Chagasic Cardiopathy

Immunopathologic and Morphologic Studies in Myocardial Biopsies

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Immunopathologic and morphologic studies at the light and transmission electron microscope levels were carried out in myocardial biopsies of 4 chagasic individuals with circulating antibodies reacting with plasma membrane of striated muscle and endothelial cells (EVI antibody). Two cases did not present clinical evidences of heart involvement, and 2 cases showed chronic heart disease. *In vivo* deposits of immunoglobulins were found at the plasma membrane of working myocardial cells and endothelial cells. The cytologic location of the *in vivo* bound γ-globulin was coincident with the specificity of the EVI antibody. Ultrastructural studies showed intracellular alterations compatible with hypoxia of the fibers; these lesions, although they were more severe in the 2 cases with heart disease, were also present in the asymptomatic individuals. These results are congruent with a possible pathogenic effect of the EVI antibody. In 2 patients with Chagas' heart disease, foci of mononuclear infiltrates were examined by transmission electron microscopy. At that level, a close relationship between lymphocytes and muscle cells was observed, with imbrication of the plasma membranes and disappearance of the basal laminae. In the neighborhood of the lymphocytes, definite muscle cell abnormalities were found. These observations are also congruent with the recently suggested possibility that a lymphocyte-mediated immune response against heart tissue may participate in some of the pathogenetic mechanisms of chronic chagasic cardiopathy. (Am J Pathol 86:533–544, 1977)

ALTHOUGH AUTOIMMUNE PHENOMENA involving the heart were recently observed in both human and experimental Chagas' disease,[1-4] the pathogenetic mechanism of the chronic human cardiopathy is not established at the present time.

In recent studies, an antibody reacting with plasma membrane of striated muscle and endothelial cells (EVI antibody) has been described in Chagas' disease.[1-2] In a more recent report, evidence was presented suggesting that this antibody can interact *in vivo* with skeletal muscle;[3] in addition, EVI-positive chagasic individuals presented morphologic alterations in this tissue.[3]

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On the other hand, the appearance of a lymphocyte-mediated immune response against myocardial cells in experimental Chagas' disease has been recently demonstrated.[4]

In this study, the presence of *in vivo* bound immunoglobulins in myocardial biopsies of 4 EVI-positive chagasic individuals is presented. In addition, morphologic observations which are congruent with the possible participation of a lymphocytotoxic effect on heart tissue in some of the pathogenetic mechanisms of chronic Chagas' heart disease are shown.

Materials and Methods

Myocardial Samples

Patients

Four EVI–positive individuals chronically infected with *Trypanosoma cruzi* were studied. EVI positivity was defined by means of the indirect immunofluorescent technique.[1] All cases fulfilled the epidemiologic and serologic conditions required for the diagnosis of *T. cruzi* infection.[1] Cases 1 and 2 (8 and 39 years of age, respectively) presented an interatrial septal defect, confirmed by hemodynamic and angiocardiographic procedures. Case 2 presented a normal cineangiocardiographic study performed on account of his age. In both cases, no signs or symptoms of left ventricle involvement which could be imputable to Chagas' disease were observed. Atrial appendage and left ventricle samples were obtained during the corrective surgical procedure of their congenital heart diseases. The samples were obtained immediately after the heart was arrested, by means of a cold knife (atrial appendage) or a Vim-Silverman needle (left ventricle), and processed as mentioned below. Interatrial septal defect was selected because this condition does not overload the left ventricle. Cases 3 and 4 (28 and 31 years old, respectively) presented an advanced chagasic cardiopathy with cardiomegaly and severe A-V conduction disturbance. Cold knife samples were obtained from the left ventricle free wall when a subepicardial electrode pacemaker was implanted.

The clinical material was selected in this way in order to have patients with chronic *T. cruzi* infection in whom clinical myocardial involvement is not yet observable, and patients with a well-developed chronic heart disease.

Controls

Atrial and left ventricle biopsies were performed during surgery in a 17-year-old girl from a nonendemic area with an interatrial septal defect. This case presented negative serology for Chagas' disease.

In addition, as control to the indirect immunofluorescence tests, left ventricle samples were also obtained immediately postmortem from 5 individuals without chagasic infection or obvious heart disease. The myocardial samples were carefully divided into three pieces which were employed for immunofluorescent studies, ultrastructural immunochemical procedures, and light and conventional transmission electron microscopy, respectively, as mentioned below.

