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PATHOGENICITY TO MICE OF SOME STRAINS OF *TRYPANOSOMA CRUZI* ISOLATED FROM WILD ANIMALS OF BRAZIL

Maria Paumgartten DEANE, Thales de BRITO and Leonidas M. DEANE

SUMMARY

The Authors compare the pathogenicity and histotropism of four strains of *Trypanosoma cruzi* from wild mammals of Brazil, in mice inoculated intraperitoneally and intragastrically with cultures.

The strains were isolated from a wild rat (*Nectomys squamipes*), a squirrel-monkey (*Saimiri sciureus*), an opossum (*Didelphis marsupialis*) and a bat (*Eumops abrasus*). The first three were obtained in an area where no human cases of Chagas' disease have been recorded and belong to immunological type B, of NUSSENZWEIG et al., while the fourth came from an endemic area of the disease and belongs to immunological type A (which includes virulent human strains).

A definite difference in virulence was observed between the strains, but with no correlation with the immunological types or geographical areas. All strains produced more severe infections by intraperitoneal inoculation than by the gastric route. No differences were noted as to histotropism and type of lesions determined by the various strains.

INTRODUCTION

Among the *cruzi*-like trypanosomes isolated by one of us (L.M. DEANE) from wild animals of Brazil and others obtained from various Brazilian sources, differences in virulence for laboratory mice go from the apparently total lack of infectivity of some of the bat strains, to the very high virulence of the "Y" human strain. This has been learned through the study of some strains [4, 6, 16, 20] (L. M. DEANE, unpublished data) and the routine efforts to passage all of them in mice for isolation and maintainance. It has been also observed that, despite some overlapping variation, the strains from wild animals usually produce milder infections than those isolated from human cases of Chagas' disease. However, the pathogenicity of those "wild" trypanosomes had not been studied before on a comparative basis.

The presence of distinct antigenic fractions among several of our strains of *Trypanosoma cruzi* was recently demonstrated through agglutination, electrophoretic and gel precipitation techniques [13, 14] (NUSSENZWEIG, personal communication). The strains were, accordingly, grouped in immunological "types": those of a domestic origin (human and domestic triatoma bugs) and — curiously enough — two isolated from bats (of two species) belonged to antigenic type A, while all the others, isolated from 7 species of wild animals, belonged to types B or C. A comparison of the degree of parasitemia and cross immunity produced by some strains of types A and B was also made [15]. It was not possible to clearly separate these types by means of protection tests but, here again, the "wild" strains were less virulent, producing patent parasitemia in only 10% of the mice during the immunizing inoculations, while with the human strains a patent infection was observed in 76% of the animals. The inoculum consisted of cultural forms in all cases.

Faculdade de Medicina da Universidade de São Paulo — Instituto de Medicina Tropical — Departamento de Parasitologia (Prof. A. D. F. Amaral).

Due to the above observations and others related to the epidemiology and pathology of *T. cruzi* infections, the differences in pathogenicity of various wild strains seemed worth of more investigation. The work here described represents a trial to compare them not only on the basis of parasitism, but also as to histotropism and possible adaptations to unusual transmission mechanisms.

MATERIAL AND METHODS

Four strains of *T. cruzi* were used:

1) 8717, from the wild rat *Nectomys squamipes;*
2) 3014, from the squirrel-monkey *Saimiri sciureus sciureus;*
3) 8857, from the opossum *Didelphis marsupialis marsupialis* and
4) M, from the bat *Eumops abrasus abrasus.*

The original hosts of the first three strains were captured in the Amazon Region (State of Pará), while the bat that furnished the M strain was caught in Ribeirão Prêto (State of São Paulo).

Since some of the strains were known to produce only light and transient parasitemias on which we could not depend for a regular supply of blood trypanosomes, cultures were used for the inoculations. Except for strain 3014, which had been in culture for only 5 months, the others had been maintained on blood-agar media for 4 years. The first isolation in culture was made with blood of the host or of mice infected after 1 passage in other mice or in triatomid bugs.

