

TRYPANOSOMA CRUZI AND EXPERIMENTAL CHAGAS' DISEASE: CHARACTERIZATION OF A STOCK ISOLATED FROM A PATIENT WITH ASSOCIATED DIGESTIVE AND CARDIAC FORM

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A new Trypanosoma cruzi stock isolated from a patient in the chronic phase of Chagas' disease with the digestive and cardiac form of the disease was characterized by experimental infection in isogenic, susceptible, A/Sn strain mice. Parasitemia curves showed up to 1.7×10^6 parasites/ml and no mortality was observed up to 300 days post infection. Specific IgM was found in mice in the acute phase up to 40 days and also in the chronic phase. IgG antibodies were detected in the acute and chronic phase. Histopathology examination demonstrated myotropism to the digestive tract muscle layers and to the heart.

Key-words: Trypanosoma cruzi strain. Chagas' disease. Experimental infection. Serology. Histopathology.

Trypanosoma cruzi is an hemoflagellate protozoan that causes Chagas' disease which produces different clinical manifestations in humans. Many patients remain asymptomatic in the indeterminate form of the disease. Amongst those symptomatic patients who develop lesions 10-20 years after the infection, 20-30% present the cardiac form²² of the disease and around 7% have megaesophagus⁶. The prevalence of patients with megacolon is not clear up to now.

Besides the genetic variation of the human host, *T. cruzi* has demonstrated to present intra-specific variation^{1 2 12}. Infective population of parasites may have marked differences in relation with tissue tropism, parasitemia, pathogenicity and virulence. This heterogeneity in the *T. cruzi* population may contribute to the different clinical manifestations observed in the human population^{3 11}. A possible link between unique characteristics of the parasite population and the clinical manifestation of the host has been searched for,

after several studies^{7 18 25}. The heterogeneity of the parasite population could explain the predominance of certain clinical forms in different geographical regions^{21 24}. However the extent to which the parasite population contributes to the clinical manifestations of the disease is not clear. Classification of *T. cruzi* strains has been based on various criteria such as virulence, pathogenicity, morphology, isoenzymes, tissue tropism, parasitemia, immune response of the host, antigenic composition and susceptibility to various chemotherapeutic compounds^{1 13}.

The study summarizes results concerning experimental infection with a stock of *T. cruzi* isolated from a patient with the associated (cardiac and digestive) form of Chagas' disease. The experimental infection was assessed in terms of parasitemia, mortality, antibody production of the host and histopathological lesions.

MATERIAL AND METHODS

Source of parasites

Triatomine bugs from xenodiagnosis of A.R.O., 35 years old, from an endemic region (Pirenópolis, State of Goiás), clinically, serologically and parasitologically diagnosed to have Chagas' disease was employed as the source of parasites used. Clinical manifestations of the disease included chagasic

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cardiopathy characterized on the EKG by right bundle branch block and anterior left bundle branch block. This patient had a normal chest X-ray and was radiologically classified to have megaesophagus (group III) and dolico-megacolon. Serological reactions for American trypanosomiasis were positive by indirect hemagglutination, indirect immunofluorescence, direct agglutination with 2-mercaptoethanol and ELISA. The patient was submitted to xenodiagnosis employing 40 *Triatoma infestans* bugs. Thirty and sixty days after the bloodmeal 5 out of 34 bugs were demonstrated to be positive.

Obtention of parasites

Infective metacyclic trypomastigote forms were obtained by compression of the posterior gut of positive *T. infestans* bugs. A suspension of parasites diluted in saline solution was inoculated intraperitoneally into susceptible A/Sn mice (4-6 weeks old, both sex). After 4 reinoculations a high number of parasites was obtained and used both for the experiment and for cryopreservation with 10% FCS (Microbiologica, Rio de Janeiro), 10% glycerol (Merck, Rio de Janeiro) and completed with RPMI-1640 (GIBCO, USA).

Experimental infection

Three groups of 7 male A/Sn mice, 4 to 6 weeks old, weighing between 13 and 18g were used. *T. cruzi* trypomastigotes were obtained by bleeding infected mice by the retro-orbital sinus. Each group of animals was infected intraperitoneally (I.P.) with different inocula which were as follows: 1,000 forms/mouse in group I, 10,000 forms/mouse in group II and 100,000 forms/mouse in group III. Four animals in the same conditions inoculated with 0.2ml of saline I.P. were used as a control group.

