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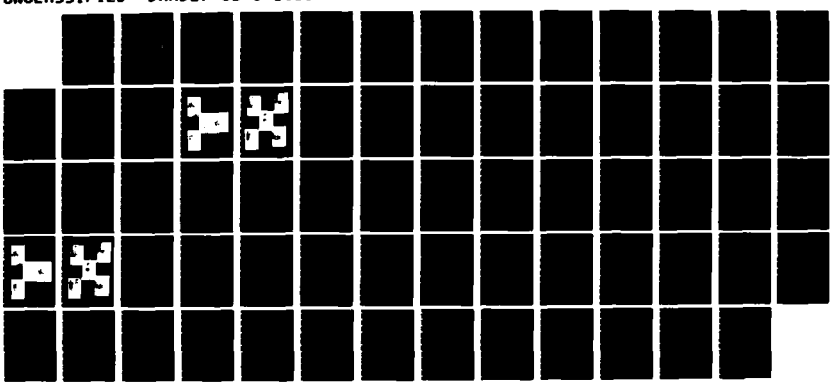

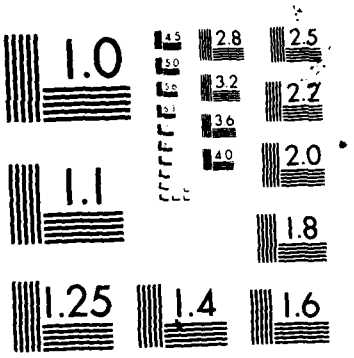

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ANNUAL SUMMARY REPORT

SEPT. 1983

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Testing Experimental Compounds Against Leishmaniansis in Laboratory Animal Model Systems

ANNUAL SUMMARY REPORT

Jan S. Keithly, Ph.D.

SEPTEMBER 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick
Frederick, Maryland 21701

Contract No. DAMD 17-83-C-3039

Cornell University Medical College
New York, New York 10021

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Testing Experimental compounds against Leishmaniasis in Laboratory Animal Models		5. TYPE OF REPORT & PERIOD COVERED Annual Summary Report 1 12/15/82 to 9/01/83
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Jan S. Keithly, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD 17-83-C-3039
9. PERFORMING ORGANIZATION NAME AND ADDRESS Cornell University Medical College Department of Medicine 1300 York Avenue, New York 10021		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS --62770A.3M162770A871.AF. 112.
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE September 1983
		13. NUMBER OF PAGES 47
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

leishmaniasis	hamster	amastigote
cutaneous	Pentostam	promastigote
mucocutaneous	drugs	log
visceral	therapeutic index	stationary
mouse	lesion	suppression

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Pentostam and parasite dose responses have been determined for, and three WRAIR experimental compounds have been tested against visceral and mucocutaneous leishmaniasis. In addition, the conditions for screening compounds against mucocutaneous leishmaniasis caused by Leishmania braziliensis braziliensis M2904 (WR464) in male BALB/cByJ mice are presented.

The major findings for screening against L. donovani Khartoum are:

1. Amastigotes, stationary primary (PCP) or subcultured (SP) promastigo-tes from Schneider's drosophila medium (cSDM) yield equivalent

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liver burdens in mice. This allows 3 separate screening experiments from one hamster donor, increasing efficiency of testing.

2. The ED90 for Pentostam in this model is 10 mg/kg/day (mkd) x 5. This is 5 times less than previously determined for Sudan strain L. donovani.
3. A single total dose of Pentostam is as effective as multiple doses.
4. Parasitic cure is obtained at 80 mkd x 5.
5. None of the three experimental compounds tested is competitive with Pentostam, although they are better tolerated than previously tested drugs
6. Male mice are more sensitive to drug treatment than are females.

The major findings for mucocutaneous leishmaniasis in both models presented here are:

1. Mucocutaneous leishmaniasis (MCL) caused by L. b. braziliensis can be routinely produced in mice when the following criteria are met:
   a. Amastigotes from mouse rather than hamster lesions are used.
   b. Amastigotes are allowed to transform on blood agar overlaid with cSDM.
   c. Promastigotes are harvested at stationary phase and resuspended in cSDM for injection.
   d. Tail base injections are used rather than footpads.
   e. Male rather than female mice are used.
2. MCL caused by L. b. guyanensis in BALB/cByJ mice is influenced by:
   a. Parasite genotype
   b. Host immune status
   c. Sex of host
   d. Parasite dose
3. In both these models of MCL, doses of 3 to 5 x 10^7 are necessary.
4. Pentostam dose responses to MCL are currently in progress for both MCL models, and await completion of parasite dose response SOP.
5. None of the experimental compounds tested was effective in suppressing L. b. guyanensis amastigote-infected mice. Two of these compounds are more toxic in this model than in visceral infections.

In this report, the Standing Operating Procedures for both visceral and mucocutaneous infections in BALB/cByJ mice have been revised. Secondary screening of WRAIR compounds is now possible in two models of MCL in mice.

FOREWARD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals", prepared by the Committees on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Summary

In this report, Parasite and Pentostam Dose Responses have been determined for, and three WRAIR experimental compounds have been tested against visceral (Leishmania donovani Khartoum WR 120) and mucocutaneous (L. braziliensis guyanensis and L. b. braziliensis) leishmaniasis. In addition, the conditions for screening compounds against mucocutaneous leishmaniasis caused by L. b. braziliensis in male BALB/cByJ mice are presented.

The major findings for screening in visceral leishmaniasis are:

1. Amastigotes, stationary primary (PCP) or subcultured (SP) promastigotes from Schneider's drosophila medium (cSDM) yield equivalent liver burdens in BALB/cByJ mice. This allows 3 separate screening experiments from one hamster donor, increasing efficiency of testing.

2. The ED90 for Pentostam against L. donovani Khartoum in BALB/cByJ mice is 10 mkd x 5 days. This is five times less than previously determined for 1S Sudan L. donovani in this model.

3. A single total dose of Pentostam is as suppressive as multiple doses in decreasing parasite burdens in mice due to infection with L. donovani.

4. Parasitic cure is obtained only at 80 mkd x 5, as measured by culture of spleen homogenates.

5. All three experimental compounds (BG 14472, BG 70112, and BE 55795) are better tolerated than 6 of 8 compounds previously tested. Two are toxic as shown by decreased spleen weights. Suppression of liver parasite burdens is being calculated. Based upon the low ED90 of Pentostam against visceral leishmaniasis in this model, it is doubtful these compounds will be competitive.

6. Male mice are more sensitive to treatment with Pentostam and these experimental compounds, than are female mice. Therefore, screening should include both sexes.

The major findings for mucocutaneous leishmaniasis in both models presented here are:

1. Mucocutaneous leishmaniasis (MCL) caused by Leishmania braziliensis guyanensis in BALB/cByJ mice is influenced by:
   a. Parasite genotype - as measured by infectivity of promastigote clones for mice.
   b. Host immune status - especially T-dependent cell-mediated response as measured by spleen cell transformation and plaque-forming assays.
   c. Sex of host - as measured by infectivity, percent and persistence of infection.
   d. Parasite dose - as measured by time to lesion formation and size of lesions.

Summary: part 2

2. MCL caused by L. b. braziliensis can be routinely produced in mice when the following criteria are met:

a. Amastigotes from mouse rather than hamster lesions are used.
b. Amastigotes are allowed to transform on blood agar overlaid with cSDM, and then gradually adapted to growth in cSDM alone.
c. Promastigotes are harvested at stationary phase (days 5-6) and then are resuspended in cSDM for injection
d. Tail base injections are used rather than footpad injections.
e. Male rather than female BALB/cByJ mice are used.

3. In both models of MCL, doses of 3 to 5 x 10^7 amastigotes or promastigotes are preferred.

4. Pentostam dose responses to MCL are yet to be determined, but should include:

a. Male and female mice.
b. Early (3-5 week) and late (5-7 week) lesions.
c. One, two, and three week treatment regimes.
d. Amastigote and stationary phase PCP- and SP-infected mice.
e. A wide range of Pentostam doses (800 to 50 mkd)

5. None of the 3 experimental compounds tested was effective in suppressing L. b. guyanensis amastigote-infected mice. Two of three compounds were toxic in this model at levels tolerated in the visceral model. Possible reasons include:

a. Sex of host
b. Toxic effect of drug upon spleen combining with depressed T-cell function in 3-week infected mice to synergistically enhance toxicity.
c. Direct physiological or pathological effect of this subspecies complex for BALB/cByJ mice.

In this report, we have accomplished approximately 85% of our goals to determine or revise the Standing Operating Procedure for visceral and mucocutaneous models for secondary screening WRAIR experimental compounds. Our next report will include data for cutaneous L. mexicana mexicana and will complete data for these two models currently being evaluated.

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Contract DAMD 17-82-C-3039

I. Objective

To serve as a secondary screen for promising WRAIR experimental compounds against cutaneous, mucocutaneous, and visceral leishmaniasis in BALB/c mice. Specifically to test 10 compounds a year against Leishmania mexicana mexicana, L. braziliensis panamensis (or guyanensis), and L. donovani and to test 5 compounds a year against L. braziliensis braziliensis.

II. Background

Human leishmaniases are severely debilitating and affect about 100 million people. Their public health importance was recognized when the WHO Special Program included them among its 6 major diseases, and when the NIH-NIAID initiated its Collaborative Program for Training in Tropical Disease. Cutaneous disease in the Americas has always been a problem among U.S. Army personnel (63,65,68). In light of recent events in Central and South America, and the continued interest of the United States in the security of civilians and military in this hemisphere, cutaneous and mucocutaneous leishmaniasis, for which only microbistatic therapy is available, may become critical considerations in economic development and peace-keeping. Evidence also strongly suggests endemic areas of leishmaniasis to occur in southwestern U.S.A. (6,56), and the number of imported civilian cases continues to increase (9,39,57,58, personal observations).

Current therapy still involves the use of antimonials, arsenicals, and other toxic heavy metal drugs. Although these are generally effective against visceral leishmaniasis, they vary in efficacy against American cutaneous and mucocutaneous leishmaniasis (10,41,54). Rational approaches to chemotherapy are being developed (61,62,68). One of these uses liposomes to deliver high concentrations of drug into parasite-containing phagocytic vacuoles (4,15), and was developed in part by WRAIR personnel.

Although the 5-nitroimidazole, metronidazole, is not effective against kinetoplastids (33,66), 2-nitroimidazoles (Radanil) and 5-nitrofurans (Lampit) are (49,24,25). The lepidines, especially WR 6026, can be 700 times as effective as standard antimonials against experimental infections of L. donovani (4), and will enter clinical trial this year. These compounds probably function in disrupting the respiratory chain or pyrimidine biosynthesis. A new compound, alpha difluromethylornithine (RMI 71,782), is a highly specific inhibitor of polyamine biosynthesis in African trypanosomes (7), and is effective alone and in combination with Bleomycin against visceral leishmaniasis (JSK, unpublished observations), but is not against cutaneous leishmaniasis. Combination therapies of d-DFMO compounds with high and low doses

of Pentostam, Clindamycin, or boronic acid compounds against L.m. mexicana and L. donovani Khartoum in our laboratory were however uniformly negative (36).

Previous Progress

Over a two year period under contract DAMD 17-80-C-0061, eight WRAIR compounds were tested against two subspecies of L.m. mexicana, L. donovani or L. braziliensis panamensis (Table 1).

Table 1

Compound	LD50 (mkd)	Pentostam Index		Therapeutic Index
		Visceral	Cutaneous Mucocutaneous	
Pentostam Sb^V	>800	-	-	160.00
WR 2975	125	1.40	<1.00	2.00
2116-66	24	3.10	1.06	1.00
227-495	18	5.8	1.00	1.50
219-423	14	7.8	6.20	1.55
242-511	12	7.2	2.50	1.20
241-317	10	11.8	<1.00	1.66

In the visceral model, all WRAIR experimental compounds were as active as the standard antimonial (Pentostam, Burroughs-Wellcome, Beckenham, England) in decreasing spleen and liver parasite burdens, as shown by Pentostam Indices (PI) 1.4 to 11.8. However, spleens from these mice were all culture positive. Cutaneous and mucocutaneous infections were only slightly altered by experimental drug treatment (PI $\leq$1.00 to 6.20). When the Therapeutic Indices of these compounds were compared with Pentostam, none of them was competitive (TI 1.20 to 2.00 v/s 160). Pentostam was 100x more tolerated, and parasitic cures resulted. The LD 50 of Pentostam was >800 mg/kg/day (mkd) pentavalent antimony (Sb^V), whereas that for WRAIR experimental drugs was 10 to 125 mkd. Therefore, based upon these data, none of these compounds showed promise for further development.

III. Scope of Work: Specific Aims

This proposal is a continuation of a two year, ongoing core program for screening WRAIR experimental compounds against American cutaneous and mucocutaneous leishmaniasis (DAMD 17-80-C-3039), and represented a change in scope of work from DAMD-80-C-0061 in two ways:

1) The number of Leishmania subspecies of tested was

decreased to concentrate efforts on prototype subspecies of cutaneous (L. m. mexicana), mucocutaneous (L. b. braziliensis, L. b. panamensis, or L.b. guyanensis), and visceral (L. donovani) disease.

2) Three alternate Protocols for testing were proposed (Table 2), and Protocol C was selected for funding.

Table 2

Subspecies of Leishmania	Number of Compounds		
	A	B	C
Leishmania donovani	10	20	10
L. mexicana mexicana	20	10	10
L. brasiliensis panamensis	20	10	10
L. brasiliensis brasiliensis	5	5	5

Of the possible models for cutaneous disease available, we selected American leishmaniases because of the involvement of the U.S. Army in peace-keeping in this hemisphere. We assigned highest priority to these Leishmania because self-healing is not commonly encountered, and effective treatment is unavailable.

Two years ago we successfully developed in inbred BALB/c mice working models of each Leishmania species except L. b. braziliensis. In 1982, we were able to infect mice with this mucocutaneous subspecies. To our knowledge, this is the first instance of a well-characterized strain of L. b. braziliensis being established in BALB/c mice. Mice have subsequently been infected with amastigotes from lesions of these mice, and as expected, virulence appears greater upon second passage in them (Plate I). Although we originally did not consider these tail base lesions to be mucosal, we have since seen that L.b. guyanensis amastigotes and cloned promastigotes cause invasion of cartilage, loss of the tail, and metastasis to other sites (Plate II).