For each animal the inoculum was 1 ml of a rich suspension (in saline) of flagellates taken from 10-15 day old subcultures selected before use on the basis of abundance of active metacyclic trypanosomes.

The animals used were white mice from the colony maintained at the University of São Paulo Medical School, and known to be very susceptible to various strains of *T. cruzi.* Twelve mice, all females, weighing about 20 g each, were used for each trypanosome strain: half of them were inoculated through the peritoneum, the other half by the intragastric route with the help of a plastic tube.

Diet and ambient temperature and humidity (around 25°C and 80-90%, respectively) were the same for all mice throughout the experiments.

Parasitemia was determined at regular intervals, in a standard sample of blood under a standard coverslip. The number of trypanosomes was recorded per 100 microscopic fields — 20 on each corner and 20 on the center of the coverslip, as suggested by BRENER [3]. If these fields were negative the whole preparation was examined.

On the 15th day, half of the mice in each group was necropsied after being submitted to xenodiagnosis; the same was done with the other half on the 30th day.

For the xenodiagnosis 6 fifth instar nymphs of *Rhodnius prolixus* were engorged on each mouse. Dissection and examination of the bugs were done about 30 days after the feeding.

Heart, lungs, encephalum, eosophagus, stomach, small and large intestines, liver, spleen, kidneys, skeletal muscles, lymph nodes and peritoneum were wholly or partially fixed in Helly's or Bouin's, sectioned to 5-6 micra and stained by hematoxylin-eosin or Maximow's.

After a first look through the sections it became evident that there were definite differences in the amount of tissue damage among the various groups. It was then decided to classify the material according to the degree of total damage found, as follows:

Degree	*Amount of damage*
I	Discrete lesions limited to liver, spleen and lymph nodes.
II	Discrete lesions extending to other organs, chiefly heart and digestive tract.
III	Extensive lesions in liver and mostly discrete lesions in other organs, including sometimes the CNS.
IV	Extensive lesions in most of the organs examined.

All the material was then examined and classified, care being taken to cover as much as possible equivalent areas of each organ for all animals.

Tissue parasites were not taken into consideration when the degree of damage were defined, but their presence was registered for each organ examined.

To secure unbiased comparison, only numbers and letters were used to label the material. The pathologist among the authors (T. DE BRITO) was unaware of the origin of the strains, their antigenic type and previous observations on their behaviour, when he examined the sections and made his classification. So were the laboratory technicians who examined the blood and the triatoma bugs.

RESULTS

1) *Parasitemia* — This, as detected directly by blood examination and indirectly through xenodiagnosis, is given in Table I. Since only mice inoculated with strain 8717 showed patent parasitemia, the results of trypanosome counting are not given in the Table. They were as follows, according to the average number of trypanosomes per 100 fields:

	Intraperitoneal route	*Intragastric route*
5th day	5.4	1.0
10th day	1.3	1.0
15th day	1.0	—
30th day	—	—

As it may be readily seen by examining the data in Table I, there was a definite gradation in the infection produced by the various strains as expressed by parasitemia. The only death probably due to infection occurred (on the 28th day) in the group infected through the peritoneum with the wild rat strain 8717. This strain infected 100% of the mice (as revealed by xenodiagnosis) by both inoculation routes. Also a higher percentage of bugs were positive among those fed on the mice inoculated with strain 8717 than among the other groups.

Strains 3014 and 8857 were decidedly less virulent than the above. Both infected 100% of the mice through the peritoneum, producing, however, lower parasitemia than strain 8717, since no parasites were found by direct blood examination and the proportions of infected bugs were lower. Also xenodiagnosis detected infection in only 50 and 67% of the mice inoculated with these strains by the gastric route.

The bat strain M, in this experiment, did not produce any parasitemia that could be detected by these means.

