ARTÍCULO ORIGINAL

Effects of Acute Chagas’ Disease on Mice
Central Nervous System

ANA LUGO DE YARBUH*, SONIA ARAUJO*, CESARE COLASANTE**,
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ABSTRACT

This study has been done to evaluate the central nervous system (CNS) of mice infected with Trypanosoma cruzi and its relationships with the irreversible decrease of motor activity of the rear limbs during acute Chagas’ disease. The course of the present study shows the in vivo behaviour of three parasites strains which were isolated from different sources and geographical areas, with the purpose of explaining the parasitemia, mortality rate, clinical, pathological and histopathological changes in the CNS of infected mice. The mice were injected intraperitoneally with $5 \times 10^3$ bloodstreams of different T. cruzi strains. The mice infected with PR and ASM strains from Venezuela, showed low parasitemia and high mortality, while the Y strain produced higher parasitemia levels. At the 30th day post-infection both left parietal brain cortex (LPC) and spinal cord (SC) were sectioned, stained with hematoxilin and eosin (H-E) and examined by means of confocal light microscopy. At this time, the pathology of the CNS exhibited focal infiltrates of monocytes, lymphocytes, plasmocytes, polymorphonuclear cells and loss of neuronas and motoneurons. The sections of LPC of infected mice with ASM strain, showed loss neuronal, parasites and abundant T. cruzi antigen deposits in the proximity of the swollen neurons. The sections of SC stained with Enolase-Avidin-Biotin-Peroxidase showed a reduction in the average number of neurons of the cervical region (CR) of the infected mice with PR, ASM and Y strains. Sections stained with Propidium Ioduro (IP) showed a reduction of the number of motoneurons in all regions of the SC, with a significant difference between groups infected with different T. cruzi strains and control uninfected mice ($P < 0.05$). This study established a correlation between the parasitism in the proximity to inflammatory cells, together the appearance of T. cruzi antigen and neuronal destruction in the brain. Therefore it can be concluded that the changes in CNS may be attributed to early parasitism in nervous tissue, which occur in a few days, involving clinico-pathological manifestations, which produced alterations of the mobility with paralysis of the rear limbs and death in 100% of mice with acute infection produced by PR and ASM-T. cruzi strains from Venezuela.

Key words: Brain, Spinal Cord, Neurons, Motoneurons, Acute Chagas’ disease.

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INTRODUCTION

It is known that Chagas’ disease (American trypanosomiasis) is caused by a flagellate protozoan parasite Trypanosoma cruzi, and transmitted to human and other vertebrate hosts by blood-sucking triatominae insects from the family Reduviidae. The disease affects 18 million people in regions of Latin America, and in Venezuela is a public health problem affecting approximately 10% of the rural and suburban populations.