It has been one year since we introduced L.b. braziliensis and L.b. guyanensis successfully into BALB/c mice. In this report we will present dose response and screening data for L.b. guyanensis, and infectivity data for L.b. braziliensis M2904 in this mouse model.

The approved protocol (C) places equal emphasis on all models for testing. Our laboratory will screen promising WRAIR experimental compounds against cutaneous, mucocutaneous, and visceral leishmaniasis in BALB/c mice. Specifically, we will test 10 compounds a year against Leishmania mexicana mexicana, L.

PLATE I

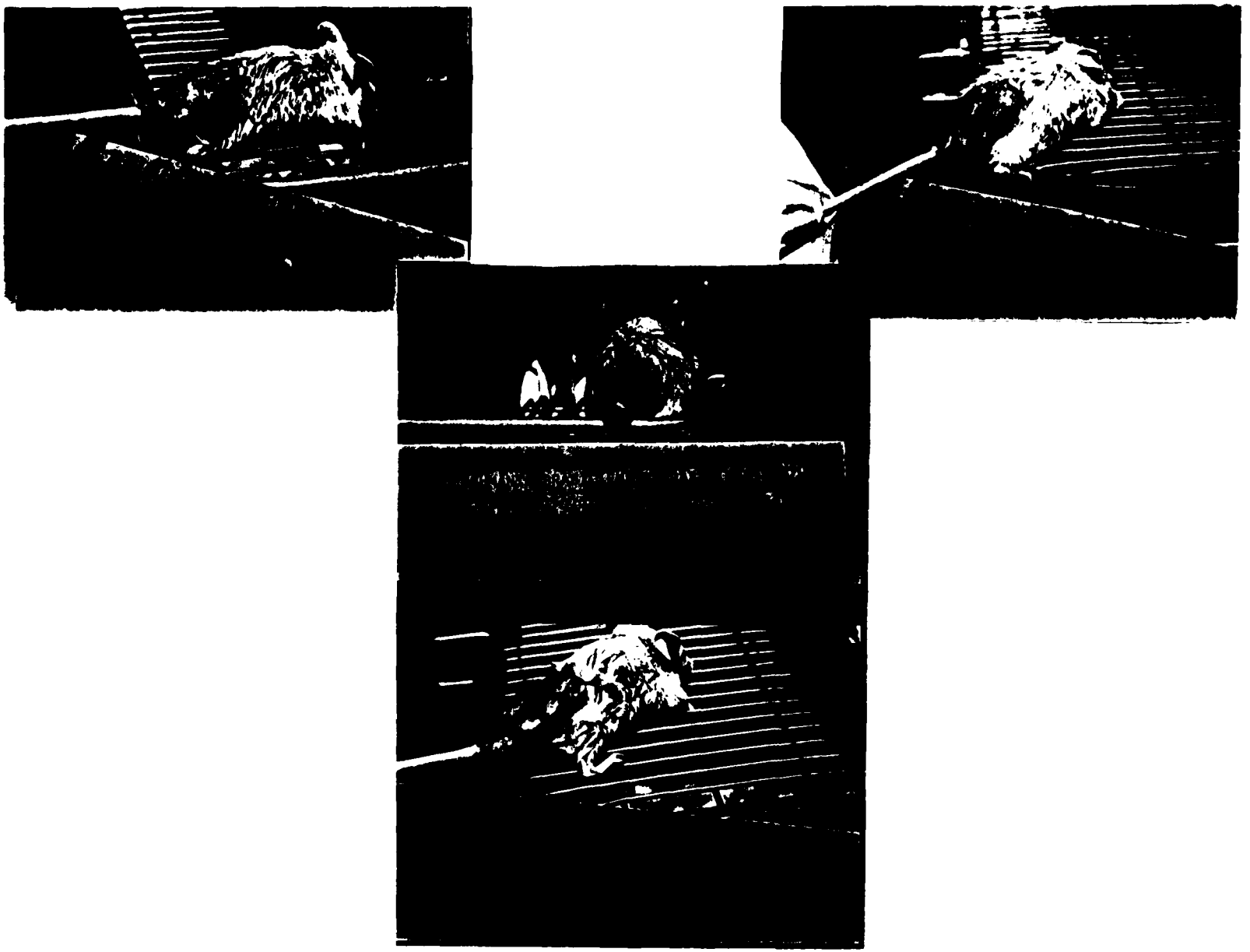
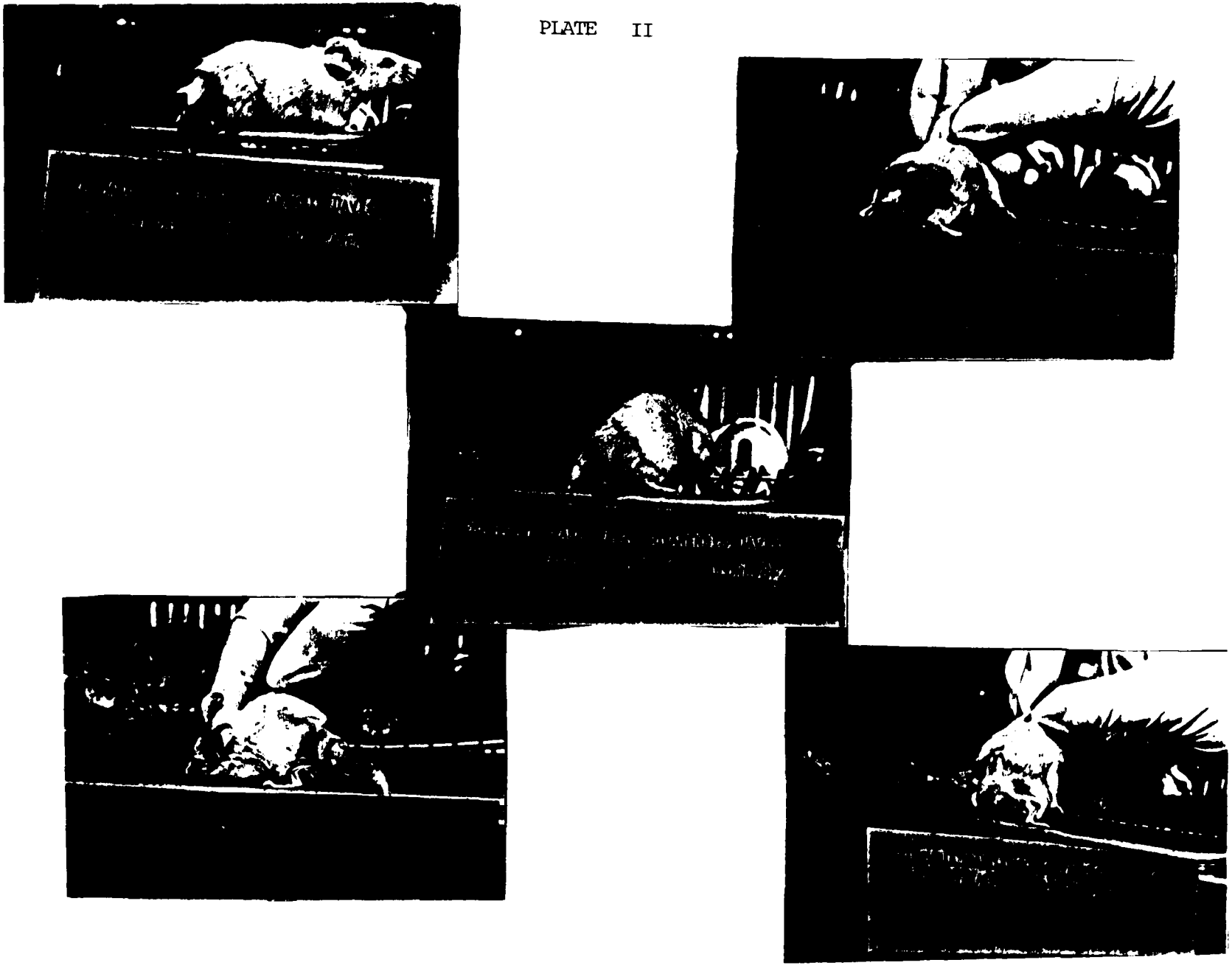


PLATE II



braziliensis panamensis (or guyanensis), and L. donovani; and 5 compounds a year against L. braziliensis braziliensis.

Methodology

1. Parasites. The subspecies of Leishmania previously tested were L. donovani Sudan 1S, L.m. mexicana WR183, L.m. amazonensis WR 303, L.b. panamensis WR120, and L.b. guyanensis Davis. The parasites currently being tested are L. donovani Khartoum WR 130, L.m. mexicana WR 183, L.b. guyanensis Davis, and L.b. braziliensis WR 464 (M 2904). These are all prototypes of leishmania in the Americas, and all have been validated by isoenzyme analysis, DNA buoyant density analysis, monoclonal antibody typing, and infectivity for sandflies.

2. In Vivo Screening

a. Visceral Leishmaniasis

Six groups of 6 mice will be infected intracardially either with a 0.1 ml spleen suspension containing 10 to 50 million amastigotes of L. donovani obtained from heavily infected spleens of outbred hamsters (LVG:LAK) inoculated 4 to 6 weeks earlier, or sham-infected with a normal spleen suspension in HBSS. Experimental compounds will be given subcutaneously 7 days after infection in 4-fold dilutions for five days, the doses to be determined from toxicity data provided by WRAIR, or to be determined empirically for each compound. Suppression will be measured at necropsy 14 days after infection (7 days post-treatment) as described previously (32,60), by examining impressions of the liver and spleen, and by counting the number of parasites per liver or spleen cell nucleus after a method of Stauber (59). Randomly selected spleens will be checked by culturing spleen homogenates of 3/6 mice (32).

The protocols for screening against L. donovani in BALBc mice in this proposal differed from the DAMD 17-80-C-0016 in these ways:

i). Animal Groups and Drug Doses

a). Three dilutions of drug (not 4) will be given daily for 5 days. Previous toxicity data will be be requested from WRAIR for each experimental drug to serve as a basis for dosing. If toxicity data are available, 3 groups of 3 sham-infected mice each will be subcutaneously injected and the Maximum Tolerated Dose (MTD) will serve as the upper limit of drug to be tested. If previous toxicity data are unavailable, then previous experience will be used as an empirical guide. For other WRAIR drugs, the LD50 for mice ranged from 5 to 150 mkd x 5 days. The four most active experimental compounds had an LD50 between 10 and 50 mkd. Therefore, initially 4-fold dilutions at

5, 20, and 80 mkd x 5 will be tested. Three rather than 5 or 6 mice per group have been sufficient for predicting drug toxicity. Greater numbers are redundant and costly.

b). Based upon the ED90 for Pentostam in visceral infections (Annual Report 1981-82), only one group of six mice at 100 Sb^V mkd x 5 will serve as a standard control. However, for comparative purposes, a lower dose within the effective range of the experimental compound being tested will be included, eg. its MTD or the ED50 for Pentostam.

c). One group of 6 mice will serve as saline (untreated) controls, whereas experimental drugs will be tested in three groups of 6 mice each. A revised table for screening drugs against L. donovani in BALB/c mice was determined.

TABLE 3
Numbers of BALB/c Mice

mkd	Toxicity	Test Drug	Pentostam	Saline Control
5	3	6	-	-
20	3	6	6	6
80	3	6	-	-
100	-	-	6	-

ii). Analysis of Data. In addition to a Pentostam Index, a Therapeutic Index will be calculated. In both model systems, the anti-leishmanial activity as shown by these indices, will be calculated as follows:

$$\text{Pentostam index} = \frac{\text{ED90 for Pentostam}}{\text{ED90 for Experimental Drug}}$$

$$\text{Therapeutic Index} = \frac{\text{LD50 Experimental Drug}}{\text{ED50 Experimental Drug}}$$

In the former, comparisons are based upon the weight of antimony in the standard, and upon the total molecular weight of the test compound less its salt. A 90% Effective Dose (ED) is determined by plotting on log probit paper percent parasite suppression, as

measured by LDUs [(amastigotes/1000 liver cell nuclei) x mg wt liver], against the milligrams of drug given per kilogram body weight. A Pentostam index greater than one indicates the test compound is more active than Pentostam.

The Therapeutic Index is the ratio between the Median Lethal Dose or Median Toxic Dose and Median Effective Dose (LD50 or TD50/ED50) of any compound. The greater the therapeutic index, the safer the drug. Since one of the major goals of this core screening program is to find a highly active, non-toxic drug, an index which considers toxicity is necessary for proper evaluation.

Significance will be measured by 1 way analysis of variance. Data will be summarized in tabular form and dose response of experimental drug and pentostam plotted on log probit paper.

b. Cutaneous and Mucocutaneous Leishmaniasis

Six groups of 6 experimental mice will be infected or sham-infected intradermally (ID) into the depilated base of the tail with a 0.1 ml suspension containing 100 million infective promastigotes harvested from complete Schneider's medium (cSDM = SDM + 15% HIFBS (Hyclone Sterile Systems, Logan, Utah) + 100 U/ml penicillin + 100 mg/ml streptomycin) (34), or with 10 million amastigotes obtained from heavily-infected mouse lesions (33,69). After palpable lesions developed drugs will be given subcutaneously for 5 days each of three weeks. Observation and evaluation of lesions will be made weekly.

Suppression will be assessed by comparing percent change in lesion size and median time for lesion regression. All animals will be necropsied six weeks post-treatment as previously described (33), in which lesions are aseptically removed, weighed, homogenized, and serially diluted for counting and inoculating cultures. Confirming assays will include counting the number of viable parasites in the lesion by serial dilution of homogenates into medium. Lesions will be homogenized in one to 5 ml cSDM, diluted 10, 50, and 100 times, and will be incubated at 27^{o} C for several days. Numbers of parasites are determined daily by removing and diluting a 0.1 ml sample in HBSS to which erythrosin B is added, and counting under phase optics using a hemocytometer. Since the generation times in this medium are known for each Leishmania subspecies, the total parasite number in the lesion at necropsy could be calculated as follows:

$$\text{Initial Number Parasites} = \frac{\text{Number Parasites Counted}}{2^{kt}} \times \frac{1}{\overline{\text{Fraction Inoculated}}}$$

where: k = reciprocal of generation time
t = days in culture

Presence of amastigotes in the viscera of random animals will be assessed by liver and spleen impression smears, and by culturing spleen suspensions in cSDM. Two confirming assays will be used - colony formation upon blood agar plates (CFUs) and µL dilutions.