Direct Immunofluorescence Technique

Two-micron cryostat sections of the myocardial biopsies were washed for 10 minutes in phosphate-buffered saline (PBS), pH 7.2, and directly treated for 40 minutes with the adequate dilution of the fluoresceinated antisera, followed by three washes in PBS.

Biopsies were studied with goat γ-globulin antihuman γ-globulin labeled with fluorescein,[1] employed as previously described,[1-2] and with a fluorescein-labeled goat anti-human β_1C-A antiserum commercially obtained (Hyland Division Travenol, Los Angeles, Calif.); the anti-β_1C-A antiserum was employed diluted 1 : 6. Both antisera were previously absorbed with mouse liver powder and bovine skeletal and heart muscle powder and with A+–B+ human red blood cells.

Tissue sections were also studied after being washed for 2 hours in citrate-buffered saline, pH 3.2;[1] as control, another section was treated during the same time with PBS.

"Blocking" experiments were done with the same goat antiimmunoglobulins antiserum, but unlabeled. It was applied for 40 minutes, prior to applying the labeled one. Readings were peformed with a Carl Zeiss microscope (West Germany), fitted with an epicondenser fluorescence equipment and with phase contrast. Readings were performed by two observers, and the intensity of fluorescence was graded from 1+ to 3+.

Ultrastructural Immunochemical Procedures

The presence of autologous γ-globulin in the myocardial samples was investigated by means of goat antihuman immunoglobulins labeled with peroxidase. The treatment of the tissue sections and labeling of antiserum were performed as previously described.[2]

Transmission Electron and Light Microscopy Procedures

The heart tissue samples were fixed for 6 hours in cold (4 C) 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, rinsed in the same buffer, postfixed in osmium tetroxide, and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope. Thick sections (1 μ) were stained with alkaline toluidine blue and studied by light microscopy.

Results

The immunopathologic findings are summarized in Table 1. It can be observed that *in vivo* bound immunoglobulins were present in the four left ventricle biopsies and in one of the two right atrium biopsies. β_1C-A was present in three of four ventricles and in the atrium with fixed autologous immunoglobulins; the positive staining was of less intensity

Table 1—Clinical Characteristics and Immunopathologic Findings in Myocardial Biopsies of 4 Chagasic Patients

Case No. and age	Diagnosis	Tissue samples obtained	Immunopathologic Studies: D-IFL Ig	D-IFL C	D-USICH Ig
1 (LS) 8 yrs	IASD, chagasic infection	Right atrium	–	–	–
		Left ventricle	2+S, 2+ BV	–	+S, +BV
2 (CB) 39 yrs	IASD; chagasic infection	Right atrium	1+ S, 3+ BV	1+ S, 1+ BV	+S, +BV
		Left ventricle	1+ S, 3+ BV	1+ S, 1+ BV	+S, +BV
3 (RP) 28 yrs	Chagasic cardiopathy	Left ventricle	2+ S, 2+ BV	1+ S, 1+ BV	+S, +BV
4 (CL) 31 yrs	Chagasic cardiopathy	Left ventricle	2+ S, 2+ BV	1+ S, 1+ BV	+S, +BV

BV = blood vessels, D-IFL = direct immunofluorescence, D-USICH = direct ultrastructural immunochemistry, S = sarcolemma, IASD = interatrial septal defect.

(Table 1). The controls did not show *in vivo* bound immunoglobulins or C3. The positive staining was observed in the sarcolemmal area (Figure 1) and distributed in a focal manner. In 1 subject (Case 2) deposits in the sarcolemmal area were extremely focal and of low intensity, but the inner part of the coronary blood vessels showed important deposits (Figure 2). The positive staining could be blocked by preincubation of the sections with the unlabeled goat antihuman γ-globulin antiserum. Treatment of sections with acid buffer resulted in a marked decrease in the intensity of the fluorescence. Control washes with PBS had no effect. Granular deposits of immunoglobulins along heart fibers, as observed in *T. brucei*–infected mice,[5] were not observed in our cases.

Ultrastructural immunochemical procedures confirmed and extended the observations performed by the immunofluorescent technique (Table 1); it was observed that the *in vivo* bound immunoglobulins demonstrable by immunofluorescence methods were located at the plasma membrane of the working myocardial cells and the vascular endothelial cells (Figures 3 and 4); blocking experiments abolished the reaction, and controls performed with peroxidase-labeled anti-rat IgG antiserum or with free peroxidase gave negative results.

