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Comparative Experimental Infections with Costa Rican Strains of *Leishmania braziliensis* Vianna, 1911

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Experimental cutaneous infection with *Leishmania braziliensis* has been attempted, to determine the relative susceptibility of animals to the parasite. Dogs, hamsters and mice frequently were used as experimental hosts, injecting them by different routes with various strains. Literature on experimental infections with *L. braziliensis* was reviewed by PÊSSOA & BARRETO (1948). The various aspects of the host-parasite relationships of this leishmania are not well defined. The present paper is intended to clarify some of these aspects with particular emphasis on the comparative virulence of several Costa Rican strains of *L. braziliensis*. Preliminary notes on the subject have been published (ZELEDÓN & BLANCO, 1963; ZELEDÓN & BLANCO, 1965; ZELEDÓN, MONGE & BLANCO, 1965).

1. Experiments

General considerations

The characteristics of strains used in the present experiments are presented in Table 1. All strains were isolated from human cutaneous leishmaniasis in Costa Rica by the authors except strain O-CR, which was isolated by Dr. Alfonso Trejos. All were maintained since isolation in Senekjie's medium (TOBIE & REES, 1948) at room temperature and transferred monthly. A total of 430 adult hamsters of both sexes, weighing between 90 to 130 gm, were employed to study the virulence of the strains plus some particular aspects of the behaviour of strain O-CR. Furthermore sixty adult male mice (C_3H strain) were injected with strain O-CR. The promastigote forms were cultured in Roux bottles in the above-mentioned medium, harvested just before the end of exponential phase of growth and washed once in Locke's solution before inoculation.

Comparative infections

In general the hamsters were divided in groups of five for each experiment (from 3 to 10 in some cases) and injected subcuta-

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TABLE 1

Main characteristics of the strains used for the present work

Strain identification	Date of isolation	Type of lesion	Time of the strain in culture at moment of starting experiments
0-CR	1957	Ulcerous cutaneous (?)	6 years
1-CR	April, 1964	Ulcerous cutaneous	3 months
2-CR	November, 1960	Ulcerous cutaneous	4 years
3-CR	July, 1961	Ulcerous cutaneous	2 years
4-CR	May, 1962	Dry Verrucous	7 months
5-CR	January, 1965	Ulcerous cutaneous	2 months
6-CR	April, 1965	Ulcerous cutaneous	2 months

neously in the nose unless otherwise indicated, with 0.5 ml to 1 ml of inocula. These experiments are summarized in Table 2. The nature of the lesions was confirmed by observing thin smears and, in some cases, paraffin sections.

Some special experiments with strain O-CR

Strain O-CR was chosen to perform a series of other experiments. The route of injection was varied to determine the areas of the skin susceptible to infection. Subcutaneous temperatures of the injected areas were taken from groups of 12 hamsters with an electric thermometer with a needle applicator (Electrolaboratoriet,

TABLE 2

Summary of experiments on comparative infections

No of experiments performed	Strain	Inocula (Promastigotes $\times 10^6$)	No of animals employed	Route
4	0-CR	56–150	25	nose
1	1-CR	80	5	nose
1	1-CR	86	5	foot
2	2-CR	100–383	15	nose
1	2-CR	107	5	foot
1	2-CR	amastigotes	3	nose
4	3-CR	98–150	26	nose
1	3-CR	90	5	foot
1	3-CR	48	5	ear
2	4-CR	50–125	15	nose
1	5-CR	86	5	nose
3	6-CR	71–122	20	nose

Copenhagen). As a temperature difference was observed in two areas 1.5 to 2 cm apart on the hind leg, it was decided to perform two experiments by injecting simultaneously two groups of animals below (naked) and above (haired) the tarso-tibial articulation.

Furthermore, the effect of number of promastigotes was observed in three experiments. All these experiments are summarized in Table 3. Other experiments are described below.

TABLE 3

Summary of some special experiments with strain 0-CR

Name of experiment	Experiment No.	Inocula (Promastigotes $\times 10^6$)	No. of animals employed	Routes
Route of injection	1	180	30	nose, foot, ear, tail, middle dorsum, middle ventral area *
Route of injection	2	250	25	same omitting ear
Route of injection	3	157 to 1350	9	intra peritoneal
Route of injection	4	5.5 to 14.5 amastigotes	12	intra peritoneal
Injection in two areas of hind leg	1	200	10	below and above
	2	20	10	tarso-tibial articulation **
Effect of numbers	1	1–5–25–50	25	nose
Effect of numbers	2	0.02–0.1–0.5–2.5–12.5	25	nose
Effect of numbers	3	0.00001–0.0001–0.001–0.01–0.1	35	nose

* Respective subcutaneous temperatures, plus or minus standard deviation, in same order: 32.6 ± 0.9; 30.0 ± 1.5; 28.4 ± 0.7; 30.8 ± 0.9; 36.6 ± 0.6; 36.7 ± 0.7°C. Room temperature: 24.0°C.

** Subcutaneous temperatures, same order: 24.4 ± 0.6 and 34.9 ± 0.9°C. Room temperature: 20.5°C.

Effect of the age of the promastigotes on infectivity

Strain 3-CR was chosen for these experiments. In the first experiment 30 females in groups of 5 were injected with culture forms of 5, 6, 7, 8, 9 and 10 days of age (peak of the growth curve at the 9th day). The inocula varied between 120 to 175×10^6 promastigotes in the nose per animal. In a second experiment three groups of 5 females each were injected with 109 to 124×10^6 promastigotes from cultures of 5, 6 and 8 days of age, also in the nose.

