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EFFECT OF DIHYDROFOLATE REDUCTASE INHIBITORS ON EXPERIMENTAL CUTANEOUS LEISHMANIASIS, WITH ESPECIAL EMPHASIS ON *LEISHMANIA* ISOLATES FROM LATIN-AMERICA

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SUMMARY

The course of infection in hamsters and their response to treatment have been studied with 7 isolates from Latin-American cutaneous leishmaniasis. Treatment with anti-folic reductase drugs, pyrimethamine, cycloguanil pamoate and trimethoprim and combination of pyrimethamine and trimethoprim with sulphadiazine was compared with treatment with sodium stibogluconate and paromomycin. The response of the Latin-American isolates to these drugs has been compared with that of 2 Asiatic isolates of *Leishmania tropica*. There was no significant response of either the Latin-American or Asiatic isolates to the folic reductase inhibitors or their combination with sulphonamides. Sodium stibogluconate was highly effective in suppressing the infection with all but one isolate. The course of infection with the Latin-American isolate of *Leishmania* varied from "slow" to "fast". This was not correlated with the type of *in vitro* growth as postulated by LAINSON and SHAW. However the attenuation effect of continuous cultivation could interfere with the interpretation of results on the relationship of virulence and *in vitro* cultivation.

INTRODUCTION

Conflicting clinical reports have been published regarding the efficacy of pyrimethamine against cutaneous leishmaniasis (NEAL [8]; JOHNSON [4]; VEGAS & FURTADO [13] and CALLE & VELASQUEZ [1] whereas the laboratory reports have shown pyrimethamine to be inactive against experimental infections. Cycloguanil pamoate (Camolar) has also been used clinically, with varying degrees of success (SALEM et al.[12]; WALTON et al.[14]; KURBAN et al.[5] and PENA-CHAVARRIA et al.[10]).

The present paper is an investigation of the effect of the anti-folic reductase drugs, pyrimethamine, and cycloguanil against cutaneous leishmaniasis with emphasis upon Latin-American isolates of *Leishmania*. The drugs studied have been extended to include trimethoprim and the combination of the pyrimidines with sulphonamides.

MATERIAL AND METHODS

i) *Isolates and cultivation*

Five new isolates from clinical cases of cutaneous leishmaniasis were kindly sent by Dr. B. C. WALTON while working at the Gorgas Memorial Laboratory, Panama. These five isolates with details of clinical history are as follows:

1. *Courtwright* — Isolated in Panama, 1967, from a skin lesion contracted in Panama. Rapid response to Camolar. Culture received, 19th May, 1969;

2. *Boynton* — Isolated in Panama, 1967, from cutaneous lesion contracted in Panama. Slow response to Camolar. Culture received, 8th April, 1969;

3. *Atenzana* — Isolated in Panama, 1969, from a Bolivian patient with advanced

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espundia. Culture received, 19th May, 1969;

4. *Clonts* — Isolated in Panama 1969 from skin lesion contracted locally. Culture received, 8th April, 1969;

5. *Baker* — Isolated in Panama 1967 from skin lesion contracted in Panama. Culture received, 8th April, 1969.

Two further Latin-American isolates used in these experiments were a recent isolate from Peru given by Dr. L. G. GOODWIN and an isolate from Costa Rica which had been in laboratory use for some years, from Dr. R. ZELEDON. Details of these are:

6. *L-280* — Isolated from a case described as uta, mucocutaneous variety in Peru, 1966. Culture received on 24th November, 1966;

7. *O-CR-B* — Isolated in Costa Rica in 1967 from a cutaneous ulcer (ZELEDON, BLANCO & MONGE 1969) [15]. Culture received, 19th February, 1969.

The results with these seven isolates were compared with results with two Asiatic isolates. The Asiatic isolates are:

8. *L. tropica major* isolate *P.* — This isolate was isolated in 1959 from Uzbeck U.S.S.R. For further details see NEAL, 1964;

9. *L. tropica Baghdad* — Isolated in 1962 from lesion on the lip, contracted in Baghdad. Culture received in 1962 from the late Professor S. ADLER.

All these isolates with one exception grew profusely on blood agar medium. Eleven mls of blood agar (10% defibrinated fresh rabbits blood in nutrient agar) were sloped in 100 ml screw-capped "medical flat" bottles with 5 ml of glucose-saline as overlay. Cultures were passaged twice per week. The isolate which would not grow well in blood agar was *L-280*. This isolate grew better in Adlers' semi-solid medium.

