Immunopathologic and Morphologic Studies of Skeletal Muscle in Chagas' Disease

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Skeletal muscle biopsies from 21 individuals infected with *Trypanosoma cruzi* were studied my means of immunofluorescence, ultrastructural immunochemical, light and electron microscopic, and histochemical procedures. In 12 cases, definite morphologic alterations were found. These alterations were coincident with the presence of circulating antibodies against the plasma membrane of striated muscle fibers and endothelial cells (EVI antibodies). In almost all cases the lesions also presented autologous immunoglobulins bound to the plasma membrane of muscle fibers and endothelial cells. Interstitial inflammatory exudate was not observed in the diseased muscle. On the basis of these observations, it is suggested that the EVI antibody is related to some of the pathogenetic mechanisms of skeletal muscle damage in Chagas' disease. (Am J Pathol 80:153–162, 1975)

ANTIBODIES reacting with the plasma membrane of skeletal muscle and endothelial cells (EVI antibody) have been described in 50% of asymptomatic individual infected with *Trypanosoma cruzi* and in 95% of humans with chagasic cardiopathy.[1-2] In addition, in EVI-positive chagasic individuals the presence of autologous γ -globulins bound to the skeletal muscle was demonstrated.[2]

On the basis of these observations it was planned to search for skeletal muscle alterations in chagasic patients. In the present report, the results of light and electron microscopic, histochemical, and immunohistochemical studies on muscle samples obtained of 21 humans infected with *T. cruzi* are described.

Materials and Methods

Patients

Twenty-one chagasic individuals were included; 14 were EVI positive and 7 were EVI negative, as defined by the indirect immunofluorescence test.[2] Six of the 14 EVI-positive

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cases had heart involvement; none of the EVI-negative individuals had heart disease. Clinical and serologic criteria for diagnosis was presented elsewhere.[2] In the 21 cases selected, metabolic and toxic diseases were ruled out. When possible, the presence of obvious antecedents of familial myopathies was also ruled out.

The patients had not received any medication for at least 2 years before skeletal muscle biopsy samples were obtained, and they were not treated with anti-*T. cruzi* drugs. Clinical evidences of functional failure of the skeletal muscle system were not present in any case.

Clinical elements of obvious heart failure were absent in all cases with cardiopathy.

Muscle biopsies were obtained from the biceps muscle by means of a minimal surgical procedure.

Each muscle sample was divided into four fragments. One of them was immediately frozen at −20 C and employed for immunofluorescence and histochemical studies. Another one was fixed in cold (4 C) 10% buffered formaldehyde and processed for ultrastructural immunohistochemistry as described below. A third fragment was fixed in cold (4 C) 5% glutaraldehyde in phosphate buffer, pH 7.4, in order to perform an ultrastructural study. The last piece of muscle was fixed in 10% neutral formaldehyde and processed for routine histologic technics.

The patients studied in the present report were classified as EVI positive or EVI negative by two observers. Then, they were coded and studied by means of the light and electron microscopic, ultrastructural immunochemical and histochemical methods by two other observers who proceeded without knowledge of the result of the EVI immunofluorescence test or the clinical diagnosis of each case.

Immunofluorescence Techniques

Two-micron cryostat sections of the skeletal muscle biopsies were washed during 10 minutes in phosphate-buffered saline (PBS), pH 7.2, and treated with fluorescein-labeled antihuman γ-globulin antiserum (IgG, IgA, IgM) prepared in a goat, according to the method of Nairn.[3] The same batch as in previous studies[2] was used. Other sections were also treated with fluorescein-labeled goat γ-globulin antihuman C3 (Hyland Laboratories, Los Angeles, Calif.), and with rabbit globulin antihuman fibrinogen labeled with fluorescein.[2] Details of the procedure were reported elsewhere.[2] Skeletal muscle biopsies were studied unfixed and also after washing them for 2 hours in a coplin jar with citrate buffer at pH 3.2.[2] As control, other sections were washed for 2 hours in PBS. Sections in which γ-globulin bound *in vivo* was removed by acid elution were then used in an autologous indirect immunofluorescent test, employing in each case the patient's own serum in a 1:10 dilution. As a control, normal human sera were used.

