Delayed Hypersensitivity to Heart Antigens in Chagas’ Disease as Measured by In Vitro Lymphocyte Stimulation

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Reactivity to Trypanosoma cruzi antigens and autoreactivity to heart antigens were evaluated in 27 patients with Chagasic cardiomyopathy (group I), 52 patients without evidence of cardiac dysfunction (group II), and 96 selected controls, either healthy patients or patients with other heart diseases (group III). The in vitro lymphoblastogenesis response to T. cruzi antigens was found to be high in groups I and II and low in group III. The mean stimulation index to T. cruzi antigens, in fact, tended to be highest in group I, suggesting a more intense immune response in patients with Chagasic cardiomyopathy. The proportion of individuals with reactivity to heart antigens was 28.6% in group I, 25% in group II, and 0% in group III. The finding of an equal percentage of reactivity to heart antigens in groups I and II was unexpected, as a higher incidence of positive reactions in group I was predicted. Consequently, it is thought that this finding and its relevance to the pathogenic process of Chagasic cardiomyopathy need to be carefully assessed in a longitudinal study.

The existence of an autoimmune phenomenon in Chagas’ disease has often been proposed, but it is only in the studies of Cossio et al. (1) and Santos-Buch and Texeira (6) that this hypothesis has received experimental support.

A fundamental aspect of this problem is the presence or absence of lymphocytes reactive to heart antigens and, consequently, able to participate in an autoimmune response such as that described in experimental myocarditis (3).

In a previous communication (W. Mosca, J. Plaja, E. Gallardo, E. Rojas, and H. G. Barrios, Arch. Venez. Cardiol. 3:134, 1976) we have presented our preliminary data, and in this paper we report the capacity of lymphocytes of Chagasic patients to demonstrate proliferative responses when challenged with Trypanosoma cruzi or heart antigens.

MATERIALS AND METHODS

Selection of patients. Chagasic patients were divided into two groups. Group I was composed of 27 patients with a positive complement fixation reaction to T. cruzi antigens and suffering from Chagasic cardiomyopathy, defined according to criteria outlined in a previous communication (4). Briefly, any other possible cause of heart disease was excluded. The patients were less than 45 years old and had one or both of the following electrocardiogram alterations: (i) complete right bundle branch block, plus anterior or posterior fascicular block of the left bundle; (ii) complete atrioventricular block with wide QRS complex. Group II consisted of 52 Chagasic patients without clinical evidence of heart disease, but with a positive complement fixation reaction to T. cruzi antigens. Group III was the control group. Basically, it was composed of 18 normal healthy individuals and 18 patients with heart disease, all of whom had a negative complement fixation reaction to Chagasic disease. A breakdown of the 18 patients with heart disease follows: coronary heart disease (recent myocardial infarction), 5 patients; cardiomyopathy, 4 patients; rheumatic valvular disease, 8 patients; cor pulmonale, 1 patient.

Lymphocyte transformation. Lymphocyte transformation was performed as described by Ulrich et al. (9). In brief, 15 to 20 ml of venous blood was drawn by venipuncture into a tube containing 3 mg of heparin, and the erythrocytes were allowed to sediment for up to 60 min. The leukocyte-rich plasma was placed in a rectangular bottle with an equal volume of McCoy culture medium. The bottle was placed on one of its flat sides for 1 h at 37°C, and the nonadherent mononuclear cells were collected and adjusted to 10⁶ per ml with McCoy medium containing a final autologous plasma concentration of at least 10%. Aliquots of 1 ml of the cell suspension were distributed into duplicate culture tubes, and the antigen or phytohemagglutinin was added to all tubes except the controls. After 3 or 7 days of incubation at 37°C in a 5% CO₂-95% air atmosphere, 1 μCi of tritiated thymidine was added to each tube. After incubation for a further 18 h, the tubes were processed, and thymidine incorporation was determined in a Tri-Carb liquid scintillation spectrometer.

Results are expressed as net counts per minute, determined by subtracting the counts per minute of
the control tubes from those of the tubes with antigen. A stimulation index (SI) was also obtained by dividing the average counts of tubes with antigen by the average counts of control tubes. Any SI value equal to or greater than 3.5 was, arbitrarily, considered to be indicative of reactivity against the antigen.

**Phytohemagglutinin.** Phytohemagglutinin P (Difco Laboratories) was used at a concentration of 20 μg per tube, when feasible, as an index of lymphocyte function. The tubes were processed after 72 h of incubation and a 6-h pulse of tritiated thymidine.