TABLE I

Parasitemia in mice inoculated with 4 "wild" strains of *Trypanosoma cruzi*, as detected by blood examination and xenodiagnosis (12 mice for each strain, 6 for each route of inoculation)

Trypanosome strains	Route of inoculation (X)	Blood examination: % mice positive at intervals, in days, after inoculation				Xenodiagnosis % positives	
		5th	10th	15th	30th	mice	bugs
Wild rat (8717)	P*	83	67	17	—	100	88
	G	17	17	—	—	100	89
Squirrel-monkey (3014)	P	...	—	—	—	100	60
	G	...	—	—	—	50	27
Opossum (8857)	P	—	—	—	—	100	56
	G	—	—	—	—	67	18
Bat (M)	P**	—	—	—	—	—	—
	G	—	—	—	—	—	—

(X) P = Intraperitoneal; G = Intragastric.
* 1 mouse of this group died on the 28th day.
** 1 mouse of this group died on the 2nd day.

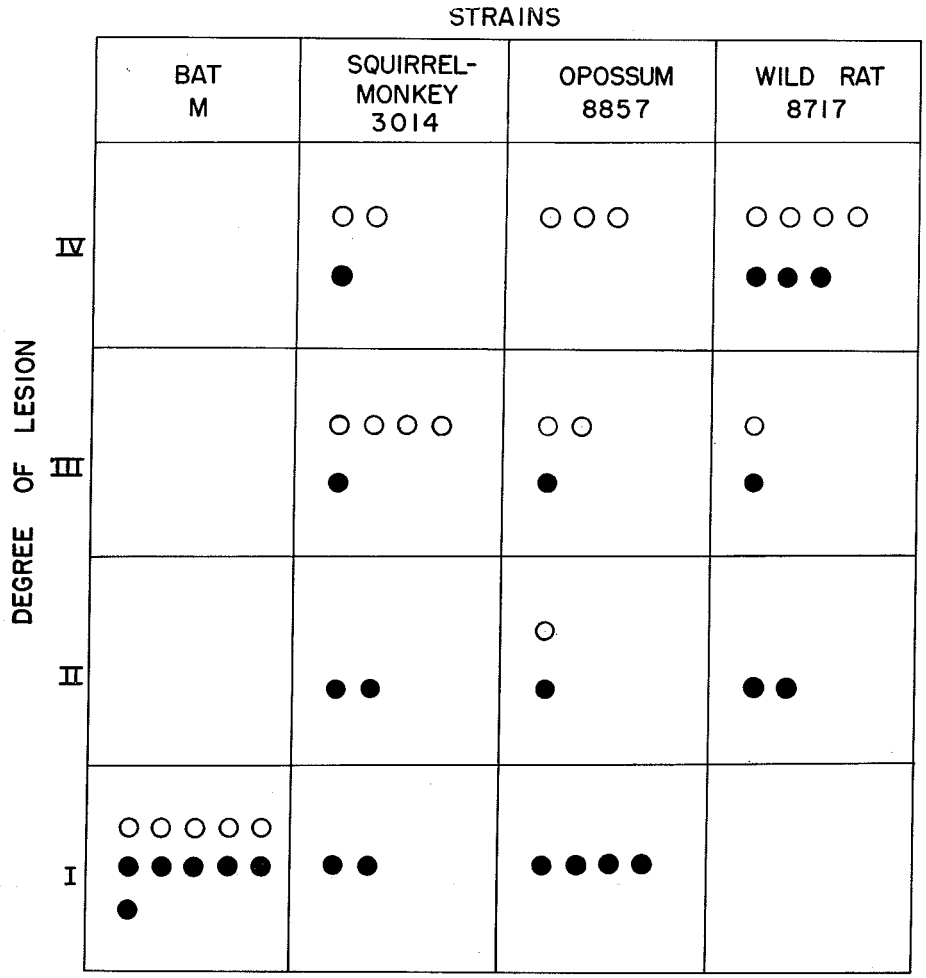


Fig. 1 — Degree of lesion observed by histological examination of viscera of mice inoculated from wild animals of Brazil. Each circle corresponds to one mouse; whites, inoculated intraperitoneally, blacks intragastrically.

