrast to the peak responses observed after stimulation with 4×10^6 organisms (Table 3). Such variations, no doubt, result from the fact that the number of promastigotes in a given suspension used to prepare sonicated antigen was only approximate, in spite of efforts to ensure accuracy in these estimations.

Three experiments demonstrated that blastogenic responses by immune spleen cells to antigen complexed with autologous immune serum were partially enhanced in only one of three experiments compared to responses by cells incubated with antigen alone (data not shown). Immune serum by itself did not stimulate ^{3}H-TdR uptake by cells when compared to untreated controls.

DISCUSSION

The in vivo results indicate that the intradermal vaccination of sonicated *L. b. panamensis* promastigotes was protective at a highly significant level. The fact that only 56.2% of animals were

TABLE 3

The in vitro blastogenic response by splenic lymphocytes from Mystromys albacaudatus *to incubation with various concentrations of* L. braziliensis panamensis *antigen**

Number promastigotes used to generate antigen	Control animal			Vaccinated animal		
	CPM†	Change‡	BTI§	CPM†	Change‡	BTI§
1×10^8	1,859 ± 490	−929	0.7	6,058 ± 492	−1,223	0.8
2×10^7	4,583 ± 1,980	+1,795	1.6	16,157 ± 1,510	+8,876	2.2
4×10^6	6,966 ± 2,214	+4,178	2.5	66,588 ± 8,589	+59,307	0.1
8×10^5	6,870 ± 1,690	−4,082	2.5	46,937 ± 5,113	+39,656	6.4
1.5×10^5	4,808 ± 1,209	+2,020	1.7	43,701 ± 5,855	+36,420	6.0
3×10^4	3,264 ± 1,756	+476	1.2	22,195 ± 2,263	+14,914	3.0
Medium only	2,788 ± 688	—	—	7,281 ± 2,603	—	—

* Blastogenic response shown here represents uptake of ^{3}H-TdR by cells tested at 3×10^5/well. Similar dose responses were seen at 1×10^5/well.
† CPM = counts per minute; $\bar{x} \pm$ SD of triplicate samples.
‡ Change in measured cpm relative to cells in medium control.
§ BTI = blast transformation index (see Methods).

protected could be explained by a number of factors. Perhaps intramuscular injections as used by Mayrink et al. are more immunogenic than the intradermal route used in our study.[5] Exposure to a more purified antigen preparation might also have improved protection.[10]

The use of specific subcellular components of leishmania organisms has been shown to determine the development of cellular and humoral response and the induction of protective immunity in the guinea pig model.[11] Perhaps the isolation of specific and highly purified *L. b. panamensis* antigens and their use as vaccine would provide more protective immunity in the white-tailed rat model. Furthermore, the addition of immune adjuvants, such as BCG or Brewer's yeast, might improve immune response and resultant protection. Whether methods for killing promastigotes other than sonication, such as by use of heat, would create a more immunogenic antigen is a subject of another study, as is the evaluation of the efficacy of a live attenuated vaccine.

Development of immunity to the parasites was evident in the type of skin ulcers which developed at the site of vaccination. These ulcers were uniformly smaller at 6 and 12 weeks PI than those of control animals. The ulcers of vaccinated animals healed more rapidly than those of control animals. However, rate of healing was difficult to evaluate because of the frequency of secondary infection in the original lesions, usually by a non-beta-hemolytic streptococcal organism. Secondary infection might be greatly reduced by decreasing the amount and number of applications of depilatory cream and water.

Delayed hypersensitivity is felt to be a feature common to cutaneous leishmaniasis, which may be exhibited by a positive Montenegro skin test. Further, Bryceson et al. demonstrated that lymphocytes taken from guinea pigs infected with *L. enriettii* could be stimulated in vitro by leishmanial antigens.[12] Tremonte and Walton used blast transformation as a measure of delayed hypersensitivity in human and reported positive results in 10 of 12 patients with active *L. braziliensis* infections.[13] Other have published less consistent results demonstrating a varying response rate in small groups of patients with not only healed and active cutaneous leishmania, but also kala-azar and diffuse cutaneous leishmaniasis.[14] Witztum et al. demonstrated a difference in blast transformation in different stages of cutaneous leishmaniasis.[15] They demonstrated marked stimulation of peripheral lymphocytes in all convalescent patients (N = 5) by a specific leishmanial antigen. Also of great interest is that two of five exposed, but uninfected, laboratory workers, demonstrated marked stimulation of lymphocytes in vitro after exposure to specific leishmanial antigens. These laboratory workers did not, however, demonstrate measurable cell-mediated immunity when skin-tested.