In the 2 subjects (Cases 1 and 2) without clinical chagasic cardiopathy morphologic modifications of similar magnitude were found. At the light microscopic level the only alteration consisted of an increase in the amount of lipofuscin granules. The increase was significant, especially if it is considered that in Case 1 the subject was only 8 years old.

With the electron microscope, minor but definite alterations of the left ventricle cells were found. The nuclei, myofibrils, and sarcoplasmic reticulum appeared normal on morphologic basis. The most altered components appeared to be the mitochondria. Almost normal in size and shape, they appeared abnormally distributed within the muscle fiber. Large clusters containing many mitochondria were frequently seen (Figure 5). On the other hand, many myofibrillar areas appeared devoid of these organelles. The mitochondrial clusters were not connected with the perinuclear zone and could be seen at any place of the heart muscle cells. The amount of glycogen was also increased, and discrete areas of sarcoplasm appeared to be occupied by this substance, with no other cell component (Figure 6). The sarcolemma did not show alterations.

In the 2 patients with chagasic cardiopathy (Cases 3 and 4) the light microscopy study showed an increase in the number of lipofuscin granules and the presence of interstitial mononuclear infiltrates. With the electron microscope the heart muscle cell alterations were similar to those found in Cases 1 and 2 but were much more severe. The mononuclear infiltrates

appeared to be composed of macrophages and a large amount of lymphocytes with scarce plasma cells (Figure 7). No polymorphonuclear leukocytes were found.

A remarkable finding was the close relationship observed between the lymphocytes and the myocardial cells. At some places a close adherence of lymphocytes was seen, and occasionally, cell expansions got deeply into invaginations of the myocardial plasma membrane (Figures 8 and 9). At this place, no basal lamina was seen, and between the plasma membrane of both the lymphocyte and the heart cell a narrow space of a few Ångstrom units in thickness was present (Figure 9). In other areas, where no close contact was present between both kind of cells, the lymphocytes presented cytoplasmic expansions in the surface which pointed towards the working myocardial cells. At that level, the latter showed abnormalities of the plasma membrane and subsarcolemmal areas, consisting of swelling with localized cytoplasmic protrusions towards the interstitium (Figure 10).

In none of the cases examined were *T. cruzi* found, either at the light or the electron microscopic levels.

Discussion

In the present report, *in vivo* bound immunoglobulins in left ventricle myocardial biopsies of EVI-positive chagasic individuals were demonstrated. The location of the deposits in the plasma membrane of working myocardial cells and endothelial cells, as observed by ultrastructural immunochemical methods, is coincident with the specificity of the EVI antibody.[2] This observation suggests that the EVI antibody interacts *in vivo* with the target cells of the heart, making it necessary to further consider that this antibody might have a pathogenic effect.[1-2] It is interesting to note that although EVI antibody fixed C *in vitro*,[1] *in vivo* bound C3 was observed with a lesser intensity and, in 1 case, was absent. These findings are similar to the observations performed in the skeletal muscle of EVI-positive chagasic individuals.[3]

The possible mechanism of tissue damage by EVI antibody cannot be deduced with the present evidence. The morphologic observations suggest metabolic alterations of the fibers, as those reported in chronic hypoxia, in both experimental conditions and human diseases.[6] Since immunoglobulins are bound to the plasma membrane of the myocardial and endothelial cells, it appears reasonable to speculate that they might interfere with some of the transmembrane difusion and transport processes.

On account of the presence of *in vivo* bound γ-globulin and C, an

immunologically mediated inflammatory reaction with a consequent deterioration of the heart muscle fibers could be expected. However, polymorphonuclear cell exudates, which could be present through the activation of the C system,[7-8] were not observed in our cases; the limitations which emerge on account of the small quantity of biopsy tissue examined should be considered.

Lymphocytic infiltrates are classically described in the myocardium of individuals who died of a chagasic cardiopathy.[9] However, the exact significance of these lymphocytes is not clearly established.

