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REVERSIBILITY OF CARDIAC FIBROSIS IN MICE CHRONICALLY INFECTED WITH *TRYPANOSOMA CRUZI*, UNDER SPECIFIC CHEMOTHERAPY

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This investigation was performed to verify the effect of specific chemotherapy (Benznidazole or MK-436) on the inflammatory and fibrotic cardiac alterations in mice chronically infected with the strains 21 SF (Type II) and Colombian (Type III) of Trypanosoma cruzi. *To obtain chronically infected mice, two groups of 100 Swiss mice each, were infected with either the 21 SF or the Colombian strain (2×10^4 and 5×10^4 blood forms respectively). The rate of morality in the acute phase was of 80% for both groups. Twenty surviving mice chronically infected with the 21 SF strain and 20 with the Colombian strain were then divided in treated and untreated groups. Excluding those that died during the course of treatment, 14 mice chronically infected with the 21 SF strain and 15 with the Colombian strain were finally evaluated in the present study. Chemotherapy was performed with Benznidazole (N-benzil-2-nitro-1-imidazolacetamide) in the dose of 100 mg/k.b.w/day, for 60 days, or with the MK-436 (3(1-methyl-5 nitroimidazol-2-yl) in two daily doses of 250 mg/ k.b.w, for 20 days. Parasitological cure tests were performed (xenodiagnosis, haemoculture, subinoculation of the blood into newborn mice), and serological indirect immunofluorescence test. The treated and untreated mice as well as intact controls were killed at different periods after treatment and the heart were submitted to histopathological study with hematoxilin-eosin and picrosirius staining; ultrastructural study; collagen immunotyping, fibronectin and laminin identification by immunofluorescence tests. Results: the untreated controls either infected with 21 SF or Colombian strain, showed inflammatory and fibrotic alterations that were mild to moderate with the 21 SF strain and intense with the Colombian strain. Redpicrosirius staining showed bundles of collagen in the interstitial space and around cardiac fibers. Increased deposits of matritial components and collagen fibers, macrophages and fibroblasts appeared at the ultrastructural examination. Deposits of fibronectin, laminin, pro-III and IV collagens were seen, most intense in those infected with the Colombian strain. Treated mice, parasitologically cured, presented clear-cut regression of the inflammatory lesions and of the interstitial matrix thickening. Mice infected with the Colombian strain and treated with MK-436, was parasitologically cured in 5/6 cases and showed mild inflammatory infiltration and fibrosis. The mice treated with Benznidazole (Colombian strain) did not cure and showed moderate fibrosis and inflammation. Treatment of the mice infected with the 21 SF with Benznidazole determined parasitological cure of all animals, that showed mild inflammation and fibrosis of the myocardium. The cured mice of all groups and treated but uncured showed collagen degradation at electronmicroscopy and decrease of the immunofluorescence pattern of the matrix.*

Key words: *Trypanosoma cruzi* – chronic myocarditis – collagen immunotyping – fibronectin – laminin – chemotherapy

The murine model of chronic Chagas' myocardiopathy due to *Trypanosoma cruzi* infection mimics the aspects seen in humans and appears as a progressive inflammatory and fibrogenic process (Andrade & Grimaud, 1986). Deposits of laminin, fibronectin and Types III, Pro-III and IV collagens have been demonstrated in a sequential study from the subacute to late chronic infection (Andrade et al., 1989c) with correlation between fibrogenesis and inflammatory cell infiltration.

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In the present paper we attempt to evaluate the effect of curative chemotherapy on cardiac fibrosis in mice chronicaly infected with *T. cruzi*. Two drugs with specific anti-*T. cruzi* action were used: a) Benznidazole (N-benzyl-2-nitro-1-imidazoleacetamide) a drug clinically used (Brener, 1984) that has been effective against the infection with Type II strains of *T. cruzi* (Andrade, 1985; Andrade et al., 1985); b) MK-436 (2-substituted-5-nitroimidazole) only experimentally used (Murray et al., 1983) and active in the treatment of mice infected with Type III strains that are resistant to Benznidazole and Nifurtimox (Andrade et al., 1985; Andrade et al., 1989a).

The dynamics of myocardial fibrosis were morphologically analysed and evidence of involution of both inflammatory and fibrotic changes was obtained after curative chemotherapy.