In both the direct and the autologous immunofluorescence test, blocking experiments were performed by applying the same goat antihuman γ-globulin antiserum, but unlabeled, before applying the labeled antiserum.

For studying the action of the acid buffer on the EVI antigen, murine heart and skeletal muscle cryostat sections were washed for 2 hours with this buffer and employed in the indirect immunofluorescent test with an EVI-positive chagasic serum.

Histochemical Techniques

In unfixed tissue sections, 2 μ in thickness, obtained with a cryostat, succinate dehydrogenase, muscle phosphorylase, and adenosine triphosphatase [4] activities were investigated.

Ultrastructural Immunohistochemistry

The presence of autologous γ-globulin in the muscle samples was investigated by means of rabbit antihuman globulin labeled with peroxidase.

The treatment of the tissue sections and labeling of the antisera was performed as previously described.[1]

Ultrastructural Studies

The samples fixed in glutaraldehyde for 4 hours were rinsed overnight in phosphate buffer and postfixed for 1 hour in 1% osmium tetroxide. The material was embedded in Araldite.

Thin sections were stained with uranium acetate and lead citrate and examined with a Philips 200 electron microscope.

Histologic Studies

The fragments of muscle fixed in 10% formaldehyde were embedded in paraffin, cut 5 μ thick, and stained by the hematoxylin and eosin, periodic acid-Schiff and Masson's trichrome methods.

Results

Immunofluorescent Study

All 14 EVI-positive chagasic individuals presented γ -globulin bound to the skeletal muscle, as it has been previously described[2] (Figure 1, Table 1).

It is interesting to note that C3 was observed with the same localization as that of the immunoglobulins in 9 of the 14 cases, and that it was almost always with a less intense pattern.

Table 1—Immunologic and Morphologic Findings in Chagasic Individuals

Patient	EVI	Bound Ig seen by IF*	Bound C3 seen by IF*	Bound Ig seen by UIF*	Degree of alteration†
IF	+	++	+	+	Moderate
LC	+	+++	++	+	Mild
HR	+	+	—	+	Intense
EM	+	++	+	+	Intense
ET	+	++	—	+	Normal
IP	+	+	+	+	Mild
IN	+	+	+	+	Mild
ME	+	++	—	+	Mild
NS	+	++	+	+	Normal
CL	+	+	—	+	Intense
LS	+	+	—	+	Mild
RP	+	+++	+	+	Intense
CB	+	+	+	+	Mild
JI	+	+++	+	+	Moderate
AL	—	—	—	—	Normal
RV	—	—	—	—	Normal
HS	—	—	—	—	Normal
MR	—	—	—	—	Normal
NS	—	—	—	—	Normal
HR	—	—	—	—	Normal
FM	—	—	—	—	Normal

* Intensity of deposits was graded from + to +++.
† As seen by light microscopy.
IF = immunofluorescence techniques, UIF = Ultrastructural immunohistochemical techniques.

No γ -globulin or C3 bound to the skeletal muscle was observed in the EVI-negative chagasic patients.

In all cases, negative results were obtained with the fibrinogen antiserum.

Acid elution removed the γ -globulin bound *in vivo;* control washes performed with PBS had no effect. When the acid-eluted sections were used in an autologous reaction in the indirect immunofluorescence test, employing in each case the patient's own serum, a positive reaction was obtained with a pattern similar to that observed with the direct immunofluorescent technique. The staining was observed near the sarcolemmal area, with projections into the interstitium. Blocking experiments abolished the fluorescence. Controls performed with normal human sera gave negative results. Murine heart and skeletal muscle sections previously treated for 2 hours with the acid buffer were an adequate substrate for an EVI test employing the indirect immunofluorescence technic, showing that the EVI antigen(s) is not substantially affected by this treatment.

Histologic Studies

All the EVI-negative patients presented normal muscles, on morphologic basis, as did 2 of the 14 EVI-positive patients (Table 1).

