Myocardial Changes in Acute *Trypanosoma cruzi* Infection

Ultrastructural Evidence of Immune Damage and the Role of Microangiopathy

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Histological and ultrastructural studies of the hearts of dogs sacrificed 18 to 26 days after intraperitoneal inoculation with 4 × 10⁵ blood forms of the 12 SF strain of Trypanosoma cruzi/kg of body weight disclosed myocarditis characterized by parasitic invasion of some myocytes, damage and necrosis of nonparasitized myocytes, and interstitial infiltration by mononuclear cells. Nonparasitized myocytes showed alterations ranging from mild edema to severe myocytolysis. These changes often were accompanied by contacts of myocytes with lymphocytes (both granular and agranular) and macrophages. These contacts were characterized by focal loss of the myocyte basement membrane and close approximation of the plasma membranes of the two cells. Contacts between lymphocytes and capillary endothelial cells were also frequent. Platelet aggregates and fibrin microthrombi were observed in some capillaries. Our findings suggest that immune effector cells play a major role in the pathogenesis of the myocyte damage and the microangiopathy in acute Chagas' disease. (Am J Pathol 1994, 144: 1403–1411)

Myocarditis is the most important finding in acute infection caused by *Trypanosoma cruzi*. The pathogenesis of the myocarditis caused by acute infection with *T. cruzi* is not fully understood. Parasite-related fac-

tors are certainly involved. Multiplying intracellular forms of the parasites (amastigote forms) can provoke rupture of the myocytes and the disintegrating parasite and host cell products can induce inflammation, which can be amplified by the host immune system. However, although parasite-related lesions remain focal or multifocal within several organs where parasites may localize and multiply, the changes in the heart tend to become diffuse, more severe than elsewhere, and out of proportion to the number of parasites locally present.[1]

In addition to inflammation, changes suggestive of ischemic injury to the heart muscle also are usually present. These lesions can be detected electrocardiographically. Variable degrees of T wave and ST segment alterations and low QRS voltage are frequently present. Changes suggestive of acute myocardial infarction also have been documented in both humans[2] and experimental animals.[3] Morphologically, focal degenerative changes and necrosis of nonparasitized myocytes are prominent features.[4,5] They can play a key role in inducing myocardial failure and mortality. In more commonly observed nonfatal cases, necrosis involving the conducting system and intracardiac ganglion cells may lead to serious consequences long after the acute lesions have subsided.[6]

Possible causes of the myocardial injury associated with acute Chagas' disease include: 1) immunological injury after adsorption of *T. cruzi* antigens on nonparasitized cells,[7] 2) ischemic injury due to platelet aggregation and obstruction of myocardial capillaries,[8,9] and 3) direct or antibody-mediated cytotoxic damage by inflammatory and immune effector cells,

including lymphocytes, neutrophils, eosinophils, macrophages, and mast cells.[10-16]

During the course of ultrastructural studies of the hearts of dogs with experimental *T. cruzi* infection, we have observed interaction of immunologically competent cells with endothelium and myocytes. These findings indicate a complex damage mechanism involving both parasite and host factors and suggest that immune-mediated cellular injury plays a major role in inducing damage and necrosis of nonparasitized cells. A description of these observations forms the basis of the present report.

Materials and Methods

Eight mongrel dogs weighing 1000 to 1900 g were used. They were 2-months old when they were inoculated intraperitoneally with mouse blood containing *T. cruzi* trypomastigotes of the 12 SF strain. The inoculum totalled 4×10^5 blood forms of trypanosomes/kg of body weight. Parasitemia was evaluated weekly. Three dogs were sacrificed on the 18th day, 2 on the 21st day, and 3 on the 26th day after inoculation. At the time of sacrifice, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital. Blood was then collected for the indirect immunofluorescence tests for the detection of *T. cruzi* antibodies, and an ECG was obtained. Pre-inoculation ECGs and blood collection were made in all animals.

Complete autopsies were performed on each dog. Small samples from the right atrium, the walls of the right and left ventricles, and the upper muscular part of the ventricular septum were immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 hours then washed several times in buffer and postfixed with 1% osmium tetroxide in 0.15 M cacodylate buffer. After dehydration, the tissues were embedded in polybed resin. One-micron thick sections were stained with toluidine blue and studied by light microscopy to select areas for ultrathin sectioning. The ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a JEOL 1200 EX electron microscope at 60 kV.