MATERIALS AND METHODS

Two groups of 100 Swiss mice each, weighing 18 to 20 g were infected respectively with the 21 SF strain of *T. cruzi* (São Felipe-Bahia), classified as Type II and the Colombian strain, classified as Type III, according with its morphobiological behaviour (Andrade, 1974, 1985), and isoenzyme patterns (Andrade et al., 1983). The inocula were of 2×10^4 blood forms for the 21 SF strain and 5×10^4 for the Colombian strain. The acute phase of infection was evaluated by parasitemia and mortality. It was detected 80% of mortality in the acute phase for both strains. Twenty surviving mice, chronically infected with the 21 SF strain were in part treated with Benznidazole from the 175th day of infection and in part maintained as untreated controls; excluding the mice that died during the course of treatment, 14 mice chronically infected with 21 SF strain were utilized in this study, beeing 7 treated and 7 untreated controls; 6 uninfected controls were also used, (Table I). They were killed after 30, 55, 65 and 85 days after the first dose of Benznidazole simultaneously with untreated infected controls and uninfected controls. As for the Colombian strain, 20 surviving mice, chronically infected, were in part submitted to treatment with Benznidazole and in part with MK-436; treatment with Benznidazole was from the 90th and 157th days after infection and the animals were killed 150 days thereafter. Treatment with MK-436 began at the 157th days after infection and the mice were killed 65 days after the first dose. Infected untreated controls and uninfected controls were also studied. Excluding those that died during the course of treatment, 15 mice chronically infected with the Colombian strain were used in the present study (Table I).

Schedules of treatment – Benznidazole (N-benzil-2-nitro-1 imidazoleacetamine) was administered as previously described (Andrade et al., 1985) by gavage in the daily dose of 100 mg/k.b.w. during 60 days. The MK-436 (3(1-methyl-5 nitroimidazol-2-yl) was administered by gavage in two daily doses of 250 mg/k.b.w., a course already established to be effective (Murray et al., 1983).

Cure tests – Parasitological tests consisted of: xenodiagnosis with five 4th-5th stage *Rhodnius prolixus* nymphs, haemoculture and subinoculation of the blood into newborn mice.

Indirect immunofluorescence (IIFT) was performed according to Camargo (1966) with serum dilutions from 1:10 to 1:80 and anti-mouse IgG fluorescein conjugate at a dilution of 1:80.

Methodology of study – After complete autopsies the heart was submitted to histopathological and ultrastructural study, collagen immunotyping and identification of laminin and fibronectin. Histopathological study was performed in formalin fixed and paraffin embedded sections, stained with hematoxylin and eosin. The picrosirius method for collegen was performed in paraffin section using an aquous saturaded solutions of picric acid plus 0.1% fast green and 0.1% red sirius (Junqueira et al., 1979).

Ultrastructural study – Small pieces of heart tissue were fixed with 2% glutaraldehyde in 0.1M cacodylate buffer solution, pH 7.4 and post fixed with osmium tetroxide in 0.15M cacodylate buffer solution, pH 7.4. After dehydration and Epon embedding ultrathin sections were obtained and contrasted with uranyl acetate and lead citrate. Examination was performed in an EM 300 Phillips microscope.

Immunolabelling of collagen and identification of laminin and fibronectin used specific purified antibodies from immunized New

TABLE I

General data on mice chronically infected with *Trypanosoma cruzi* submitted to chemotherapy

T. cruzi strain (types)	Treated mice (No.)	Treatment				Untreated controls (No.)	Uninfected controls (No.)
		Chemotherapy (*a*)	Initial dose (days)[b]	Duration (days)	Sacrifice (days after 1st dose)		
21 SF (II)	7	Benz	175	60	30-55-65-85	7	6
Colombian (III)	4	Benz	90-157	60	150	2	2
	6	MK-436	90-157	20	65	3	3

a: Benznidazole (N-benzyl-1-2 nitro 1-imidazoleacetamida) and MK-436 (2,5-nitroimidazole).
b: days post infection at which treatment was started.

TABLE II

Inflammatory and fibrotic cardiac alterations in mice chronically infected with *Trypanosoma cruzi*, Colombian strain – Untreated controls

Ident. (No.)	Duration of infection (days)	Histopathology		Electron microscopy				Interstitial matrix			
		miocarditis	fibrosis	collagen	inflam. cell	fibroblasts	fibronectin	laminin	collagen types[a]		
									III	pro III	IV
F-62	155	++ d	++	++	+	+	– – –	– – –	– – –	– – –	– – –
F-63	155	++ d	++	+++	++	+	– – –	– – –	– – –	– – –	– – –
F-67	240	++ f	+	++	n	+	+	++	+++	+++	++
F-73	240	+++d	+++	+++	+++	+	+	+	+	+++	na
F-74	240	+++d	+++	++	++	+	+	+	+	++	– –

a: Type I collagen was unaltered in all cases.
d: diffuse; f: focal; n: negative; na; not altered.
– – – not determined.

