Immunopathology of Experimental Chagas’ Disease: Binding of T Cells to Trypanosoma cruzi-Infected Heart Tissue

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The immunopathology of Chagas’ disease was studied in the experimental model of chronic infection in C57BL/10JTor mice. Sublethal infection with Trypanosoma cruzi, Y strain, induced specific antibodies and a delayed hypersensitivity response to parasite antigens. Mice developed chronic chagasic myocarditis but not skeletal muscle myositis. Binding of T cells to infected heart tissue was investigated during short-term cocultivation of lymphocytes with heart cryostat sections. T cells from infected mice and from normal controls bound equally to myocardium and liver sections from both infected and normal mice. A search in depth was attempted with cells heavily tagged with 99mTc. Labeled T cells from chagasic mice bound to both normal and infected myocardium slices. 99mTc-labeled T cells from controls gave the same binding values. Glass-adherent spleen cells behaved identically to T cells. Prior treatment of the tissue with serum from chronically infected mice did not increase the number of binding cells. Peritoneal macrophages tagged with 99mTc-sulfur colloid also bound to infected myocardium slices. The binding of macrophages was not changed by pretreatment of infected tissue with anti-T. cruzi antibodies. In short, this study did not detect any population of T cells or macrophages which could bind specifically to infected heart tissue to initiate an autoreactive process.

Chagas’ disease is a serious problem that affects millions of people in Latin America, where it is endemic. This disease is caused by Trypanosoma cruzi, an intracellular hemoflagellate protozoan transmitted by triatomine bugs, during blood transfusion, or at birth. Both in humans and in experimental models, infection produces an acute illness which is followed by a long-lasting chronic phase. In human beings, irreversible lesions of the heart, esophagus, and colon with often undetectable parasite levels (subpatent parasitemia) are the main findings (1).

The histopathology of chronic chagasic myocarditis shows a mononuclear cell infiltrate closely associated with damaged cardiac fibers in the absence of intracellular parasites. In areas of parasite nests, however, slight focal inflammation and light degenerative processes can be seen. This picture led to an assumption that chronic myocarditis could be generated by continuous infection of neighboring cells and that parasites disappear due to the onset of the immune response (27). Studies on experimental infection have suggested that T. cruzi-primed rabbit lymphocytes damage rabbit fetal heart cells in an immunologically cross-reactive fashion (13) and that the same phenomenon could be implicated in the genesis of chronic human chagasic myocardopathy (25). However, heart cells could also be damaged in vivo by the generation of autoreactive T cells due to parasite modification of the host cells (20). Nevertheless, it has not been possible to detect either specificity of the T-cell proliferative response to heart antigen (26) or the proliferation of a possible self-reactive T cell (8). This body of information makes the self-reactivity in Chagas’ disease a matter of controversy.

This work was outlined to study, in genetically defined mice, the binding of T cells or macrophages to infected heart tissue, because this is the first step required for an autoreactivity or antibody-dependent cellular cytotoxicity (ADCC) reaction.

MATERIALS AND METHODS

Mice. Eight-week-old male C57BL/10JTor (B10) mice were obtained from our breeding facilities. They were originally derived from pedigreed pairs obtained from Jackson Laboratories, Bar Harbor, Maine, and maintained by rigid inbreeding in Tor Colonies (S. T. Torres, Universidade Federal Fluminense, Rio de Janeiro, Brazil). Outbred Swiss 55 mice (Sw55/Tor), randomly bred in closed colonies, were also used. They were fed commercial pellets and acidified water ad libitum.

Parasites and infection. The Y strain of T. cruzi was used. Parasites were maintained by serial intraperitoneal passages in Sw55/Tor mice. Bloodstream trypomastigote forms were purified from blood cells by density centrifugation on Ficoll-meglumin iothalmate (density, 1.078) as previously described (17). Parasites were washed twice in RPMI 1640 medium supplemented with 10% fetal calf serum (Microbiologica S.A., Rio de Janeiro, Brazil) and injected subcutaneously into B10 mice. An inoculum containing 5,000 parasites is able to induce a short acute infection followed by a long chronic phase that lasts for more than 90 days in the surviving mice (21).