Since the discovery of the intracellular forms of the parasites, a large number of studies have been carried out to analyze diverse manifestations produced in mammalian hosts using T. cruzi strains from different geographical regions, where the parasite circulates between wild and domestic reservoirs, triatomine vectors, and naturally infected humans. Both in humans and in experimental animals, this disease progresses through an initial acute phase characterized by high parasitemia in the bloodstream, followed by invasion of parasites into different tissues of the body. In this sense, the structures most frequently affected are the cardiac tissue, digestive tract and skeletal muscle. In addition, other reports have been shown, regarding lesions in the nervous tissue of the infected animals with T. cruzi, which induced earlier stages of pathological processes and parasites in infected mice CNS. T. cruzi strains are likely to play a role in the onset involving the tissue colonization and the establishment of lesions caused by direct action of parasites, it produce initial alterations to the mononuclear phagocytic system, followed by a process of progressive damage in the tissue infected with highly virulent strain of T. cruzi. According to de Souza et al (1996), its inflammatory changes in tissue infected with T. cruzi has been also involved with the neuronal damage during acute infection. Pathologically, neuronal degeneration exhibit focal lymphocytic infiltrates and fatal neurological complications during acute phase. In this sense, T. cruzi induced chronic leptomeningitis in spinal cord with infiltrates limited to spinal roots and dorsal ganglia in animal infected. These authors have suggest that T. cruzi strain-dependent mechanisms are involved, and some strains have cardio tropic and neurotropic tendency, affecting not only cardiac and intestinal structures but also neurological structures, as was detected in encephalon chronic inflammation of infected mice. Andrade et al (1997) considered that the biological types of strains correlate with different histopathological lesions considering cardiac involvement and neuronal lesions. These findings suggest that the biological behavior together with isoenzymes patterns and pathological pictures in the vertebrate host, can be an important tool for establishing correlation between strains. In fact, T. cruzi is formed by groups of heterogeneous sub-population, which present specific correlation between strains behaviour and antigenic differences in the parasite strains from various sources and different geographical areas. In this study we have produced infections with three T. cruzi strains from distinct regional areas, with the purpose to analyse two local strain of T. cruzi harvested from different sources and geographic areas, with proven pathogenic potential, which caused parasitism in the nervous central system and clinico-pathological manifestations, related with decrease of the locomotor activity and paralysis of the rear limbs and death of the mice while still in the acute phase of Chagas’ disease.

MATERIALS AND METHODS

T. cruzi strains: The following T. cruzi strains were used in the experimental infection: 1) M/DID/Ve/99/PR isolated from naturally infected opossum (Didelphis marsupialis), a primary reservoir collected in highly urbanized environment in Caracas’ valley, Venezuela; 2) M/HOM/Ve93/ASM strain isolated from a patient acutely infected in an endemic area of Barinas state, Venezuela. Both strains have been kept in male mice NMRI regularly inoculated with bloodstream of T. cruzi. All strains have been kept in male mice NMRI regularly inoculated with bloodstream of T. cruzi.

Infection the mice: A total of 20 young (30 days old) male mice NMRI, weighing 20 g were used in this study. The animals were divided into four groups (5 mice/group). Each mouse was intraperitoneally inoculated with 0.1 ml of mouse blood containing 5.10⁷ trypomastigotes from the different T. cruzi strains, except the control healthy group (C) which was injected with saline solution.
Parasitologic evaluation and histopathology:
The parasitemia levels were determined daily using 7 µl of peripheral blood drawn from the mouse tail during acute phase from day 15 post-inoculation (pi). At the 30th day pi each mouse was intracardially bled and perfused with Ringer’s solution and then all the animals were examined by a complete autopsy. To evaluate tissue damage, fragments of brain and spinal cord of infected mice were dissected out, formalin-fixed and paraffin-embedded. Tissue sections of 7 µm were stained with hematoxylin and Eosin and examined microscopically. Inflammatory infiltrate, *T. cruzi* antigen and neuronal destruction were confirmed by immunocytochemical staining.

Detection of *T. cruzi* antigen in the brain and spinal cord: Peroxidase anti Peroxidase (PAP) and indirect immunofluorescence (IFI) assays, were used in the analysis of the histological sections of LPC, CR, LR and SR of the SC. Its were hydrated, incubated with 0.3% hydrogen peroxide in methanol for 30 min in order to block endogenous peroxidase activity, and then incubated with rabbit anti-*T. cruzi* serum at 1:30 dilution in (0.15 M NaCl, 50 mM phosphate buffer with 0.1% Triton X-100, pH 7.2; solution A) for 1h, followed by goat anti-rabbit IgG peroxidase conjugated (Sigma, St Louis. USA) at 1:500 in solution A for 45 min. Sections were stained with 3-3 Tetracloruro Diaminobencidine (Sigma, St Louis. USA) for 7 min and counterstain with Mayers’ hematoxylin, dehydrated with isopropil alcohol and mounted in MAR-TEX. The Immunofluorescent label was performed as follows: Sections the LPC and SC were incubated with rabbit anti-*T. cruzi* serum (1:30 dilution) for 1 h, followed by incubation with goat anti-rabbit IgG fluorescein isothiocyanate conjugated (Sigma, St Louis. USA) at 1:500 in solution A for 45 min. Then, incubation with biotilated goat anti-rabbit IgG (Vector, Inc. USA) at 1:30 dilution in solution A for 45 min. Washed with solution A and incubated with Avidin-biotin complex (Vector, Inc. USA) applied for 30 min, followed by 20 mg of 3-amino-9-etilcarbazole and N-N dimetilformamide (Sigma, St. Louis. USA) in acetate buffer 50 mM at pH 5.0, and 25 µl of hydrogen peroxide at 30% for 10 min and counterstain with Mayer’s hematoxylin, hydrated in isopropil alcohol and mounted in MAR-TEX for microscopy observation.

