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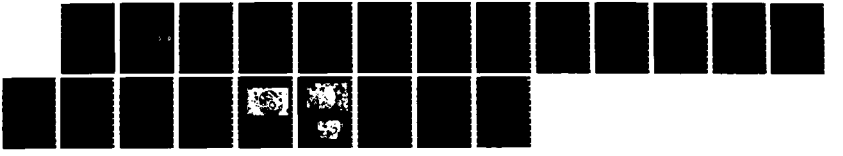

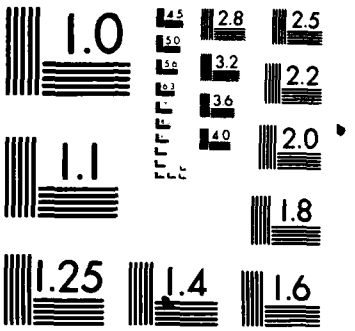

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CHEMOTHERAPY OF LEISHMANIASIS

Final Report

by

Wallace Peters, MD, DSc.

December 1982

Supported by

U.S.ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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London School of Hygiene and Tropical Medicine
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LEISHMANIA, CHEMOTHERAPY, ANTIMONIALS, ANTIBIOTICS, 8-AMINOQUINOLINES

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

Standard Leishmania strains used in this laboratory have been characterised by their isoenzymes The relationship of antimonial treatment of L.donovani-infected BALB/c mice and host immunity has been established. Treatment very early or very late in the infection is optimal ie before parasite numbers have greatly increased, or after the establishment of cell-mediated immunity.

Rifampicin and isoniazid exhibit a marked potentiation against

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20. ABSTRACT (contd.)

L.mexicana amazonensis in mice. No potentiation is seen in mice infected with L.donovani.

L.major, L.m.amazonensis and L.donovani in macrophage cultures are used to evaluate candidate antileishmanial drugs, using sodium stibogluconate (Pentostam) as standard. Of a selection of anti-mycobacteria drugs tested so far, clofazimine has shown marked activity, mostly against L.major but also against the other species. Oxytetracycline also shows some activity against L.major in this system. The new 8-aminoquinoline WR 6026 has proved very active, with a Pentostam index of 10 against L.donovani, 7 against L.m.amazonensis and 29 against L.major.

In mode of action studies we are developing an amastigote system to measure respiration. Developmental difficulties relate mainly to the preparation of sufficient numbers of amastigotes of the same three species of Leishmania. An ultrastructural study on the effect of WR 6026 on L.m.amazonensis in mice has shown that the drug induces membrane changes in the outer membrane of the amastigotes within the flagellar pocket and some evidence of early changes in the mitochondria and kinetoplast are present. No damage to nuclear structures has been seen so far. These studies continue.

TABLE OF CONTENTS

INTRODUCTION

At the commencement of this contract it was envisaged that the proposed research programme would run for a period of two to three years. As a result of a change of policy regarding the award of research contracts, our request for continued support submitted in September 1981 was not eligible for consideration although our initial contract was extended. Unfortunately, owing to the late receipt of the invitation to tender for renewal, it was not possible to apply for continued funding for the project. During the period covered by this report much of the work has been of a developmental nature and has not yielded all of the information we had anticipated producing over the total planned project period. In particular ultrastructural studies on the lepidine series of 8-aminoquinolines are still at an early stage. In view of the importance we attach to these studies it is our intention to continue our planned experiments and a supplement to this report will be submitted when the work is completed.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee 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).

SCIENTIFIC ACTIVITIES

1. CHEMOTHERAPY

1.1 Biochemical identities of standard strains.

In our Summarising Report (1979) of the work carried out in Liverpool, we drew attention to the importance of precisely identifying the strains used with the aid of biochemical taxonomy. A considerable amount of work has now been done in London on this subject and an extensive battery of tests for the presence of parasite isoenzymes is in use. This work is still in progress but it is already apparent that some revision of the nomenclature that we have previously used is necessary. Accordingly, we have now adopted the standard international identity codes proposed by WHO and the current identity classification of our standard Old World strains of *Leishmania* is shown in Table 1. It will be noted that the only major change that has occurred is with regard to our standard visceral strain (previously designated *L.infantum* LV9). In accordance with current views and as confirmed by the isoenzyme data we have obtained, this strain is now considered to be a member of the *L.donovani* complex. Work is continuing on the biochemial taxonomy of *Leishmania* and to avoid the inherent confusion that has previously arisen from premature classification of enzyme types we have adopted a temporary alphabetical categorisation of enzyme patterns. A definitive classification of isoenzyme patterns will be made when our studies, including comparative studies of New World strains, are completed. A comparison of isoenzyme patterns of *L.major* LV39, *L.donovani* DD8, *L.donovani* LV9 and *L.infantum* LEM78 by starch gel electrophoresis is shown in Fig.1.