Parasitemia and mortality

Daily examinations of parasitemia were performed up to 14 days of the infection and then each other day until negativation was observed. Parasites were counted according to the technique proposed by Brener² which analyses 5 μ l of infected

blood under a coverslip of 22 x 22mm with 400x magnification. Mortality was checked daily.

Serological profile

Animals were bled by the retro-orbital sinus on days 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210 and 300 post infection. Blood from at least 3 animals from each group was pooled and sera were tested by indirect immunofluorescence employing antimouse IgM (Cappel Laboratories, Cochranville, USA) conjugated with rhodamine and antimouse IgG (Pasteur Institute, Paris) conjugated with fluorescein. Sera were diluted from 1:5 up to 1:640 for IgM and from 1:20 up to 1:640 for IgG.

Histopathological examinations

One animal from each group was sacrificed on days 40, 60, 90, 180, 210, 300 and 420 (only one animal of group I) employing ether. Tissue samples were fixed with buffered formaldehyde 10% (v/v), pH 7.0, cut into 4 μ m thick, stained by hematoxylin-eosin and analysed with magnifications of 20, 40 and 100x whenever necessary. Under suspicion of fibrosis, trichromic Masson staining was employed for better identification. Analysis of tissues included the identification of parasites, intensity of parasitism and type of inflammatory reaction. The following organs and tissues were examined: liver, spleen, lymph nodes, lungs, kidneys, testis, epididymus, adrenal glands, fat tissue (mesentery), esophagus, stomach, small intestine and bowel, striated muscle and heart (the whole organ).

RESULTS

Parasitemia and peak of infection

Parasites were found in animals of group I (lowest inoculum) on the 10th day post infection; in group II (intermediate inoculum) there was one positive animal on the first day post infection; in group III (highest inoculum) all animals were positive on the first day post infection (Figure 1). A direct correlation was observed between the inoculum and peak of parasitemia: the highest the inoculum, the highest and earliest peak of parasitemia

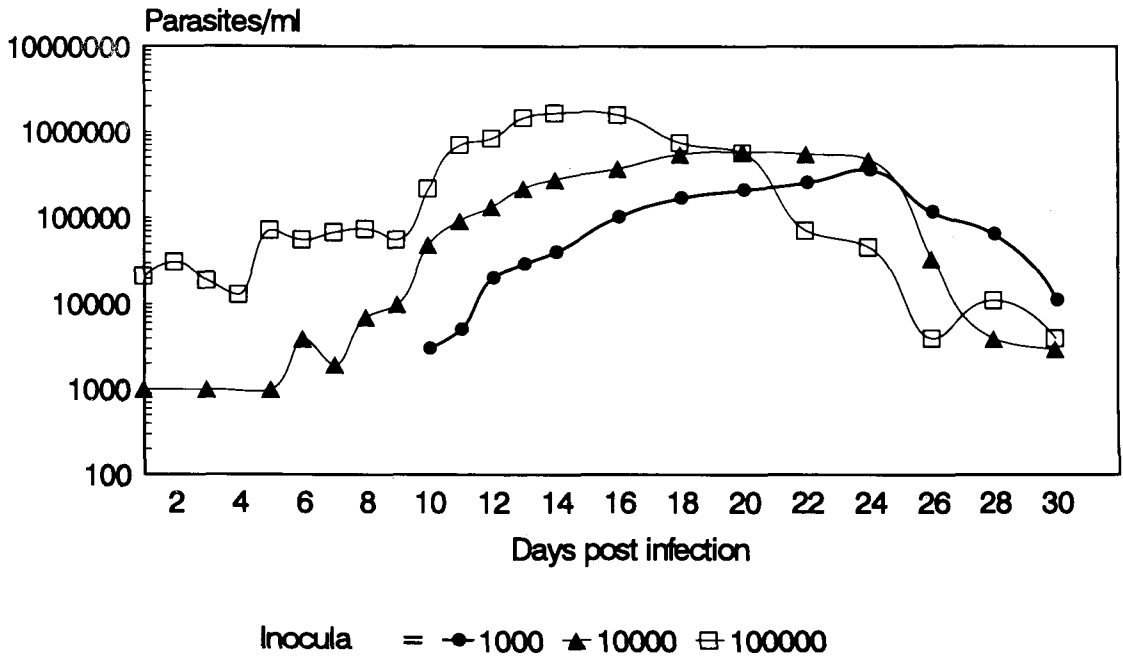


Figure 1 - Parasitemia curves with different inocula.