The protocol for screening against cutaneous and mucocutaneous species in BALB/c mice differs from DAMD 17-80-C-0016 in these ways:

i). The inoculum has been increased from 0.05 ml to 0.1 ml containing either one to 10 million amastigotes from mouse lesions, or 100 million promastigotes harvested from cSDM.

ii). Drugs will be administered as outlined in the RFQ, except that the period of treatment will be 5 days per week for two or three consecutive weeks. This is the minimum time required to evince 50 to 75% suppression of lesions using Pentostam. This regime also more closely resembles recommended treatment of humans (600 mg/kg Pentostam IM or IV over 6 to 10 days).

iii). Animal Groups and Assays

Three groups of 6 mice each will be necropsied six weeks post-infection. This eliminates duplication of effort and is cost effective. By treating mice 2 to 3 weeks and following changes in lesion size weekly, we have been able to effectively compare the efficacy of any test compound with Pentostam without group necropsies at two and four weeks.

IV. Revised Scope of Proposal (February 1983 with COTR Cpt. Patrick B. McGreevy)

The basic scope of the work will remain the same, except that during the first six months of Year 1 an effort to develop a Standing Operating Procedure for each model, especially the muco-cutaneous model, will be made. Specifically, the following will be evaluated:

1. Parasite
   a. Source
      (1. Amastigotes: mouse versus hamster
      (2. Promastigotes: log versus stationary phase

   b. Inoculum: cSDM, BA + HBSS, BA + cSDM

2. Infection
   a. Site: nose, tail base, footpad
   b. Presentation: nodule versus ulcer
   c. Parasite burden: numbers versus size lesion

3. Drugs
   a. Route: oral versus subcutaneous
   b. Dosing: single versus multiple
   c. Treatment Time: lesion size versus weeks infected

4. Host
   a. Age
   b. Sex
   c. Genetic Background

Based upon these considerations, we have divided our Progress Report for each of the models first nine months into the following:

a. Dose Response to Infection.
b. Pentostam Dose Response (PDR)
c. Experimental Compounds Tested.
d. Revised Standard Operating Procedure.

Each contains a brief background, results, and discussion section.

V. *In Vivo* Screening of Drugs Against Visceral Leishmaniasis (*L. donovani* Khartoum)

Statement of the Problems

A. To determine the optimum dose and stage of parasite for screening - Parasite Dose Response
B. To determine the Pentostam Dose Response to infection with *L. donovani* Khartoum amastigotes and promastigotes.
C. To screen three new WRAIR compounds against visceral leishmaniasis.
D. To optimize the Standing Operating Procedure for this model.

A. Parasite Dose Response

1. Background

Using the 1S Sudan strain of *L. donovani* (72) we had previously determined that stationary phase promastigotes were more infective for hamsters and mice than were log phase cells (32), and that cSDM was superior to blood agar in maintaining infectivity of subcultured cells. However, we had not systematically examined these questions for *L. donovani* Khartoum, because prior to 1982 we had used Sudan 1S strain for testing. Since RFQ 0018 serves as a second testing model (murine) in support of the primary hamster screen against *L. donovani* Khartoum, a systematic reevaluation was begun.

2. Results.

In this report, we show that:

1. Splenic amastigotes of *L. donovani* Khartoum or 6 day stationary phase primary (PCP) and first subculture (SP) promastigotes from cSDM are identically infective in a non-linear, dose dependent manner from 5×10^6 to 3×10^8 (Table 4). Three day log phase promastigotes are 300 to 700 less infective (Table 4), and hardly give reliable LDU below 5×10^7 cells. These results confirm our earlier infectivity studies for *L. donovani* 1S Sudan in BALB/c mice (34) and outbred hamsters (32).
2. Spleen weights of amastigote-infected BALB/c mice are significantly greater in mice infected with PCP and stationary phase SP. Stationary phase SP are more infective than log phase SP at all doses tested (Table 5).
3. Liver weights are not significantly different whether mice are infected with amastigotes or promastigotes (Table 6).
4. In mice infected with stationary PCP and SP, spleen and liver weights increase significantly between weeks 2 and 3. Increased weights are dose related; log phase SP do not cause significant weight changes except at doses 10x higher (5×10^7) than stationary phase cells.

TABLE 4

PARASITE DOSE RESPONSE: Amastigotes in Liver (LDUs) of BALB/cByJ Mice Infected with Leishmania donovani Khartoum

No. Parasites	Amastigotes	Promastigotes		
		Stationary		Log
		PCP	sP	sP
3×10^8	nd	nd	4935±2908	1403 ± 976
1×10^8	2783 ± 979	2487 ± 634	2642±1674	147
5×10^7	nd	1802 ± 1307	752± 192	65
1×10^7	3476 ± 785	3601 ± 901	2278± 114	3
5×10^6	nd	2786 ± 874	1568± 637	2± 1
1×10^6	55 ± 17	nd	nd	nd
5×10^5	0	nd	nd	nd
1×10^5	0	nd	nd	nd
1×10^4	0.2 ± 0.5	nd	nd	nd

No significant difference between Amastigotes and sP_1

Significant difference between Stationary and Log sP_1

TABLE 5

PARASITE DOSE RESPONSE: Spleen Weights*of BALB/cByJ

Mice Infected with Leishmania donovani Khartoum

No. Parasites	Amastigotes	Promastigotes		
		Stationary		Log
		PCP[1]	sP[2]	sP
3×10^8	nd	nd	197±42	137± 20
1×10^8	320 ± 54	nd	181±6	117± 6
5×10^7	nd	213 ± 87	140±12	105± 4
1×10^7	243 ± 24	170 ± 65	159±129	129± 14
5×10^6	nd	98 ± 26	145±25	98± 16
1×10^6	178 ± 25	123 ± 30	nd	nd
5×10^5	nd	123 ± 29	nd	nd
1×10^5	143 ± 22	104 ± 18	nd	nd
1×10^4	118 ± 13	nd	nd	nd

TABLE 6

PARASITE DOSE RESPONSE: Liver Weights*of BALB/cByJ

Mice Infected with Leishmania donovani Khartoum

No. Parasites	Amastigotes	Promastigotes		
		Stationary		Log
		PCP	sP	sP
3×10^8	nd	nd	1463±73	1821± 261
10^8	1741 ± 100	nd	1498±26	1637± 32
5×10^7	nd	1564 ± 421	1412±38	1352± 33
10^7	1661 ± 286	1623 ± 213	1477±31	1728± 11
5×10^6	nd	1157 ± 165	1444±190	1507± 112
10^6	1515 ± 173	1254 ± 299	nd	nd
5×10^5	nd	1258 ± 123	nd	nd
10^5	1299 ± 108	1488 ± 168	nd	nd
10^4	1347 ± 164	nd	nd	nd

* mg

[1] PCP = Primary Culture Promastigotes

[2] sP = First subculture promastigotes; log = 3 days, stationary = 6 days in cSDM.

3. Discussion.

These results suggest that stationary PCP or SP promastigotes of L. donovani Khartoum grown in cSDM are fully-infective, and can be used for drug screening at 10^6 - 10^8 equally well. They also indicate that log phase SP are poorly infective and cannot be used except at $>10^8$ cells. These data indicate we can use as few as 10^6 amastigotes (PCP and SP) from a single donor hamster for drug screening with confidence, and that these would give equivalent burdens for evaluating drugs.

Amastigotes, PCP, and SP induce dose-dependent splenomegaly, but no hepatomegaly in BALB/cByJ mice during the first 3 weeks of infection (Table 5,6). These results indicate that spleen enlargement is correlated with immune responsiveness to parasite antigen and is dose dependent. They also support previous in vitro and in vivo data showing that spleen cell responsiveness to antigen- and mitogen-stimulation is correlated with parasite burdens (42), and that BALB/c can respond normally at least until week 4 (42). At that time, a T-cell dysfunction, probably an influx of T-suppressor cells occurs, which renders the host immunodeficient (73).

Conclusion.

At doses above 10^6, amastigotes, PCP, or SP may reliably and equivalently be used in 14 day drug screening against L. donovani Khartoum.

B. Pentostam Dose Response (PDR)

1. Background

Since the early 1900's, antimonials have been used to treat Kala-azar (12). Pentavalent antimonials (Pentostam, Glucantime) were superior to trivalent compounds (tartar emetic) because of their reduced toxicity and greater potency, thus reducing treatment times and drug amounts (61). Multiple courses IM or IV for 10 days were thought necessary for obtaining a positive plasma antimony balance (61).

Experimental animal studies have indicated preferential accumulation of antimony in spleens and livers of hamsters (12), and recently in vitro accumulation of ^{125}I-Pentostam in phagolysosomes containing amastigotes of L. donovani and L.m. mexicana (19). In vivo and in vitro evidence suggests uptake of Pentostam into glycosomes of L.m. mexicana amastigotes (33). The mode of action of antimonials may be inhibition of the sulfhydryl enzyme phosphofructokinase. This enzyme is important for glycolysis and is localized in glycosomes (24).

Pentavalent antimonials are effective against human (39) and experimental animal infections with L. donovani (13,28,60), but Pentostam resistance occurs (39). Relapses in humans (5) and incomplete parasitological cures in animals are frequent (28). Improved therapy using liposome encapsulated Pentostam is promising (4), but FDA regulations concerning standardization of

vesicles will probably slow development of this treatment regime. Until less toxic more efficacious drugs are identified, Pentostam or Glucantime remain the drugs of choice.

Toxicity for man and animals is minimal using pentavalent antimonials (61). In Kenya, the recommended human dose has recently been changed from 10 to 20 mg Sb^V/kg body weight for 4 weeks, based upon a controlled study in children and adults (5). At a recent World Health Organization workshop, this recommended dose has been adopted, to a maximum of 850 mg Sb^V, over 4 weeks (28). Although none of the 54 patients in this study had symptoms or signs of toxicity due to antimony, caution was urged in accepting this dosage unequivocally. In a recent study in Brazil (P. Marsden, pers. comm.), reversible renal toxicity was noted after this treatment regime. Similar nephrotic problems had been reported previously for African Kala-azar patients (39).

Because of the difficulty in treating patients for extended periods by hospitalization, it has been suggested that a single dose of Pentavalent antimony be given once weekly. Discomfort at injection sites is the major side effect, and there are no published studies to date detailing single versus multiple treatment regimes. Although some pentavalent antimonials may be given orally (12), because of irritation and rapid excretion, most regimes use IM or IV routes (61).

Pentostam and Glucantime are well-tolerated in expermental animal models of visceral leishmaniasis. Both hamsters and BALB/c mice (Charles River-Lakeview) tolerated 832 mkd once daily for 5 days. Non-human primates tolerate Glucantime less well. The ED99 for Glucantime in owl monkeys was 104 mkd IM once daily on days 10 through 19 post infection (MTD = 125 mkd, LD100 = 250 mkd) (28).

Primary screening against L. donovani Khartoum in hamsters showed the ED90 of Glucantime to be 832 mkd. Liposome encapsulation of Glucantime (4) or Pentostam (13) increased drug efficacy in hamsters 276 and 700x, respectively. Using a 14 day screen in BALB/c mice against L. donovani Sudan 1S, we determined the ED50 and 95 to be 29 and 58 mkd, respectively (Annual Report 1981). In preliminary trials Pentostam was 30 - 100x more effective than Glucantime in BALB/c mice (Annual Report 1981). Therefore, we have used Pentostam as our standard pentavalent antimonial.

2. Results.

In this report, using IC injection of 10^7 promastigotes in a 14 day PDR assay we show that:

a. The ED 50 and ED 90 for Pentostam in male or female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) are 1.2 and 10 mkd, respectively, when given once daily for 5 days one week after infection (Figs. 1,2).