Astroglias: Sections the LPC and SC were incubated with polyclonal anti-Glial Fibrillar Acidic Protein (GFAP) (Sigma, St Louis. USA) 1:80 warmight at 4°C, washed with PBS pH, 7.2 and incubated with anti-rabbit IgG- FITC (Sigma, St Louis. USA) and Propidium Iodide 1:1000 1 h, washed with PBS pH 7.2 and mounted in glycerin 9:1 for microscopy observation.

**Neurons Immunolabeling:** Sections the LPC and CR, LR and SR were stained either with Enolase-Avidin-Biotin-Peroxidase (EABP). Sections were incubated at room temperature with rabbit anti-enolase (Sigma, St. Louis. USA) serum 1:1000 dilution in solution A supplemented with 5% goat normal serum for 45 min and with rabbit anti-*T. cruzi* serum (1:500) for 1 h. Then, incubation with biotilated goat anti-rabbit IgG (Vector, Inc. USA) at 1:30 dilution in solution A for 45 min. Washed with solution A and incubated with Avidin-biotin complex (Vector, Inc. USA) applied for 30 min, followed by 20 mg of 3-amino-9-etilcarbazole and N-N dimetilformamide (Sigma, St. Louis. USA) in acetate buffer 50 mM at pH 5.0, and 25 µl of hydrogen peroxide at 30% for 10 min and counterstain with Mayer’s hematoxylin, hydrated in isopropil alcohol and mounted in MAR-TEX for microscopy observation.

**Motoneurons label:** Sections the CR, LR and SC were stained with FITC-nuclear marker Propidium Iodide (PI) (Molecular Probe, Inc. USA) at 1:1000 dilution for 1 h, mounted in 95% glycerol-PBS. The sample tissues were analyzed using an Olympus fluorescent inverted microscope coupled to a Laser Confocal system Olympus Fluoview II. The data were analysed using Dunca’s 2x2 multiple comparison or one-way analysis of variance.

**RESULTS**

**Level of Parasitemia:** The experimental infection of NMRI mice with PR and ASM *T. cruzi* strains resulted in lows parasitism between days 19 and 30 pi, with values represented by 28.53 ± 8.5 and 22.19 ± 7.2; 19.02 ± 5.3 and 14.26 ± 4.8 trips./mm³ of blood circulating respectively. When mice of the same inbred strain were infected with *T. cruzi* Y strain, there was a high numbers parasites between 26.15 ± 8.1 and the highest level 596.75 ± 87.5 trips./mm³ of blood at the end of the acute infection (Figure 1).

**Clinical aspects:** Earlier stages of pathological processes were caused in mice infected with PR and ASM *T. cruzi* strains between the 20th and 30th day pi. The results shown loss of the mobility of the rear limbs, loss of weight, rectal proluxus, continuous deharrea and death while still in the acute Chagas’ disease. The necropsy of the mice...
revealed abdominal swelling with peritoneal exudate, swollen urinary bladder and hepatosplenomegaly.