TABLE II

Mice inoculated with 4 "wild" strains of *T. cruzi*, classified according to degree of tissue damage (12 mice for each strain, 6 for each route of inoculation)

Trypanosome strain	Route of inoculation (X)	% of mice by degree of tissue damage			
		I	II	III	IV
Wild rat (8717)	P**	—	—	20	80
	G	—	33	17	50
Squirrel-monkey (3014)	P	—	—	67	33
	G	33	33	17	17
Opossum (8857)	P	—	17	33	50
	G	67	17	17	—
Bat (M)	P**	100	—	—	—
	G	100	—	—	—

(X) P = Intraperitoneal; G = Intragastric.
** in this group 1 mouse died and was not examined.

TABLE III

Mice inoculated with 4 "wild" strains of *T. cruzi*, by the intraperitoneal route. Distribution of lesions and parasites (6 mice for each strain)

Trypanosome strain	Lesions or parasites	% of mice with lesions or parasites						
		Liver	Spleen & lymph nodes	Digestive tract	Heart	CNS	Other**	Any organ
Wild rat (8717)*	Lesions	100	80	100	100	40	100	100
	Parasites	—	—	100	60	—	40	100
Squirrel-monkey (3014)	Lesions	100	100	100	100	—	100	100
	Parasites	—	—	17	50	—	17	50
Opossum (8857)	Lesions	100	83	100	100	17	83	100
	Parasites	—	—	17	17	—	17	50
Bat (M)*	Lesions	100	100	—	—	—	—	100
	Parasites	—	—	—	—	—	—	—

* 1 mouse died and was not examined.
** These include peritoneum, skeletal muscles and pancreas.

TABLE IV

Mice inoculated with 4 "wild" strains of *T. cruzi*, by the intragastric route. Distribution of lesions and parasites (6 mice for each strain)

Trypanosome strain	Lesions or parasites	% of mice with lesions or parasites						
		Liver	Spleen & lymph nodes	Digestive tract	Heart	CNS	Other*	Any organ
Wild rat (8717)	Lesions	100	100	100	83	67	100	100
	Parasites	—	—	17	—	17	—	33
Squirrel-monkey (3014)	Lesions	100	100	50	83	17	83	100
	Parasites	—	—	—	17	—	—	17
Opossum (8857)	Lesions	100	100	33	33	33	33	100
	Parasites	—	—	—	—	—	—	—
Bat (M)	Lesions	100	100	—	—	—	—	100
	Parasites	—	—	—	—	—	—	—

* These include peritoneum, skeletal muscles and pancreas.

2) *Pathology* — Results of the histological examination are found in Tables II to IV and Figure 1. In the first of these the animals are classified according to the degree of tissue damage, following the already mentioned criteria.

As it can be readily seen, strain 8717 produced more tissue damage in more organs and a greater number of mice than the other strains. This is also true if the groups are compared according to the inoculation route.

Here again strains 3014 and 8857 seem to be of comparable virulence. Leishmania nidi or free leishmanoid parasites were seen in mice inoculated with each of the strains 8717, 3014 and 8857, although they were more frequent in the first (64%) than among the other two (33 and 25%). They were also much more abundant among the mice inoculated through the peritoneum than among those inoculated by the gastric route, the proportions being, in percentages: 100 and 33, 50 and 17, 50 and 0 for each case.

This is perhaps due to slower invasion of tissues when the parasites are introduced into the stomach since it was noticed that among the animals infected in this way both lesions and parasites were more frequent in those killed on the 30th day of infection than in the group killed on the 15th day. The contrary was observed for the mice inoculated intraperitoneally.

In comparing the inoculation routes it is also interesting to note that the proportion of mice found with lesions in central nervous system was higher among those infected by the gastric route than among the others. With this exception, the intraperitoneal inoculation resulted in more extensive lesions.