(group III 1.7×10^6 parasites/ml on the day 14th). A single peak was observed in each group. Peak of parasitemia in group II occurred on the 20th day post infection with 0.58×10^6 parasites/ml; in group I parasitemia peaked on the 24th day with 0.35×10^6 parasites/ml. Following the peak there was negativation of the parasitemia which started on 24th day post infection in group II and III and on 28th day in animals from group I. Complete negativation was observed on the 46th and 50th day post infection in the animals infected with the intermediate and lowest inocula respectively. One animal belonging to the group of the highest inoculum remained positive up to 88 days post infection. There was no mortality during the acute phase. One animal from the group III and another from group II died on days 316 and 390 post infection, respectively. Control mice were alive up to 420 day when they were sacrificed and their organs examined.

Serological profile

Specific IgM antibodies were detected 5 days after infection in group I and II (titer 1/10). For the

lowest inoculum they persisted through all the observation with peaks on days 15, 30, 90 and 300 (Figure 2).

IgG antibodies were demonstrated earlier (30 days) in the group of highest inoculum - group III - (titer 1/20). The other groups (I and II) became positive after 50 days of infection (Figure 3) and remained positive for all samples tested.

Histopathological examination

Parasite pseudocysts were found in the heart, kidney, fat tissue and digestive tract in animals which were sacrificed up to 60 days after infection (Table 1). Heterogeneous patterns of reaction were observed amongst different organs and between different sections within a single organ examined.

Most prominent inflammatory lesions were observed in the muscle layer of digestive tract (Figure 4). These alterations were of the same intensity along the esophagus, stomach, small and large intestine. Infiltrate was mostly lymphohistioplasmacytic however neutrophils and eosinophils were detected in certain areas. Inflammatory response was always in foci and not

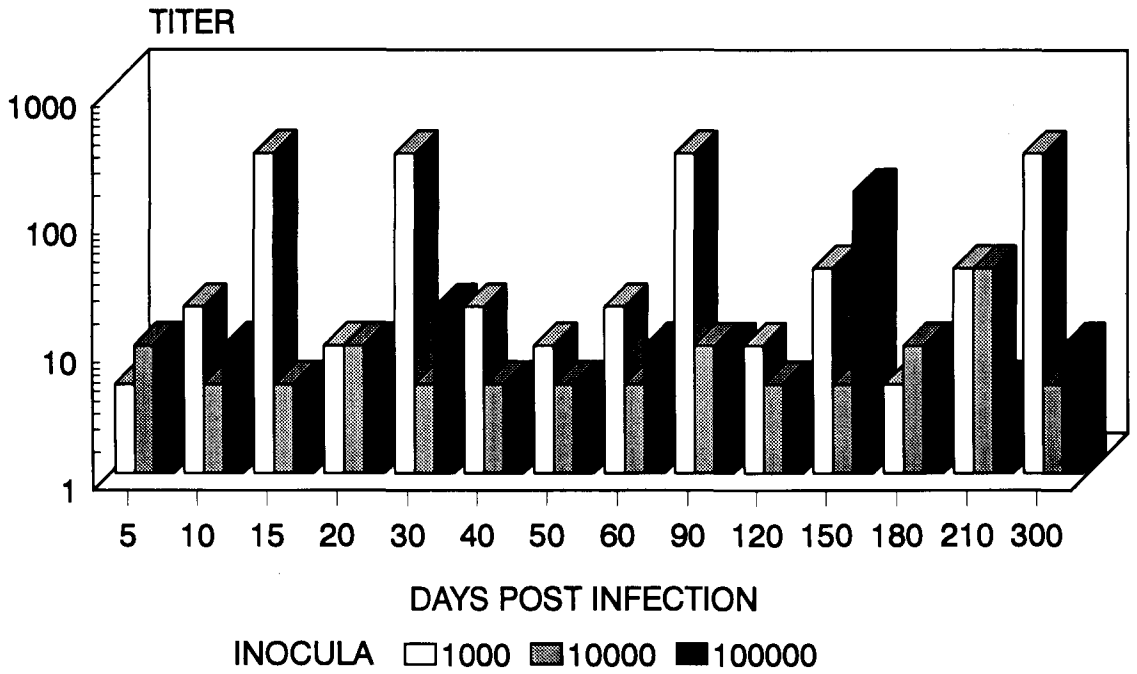


Figure 2 - IgM antibodies detected in the acute and chronic phase.