In the remaining 12 EVI-positive patients, several degrees of muscle alterations could be seen. The modifications were focal, and deeply altered areas appeared alongside normal-appearing ones. In all cases, isolated hyalinized fibers could be seen. In other places a marked nuclear activity was observed with an increase in the number of nuclei, which often were located in the center of the muscle fibers. Large rows of centrally located nuclei were frequently to be seen (Figure 2). Occasionally, sprouting buds containing numerous nuclei were observed (Figure 3). No interstitial inflammatory exudate was seen, and in spite of the thorough search of at least 10 sections in each patient, no *T. cruzi* could be found in the muscles examined.

Although the alterations were similar in all the 12 cases, the intensity was variable (Table 1). Three cases showed deeply altered muscles, and in one of them, frankly atrophic lesions with the replacement of the muscle fibers by fat cells was observed. In the remainder, the lesions, although definite, were minimal or moderate, and most of the fibers presented a normal appearance.

Ultrastructural Studies

All the EVI-negative patients presented normal-appearing fibers. In EVI-positive patients, two kinds of lesions could be found. One of them

was extended to most of the fibers and consisted of a marked increase in the number of lysosomes, especially with a subsarcolemmal localization, and in the deposit of numerous fat globules (Figure 4).

The amount of glycogen was also increased, and abundant granules could be seen occupying discrete sarcoplasmic areas. This alteration was present in all the EVI-positive cases, even in those with normal-appearing muscles as shown by the light microscope study.

The second kind of lesion was focal and was represented by the presence of structurally disorganized fibers with variable degrees of myofilament and organelle atrophy (Figure 5).

Ultrastructural Immunohistochemistry

In all the EVI-positive patients who presented bound immunofluorescence as revealed by the immunofluorescence techniques, autologous γ -globulin could be seen alongside the plasma membrane of the striped fibers and the endothelial cells as demonstrated by positive peroxidase activity (Figure 6). The enzyme reaction, although focal, showed an accurate localization, and no activity could be seen in other muscle structures.

No clear relationship could be found between the deposits of autologous γ -globulins and the presence of lesions.

In the control studies performed on samples treated previously with unlabeled antibodies and in the unincubated sections, no enzyme reaction could be seen.

Histochemistry

The succinate dehydrogenase and ATPase activities did not show alterations in either EVI-positive or EVI-negative patients. On the contrary, phosphorylase activity appeared abnormal in the EVI-positive patients. In them, no clear distinction between Type I and Type II fibers could be seen with this method (Figure 7), as if a decrease of the enzyme activity was present in Type II fibers.

Discussion

Our results indicate that in a significant proportion of *T. cruzi*-infected humans, definite skeletal muscle abnormalities can be observed. These alterations are focal and of variable intensity. At the light and ultrastructural levels, the modifications appear nonspecific, since they can also be found in other muscle diseases,[5,6] and suggest the presence of degenerative and reparative process.

With the electron microscope two kinds of alterations were found. One,

extending the light microscope observations, was focal and consisted of different degrees of muscle fiber disorganization and atrophy with loss of the cell components. The other involved a large number of fibers and consisted of an increase in the number of lysosomes and in the appearance of different amounts of fat. These modifications suggest the presence of metabolic alterations of the muscle fibers. This interpretation would be supported by the results of the histochemical study, with a decrease of muscle phosphorylase activity, with no clear distinction between both kinds of muscle fibers, as can be established with this technique.

Although other factors responsible for muscle alterations cannot be completely discarded in our material, the high prevalence of these modifications in *T. cruzi*-infected patients, the lack of toxic, metabolic, and ischemic diseases, as well as the absence of hereditary antecedents, makes it reasonable to suppose that the lesions are associated with *T. cruzi* infection. The absence of *T. cruzi* in the lesions makes it reasonable to postulate the actions of an indirect pathogenetic mechanism. It is noteworthy that the lesions were observed only in the EVI-positive chagasic individuals.

In addition, all the diseased muscles presented autologous γ -globulin bound to the muscle fibers. Ultrastructural immunohistochemical procedures showed that these globulins were located on the muscle fiber plasma membrane. In a recent report,[1] it has been shown that EVI antibodies react with those structures, posing the possibility that the deposited γ -globulin represents the *in vivo* fixation of that antibody.