Effect of fasting of promastigotes on infectivity

Two experiments were set up to show the effect of fasting on the infectivity of promastigotes (O-CR). In the first experiment, 35 females in groups of 5 were injected each subcutaneously in the nose with 127×10^6 flagellates suspended in Krebs-Ringer-Phosphate (pH 7.2), and which had been previously subjected to a conventional experiment in a Warburg apparatus at 30°C (ZELEDÓN, 1960). The groups were injected at the end of the first and succeeding hours, to and including the 6th. The last group of five animals was inoculated with a suspension to which a glucose solution (final concentration 0.01 M) had been tipped from the lateral arm of the flask at the end of the fifth hour. Respiratory measurements were also noted at the end of each hour. Nitrogen content of the organisms was determined by LANG's method (1958). The second experiment was similar except that the flagellates had been starved an additional 15 hours at 5°C. The animals (25 males in groups of five) were injected after the 1st, 2nd, 3rd and 5th hours, and the last group (control) was injected with flagellates in the presence of glucose after the 3rd hour of the Warburg experiment.

Reinoculation of animals

Two types of experiments were performed in order to test the hypothesis that a previous injection of living promastigotes will confer immunity to an animal for a challenging dose. The methods for these experiments are summarized in Table 4.

TABLE 4

Reincoculation of hamsters with strain 0-CR in the nose

Name of Previous Experiment	Experiment No.	Strain used previously and inoculum (flagellates $\times 10^6$)	Challenging inoculum (flagellates $\times 10^6$)	Time elicited after first injection (months)	No. of animals tested	No. of control animals
Route of injection (ventral and dorsal routes)	1	0-CR (250)	98	7	10	5
Route of injection (ventral and dorsal routes)	2	0-CR (180)	98	4	10	5
Effect of the age	1	3-CR (120–175)	127	$3^1/_2$	10	10

Liver and spleen search for leishmaniae

A total of 195 of the above hamsters were killed at different intervals after having been inoculated in the nose with strain O-CR in order to demonstrate visceral infection by culture and direct microscopic observations of the organs. The same type of investigation was done in 10 animals inoculated with strain 4-CR (11 to 15 months later); 3 animals of strain 3-CR (after 3 to 13 months); 2 animals of strain 6-CR (after 3 months) and 1 animal of strain 2-CR (after 13 months). Half of the spleen and about a half of the left lobule of the liver were triturated separately in mortars with 2.5 ml of physiological saline containing 500 U of Penicillin and 0.625 mg/ml of Streptomycin. From 0.15 to 0.2 ml of each suspension was inoculated into each of 5 tubes with Senekjie's medium. The cultures were observed for 3 to 4 weeks. Thin smears were made from both organs in all animals and stained with Giemsa; paraffin sections were also made in 56 animals of strain O-CR and stained with Hematoxylin-Eosin. In two instances, a total of six hamsters were inoculated in the nose with strain O-CR either with liver or spleen macerate from 2 hamsters in which smears and cultures were positive.

Infections in mice

The infectivity of strain O-CR was also tested in C_3H mice. Five experiments were performed using groups of 5, 10 or 20 males weighing from 22 to 27 gm. In two instances the animals were injected with culture forms and in three with tissue forms from hamsters. The number of promastigotes employed were: 40×10^6 (base of the tail) and 300×10^6 (nose). The number of amastigotes were: 6×10^6 (i.p.) and 4×10^6 (nose and base of tail).

2. Results

Comparative infections and characteristics of strains

Strain O-CR exhibited high virulence in all cases through the entire period of the experiments on which this strain was used (approximately 4 years). Nodules appeared two or three weeks later at the site of the injection with rapid increase in size, ulceration or production of crust, with no tendency to heal or fibrose (Figs. 1, 2, 3, 12, 13, 14, and 15). Metastases in the fore and hind feet and tail, and in a few cases in the lips, eyelids and perianal regions, were commonly observed, 4 to 14 months later (Fig. 7 and

16). In one case histological sections showed invasion of the mucosa of the nose cartilage. No difference in susceptibility of sexes to this strain was observed. Strain 1-CR showed low virulence producing small, but well defined, nodules on the nose from two weeks to one month after injection, with a tendency toward healing or chronicity with extensive fibrosis after 3 months. It was more difficult to produce lesions in the foot with this strain, and only one animal showed a nodule that persisted for 4 months when the animal died of unknown cause. Strain 2-CR produced very small chronic nodules in 3 animals out of 15 after a long incubation period of 9 months or more (Fig. 6); early mortality was high among these animals. Of the 5 animals injected in the foot, 3 showed small lesions, 9 to 11 months later, with no tendency to grow. The three males infected with tissue forms showed similar characteristics, but a shorter period of incubation (2 months). Strain 3-CR exhibited low virulence producing small nodules in a few animals after a year or more with a tendency to disappear, although in 5 hamsters the small nodules persisted until they died (16 to 24 months). It was not possible to infect any animal with this strain in the foot or ear (16 months of observation). Strain 4-CR was able to infect hamsters producing evident lesions with a marked tendency to become chronic after 4 or more months of infection (Fig. 4). With time these lesions became fibrotic with extensive invasion of other areas of the face, including the area around the eyes, which appeared as healed areas without hair.

In one case the lesion exposed the nasal septum (Fig. 5), as also occurred in one animal infected with strain O-CR, and in 2 animals metastases to the fore and hind feet were observed after 8 months. With strain 5-CR the animals presented small but defined nodules after one month with a tendency to decrease and heal after the third month. Strain 6-CR produced similar lesions to those produced by strain O-CR, but with a tendency to become smaller after a few months and finally become chronic, producing extensive fibrosis. One animal of the first experiment showed a metastasis to the left hind foot 14 months after injection. The 3rd experiment with this strain, performed 10 months after the first, showed a tendency of the strain to lose virulence since the lesions were smaller and with an increased tendency toward spontaneous cure in some animals. Histological sections of lesions produced by strain O-CR showed extensive areas of vacuolated histiocytes, full of parasites (histiocytoma) (Fig. 18).