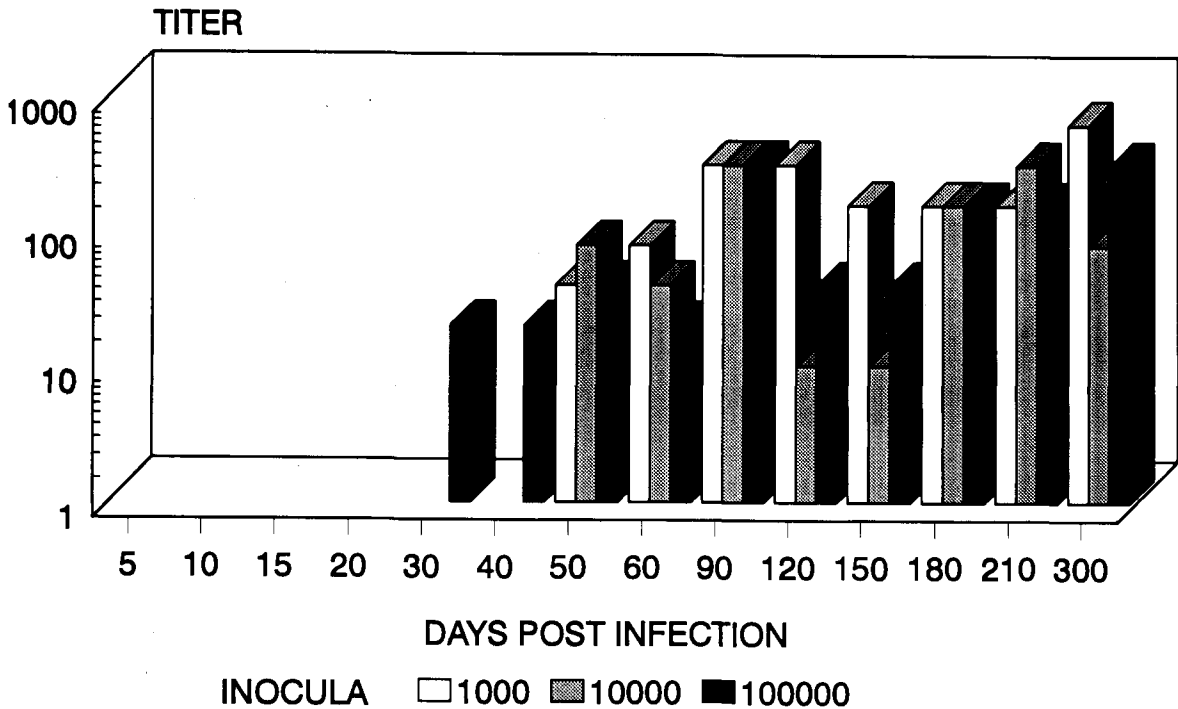


Figure 3 - IgG antibodies: high levels in the chronic phase.

Table 1 - Inflammatory response and parasitemia.

Organ days/inoc	Heart	Striat muscle	Tongue	Eso-phagus	Stomach	Small intest.	Colon	Kidney	Fat tissue	Liver Kupffer cells	Spleen plasma cells	Lungs	L.node plasma cells
40d 10 ³	+++ PP	+	-	+++ PPP	+++ PPP	+	+	-	++	++	-	-	-
10 ⁴	+	+	-	-	-	-	+	-	-	+++	-	-	-
10 ⁵	+++ PPP	-	-	+	-	+	+	-	++	-	-	+	-
60d 10 ³	+	-	-	+	+	++	++	-	+++	+	+	-	+
10 ⁴	++	++	-	++	-	++	++	-	-	+	+	-	++
10 ⁵	+++	+	-	+	-	+	-		+	-	-	-	-
						PP		P	PPP				
90d 10 ³	+	+	-	-	-	+	+++	-	+++	-	-	-	++
10 ⁴	++	+	-	+	-	+	+	-	+	+++	-	-	-
10 ⁵	++ F	+	+	+++	+++	+++	+++	-	+	-	-	+	-
180d 10 ³	+	+	++	+++	+++	+++	+++	-	-	-	-	-	-
10 ⁴	+	++	+	-	-	+	+	+	+	+	-	-	+
10 ⁵	++	+	+	+	-	+	+	-	-	-	-	-	-
210d 10 ³	+	+	+	-	-	+++	+++	+	+	+++	-	-	-
10 ⁴	++ F	+	-	-	-	+++	+++	-	+	+++	-	-	-
10 ⁵	+++	-	-	-	-	+++	+++	-	+++	+++	-	-	-
10 ³	+	+	-	+	+	++	++	-	++	+	-	-	-
300d 10 ⁴	+	+	-	+	+	++	++	-	+	+	-	-	-
10 ⁵	+	+	-	+	+	+	+	-	++	+	+	-	+
420d 10 ³	+	+	-	++	+	+	++	-	+	+	-	-	-