The association of all these findings makes it reasonable to postulate that EVI antibodies play some role in the development of the muscle alterations in Chagas' disease. The possible mechanism of tissue damage by this antibody cannot be deduced with the present evidence. The morphologic and histochemical observation suggests metabolic alterations. Since immunoglobulins are bound to the plasma membrane of the muscle cells, it appears reasonable to postulate that they could interfere with some of the transmembrane diffusion and transport proceses.

It is interesting to notice that although EVI antibody fixes complement *in vitro*,[2] C3 bound *in vivo* was observed with a lesser frequency and intensity. Although the explanation of this observation appears difficult, it is congruent with the lack of inflammatory exudate in all the muscles examined.

Recently, Santos-Buch and Teixeira [7] 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 cell-mediated immunity. In addition, these rabbits develop heart and muscle alterations and an antimuscle antibody.[8]

However, in our material the lack of inflammatory exudate or lymphocyte infiltration in all the muscle biopsies examined would indicate that, in the development of skeletal muscle lesions, lymphocyte-mediated immunity does not play a major role.

According to evidence presented in this paper, it can be assumed that a causal relationship exists between the presence of EVI antibodies in the serum of chagasic patients and the development of muscle lesions.

References

1. Cossio PM, Laguens RP, Diez C, Szarfman A, Segal A, Arana RM: Chagasic cardiopathy: Antibodies reacting with plasma membrane of striated muscle and endothelial cells. Circulation 50:1252–1259, 1974
2. Cossio PM, Diez C, Szarfman A, Kreutzer E, Candiolo B, Arana RM: Chagasic cardiopathy: Demonstration of a serum gamma globulin factor which reacts with endocardium and vascular structures. Circulation 49:13–21, 1974
3. Nairn R: Standardization in immunofluorescence. Clin Exp Immunol 3:465–476, 1968
4. Hughes JT: Pathology of muscle. Philadelphia, WB Saunders Co, 1974, pp 204–205
5. Fishmann DA, Meltzer HY, Poppei RW: The ultrastructure of human skeletal muscle: Variation from archetypal morphology. The Striated Muscle. Edited by CM Pearson, EK Mostofi, Baltimore, The Williams and Wilkins Co, 1973, pp 58–76
6. Price HM: Ultrastructural pathologic characteristics of the skeletal muscle fibers: An introductory survey.[5] pp 144–184
7. Santos-Buch CA, Teixeira ARL: The immunology of experimental Chagas' disease. III. Rejection of allogenic heart cells in vitro. J Exp Med 140:38–53, 1974
8. Teixeira ARL, Teixeira ML, Santos-Buch CA: The immunology of experimental Chagas' disease. IV. Production of lesions in rabbits similar to those of chronic Chagas' disease in man. Am J Pathol 80:163–180, 1975

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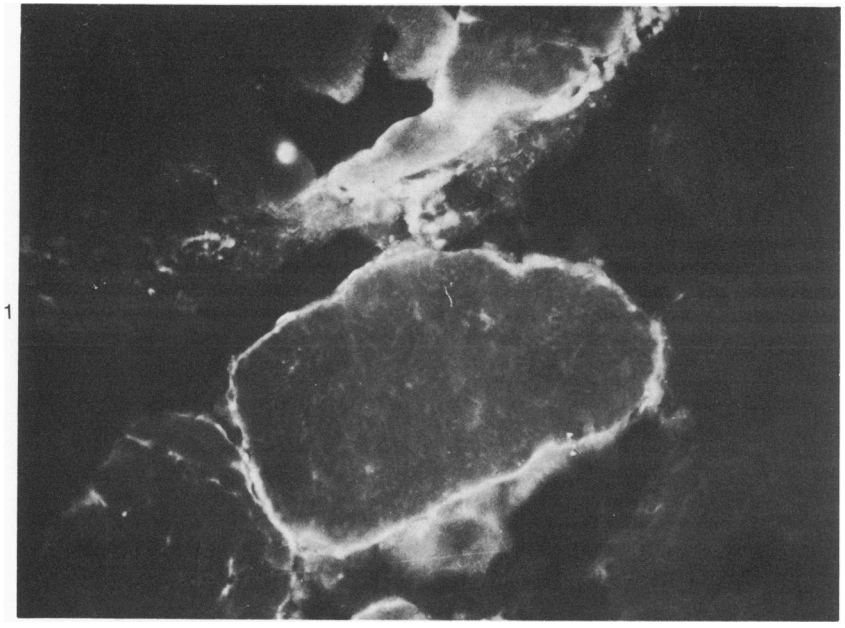
[*Illustrations follow*]



