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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 an ulcerogenic dose of L. tropica (Jericho) and L. braziliensis (panamensis) was 2 x 10^6 promastigotes. Preliminary results indicated that L. tropica (Jericho) infected M. albacaudatus may develop immunity to infection with not only the homologus strain but also against L. braziliensis panamensis.

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

First Annual Report

Bruce E. Beacham, M.D.

September 1979

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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University of Virginia
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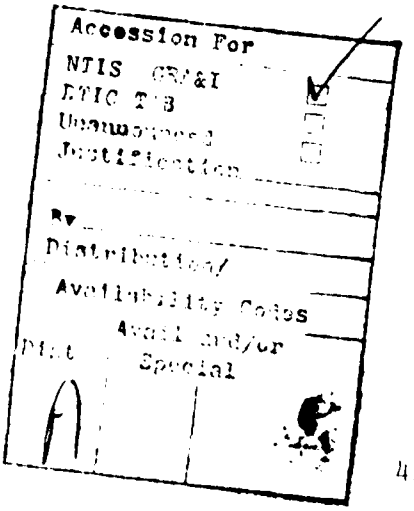
ABSTRACT

It has been observed that after recovery from Leishmania mexicana infection Rhesus monkeys are resistant to challenge by Leishmania b. braziliensis but not Leishmania b. panamensis (Lainson and Bray 1966). Since this report a successful human leishmanial vaccine for L. tropica has been developed (Naggan, et al, 1970/1972) and an animal model has been described for cutaneous leishmaniasis (1977 American Society of Parasitology Meetings -- Dr. Larry Hendricks). We are investigating whether the immunization of Mystromys albacaudatus by L. tropica (Jericho strain) adequately protects these animals against L. b. braziliensis.

Methods have been outlined for storage and reconstitution of the various leishmania strains which will be used as a vaccine. The strains include L. tropica (Jericho), L. braziliensis and L. panamensis, all of which have been isolated from human cases and cryogenically stored.

Initially we investigated cross immunity utilizing three central experiments: (1) To establish the infective dose of L. tropica (Jericho) promastigotes and L. b. braziliensis promastigotes needed to infect 50 percent of Mystromys albacaudatus; (2) To establish the approximate length of time needed for immunity to develop after initial immunization with L. tropica (Jericho) (animals were rechallenged with an homologous strain of L. tropica (Jericho) at monthly intervals after self-healing of the initial ulcer); (3) To test the immunogenicity of a variety of dosages of L. tropica (Jericho) promastigotes when challenged with L. b. panamensis and L. b. braziliensis promastigotes.

Thus far, we have established an optimal ulcerogenic dose of L. tropica (Jericho) newly isolated strain and L. b. braziliensis (panamensis) to be 2×10^6 promastigotes. The incubation period depends 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. 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. b. panamensis.



FOREWORD

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 preliminary evidence that cross immunity does exist utilizing M. albacaudatus as an animal model. The preliminary nature of the following report must be underscored because animals which have been challenged must be observed clinically for adequate periods and biopsies and cultures must be obtained before refractoriness to challenge can be claimed.

Although the search for a vaccine has been unsuccessful to date, it is hoped that future results from this study may further contribute understanding to the development of such immunotherapy.

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).

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I. STATEMENT OF THE PROBLEM

If suitable experimental animals are successfully vaccinated with promastigotes of L. tropica (Jericho) solid immunity will develop to challenge L. b. braziliensis or L. b. panamensis promastigotes. 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.

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, Babesciosis 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 differenct 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. b. braziliensis but were easily infected by L. b. 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 vaccine 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, Nasgan, 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 attach 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 protozoal disease has centered around experience with Plasmodium falciparum malaria in man (Cohen and Sadun, 1976); McGregor and Carrington, 1961). The antibody is directed against the merozoites and prevents the reinvasion of the red blood cell by blocking the attachment of the parasite to the erythrocyte membrane. However these antibodies are variant-specific and substantial problems were encountered in the development of a vaccination program against malaria (Brown, 1976).

Active immunization has been investigated in protozoal diseases 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 desireable 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, 1971/1972; Skov and Twohy, 1974). 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 might 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 (Nusenzweig, Vanderbergand 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 intradermally, 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 inoculation.

In order to maintain the ulcers produced during vaccination, the area surround the ulcer must be depilated by shaving with a #40 shaving head, followed by a 30-second massage using a cream depilatory 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 (1973), 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 panamensis 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. b. panamensis and L. b. braziliensis.

The precise experiments were:

1. 10 animals innoculated with $0.5x10^6$ L. tropica (Jericho)
   10 animals innoculated with $1x10^6$ L. tropica (Jericho)
   10 animals innoculated with $2x10^6$ L. tropica (Jericho)

   10 animals innoculated with $1x10^6$ L. braziliensis panamensis
   10 animals innoculated with $2x10^6$ L. braziliensis panamensis

2. Sham controls innoculated with vehicle and challenged with L. braziliensis (10 animals).

3. Forty animals innoculated with $2x10^6$ L. tropica (Jericho):

	healed lesions	challenge with $2x10^6$ L. braziliensis panamensis
3 months		
	without lesions	challenge with $2x10^6$ L. braziliensis panamensis

Forty animals innoculated with $2x10^6$ L. tropica (Jericho):

	2nd innoculation $2x10^6$ L. tropica	challenge with $2x10^6$
3 months	healed lesions or none at all	L. braziliensis panamensis

Additional studies which would be of great interest would be to compare the immunogenicity of newly isolated L. tropica (Jericho) to our older, cryogenically stored material. The newly isolated promastigotes could be obtained from Israel from Dr. Greenblatt of the University of Hadassah.

IV. RESULTS WITH DISCUSSION OF RESULTS

Secondary to various delays in the onset of this project, such as late delivery dates of equipment secondary to gas shortages and the unexpected long healing period of many of the ulcers, only preliminary results are available at this time. Thus far, as depicted in Table I, we have established an

TABLE I

Initial innoculation of Nystromys albacaudatus with L. tropica (Jericho) and L. braziliensis panamensis for determination of optimal lose

Number of Animals	Species and Strain	Inoculum 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	$2x10^7$	4	4 weeks	21 weeks

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 promastigotes 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 ten Hystrongs albacaudatus were inoculated with media without subsequent ulceration. There came ten 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 Hystromys 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 38 days with .2cc L. tropica (Jericho new strain) with lesions ranging from 2mm to 1.3cm 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 Hystromys 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 challenging agent.

V. CONCLUSIONS

As one can now see, we have some data primarily in vivo which may support the existence of cross immunity between L. tropica (Jericho) and L. braziliensis panamensis. It also is obvious that we need to await the bulk of the data which will not be available for as long as eight to ten months. The fact that this data will not be available until then is primarily because of the longer than predicted time for ulcer healing.

VI. RECOMMENDATIONS

The University of Virginia School of Medicine has developed a geographic medicine program with personnel support through the Rockefeller Foundation. This program, administered through the Department of Medicine, is actively engaged in basic and clinical research in Northeastern Brazil,

TABLE II

Initial innoculation of Mystromys albacaudatus with
L. tropica (Jericho old & new) and L. braziliensis panamensis

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

TABLE III

Nystromys albacaudatus innoculated 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

known to be endemic for cutaneous and visceral leishmaniasis. This setting, of course, will provide a source of patients who will provide opportunities for further in vitro and in vivo studies.

The encouraging in vivo results thus far, as well as the possibility for field work in the endemic areas of leishmaniasis, should underscore the importance of the continued support of this work.

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