Evaluation of chronic infection. Mice were killed 3 months after infection, and the blood, the heart, and skeletal muscle samples were collected, minced, fixed in neutral buffered Formalin, and stained with hematoxylin-eosin (HE).

Antibody production against T. cruzi was evaluated by passive hemagglutination with sheep erythrocytes coated with parasite antigen by the glutaraldehyde method (3). Antigen was extracted from epimastigote forms by incubation in sodium bicarbonate (pH 9.6) at 37°C for 3 days. The preparation was cleared by centrifugation (10,000 × g, 4°C, 3588
30 min) and the protein content was determined (1.2 mg/ml) by the method of Lowry et al. (13a). Indirect immunofluorescence tests were carried out on smears of epimastigote forms with fluorescein isothiocyanate conjugates of rabbit anti-mouse immunoglobulin G (IgG) antiserum (Miles Laboratories Inc., Kankakee, Ill.) as the second antibody. Sera from chronically infected mice were pooled for use as the source of immune serum. The titer of anti- T. cruzi antibody used in binding experiments was 1:160.

The delayed-type hypersensitivity (DTH) response to T. cruzi antigen was evaluated by the ear swelling test as described before (22). Mice were challenged intradermally in the ear pinna with 10 μg (10 μl) of parasite antigen with a Hamilton syringe. DTH was measured at 24 and 48 h with a dial gauge micrometer of flat interfaces (Mitutoyo Ind., Brazil, catalog no. 7301). Swelling was calculated by the difference between the thickness of injected ear and the average thickness of uninjected normal ears. Readings were recorded as units of 10^-3 in. Data were analyzed by one-way analysis of variance and t test between means.

**Effector cells.** RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), 10% fetal calf serum (Microbiologica), 2.5% mM L-glutamine (Sigma), 40 μg of gentamicin per ml, and 10 μg of streptomycin per ml was used in all experiments and is referred to hereafter as medium.

Spleen cells from chronically infected B10 mice were allowed to adhere to plastic culture dishes in serial 1-h steps at 37°C to retain most macrophages and dendritic cells. Nonadherent cells were passed through a nylon wool column with a 10-ml eluate volume. Nylon wool-nonadherent cells were used as a source of T cells. The purity of the preparation exceeded 90% Thy.1.2* cells as scored by Thy.1.2 antiserum plus rabbit complement (Cedarlane Laboratories, Ontario, Canada).

Mouse macrophages were obtained by peritoneal washings with 5 ml of medium. Cells were plated on plastic culture dishes for 1 h at 37°C, washed twice to remove nonadherent cells, and scraped gently with a rubber policeman.

**Labeling effector cells.** T lymphocytes were labeled with 99mTc. This isotope was chosen for its ideal tracer properties (100% gamma abundance of 150 keV). The method included prior incubation of cells with a technetium reducing agent to favor isotope binding to cytosol material, since the native technetium (pertechnetate, +7 oxidation state) is highly stable and does not bind to proteins. Spleen cells (3 x 10^7) were then incubated in medium containing 30 μg of stannous glucoheptonate (GHA; Charles E. Frosst & Co., Montreal, Canada) for 15 min at 37°C. The cells were washed once to remove the excess GHA and suspended in 2 ml of medium containing 1 mCi of [99mTc]pertechnetate eluted from a 99Mo,99mTc generator (Mallinckrodt Inc., St. Louis, Mo.). After 30 min at 37°C, cells were washed five times (500 x g, 5 min) in 5 ml of medium and tested. [99mTc]GHA is a harmless product used as a radiopharmaceutical agent for kidney and brain imaging. Labeled cells maintained viability throughout the studies as assessed by trypan blue dye exclusion. Labeling yield was greater than 10% (equivalent to 30,000 cps per 10^6 cells).