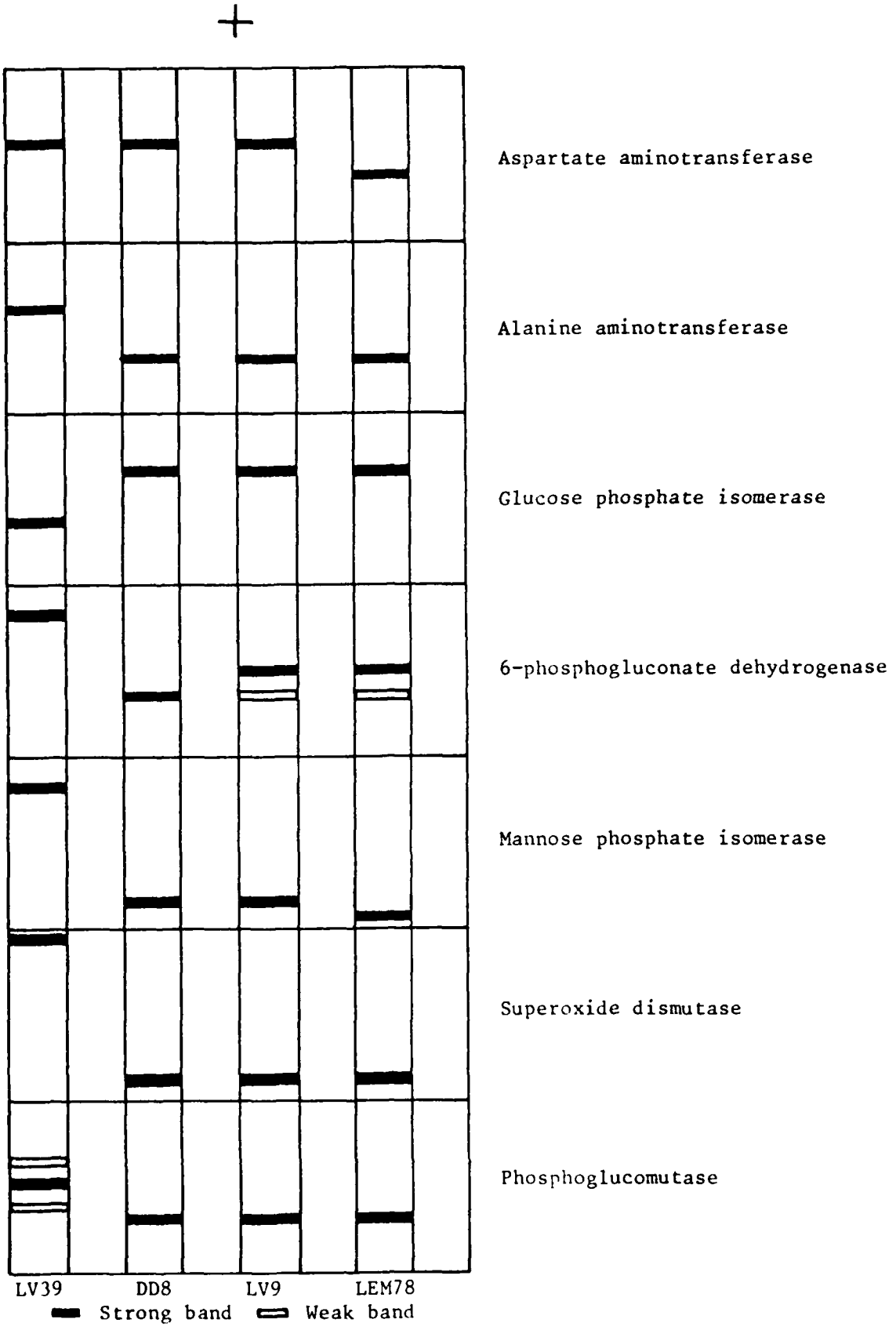


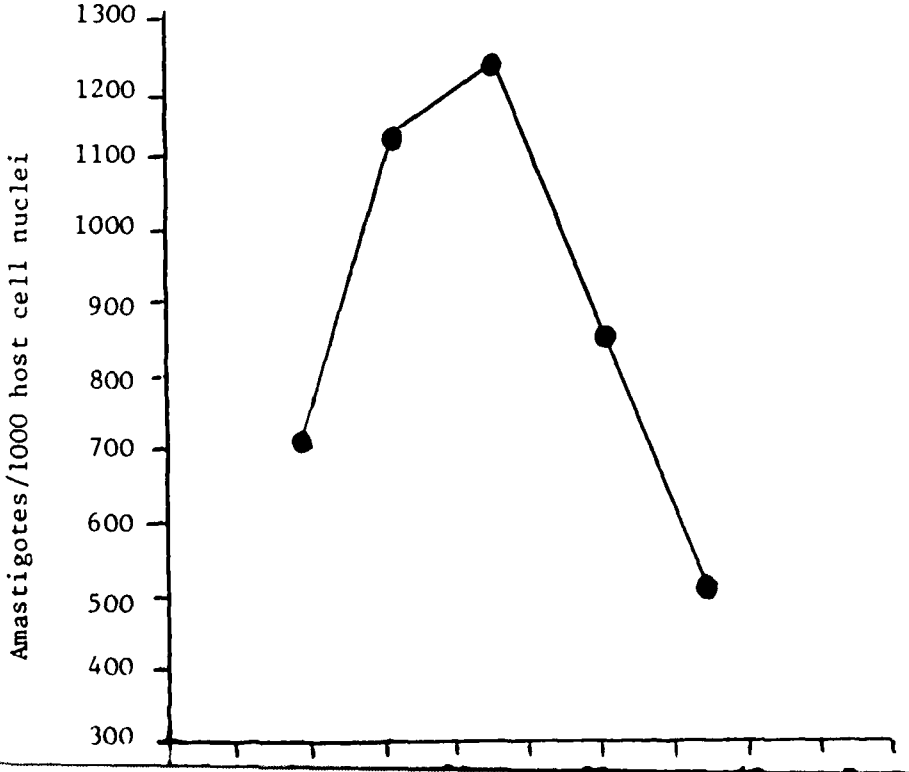
Figure 1. A comparison of isoenzyme patterns of L.major LV39, L.donovani DD8, L.donovani LV9 and L.infantum LEM78 by starch gel electrophoresis.