In other areas, groups of plasma cells and lymphocytes, sometimes surrounding histiocytes, or as a continuous exudate, were observed. In sections of nodules produced by strain 2-CR, no

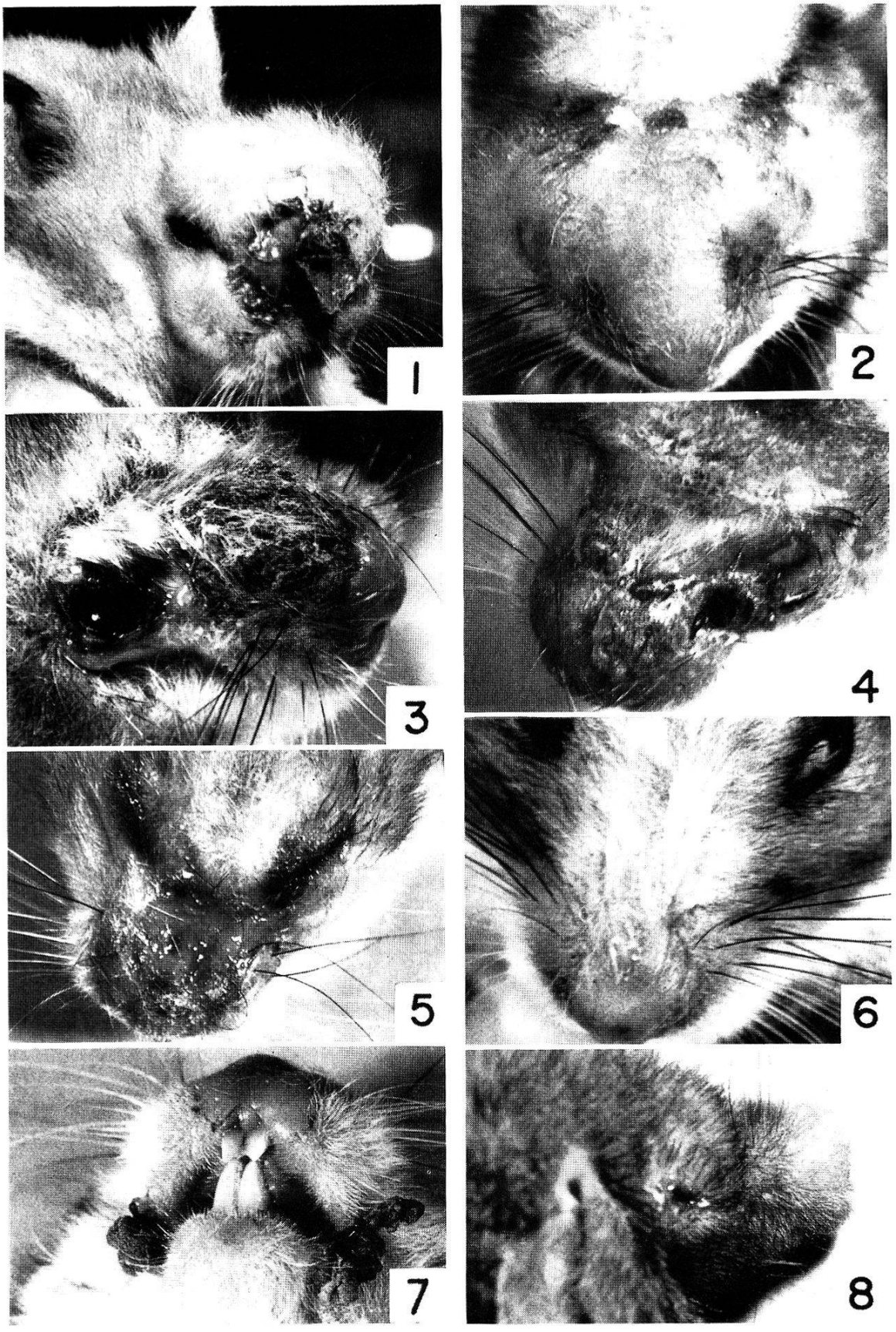

Fig. 1. Hamster with a tumor type lesion of 2 months duration with ulceration on one side produced after subcutaneous injection in the nose of 150×10^6 promastigotes of strain O-CR.

Fig. 2. Tumor type of close lesion in hamster produced by strain O-CR after 14 months of subcutaneous inoculation of 360×10^6 promastigotes in the nose.

vacuolated histiocytes were seen and the reaction was more of the tuberculoid type and parasites were scarce.

Route of injection

All the hamsters injected with O-CR showed lesions on the nose, foot, ear and tail. However, the animals could not be infected in the dorsal or ventral areas. The animals injected i.p. with culture forms presented nasal nodules starting 2 to 7 months after the injection. Some of these lesions metastazied after several months. In one case a slight periorchitis was observed after 6 months, which progressed no further. On the other hand, the animals injected with tissue forms developed lesions in several areas with well marked periorchitis 3 or 4 months after injection, resulting in considerable scrotal edema with or without ulceration and necrosis of the scrotum (Figs. 9 and 11) with a typical microscopic picture of histiocytes full of parasites. Approximately at the same time, nodules in the feet were observed and in some cases lesions in the tail, preputial and perianal regions. The subcutaneous scrotal temperature was 34.7 ± 0.7 at 22.5°C.

Injection in two places of the hind leg

In the first experiment, lesions were evident one month after injection in the five animals injected in the foot below the articulation (Fig. 13). One animal, in the group injected above the articulation, showed a small initial lesion after a similar period. Three months later, 4 animals of the latter group showed lesions (Fig. 15), and one of them cured spontaneously sometime later. In the second experiment, with a much lower inoculum, the lesions were evident only in two animals of the group injected below the articulation, 2 months after the injection; one month later, continuous and progressive lesions were evident in 4 of these animals (the fifth one