Fig. 1

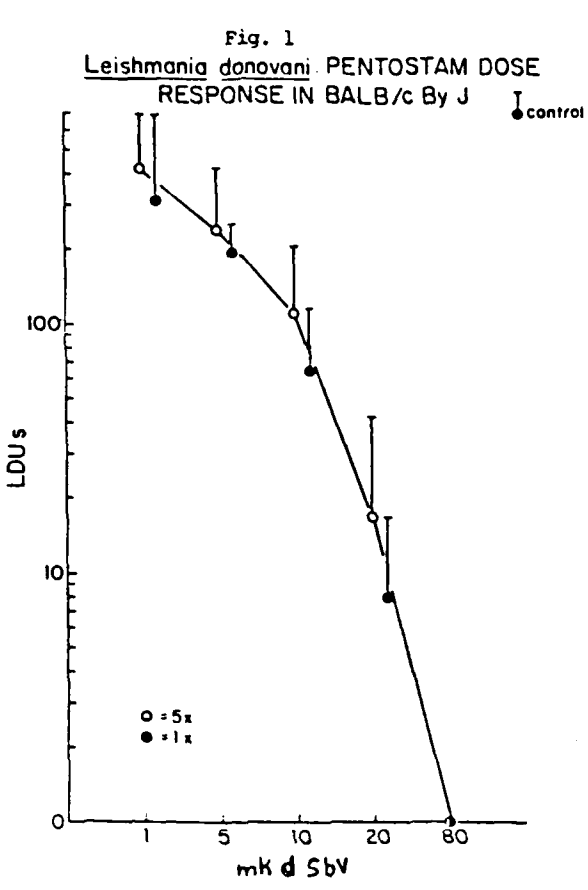
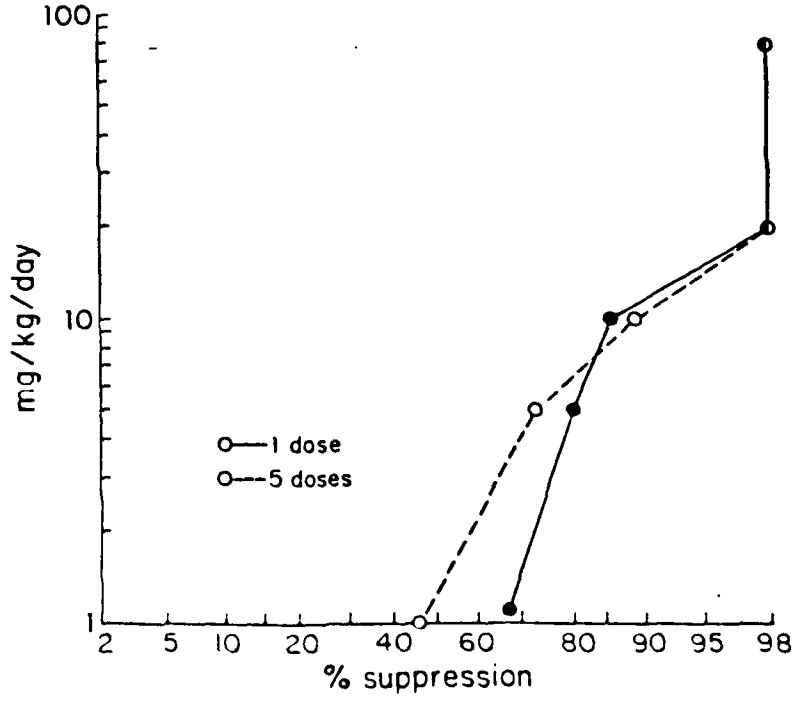

Leishmania donovani PENTOSTAM DOSE RESPONSE IN BALB/c By J
control
100
10
0
LDUs
o = 5x
● = 1x
1
5
10
20
80
mK d SbV

Fig. 2


PENTOSTAM DOSE RESPONSE: EFFECT OF SINGLE AND MULTIPLE DOSES AGAINST Leishmania donovani IN ♀ BALB/c By J MICE
100
10
1
mg/kg/day
1 dose
5 doses
2
5
10
20
40
60
80
90
95
98
% suppression

b. A single dose of 10, 20, or 80 mkd Pentostam is as efficacious as 5 daily doses of each in supressing liver amastigotes 85-100% (Figs. 1,2). At 1 or 5 mkd, a single dose is more suppressive than multiple doses (Fig. 1), but these differences are not significant ($p < 0.05$, Fig. 2).

c. Parasitic cure was obtained only at 80 mkd, as measured by cultures of spleen homogenates (Table 7).

d. The MTD for Pentostam in this model is >400 mkd; the LD100 is 600-800 mkd.

3. Discussion

The ED50 (1.2 mkd) and ED90 (10 mkd) for Pentostam against L. donovani Khartoum in BALB/c mice reported here are considerably lower than those previously reported by us for L. donovani 1S Sudan in BALB/c mice (29 mkd and 58 mkd, respectively) and by others (12) for outbred Swiss mice (94 mkd x 6d. Since the infectivity of Khartoum and Sudan strains for hamsters (72) and for mice (34) is identical, the differences in efficacy are probably of host origin. Inbred BALB/c mice from Charles River-Lakeview are now of dubious genotype (59), and Swiss outbreds have never been good hosts for L. donovani (12). In vivo, differences in immune status between mice susceptible and resistant to visceral leishmaniasis are well-documented (16), and immune status of guinea pigs affects the outcome of treatment with Pentostam during L. enrietti infection (47). Therefore, it is reasonable to assume host genotype and immunity also play a role in how well Pentostam controls visceral leishmaniasis.

However, it is also possible that the Khartoum and Sudan strains of L. donovani have evolved different infectivities for BALB/c ByJ mice since their initial isolation, and different sensitivities to Pentostam. Greater drug concentrations may be required in moderately to highly resistant mice to eliminate parasites, because of differences in uptake and concentration of drug into host macrophages (19). In this study, parasitological cures were obtained only at 80 mkd, as measured by culturing (Table 7). We know the culturing data are reproducible and reliable, but we think our CFU data (from one PDR) are too preliminary to yet reach a general conclusion. Additional screening data should establish its usefulness. Percent suppression as measured by 1, 10, and 100 µl dilutions of spleen homogenates directly correlate liver LDUs from organ impressions (data not shown).

The CDC recommended course of treatment against Kala-azar was 6 daily injections of Pentostam IM 600 mg/kg over 6-10 days, but the new recommendation by the WHO (20 mg/kg/day for 4 weeks, up to 800 mkd) may alter this recommendation. Since part of the difficulty in Kala-azar patient care is the extended time of

TABLE 7

PENTOSTAM DOSE RESPONSE: Percent (+) Cultures and CFU[1] after single and multiple treatment of Leishmania donovani in BALB/cByJ

TREATMENT (mkd)	% Positive Cultures		CFU	
	1x	5x	1x	5x
None (control)	100	50	200	8
Pentostam				
80	0	0	140	6
20	50	100	96	204
10	100	50	-	17
5	100	100	-	65
1	100	100	125	1

[1]CFU = colony-forming units on blood agar plates

treatment, and repeated courses, a single drug dose would be welcome (28). To date, there have been no published studies comparing single and multiple doses in non-human primates or humans.

In hamsters, a single SC dose of 50-200 mg/kg significantly reduced spleen burdens in a one week assay (12). Our data for BALB/cByJ mice show a single dose of 80 mkd Pentostam is equivalent to 5 daily doses in suppressing liver burdens 46-100% (Figs. 1,2). Although pentavalent antimonials achieve high serum levels in humans when given IM or IV over a course of 10 days, virtually nothing is known about the amounts of Sb^V in tissues (5). Our data suggest a single high dose once weekly may be worth pursuing (Figs. 1,2). It is possible, however, that other mammals, and particularly humans, will not respond the same way.

3. PDR Conclusions.

An ED 90 and 50 of 11.5 and 1.2 mkd for multiple doses of Pentostam have been established in this model, and are considerably lower than those previously reported by us for L. donovani 1S Sudan in BALB/c mice (29 mkd and 58 mkd, respectively), and by others for outbred Swiss mice (94 mkd x 6d) (60). For single dosing, we propose to extend the PDR in order to generate lower Effective Doses so we will have low single and multiple doses with which to compare any new experimental compound.

C. WRAIR Screen of Compounds BG 14472, BG 70112, and BE 55795.

1. Background and Previous Results. From 1980-1982, eight WRAIR compounds were tested in our BALB/c visceral leishmniasis model (34). None of these compounds was competitive with Pentostam (Table 1). Of these, 5 were 8-aminoquinolines, two were imidazole derivatives, and one was the antimalarial primaquine phosphate. All had a low Therapeutic and Pentostam Index, and three aminoquinolines caused mild to moderately toxic symptoms including i) bleeding at injection site, ii) tachynea, iii) internal hemolysis, and iv) hyperactivity.

Aminoquinolines with substitutions at the 3 and 5 methyl groups are especially active in vivo (35) and in vitro (42). Although their mode of action is not yet understood, it is suggested they interfere with mitochondrial respiratory ubiquinones (24).

Ketoconazole and its hydrolysate were also inactive against visceral leishmaniasis in BALB/c mice (Annual Report 1981). Hemoflagellates synthesize large amounts of plasma membrane ergosterol in serum-free media. Ketoconazole and miconazole actively disrupt ergosterol synthesis in a variety of bacteria, fungi, and yeasts both in vivo and in vitro (14). An acid hydrolysate of ketoconzole kills L. tropica major amastigotes and promastigotes in vitro (15). It is proposed that the shift from

unsaturated, long-chain to saturated, short-chain fatty acids, and the general displacement of membrane lipids may be responsible for the in vitro effects of these imidazoles against leishmania (14) and Trypanosoma cruzi (15). Recently, ketoconazole was shown to inhibit sterol biosynthesis in vitro, both in the presence and absence of serum, at the level of demethylation (15). However, the intracellular uptake and inhibition of amastigotes by this drug was not tested. Under natural conditions within a host, cholesterol is probably preferentially used by these protozoa.

2. Results

Three experimental WRAIR compounds were received for testing 5/31/83. Toxicity data in outbred Swiss Webster mice were received 6/06/83 (pers. comm. Cpt. P.B. McGreevy). Based upon these data, it was decided to test their toxicity in BALB/cByJ mice in single and multiple doses as follows:

	BG 14472	BG 70112	BE 55795
mkd:	320	640	320
	160	320	160
	80	160	80
	40	80	40
	20	40	20

The route of administration for experimental drugs was changed from SC to PO, as per discussion with COTR, March, 1983. All compounds were moderately soluble in water, have been freshly prepared in 0.5 ml (w/v) doses, and were administered by gavage in single or 5 daily doses. From the first experiment (Table 8), it is apparent that the LD 50 for a single dose of BG 14472 is between 80-160 mg/kg, and that a total of 320 mg/kg is tolerated if given over 5 days (64 mkd). These results fall within the expected range of data received from WRAIR on outbred mice. It is apparent that at 160 or 320 mg/kg spleen weights are suppressed from normal controls 35 and 50%, respectively. Therefore these higher doses may not be tolerated in cutaneous repeated treatments. In fact, using 7-week old sham-infected males, a single 80 mg/kg dose killed 66% of the mice. In the first experiment this dose was not lethal, but the mice were one month older females. Therefore, as previously mentioned, for any drug screen, mice of identical sex and age must be used. Males apparently tolerate the drug less well.

TABLE 8

TOXICITY AND EFFECT OF BG 14472 AGAINST
LEISHMANIA DONOVANI KHARTOUM IN BALB/cByJ MICE

COMPOUND	Dose (mg/kg)	% SURVIVAL	ORGAN WEIGHTS ± SD (mg) SPLEEN	LIVER
BG 14472	160	0	161 ± 123	1587 ± 323
	80	100	243 ± 84	1359 ± 255
	40	100	231 ± 127	1465 ± 349
	20	100	386 ± 61	1625 ± 108
	320 ÷ 5	100	124 ± 42	1377 ± 195
	160 ÷ 5	100	222 ± 201	1267 ± 1024
	80 ÷ 5	100	215 ± 108	1569 ± 253
	40 ÷ 5	100	352 ± 208	1677 ± 428
H_2O Control		100	251 ± 159	1536 ± 230

Based upon these toxicity data, 6 groups of 5 female mice each were infected IC with 10^7 amastigotes to test the efficacy of BG 14472 against visceral leishmaniasis. One week later groups were treated with 128, 80, or 40 mg/kg over 5 days. High (800 mkd) and low dose (50 mkd) Pentostam controls were included. By day 2, all mice at 800 mkd Pentostam had died. (This dose had previously been tolerated, Annual Report, 1982). Mice were necropsied 8/29/83. Spleen and liver weights of treated mice are not significantly different from water controls (Table 9) and no mice died at the high experimental drug dose (128 mg/kg). LDUs and percent suppression are being calculated.

Compound BG 70112 was tested for toxicity in 11 week old sham-infected female BALB/c at three single doses and one multiple dose (Table 10). These data indicate the LD 25 to be 480-640 mg/kg, and that the LD 50 is probably >640 mg/kg as reported by WRAIR for outbred hosts.

Based upon these data, BG 70112 was tested in 11 week old female BALB/c as before at total doses of 592, 480, and 320 mg/kg. Spleen weight of the two highest doses are significantly suppressed from water controls, as are all liver weights ($p < 0.05$) (Table 10). Thus BG 70112, as measured by organ weight change, is also toxic to BALB/c mice. LDUs and percent suppression are being calculated.

Experimental drug BE 55795 was tested for toxicity in three single doses and one multiple dose (Table 11). These data indicate the LD 50 to be 320 mg/kg, and agree in general with data furnished us by WRAIR (LD 50 = >160). There is exact correlation between single and multiple doses that the LD 50 is 320-400 mkd (Table II). To test the efficacy of BE 55795 against L. donovani Khartoum, three groups of mice each received multiple doses of 20-320 mg/kg over 5 days. There were no significant differences in spleen or liver weights with BE 55795, indicating little if any toxicity of this compound (Table 10). LDUs and percent suppression are being calculated. In a second test, mice infected with 10^7 amastigotes were given 320, 160, and 80 mg/kg BE 55795 daily for 5 doses. Pentostam controls received 400 and 50 mkd, respectively. This experiment is in progress.

(4. Conclusions. Two of 3 experimental compounds sent for testing are toxic at higher doses, as shown by spleen weight loss. The LD 50 for each are:

Compound	LD 50
BG 14472	>80
BG 70112	>480
BE 55795	320-400

TABLE 9

EFFECT OF EXPERIMENTAL COMPOUNDS ON LEISHMANIA DONOVANI

KHARTOUM IN BALB/c ByJ MICE

COMPOUND	DOSE* (mg/kg)	ORGAN WEIGHTS ± SD SPLEEN (mg)	LIVER	LDU
BG 14472	128	147 ± 36	1254 ± 377	
	80	153 ± 21	1294 ± 49	
	40	158 ± 19	1367 ± 114	
BG 70112	480	94 ± 12	923 ± 128	
	592	88 ± 21	918 ± 151	
	320	160 ± 16	970 ± 155	
BE 55795	320	141 ± 18	1453 ± 142	
	160	155 ± 8	1364 ± 114	
	80	164 ± 28	1420 ± 49	
Pentostam	50	179 ± 14	1288 ± 93	
H_2O	-	172 28	1443 98	

* mg/kg divided into 5 equal daily doses

TABLE 10

TOXICITY OF BG 70112 AND BE 55795 IN SHAM-INFECTED BALB/c ByJ MICE

COMPOUND	DOSE (mg/kg)	DEATHS
BG 70112	1280	1/4
	640	1/4
	320	0/4
	800 : 5	1/4
H_2O		0/4
BE 55795	640	4/4
	320	2/4
	160	0/4
	400 : 5	2/4
H_2O		0/4

TABLE 11

EFFECT OF BE 55795 ON LEISHMANIA DONOVANI
KHARTOUM IN BALB/c ByJ MICE

COMPOUND	DOSE (mg/kg)	ORGAN WEIGHTS (mg) ± SD	
		SPLEEN	LIVER
BE 55795	160	105 ± 6	1531 ± 123
	80	107 ± 6	1392 ± 49
	40	166 ± 32	1453 ± 13
	320 ÷ 5	89 ± 23	1436 ± 68
	160 ÷ 5	(121)	(1363) one animal surviving
	80 ÷ 5	115± 22	1219 112
	40 ÷ 5	136± 50	1332 69
	20 ÷ 5	107± 8	1306 ± 88
H_2O	—	101± 17	1235 ± 59

All are well-tolerated PO. Suppression of liver parasite burdens is being calculated and will be included in our next report. Seven week old male mice are more sensitive to BG 70112 than are 11 week old females. All three of these new WRAIR compounds are only marginally better tolerated (80-640 mkd) than 6 of 8 compounds previously tested (10-125 mkd), showing suppression at toxic levels. Therefore, we do not think these 3 drugs show promise against visceral leishmaniasis.