The remainder of each heart was fixed in 10% buffered formalin. Selected blocks were embedded in paraffin. Sections of these blocks were stained with hematoxylin and eosin (H&E) Giemsa, and the periodic acid methenamine-silver (PAM) method of Yajima and Aihara[17] for the demonstration of basement membranes.

Results

Clinical and Gross Anatomical Findings

All dogs had serum antibodies against *T. cruzi* and in all of them parasitemia was demonstrated at the time of sacrifice. Moderate bradycardia, first degree AV block, inversion of T waves, and nonspecific ST segment alterations were the main electrocardiographic changes found. There was no clinical or pathological evidence of congestive heart failure in any of the animals, although a small amount of ascitic fluid and/or a mild pericardial effusion were observed in a few of them. The hearts were flabby but not enlarged. The chambers were free of thrombi.

Light Microscopic Findings

Moderate to severe, diffuse myocarditis was present in all animals and was most evident in sections taken from the right atrium, free wall of the right ventricle, and upper portion of the ventricular septum. Multiplying forms of *T. cruzi* were rarely observed within myocytes. Inflammatory cells were predominantly macrophages and lymphocytes. Plasma cells, mast cells, neutrophils, and eosinophils were seen in small numbers. Lymphocytes and macrophages were frequently in close contact with damaged myocytes, sometimes surrounding fragments of cells. These contacts involved ordinary myocytes (Figures 1 and 2) and cells of the AV conducting system (Figure 2). Some of the myocytes were dissociated and the interstitial tissue was edematous. Groups of myocytes sometimes had disappeared from focal areas and replaced by accumulations of inflammatory cells,
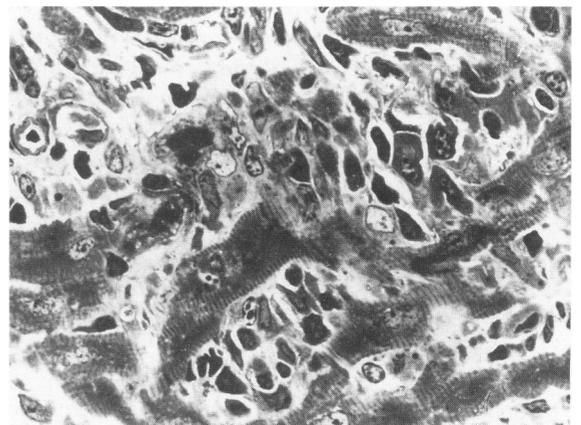

Figure 1. *Light micrographs showing representative aspects of acute Chagas' myocarditis in the dog. View showing the intimate association of mononuclear cells with damaged myocytes. H&E stain. (×200).*
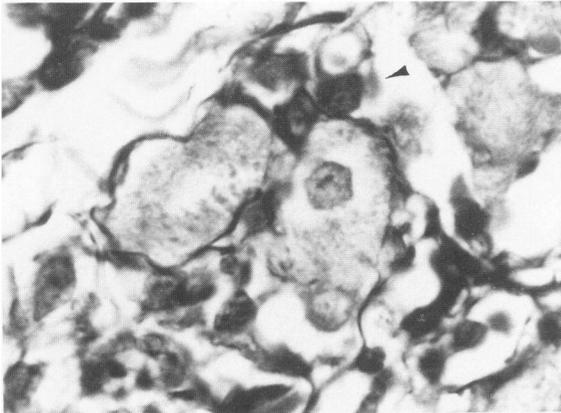
Figure 2. *Section of atrioventricular node stained with the periodic acid-methenamine silver method for demonstrating basement membranes. A lymphocyte (**arrowhead**) is in close contact with the basement membrane of a nodal myocyte (×400).*

among which tiny remnants of myocytes were observed. The basement membranes of myocytes and vascular structures were clearly demonstrated in preparations stained by the PAM method. Close contacts between mononuclear cells and myocytes were also evident in these preparations (Figure 2).

Ultrastructural Findings

In myocytes containing *T. cruzi* amastigotes, the parasites pushed aside the contractile elements and the mitochondria, but these structures appeared well preserved (Figure 3). Inflammatory cells were present in the vicinity of the parasitized cells but were not in close contact with them.