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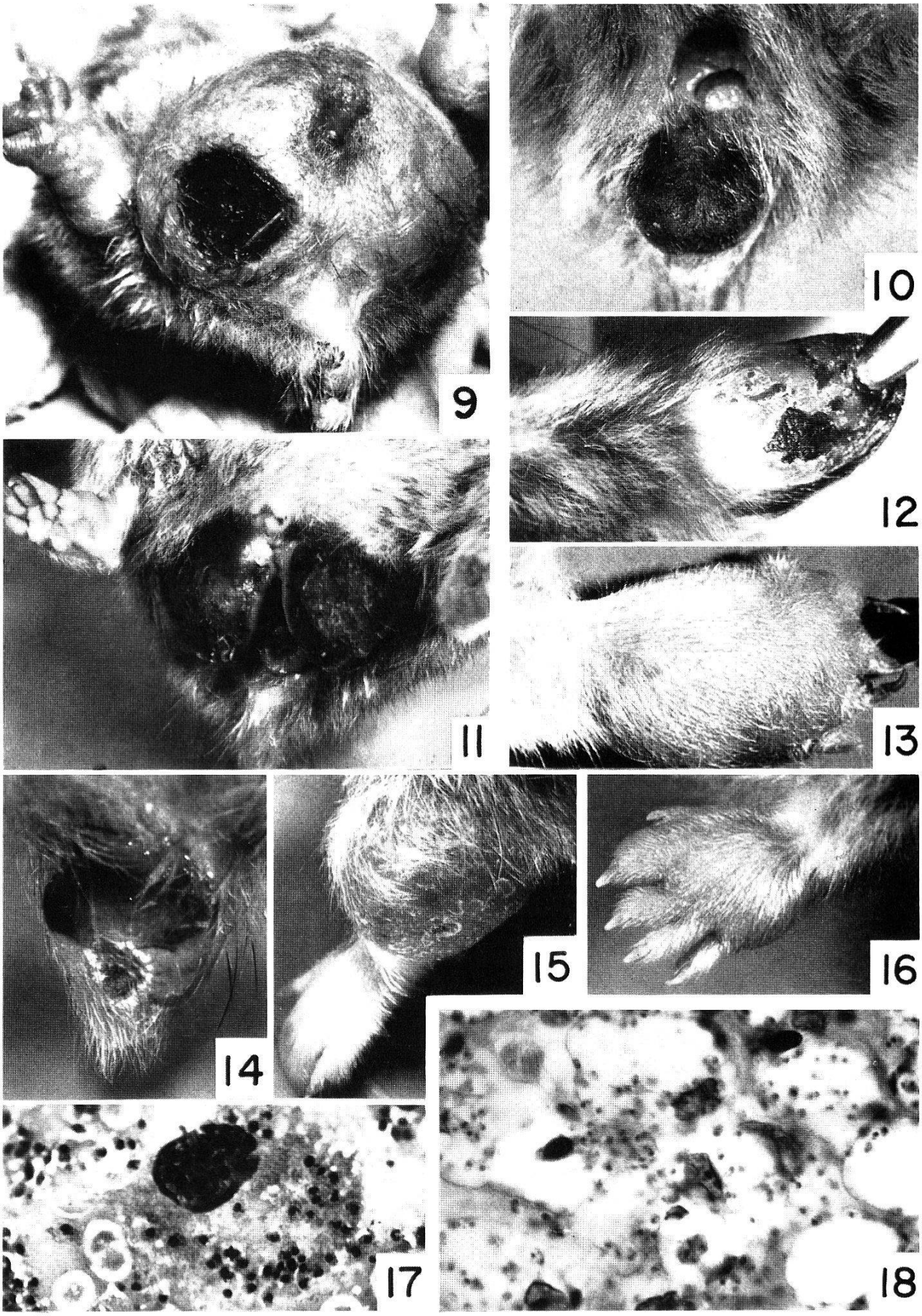
Fig. 3. Lesion with crust in hamster produced by strain O-CR, after 14 months of subcutaneous inoculation in the nose with 360×10^6 promastigotes.
Fig. 4. Dry type of lesion in hamster produced by strain 4-CR after 13 months of the injection of 125×10^6 promastigotes in the nose.
Fig. 5. Ulcerated lesion of the nose of a hamster with exposure of the nasal septum produced by strain 4-CR after 12 months of local inoculation with 125×10^6 promastigotes.
Fig. 6. Small and discrete lesion produced by strain 2-CR in a hamster 12 months after subcutaneous inoculation in the nose with 383×10^6 promastigotes.
Fig. 7. Curious pedunculated hyperkeratosic metastatic lesions of the mouth produced by strain O-CR. The lesions appeared 12 months after the injection of 360×10^6 promastigotes in the nose.
Fig. 8. Tumor type lesion of the nose in a mouse after 19 months of subcutaneous injection of 5.8×10^6 amastigotes of strain O-CR from hamster.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died after 6 weeks without showing a lesion). Of the animals inoculated above the articulation only one showed a small lesion after 3 months and was cured by the end of the 13th month. Between the 6th and the 7th month, 3 animals of this group died without showing an infection. A second hamster showed a lesion after 7 and $^1/_2$ months, but it was healed by the 9th month.

Effect of number of promastigotes on infection

There was good correlation between the number of flagellates and the time of appearance of lesions. The smaller the number of promastigotes, the longer the period of incubation. In the first and second experiments the incubation period varied between 1 and 5 months (first hamster to show an initial lesion). In the 3rd experiment the prepatent period varied between $1^1/_2$ and $16^1/_2$ months. With the smallest inoculum (10 promastigotes) only a very small nodule, which did not grow, appeared in one hamster, while with 10^2 promastigotes only two animals showed lesions (small nodules) that persisted. With 10^3 promastigotes 4 animals became infected, indicating that between 100 and 1000 flagellates would be necessary to infect a hamster with this strain when injected subcutaneously in the nose. Figure 19 presents these results and shows clearly that the prepatent period, based on the first animal of each group to show a lesion, is inversely proportional to the logarithm of the number of promastigotes injected. Nevertheless, there was a wide variation in the incubation periods among the individuals of the groups.

Fig. 9. Large scrotal lesion in the hamster, with ulceration, after 7 months of the injection of 14.5×10^6 amastigotes i.p. of strain O-CR. The feet and tail also show lesions.

Fig. 10. Lesions of the scrotum, prepuce and penis in a mouse after 19 months of inoculation with 5.8×10^6 amastigotes i.p. of strain O-CR.

Fig. 11. Scrotum of hamster with necrosis produced 5 months after injection with 5.8×10^6 amastigotes of strain O-CR.

Fig. 12. Lesion of the ear after 4 months of local inoculation of 100×10^6 promastigotes of strain O-CR.

Fig. 13. Tumoral lesion of the leg observed after 14 months of injection of 115×10^6 promastigotes of strain O-CR.