**Histopathological evaluation:** The analysis based with hematoxilin and eosin stained on the sections of the LPC and SC of the spinal cord, showed inflammatory cells as monocyte, macrophages, plasmocytes and predominantly lymphocytes (Table 1). In some sections of the nervous tissue the inflammation areas was concentrated around of the microvessels. The inflammatory infiltrate was greater in the LPC of the mice infected with ASM strain. In this region of the brain the comparison of lymphocytes between infected and uninfected mice, was statistically significant (P < 0.05). The nervous tissue showed *Trypanosoma cruzi* amastigotes, small foci of tisular alteration with necrosis in 15% and 45% the LPC of mice infected with PR and ASM respectively.

**Immunocitochemical analysis:** Both PAP and IFI assays indicated amastigotes and abundant *Trypanosoma cruzi* antigens deposits in sections of the LPC and LR of the SC of mice infected with PR and ASM strains. Its sections tissue also showed *Trypanosoma cruzi* positive reaction around the neurons (Figure 2 and 3).

**Evaluation of neurons and motoneurons in brain and spinal cord:** In the assay with EABP, the average number of neurons in the cervical (CR), lumbar (LR) and sacral (SR) regions was quantified. In the CR of the three groups of infected mice the number of neurons was reduced to 40.96 ± 14.95 neurons, compared with 103.1 ± 10.81 neurons in uninfected mice. In the LR the infection with ASM strain reduced the number to 64.45 ± 6.35 neurons compared
Figure 3. Histological sections of the spinal cord that show: A) antigen of T. cruzi around of the neurons and B) antigen dispersed in the lumbar region of infected mice with ASM strain (PAP stained, magnification 490x, 1.250x).

Figure 4. The bars show means ± Standard Deviation of neurons numbers in the spinal cord regions of the groups of infected mice with PR, ASM and Y Trypanosoma cruzi strains and uninfected animals. Values are the mean ± S.D of neurons in the spinal cord for group of five animals each. The assay represent a significant difference between groups infected with different strains and control uninfected mice: P < 0.05.

Figure 5. The bars show means ± S.D. of motoneurons numbers in the spinal cord regions of infected mice with PR, ASM and Y Trypanosoma cruzi strains and uninfected mice. Values are the mean ± S.D of motoneurons in the spinal cord for group of five animals each. The assay represent a significant difference between groups infected with different strains and control uninfected mice: P < 0.05.

Table 1. Average number of lymphocytes in the Left parietal cortex (LPC) and spinal cord regions of infected mice with three Trypanosoma cruzi strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cervical</th>
<th>Lumbar</th>
<th>Sacral</th>
<th>(LPC)</th>
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<tr>
<td>PR</td>
<td>32.10 ± (4.76)</td>
<td>36.82 ± (5.01)</td>
<td>25.66 ± (4.42)</td>
<td>31.54 ± (4.73)</td>
</tr>
<tr>
<td>ASM</td>
<td>32.46 ± (2.30)</td>
<td>33.45 ± (4.03)</td>
<td>23.44 ± (4.57)</td>
<td>32.25 ± (2.53)</td>
</tr>
<tr>
<td>Y</td>
<td>31.08 ± (4.32)</td>
<td>33.81 ± (3.66)</td>
<td>36.02 ± (3.70)</td>
<td>34.34 ± (3.67)</td>
</tr>
<tr>
<td>Healthy mice</td>
<td>18.48 ± (3.76)</td>
<td>12.96 ± (2.84)</td>
<td>14.76 ± (3.76)</td>
<td>12.96 ± (3.39)</td>
</tr>
</tbody>
</table>

Data are values means ± Standard Desviation of Lymphocytes Comparing Infected mice with uninfected mice: P < 0.05
with 80.71 ± 10.79 neurons in uninfected mice. In the SR values were between 42.2 ± 3.92 and 67.16 ± 11.69 neurons compared with 70.94 ± neurons in uninfected mice. These values were statistically different (P <0.05 >). In the histological sections of the LPC the reduction of numbers neurons was higher in mice infected with PR strain, with 214.12 ± 46.04 neurons as compared with 352.60 ± 51.27 neurons in uninfected mice, being that statistically significantly P < 0.05 (Table 2, Figure 4).