Recently, Santos-Buch and Teixeira demonstrated that normal rabbit heart cells in culture can be destroyed with sensitized lymphocytes obtained from rabbits inoculated with *T. cruzi* or immunized with subcellular fractions of this agent, suggesting that heart tissue damage in Chagas' disease could be due to a lymphocyte-mediated immune mechanism.[4] In addition, these rabbits develop heart and skeletal muscle alterations with the appearance of lymphocytic infiltrates in the myocardium.[10]

In the present report it was possible to examine, with transmission electron microscopy, foci of lymphocytic infiltrates in the left ventricle of 2 patients with chronic Chagas' heart disease. At that level, a close relationship between lymphocytes and muscle cells was observed with imbrication of the plasma membranes and disappearance of the basal laminae. Also, in the neighborhood of the lymphocytes, definite muscle cell abnormalities were found. Although there are several alternative reasons why lymphocytes might be in those locations, these findings are congruent with the possibility of an *in vivo* lymphocytotoxic effect over the working myocardial cells and with the participation of a lymphocyte-mediated immune response against myocardial fibers in some of the pathogenetic mechanisms of chronic chagasic cardiopathy in humans, as was suggested after studying the experimental infection of the rabbit.[4,10] Further studies concerning *in vitro* tests of cell-mediated immunity with heart antigens involving asymptomatic individuals infected by the *T. cruzi* and patients with chronic chagasic cardiopathy seems warranted for further clarification of this point.

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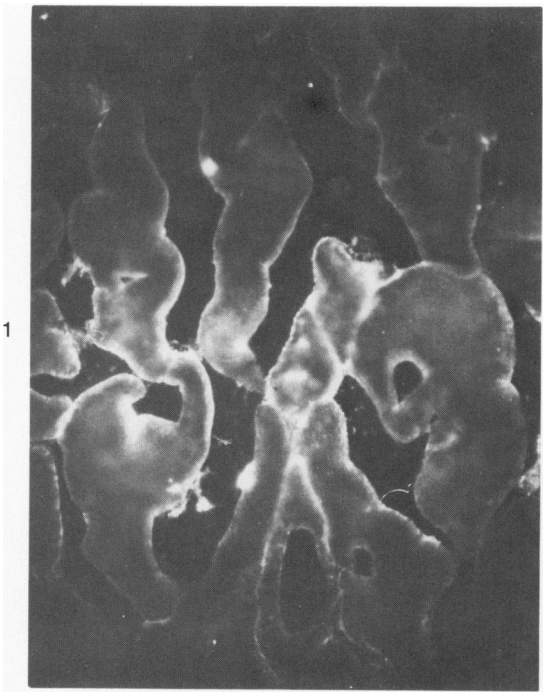
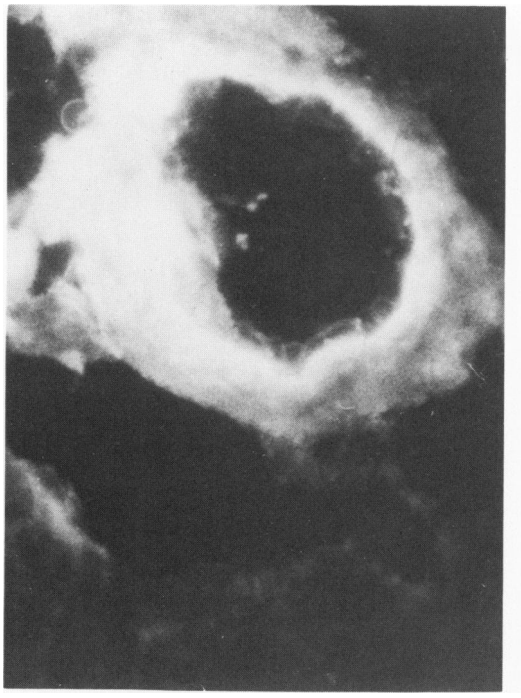
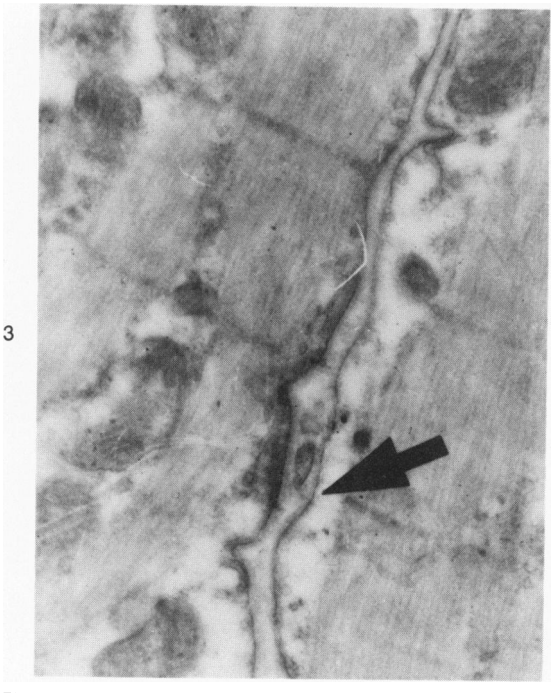
[*Illustrations follow*]