Species	ASAT	ALAT	GPI	6PGD	MPI	SOD	PGM
L.major LV39 R/SU/1959/NEAL-P	a	a	a	a	a	a	a
L.donovani LV9 MAN/ET/1967/L82LV9	a	b	b	b	b	b	b
L.donovani DD8 MAN/IN/1980/DD8	a	b	b	d	b	b	b
L.infantum C/TN/1978/LEM78	c	b	b	c	c	b	b

Table 1. A comparison of some important enzymes of L.major LV39 and L.donovani LV9 with L.donovani and L.infantum marker strains

1.2 The effect of Pentostam on L.donovani infections of varying duration.

As a result of an observation that we had made in our in vitro dog sarcoma cell system that none of the drugs known to be effective in man had any significant effect, except at phenomenally high dosage, we postulated that drug action may be based on an interaction between the drug and host immunity. In order to test this hypothesis we decided to investigate the effect of treating L.donovani infections of varying duration with a fixed dose of Pentostam (400 mg/kg x 5 sc). The BALB/c mouse: L.donovani model was selected as we knew it to be a self-limiting infection, indicating a good immune response in the host (Figure 2).



When an infection of nine days duration was treated in this way, there was a very marked response with an excess of 90 percent suppression of infection, as measured by the number of amastigotes/1000 host cell nuclei. This level of response fell considerably when treatment was commenced at 16 days (63 percent suppression) and 23 days (28 percent suppression). When a 30 day infection was subjected to this regimen the response reverted to that of the nine day infection and a 37 day infection showed a further slight improvement with 94 percent suppression (Figure 3).

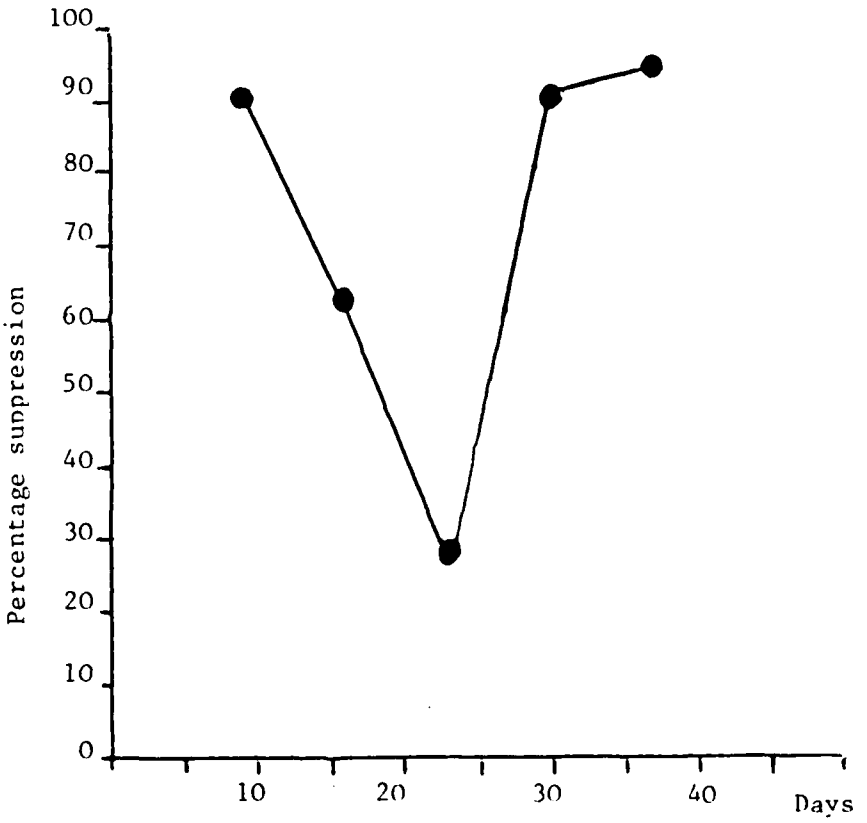


Figure 3 Effects of administering 400 mg/kg x 5 sc of Pentostam to BALB/c mice at varying intervals after infection with L.donovani LV9.

From these results it may be inferred that the relationship between drug action and host immune response, which we envisaged, does exist in the case of Pentostam. In the early stages of the infection, when the parasites are actively multiplying, there is a very marked progressive reduction in the response to Pentostam. In fact, a course of five subcutaneous doses of 400 mg/kg begun 23 days post infection has only 25 percent of the effect shown by the same regimen commenced nine days after infection.

However, when the host's immune system is actively combating the parasites in the later stages of infection, the response to this treatment is as great as or even greater than the effect observed at the earliest treatment time.