Fig. 14. Ulcerated lesion of the tail after 3 months of local inoculation of 180×10^6 promastigotes of strain O-CR.

Fig. 15. Large lesion of $7^1/_2$ months produced above the tibio-tarsal articulation with high inoculum (200×10^6 promastigotes) with strain O-CR.

Fig. 16. Hamster with metastatic lesion of the foot after 6 months of nasal infection with strain O-CR.

Fig. 17. Smear of nasal lesion from hamster, produced by strain O-CR, after Giemsa staining, 880 ×.

Fig. 18. Histological section showing characteristic appearance of nasal lesion from hamster, produced by strain O-CR, 880 ×.

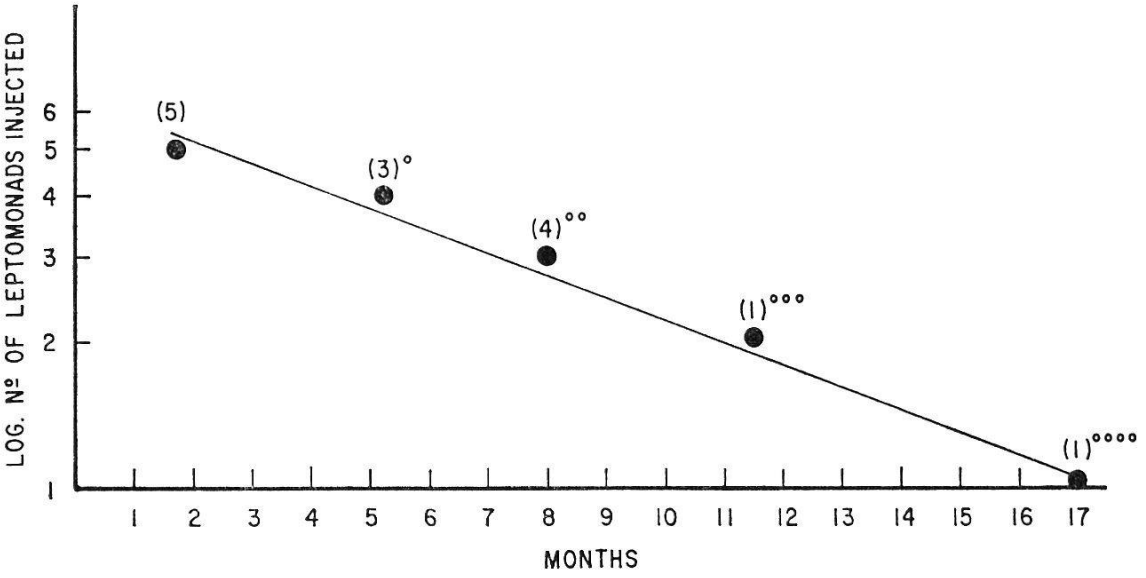

Fig. 19. Time of the first hamster of each group to initiate lesions against the logarithm 10 of the inoculum. In parenthesis number of animals of each group that became infected. °: In 2 animals there was a regression of lesions at month 17th. °°: In 1 animal there was a regression of lesion at month 17th. °°°: Died at the 17th month with small lesion. °°°°: Died at the 22nd month with very small nodule.

Effect of the age of the culture on infectivity

The age of the culture in strain 3-CR had no effect on the low virulence pattern of this strain. In the two experiments performed, only a few animals of different groups showed small nodules after several months of infection, independent of the age of the promastigotes.

Effect of fasting of promastigotes on infectivity

In the first experiment the virulence of the promastigotes suffered no change, and all groups showed lesions at the same time and with the same intensity. Respiration dropped from 118 μl O_2/mg N/hour at the end of the first hour to 72 μl at the end of the sixth. In the second experiment, the animals injected after the first and second hour and the control group with glucose, showed lesions 6 weeks later, while in the animals injected after four and five hours the lesions started only after 3 months. Respiratory rates were respectively: 21, 7, 4, 5 and 10 μl O_2/mg N/hour for the control.

Reinoculation of animals

The groups of animals injected previously in the dorsal and ventral areas and challenged with the homologous strains presented lesions without difficulty. Nevertheless, the animals injected 7 months after the first inoculum produced lesions with longer