Degenerative and necrotic changes were frequently observed in nonparasitized myocytes (Figure 4). Zones of lytic necrosis of myocytes were adjacent to myocytes exhibiting relatively well preserved

structures. Myocytes in far advanced stages of disintegration, with a few barely visible Z bands and intercalated disks and a few mitochondria, were adjacent to normal myocytes showing good preservation of myofibrils, mitochondria, T tubules, and sarcoplasmic reticulum. A few inflammatory cells were also seen between preserved myocytes (Figure 4). In myocytes showing mild to moderate degenerative changes, the mitochondria were the most normal-appearing organelles, rarely showing swelling and distortion of the cristae. Focal dilatation and vesiculation of T tubules and sarcoplasmic reticulum were frequently seen in such cells, often in association with fragmentation, clumping, or lysis of myofibrils. The intercellular junctions of myocytes often were a preferential site of damage with widening of the intercellular space and dissociation of the desmosomal attachments. Other milder and more frequent changes included increased numbers of lipid droplets and lysosomes, formation of myelin figures, and deposition of lipofuscin granules in the perinuclear areas. The sarcolemma had a wavy appearance, with frequent subsarcolemmal vesicles. Zones of myofibrillar hypercontraction were often evident.

Lymphocytes and macrophages were frequently in close apposition to myocytes. The contact between a lymphocyte and a myocyte (Figure 5) often involved multiple areas of approximation of the plasma membrane of the two cells. Several lymphocytes frequently made contact with a single myocyte. Large granular lymphocytes were characterized by their content of small (<0.5 µ in size) cytoplasmic granules that were composed of moderately electron dense material and limited by single membranes. Small lymphocytes had small rims of cytoplasm in which few organelles were present. The nuclei of large granular lymphocytes
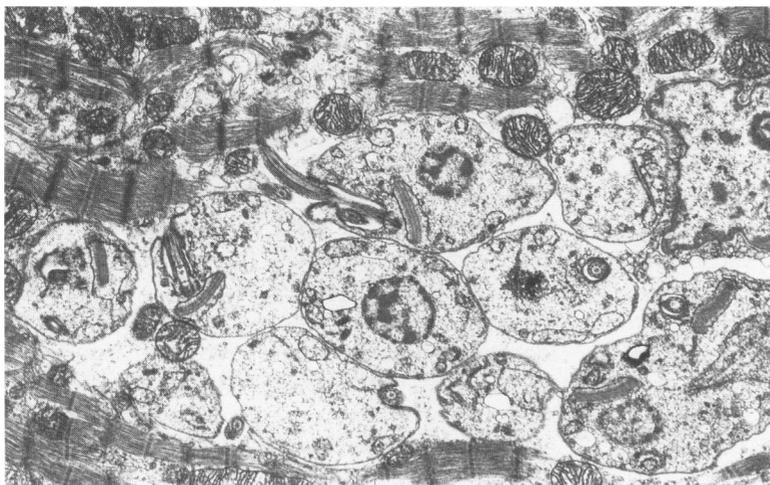

Figure 3. *Electron micrograph of parasitized myocytes that contains many amastigotes. Mild intracellular edema and mitochondrial swelling are present (×6000).*
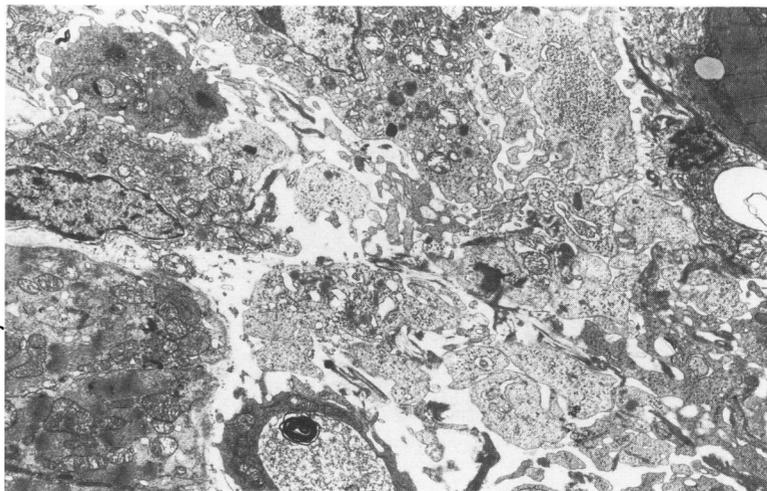
Figure 4. *Area of focal necrosis involving several myocytes, some of which are associated with macrophages and lymphocytes and with deposits of fibrin-like material in the interstitium (×4000).*
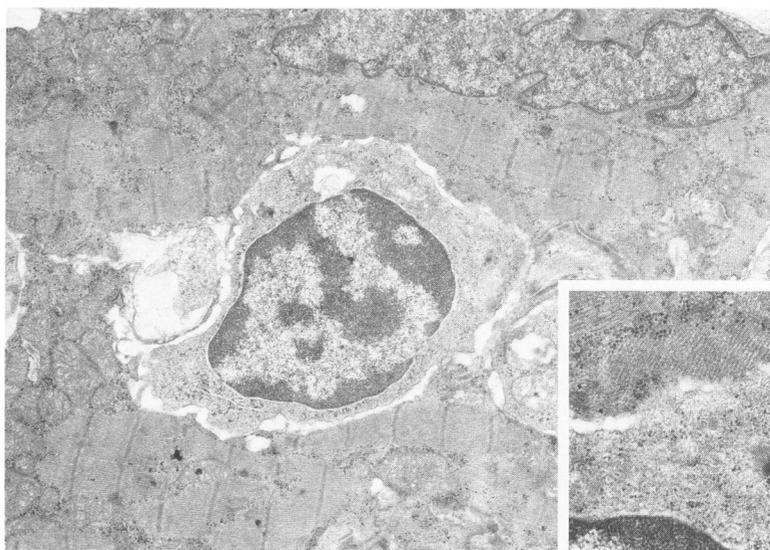