In this context, it is interesting to consider the notable lack of success of Pentostam and indeed any other drug treatment against those leishmanial infections, such as disseminated cutaneous leishmaniasis, which occur in immunologically incompetent individuals.

1.3 Rifampicin/Isoniazid combination studies.

During the course of the antileishmanial drug screening programme carried out in Liverpool it had been established that rifampicin had a modest degree of activity against Leishmania both in vitro and in vivo.

As a result of a report from Brazil in 1980 regarding a patient with long standing diffuse cutaneous leishmaniasis, which classical antileishmanial drugs had failed to cure, who had shown striking remission of his leishmanial skin lesions after receiving treatment for an intercurrent mycobacterial infection, we decided to renew our previous experiments with rifampicin and to extend them to include observations on isoniazid and para-aminosalicylic acid (PAS). Coincidentally, at the time of receiving the report on this patient, we were running L.m.amazonensis M1132 in hamsters at Winches Farm Field Station. As this was the strain which had been isolated from this particular patient in 1969 it was selected as the parasite to be used for these studies. The experiments were performed according to our normal techniques using random bred albino mice (Tuck's TFW strain). The results of these tests confirmed our earlier findings with rifampicin and showed a total lack of activity with PAS at the maximum tolerated dose (MTD). Isoniazid, however, proved to be fairly active although not completely so at the MTD (Table 2).

Drug	ED_{50}	ED_{90}	MTD
Rifampicin	200	300	> 300
Isoniazid	25	180	< 300
PAS	Inactive	Inactive	1000

Table 2 Effect of therapy on leishmanial infection (L.m.amazonensis M1132) in TFW mice. (Doses - mg/kg/day).

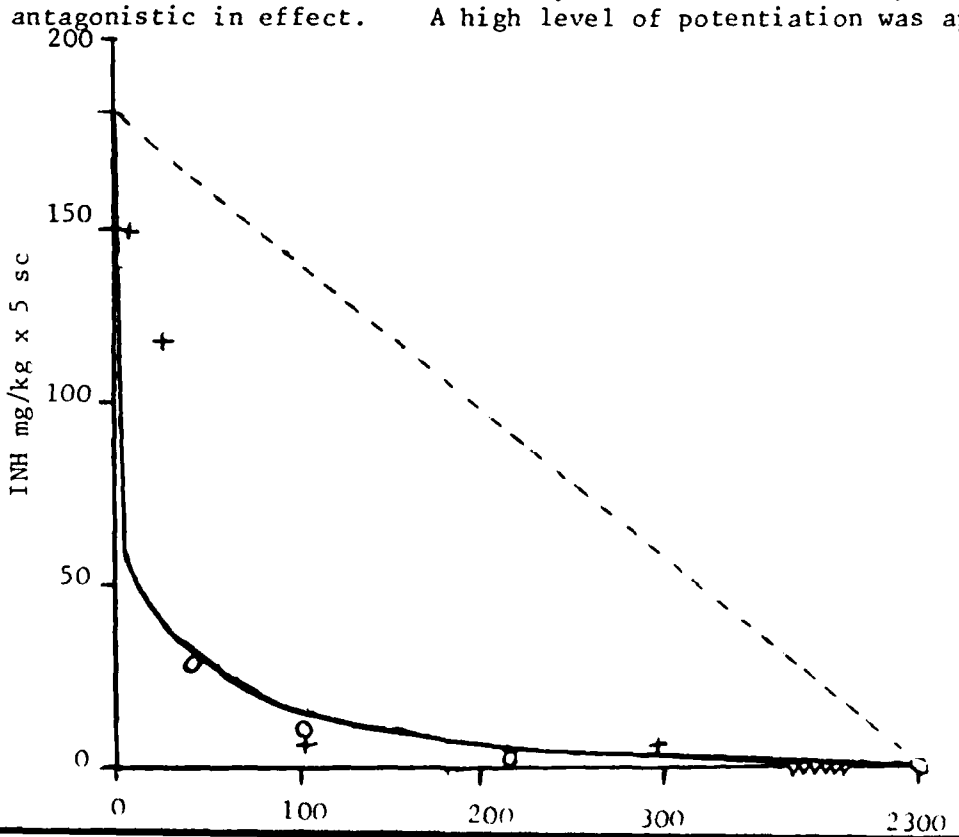
In the light of these observations we repeated the experiment, this time administering rifampicin or isoniazid either alone or in mixtures of varying proportions. The results are shown in Table 3. From the data obtained the ED_{50} and ED_{90} levels of the individual drugs and their combinations were calculated.

Drugs	Rifampicin(R)		Isoniazid(INH)	
	ED_{50}	ED_{90}	ED_{50}	ED_{90}
R alone	200	c.2300*	---	---
R+3mg/kg INH	11.3	225	---	---
R+10mg/kg INH	9.5	100	---	---
R+30mg/kg INH	5.8	50	---	---
INH alone	---	---	25	180
INH+10mg/kg R	---	---	7	150
INH+30mg/kg R	---	---	4	120
INH+100mg/kg R	---	---	0.25	5.5
INH+300mg/kg R	---	---	0.25	5.5

Table 3 Effect of combination of rifampicin and isoniazid in varying proportions on *L.m.amazonensis* (M1132) infections in TFW mice.

*Graphically interpolated result

These values were used to plot a graph (Figure 4) to determine whether the combination of the two compounds was additive, synergistic or antagonistic in effect. A high level of potentiation was apparent.