D. Standard Operating Procedure: Revisions.

The protocols for screening compounds against L. donovani Khartoum in BALB/cByJ mice will be modified from the original proposal DAMD 17-82-C-3039, page 5, in these ways:

1 Parasites 10^7 amastigotes, stationary PCP or SP from cSDM will be used as dose response data show these to yield equivalent burdens in our 14 day assay. This will allow three separate screens from one hamster donor, thus increasing efficiency of screening. Using 6 groups of 6 mice each/drug, three drugs could be simultaneously tested against amastigote-infected mice from one hamster donor. These drugs could then be tested a second and third time using stationary PCP and SP, respectively.

2 Hosts: Eleven week old female BALB/cByJ mice have been used for routine screening. Toxicity of Pentostam for 20-30 g males and females at 7, 9, 11, and 13 weeks should be determined in order to predict differences in drug efficacy based upon age and sex. An SOP should then select the optimum age for testing experimental compounds in both males and females.

3 Drugs:

a. Route - the oral route has been selected for experimental compounds as per COTR discussion. Pentostam will be given SC as it has shown no activity PO in this model.

b. Dosage - single and multiple high (ED 90) and low (ED 50) dose Pentostam will be compared with single and multiple doses of experimental drugs. This will allow experimental determinations of whether improved therapy might be possible by using single drug doses.

c. New drugs will be compared with Pentostam at ED 50 and 90. The MTD and LD 50 determined so that both a Pentostam and Therapeutic Index can be calculated.

VI. *In Vivo* Screening against Mucocutaneous Leishmaniasis (MCL)

Background and Previous Results

Mucocutaneous leishmaniasis in the Americas is now known to be caused both by members of the *Leishmania braziliensis* complex (38) and *L. mexicana* complex (8). In the Old World, mucosal disease also results from infection with *L. donovani* (1) and *L. tropica major* (2). Mucosal destruction appears related to host genetic background (64) and immune status (8). It also appears to be related to parasite genetic factors, as revealed by analysis of cloned *L. braziliensis guyanensis* (data, this report).

Recent cross-immunization (37) and incidence (41,75) studies indicate that *L.b. guyanensis* is an important mucosal disease (37). This subspecies cross-protects primates against other *L. braziliensis* subspecies and *L.m. mexicana* (37).

Incidence of and severity known mucosal disease in the Americas varies. In Colombia, 20% of all persons surveyed are infected with *L.b. braziliensis* which is sensitive to low doses of Pentostam (R), whereas in Tres Bracos, Bahia, Brazil 2% of patients are infected with *L.b. braziliensis* resistant to antimonials (54,75.)

Patients with strongly positive Montenegro tests to MCL revert to negative upon spontaneous or intervened cure (41). In Bolivia, 55% of 54 patients with multiple mucocutaneous lesions (MMC) had circulating immune complexes (CIC) and Anti-IgG antibodies (20), whereas 33 other patients with single MCL or cutaneous lesions did not. There was a significant correlation between these two parameters ($r = 0.63$; $p < 0.001$), but in only 3 of 16 patients could part of the CIC be identified as anti-IgG antibodies (20). No change in CIC levels occurred after Glucantime treatment, but skin tests reverted to (-) as usual following cure (20). These data suggest that CIC and anti-IgG Ab are associated with severe MCL, and may be used to predict those at high risk for mucosal destruction (20).

Patients infected with *L.b. braziliensis* or *L.b. panamensis* who are slower to develop response to treatment seem to relapse less frequently (53). These initial lesions contain a mixture of histiocytes, plasma cells, and neutrophils (53); lymphocytes appear later (53). Initial response may also depend upon size and site of the inoculum (37). High doses at one or more sites may render the host immunotolerant. This is most probably the case in "pian bois" where vectors are anthoprophilic and multiple lesions (>40) abound (37).

Whether environmental temperature has any effect upon predilection for mucous membranes of the oropharyngeal cavity is still debated. Certainly, there is recent evidence that infection of

the human nose can cause mucosal disease as early as 1 month post-infection (2), and that systemic spread of L.b. braziliensis (26) and L.m. amazonensis (8) can yield MCL within 3 to 12 months in experimental animals (65). the latter can be directly correlated with T-cell dysfunction (8). If CIC and anti-IgG Ab play a role, the vascular network and structure of the basement membrane in the nose may be as important as temperature for mucosal destruction. There many factors, including size and charge, which influence local pathogenesis in immune complexes (18). It is also possible that MCL caused by L.b. braziliensis will be found due to inoculation into that site, and that mucosal destruction by subspecies of L. mexicana will be due to immune dysfunction.

The search for new lead compounds against MCL has been hampered by two problems: 1) prototypes of L.b. braziliensis, the putative agent of most MCL and 2) reliable experimental animal models of L.b. guyanensis and L.b. braziliensis infections.

Until recently, prototypes of these subspecies were uncertain. Chiefly though the efforts of Pratt (43), Chance (76), Kreutzer (77), and Lainson and Shaw (38) biochemical and biological identification of these subspecies has been determined.

In South America, the natural reservoirs of these disease are cricetid and echimyd rodents, marsupials, and edentates (38,44). Experimental inbred hosts which will allow examination of immunoological and genetic parameters are murid rodents (27). The primary point for screening is a valid host in which relatively rapid, reliable lesions can be produced. Inbred mice (murids) of various genotypes serve this purpose. Althougn BALB/c mice are uniquely susceptible to most species of Leishmania (27), L.b. guyanensis and L.b. braziliensis have presented special problems.

Neither of these subspecies had been routinely passaged in animals for some years, and L.b. braziliensis was especially refractory to inbred mice. Outbred hamsters could be infected, but lesions were small, slow developing, and parasites few (69, 38; pers. observations).

Leishmania braziliensis braziliensis M2904 (WR464) was isolated in 1975 from a cutaneous leg lesion by inoculating hamsters and by culturing in NNN (78). Lesions in hamster noses were small and self-limiting. Parasites were animal passaged every 9-12 months and were cultured continuously in NNN. In late 1981, we obtained duplicate cultures of L.b. braziliensis M2904 from Dr. Diane McMahon-Pratt, Harvard Medical School on blood agar slants. [This subspecies is being used as the prototype L.b. braziliensis based upon its initial isolation characteristics and upon its nDNA and kDNA buoyant density, iso-enzymes, reaction with monoclonal antibody and development in sandflies (43).] Each of these was grown in 250 ml BA flasks overlaid with 20-25 ml cSDM. Approximately 0.05 ml or 0.1 ml containing 10^7 promastigotes were inoculated ID into the nose or shaved tail base of 5

male outbred LVG:LAK hamsters (Charles River, Lakeview) or inbred BALB/c ByJ mice (Jackson Labs, Bar Harbor, Maine). During 1982, two subsequent amastigote passages from mouse to mouse after 6 and 3 months, respectively, resulted in reproducible lesions in 100% of the second group of mice (Plate I). To our knowledge, this is the first instance of a biochemicall characterized strain of L.b. braziliensis routinely producing lesions in an animal model.

Leishmania braziliensis guyanensis Davis was originally isolated from an ornithologist traveling in Surinam (French Guiana) who presented with multiple lesions at New York Hospital (1980). Punch biopsis from arm were obtained and promastigotes isolated within 3 days in cSDM. This strain was typed by monoclonal antibodies (43) and DNA buoyant density analysis (D. Barker, pers. com.) to be L.b. guyanensis. Stabilates of the initial isolate are maintained in liquid N_2, and parasites have been routinely passaged in BALB/c ByJ mice and outbred hamsters since isolation. In Fall, 1982, a stabilate was thawed and promastigotes were grown in cSDM for the purpose of cloning. Growth in cSDM is rapid (generation time 11-13 hours) and rosetting is common. Mice were infected with wild type and cloned organisms in November 1982.

In this report, we show that

i. mouse lesions are a better source of amastigotes than are hamster lesions
ii. transformations in culture requires a complex blood source
iii. infectivity is greatest when insect medium is diluent
iv. tail base is superior to footpad for lesion development
v. intermediate doses (10^7) are superior to high (10^8) or low (10^6) doses for rapid reproducible lesion development.

Previously we had tested six WRAIR compounds against L.b. panamensis (WR120) in BALB/c mice, but none of these was competitive with Pentostam (Final Report, DAMD 17-80-C-0016). We had also tested Pentostam systemically and in combination with topical creams against this subspecies and L.b. guyanensis Davis. Topical application of Pentostam cream did not improve therapy in either of these MCL models.

To date, then, none of the experimental compounds tested in animal models (74) or in humans (54) have Therapeutic Indices competitive with pentavalent antimonials (41). As mentioned before, most of the 20% patients with MCL in Colombia respond well to antimony (75), as do patients in the Old World wth MCL caused by L. donovani ( ) and L. tropica (2). Cases of human MCL resistant to antimony (Glucantime) are only sometimes cured by treatment with Amphotericin (54). These patients also have many side effects, and many refuse further treatment (54). Nifurtimox causes improvement in some cases (23), but it is generally unreliable. The 8-aminoquinaline Moxipraquine (11) was suppressive against Trypanosoma cruzi, L. mexicana and L. braziliensis as long as treatment was applied (11), but experimental

PLATE I

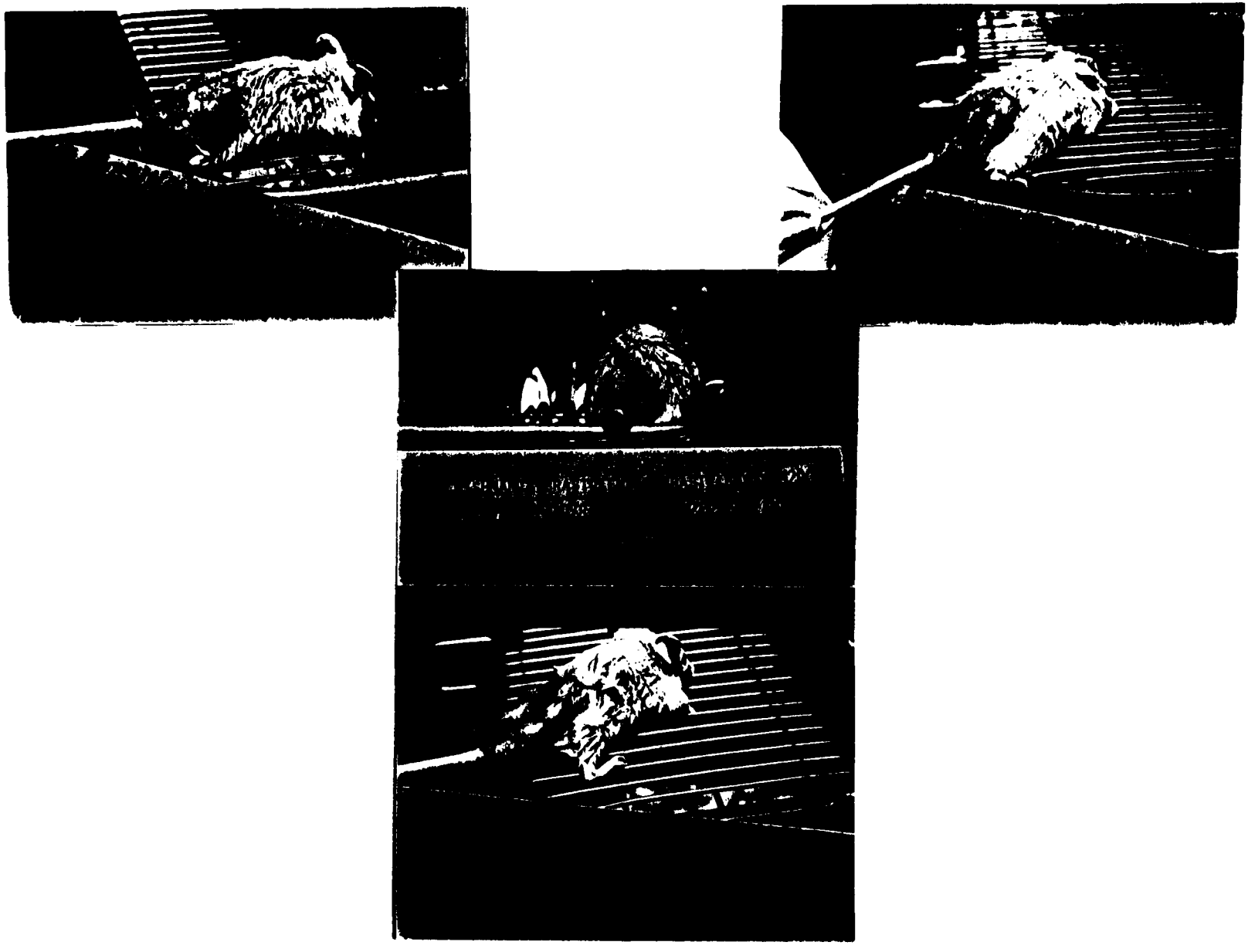
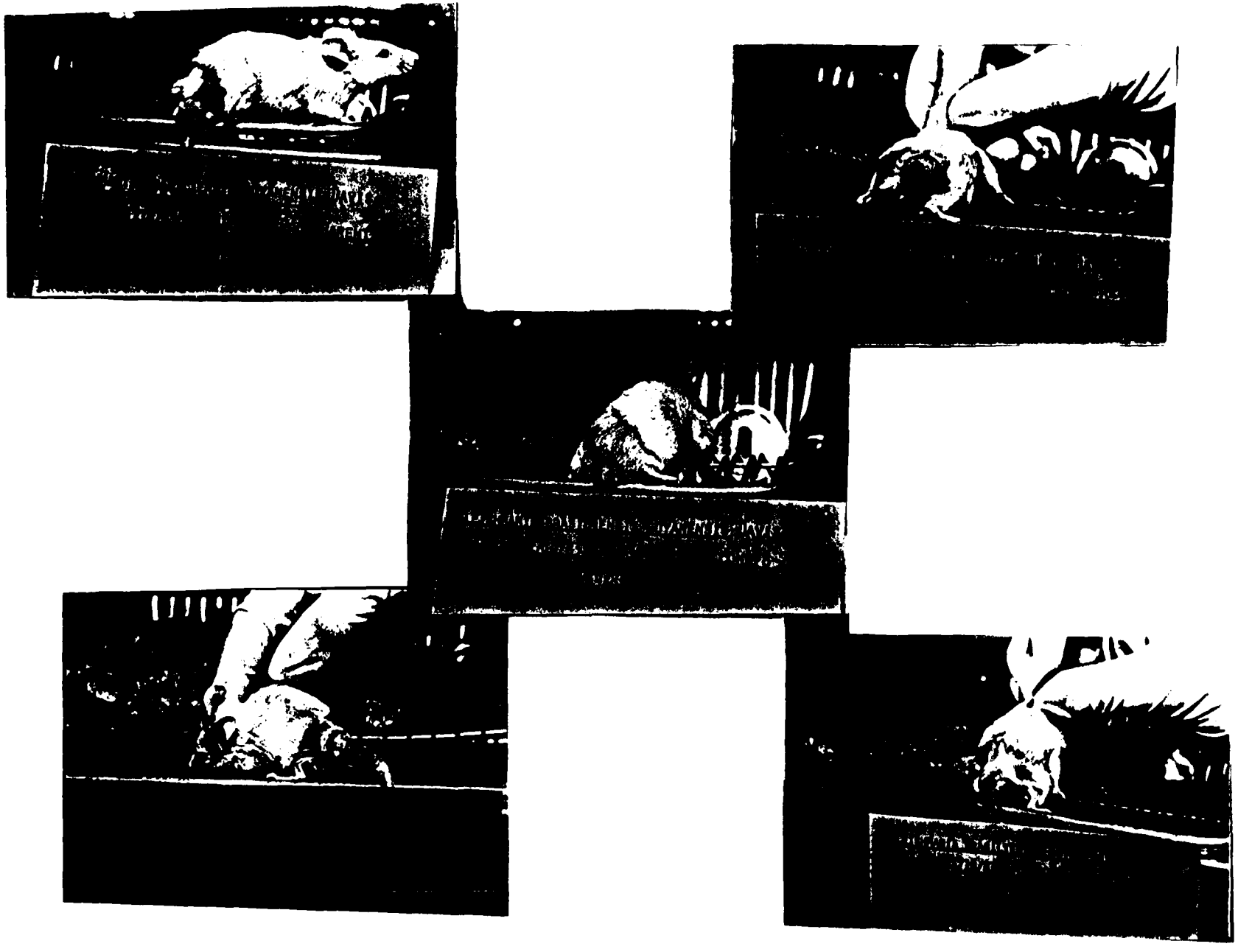