Zealand rabbits or goats injected with the following antigens: fibronectin from plasma; laminin from EHS mouse tumour; Type I collagen from fibrotic human liver, Type III collagen, from human placenta; pro-III, from a pepsin digest of fetal calf serum as described by Rhodes & Miller (1978) and Type IV collagen from bovine lens. Human plasma fibronectin was prepared by affinity chromatography using gelatinsepharose 4b according to Engvall & Rouslahti (1977), purified by DE cellulose chromatography and verified by SDS-polyacrilamide electrophoresis. Laminin was provided by G. Martin, NIH, Bethesda, MD, USA. Details on the techniques for obtaining monospecific antibodies and for the control of antibody purity, have been described elsewhere (Grimaud et al., 1980; Andrade et al., 1989c). Indirect immunofluorescence was performed on 6 μm thick cryostat sections treated with purified antibodies (0.005-0.02 mg/ml) and a fluorescein isothyocyanate (FITC) labelled sheep antirabbit or rabbit anti-goat IgG globulin (Institut Pasteur de Lyon-France, code 74561 and Nordic RAG-FITC). To identify the presence of interspecific immunoglobulins in the tissues immunofluorescent reactions were controlled by nonimmune rabbit serum. The results were negative.

RESULTS

General data – The parasitemia in the acute phase of the infection with either the 21 SF and the Colombian strain reproduced the Types II and III strains, respectively (Andrade, 1985) and became negative in the chronic infection.

TABLE III

Effect of chemotherapy with Benznidazole or MK-436 on the inflammatory and fibrotic cardiac lesions of mice chronically infected with *Trypanosoma cruzi* – Colombian Strain

Ident. (No.)	Initial dose (days of infection)	Sacrifice (days after 1st dose)	Chemo-therapy	Cure tests *a*	Histopathology		Electron microscopy			Interstitial matrix				
					miocar-ditis	fibro-sis	colla-gen	inflam. cells	fibro-blasts	fibro-nec-tin	lami-nin	col. types[b]		
												III	pro III	IV
F-59	90	65	MK-436	neg	+ d	+	+	+	+	– – – – – – – – – – – – – – – –				
F-60	90	65	MK-436	neg	+ d	n	+	+	n	– – – – – – – – – – – – – – – –				
F-61	90	65	MK-436	neg	+ d	+	+	+	+	– – – – – – – – – – – – – – – –				
F-70	157	65	MK-436	neg	++d	+	+	+	n	na	+	+	+	na
F-71	157	65	MK-436	pos	+ d	n	+	n	+	+	+	+	+	+
F-72	157	65	MK-436	neg	+ f	n	+	n	n	+	+	– – – – – – – –		
F-65	90	150	Benz	pos	++d	++	++	n	+	+	na	+	++	na
F-66	90	150	Benz	pos	++d	++	+	n	+	+	+	na	+	na
F-68	157	150	Benz	pos	+ f	n	+	n	+	++	+	na	+	na
F-69	157	150	Benz	pos	n	n	+	+	+	+	+	+	+	+

a: parasitological cure tests: xenodiagnosis, subinoculation, haemoculture.
b: type I collagen was normal in all case.
n: negative; na: not altered; f: focal; d: diffuse.
– – – not determined.

TABLE IV

Cardiac interstitial matrix alterations in mice infected with *Trypanosoma cruzi* – 21 SF strain

Ident. (No.)	Exper. group treated/ untreated	Days after 1st dose	Histopathology		Electron microscopy			Interstitial matrix			
			inflam.	fibr.	colla-gen	inflam. cells	fibro-blasts	fibro-nectin	collagen types[a]		
									III	pro III	IV
F-41	untreated	30[b]	+ d	+	+	+	n	+	na	na	na
F-42	untreated	30[b]	+ d	n	+	n	n	na	na	na	+
F-46	untreated	55[b]	+ d	+	+	+	+	++	++	++	+
F-47	untreated	55[b]	+ d	n	+	+	n	++	+	++	+
F-51	untreated	65[b]	++f	n	+	n	n	– –	na	++	++
F-52	untreated	65[b]	++f	n	+	+	+	+	+	+	+
F-56	untreated	85[b]	++f	n	n	n	n	na	+	+	na
F-39	treated[c]	30	+ f	+	+	n	n	+	+	++	+
F-40	treated[c]	30	+ f	+	+	+	n	na	na	na	na
F-44	treated[c]	55	+ f	n	+	n	+	+	+	+	+
F-45	treated[c]	55	+ f	n	+	n	+	+	+	+	+
F-49	treated[c]	65	+ f	+	+	n	+	+	+	+	na
F-50	treated[c]	65	+ f	+	+	+	n	+	+	++	na
F-55	treated[c]	85	+ f	n	n	n	n	+	+	+	+

a: type I collagen was unalter in all cases.
b: sacrificed at the same day as the treated mice.
c: all treated mice were parasitologically cured.
n: negative; na: not altered; f: focal; d: diffuse.
– – not determined.