In order to preserve the virulence of the culture, each isolate was frozen down in liquid nitrogen vapour (-196°C) as soon as possible. At the time of writing, frozen material of each isolate is still being stored in liquid nitrogen. Hamsters were inoculated within 2 to 3 weeks of receipt of the Latin-American isolates.

Inocula were prepared by pooling the overlay from several cultures at the end of the logarithmic phase of growth. The number of promastigotes were determined by haemocytometer counts. Before inoculation the promastigote suspensions were standardized to contain 1 x 10^8 cells/ml.

ii) *Infection of animals*

Preliminary studies confirmed previous work that hamsters were more susceptible to infection than mice and that good infections were generally observed after the inoculation of 4 x 10^6 or more flagellates. Since the main object of this present work was to study the chemotherapeutic response of newly isolated parasites, no further preliminary work was done. As soon as the cultures were growing well and animals became available, inocula of each isolate were prepared containing 1 x 10^8 cells/ml. Animals were inoculated with 1 x 10^7 promastigotes by injecting 0.1 ml into the dorsal tip of the nose in the case of hamsters, and about 1 cm above the root of the tail in mice.

Between 50 to 80 animals were inoculated with each isolate. After inoculation, they were divided into groups of 8 to 10 animals and dosed with the appropriate drug. The experimental animals were kept in a ventilated animal room maintained at about 24°C.

In the case of the isolate *L-280*, since it seemed likely to be difficult to obtain sufficiente parasites from culture to infect hamsters, the inocula were prepared from hamsters previously infected from culture. The lesions from 15 hamsters were excised, ground up in 9 ml of saline, filtered free of coarse particles and 0.1 ml inoculated in the nose of each hamster.

Hamsters were obtained from a single breeder and at the commencement of each experiment weighed about 50 g. Mice were similar to those used previously (NEAL, 1970) and initially weighed 20 g.

iii) *Treatment*

A standard protocol was used which compared the effects of the antifolate compounds pyrimethamine and cycloguanil with the standard compounds sodium stibogluconate and paromomycin. Paromomycin was used as the sulphate salt, while pyrimethamine was the base. Cycloguanil was obtained from Dr. P. E. THOMPSON (Parke-Davis) as a suspension of the pamoate salt in castor oil — benzyl benzoate at 140 mg base/ml (batch no. Rx 344250). Sodium stibogluconate (Pentostam) was manufactured by Burroughs Wellcome & Co.

The plan of each experiment with each isolate was as follows:

Drug	Dose Level mg base/kg x 5	Route of administration
1. Untreated	None	—
2. Cycloguanil pamoate	400	Sub-cutaneous
3. Sodium stibogluconate	400 (*)	Sub-cutaneous
4. Pyrimethamine	50	Oral
5. Paromomycin	10	Sub-cutaneous

(*) The dosage of sodium stibogluconate was expressed as mg of antimony

Treatment of the animals was started about 6 hours after the inoculation of parasites. Subcutaneous injections were given dorsally between the shoulder blades. Treatment was continued daily for a further 4 days.

iv) *Assessment of Infection*

The infection was followed by recording the development of a cutaneous lesion at the site of inoculation of promastigotes. The scale of lesion development was identical to that used previously (NEAL [8]).

No thickening of skin 0
Slight thickening of skin 1
Definite thickening 2
Large area of thickening or nodule measuring 5 mm or less in diameter 3
Area of thickening or nodule measuring more than 5 mm in diameter 4

In general, the presence or absence of parasites was determined only after the completion of experiments. Smears were prepared from the lesions, stained with Giemsa stain and examined microscopically for the presence of amastigotes.

The animals were examined weekly and the lesion score recorded for each animal. The development of the lesion of treated and untreated animals was followed until the lesions had reached their maximum size, usually between 1 and 2 months.

A simple method of classifying the drug response was devised. At the time of maximum development of the untreated group, the lesion size of the treated groups was compared to the untreated group and assigned to one of the following classes of drug activity.

RESULTS

i) *Course of infection in hamsters*

The rate of lesion development of the Latin-American isolates in hamsters inoculat-

Average lesion score of treated group	Class of drug activity
Equal or greater than the controls	0 (drug inactive)
Equal to or 2/3 that of the controls	+
1/3 to 2/3 that of the controls	++
Negative to 1/3 that of the controls	+++ (maximum drug activity)

ed with 1 x 70^7 promastigotes is shown in Table I.