PLATE II



animals were never cured (//). While this compound was in clinical trial, it was discovered to be teratogenic in rats and rabbits, and so its development as an alternative to antimonial discontinued (//).

Based upon preliminary data against L.b. guyanensis (and L. donovani) in this report, although BG 14472, BG 70112, and BE 55795 are better tolerated than those 6 previously tested, none appears competitive with Pentostam and all cause body and organ loss at the higher doses necessary to suppress lesion development and parasite burdens. Therefore, although we have two new models of MCL in which to test experimental WRAIR compounds, it appears that these three are not promising leads.

A. Parasite Dose Response

1. Background

Previously we had shown (Annual Report, 1981) that both amastigotes and promastigotes of L. braziliensis panamensis (WR 120) will yield palpable lesions in BALB/c mice. Rapid and reliable lesions for screening occurred when 0.1 ml cells were inoculated ID at the tail base. Approximately 3-6 weeks were necessary for 10^7 amastigotes or promastigotes to yield lesions for screening. Lesions of L.b. panamensis (WR 120) were small and slow to ulcerate. Replacement of WR120 with another isolate WR 104, failed to produce reliable lesions in BALB/c ByJ mice. The immediate availability of L.b. guyanensis in BALB/c ByJ mice and its biochemical classification as a prototype for MCL led to the decision to substitute this subspecies during the first year of Contract DAMD 17-83-C-3039.

2. Results

a. Leishmania braziliensis guyanensis Davis.

In cloning experiments amastigote-infected female BALB/c ByJ mice develop lesions within one week (Fig. 3,4) and 100% of the mice are infected by week three (Table 12). Wild type promastigotes (10^7) cultivated in cSDM are 2 logs less infective than amastigotes (10^5) (Fig. 3). Only 70% of wild type promastigote-infected mice develop lesions (Table 12). Lesions of this subspecies generally are large spreading and wet. Central ulcerations occur between 5-7 weeks and lesions rarely heal (Plate I). Infective clones of this subspecies cause tail loss and metastasis to skin and ears (Plate II). Visceralization has not been detected.

If time to lesion development and lesion size are used as indicators of pathogenicity, amastigotes of L.b. guyanensis are 100x more infective than wild-type promastigotes (inoculum = 10^5 and 10^7, respectively), and consistently yield higher precent infection (100%) than do promastigotes (0-80%) (Table 12). These results agree with previous infectivity data for amastigotes and

INFECTIVITY OF CLONED *Leishmania braziliensis guyanensis* FOR BALB/cByJ MICE

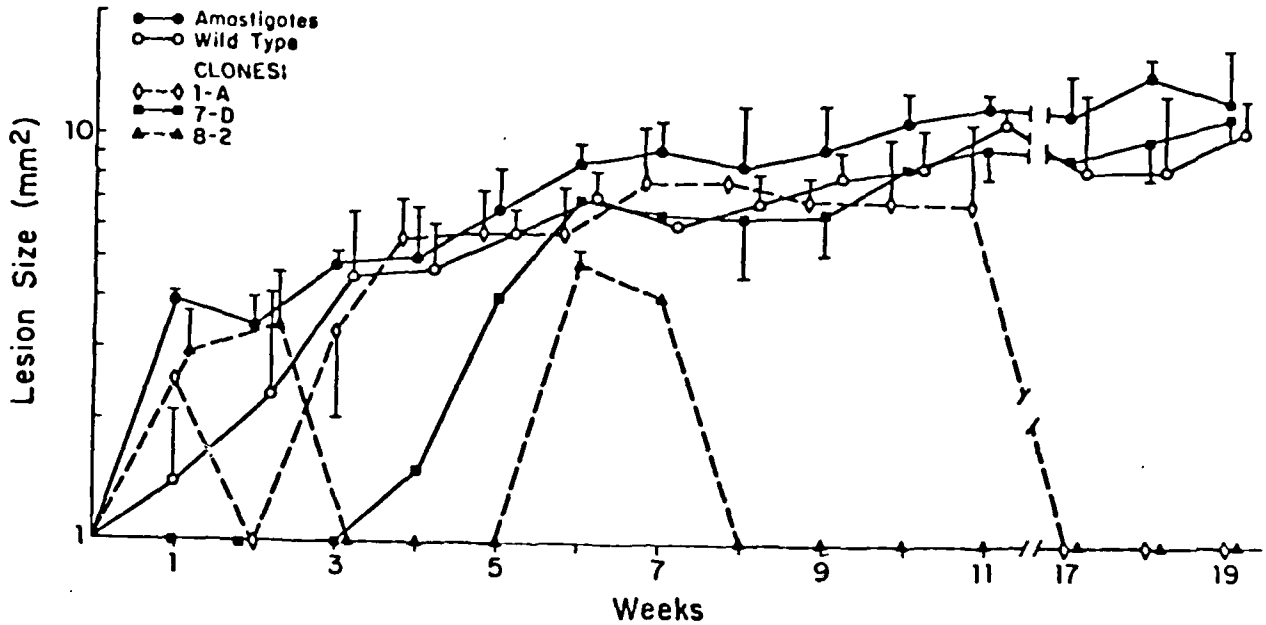


FIGURE 3

VARIATION IN *Leishmania b. guyanensis* SUBCLONE INFECTIVITY FOR BALB/cByJ MICE

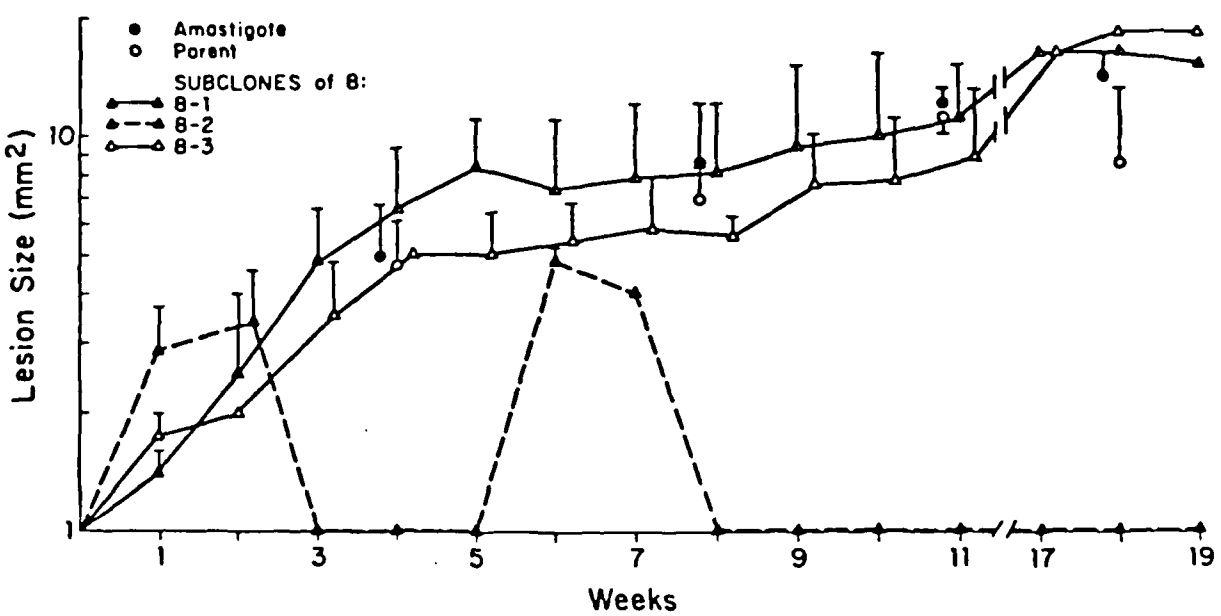


FIGURE 4

TABLE 12

PERCENT BALB/c ByJ INFECTED WITH *LEISHMANIA BRAZILIENSIS GUYANENSIS*[1,2]

	WEEKS POST INFECTION			
CELL TYPE	2	4	8	10
AMASTIGOTES	30	100	100	100
PROMASTIGOTES:				
WILD TYPE	30	40	70	70
CLONE 8-1	60	40	40	40
8-2	80	0	0	0
8-3	16	50	66	66
5-1	60	40	40	20
5-2	20	20	40	40
7-D	0	20	40	40
1-A	0	80	60	80

[1] INOCULUM = 10^5 OR 10^7 VIABLE AMASTIGOTES OR PROMASTIGOTES, RESPECTIVELY

[2] (N) = 5-7 MICE PER GROUP

promastigotes of L. donovani (32), L. tropica and L.m. mexicana (27,69).

Three weeks post-infection patterns of infectivity of promastigote clones for BALB/c mice (Fig. 3,4) show that at least three clones (8-1, 8-3, and 7-D) mimic the course of infection of their wild type parent. Clone 7-D has, however, a much lower percent infectivity (20-40%) than do clones 8-1 and 8-3 (66 and 50%, respectively). Both clone 1-A and 8-2 show cyclic infectivity patterns (Figs 3,4), and eventually both of these are unable to sustain infections in BALB/c mice.

Although the preliminary studies to characterize the behavior of clones in vivo were not designed to test immunological parameters during acute and chronic infection, by 17 weeks post-infection deaths, metastic lesions and erosion of tails prompted us to test the immune responsiveness of these mice by measuring lymphocyte proliferative responses to Con A and L.b. guyanensis antigen, and by using a plaque forming cell assay (PFC).

To test T-cell function, three mice each of clone 5-2, wild type, and age and sex matched normal controls were infected with 500 µg DNP.BGG in 0.2cc complete Freunds adjuvant. The hapten carrier 2,4-dinitrophenyl sulfonic acid (Sigma) was reacted with bovine ɣ-globulin (BGG, Miles-Yeda, Kankakee, Illinois), and averaged 44 DNP groups per BGG molecule (52).

A slide modification of the Jerne-Nordin assay (30) was used to detect antibody-reacting cells in mouse spleens 9 days after immunizations. Rabbit anti-mouse IgG at 1:200 was used to develop indirect PFC. Lyophilized guinea pig serum (GIBCO) was dissolved in Millipore-filtered triple-distilled water and was used 1:20 as a source of complement (C).

Wild type promastigotes show no evidence of altered immune response to a T-dependent heterologous antigen (DNP-BGG), whereas moderately virulent clone 5-2 has a defect in its ability to switch from Igm to IgG (Table 13). Some T-cell function of this clone is normal, i.e. its TD IgM response to DNP-BGG.

These data show that immune responsiveness to TD heterologous antigens in susceptible mice depends to some degree upon the parasite genotype.

We had previously determined that stationary phase promastigotes cultivated in cSDM of L.m. mexicana and L.m. amazonensis were more infective for mice and hamsters, than were log phase promastigotes (Annual Report 1981). In cSDM, L.b. guyanensis reached stationary phase between days 4 and 5. These results agree essentially with other published results for cutaneous and mucocutaneous strains grown in insect-based media (69). We routinely harvest for infection and transfer on day 5.

