HUMORAL NITRIC OXIDE LEVELS AND ANTIBODY IMMUNE RESPONSE OF SYMPTOMATIC AND INDETERMINATE CHAGAS’ DISEASE PATIENTS TO COMMERCIAL AND AUTOCHTHONOUS TRYPANOSOMA CRUZI ANTIGEN

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Abstract. We report here the evaluation of chagasic patients for the presence and/or severity of the disease, antibody to Trypanosoma cruzi, and nitric oxide (NO) serum levels. Serum samples tested by ELISA with autochthonous and commercial antigen revealed that 10% and 7.5% of the individuals were anti-T. cruzi antibody-positive, respectively. Ten of 21 seropositive individuals had no clinical signs, the other 11 cases presented cardiomyopathy and/or mega-gastrointestinal syndromes, and three patients presented a combined form. A statistical difference (P < 0.001) in antibody titer between asymptomatic and symptomatic patients with autochthonous antigen was detected, and serum NO levels was found to be three times higher in cases than in controls. These results suggest that it is recommended to use a sole source of antigen (autochthonous) for the serodiagnosis of Chagas’ disease, and that the pathogenic role of NO in this disease should be evaluated.

Chagas’ disease, a zoonosis produced by Trypanosoma cruzi infection, is a major cause of morbidity and mortality in Latin America. In most endemic countries, the disease is recognized as an important public health problem and is receiving increasing priority for its control. The World Health Organization estimates that 16–18 million people are currently infected with T. cruzi, 2–3 million may already have developed chronic complications, while more than three million are at risk of developing chronic Chagas’ disease in the future. Mortality due to Chagas’ disease is difficult to estimate, but extrapolation from longitudinal studies suggests that the disease would cause more than 5,000 deaths per year.

The risk of infection with T. cruzi is directly related to socioeconomic factors. The parasite is mainly transmitted by blood-sucking triatomin bugs, which find a favorable habitat in wall fissures of poor-quality houses in many rural areas and in unplanned urban development. Furthermore, rural to urban migration is a contributing factor to spread of the infection by nonvectorial means. Epidemiologic results from a National Serologic Survey carried out in Mexico showed that less than 1% of the general population were seropositive. However, studies conducted in selected rural villages revealed a prevalence that varies from 8% to 85%. Thus, it is quite possible that there are endemic areas where the prevalence is higher than national mean.

Human infection is characterized by two main stages, acute and chronic. Acute disease predominantly affects children less than five years of age, and causes a febrile illness with local inflammation at the inoculation site. Parasites replicate intracellularly as amastigotes and then are distributed widely as blood-borne trypomastigotes. Once established, T. cruzi results in a lifelong chronic infection, and most chronically infected individuals have no clinical manifestations; thus, they have an indeterminate infection.

Pathogenic mechanisms responsible for the symptomatic forms of chronic Chagas’ disease are not known, but there is evidence suggesting that immune phenomena may be involved. Acute infection causes a vigorous polyclonal B and T cell activation, followed by a brief phase of generalized immunosuppression associated with the inflammatory response in host tissues. Therefore, it is possible that nonspecific mechanisms play an important role in protecting the host, and either an exaggerated or a less than a normal immune response to the parasite might be associated with the development of chronic disease. Both humoral and cell-mediated immune response to T. cruzi antigens are present during the chronic phase of Chagas’ disease. However, despite the immune response, parasites persist in blood and tissues. Studies attempting to correlate the level of T. cruzi antibody response to the clinical spectrum of Chagas’ disease have not identified a significant difference between the antibody response of indeterminate and symptomatic patients.

In vitro studies have shown that reactive nitrogen intermediates, nitrite and nitrate, play an important role in the control of intracellular growth of T. cruzi in gamma-interferon–activated macrophages. Control of other parasite protozoa by macrophage-derived nitric oxide (NO) has been reported for Leishmania spp., T. musculi, and T. brucei. On the other hand, T. cruzi infection up-regulates the release of macrophage-derived NO independently of the respiratory burst.

In vivo studies using animal models have shown that plasma levels of NO correlate with parasitemia in experimental malaria. It has been reported that human patients infected with Plasmodium falciparum and P. vivax show increased NO levels, and that the observed plasma levels of NO during malaria are related to disease severity and directly affected by the degree of immunity to the disease.