Figure 5. *Two myocytes show deep cytoplasmic indentations at points of contact with an agranular lymphocyte. In some of the points of contact between these cells (inset) there is myofibrillar damage and focal dissolution of the basement membrane (×5,000, inset, ×12,000).*

were large and indented, whereas those of small lymphocytes were round and contained densely packed chromatin. Both small and large granular and agranular lymphocytes were involved in these contacts. Large granular lymphocytes sometimes had extensive areas of contact with the myocytes, projecting deeply into indentations of myocyte cytoplasm. The basement membranes of myocytes often were focally damaged or absent at the points of contact with lymphocytes. Small fragments of disintegrating myocytes were seen within phagocytic vacuoles in the cytoplasm of macrophages. Portions of myocytes frequently appeared to be isolated and were surrounded by numerous filopodia of macrophages. In some points of contact with the filopodia of macrophages, the myocytes showed vacuolization and focal damage of membranes (Figure 6). Cell-to-cell

attachments consisting of subplasmalemmal linear densities were observed in some of the macrophages. These structures appeared similar to those that have been described in macrophages in other tissues.[18]

The myocardial capillaries were dilated and the endothelial cells showed evidence of increased pinocytosis. Both large granular and agranular lymphocytes were seen within the capillary lumina with multiple points of adhesion to the surfaces of the endothelial cells (Figure 7). Sometimes such attachment coexisted with severe degenerative changes of the endothelial lining with cytoplasmic swelling, mitochondrial destruction, and vesicle formation (Figure 8). Capillaries containing aggregated clumped platelets and fibrin thrombi were observed adjacent to others in which the lumina were widely open (Figures 9
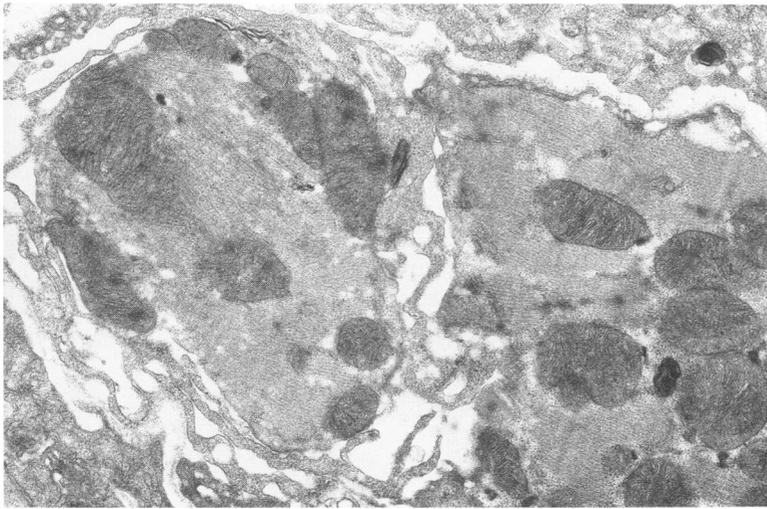
Figure 6. *A portion of a damaged myocyte is surrounded by numerous filopodia of macrophages. Several areas of membrane-to-membrane contact are coincident with zones of focal damage of the myocyte (×36,000).*
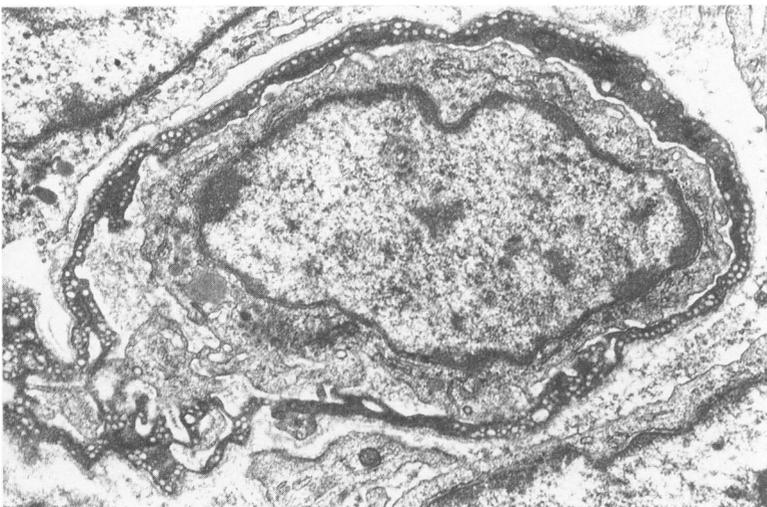