**Antigens.** (i) **Antigen A.** Epimastigotes of *T. cruzi* strain Y, kindly supplied by A. Velazquez of the Institute of Tropical Medicine, Central University of Caracas, were cultured in medium 199 plus 2% fetal calf serum, separated by centrifugation, washed twice with Hanks solution, resuspended at a concentration of 30 × 10^6 per ml, and then autoclaved for 10 min at 15 lb/in^2^. A 0.05-ml volume of this preparation per lymphocyte culture tube was used.

(ii) **Antigen B.** The same procedure as for antigen A was followed, except that the epimastigotes were freeze-thawed 10 times and centrifuged at 650 × g for 15 min. The supernant was withdrawn and used as the antigen at a concentration of 75 μg of protein per tube.

(iii) **Antigen TCS (T. cruzi sediment).** The sediment of antigen B was resuspended in phosphate-buffered saline and used at a concentration of 75 μg of protein per tube.

(iv) **Heart antigen.** To prepare heart antigen, young rats were sacrificed and the hearts were removed. Both atria and valve were removed, and the remaining ventricular mass was freeze-thawed 10 times and, after the last thaw, left to sediment for 15 min. The supernatant was collected and used as the antigen at a concentration of 100 μg per lymphocyte culture tube.

The protein concentrations of the antigen preparations were determined by a modification of the method of Lowry et al. (5).

**Statistical methods.** A statistical analysis was performed by the chi-square and Mann-Whitney U tests.

**RESULTS**

**Response to *T. cruzi* antigens.** (i) **SI to antigen A.** A total of 86% of the patients in group I, 78% in group II, and only 5.9% in group III gave positive (SI, 3.5) responses to the *T. cruzi* antigen A preparation (Table 1). An interesting observation is that the SI tended to be higher (although not statistically significant) in group I than in group II. This observation was also true for each of the *T. cruzi* antigens tested.

(ii) **SI to antigen B.** Against antigen B, 72% of the patients in group I, 67% in group II, and 0% in group III exhibited positive SIs.

(iii) **SI to antigen TCS.** A total of 80% of the patients in group I, 80.5% in group II, and 0% in group III were positive for antigen TCS.

Considering positive responses to any one of these *T. cruzi* antigen preparations, 96% of group I, 90% of group II, and 5.9% of group III patients showed positive responses.

A statistical analysis of the net counts per minute in response to the various *T. cruzi* antigen preparations is reported in Table 2. No significant differences were found when the net counts per minute to antigens A, B, and TCS of group I were compared with those of group II, probably due to the great individual variation in the response. Responses in group III, however, were much lower.

To evaluate any possible difference in the base lines of the groups studied, the counts per minute in the control tubes for groups I, II, and III were compared; no difference was demonstrated (775 ± 586, 1,506 ± 1,817, and 1,340 ± 1,437 cpm for groups I, II, and III, respectively).

**Response to heart antigens.** Positive SIs to heart antigens were obtained in 28.6% of the patients in group I, 25% in group II, and 0% in group III (Table 1). The higher incidence of reactivity in patients of groups I and II than in the controls was statistically significant. These findings are particularly relevant when it is considered that patients with different forms of non-Chagasic cardiopathy were included in the control group to evaluate the presence of any non-specific responses.

**Table 1. SIs to *T. cruzi* and heart antigens**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% +</td>
<td>SI</td>
<td>n</td>
</tr>
<tr>
<td>A</td>
<td>85.7±</td>
<td>18.8 ± 17</td>
<td>27</td>
</tr>
<tr>
<td>B</td>
<td>72.0±</td>
<td>12.7 ± 13</td>
<td>25</td>
</tr>
<tr>
<td>TCS</td>
<td>80.5±</td>
<td>17.2 ± 22</td>
<td>25</td>
</tr>
<tr>
<td>A or B or TCS</td>
<td>96.4</td>
<td>6.4 ± 3.5</td>
<td>27</td>
</tr>
<tr>
<td>HA</td>
<td>28.6±</td>
<td>6.4 ± 3.5</td>
<td>27</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation.
+ P < 0.001.
* Percentage of patients positive to at least one of the *T. cruzi* antigen preparations.
+ HA, Heart antigen.
+ P < 0.005.
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S5 and net counts per minute against heart antigens were found to be significantly different between groups I, II, and III. A statistical analysis was conducted using a two-tailed t-test, and the results are presented in Table 2.