TABLE 13A

HAPTEN-AUGMENTED (TNP-BGG)[a] PFC PRIMARY RESPONSE OF BALB/cByJ MICE INFECTED[b] WITH LEISHMANIA BRASILIENSIS GUYANENSIS

LEISHMANIA	Indirect (IgM)	Direct (IgG)
None (Control)	12.8 ±	32.4 ±
Wild Type	12.4 ±	18.1 ±
Clone 5-2	12.7 ± 4.6	1.9 ± 3.3

[a] T-dependent response using 500 µg in CFA injected IO 9 days prior to assay

[b] Five month-infection (20 weeks)

TABLE 13B

CHRONIC MUCOCUTANEOUS INFECTION in BALB/cByJ Mice: Response of Spleen Cells to Concanavalin A (T-dependent) Stimulation

Group	CPM/ Con A (cpms)	Control	Total	Lesion
Normal	170,000	450	169,550	
Amastigotes	20,000	350	19,650 *	+
Pros	70,000	3500	66,500	+
8-1	28,000	5000	23,000 *	-
8-2	80,000	700	79,300	-
* 8-3	5,500	2000	3,500	+
1-A	18,000	1000	17,000	-
7-0	40,000	1500	38,500	+

In a preliminary dose response test (data not shown), five groups of 4 female BALB/c ByJ mice each were infected ID at the shaved tail base with 8 day PCP harvested from cSDM. We had switched to female mice because the course of infection with L. tropica, L.m. mexicana, and L.m. amazonensis appeared to be identical with that seen in males (27). In this experiment over 10 weeks, all animals gained weight and there were no significant differences between spleen, liver, or body weights at necropsy. By three weeks post-infection, 50-100% of the mice infected with 10^6 to 10^7 PCP were positive whereas mice receiving 5 x 10^7 or 1 x 10^8 cells were negative. At the time, we attributed these results, in part to use of 8 day PCP (too old). We were also suspicious that the female mice might yield a more variable course upon infection with L.b. guyanensis than males.

In two separate recent experiments, we were able to confirm our observations that intermediate doses of L.b. guyanensis gave best results, and that male mice consistently yielded palpable lesion in all mice by week three. Seven to nine week old male BALB/c ByJ mice infected with varying doses of either amastigotes (Fig. 5) or sP were 75-100% infected by weeks 2-4 (Fig. 6). By week three, an inoculum of 5 x 10^7 amastigotes or sP yields lesions between 5-7 mm^2 (Fig 5,6) in 100% of thes mice. Therefore this dose seems optimal for screening compounds against L.b. guyanensis in this model. It also indicates that sP are as infective as amastigotes when grown in cSDM and harvested from stationary phase (day 5).

b. Leishmania braziliensis braziliensis

As with L.b. guyanensis, preliminary dose response data for L.b. braziliensis in BALB/c ByJ mice were poor. Five groups of 4 female BALB/c ByJ mice each were infected ID at the tail base with 6-day old S_3P harvested from cSDM, resuspended in HBSS for injection. At the end of 10 weeks, only 1/24 mice (10^8) was still positive. Few animals appeared positive at any time, and only in the high dose groups during the first three weeks. Upon footpad challenge three months later with 1 x 10^7 promastigotes suspended in 0.05 SDM, mice from each dose response group developed lesions which persisted. Later, 2/3 mice started to spontaneously heal, but the uninfected initial control mouse (LF) did not (Table 14). There was no significant correlation between initial infecting dose and time apparent to healing upon challenge. These results suggest, but do not prove, that initial infection without lesion formation, does not protect against homologous challenge with L.b. braziliensis. These data support previous observations that primates are protected when challenged with homologous L. braziliensis subspecies if lesions develop, ulcerate, and cure (37). All these footpad lesions were culture positive at necropsy, whereas skin snips from tail base were not. Footpad popliteal draining lymph nodes were enlarged, but spleens were culture negative. Metastasis was not detected.

TABLE 14

RESPONSES OF BALB/c ByJ MICE INFECTED WITH VARYING DOSES OF *LEISHMANIA BRAZILIENSIS BRAZILIENSIS* TO HOMOLOGOUS CHALLENGE INTO FOOTPADS[1]

	Weeks after challenge				
ANIMAL	1	2	4	6	7
LF	+ 0.46	+ 0.34	+ 0.89	+ 1.20	+ 2.28
LR	+ 0.16	- 0.12	+ 1.62	+ 1.38	+ 0.18
RR	+ 0.63	+ 1.21	+ 1.28	+ 0.99	+ 1.30
RM	+ 0.12	+ 0.53	+ 1.29	+ 1.24	+ 0.17

[1]mm change from normal contralateral footpad

FIG. 5

BALB/c By J RESPONSE TO VARYING DOSES OF
Leishmania brasiliensis guyanensis AMASTIGOTES

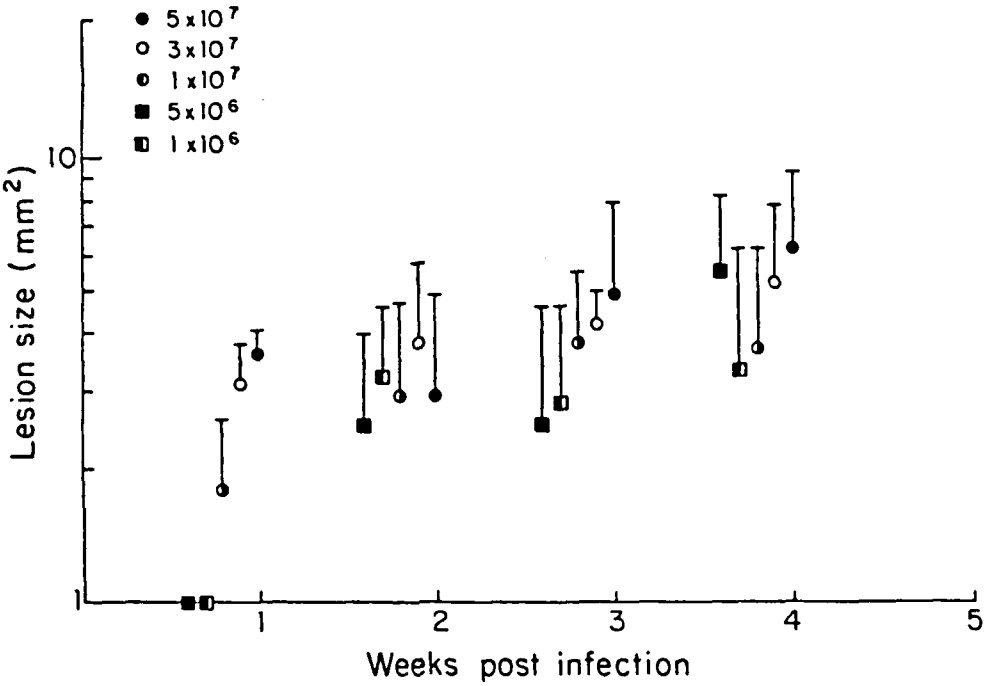
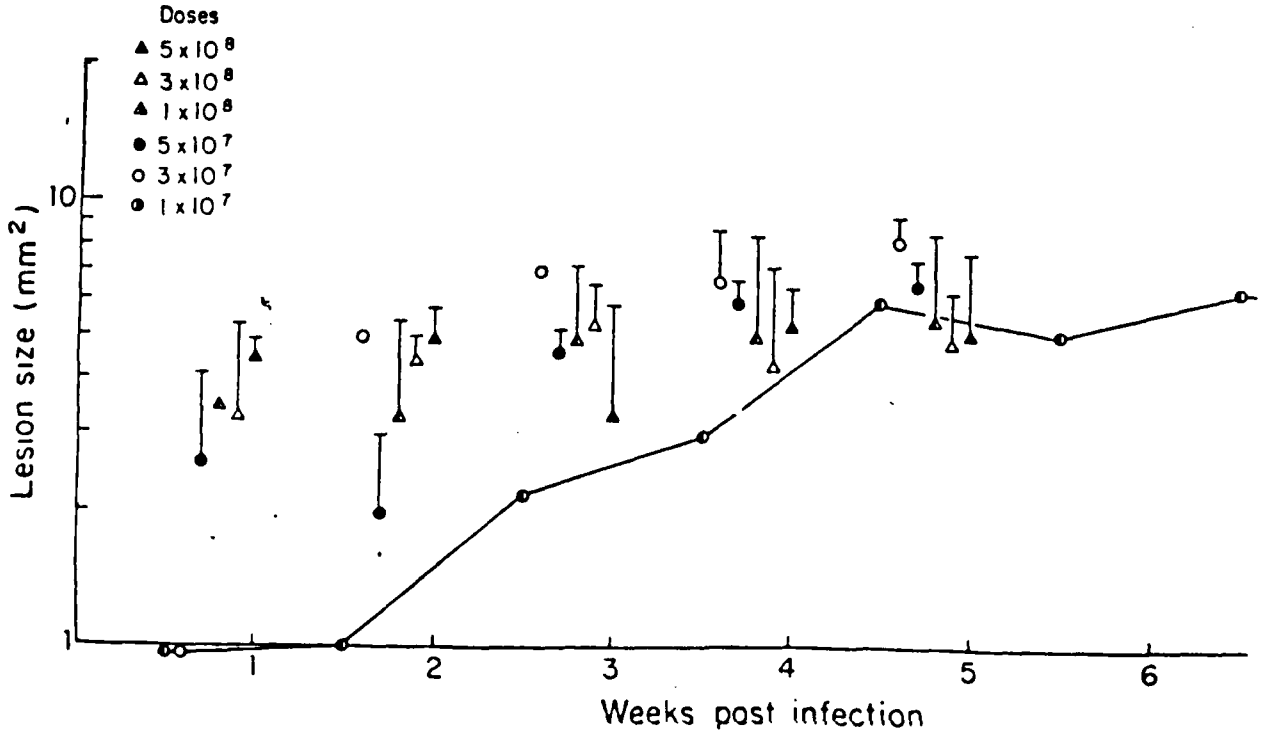


Fig. 6

BALB/c ByJ RESPONSE TO VARYING DOSES OF
Leishmania brasiliensis guyanensis PROMASTIGOTES



We attribute lack of lesion development upon initial infection in part to 1) use of HBSS as diluent 2) use of female hosts, and 3) use of S_3P promastigotes from cSDM. During 1982 we had successfully passaged L.b. braziliensis from mouse to mouse using amastigotes (Plate I). Each time the percent infection increased and time to lesion formation decreased. However all attempts to initiate promastigote cultures from these lesion amastigotes were unsuccessful.

Subsequently, a series of various media were prepared to test what factors were essential for transformation of L.b. braziliensis isolates into promastigotes. In our hands, only blood agar (BA) overlaid either with cSDM or HBSS yielded promastigotes. Initial growth was better in BA overlaid with cSDM but cells in BA + HBSS soon reached equivalent numbers. Each of these media could be substituted by cSDM alone after 2 to 3 adaptive passages into larger and larger volumes (2, 15-20, 50, 100 ml).

From these experiments, we determined that rapid, reproducible and sizable lesions in mice infected with promastigotes of L.b. braziliensis M2904 is influenced by diluent, dose, and sex of host (Table 15). Best results are obtained in male BALB/c ByJ mice infected at the tail base with 10^7 to 10^8 promastigotes suspended in cSDM (100% infection, 4-8 mm^2 lesions at 5 weeks) (Table 15). Female BALB/c ByJ mice infected either with 10^7 cells cultivated in BA + HBSS or cSDM apparently control this infection, as shown by a decrease in lesion size, even though 60-100 percent of the mice remain infected at 5 weeks (Table 15).

Based upon these data, we recommend using:

i) 7-9 week old male mice for

ii) 5 day promastigotes grown in cSDM after transformation in BA + cSDM

iii) doses of $>10^7$ for drug screening against L.b. braziliensis. To confirm that these differences are significant, we are planning a series of parasite dose responses in both male and female BALB/c ByJ mice comparing infectivity of promastigotes harvested from cSDM with those harvested from BA + cSDM and BA + HBSS-. To test the effect of diluent on these cells, each will be suspended either in HBSS or SDM for injection.

Amastigotes of L.b. braziliensis from hamster noses did not transform in any of six media tested. A preliminary comparison of tail base and footpad injections using amastigotes from mouse or hamsters lesions shows that i) only mouse lesions are an effective source, and that ii) tail base is superior to footpad injection (Table 16).

Table 16

	Mouse Origin		Hamster Origin	
	Tail (mm)	Footpad (% Positive)	Tail (mm)	Footpad (% Positive)
6wks	5.2± 1	33	0	0
8wks	5.7± 2	33	0	0
10wks	7.7± 1	33	0	0

TABLE 15

EFFECT OF CULTURE CONDITIONS AND SEX OF HOST UPON INFECTIVITY OF LEISHMANIA BRAZILIENSIS BRAZILIENSIS FOR BALB/c MICE

Sex	Medium	Dose	% Infected weeks					Lesion Size at Necropsy (mm^2)	Number Mice
			1	2	3	4	5		
♂	HBSS-	10^7	0	0	17	0	42	0.5	12
♂	Sch	10^7	50	-	58	33	42	0.5	12
♂	Sch	10^7	80	80	60	80	100	4.0 ± 4	10
♂	Sch	10^8	100	80	70	50	100	4.4 ± 4	10
♀	BA/HBSS	10^7	13	33	52	47	100	1.3 ± 2	15
♀	BA/Sch	10^7	70	90	60	50	60	3 ± 2	10
		Lesion Size	6.4 ± 0.8	5 ± 2	3.4 ± 3	4 ± 3	2.3 ± 3		

Discussion.

These results clearly show that 1) parasite genotype 2) conditions of amastigote transformation and promastigote cultivation and 3) sex of host all influence the outcome of L.b. guyanensis infection in BALB/c mice.