Figure 7. *A large lymphocyte completely fills the lumen of a capillary. There are focal areas of close membrane-to-membrane apposition between the lymphocyte and the endothelial cells. The latter exhibit increased numbers of pinocytotic vesicles. The lymphocyte has an indented nucleus and some granules concentrated in one pole of the cell (×60,000).*

and 10). Segments of myelinated and nonmyelinated nerves were found in the edematous connective tissue of the myocardium, sometimes in close proximity to inflammatory cells. Changes in these structures were mild and consisted of swelling of Schwann cells and increased sizes and numbers of dark granules and vesicles.

Discussion

The dog is an excellent model for the study of the pathology of Chagas' disease.[3] As in humans,[19] the infection in the dog is accompanied by severe myocardial involvement, even when the intracellular parasites are not numerous. The mechanisms by which acute myocarditis develops in Chagas' disease remain uncertain. The results of this study strongly suggest that much of this acute damage is caused by immunological phenomena and is mediated by contacts between immune effector cells and cardiac myocytes and endothelial cells. In agreement with previous reports,[4] the observations in this study show that the degree of cardiac damage is far greater than can be accounted for on the basis of parasitic invasion of the myocytes. The myocarditis is characterized by mononuclear cell infiltration of the interstitial tissues and small focal areas of myocyte damage and necrosis. The initial lesions in myocytes consist of swelling and disruption of the T tubules and the sarcoplasmic reticulum and lysis of the myofibrils but with relatively mild changes in the mitochondria. These lesions progress to eventual, complete necrosis of the myocytes. The cytotoxic nature of many of the lesions observed in this study is suggested by

Figure 8. *Two lymphocytes are attached to the endothelial lining of a venule. The endothelial cells appear severely damaged, with cytoplasmic edema and vacuolization and mitochondrial swelling and cristolysis (×4000).*

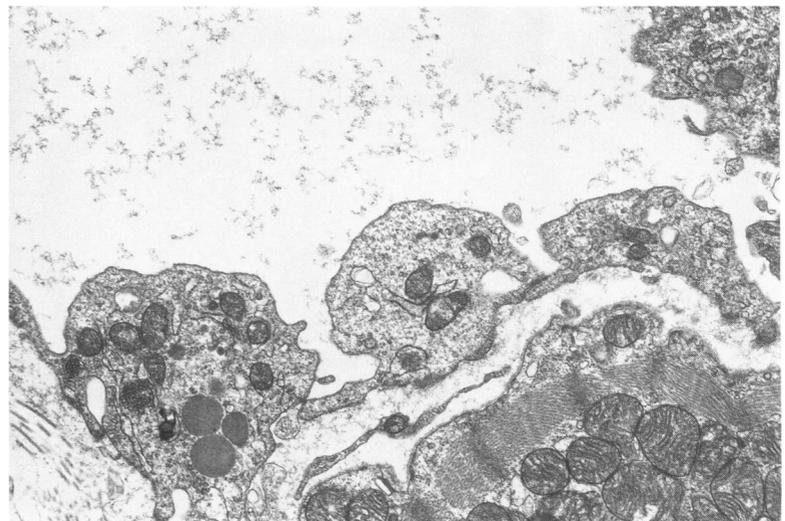Figure 9. *Dilated capillary contains aggregate of platelets (×24,000).*