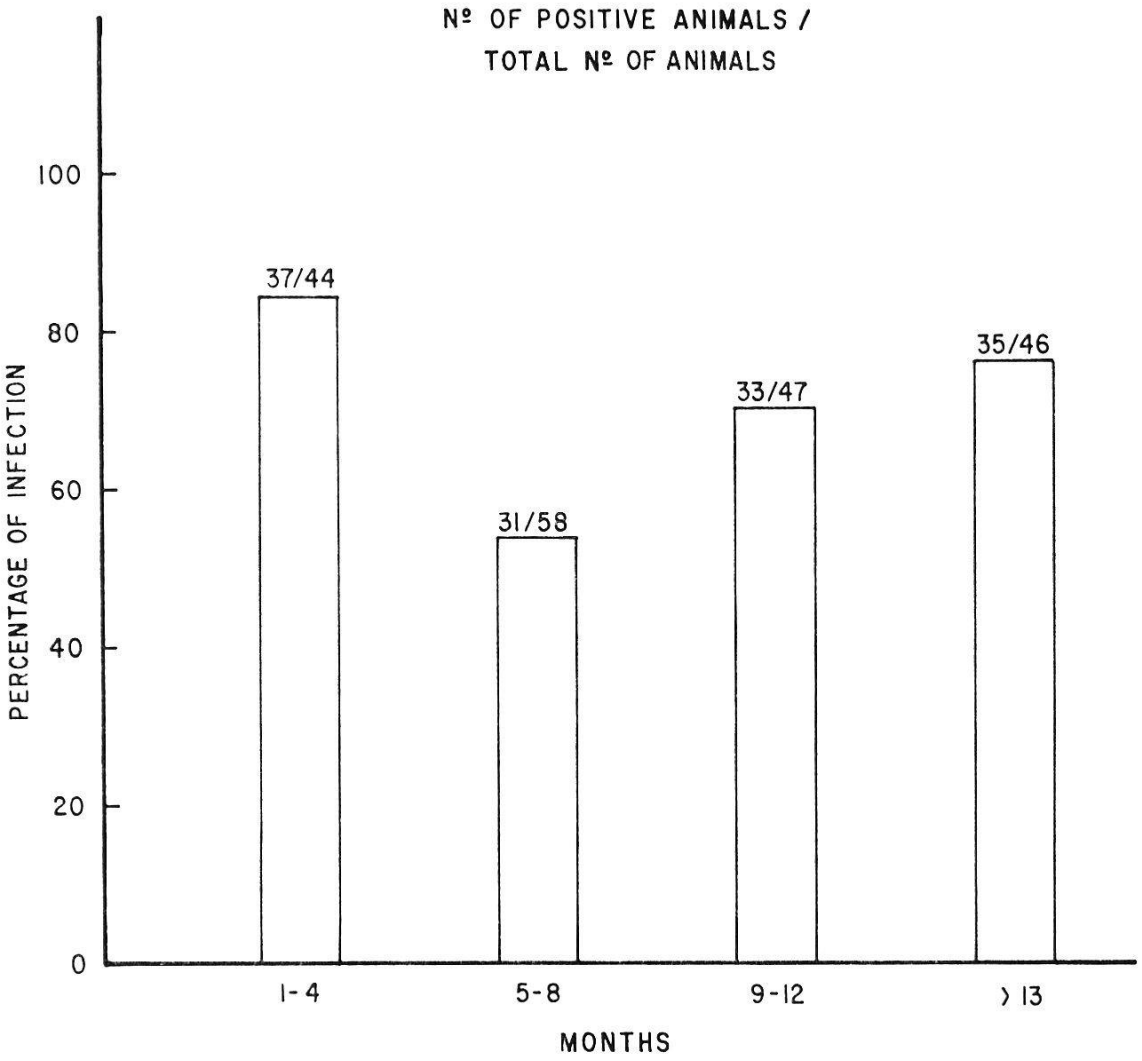

Fig. 20. Graphical representation of the number of animals with positive cultures of liver and/or spleen during the course of the infection.

incubation periods than the controls, and 3 of the group injected in the dorsal region and 1 of the group injected in the ventral areas showed no lesions. In animals reinoculated with an heterologous strain (previously injected with 3-CR), longer incubation periods were also observed in relation to the control group, and one hamster of the reinoculated group never showed an infection with strain O-CR.

Liver and spleen search for leishmaniae

Results of the cultures of liver and spleen are summarized in Table 5 and 6. In no case were the differences observed between the sexes statistically significant. In the majority of the cases the culture tubes were positive the first or second week after inoculation. Fifty-two animals showed amastigotes in thin smears and/or tissue sections and, with the exception of 3 animals on which parasites were easily found, only a few leishmaniae were seen. At times

TABLE 5

Results of the cultures of liver and spleen of hamsters infected subcutaneously in the nose with promastigotes of strain 0-CR

Sex	No of animals	Total of positives	%	No of positives only in spleen	%	No of positives only in liver	%	No of positives in spleen and liver at the same time	%
Males	119	85	71.4	16 *	13.5	13 *	10.9	56	47.0
Females	76	51	67.1	17	22.4	9	11.8	25	32.9
Total	195	136	69.7	33	16.9	22	11.3	81	41.5

* Cultures contaminated in one animal.

TABLE 6

Results of cultures of liver and spleen of hamsters infected subcutaneously in the nose with promastigotes of strain 0-CR in relation to time of infection

Time of infection	No of animals positive / Total No animals	% positives	% positives only in spleen	% positives only in liver	% positives in spleen and liver
1–2 months	15/18	83.3	16.7	27.8	38.9
3–4 months	22/26	84.6	3.8	23.1	57.7
5–6 months	16/30	53.3	20.0	3.3	30.0
7–8 months	15/28	53.6	21.4	7.1	25.0
9–10 months	20/28	71.4	17.9	7.1	46.4
11–12 months	13/19	68.4	26.3	5.3	36.8
13–14 months	8/11	72.7	9.1	9.1	54.5
15–16 months	19/21	90.4	19.0	19.0	52.4
17–18 months	8/11	72.7	18.2	0.0	54.5

degenerate amastigotes or parasites with picnotic nuclei were observed. In 10 animals the smears were positive and the cultures were negative indicating that the forms were dead; in 39 animals the smears were positive as were the cultures; in 97 animals only the culture was positive and in 49, both culture and smears were negative. Fifty-two of the 56 animals histologically examined were positive by the culture method. In 25 of these animals amastigotes were found in smears, while in 8, parasites were also found in reticuloendothelial cells in tissue sections (in 3 of these there was no correlation with the smear). The percentage of animals with visceral involvement during different periods of time after infection as revealed by the culture method, is presented in Figure 20. The animals inoculated with liver and/or spleen macerates in the

nose showed typical lesions 2 to 4 weeks later. The cultures from animals inoculated with strain 3-CR, 6-CR and 2-CR were negative in spite of the fact that in two cases (one for strain 3-CR and one for 2-CR) a few degenerate parasites were observed in smears. With strain 4-CR only in one case the culture method was positive and in another the smear was positive but the culture was negative.

Infection in mice

It was more difficult to infect mice than hamsters, and the incubation periods were longer. The 10 mice injected in the nose with promastigotes did not show clear nodules up to 19 months of observation while 5 of the 10 animals injected in the base of the tail showed nodules starting at the 10th month persisting up to the 15th month after which they died accidentally.

The mice injected in the nose with tissue forms showed nodules (Fig. 8) with large numbers of amastigotes between 8 to 10 months, whereas parasites were found after 12 months in the mice injected with tissue forms in the base of the tail. In some of these animals the macroscopic lesions were discrete, consisting of an infiltrated, depigmented area. In 3 of these animals, thin smears showed parasites. Three of the 10 animals injected i.p. showed periorchitis 11 months after injection, and in one of these the scrotum became ulcerated while another animal also presented lesions in the nose, right foot, prepuce and penis (Fig. 10). In some of the animals injected with tissue forms, either in the nose or i.p., smears of liver and spleen showed few amastigotes, and the parasites were recovered in a few instances by the culture method from these organs. In the lesions of mice it was common to observe a real 'carpet' of parasites in extensive areas of necrosis with rather poor inflammatory reaction.