In this work, we report the results of immunoresponse studies in chagasic patients who were serologically and clinically evaluated. Serum levels of NO are also reported. To study antibody titer, we used autochthonous parasite crude antigen and commercially available T. cruzi antigen.
POPOPULATION AND METHODS

Study area. The study was carried out in Molcaxac, a rural settlement located 86 km southeast of Puebla City, Puebla, Mexico. No cases of Chagas’ disease have been reported in this community in spite of the existence of socioeconomic and ecologic conditions that promote the development of the disease. It has a warm climate and a mean annual temperature of 22°C, and it is located 1,800 m above sea level. Vegetation in most of the area is desert type.

The procedures for serum collection and clinical studies followed the guidelines laid down by Helsinki Convention and were approved by the Scientific Committee of the Centro Médico Nacional Manuel Ávila Camacho del Instituto Mexicano del Seguro Social. All information was given to the participants and their written consent was obtained (from parents in the case of children when necessary) prior to the study.

Parasite. The parasite was isolated from the feces of an intradomiciliary triatomine bug that was taxonomically identified as Triatoma barberi. The isolated parasite was identified as T. cruzi, and it was cultured and propagated in liver infusion tryptose medium supplemented with 10% fetal calf serum.

Serologic studies. Serum samples from 281 people (males and females) selected from the homodemic cross-sectional study were analyzed for parasite antibodies using three independent assay techniques: an indirect hemagglutination (IHA) test, an indirect immunofluorescence (IFA) test, and an ELISA. Positive and negative controls were included in each test. A positive result was defined as titer > 1:64 for the IHA test (Chagastest HAI; Wiener Laboratories Group, Rosario, Argentina), > 1:32 for the IFA test, and an ELISA value > 0.200 with the autochthonous T. cruzi antigens. Soluble epimastigote antigen was prepared as described.24 A value > 0.150 in the ELISA using a commercially available kit (Chagastest ELISA, Wiener Laboratories) was considered positive. Results obtained with this commercial kit were confirmed by positive and negative serum controls provided by the manufacturer.

Clinical studies. Sex- and age-matched seropositive and seronegative subjects were enrolled in a case-control design to examine their clinical status. All individuals were blindly evaluated by a physician who administered an epidemiologic questionnaire and conducted a physical examination. Control cases were evaluated to detect autoimmune diseases or seropositivity to Toxoplasma gondii. A 12-lead electrocardiogram (ECG), a heart roentgenogram (two m distance) to determine the cardiothoracic index (CI < 0.5 cm), and radiologic imaging of the esophagus and colon were obtained using conventional techniques. Prevalent cases were classified as indeterminate if they were asymptomatic and had no alterations or evidence of lesions, and symptomatic if there were clinical features of cardiac (CI > 0.5 cm, complete right bundle branch block [CRB], incomplete left bundle branch block [ILB], first degree atrioventricular block [IVB]) or gastrointestinal (megacolon [MECLN] or megaesophagus [MESPH]) diseases, and had documented changes on the ECG or esophageal or colon dilatation.

Measurement of serum levels of NO. The NO levels were evaluated by measuring nitrite in serum samples by the Griess reaction.25 Nitrate was reduced to nitrite using spongy cadmium. The absorbances were measured at 540 nm in a microplate ELISA reader (Multiskan MS; Labsystems OY, Helsinki, Finland). Sodium nitrite was used as a standard. The control group was chosen from the cross-sectional study in the serologic studies. Seropositive individuals were considered as cases and seronegative individuals from that community, with negative serology for Toxoplasma gondii and without autoimmune diseases, were paired by age and sex.

RESULTS

The study population consisted of 658 inhabitants and serologic tests were conducted only in 281 subjects selected by simple aleatory corresponding to four sections of Molcaxac, Puebla, Mexico. Serologic screening by the ELISA with both commercial and autochthonous antigen revealed that 21 (7.5%) and 28 (10%) individuals were anti-T. cruzi antibody positive, respectively. Subjects positive for autochthonous antigen were also positive by the IHA test and only two were negative by the IFA test. Among the 28 seropositive individuals, one person died of complicated nephropathy, another had prostatic adenoma, three individuals were seropositive for Toxoplasma gondii (optical density > 0.300), and two individuals refused to participate in the study. Therefore, the group of cases was composed of 21 individuals, all of whom were born and lived in this community.

To compare the anti-T. cruzi antibody levels against commercial and autochthonous antigen, sera from positive individuals were evaluated by the ELISA using the same serum dilution. Results obtained from symptomatic and asymptomatic patients are plotted in Figure 1. Antibody levels to autochthonous antigen were always higher than to commercial antigen in 14 individuals, lower in five, and the same in two of 21. On the other hand, four of 11 symptomatic patients and three of 10 asymptomatic individuals were negative to commercial antigen (Figure 1A and B, respectively).