Peritoneal macrophages were labeled by 99mTc-sulfur colloid (TcSC). This technique is specific for labeling phagocytic cells, usually yielding about 60% isotope intake (7). TcSC was prepared by heating [99mTc]pertechnetate with sodium thiosulfate as described previously (18). Macrophages (10^7 cells) were incubated in 2 ml of medium with 1 mCi of TcSC at 37°C for 30 min with gentle shaking, followed by five washings as above.

**Binding of T cells or macrophages to heart tissue.** Binding studies were performed in three ways: (i) binding of unlabeled T cells and glass-adherent cells from spleens of infected mice to heart cryostat sections of normal or chronically infected donors in an assay performed with sections treated or not by immune sera; (ii) binding of 99mTc-labeled T cells from normal or chronically infected donors to myocardial slices from normal or chronically infected mice; and (iii) binding of 99mTc-labeled peritoneal macrophages from chronically infected mice to myocardium slices from chronically infected counterparts.

Cocultivation chambers were made on microslides with plastic rings sealed with silicon rubber. Cryostat sections

![FIG. 1. Heart histopathology 3 months after infection of B10 mice with the Y strain of T. cruzi. Sparse mononuclear infiltrate and a pseudocyst of amastigotes (arrow) can be seen in the section. H+E. Magnification, ×100.](http://iai.asm.org/)
was removed, washed in medium, and frozen at −20°C for 2 h. The heart was cut in diagonal slices (6-mm diameter by 1 mm thickness) which were used to fill the bottoms of the tubes. About 10⁷ (1 ml) ⁹⁹ᵐTc-labeled cells were then added, and the tubes were centrifuged at 500 × g for 1 min. When macrophages were tested, heart slices were incubated with 1:10 immune serum at 37°C for 30 min and washed once before cell addition. Reaction tubes were incubated for 3 h in four replicates. The number of heart-bound cells was calculated by counting cells on heart slices after four washings. Results were expressed as percent bound cells. Nonspecific absorption by heart tissue of isotope leaked from effector cells was determined previously by labeling the slice, with the isotope activity equal to that released by effector cells at the time of incubation. Experimental values were corrected by subtracting the nonspecific release value from the original readings.

**RESULTS**

**Development of chronic infection.** Heart histopathology 3 months after infection showed sparse mononuclear cell infiltrate and scanty pseudocystlike nests of amastigote forms in myocardium and adipose tissue (Fig. 1), whereas skeletal muscle myositis was not detected. The cellular immune response to parasites evaluated by the DTH reaction evoked by parasite antigen is shown in Fig. 2 (top). Infected mice showed 27.7 ± 4 units (mean ± standard error) and control mice showed 16.3 ± 2.6 units at 24 h (P < 0.05). The difference between normal and infected mice at 48 h was not statistically significant. The specific IgG antibody titer detected by passive hemagglutination ranged from 1:16 to 1:128 (geometric mean of log-transformed data, 1:40), and that detected by immunofluorescence ranged from 1:40 to 1:640 (mean titer, 1:80) (Fig. 2, bottom).

**Binding of T cells to heart sections.** Tissue-adherent cells were scored in fields at 400× magnification, and the values were arbitrarily ranked in four ranges: <10, 10 to 20, 20 to 40, and >50 cells per 100 fields. Table 1 shows that T cells from infected mice bound to both infected and noninfected heart tissue. In fact, there was a slightly greater binding of these cells to normal tissue than to infected tissue. T cells from both normal and infected mice showed the same pattern of binding. Figure 3 shows T cells bound to myocardium fibers at several points after cocultivation; there were clear modifications of effector cells by the binding, like a

![Fig. 2](http://iai.asm.org/) (top) DTH response to 10 μg of *T. cruzi* antigen in infected mice (solid bars) and control (striped bars). Mice were tested 80 days after infection. Difference at 24 h was significant (P < 0.05, one-way analysis of variance and t test). (Bottom) IgG antibody (Ab) response to parasite by immunofluorescence (IF) and hemagglutination (HA); titer varied from 1:40 to 1:640 and from 1:16 to 1:128, respectively. Heavy bars represent the mean of log-transformed data. 10⁻⁴ pol. is 10⁻⁴ in.