It is clear that the isolates differ considerably in their infectivity and extent and rate of development. Two isolates (*Courtwright* and *O-CR-B*) infected all inoculated hamsters and all had large cutaneous lesions.

At the other extreme, one isolate (*Atenzana*) was poorly infective: only one hamster out of seven inoculated showed a significant lesion (maximum lesion of this animal scored 2.0), while two other animals had a very low grade (maximum lesion score 1.0) cutaneous lesion. In general, lesions of the Latin-American *Leishmania* isolates reached their maximum size within the same time interval of 5 to 7 weeks (see Table I).

For comparison with the hamster infection with Latin-American isolates, the infectivity and rate of development of two Asiatic isolates of *L. tropica* in hamsters and mice are given in Table II.

From these results it is clear that the two most virulent Latin-American isolates were equivalent to the Asiatic strains in their course of infection in laboratory animals.

The results with two isolates (*O-CR-B* in hamsters and *P* in mice) show that the slope of the curve was very shallow at the end of the logarithmic growth phase. This resulted

TABLE I

Course of infection of Latin-American isolates of cutaneous leishmaniasis in hamsters

Isolate	Length of experiment (weeks)	no. hamsters with lesions / no. inoculated	no. of hamsters with parasites / no. examined	Time to reach maximum average lesion score (weeks)	Maximum average lesion score
Courtwright	14	8/8	4/4	6	3.0
Boynton	13	8/8	7/8	5	1.9
Atenzana	10	3/7	3/7	7	0.6
Clonts	13	7/8	7/8	6	1.6
Baker	14	8/8	4/8 (*)	5	2.1
O-CR-B	18	7/7	7/7	6 (+)	3.9
L-280	17	17/17	17/17	7	4.0

(*) Examined week 14
(+) See text above

TABLE II

Course of infection of Asiatic isolates of *L. tropica* in hamsters and mice

Isolate	Host	Length of experiment (weeks)	no. animals with lesions / no. inoculated	no. animals with parasites / no. examined	Time to reach maximum average lesion score (weeks)	Maximum average lesion score
P	Hamster	14	8/8	7/7	4	4.0
Baghdad	Hamster	17	8/8	8/8	8	3.7
P	Mouse	19	10/10	10/10	4 to 15 (+)	3.6

(+) See text p. 344

in an apparently long period to reach maximum average lesion score. The end of the logarithmic phase occurred at about 6 and 8 weeks respectively. Further experiments with *L. tropica major P* in mice showed a shorter incubation period.

TABLE III

Time at which average lesion scores of hamster infections started to decrease

Isolate	Time (weeks)
Latin-American *Leishmania*	
Courtwright	9
Boynton	6
Atenzana	>10 (*)
Clonts	7
Baker	14
O-CR-B	14
L-280	13
Asiatic *L. tropica*	
P	10
Baghdad	11

(*) maximum period of observation

The lesions produced by the Latin-American isolates in hamsters were observed as dry plaques or nodules. No ulceration with formation of open sores, as seen with *L. tropica major P* in hamsters or mice, was observed. Limited observation during these experiments with Latin American isolates did not reveal a massive spread of parasites to the liver or spleen. Microscopical observations on the latter tissues were always negative, but occasional cultures were positive.

After 6 weeks, the lesions began to diminish in size and at the completion of experiments, a few hamsters had resolved their lesions. In general, the more virulent strains took longer time to begin to heal than less virulent strains (Table III). Although isolate *Atenzana* is given as a long lasting infection in Table III this conclusion is based on the only one hamster with a definite lesion.

ii) *Effect of treatment*

The effect of treatment was analysed at the time when the lesions of untreated groups had reached maximum size, as recorded in Tables I and II. No analysis is attempted of drug treatment of infections due to the *Atenzana*, owing to the low infectivity of this isolate.

If the treatment was effective, the average lesion size of the treated groups would be much smaller than the untreated group. Thus,

treatment with sodium stibogluconate was very effective and inhibited the development of cutaneous lesions after inoculation with five out of the six Latin-American isolates tested (Table IV). However at the end of the period of observation up to 14 weeks a variable proportion of the treated hamsters had developed cutaneous lesions (see Table V). Paromomycin was less effective than sodium stibogluconate against all isolates.