Our in vitro results confirm that splenic lymphocytes from vaccinated animals are markedly stimulated when exposed to homologous leishmanial antigens. It is recognized, however, that the correlation of splenic lymphocytic proliferative response to leishmanial antigens to delayed type hypersensitivity (DTH) may not be analogous since proliferative response can be correlated to anti-

body production, as well as DTH. Since antibodies are produced in these animals, perhaps the measurement of DTH would be valuable in evaluating protective immunity in our animal model. However, our goal was the development of an in vitro model to monitor in vivo immunization and protection derived from homologous antigen. Fifty-six percent of vaccinated animals were protected, and it is likely that this protection was provided by cell-mediated immunity. Although DTH may more specifically correlate with protection, proliferative response demonstrated by our experiments also appear to correlate with protection.

The dose-dependent response by the lymphocytes, as shown in Table 3, is consistent with the response, being immunologic in nature. The high concentrations of antigen actually showed a depressed response, as compared to controls, and suggests that a suppressor (or cells) was activated which directly (or indirectly by a soluble substance) inhibited the blastogenic response of other immune reactive cells in the suspensions. A similar inhibition of cell-mediated immune response caused by the generation of a specific T suppressor cell population and resulting in the increased susceptibility of the BALB/C mouse strain to *Leishmania tropica* infection has recently been postulated.[16]

As in our in vivo study, our ability to accurately calculate the concentration of antigen was hindered by the method that was utilized. Because our blast transformation studies used splenic lymphocytes, and did not utilize peripheral blood lymphocytes, comparisons to the previously mentioned studies is difficult. Since our animals were not vaccinated with live vaccines, perhaps the in vitro recognition of leishmanial antigens could be more closely correlated to the above mentioned laboratory workers who were exposed but not infected. Thus, even better lymphocyte stimulation might be expected if the animals could be infected with attenuated liver leishmanial organisms. Greater stimulation of lymphocytes with the addition of immune sera to provide stimulating complexes might also be expected, as was recently reported in human studies.[17] These investigators showed that the addition of specific antibodies to an antigenic suspension greatly enhanced subsequent proliferative response by immune cells to that antigen. In contrast to these results, we observed in three experiments that proliferative responses by immune spleen cells to antigen complexed with autologous immune serum were partially enhanced in only one of three experiments compared to response by cells incubated with antigen alone (data not shown). Immune serum by itself did not stimulate ^{3}H-TdR uptake by cells when compared to untreated controls.

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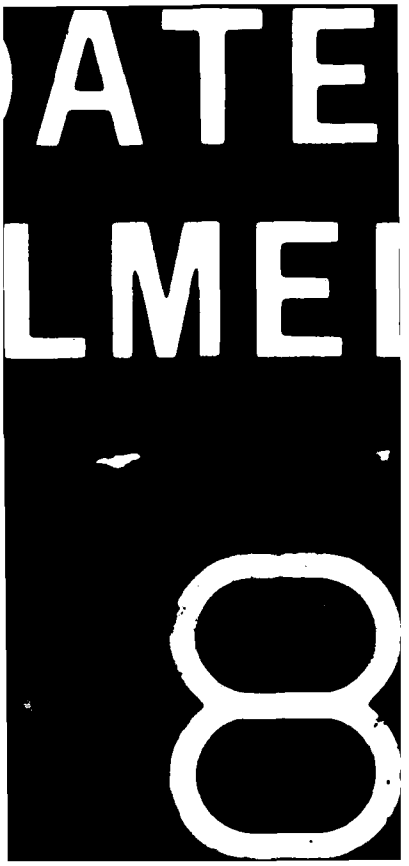
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