Ten of 21 seropositive individuals had no manifestations of Chagas’ disease (indeterminate group), and the other 11 cases had cardiomyopathy (conduction system disturbances) and/or mega-gastrointestinal syndromes (symptomatic group). Among the symptomatic cases, seven patients of both sexes, ranging in ages from 23 to 86 years, showed evidence of CRB and ILB (observed in four patients) (Table 1). Among these patients, two showed dyspnea grade I, one showed dyspnea grade II, and the remaining four were normal. Two patients were IFA negative (no. 3231 and no. 4411), although patient no. 3231 showed the highest IHA titer and patient no. 4411 showed the lowest titer in the same essay. The ELISA value of patient no. 4411 was in the range of the controls. To confirm the diagnosis of Chagas’ disease, a xenodiagnosis test was conducted in this and other patients from the same group. Xenodiagnosis was negative in patient no. 3231 and positive in patients no. 3433 and no. 4411, which indicates that patient no. 4411, in spite of having active infection, had a low antibody titer.

Radiologic imaging of gastrointestinal tract showed mega-esophagus Grade 1 with delayed emptying time (> 60 sec) and an enlargement up to 4.5 cm of the esophagus diameter.
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in five patients, and megacolon with an enlargement from 5 to 9.5 cm in six patients (Table 2).

Only three patients of the symptomatic group were referred to as the combined form, characterized by the presence of both cardiomyopathy and megaesophagus and/or megacolon (cases 3232, 3433, and 4441). Sex- and age-matched control groups for cases did not show evidences for alterations in the ECG and a radiograph with barium swallow.

Optical density analysis of data obtained from the ELISA using autochthonous antigen showed a statistical difference between asymptomatic and symptomatic patients \( (P < 0.001) \) (Figure 2). However, there was no difference between the two symptomatic forms. Mean \( \pm \) SD values were 0.586 \( \pm \) 0.218 for cardiomyopathy patients, 0.635 \( \pm \) 0.234 for mega-gastrointestinal patients, and 0.260 \( \pm \) 0.040 for asymptomatic individuals. On the other hand, there was no difference between asymptomatic and symptomatic patients when commercial antigen was used.

When serum levels of NO (nitrate plus nitrite) were measured in healthy people and chagasic patients, cases showed NO levels three times higher than the controls (Table 3). However, no statistical difference could be detected between asymptomatic and symptomatic patients or megagastrointestinal and cardiomyopathy patients. Although patients with the combined form showed the higher levels of NO, no significant correlations among serum NO level and blood pressure, parasitemia or antibody titer were found in symptomatic patients.

DISCUSSION

Data presented in this report showed seroprevalences of 7.5% and 10% to \( T. cruzi \) (in Molacaxac Puebla) when using commercial and autochthonous antigens, respectively. This result suggests that the differential recognition could be due to different antigenic compositions between the two antigen

### TABLE 1

<p>| Antibody titer and clinical and radiologic studies of cases with cardiac symptoms* |
|---------------------------------|-----------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IHA†</th>
<th>IFA‡</th>
<th>ELISA</th>
<th>CI (cm)</th>
<th>ECG§</th>
<th>Dyspnea¶</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1123</td>
<td>++</td>
<td>+++</td>
<td>0.609</td>
<td>&lt;0.5</td>
<td>CRB-ILB</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>2721</td>
<td>++</td>
<td>++</td>
<td>0.762</td>
<td>&lt;0.5</td>
<td>CRB-IVB</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>3232</td>
<td>++</td>
<td>+++</td>
<td>0.798</td>
<td>&lt;0.5</td>
<td>CRB</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>3433</td>
<td>++</td>
<td>++</td>
<td>0.563</td>
<td>&lt;0.5</td>
<td>CRB-ILB</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4441</td>
<td>+++</td>
<td>++</td>
<td>0.687</td>
<td>&lt;0.5</td>
<td>CRB-ILB</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>3231</td>
<td>+++</td>
<td>–</td>
<td>0.356</td>
<td>&lt;0.5</td>
<td>CRB</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4411</td>
<td>+</td>
<td>–</td>
<td>0.201</td>
<td>&lt;0.5</td>
<td>CRB-ILB</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

* IHA = indirect hemagglutination; IFA = indirect immunofluorescence; CI = cardiothoracic index; ECG = electrocardiogram.
† +, ++, and +++ in the IHA are a low, moderate, and high hemagglutination titer, respectively.
‡ –, +, and ++ in the IFA are a negative, moderate, and high fluorescence response, respectively.
§ CRB = complete right bundle branch block; ILB = incomplete left bundle branch block.
¶ Dyspnea = grade I and grade II have slight and moderate breath difficulty, respectively. N = normal.