The ordinate and abscissa show the daily doses in mg/kg given for five consecutive days from the day of infection. The points show the ED_{90} when isoniazid is used alone or with various doses of rifampicin(+) and when rifampicin is used alone or with various doses of isoniazid (0). A simple additive effect would be present if the points fell on the dotted line joining the ED_{90}s of the two compounds used alone. The curve below this line indicates a high level of potentiation between rifampicin and isoniazid.

Further tests have been carried out to examine the response of L.donovani LV9 to combinations of rifampicin and isoniazid. The ED_{50} and ED_{90} values obtained are shown in Table 4 and it is clear that L.donovani is appreciably more sensitive than L.m.amazonensis to rifampicin.

Drugs	Rifampicin		Isoniazid	
	ED_{50}	ED_{90}	ED_{50}	ED_{90}
R alone	15	185	---	---
R+10mg/kg INH		160	---	---
R+30mg/kg INH		150	---	---
R+100mg/kg INH		85	---	---
INH alone	---	---	10	160
INH+10mg/kg R	---	---		130
INH+30mg/kg R	---	---		150
INH+100mg/kg R	---	---		105
INH+300mg/kg R	---	---		22

Table 4 Effect of rifampicin and isoniazid alone and in combination against L.donovani in BALB/c mice.

When these results are plotted as previously described it becomes apparent that the effect of the combination is not a synergistic one but simply additive (Figure 5).

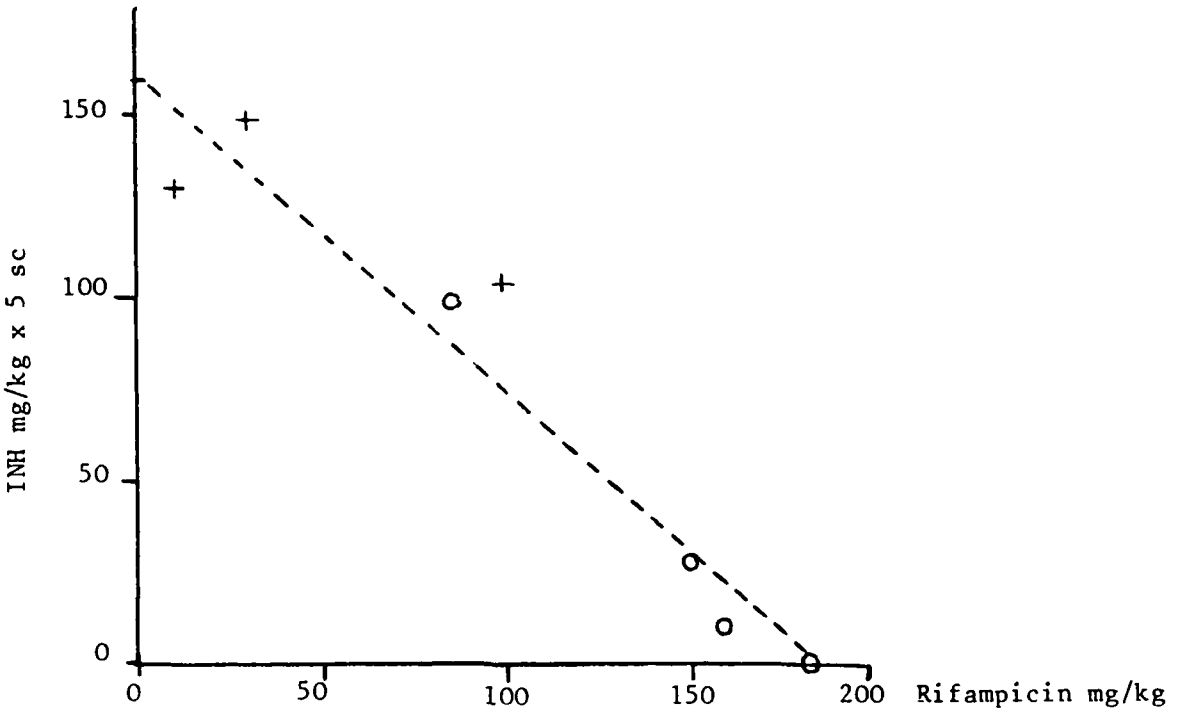


Figure 5 Graph showing interaction between rifampicin and isoniazid against L.donovani LV9 in BALB/c mice.

It should be noted that our publication in Lancet referring to this work was specifically describing work with one strain of L.m.amazonensis and indicated that studies on other Leishmania were required. A paper describing the results of our experiments with L.donovani is in preparation and this confirms the experience of clinicians who have had correspondence published in Lancet, subsequent to our initial paper, indicating the failure of human cases of kala azar to respond to combined rifampicin/isoniazid therapy.