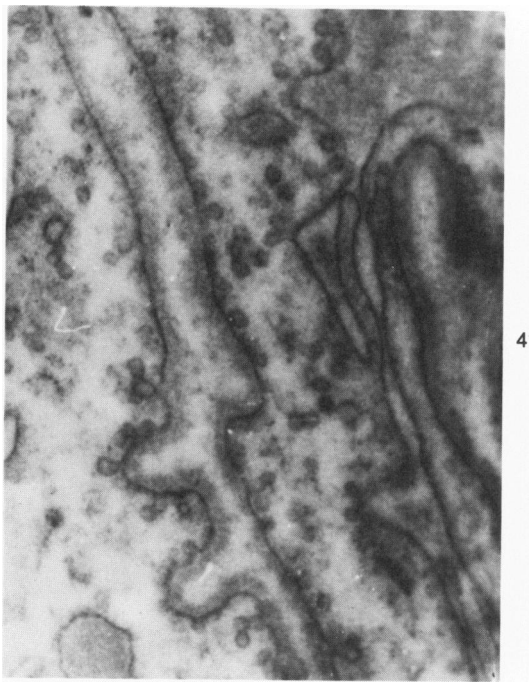
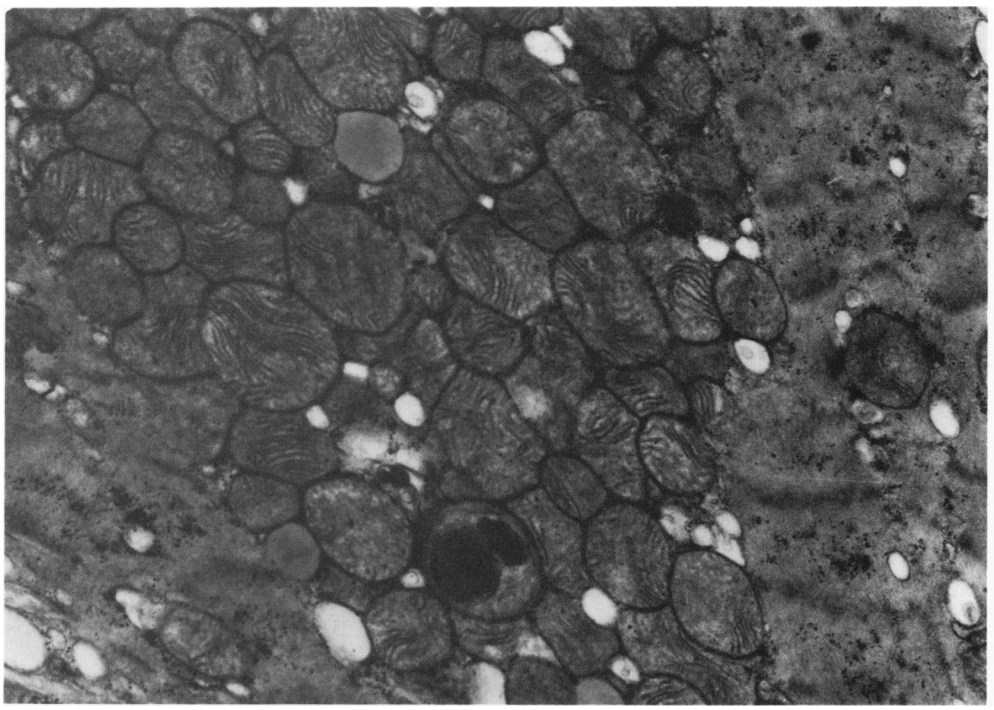