As for the bat strain M, it was again decidedly less virulent than the others and no parasites could be demonstrated in the sections.

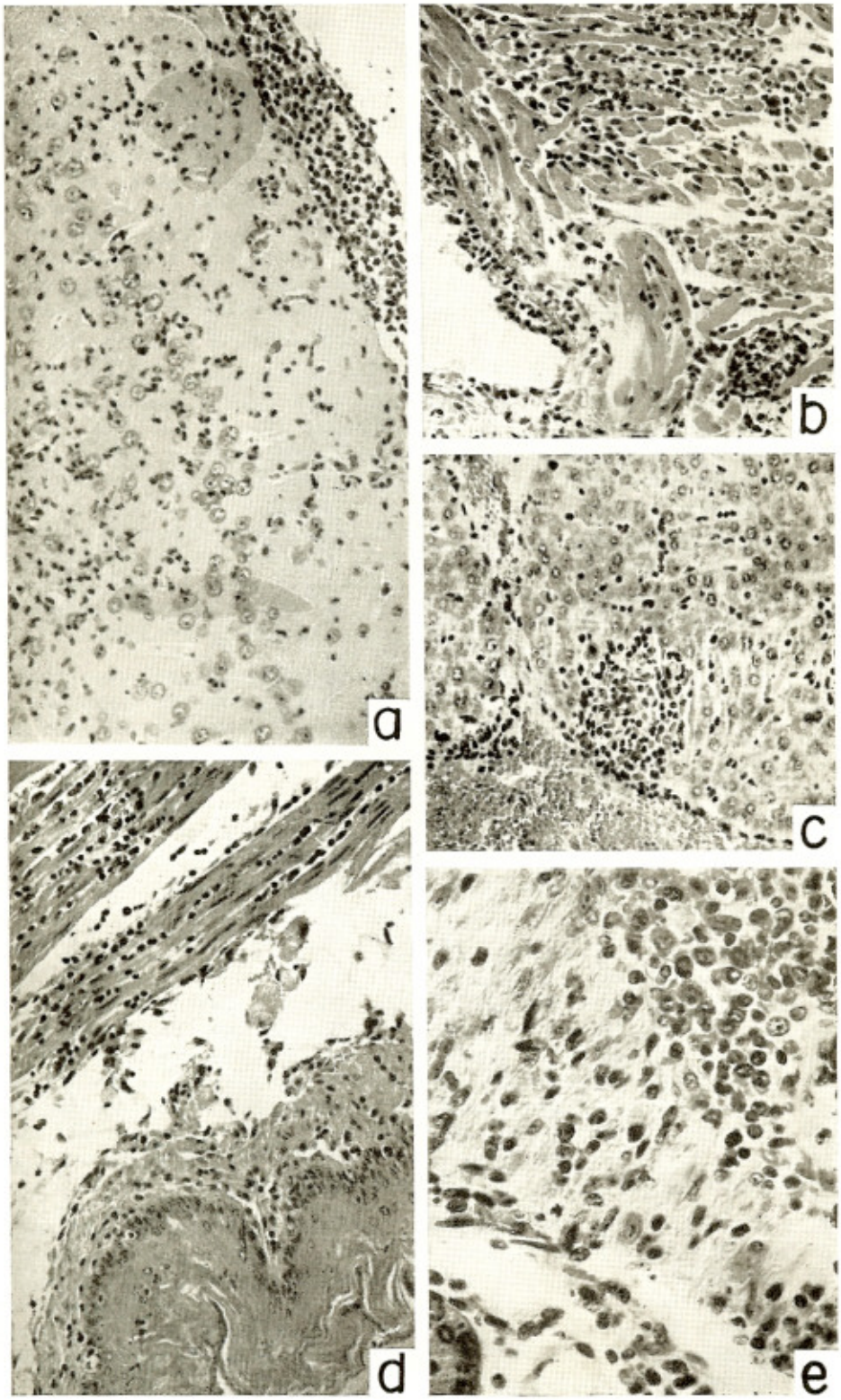
3) *Brief description of the histological lesions* — In the *liver* of every mouse an hepatitis was found. There was great proliferation of the Kupffer's cells, sometimes forming intra-sinusoidal aggregates, and a portal mononuclear infiltrate, essentially of round histiocytes with notched nucleus and basophilic cytoplasm. In 5 cases there was a granulomatous arrangement of the periportal infiltrate, with reticular cells in the center but no giant cells (Fig. 2, *c*). The hepatic cells were usually preserved, presenting only occasional and slight degenerative processes.