### TABLE 2

<p>| Antibody titer and radiologic imaging of the gastrointestinal tract of cases with intestinal symptoms* |
|---------------------------------|-----------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IHA†</th>
<th>IFA‡</th>
<th>ELISA</th>
<th>MECLN</th>
<th>MESPH</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3433</td>
<td>++</td>
<td>++</td>
<td>0.563</td>
<td>&gt;9.5 cm</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>3232</td>
<td>++</td>
<td>+++</td>
<td>0.798</td>
<td>&gt;7.0 cm</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>2761</td>
<td>++</td>
<td>+</td>
<td>0.211</td>
<td>&gt;8.0 cm</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>1922</td>
<td>++</td>
<td>+</td>
<td>0.681</td>
<td>&gt;6.8 cm</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>2104</td>
<td>++</td>
<td>+++</td>
<td>0.878</td>
<td>&gt;5.0 cm</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>4441</td>
<td>++</td>
<td>++</td>
<td>0.687</td>
<td>N</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>4522</td>
<td>++</td>
<td>++</td>
<td>0.793</td>
<td>&gt;5.0 cm</td>
<td>GI</td>
<td></td>
</tr>
</tbody>
</table>

* IHA = indirect hemagglutination; IFA = indirect immunofluorescence; MECLN = megacolon; MESPH = megasphagus; GI = grade I; N = normal.
† +, ++, and +++ in the IFA are a low, moderate, and high fluorescence response, respectively.
preparations. It has been reported that several parasite isolates showed different antibody recognition patterns when assayed with monoclonal as well as polyclonal antibodies.26–28 The 2.5% more seropositive individuals detected in this study using autochthonous antigen indicates that for serodiagnosis, the ELISA antigen should be prepared from autochthonous or regional T. cruzi isolates, or recombinant proteins that exhibit antigenic cross-reactivity with several isolates.

The observed differences between the two heterologous antigens could also be due to the method used to prepare the antigenic lysates, storage conditions, or age of the commercial assay. All of these parameters are known to modify ELISA. The results obtained in that study did not reveal any significant difference in serum levels of antibody to T. cruzi between both groups. The CMI results were obtained using a trypomastigote antigen lysate prepared from the CL strain and the ELISA data using a commercially available kit. These observations and the results obtained in the present work suggest that for immunologic studies concerning the humoral and cellular immune response, a sole source of antigen should be used.

The role of NO in the development of intracellular parasite infections and associated diseases has been an area of intense study. In recent years, several investigators have described different aspects of the generation of NO and its possible impact as a pathologic or protective component in the development of cerebral malaria.20,22,33–35

Results obtained in the present report showed a significant difference in serum NO levels between healthy people and chagasic patients (symptomatic and asymptomatic). It has also been reported that patients with P. falciparum and P. vivax malaria as well as mice with P. winckei infection show increased plasma levels of nitrate and nitrite.20–22 Increased plasma levels of NO has been associated with the severity of cerebral malaria.20,36 However, in chagasic patients, we did not find a significant difference between patients with indeterminate and chronic disease. Therefore, serum levels of NO in T. cruzi-infected people do not have a predictive value for chronic disease. Since it is known that serum levels of NO can be influenced by dietary intake, the people in this study (healthy and chagasic) were chosen from the same living area, and in some cases, from the same family.

The role that NO could play in development of chronic Chagas’ disease is not clear. It has been found infiltrating
immune cells in heart tissues of patients with chronic disease and in animal models. These immune cells, such as macrophages and T cells, could induce the generation of cytokines in heart muscles, which in turn could affect normal cardiac function through the induction of NO production.

Several in vitro studies showed that cytokines generated by activated immune cells caused an increase in NO via induction of NO synthase in rat isolated myocytes, which results in a direct negative inotropic effect. In isolated heart preparations of rats, perfusion with interleukin-1 and tumor necrosis factor-α caused a depression of contractile function that could be prevented by the addition of NO synthase inhibitor, Nω-nitro-L-arginine methyl ester or by pretreatment with cycloheximide. On the other hand, it has been shown that T. cruzi infection of normal and interferon-gamma–activated macrophages up-regulate the production of NO. Local production of NO may affect the normal function of the different organs involved in chronic Chagas’ disease. It has recently been reported that NO may be involved in the neuronal destruction of myenteric plexus in acute experimental T. cruzi infection. Further studies are necessary to evaluate the role that NO could play in the development of the disease.

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