Figure 1—Direct immunofluorescence test. Myocardial section (left ventricle) of Case 4 treated with a fluorescein-labeled goat γ-globulin antihuman γ^Lglobulin. Note the positive staining in the sarcolemmal area. (Original magnification $\times$ 250). **Figure 2**—Direct immunofluorescence test. Myocardial section (right atrium) of Case 2, treated with a fluorescein-labeled goat γ-globulin antihuman γ-globulin. Note the positive staining in a coronary artery. Although the positive reaction is more intense at the inner part of the blood vessel, the wall also shows a positive staining. No significant amounts of *in vivo* bound immunoglobulins can be observed in myocardial fibers in this area. (Original magnification $\times$ 400) **Figure 3**—Electron micrograph of two ventricular-myocardial cells after incubation with peroxidase-labeled antihuman γ-globulin. The plasmalemma (*arrow*) shows a positive reaction for the enzyme, indicating the presence of bound autologous γ-globulin. ($\times$ 14,000) **Figure 4**—Electron micrograph of a ventricular-myocardial sample after incubation as in Figure 3. The endothelial cell plasma membrane shows a positive enzyme reaction. ($\times$ 26,000)

Figure 5—Electron micrograph of left ventricle myocardium of a chagasic patient. Large clusters of peripherically located mitochondria can be seen. These organelles show decrease in the electron density of the matrix and abnormally implanted cristae. (× 23,000)

Figure 6—Low power electron micrograph of a ventricular-muscle cell from a chagasic patient. Large areas containing glycogen and lipofuscin granules (*arrow*) can be seen. *G* = glycogen. (× 5000)

Figure 7—Low power electron micrograph of a left ventricle interstitial mononuclear infilrate. Most of the cells appear to be small lymphocytes. (× 4500)



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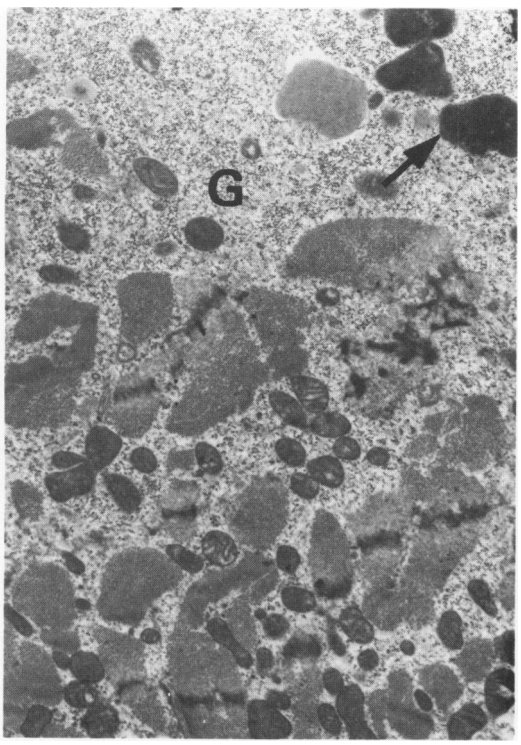

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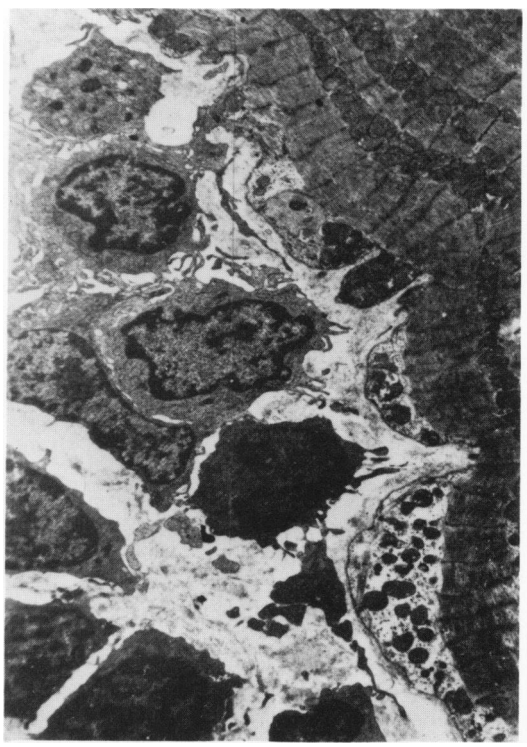