3. Discussion

It can be inferred that in general there is an inverse correlation between the time the strain has been in culture and the infectivity of the strain. Strains recently isolated will infect hamsters more easily with shorter incubation periods. CUNHA (1944) has pointed out that only cultures of *L. braziliensis* recently isolated (no more than 4 months) will infect susceptible animals. In our case one exception to this rule was evident since strain O-CR, in spite of being old, maintains a high infectivity and virulence, in fact, the highest of all strains tested. Strain O-CR also showed a tendency

to disseminate and invade other areas of the animals and this was observed to lesser degree with strains 4-CR and 6-CR. Unfortunately it was not possible to trace the exact type of clinical case from which strain O-CR was isolated since the record has been lost, but we have reasons to believe that its origin was from an ulcerative cutaneous case, the most common type of the disease in Costa Rica.

The choice of many authors to inject hamsters and other animals in the tip of the nose seems to be justified since this is one of the most susceptible areas of the skin. In our experiments it was possible to infect animals with a low virulence strain (3-CR) only in the nose. Furthermore, in the i.p. inoculation with culture forms the lesions appeared first in the nasal regions. The areas of the skin susceptible to an infection can be well correlated with temperature. Those places with subcutaneous temperature around 30°C can be infected easily with a virulent strain. Those areas covered by hair, several degrees higher, are more difficult or impossible to infect. This phenomenon is well illustrated when the injection is made in two contiguous areas of the leg with different temperatures. With larger inocula, an infection in the warmer zone can be forced out, after longer periods of incubation, even though there is a marked tendency to spontaneous cure after sometime. With smaller inocula this difference becomes more clear cut since an infection is produced, much more easily, or only, on the cooler zone. Metastases to the same cooler areas of the body of the animal including the ears have been observed by other workers (GUIMARÃES, 1951 c; LAINSON, 1965; COELHO & COUTINHO-ABATH, 1965). In our experiments we attribute the absence of metastases to the ear to poor irrigation of the same, particularly under the conditions of our laboratory with cooler weather around the year. REY (1943) made histological studies of the injection sites in hamsters inoculated in the anterior abdominal wall with a Peruvian strain. REY attributed the failure to produce infection to digestion of the parasites by macrophages, and to local production of an hypothetical parasiticidal substance. Unfortunately, REY did not inject hamsters in other areas of the skin with lower temperature, since the comparison of these sections could have shed light on the role of this factor in the infection process by the promastigotes. This relationship of skin temperature and leishmania infection has also been studied in the guinea pig leishmania *(L. enriettii)* by PEREIRA et al. (1958) and by CASTRO (1960), who were unable to infect animals at sites where the subcutaneous temperature was above 36°C. Furthermore, CASTRO & PINTO (1960) have demonstrated that *L. enriettii* would not grow at 37°C in tissue culture, whereas it does grow at 32–34°C. Similar findings have been obtained in our laboratory with the O-CR strain

(ZELEDÓN & MONGE, unpublished). GREENBLATT & GLASER (1965) have shown that there is some sort of cell damage when *L. enriettii* is brought to 37°C, as indicated by the leakage of several important elements. Another observation which indicates that temperature is probably the most important element in the invasion of a skin site by leishmaniae is the sensitivity of the parasite to heat treatment. Healing or regression of lesions, or even complete disappearance, has been accomplished at 37°C by PEREIRA et al. (1958) in guinea pig leishmaniasis, and by ZELEDÓN, MONGE & BLANCO (1965) in hamsters infected with *L. braziliensis*.

The use of amastigotes as an inoculum permits the parasite to establish itself better at intermediate or relatively high temperatures, such as those occurring in the scrotum or internal organs. The type of lesion described here in hamsters injected i.p. with amastigotes as a periorchitis corresponds in its pathology to the lesions described in greater detail by TORRES et al. (1948) in male guinea pig leishmaniasis, and by GUIMARÃES (1951 d) in hamsters infected with *L. braziliensis*.

It has been shown that with small inocula, even with a highly infective strain like O-CR, the incubation period is prolonged for several months. Whether the flagellates can increase their virulence during their phase in the *Phlebotomus* thus shortening the incubation periods, requires further study. Quantitative studies on infections with *Phlebotomus* forms are lacking.

The hypothesis that physiological changes occurring in the flagellates during the growth in culture could increase the infectivity, was not confirmed with strain 3-CR. One interesting finding was that fasting promastigotes will show a decreased infectivity that is not only explained by a lowering of metabolic rate, as indicated by oxygen uptake, but, probably, also by the depletion of certain substances from the cells not used in respiration and necessary to start multiplication and invasion. These points have been discussed further by ZELEDÓN & MONGE (1967).

Reinoculation of animals previously injected in warmer areas of the skin showed that they were susceptible to the infection with some degree of acquired immunity. Vaccination with *L. braziliensis* has been done before with different degrees of success (cf. LAINSON & BRAY, 1966).

As far as the internal dissemination of the parasites is concerned, it now seems clear that the deposition of amastigotes in different tissues is probably a constant fact starting at an early phase of infection. The blood stream may take parasites to different territories (PARAENSE, 1953) and this dissemination easily can be demonstrated by direct culture of the blood mainly during the

period of metastasis (COELHO & COUTINHO-ABATH, 1965). The parasites may multiply in several places according to certain factors. We believe that temperature is the most important barrier that will slow or prevent proliferation in certain sites of the skin or internal organs like liver and spleen. Amastigotes probably remain alive in unfavourable sites for a certain period of time, after which they begin to degenerate. These parasites are responsible for the positive cultures or positive infections with materials from liver and/or spleen. It is possible that different strains may differ in their tolerance of unfavourable conditions occurring in the internal organs. Also factors inherent to the host and to the duration of the infection will influence this interesting aspect of the host-parasite relationships. PARAENSE (1953) observed that *L. enriettii* forms that entered the internal organs of guinea pigs were quickly destroyed, and the few parasites observed in liver and spleen showed degenerative changes. Further support to our ideas is given by the fact that visceral involvement seems to be more common and heavier when the infection is started with amastigotes (MUNIZ, 1953; COELHO & COUTINHO-ABATH, 1965).