Paromomycin was used against two isolates, *O-CR-B* and *L-280* at the ten fold higher dose level of 100 mg/kg x 5. The antibiotic at the higher dose level showed ++ activity against infections with isolate *O-CR-B* but no activity against infections with isolate *L-280* at either dose level.

The antifolate drugs, cycloguanil pamoate and pyrimethamine were more variable in their effect than the other drugs (Table IV). Against two isolates, *Boynton* and *O-CR-B*, pyrimethamine showed a moderate activity and against another isolate, *Courtwright*, slight activity. No activity was observed with this drug against the remaining three isolates. Cycloguanil pamoate showed moderate activity against isolate O-CR-B, slight activity against two isolates *Courtwright* and *Baker*, and no activity against the remaining 3 isolates. Even at their highest activity, cycloguanil pamoate and pyrimethamine were very much less effective than sodium stibogluconate.

For comparison with the Latin-American isolates, the results of treatment of hamster and mouse infections with Asiatic isolates of *L. tropica* are given in Table VI.

TABLE IV

Activity of drugs on hamsters infected with Latin-American isolates of cutaneous leishmaniasis

Isolate	Time of assessment (weeks)	Treatment						Sodium stibogluconate		Paromomycin	
		Untreated		Cycloguanil pamoate		Pyrimethamine					
		A	B	A	B	A	B	A	B	A	B
Courtwright	6	8/8	3.0	8/8	2.1	8/8	2.6	0/8	0	8/8	2.7
Boynton	5	8/8	1.9	8/8	2.0	5/8	1.0	0/8	0	7/8	2.0
Atenzana	7	3/7	0.6	1/8	0.1	6/8	1.0	5/8	1.0	3/6	0.7
Clonts	6	8/8	1.6	8/8	2.5	6/6	1.8	0/8	0	8/8	2.3
Baker	5	8/8	2.1	6/6	2.0	8/8	2.1	0/8	0	8/8	1.7
O-CR-B	6	7/7	3.6	4/4	1.5	8/9	1.7	0/8	0	6/6	2.8
L-280	6	13/13	3.8	10/10	3.9	10/10	4.0	10/10	3.3	10/10	4.0

A = Proportion of hamsters with lesions at the time of assessment
B = Average lesion score at the time of assessment

TABLE V

Number of hamsters infected with Latin-American isolates of *Leishmania* which had relapsed after treatment with sodium stibogluconate

Isolate	Time of conclusion of expt. (weeks)	no. relapsed / no. treated (average lesion score)	
Courtwright	14	0/8	(0)
Boynton	9	1/5	(0.2)
Clonts	9	0/8	(0)
Baker	14	3/8	(0.4)
O-CR-B	14	4/8	(1.1)

In these experiments, sodium stibogluconate was highly effective in suppressing the infection. However the activity of sodium stibogluconate against hamster infections with *L. tropica major P* was less than expected from previous experiments. Paromomycin was more effective against mouse infections than hamster infections with the same *Leish-*

TABLE VI

Activity of drugs on hamsters and mice infected with Asiatic isolates of cutaneous leishmaniasis

Isolate and host	Time of assessment (weeks)	Untreated		Cycloguanil pamoate		Treatment		Sodium stibogluconate		Paromomycin	
						Pyrimethamine					
		A(*)	B(*)	A	B	A	B	A	B	A	B
Hamster											
P	4	8/8	4.0	8/8	4.0	8/8	4.0	8/8	3.6	8/8	3.2
Baghdad	6	8/8	3.6	8/8	3.6	8/8	3.3	7/9	1.0	9/9	1.9
Mice											
P	9	10/10	3.2	7/7	3.3	9/9	3.2	10/10	1.1	9/10	1.1

(*) See footnote to Table IV for explanation of symbols

TABLE VII

Summary of relative effectiveness of activity of drugs in suppressing experimental cutaneous leishmaniasis

Isolate and laboratory host	Treatment			Paromomycin
	Cycloguanil pamoate	Pyrimethamine	Sodium stibogluconate	
Latin-American: hamster				
Courtwright	+	+	+++	+
Boynton	nil	++	+++	nil
Clonts	nil	nil	+++	nil
Baker	+	nil	+++	+
O-CR-B	++	++	+++	+
L-280	nil	nil	+	nil
Asiatic: hamster				
P	nil	nil	+	+
Baghdad	nil	+	++	++
Asiatic: mice				
P	nil	nil	+++	+++

mania isolate. Cycloguanil pamoate and pyrimethamine were either inactive or only slightly active against the Asiatic strains of *L. tropica*.