were mounted inside chambers and covered with 0.4 ml of culture medium. T cells (5 × 10⁷ per ml, 0.1 ml) and 0.3 ml of medium were added. *T. cruzi* antiserum (1:10) was added when indicated. Chambers were covered with cover slips and incubated at 37°C for 20 to 24 h. After tests, tissue sections were carefully washed with warm medium, fixed with buffered Formalin for 4 h, and stained with Giemsa-Lennert and HE. Sections were searched throughout at 400× magnification, and binding cells were scored by the number of microscope fields examined as described previously (6). Sections of liver and kidneys were used as controls.

The binding of labeled cells to myocardium slices was carried out in flat-bottomed 3-ml tubes. Each mouse heart

**TABLE 1. Binding of T cells to tissue sections of chagasic and normal mice**

<table>
<thead>
<tr>
<th>Target and donor status</th>
<th>No. of tissue-adherent T cells from:&lt;br&gt;Normal mice</th>
<th>Infected mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>20–40</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Infected</td>
<td>20–40</td>
<td>20–40</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>&gt;50</td>
<td>20–40</td>
</tr>
<tr>
<td>Infected</td>
<td>20–40</td>
<td>20–40</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Infected</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*Infected B10 mice were tested 70 to 80 days after sublethal infection.

*Cell number scored in 100 fields at 400× magnification.
cell-mediated cytotoxicity reaction but lacking specificity. The binding of T cells to liver sections was similarly non-specific (Fig. 3). Renal tissue was not receptive to the adherence of T cells, giving counts of fewer than 10 cells per 100 fields.

**Binding of $^{99m}$Tc-labeled cells to heart slices.** Spleen lymphocytes and peritoneal macrophages were heavily tagged with $^{99m}$Tc. The labeling yield of lymphocytes was equivalent to 30,000 cps per $10^6$ cells or 180 cpm per 100 cells, whereas labeling of macrophages was sixfold greater, on the order of 1,000 cpm per 100 cells. Hence, the binding of at least 200 T cells (0.002% of the cells added to the chamber) or 10 macrophages (0.0001% of the macrophages added) was detectable in the heart slice.

Table 2 shows the number of labeled lymphocytes bound to infected tissue after 3 h of incubation. Lymphocytes from chronically infected mice bound to both infected and non-infected heart slices (18.3 and 19.8% of the total number added, respectively). Nevertheless, lymphocytes from normal mice displayed the same pattern of binding to heart slices (19% for normal and 19.4% for infected myocardium). Macrophages bound to infected tissue in a proportion of 11.3%. Previous sensitization of heart slices with *T. cruzi* antiserum did not increase cell binding.

**DISCUSSION**

*T. cruzi* is an obligate intracellular parasite in the vertebrate host. During the acute phase of Chagas' disease,
TABLE 2. Binding of \(^{99m}\)Tc-labeled T cells and macrophages to myocardium of mice with chronic chagasic cardiomyopathy

<table>
<thead>
<tr>
<th>Cells and mouse status</th>
<th>Mean % binding (\pm SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal myocardium</td>
</tr>
<tr>
<td>T cells</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>19.0 (\pm 2.7)</td>
</tr>
<tr>
<td>Infected</td>
<td>19.8 (\pm 1.3)</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>ND</td>
</tr>
<tr>
<td>Infected plus immune serum</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{99m}\)Tc were labeled with \(^{99m}\)TcGHA. Macrophages were labeled with TcSC.