Spleen with great hyperplasia of the whole pulp, the white showing accumulations of reticular cells in the central portion of the lymph follicles, and the red with proliferation of histiocytes and mononuclear cells in the interstitium. Here were also found large cells with basophilic cytoplasm, nucleus with coarse chromatin and irregular contour, similar to megakaryocytes.

Lymph nodes enlarged, showing marked hyperplasia of the lymphoid follicles. The lining of the lymphatic sinuses was swollen, its cells falling into the lumina and presenting a secondary granulomatous arrangement. These cells have an oval and vesiculous nucleus, abundant and clear cytoplasm with indefinite contour.

In the *digestive tract* the damage, when present, was frequently severe (Fig. 2, *d* and *e*) and characterized chiefly by interstitial myositis focally distributed along the various portions. Inflammation is by elongate histiocytes and great mononuclear cells with notched nucleus and basophilic cytoplasm. Sometimes eosinophils and neutrophils were also observed in the infiltrate. The muscular fibers were thinner in the affected portions, sometimes without nucleus and with an acidophilic cytoplasm. The infiltrate is also seen around small vessels of the subserosa which present a thickened wall and a swollen endothelial lining projecting into the lumen. Inflammation was detected around parasympathetic ganglia and the neurons exhibited slight degenerative processes or nuclear picnosis, or both.

Fig. 2 — Pathological aspects in mice inoculated with strains of *Trypanosoma cruzi* isolated from wild animals of Brazil. *a* — Microglial foci in gray matter. Marked mononuclear infiltrate at the leptomeninge. Strain 8717, from wild rat, intragastric. HE stain, 140×. *b* — Interstitial myocarditis, inflammatory infiltrate being made up by mononuclear cells. Note focal area of destruction of myocardial fibres. Strain 3014, from squirrel-monkey, intraperitoneal. HE stain, 140×. *c* — Liver showing marked reticulo-endothelial hyperplasia and granuloma formation at the portal space. Strain 3014, from squirrel-monkey intraperitoneal. HE stain, 140×. *d* — Interstitial myositis of esophagus. Strain 3014, from squirrel-monkey, intraperitoneal. HE stain, 140×. *e* — Secondary involvement of the mural plexus of the bowel through interstitial myositis. In this case neurons are still preserved. Strain 8717, from wild rat, intraperitoneal. HE stain, 320×.


a
b
c
d
e

Leishmaniae were frequently found in the digestive tract, more often disposed in line, amongst the infiltrate. Typical nidi were less common, seen inside normal or altered muscular fibers.

The above described aspects were seen chiefly along the muscular layer of the oesophagus, stomach and large intestine. The lesions of the small intestine were less severe and had a more focal distribution along the muscular layer of the organ. The submucosa and the chorium of the mucosa were, in various places, oedematous and infiltrated by mononuclear cells.

Fragments of *striated muscles* around the terminal portion of the rectum, presented severe interstitial myositis and many parasite nidi inside fibers which often had a diffuse hyalinization of the cytoplasm.

The *peritoneum* also exhibited frequent lesions, varying from discrete inflammatory infiltrate similar to the above described, to areas of fat necrosis surrounded by marked chronic inflammation in which xantomatous histiocytes were seen. Isolated leishmaniae could be seen among the infiltrate.

Pancreas frequently showed acute pancreatitis, with intense edema and acinar cells atrophy. Inflammatory infiltrate was mononuclear but neutrophils could also be seen. We did not find parasites in the pancreas.