1.4 In vitro screen for antileishmanial activity.

A method for in vitro screening of compounds for the measurement of activity against Leishmania species in mouse peritoneal macrophages is being developed. The system has been developed using L.donovani LV9; therefore screening drugs against this organism has presented no new problems. It has been found possible to adapt the technique for use with L.major LV39 and L.m.amazonensis LV78. A far lower initial infection ratio of parasites to macrophages is used because of the rapid growth of these strains, and incubation of the cultures is carried out at a lower temperature (34°C rather than 37°C) to simulate conditions in a superficial lesion. In the case of L.m.amazonensis, transformation from promastigote to amastigote occurred rapidly although some of these remained extracellular. In the L.major system, however, transformation was incomplete with many extracellular promastigotes visible throughout the test period. The sensitivity of these systems to test drugs was found to be much lower than that of the L.donovani system and for this reason we are now trying an amastigote inoculum. The infection produced by L.m.amazonensis using this technique is typical of the in vivo appearance of the mexicana group - heavy infestations with parasites grouped around the periphery of large parasitophorous vacuoles. In the LV39 system there is still a tendency for a few promastigotes to appear towards the end of the test period; however, this can occur with heavy infections with L.donovani and does not appear to affect sensitivity to any significant degree. Results obtained with Pentostam by this technique are shown in Table 5.

System	ED_{50}(µg/ml)	ED_{90}(µg/ml)
LV9 - amastigotes	4.4	18.2
LV78 - amastigotes	18.2	69.2
LV78 - promastigotes	63.1	218.8
LV39 - amastigotes	26.3	120.2
LV39 - promastigotes	44.7	354.8

Table 5 Effects of Pentostam on L.donovani LV9, L.m.amazonensis LV78 and L.major LV39 in peritoneal macrophage culture.

1.5 Activity of antimycobacterial drugs in vitro.

As a consequence of our work on rifampicin and isoniazid in vivo it was decided to examine a wider range of antimycobacterial agents in the in vitro system. The results of our initial study are summarised in Table 6.

Drug	Concentration µg/ml	Activity (% suppression) LV9	LV39	LV78
Cycloserine	27	0	0	0
	81	0	0	0
Clofazimine	27	29	91	26
Dapsone	27	0	0	0
Isoniazid	27	0	0	0
	81	NT	9	NT
Oxytetracycline	27	9	0	0
	81	0	19	0
Thiambutosine	27	0	15	9
	81	NT	Toxic	NT
Rifampicin	9	NT	0	NT
	27	NT	13	NT
	81	NT	31	NT
Rifampicin + Isoniazid	4.05 + 40.5	NT	10	NT
	8.1 + 40.5	NT	6	NT
	27 + 54	NT	10	NT
	40.5 + 40.5	NT	12	NT
	54 + 27	NT	13	NT

Table 6 Activity of some antimycobacterial drugs against

Clofazimine has shown good activity in all three systems and its in vivo activity is now being assessed. It is fairly toxic to the host cells at 27 µg/ml and there is some evidence of toxicity at 9 µg/ml. However, its systemic toxicity in vivo is very low (Yawalker et al.,1979) and it becomes concentrated in the cells of the reticuloendothelial system suggesting that the therapeutic index may be high. Thiambutosine and oxytetracycline show marginal activity warranting further study in vitro. The activity of oxytetracycline against LV9 is of particular interest because it can reach high concentrations in the liver due to enterohepatic circulation (Acocella et al., 1968). Dapsone and cycloserine appear to be totally inactive. Isoniazid and rifampicin have so far been tested against LV39 and show poor levels of activity in this sytem. Few combinations have been assessed of the two drugs but so far no synergistic effects have been demonstrated.

1.6 Activity of WR6026 in vitro.

In the mouse peritoneal macrophage system WR6026 shows excellent activity although the therapeutic index is low. Interestingly the few experiments carried out initially using a promastigote inoculum of L.m.amazonensis LV78 show a similar level of activity to that shown in the amastigote system, and therefore the results for L.major LV39 were obtained using a promastigote inoculum. This is because promastigotes are sensitive to the effects of the compound; exposure of LV78 promastigotes to concentrations of WR6026 above 10 µg/ml for 24 hours results in the appearance of abnormal forms possessing multiple organelles and eventually produced complete disintegration of the parasite. It may be postulated that at sublethal dosage transformation of the parasite would also be affected. The results of these in vitro tests are summarised in Table 7.

Strain	Drug	ED_{50} µg/ml	ED_{90} µg/ml	Pentostam Index
LV9	Pentostam	4.4	17.0	
	WR6026	1.0	1.7	10
LV78	Pentostam	15.8	63.1	
	WR6026	4.0	8.7	7
LV39	Pentostam	43.6	346.7	
	WR6026	3.8	12.0	29

Table 7 Effects of WR6026 on Leishmania in mouse peritoneal macrophages in vitro compared with those of Pentostam.

1.7 Mode of action studies.

Apart from electron microscopy studies of ultrastructural changes, most biochemical investigations will require a method of isolating amastigotes free of significant host contamination, in sufficient numbers for analysis. Promastigotes were used to test the effect on respiration of drugs using an oxygen electrode, but sensitivity to pentamidine, WR6026 and respiratory inhibitors such as amytal and azide could be demonstrated only at high concentrations (150 µg/ml). The lesions of LV39 in mice are too small to provide sufficient material for amastigote isolation, therefore this work will be concentrated on L.donovani LV9 and L.m.amazonensis LV78. Much host cell contamination can be removed by saponin lysis in the case of LV9; however, LV78 has been found to be too susceptible to lysis for this technique. Conversely, poor yields of LV9 are obtained using column separation techniques in comparison to LV78 (Brazil, 1978). It may be possible to obtain relatively pure L.donovani LV9 amastigotes by saponin lysis and isopycnic centrifugation on a Percoll gradient (Hart et al.,1980), a method which has been used with some success by other workers.