ND, Not determined.

parasites invade the mononuclear phagocyte system (reticulotropism) and muscular tissue (myotropism). There is intense parasitism in the macrophage-dominant zone (red pulp and marginal zone) of the spleen (14). Lymphocyte depletion follows simultaneously with parasitization of sites in the spleen (24). Polyclonal B- and T-cell activation has been implicated in the exhaustion of B- and T-cell potential and immunosuppression found in the acute phase of the disease (5, 15). The acute disease usually lasts about 1 month in the mouse and 2 months in humans. Afterwards, due to the immune response built against the parasite, parasitism becomes subpatent, and the chronic phase, consisting of long-term myocardopathy, begins. In humans, the immune response appears to be somewhat effective since only 15 to 20% of infected people develop cardiopathy, but in mice the experimental infection seems to be more serious. Although some studies with inbred mouse strains defined as susceptible or resistant to high \(T. cruzi\) inocula had shown differences in the mortality rates and in the development of the chronic phase (2, 28), studies with very low inocula have shown high mortality in resistant strains as well (16). The study presented here, with a mouse strain relatively resistant to \(T. cruzi\), showed compromised cardiac tissue with sparse nests of amastigote forms 3 months after infection, which indicated evolution to the chronic phase. Specific IgG antibody response to the Y strain of parasites was high compared with previous results with either the same parasite strain and a B10 congenic mouse strain (19) or the same mouse strain but the Peruana, 21SF, and Colombiana strains of \(T. cruzi\) (2). A significant DTH response to parasite antigens indicated that mice were able to build a specific cellular immune response that persisted in the chronic phase, overcoming the immunosuppression that takes place during the onset of the disease (4).

Striking features of the immune response to intracellular parasites have been revealed recently. It has been demonstrated in the murine model of listeriosis that effective immunity results from the interaction of CD4\(^+\) helper-inducer and CD8\(^+\) cytolytic T cells (9). In the antituberculous immune response, the double role of these cells has also been implicated in the efficacy of host defense (10). Furthermore, CD8\(^+\) T cells are involved in protection against several intracellular pathogens including \emph{Bacteroides fragilis}, \emph{Leishmania} spp., \emph{Mycobacterium leprae}mum, \emph{Rickettsia} spp., \emph{Brucella abortus}, \emph{Theileria parva}, and malaria plasmodia (reviewed in reference 10). Evidence for a possible role of cytolytic T cells in immunity to \(T. cruzi\) was first provided by an in vitro assay in which infected fibroblasts were lysed by lymphocytes from immune mice (12).

There are some reports suggesting the participation of cytolytic lymphocytes in the generation of heart damage in chagasic hosts, involving self-reactivity induced by cross-reaction between common antigens on myocardium fibers and \(T. cruzi\) (6, 23, 25). All of these works used an allogeneic system to assess cell-mediated cytotoxicity or lymphocyte binding to infected tissue but this system may induce allo-reactive response of \(T. cruzi\)-specific T cells (8).

The present work, using a syngeneic system of a mouse strain classified as a good responder for cellular immunity, shows that the binding of T cells to infected heart tissue occurs nonspecifically and in low numbers. Other studies also did not detect a significant specific T-cell response against heart antigens (8, 26). Although low proliferative responses of antiheteroautoreactive T cells have been detected in chronically infected mice, this activity seemed to occur as a consequence of the injury induced in cardiac fibers by the intracellular parasitism (8). Recent reports have shown ADCC mediated by sera and spleen cells from chronically infected mice against cardiocytes in vitro (13). The present study, however, did not detect a significant number of spleen cells or peritoneal macrophages able to bind specifically to infected heart tissue to initiate autoimmune phenomena or an ADCC reaction.

The information acquired in the last few years about the immune response to intracellular parasites and about the pathogenesis of \(T. cruzi\) infection suggests a possible involvement of cytolytic cells in immunity to \(T. cruzi\) but has not resolved the controversy about tissue damage in Chagas' disease. The elucidation of the exact role of these cells in the generation of effective immunity, long-term disease, and lesions is crucial for developing specific vaccines. The hypothesis that Chagas' disease would be regulated and self-sustained by autoimmunity has hindered the search for a reliable vaccine. By the number of reports on this matter, there has been considerable effort to demonstrate an autoimmune phenomenon. However, if such an established theory is incorrect (reviewed in reference 11), much time is being wasted with misinterpretations.

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LITERATURE CITED