Adrenals and *kidneys* failed to show marked lesions in our cases.

Lungs showed lesions in few cases only, characterized by mononuclear inflammatory infiltrate around small bronchi and at the interstitium of the musculature. In one case a leishmania nidus was seen in the muscle fibers. In another one, inflammatory infiltrate was focal, with enlarged mononuclear cells with abundant clear cytoplasm, the nuclei with scanty chromatin, making up a granulomatous arrangement.

The *heart* was frequently damaged through an interstitial myocarditis located mainly at the atrial musculature with secondary propagation to the limit between atria and ventricles (Fig. 2, *b*). Musculature of the ventricles was sometimes focally affected. Inflammation was made up by hystiocytes with elongated nuclei, and basophilic cytoplasms with poorly delimited contour and by mononuclear cells with the morphology as described above. Neutrophils and eosinophils were seldom seen among the elements of the infiltrate. Muscle fibers showed focal lesions characterized by loss of striation and diffuse hyalin cytoplasmic change. In those fibers loss of the nucleus was also observed. Parasite nidi were frequently seen surrounded or not by interstitial inflammatory infiltrate. Identical infiltrate was seen at the fat tissue of the epicardium and at the connective tissue of the limit between atria and ventricles.

The *central nervous system*, showed acute encephalitis with or without meningeal involvement (Fig. 2, *a*). Gray matter was usually more affected and the anatomical picture was characterized by the appearance of inflammatory nodules made up mostly by microglia, with or without secondary involvement of isolated neurons which showed degenerative process of varying intensity. In one case isolated leishmaniae were observed among the cells of the infiltrate.

Meningeal involvement, when present, was focal, characterized by congestion, edema and marked inflammatory infiltrate made up by large mononuclear cells with clear and lobulated nucleus and basophilic cytoplasm. The cells were closely packed at the meninge. Cerebral vessels either close to the damaged areas or deep down in the cortex showed sometimes cuffs of mononuclear inflammatory infiltrate around their walls.

DISCUSSION

Besides the practical reason previously mentioned for not using blood trypanosomes in our experiments, there was, in favor of the cultural forms, the fact that the immunological typing of the strains was based on these forms [13, 14] (NUSSENZWEIG, personal communication).

The use of cultures has some advantages over the infected blood, since the inoculum is more close to the natural one and, on the other hand, the passage of blood forms is known to artificially enhance virulence. In our own and others [7] experience, cultures of most strains of *T. cruzi* are regularly infective, this being probably true even after

prolonged maintainance without animal passage [17].

Except for the genetical background of the inoculated mice, other factors capable of influencing the course of *T. cruzi* infections were standardized as much as possible.

In studying Mexican strains of *T. cruzi*, MAZZOTI [11, 10] observed that when they were taken directly from the triatomid bugs the infection in mice was not influenced by the number of trypanosomes present in the inoculum, although some correlation existed between small or large numbers and the course of infection when blood forms were used. In our experiments the flagellates were not counted in the inoculum (which, at least morphologically, corresponds to the intestinal contents of infected bugs), but the dosis of metacyclic trypanosomes for each animal was certainly many times the necessary to establish infection.

The results obtained clearly indicate marked differences in the infectivity of the various strains, the one from wild rat 8717 being the most, and bat strain M the least virulent. The other two were intermediate.

Strain 8717 was the object of a detailed study shortly after its isolation, and its relatively high pathogenicity for mice, together with some morphological characteristics (high proportion of thin blood forms) similar to those of some virulent human strains have been registered [6]. The pathogenicity of cultures of this strain, as judged from the histological examinations now made, seems to be comparable, although perhaps of a lesser degree, to the previously studied pathogenicity of cultures of the Y human strain [4].