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Brazil, R.P. (1978). Ann.Trop.Med.Parasitol., 72, 6, 579-580

Hart et al. (1980). Parasitology, 82, 345-355

Yawalkar et al. (1979). Lepr.Rev., 50, 135-144

1.8 Ultrastructural studies on the effects of WR6026

BABL/c mice were treated with WR6026 (x1 sc) fourteen days after infection with L.m.amazonensis. After fixation in 1.75% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, the specimen was post-fixed in 1% osmium tetroxide. Sections were prepared and stained with uranyl acetate and lead citrate.

On examination the sections revealed the presence of membrane changes in the outer membrane of the amastigotes within the flagellar pocket and some evidence of early changes in the mitochondrion and kinetoplast are present. No damage to nuclear structures are present and there is no evidence of ribosomal depopulation.

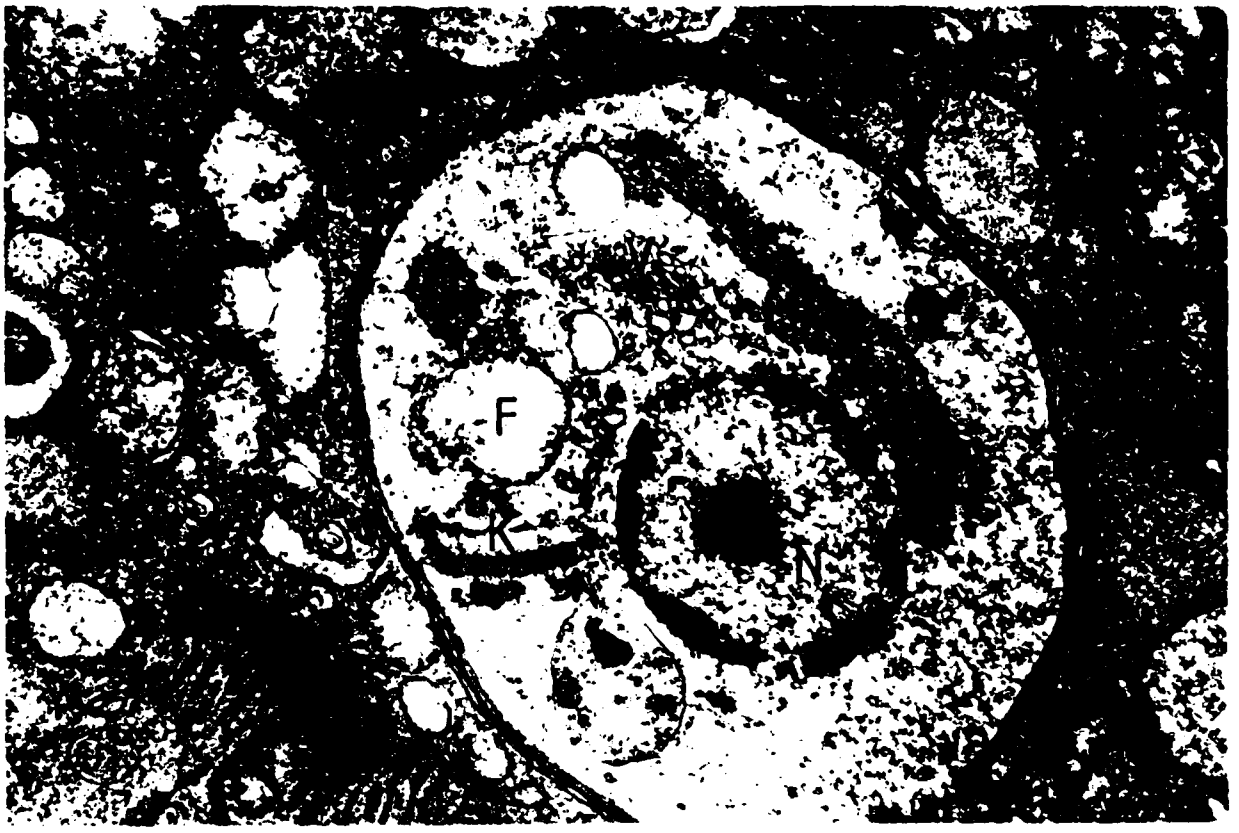

Figure 6 Amastigote in skin lesion (20 mg/kg WR6026. Lesion fixed 24 hours later). Amastigote retained characteristic ultrastructure and showing the nucleus (N) and mitochondrion (M) with no change. Slight disruption of the kinetoplast (K) is evident but the most extensive changes were observed in the large number of vesicles (V) between the flagellar pocket (F) and the Golgi region. (x 34,800).