The results on all drugs against the isolates are summarised in Table VII.

A further study of the effect of the antifolate drugs, pyrimethamine and trimethoprim, and their combination with the p-aminobenzoic acid antagonist, sulphadiazine, was made on mouse infections with the Asiatic isolate *L. tropica major P* (Table VIII).

TABLE VIII

Lesion size (average lesion score) of mice infected with *L. tropica major P*, six weeks after oral treatment with pyrimidines and sulphonamides

Treatment	Dose level mg/kg x 5	Proportion of mice with lesions	Average lesion score	Drug Activity
Untreated	—	10/10	3.2	—
Pyrimethamine	50	10/10	2.3	+
Trimethoprim	100	9/9	1.3	++
Sulphadiazine	200	8/8	2.4	+
Pyrimethamine + sulphadiazine	50 + 200	7/7	2.0	+
Trimethoprim + sulphadiazine	100 + 200	5/5	1.0	++

TABLE IX

Lesion size (average lesion score) of hamsters infected with Latin-American *Leishmania* isolate O-CR-B, seven weeks after treatment with pyrimidines and sulphonamides. All drugs except sodium stibogluconate give orally

Treatment	Dose level mg/kg x 5	Proportion of hamsters with lesions	Average lesion score	Drug Activity
Untreated	—	9/9	3.4	—
Pyrimethamine	50	9/9	3.9	0
Trimethoprim	100	10/10	3.9	0
Sulphadiazine	200	10/10	3.7	0
Pyrimethamine + sulphadiazine	50 + 200	10/10	3.4	0
Trimethoprim + sulphadiazine	100 + 200	9/9	3.2	0
Sodium stibogluconate (*)	400 mg Sb(+)/kg	0/9	0	+++

(*) Given subcutaneously
(+) Dose expressed as mg of antimony

These results show that pyrimethamine was only slightly active (+) but moderate activity (++) was observed with trimethoprim. Sulphadiazine alone was slightly active, but the combination with the pyrimidines did not result in the combined treatment showing increased activity.

The higher activity of trimethoprim compared to that of pyrimethamine was further studied with the Latin-American isolate *O-CR-B*. This experiment (see Table IX) did not confirm the activity of pyrimethamine or trimethoprim observed in the earlier experiments, though sodium stibogluconate remained highly effective. Sulphadiazine alone or in combination with pyrimidines were inactive.

TABLE X

Comparison of culturability on blood agar medium and virulence to hamsters of two *Leishmania* isolates from mucocutaneous cases

Isolate	Culturability	Virulence in hamsters
Atenzana	+++	+
L-280	+	+++

DISCUSSION

The course of infection with the Latin-American isolates is described in some detail since the results are of interest in relation to LAINSON & SHAW's [7] classification of "fast" and "slow" strains of cutaneous leishmaniasis. According to these workers there is a correlation between rate of development of lesions in hamsters and ease of growth in culture. They also consider the possibility that "slow growing" isolates are responsible for the nasopharyngeal involvement of the classic mucocutaneous leishmaniasis.

The present results show that three isolates, *Boynton, Atenzana* and *Clonts,* produced small lesions in hamsters and of these, one was a recent isolate from a case of mucocutaneous leishmaniasis (*Atenzana*). Out of the remaining isolates which produced larger lesions, isolate *L-280* was from a case of mucocutaneous leishmaniasis. The relationships of these two latter isolates are summarized in Table X. It is clear that in this small sample, there is not complete support for LAINSON & SHAW's hypothesis, since *Atenzana* grew easily in culture, while *L-280* was more difficult to grow in culture. On the other hand, it may be argued that the large lesions of *L-280* were due to the animal to animal passage that the parasite had undergone. Data on further isolates from mucocutaneous cases are required to throw light on this problem. It is perhaps hazardous to draw any more than the most general conclusions from a study of the present kind where the isolates had been maintained *in vitro* for short though variable periods. Consideration of this factor is particularly important, since earlier work had shown that loss of virulence was apparent after 10 weeks of cultivation (NEAL [8]).