The motoneuron nucleus of the SC of mice infected with PR strain of *Trypanosoma cruzi* identified with Propidium Iodide, gave values of 3.27 ± 1.05, 3.09 ± 1.20 and 2.58 ± 0.92 motoneurons in the CR, LR, and SR respectively, compared with 10.30 ± 16, 9.97 ± 1.91 and 7.70 ± 1.62 motoneurons in uninfected mice. The ASM and Y strains also reduced of number of motoneurons in the spinal cords (Figure 5). Sections of cerebral cortex and spinal cord stained with GFAP did not show histological alterations or amastigotes inside microglial cells of the animals infected with *T. cruzi* strains.

### DISCUSSION

Present investigation demonstrated that the PR and ASM *T. cruzi* parasite in the infected mice produce a similar behaviour during acute Chagas’disease as, the low virulence, marked pathological pictures and certain amount of neuronal destruction in the CNS. Its alterations were much more intense than that obtained in mice inoculated with parasites of the Y strain. Several studies have suggested that the parasitemia produced by *T. cruzi* may be considered as a parameter of comparison between the virulence and tropism in the infected animals. In this sense, some differences in the parasite strains as the host’s genetic background, biological characteristics and the behaviour in the vertebrate host, seem responsible of a wide spectrum of clinical manifestations and animal’s death during the acute phase.

The histopathological evidence in the neuronal lesions occurred in infected mice with the two types of strains from Venezuela, with patent parasitism of tissues even in a early stage of infection, demonstrated that PR and ASM *T. cruzi* strains were the most pathogenic strains, with alterations more frequent and intense in the brain and in some regions of the spinal cord. This experimental data confirms epidemiological evidences, indicating an influence of parasite strains on the histopathological lesions and clinical presentations of Chagas’disease in different geographical areas. The destruction of nervous tissue has been always associated with inflammatory exudate, as the resulting inflammatory response, and its possible that the lymphocytes and polymorphonuclear cells appeared most has been involvement with neuronal damage producing severe lesions, as occur in the peripheral and central nervous system. Some authors have suggested that when a neuron is destroyed, the process is probably mediated by a calcium-activated neutral protease, when a modification of the permeability of the cellular membrane can result in edema, due to an augmented influx of ions. In this study the morphological changes occurred in CNS of infected mice with *T. cruzi* consisted of infiltration by mononuclear lymphocytes in the proximity to the altered neurons, parasite in nervous tissue and *T. cruzi* antigen in contact with neuronal cells. In this conditions, cellular alterations founded in the nervous tissue of the mice infected, may be at least in part due the host-parasite interaction, which produced pathological processes, attributed to early parasitism of the brain and spinal cord, which may occur in a few days involving pathological processes in the mice with acute Chagas’disease. We observed both PR isolated from opossum as ASM isolated from human acute Chagas’disease, were more destructive to the neuronal tissue that Y brazilian strain. Some reports have shown that in presence of parasites and elevated levels of

<table>
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<th><em>T. cruzi</em> strain</th>
<th>Neurons ± S.D. in LPC</th>
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<tr>
<td>PR</td>
<td>214.12 ± (46.04)</td>
</tr>
<tr>
<td>ASM</td>
<td>293.09 ± (49.96)</td>
</tr>
<tr>
<td>Y</td>
<td>306.22 ± (55.74)</td>
</tr>
<tr>
<td>Healthy Mice</td>
<td>352.60 ± (51.27)</td>
</tr>
</tbody>
</table>