Table 2. Statistical analysis of net counts per minute

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Group I vs group II</th>
<th>Group I vs group III</th>
<th>Group II vs group III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Net cpm</td>
<td>Net cpm</td>
<td>Net cpm</td>
</tr>
<tr>
<td></td>
<td>(mean)</td>
<td>(mean)</td>
<td>(mean)</td>
</tr>
<tr>
<td></td>
<td>group I</td>
<td>group II</td>
<td>group I</td>
</tr>
<tr>
<td>A</td>
<td>12,598</td>
<td>14,080</td>
<td>6,123</td>
</tr>
<tr>
<td>B</td>
<td>6,074</td>
<td>7,984</td>
<td>10,102</td>
</tr>
<tr>
<td>TCS</td>
<td>12,647</td>
<td>20,239</td>
<td>12,650</td>
</tr>
<tr>
<td>HA</td>
<td>2,203</td>
<td>5,711</td>
<td>3,961</td>
</tr>
<tr>
<td>PHA</td>
<td>46,043</td>
<td>37,256</td>
<td>41,166</td>
</tr>
<tr>
<td>Control</td>
<td>775</td>
<td>586</td>
<td>1,340</td>
</tr>
</tbody>
</table>

* SD, Standard deviation.
* NS, Not significant.
* HA, Heart antigen.
* PHA, Phytohemagglutinin.

Discussions:

1. **Response to phytohemagglutinin.** No significant difference in the response to this mitogen was observed in any of the groups studied (Table 3).
2. **Autoantibodies to heart antigens.** This study further confirmed that autoantibodies to heart antigens exist in the chronic phase of Chagas' disease, as previously reported.
3. **Autoimmunity in Chagas' disease.** The presence of autoantibodies to heart antigens suggests that autoimmune processes may contribute to the pathogenesis of Chagas' disease, particularly in the chronic phase.

In conclusion, the study demonstrated that autoantibodies to heart antigens are prevalent in the chronic phase of Chagas' disease, highlighting the importance of considering autoimmune mechanisms in the disease.
It should be noted that the majority of patients in group I (71%) did not demonstrate lymphoblastogenic responses to the heart antigens. In fact, in our studies we found a lower percentage of patients who were reactive to heart antigens than did Texeira et al. (8). Those authors reported, using the leukocyte migration inhibition test with heart antigens, that all seven patients studied (two acute cases, two Chagasic cardiomyopathy patients, and three Chagasic patients without clinically apparent heart disease) were positive. This was interpreted as providing evidence for the presence of lymphocyte-mediated reactivity to heart antigens in all patients, irrespective of their clinical status. The difference between these results and ours may be due only to the small sample size used by Texeira et al.; however, it is also possible that the leukocyte migration inhibition test is more sensitive in detecting damage to the heart. It is also possible that its specificity for heart damage and its capacity to detect an active disease are low. This latter possibility is in agreement with the results of Wartenberg and Brostoff (10), who showed that leukocyte migration inhibition with heart antigens in patients with coronary disease or myocardial infarction is positive and that this positivity persists long after infarction. The positive results were interpreted as being due to a response to heart damage and nonspecific for the heart; the heart fraction responsible for this reactivity was mitochondrial, and other tissue mitochondria produced the same response. It is therefore important to note that the heart fraction used by Texeira et al. also contains mitochondria (7). It should be stressed that, to eliminate any factor that could be attributed to a nonspecific response to tissue injury or other causes, we included patients with different types of heart disease, including myocardial infarction, in our control group. None of these was positive for the heart antigen used. Consequently, it is possible that lymphocyte proliferation measures a different parameter, or is more heart specific, than the leukocyte migration inhibition test and may reflect an active autoimmune process more than a past injury.

Texeira et al. (8) also evaluated the cytotoxic effect of lymphocytes against heart cells, but this was performed with a purified T-lymphocyte population, which may not have included a series of regulatory factors of the immune response (2).

An interesting finding in our study was that the mean SI to T. cruzi antigens, although positive in both groups I and II, was higher in group I, suggesting a more intense immune response in patients with Chagasic cardiomyopathy. This observation, together with the higher net counts per minute to T. cruzi antigens in the patients with a positive SI to heart antigens in group I, is suggestive of the participation of the phenomenon of delayed hypersensitivity in the pathogenic process that produces Chagasic cardiomyopathy.

According to the reported data, lymphocyte blastogenesis could be a sensitive indicator of low-grade or incipient heart disease. Alternatively, lymphocyte blastogenesis to heart antigens may be irrelevant to the disease process.

It appears, therefore, that a significant percentage of Chagasic patients have positive lymphocyte blastogenesis to heart antigens; however, the significance of this response in the pathogenic process needs to be carefully assessed and defined in a longitudinal study.

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LITERATURE CITED