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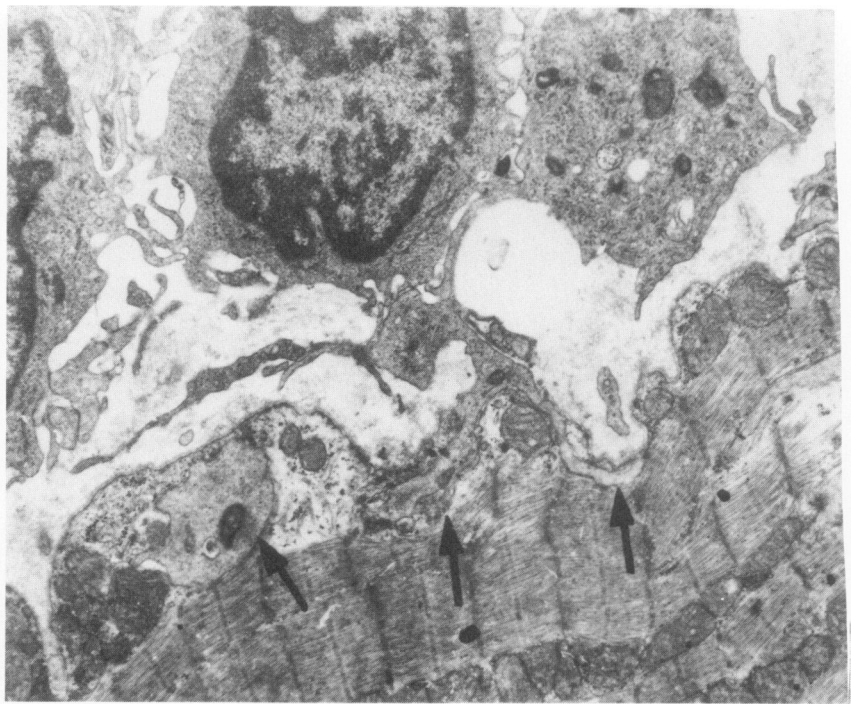

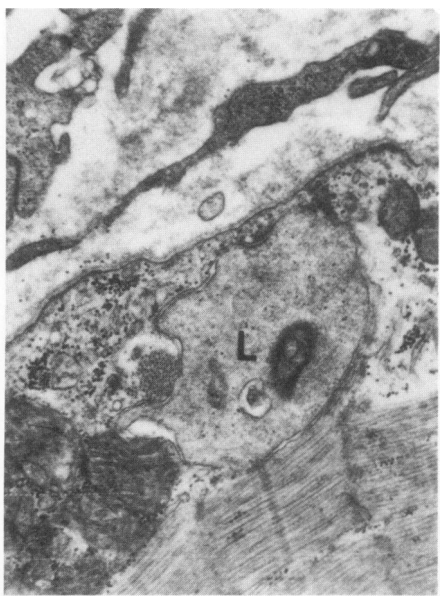

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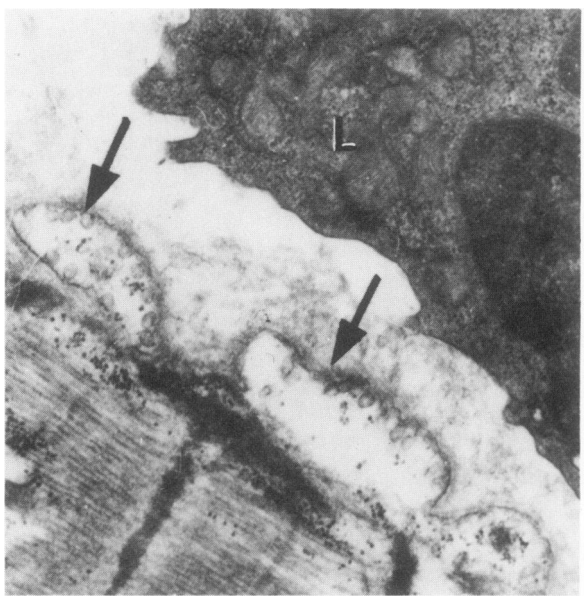

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Figure 8—Higher magnification of one of the areas of Figure 7. Between the lymphocyte prolongations and the myocardial cell a close contact exists (*arrows*). (× 14,000) **Figure 9**—Detail of Figure 8. According to the plane of sectioning, lymphocyte expansions can be seen penetrating deeply into the myocardial cell. At that level the plasma membrane of both cells appears separated by a narrow space with no basal lamina interposing between them. *L* = lymphocyte expansion. (× 28,000) **Figure 10**—In the neighborhood of a lymphocyte, the heart muscle cell shows blebs of the plasmalemma (*arrows*) with an electron-lucent sarcoplasm. *L* = lymphocyte. (× 31,000)