Turning to chemotherapy, one explanation of the successful use of pyrimethamine in Latin-America, in contrast to the failure of pyrimethamine clinically in Baghdad (RAHIM [11]) and experimentally against *L. tropica* (NEAL [8]), is to postulate a difference of sensitivity between Asiatic and Latin-American strains causing cutaneous leishmaniasis. The present experimental results show however, that there is no clear cut distinction between isolates of different geographical origin although the best response to the folic reductase inhibitors was given by the Latin-American isolates *O-CR-B* and *Boynton.* The latter isolate responded only to pyrimethamine and not to cycloguanil pamoatc. Neither of the two Asiatic strains responded to cycloguanil pamoate or pyrimethamine though there was a slight effect against *L. tropica* isolate Baghdad in both animal hosts. However confirmation of the activities of the pyrimidines was not obtained and it must be concluded that any inhibiting effect of these drugs on the parasites must be marginal.

While none of the antifolate drugs were effective, sodium stibogluconate proved very active against five of the Latin-American isolates. Initially, the infection was suppressed completely, though in a few cases lesions were beginning to develop when the experiments were terminated. It is therefore not

known whether all these animals would develop infections or whether or not the lesions would be minimal in extent.

Paromomycin which proved active against *L. tropica* mouse infections (NEAL [9]) was not effective against Latin-American strains at the dose level employed. The lack of activity of paromomycin is probably due to the use of a different host, rather than a difference between the *Leishmania* isolates. This conclusion is reached from the observation that the hamster infections with *L. tropica major P* were more resistant to treatment than the mouse infections with the same isolate. Possibly the pharmacokinetics of paromomycin is different in the hamster compared with the mouse.

The third folic reductase inhibitor, trimethoprim was more effective than pyrimethamine, against *L. tropica* infections in mice, but this activity could not be confirmed against the Costa Rica isolate (*O-CR-B*).

At the present time, there is no information on the folic reductase enzyme relating to its inhibition by 2:4-diaminopyrimidines. A study of the enzyme of amastigotes of *Leishmania* could make a valuable contribution to understanding of drug action.

It can be concluded from the present studies that no clear cut explanation has been found to account for the activity of anti-folic reductase drugs on clinical cutaneous leishmaniasis. In the case of pyrimethamine where the course of treatment is very prolonged, it is possible that the course may coincide with the infection reaching the self-limiting phase of its evolution. Cycloguanil pamoate being administered as a long-acting depot injection, might also cover the same period of time as pyrimethamine. Alternatively, the different pharmacokinetics of these drugs in man compared to that in hamsters or mice, may result in the marginal anti-leishmanial activity being more pronounced in man.

Throughout this paper the isolates have not been referred to a particular species of *Leishmania*. This is because the taxonomy of this genus is in a confused state. GARNHAM [2] gives specific status to four clinically and geographically well defined types of disease. A more comprehensive classification, incorporating laboratory data on cultivation and experimental infections, has been proposed by LAINSON & SHAW [7]. The strains used in the present work could be retrospectively fitted into this classification as members of the *Leishmania braziliensis* complex. In contrast to these two classifications Moshkovskii (1967, see HOOGSTRAAL & HEYNEMAN, 1969) refers all New World forms of cutaneous leishmaniasis as regional subtypes of *L. braziliensis*.

RESUMO

Efeito de inibidores da di-hidrofolato reductase sobre a leishmaniose cutânea experimental, com ênfase especial sobre Leishmania *isoladas na América Latina*

Estudaram-se o curso de infecção em "hamsters" e a resposta destes ao tratamento, com 7 isolamentos de leishmaniose cutânea procedentes da América Latina. Comparou-se o tratamento com drogas à base de reductase anti-fólica, pirimetamina, cicloguanil-pamoato e trimetoprim; combinação de pirimetamina e trimetoprim com sulfadiazina foi comparada ao tratamento com estibogluconato de sódio e paromomicina. A resposta desses isolamentos às drogas citadas foi comparada à de dois isolamentos asiáticos de *Leishmania tropica*.

Não se verificou resposta significativa de qualquer dos isolamentos americanos ou asiáticos aos inibidores da reductase fólica ou às suas combinações com sulfonamidas. O estibogluconato de sódio foi altamente eficaz na supressão da infecção, deixando de atuar em um único caso.

O curso da infecção com os isolamentos americanos da *Leishmania* variou de "lento" a "rápido". Isto não se correlacionou com o tipo de crescimento *in vitro* como o postularam LAINSON & SHAW. Entretanto, a atenuação ocasionada pelo cultivo contínuo poderia haver interferido com a interpretação dos resultados quanto ao relacionamento entre virulência e cultivo *in vitro*.

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