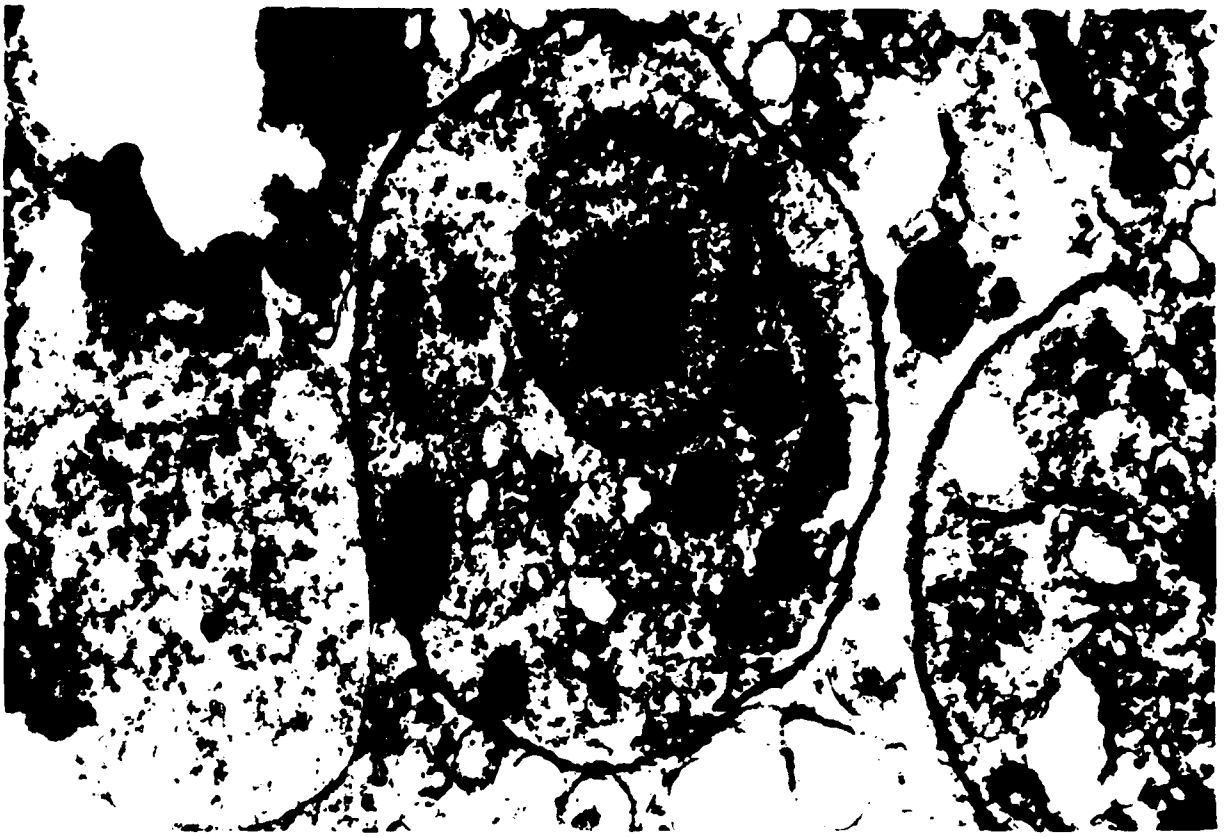

Figure 7 Amastigote in skin lesion. 15 mg/kg, single dose, lesion fixed 24 hours after dosing.
The amastigote shows characteristic shape and ultrastructure. Again there is evidence of increased numbers of vesicles (V) of variable size and shape between the flagellar pocket and Golgi region. (x 32,000)

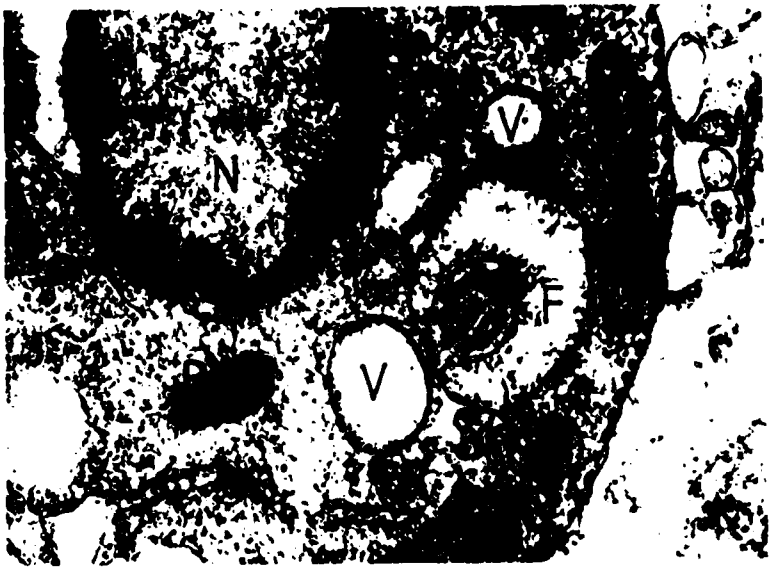

Figure 8 Amastigote in skin lesion. 15 mg/kg WR6026 sc X1. Lesion fixed 24 hours after treatment.
Some evidence of changes in the membranes of the amastigote may be seen. Vesicles (V) are forming from the flagellar pocket (F). The internal structure of the nucleus (N), mitochondrion (M), peroxisome (P) and flagellum are normal. (x 40,000)

PUBLICATIONS

The following papers have been published since the submission of the last report from Liverpool (1979).

1. Al-Gindan, Y., Omer, A.H.S., Al-Humaidan, Y., Peters, W. and Evans, D.A. (1982). A case of mucocutaneous leishmaniasis in Saudi Arabia caused by Leishmania major and its response to treatment. Clin.Exper.Dermatol. (in press).

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