On the other strains some observations have been made [15] (L. M. DEANE, unpublished data) and we knew that all — including the M — are able to produce light and transient parasitemias in mice, specially if the animals are less than 1 month old. In this experiment no parasites were detected in the mice inoculated with the M strain, by any of the methods used. We don't know if the infection did not take at all or if, the strain being of very low invasiveness, sufficient time was not allowed for the parasitism to reach patent levels.

The differences found in the present study were independent of the time the strains had been maintained "in vitro" since both the most and the least infective had been cultured during 4 years, while the one isolated only 5 months before was of intermediate virulence. They were also independent of the route of inoculation, the strain most pathogenic by the intraperitoneal route being also the most infective by the gastric route — although for each strain the first type of inoculation was more effective than the latter.

Again, no correlation was found between infectivity and geographical origin or antigenic type of the strains. The more virulent strain and the two with an intermediate virulence originated from an area (the Amazon Region) where Chagas' disease is unknown in man; these 3 strains all belong to immunological type B [13, 14, 15]. On the other hand, the bat strain M, with very low virulence, was isolated in an area where Chagas' disease is endemic and was classified within the antigenic type A, together with strains isolated from domestic sources [13].

No differences in tissue affinities were noticed among the 3 strains that produced patent tissue parasitism. The distribution of parasites and lesions suggests again that the differences among the 4 strains are due to various degrees of virulence and not to distinct tropisms.

Obviously, the fact that no parasites were seen in several organs does not mean they were really absent and it is worth mentioning that when strain 8717 was first studied, leishmaniae were found inside macrophages, in smears and sections of liver, spleen and other viscera [6]. The present data only indicate the predominant localization of the leishmaniae. All the strains seem to belong to the myotropic type that, according to BADINEZ [1] and PIZZI [18] is able to establish its intracellular cycle in the cardiac, skeletal and visceral muscles and in fat tissue, but is rare or practically absent from organs rich in cells of the reticulo-endothelial system, such as liver, spleen and lymph nodes. As a matter of fact, the histological picture seen in our material suggests that all strains were able to arouse a marked RES defence reaction which was an apparently full success

in the case of the M strain and a more or less partial failure against the more virulent strains.

Marked differences in virulence and tissue affinities among strains of *T. cruzi* have been detected by various authors [1, 2, 5, 7, 11, 12]. Through blood passages under more or less padronized conditions which include the genetical background of the hosts, it has been possible to stabilize the course of infection by some strains [19], and there are indications that those and other physiological characteristics may be constant after prolonged maintainance [8].

Of course, we don't know how all the physiological characteristics, including antigenicity, are affected by natural transmission, the trypanosomes being passaged through different species of host, both vertebrate and invertebrate, under variable environmental conditions.

Anyway, our observations seem to confirm the conclusions of HAUSHCKA *et al.*[9], that "morphological identity or physiological differences are certainly no adequate criteria for immunological affinity".

RESUMO

Patogenicidade de algumas cêpas de Trypanosoma cruzi *de animais silvestres do Brasil, em camundôngos.*

Os Autores comparam a patogenicidade e o histotropismo de quatro cêpas de *Trypanosoma cruzi* de mamíferos silvestres do Brasil, em camundongos inoculados intraperitoneal e intragàstricamente com culturas.

As cêpas provieram de um rato d'água (*Nectomys squamipes*), um macaco-de-cheiro (*Saimiri sciureus*), um gambá (*Didelphis marsupialis*) e um morcêgo (*Eumops abrasus*); as três primeiras foram obtidas numa região (Amazônia) onde não se conhecem casos humanos de doença de Chagas e pertencem ao tipo imunológico B, de NUSSENZWEIG & col.; a quarta, encontrada numa área endêmica da doença, pertence ao tipo imunológico A que inclui cêpas humanas virulentas.

Foi verificada uma nítida diferença de virulência, porém sem correlação com seu tipo imunológico ou área de proveniência. Tôdas as cêpas produziram infecções mais intensas por via intraperitoneal do que por via intragástrica. Não foram constatadas diferenças quanto ao viscerotropismo ou ao tipo de lesão causada pelas várias cêpas.

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