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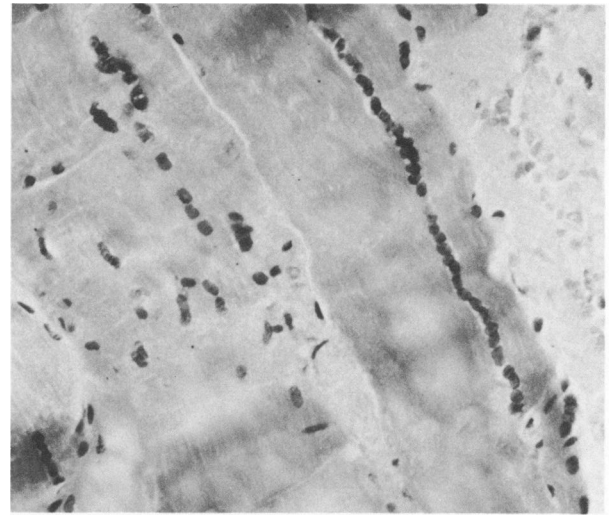

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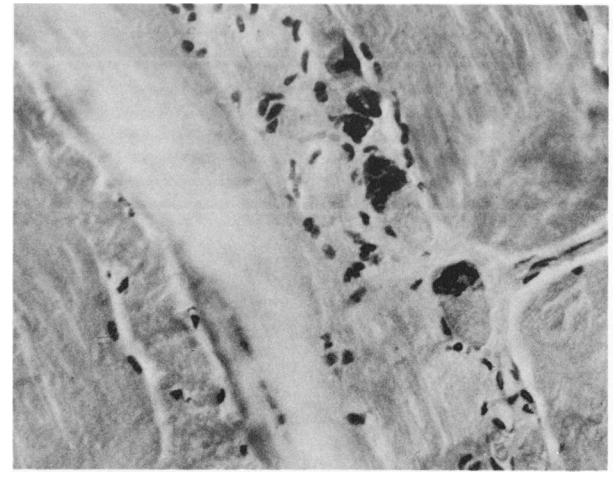