Inoc = inoculum; L. node = Lymph node;

Inflammation: + slight; ++ moderate; +++ severe; F = fibrosis.

Parasite pseudocysts: P slight; PP moderate; PPP severe.

in diffuse aspect either in the striated and heart muscles or in the walls of the hollow organs. Even where the inflammation was considered severe it had an uneven distribution having segments without any infiltration.

An increase of plasma cells was observed both in the lymph nodes and spleen. An inflammatory infiltrate ranging from slight to marked was observed in the fat tissue of all groups.

Hyperplasia of Kupffer cells with variable intensity was observed in all groups.

In spite of exhaustive search no parasites were observed in the spleen or adrenal glands.

The inflammatory infiltrate in the heart was mostly observed in the epicardium and endocardium (Figure 5). Besides the infiltrate, focal areas of necrosis were observed both in the cardiac and skeletal muscle. Discreet foci of fibrosis were observed in the animals sacrificed between 180 and 210 days (Table 1).

DISCUSSION

The *T. cruzi* strain studied was of low virulence even with the highest inoculum, since no mortality was observed up to 300 days post infection. Concerning parasitemia a direct relationship was observed between inoculum, onset of parasitemia and its peak. The presence of bloodstream forms 24h after infection could be due to non-penetrating parasites. Brener² reported 5 strains (2 isolated from chagasic patients) with bloodstream forms 24h post infection using a inoculum of 0.15×10^6 forms. The parasitemia profile observed in this study was similar to parasitemia curves obtained with other stocks of *T. cruzi* recently isolated from chronic chagasic patients^{15 16 25 26}.

Immunological response was demonstrated 5 days after inoculation, by detectable levels of specific IgM antibodies which increased progressively afterwards until 30 days. IgG antibodies were present by day 30 after infection in the group with the highest and 50 days post infection in animals with the intermediate and lowest inocula.

An unexpected result was obtained in relation to the presence of detectable IgM in mice during the chronic phase of the disease. Besides the fact that they were present in all groups of mice, they persisted throughout the time studied. Lana et al⁸

using dogs reported IgM in the chronic phase with decreasing titers from 3 to 9 months after infection; Peralta et al²⁰ using mice reported only specific IgG but not IgM during the chronic phase. According to our data, high levels of IgM were found later, in the chronic phase (10 months after infection) and titers did not decrease afterwards. Magnani et al⁹ reported IgM, IgA and IgG in the chronic phase of human disease. These data were not confirmed by studies performed by Rassi et al²³ and by Camargo and Amato Neto⁴ who did not find IgM in sera from chronic patients even with persistent positive xenodiagnosis. The presence of IgM in our infected animals may be characteristic of some strains. In support of this view a similar finding with the experimental infection of another stock⁵ was observed. The interpretation of these data which were not reported in mice before, remains to be elucidated.

A clear miotropism to skeletal and smooth muscle was shown by the histopathological examination. The heart and digestive tract had the most severe inflammatory reaction independently of inoculum size. Even injecting mice with an inoculum a hundred times higher we had the same inflammatory reaction. According to previous reports^{1 19 26} our findings confirm that inflammatory reaction and fibrosis are related to the pathogenicity of strains. Despite the inflammation no dilatation was observed in the digestive tube according to macroscopic examination. Okumura et al¹⁴ reported chronic chagasic mice with megacolon and Petana et al¹⁷ reported 2 mice with megacolon infected with stocks from natural reservoirs. Marsden et al¹⁰ reported gastric dilatation in mice infected with stocks from patients with megaesophagus. Also the origin of the strain, from naturally infected animals or humans, may influence the parasite tropism and pathogenicity of experimental animals.