In L.b. guyanensis, if 5 day stationary phase SP or PCP are harvested from cSDM, lesion size and % mice infected at three weeks are equivalent to amastigote infections (Fig. 3). Fewer than 10^8 amastigotes or promastigotes yield a greater percent of developing lesions in less time, than do >1 x 10^8 doses. Spontaneous healing frequently occurs at both higher and lower doses. Typical leaf scars result. Apparent healing at higher doses may mean the host has been rendered immunotolerant, and that parasites could be found throughout the skin. This frequently happens in disseminated cutaneous leishmaniasis (DCL), and may be one reason for the metastic spread of L.b. guyanensis in pian bois (37). In these limited experiments, however, we have not seen dose-dependent metastasis to far sites nor to viscera. Metastasis in cloned L.b. guyanensis appears related to parasite genotype (Plates I, II).

Conditions for transformation from amastigote to promastigote in Leishmania enriettii and donovani have been associated with tubulin biosynthesis (22), and in L.m. mexicana with the availability of a mixture of 6 fatty acids (24). From our data, the continuing controversy (29,55) and diffficulty in isolating L.b. braziliensis from mucosal lesions into liquid media is probably two-fold: 1) scanty amastigotes in human mucosal lesions (as in the hamster model), and 2) truly fastidious requirements for initial transformation in culture.

Our experimental data show that both Lainson and Shaw (55) and Hendricks et al. (29) are correct - aspirates collected into cSDM and inoculated onto blood agar yield uniformly positive results (Table 15). Neither L.b. guyanensis nor L.b. panamensis are this fastidious (unpublished pers. observ.). The biochemical basis for this difference is unknown. Hemin-supplemented cSDM, SDM, LIT, REIII, and Medium 199 do not furnish conditions for transformation. We have had >90% efficiency cloning L.b. guyanensis on BA using cSDM, and we think simulating natural conditions is essential to our success in both isolation of L.b. braziliensis and cloning L. b. guyanensis (34). We had previously shown cSDM superior for maintaining infectivity of promastigotes for hamsters and mice (34). These data indicate both L.b. guyanensis and L.b. braziliensis promastigotes grown and suspended in cSDM yield more rapid lesion development and greater percent of positive infections than do cells suspended in HBSS- (Table 15). For that reason we routinely resuspend cutaneous and mucocutaneous species in cSDM for animal injection. We also homogenize dilute in SDM prior to counting.

Although sex is not sited as a predisposing factor for human

MCL (41), a survey of the literature will show an incidence of 3:1 in New World patients (75). We have presented experimental data showing more rapid and reliable development of both L.b. guyanensis and L.b. braziliensis lesions in male BALB/c ByJ mice. Stauber (59) documented the difference in susceptibility of male versus female hamsters to infection with L. donovani. Because of these observations we propose testing compounds in both sexes of mice. We also suggest that hormones may influence the outcome of infection and treatment in New World MCL. Some in vitro testing of this hypothesis by WRAIR personnel might be worthwhile.

Conclusions: Amastigote and infective promastigotes of L.b. guyanensis and L.b. braziliensis can be used as valid models for screening drugs against MCL. Metastasis and invasion of cartilage occur. Course of infection is dose and diluent dependent. Reliable lesions are produced within 3-5 weeks if cell numbers are of intermediate size ($3-5 \times 10^7$), and if they are suspended in cSDM.

Male mice are better hosts for subspecies of the L. braziliensis complex, and mouse lesions are a better source of cells than are hamsters. Tail base is a preferable site of inoculation.

B. Pentostam Dose Response (PDR)

1. Background and Previous Results.

Previous data showed that lesions due to infection with amastigotes of L.b. panamensis or L.b. guyanensis (WR120) could be supressed 77-91%, respectively, if Pentostam was administered for three consecutive weeks SC at 416 Sb^V mkd (Annual Report, 1982). Neither of these doses was curative, and eight weeks post-infection (2 weeks after last treatment) lesions were culture positive and were increasing in size.

L.b. panamensis promastigote-initiated lesions were more susceptible to Pentostam suppression, than were lesions from amastigotes. Early infections (3-5 weeks) were more effectively suppressed than were later infections (7-10 weeks), but neither of these could be completely cured with repeated Pentostam treatments (Annual Report, 1981). There was no apparent difference in suppression, healing, or cure when ulcerated versus closed lesions were evaluated.

Using male inbred BALB/c mice from Charles River, Wilmington the MTD of Pentostam was 800 Sb^V x 15. Doses of 15, 23, 58, 100, and 200 Sb^V mkd for 5 or 10 days were not suppressive.

2. Results and Discussion. We have not yet begun a PDR for L.b. guyanensis or L.b. braziliensis, pending information on parasite dose response data. Based upon data in the previous section (A), we plan the following series of experiments for L.b. guyanensis

in male and female BALB/c ByJ mice.

Stationary and log phase PCP and SP promastigotes will be harvested from cSDM and will be resuspended in it at a concentration of 5 x 10^7 cells/0/1 ml. Seven groups of 5 male and female BALB/c mice each will be inoculated ID into the shaved tail base. Lesions will be allowed to develop to 5-7 mm^2 prior to screening. In males, this should occur by week three. Pentostam will be given SC once daily for 5 days over one, two, or three weeks. Lesions will be measured weekly and data evaluated as detailed previously. For comparison, similar groups of mice will be inoculated with amastigotes from the original mouse lesion and treated with Pentostam. Doses will include the MTD (800 mkd) and two-fold dilution to 50 mkd (800, 600, 400.....50).

In this manner, a PDR for each promastigote type will be determined based upon optimal dose and conditions of culture for L.b. guyanensis. Observations of weekly lesion development will include time to and number of ulcerations. So far, lesions of all male mice infected with L.b. guyanensis ulcerate between weeks 5 to 7. Later, a second protocol to test the effect of Pentostam on older, ulcerated lesions at fewer dose levels should be initiated. This would allow an extension of these data, and extrapolation to the effect of Pentostam against chronic infections in humans.

PDR against L.b. braziliensis awaits results of optimum medium and parasite dose for an SOP. As soon as these are determined, PDR will be initiated.

C. WRAIR Screen of Compounds BG 14472, BG 70112, and BE 55795.

1. Background and Previous Results.

From 1980-82, three (WR-2975, 219423, 242511) of six WRAIR compounds were tested against MCL caused by L.b. panamensis in BALB/c mice. Three additional compounds were proposed for retesting (WR 211666, 224495, and 241317), but because of low Pentostam and Therapeutic Indices in the L. donovani model, as evidenced by acute toxicity, they were not. None of these compounds was competitive with Pentostam against MCL, as mentioned previously (Table 1).

The alternate drugs tested so far against human MCL include 5-nitroimidazoles (metronidazole), 2-nitroimidazoles (benznidazole, radanil) 5-nitrofurans (nifurtimox, Lampit), 8-aminoquinolines (moxipraquine, WR6026), allopurinol, and amphotericin B (Fungizone). In most cases, these are marginally effective against MCL (nifurtimox, benznidazole) or are extremely toxic (amphotericin, moxipraquine, WR 6026) (54). Antimonials are still less toxic and better tolerated than any of these (41,68). (49,54) (10,11,25)

2. Results and Discussion

Based upon LD 50 and toxicity data for L. donovani-infected and sham-infected mice (Tables 8-11), we treated 8 groups of four male BALB/c mice each with compounds at and one dilution below their calculated LD50 (Table 17). These mice were 8 weeks old when infected ID with 10⁵ amastigotes of L.b. guyanensis Davis suspended in cSDM. When treated 3 weeks later, 100% of these mice had lesions 5 mm2 (Table 18).

Unexpectedly, 2/4 mice treated with BG 14472 died one day after receiving 64 mg/kg and another on day 5 after receiving 160 mg/kg. No mice died in the second treatment group (Total = 80 mg/kg). This dose is considerably lower than that determined for this compound in sham- or L. donovani-infected mice over the same time (Table 8), in which 64 mkd (320 mg total) was well tolerated over 5 days.

Less surprising, but disturbing, were deaths of all 4 mice treated with 160 mkd (800 total) BG 70112 (Table 18). This dose had also been well tolerated previously (1/4 deaths). Deaths in mice treated with 128 mkd (640 mg/kg) was even more unexpected, as 75% of the mice treated with a single total dose of 640 mg/kg survived, as shown by toxicity studies.

What differences can account for these unexpected deaths? Toxicity experiments used females of the same age, and mice were infected for one week only with a visceral parasite. Although one might expect intracardial injections to be more traumatic than ID infection into the tail base, parasite physiology and host immune response to infection with L.b. guyanensis may be sufficiently different to cause these differences. Host immune function influences the efficacy of Pentostam upon L. enrietti in guinea pigs (47), and in BALB/c mice T-cell response down regulates two to four weeks after infection with L.m. amazonensis(8), L. tropica (73), and L. donovani (46). Perhaps by initiating treatment three weeks post-infection, and knowing that spleen weights are affected by higher doses of these two drugs (128 mg/kg and 480 mg/kg, respectively), we synergistically stressed these mice beyond acceptable limits.

In addition, we cannot ignore the fact that male BALB/c ByJ mice are not only more susceptible to infection with L.b. guyanensis and L.b. braziliensis, as measured by lesion size and percent or persistance of infection (Table 15), but they are more sensitive to Pentostam treatment. As mentioned previously, a single dose of BG 14472 killed 66% of 7 week old male BALB/c ByJ, whereas females were unaffected by this dose.

There may be other explanations, but these results strongly suggest drug toxicity be tested in both sexes, and at various times post-infection. Ideally, immune status should be tested in these mice during infection with MCL. From these preliminary data, compound BG 70112 appears efficacious, but its Therapeutic Index is probably not competitive with Pentostam.

TABLE 17

EFFECT OF EXPERIMENTAL COMPOUNDS[1] ON *LEISHMANIA BRASILIENSIS GUYANENSIS*[2] IN BALB/c MICE

COMPOUND	mg/kg ÷ 5	ANIMALS			Necropsy Lesion Weight (mg)	WEEKS POST INFECTION							
		TOT	EXP	TOX		Percent Lesion Size				% Supression			
						1	2	3	4	1	2	3	4
BG 14472	160	4	1	3	198	107	100	112	119	0	0	0	0
	80	4	4	0	277 ± 89	126	114	123	136	0	0	0	0
BG 70112	800	4	0	4	-	121	-	-	-	0	-	-	-
	640	3	0	3	-	104	62	-	-	0	38	-	-
BE 55795	320	4	4	0	264 ± 83	107	100	105	104	0	0	0	0
	160	4	3	0	337 ± 109	128	145	148	155	0	0	0	0
BJ 58563	800	3	3	0	98 ± 46	111	92	92	87	0	8	8	13
	50	2	2	0	101 ± 9.2	109	132	135	154	0	0	0	0
H_2O		3	2	0	349 ± 111	100	127	110	97	0	0	0	3

1. Treatment = 1 week
2. Amastigotes = 3 million

TABLE 18

THE EFFECT OF EXPERIMENTAL COMPOUNDS ON MEAN LESION SIZE OF *LEISHMANIA BRASILIENSIS GUYANENSIS* INFECTED BALB/c ByJ MICE

COMPOUND	DOSE* (mg/kg)	WEEK PRETREATMENT 3	PRETREATMENT 2	PRETREATMENT 1	POST-TREATMENT 1	POST-TREATMENT 2
BG 14472	160	6.2	6.7	8.0	8.6	(8.0)
	80	5.1	6.5	7.2	8.7	8.0
BG 70112	800	6.5	7.0	5.7	7.2	all died
	640	6.6	6.8	7.2	7.6	(4.5)
BE 55795	320	7.0	7.3	8.0	8.6	8.0
	160	6.5	7.6	7.1	9.0	10.3
Pentostam	1600	6.6	6.8	6.3	7.0	5.8
	250	6.2	7.7	7.7	8.5	10.2
H_2O	-	6.5	8.0	7.5	7.5	9.5

* mg/kg divided into 5 equal daily doses

Compound BE 55795 was well-tolerated at the doses tested (Table 18), but neither dose was suppressive. Because the higher dose (320 mg/kg) is near the LD50 for mice, we also do not think this a promising lead compound for treating MCL.

Pentostam was only mildly suppressed in this test (Fig. 7, Table 19), as we were at the lower spectrum of its efficacy (160 mkd, total = 800 mg/kg) against cutaneous and mucocutaneous leishmaniasis (ED70 = 400 mkd). Even at this low dose for one week, Pentostam shows effective suppression (8-13%), whereas none of the other compounds do except at toxic levels (Table 19).

3. Conclusions

These tests need to be repeated, treating mice one, two, and three weeks after infection, and dose adjustments for compounds BG 14472 and BG 70112 need to be made. Pentostam should be administered in high (800 mkd), intermediate (200 mkd) and low (50 mkd) doses in order to overlap with experimental compounds. It is probably judicious to repeat toxicity testing for these compounds in early (3-5 week) and late (7-9 week) infections of L.b. guyanensis using male and female mice. Based upon datain this preliminary experiment, we would test each compound at two-fold dilutions below previously determined toxic levels i.e. BG 14472 at 64, 32, and 16 mg/kg and BG 70112 at 320, 160, 80 mg/kg. Since the percent suppression (if any) seen in this experiment for each of these compounds is at or near the MTD or LD50, we do not think any of these compounds shows promise for treating MCL. We would strongly reccomend this series not be pursued in light of toxicity both here and in the visceral model.

D. Standard Operating Procedure - Working Protocol

The protocols for screening compounds against L. braziliensis guyanensis and L.b. braziliensis shall include:

1. Parasites. Injections of 3 to 5 x 10^7 amastigotes or promastigotes suspended in cSDM into the tail base of mice. Mouse lesions will serve as source. Conditions of culture for transformation and growth will be confirmed. The effect of diluent on viability and infectivity of cells grown in cSDM, BA + HBSS will be determined.

2. Hosts. Both male and female BALB/c ByJ mice will be infected, and time to lesion formation and ulceration, percent and persistance of infection, and time to healing noted. If approved by COTR, immune status of each sex during infection will be monitored by in vitro lymphocyte transformation to specific and non-specific mitogens.

3. Drugs.

Toxicity. The toxicity of experimental compounds for early and late infections of MCL will be determined. Toxicity in both sexes will also be tested.

VII. References

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