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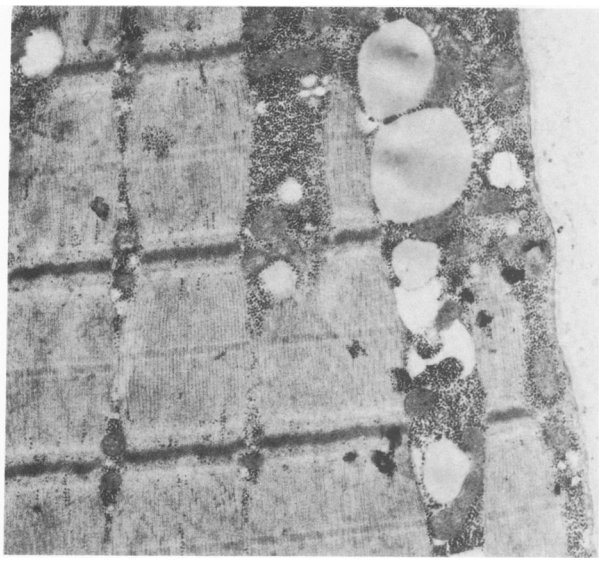
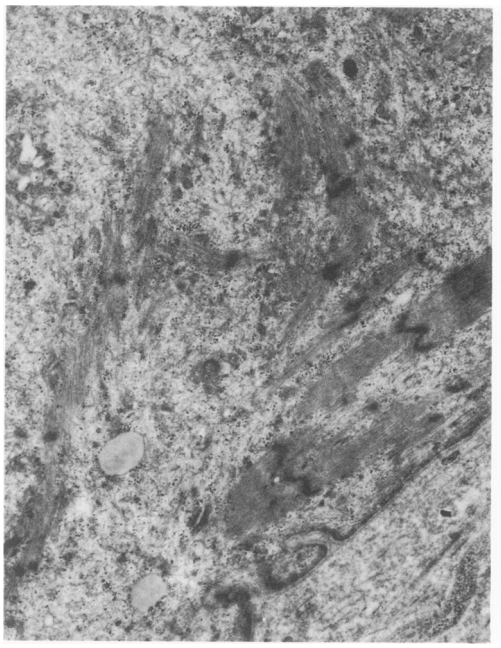
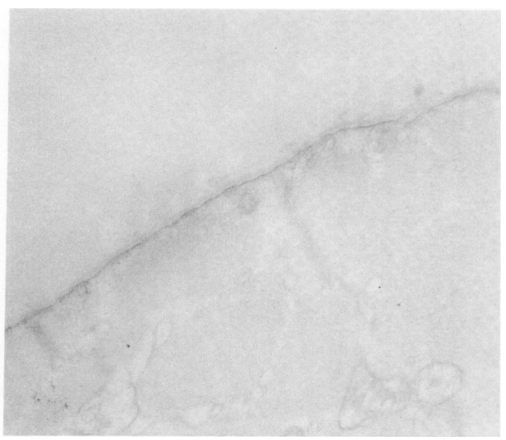
Figure 1—Fluorescence photomicrograph of a skeletal muscle biopsy section of an EVI-positive chagasic patient treated with fluoresceinated antihuman γ-globulin antiserum. Deposits of γ-globulin are observed surrounding fibers near the sarcolemmal area. (× 640) **Figure 2**—Skeletal muscle fibers showing a large row of centrally located nuclei (H&E, × 450). **Figure 3**—Sprouting buds containing numerous nuclei; at the left of the picture, a hyalinized fiber can be seen (H&E, × 450).







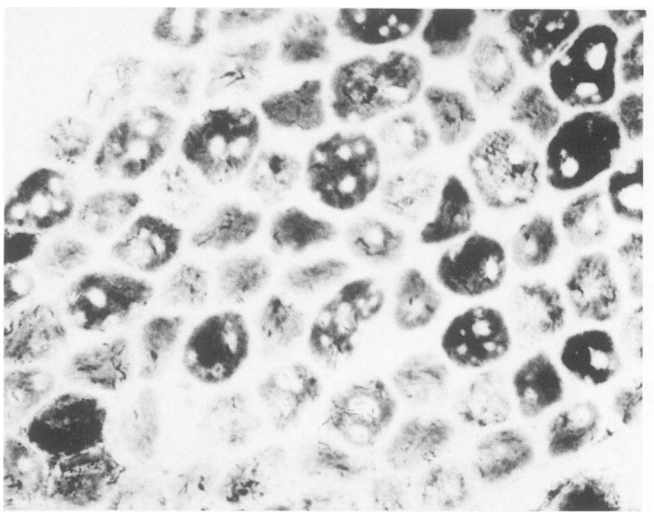


Figure 4—Electron micrograph of a skeletal muscle fiber showing numerous fat globules and an increase in the number of glycogen granules (× 8500). **Figure 5**—Electron micrograph of a skeletal muscle fiber showing a marked disorganization with a decrease in the number of myofibrils and cell organelles (× 6500). **Figure 6**—Electron micrograph of a skeletal muscle fiber incubated with peroxidase labeled antihuman γ-globulin. A positive enzyme reaction at the level of the plasma membrane can be seen. (× 8000) **Figure 7**—Muscle phosphorylase activity in an EVI-positive chagasic patient. No clear distinction between Type I and Type II fibers can be seen, and only a few muscle cells show an intense reaction. (× 250)