The patterns observed in experimental kala-azar of the hamster (we had some animals infected with the Khartoum strain of *L. donovani* for comparisons) were never seen by us during *L. braziliensis* infections. Light infections in the viscera of mice infected with *L. braziliensis* were first pointed out by FONSECA (1929) and again by GEIMAN (1940). GUIMARÃES (1951 a, 1951 b) working with Brazilian strains of *L. braziliensis* from cases of 'spundia' was able to produce a generalized acute leishmaniasis of the kala-azar type in hamsters and mice, by using amastigotes from patients or hamsters. The picture could not be produced with culture forms, and in one experiment only a few leishmania were found in the spleen of hamsters which were inoculated with material from recently isolated cultures (less than a month). Both arguments that the cases came from an area free of kala-azar and that mucosal lesions had not been observed in kala-azar can be questioned. On the other hand, GUIMARÃES (1951 c, 1951 d) observed a different behaviour with a strain from a human case of nodular leishmaniasis (diffusa form). With this strain he was able to produce very similar infections to those reported in this paper with the O-CR strain including the periorchitis (vaginalitis) when the animals were injected i.p. with amastigotes. Parasites in the internal viscera were scarce. It is not clear at present if the so-called lepromatous or diffusa type of leishmaniasis is produced, as a modified picture by *L. donovani* (SEN GUPTA, 1962) or if it is produced by a different parasite that deserves another specific name (MEDINA & ROMERO, 1962). The

clinical picture has been observed in South America and Africa (literature in GUIMARÃES, 1965), and it could possibly be produced by any of the human agents of leishmaniasis under special circumstances of the host-parasite relationships. More recently, visceral invasion of animals infected with a strain considered as *L. mexicana* have been noted by LAINSON (1965) and by COELHO & COUTINO-ABATH (1965).

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Zusammenfassung

Es wurden vergleichende Untersuchungen über die Infektion von 430 Goldhamstern mit sieben in Kultur gehaltenen Costa Rica-Stämmen von *L. braziliensis* durchgeführt. Einige andere Aspekte des Wirt-Parasit-Verhältnisses wurden beim virulentesten Stamm (O-CR) untersucht. Dieser Stamm verursachte in verschiedenen Teilen des Hamsterkörpers die Bildung großer Knoten, welche keine Tendenz zur Rückbildung zeigten; sie wurden entweder durch direkte Inoculation von Promastigoten oder durch Metastasen hervorgerufen. Die Verteilung der induzierten Läsionen und Metastasen konnte zur Hauttemperatur des betreffenden Tieres in Beziehung gesetzt werden. Andere Stämme zeigten eine geringe Virulenz, mit Ausnahme von 6-CR und 4-CR, welche stark infektiös wirkten. Die i.p. Injektion von O-DR-Mastigoten verursachte eine typische Periorchitis, welche in Geschwürbildung am Scrotum überging. Es wurde gezeigt, daß die Inkubationszeit (bestimmt durch das erste Tier, welches eine Läsion aufwies) umgekehrt proportional ist zum Logarithmus der Anzahl injizierter Promastigoten. Vorhergehende Inoculation in nicht empfängliche Regionen konnte die Inkubationszeit verlängern; bei einigen Tieren verhinderte sie die Infektion.

Promastigoten, welchen während einiger Stunden die Nahrung entzogen worden war, zeigten verminderte Infektionsfähigkeit. Positive Kulturen konnten vom ersten Monat nach der Infektion an aus Leber und/oder Milz gewonnen werden, aber i. a. wurden sehr wenig Parasiten in diesen Organen gesehen. Die Infektion von Mäusen mit dem Stamm O-CR war schwieriger; die Inkubationszeit dauerte länger und die Läsionen blieben undeutlich. Im allgemeinen bestanden die Läsionen mikroskopisch aus vakuolisierten, mit Parasiten gefüllten Histiocyten, oft durchmischt mit Exsudat von Lymphocyten oder Plasmazellen.

Résumé

Des recherches portant sur 430 hamsters ont été faites avec 7 souches de *L. braziliensis,* originaires de Costa Rica, et maintenues en culture au laboratoire. On a étudié également quelques aspects des relations entre hôtes et parasites en utilisant la souche la plus virulente (O-CR). Cette souche provoqua la formation de gros nodules dans différentes régions du corps du hamster. Ces nodules ne montrèrent aucune tendance de résorption. Leur formation fut obtenue soit par inoculation directe de formes promastigotes, soit par métastases. On put mettre en évidence une relation entre, d'une part la distribution des lésions induites et des métastases, et d'autre part la température de la peau de l'hôte en question. D'autres souches montrèrent une virulence moindre, à l'exception des souches très infectieuses 6-CR et 4-CR. L'injection de formes mastigotes O-DR causa une périorchite typique qui évolua en un ulcère du scrotum. On montra que le temps d'incubation (défini à l'aide du premier animal qui révéla une lésion) est inversement proportionnel au logarithme du nombre des formes promastigotes injectées. Des inoculations préalables, dans des régions non réceptives, augmentèrent le temps d'incubation. Des formes promastigotes ayant jeûné quelques heures avant l'injection, révélèrent une infectiosité moindre. On obtint des cultures positives en prélevant du foie et de la rate, dès le premier mois après l'infection. Peu de parasites furent cependant observés dans ces organes. L'infection de souris avec la souche D-CR fut plus difficile ; le temps d'incubation dura plus longtemps et les lésions restèrent insignifiantes. En général, l'étude microscopique des lésions révéla des histiocytes vacuolarisés, bourrés de parasites souvent mélangés à un exsudat de lymphocytes et de plasmocytes.