This stock may be classified as belonging to type II according to Andrade's parameters¹ of strain classification because of the low virulence and mortality with heart tropism. On the other hand, mortality pattern of type II strains is usually higher than the stock here described, with deaths within the first month after infection; hence, by mortality criteria, this stock could be classified as type III. Another fact that favours this stock as type III is the presence of severe intestinal lesions a fact which is

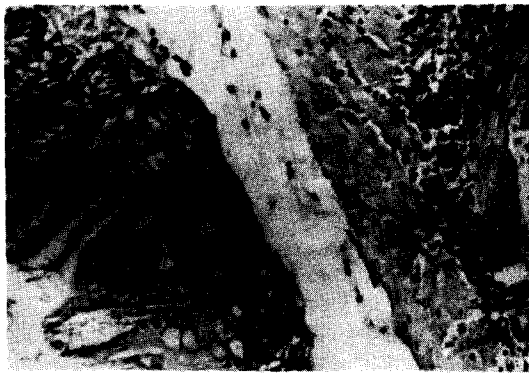
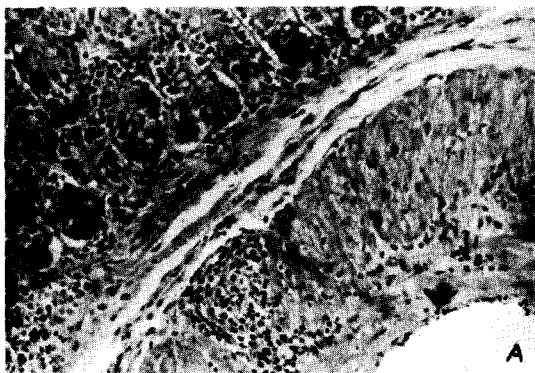


Figure 4 - Photomicrographs of small (A) and large (B) intestine in the chronic phase: severe lympho-histiocytic infiltrate mainly in muscle and serosa layers (HE) (100x).

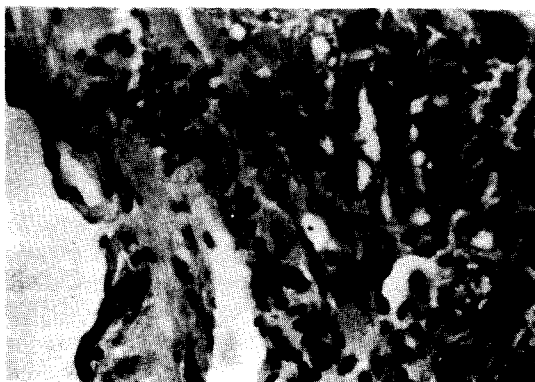


Figure 5 - Photomicrographs of heart (A and B) in the chronic phase: endocardic and myocardial lympho-histiocytic infiltrate foci (HE) (200x).

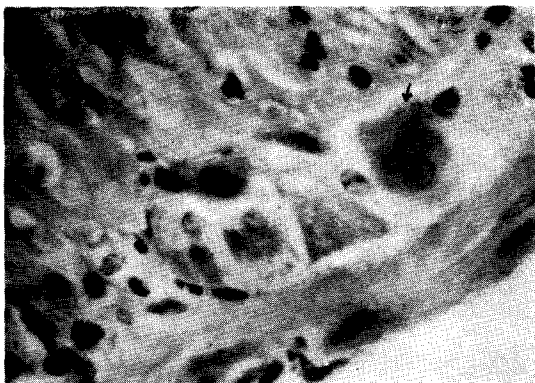


Figure 6 - Photomicrograph of intestine wall: intrinsic nervous system cells (arrow) (HE) (400x).

not described in type II strains.

A larger group of stocks isolated from the same region and from patients with well characterized clinical forms may provide further information in relation with the pathogenesis of Chagas' disease.

RESUMO

Um novo estoque de Trypanosoma cruzi isolado de paciente chagásico crônico, com a forma digestiva e cardíaca da doença, foi caracterizado através de infecção experimental em camundongos isogênicos A/Sn suscetíveis à infecção chagásica. As curvas de parasitemia mostraram picos de até $1,7 \times 10^6$ parasitas/ml não se observando

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mortalidade até 300 dias pós infecção. Anticorpos da classe IgM foram encontrados na fase aguda até 40 dias e também na fase crônica e IgG foi detectada nas fases aguda e crônica. O exame histopatológico mostrou miotropismo para músculo liso do tubo digestivo e cardíaco.

Palavras-chaves: Cepa de Trypanosoma cruzi. Doença de Chagas. Infecção experimental. Sorologia. Histopatologia.

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