their coincidence with membrane-to-membrane contacts between mononuclear inflammatory cells and cardiac myocytes and endothelial cells. Such contacts involved not only large granular lymphocytes but also small agranular lymphocytes and macrophages.

Lymphocyte-Myocyte Contacts

The structural features of the contacts between lymphocytes and cardiac myocytes found in this study are similar to those described by Deaguchi et al[20] in the initial stages of myocarditis produced in mice by infection with Coxsackie B3 virus. These findings led to the concept that two mechanisms are responsible for the initial tissue damage in this viral myocarditis: cytopathic effects induced by the invasion of myocytes by the virus and cytotoxic damage caused by

immunological events induced by the viral infection. This concept was supported by the subsequent identification of many of the contacting lymphocytes as natural killer cells,[21] and by the immunohistochemical demonstration of perforin in these cells.[22] Perforin, a potent cytolytic factor, is a component of the cytoplasmic granules of granular lymphocytes. When these lymphocytes recognize and contact a target cell they release perforin, which induces the formation of pore-like defects on the plasma membrane of the cell, thus mediating its damage or lysis.[23]

A similar type of membrane damage can result from complement-mediated reactions.[24] Contacts between large granular lymphocytes or small, cytotoxic/suppressor T lymphocytes and cardiac myocytes also have been described in the myocarditis produced in rats by the administration of large
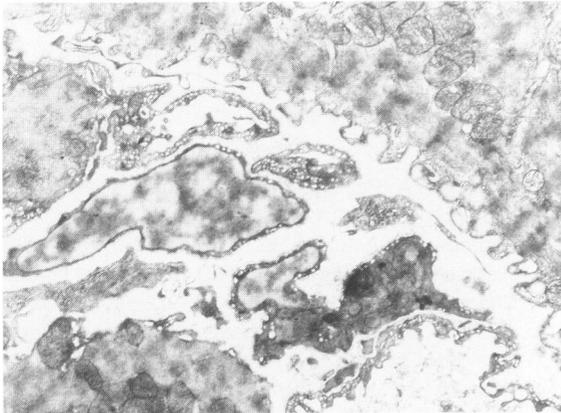
Figure 10. *A dark material with irregular densities fills the lumen of a capillary and probably represents a fibrin thrombus altered by fibrinolysis (× 4000).*

doses of interleukin-2 (IL-2).[25] The finding of lymphocyte-myocyte contacts in this study is in accord with a wealth of immunological evidence indicating the occurrence of a cell-mediated delayed type of hypersensitivity reaction in acute *T. cruzi* infection.[26–28] In this study, we found that parasitized myocytes were very few, and that nonparasitized myocytes in areas of myocardial damage were frequently in contact with lymphocytes and macrophages. It is possible that the surfaces of nonparasitized myocytes adsorb antigenic materials released from parasitized cells, thereby becoming the targets of immune effector cells. Furthermore, other studies have suggested that glycoprotein components of the cell surfaces of *T. cruzi* amastigotes elicit the formation of antibodies that can cross-react with glycoproteins normally present in the basement membranes of cardiac myocytes.[29–31]

The immunological characteristics of the lymphocytic infiltrate in acute Chagasic myocarditis is of special interest. The preliminary results of our immunohistochemical studies (using antibodies obtained from Dr. D. Gebhard, North Carolina State University) show that both CD4+ and CD8+ T lymphocytes form contacts with myocytes and endothelial cells. Unfortunately, no immunohistochemical method is available for the identification of natural killer cells in canine tissues (in contrast to the GM$_1$-ganglioside antibody, which is useful for the detection of these cells in rat and murine tissues).[32] Natural killer cells have the morphological features of large granular lymphocytes; however, the morphology of large granular lymphocytes is shared by some resting T cells and activated cytotoxic lymphocytes. For these reasons, natural killer cells cannot be identified on the basis of their morphology alone. The evidence just

reviewed can be interpreted as indicating that in acute Chagas' disease, as in Coxsackie B3 viral myocarditis, cardiac damage can be produced by invasion of the myocytes by the infecting agent as well as by immunological mechanisms in which noninfected myocytes become the targets of immune effector cells.