Data are means ± Standard Deviation of neurons in the brain of group of five animals each. Represents a significant difference between groups infected with different strains and control uninfected mice: P < 0.05.
antibodies anti-\textit{T. cruzi} in the nervous tissue, development inflammatory lesions, increase number of lymphocytes and disturb the nervous function during acute infection\textsuperscript{26-34}. In this study PR and ASM strains produced focal infiltrates of monocytes, lymphocytes, plasmocytes, polymorphonuclear cells, and loss of neurons and motoneurons in the CNS, as well as irreversible decrease of motor activity in the rear limbs in the infected mice with death of the animals during the acute phase. This pathological finding may be explained showing that parasites rupturing and releasing antigen, which may become associated with the neuronal surface membrane of the infected mice and consequently determines the course of stage pathology in these animals. Some authors have suggested a relationship between the presence of tripanosomas and neurological signs with IgM antibody index as markers of CNS invasion\textsuperscript{35}. Other reports to include cerebral trypanosomiasis in patient who had been diagnosed with epilepsy in the differential diagnosis of intracranial space-occupying lesions especially in immunosuppressed patients from endemic regions, whom developed an acute meningoencephalitis\textsuperscript{16-38} and other by demonstration of reactivation of chronic Chagas’ disease in a patients with AIDS, with high parasitemia and parasitism in CNS with 100% lymphocytes in the cerebrospinal fluid of lumbar region and a decrease in consciousness as well as of an immunodepressed patient\textsuperscript{39,40}.

In some studies, parasites have been rarely observed in neurons or found mainly in glial cell. In rats \textit{T. cruzi} proliferates in astrocytes and the number of nests and nodules varying with inoculum size, and drastic parasitemic fall at day 20 post-inoculation and forming nest devoid of enclosing membrane as described for non-glial cell\textsuperscript{24}, in this study the three strain types used in experimental infections no infected glial cells. We observed \textit{T. cruzi} affected areas occupied less than 15% in the LPC of the infected mice with PR and 45% in the infected with ASM strain, with necrosis and granuloma formation and inflammatory reaction in the LPC greater in mice infected with ASM isolated, these finding usually coincided with loss of neurons when amastigotes and antigen of \textit{T. cruzi} were observed near the swelling neurons. It is of particular interest the data presented in other studies, as neuronal destruction occurred when the parasitemia had decreased to its chronic level. Some studies suggest that the neurone destruction correlated closely with the anti-parasite response, measured by antibody suggesting that when a nest of parasites in tissue ruptures, antigens are released and can become associated with the membrane of surrounding uninfected cells\textsuperscript{41}. Our results showed that parasitism, inflammatory process and neurons and motoneuron destruction of the brain and cervical, lumbar and sacral levels of the spinal cord of infected mice, may be at least aggravating factors associated with the decrease muscular function, which produced irreversible paralysis of the rear limbs in the experimental model infected with PR and ASM strains. When parasites circulate in the blood and proliferate in several cell types specially muscle cells, the activation of an intense mononuclear inflammatory process, regulate \textit{T. cruzi} replication and neuronal destruction may be a direct effect of the parasite population. The pathogenic mechanism and neuronal lesions in mice acutely infected with \textit{T. cruzi} strains from endemic area of Venezuela, could be a matter of considerable discussion. The findings are certainly involved with some types of central neurons damage during acute Chagas’ disease. The possibility that such damage could be due to an immunological mechanism is supported by the finding of monoclonal antibody cytotoxic to mammalian neurons in vitro which labels \textit{T. cruzi} and certain types of peripheral and central nerve\textsuperscript{42}. Our results indicate that whatever mechanisms are involved in neuronal lesion in Chagas´disease when experimental animals were infected with different VenezueLAN \textit{T. cruzi} strains. In this sense, we emphasize the need for further studies other aspects of the biology, mechanism related to host-parasite interactions, and behavior of highly virulent \textit{T. cruzi} strain, from different sources and geographical areas rural where Chagas´disease is endémic.

REFERENCES


3.- VIANNA G. Contribuições para o estudo da anatomia
Acute Chagas’ Disease on Mice Central Nervous System - A. Lugo de Yarbuh


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