Myocyte-Macrophage Contacts

The significance of the macrophage-myocyte contacts observed in this study could be dual. Macrophages not only can produce injury by biochemical and immunological mechanisms, but also can phagocytize damaged myocytes. Other studies have demonstrated myocyte membrane leakage at points of contact with macrophages in acute Chagas' disease.[12] Macrophages are well known to contain large amounts of hydrolytic enzymes that can be released to cause tissue damage when these cells are activated. Activation of macrophages can also result in the release of various cytokines that can then mediate a variety of immunological and biochemical effects on other cells, particularly on T lymphocytes. The role of these interactions in acute Chagasic myocarditis remains to be determined.

Microcirculatory Alterations

Although previous light microscopic studies have described cardiac microvascular alterations in Chagas' disease,[8,9] the pathogenetic mechanism of these lesions has not been clarified. This report provides the first ultrastructural observations on the cardiac microangiopathy in Chagas' disease, and we interpret our findings as indicating that this lesion is a consequence of interactions between lymphocytes and endothelial cells. It seems likely that antigenic stimuli during the acute phase of *T. cruzi* infection lead to the activation of endothelial cells and lymphocytes, with increased expression of various endothelial cell adhesion molecules and increased adherence of leukocytes to endothelial cells and that interaction between these two types of cells results in the release of mediators that cause microvascular damage. This damage is manifested by endothelial cell swelling and degeneration and by the formation of platelet aggregates and fibrin microthrombi. Contacts between endothelial cells and T helper lymphocytes have been found in light microscopic studies of myocarditis caused by cytomegalovirus in mice.[33] Such contacts have been considered to mediate the initial

stages of cardiac damage in this type of myocarditis. Lymphocyte-endothelial cell contacts have been induced in heart, lung, and liver of rats treated with IL-2.[25] In rat skeletal muscle, IL-1, tumor necrosis factor, and endotoxin also induce a similar microangiopathy.[34] The possible toxic role of locally released cytokines in acute Chagasic myocarditis needs to be assessed in detail because of the complex interactions of these agents with lymphocytes and macrophages during the course of cell-mediated immune reactions. Nevertheless, these findings emphasize the importance of endothelial cells as targets of cytotoxic damage, mediated by immunoeffector cells, in myocarditis of various types.

Rossi et al[8] called attention to the presence of platelet aggregates in acute Chagas' myocarditis. It has been suggested that platelets play an effector role against *T. cruzi*. Platelet aggregation occurs *in vitro* around antibody-sensitized *T. cruzi* parasites and causes their lysis. This phenomenon is dependent on platelet C3b receptors.[35] Tanowitz et al[9] found that platelets of mice infected with *T. cruzi* were more sensitive than those of normal mice to aggregation induced by adenosine diphosphate and sodium arachidonate. Thromboxane A_2 levels were also elevated in the plasma of the infected mice. Tanowitz et al[9] also observed multiplying forms of *T. cruzi* within endothelial cells; however, colonization of endothelial cells by parasites has not been reported in dogs and was not observed in this study.

The consequences of cardiac microthrombosis are variable, depending on the circumstances in which they occur. Nonocclusive fibrin microthrombi have been observed in the myocardium of dogs with experimentally induced septic shock in which they do not lead to myocardial necrosis.[36] Infusion of adenosine diphosphate directly into the coronary arteries of pigs induce transient aggregation of platelets and gross cardiac infarcts.[37] In contrast, gross infarction is uncommon in patients with thrombotic thrombocytopenic purpura, despite the widespread presence of thrombi in the microcirculation.[38]

Marin-Neto et al[39] and Tanowitz et al[40] have demonstrated reduced myocardial perfusion in acute and chronic Chagas' disease and considered ischemic necrosis caused by capillary occlusion to be the main lesion in acute Chagas' myocarditis. In our study, damage and necrosis of myocytes always were associated with cellular infiltration. Thus, we observed microvascular lesions during the acute phase of Chagas' disease in dogs but their extent and severity were limited. Such alterations may have contributed to ischemic necrosis of myocytes. However, the evidence observed in the canine model indicates that

the most important cause of myocyte damage in acute Chagas' disease is direct cytotoxic injury.

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