Results of chemotherapy in chronic phase evaluated through parasitological tests, disclosed a cure rate of 100% for mice infected with the 21 SF strain, treated with Benznidazole but no cures in mice infected with the Colombian strain and treated with this drug.

For the mice infected with the Colombian strain and treated with MK-436 the parasitological evaluation was negative in 5/6 mice.

Serological tests (IIF) were positive in the treated and untreated mice (titres 1:40 to

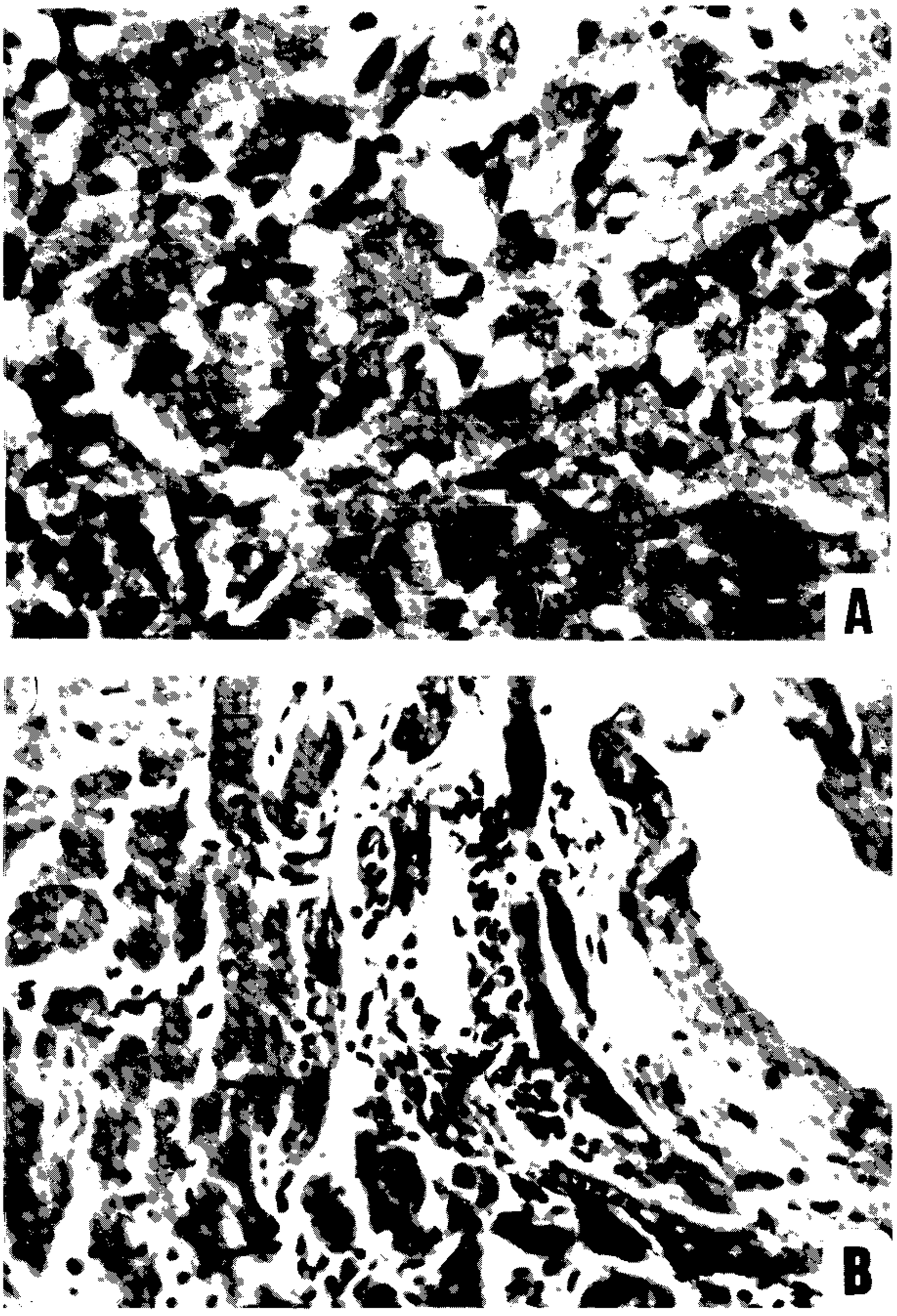

Fig. 1A - *Untreated control:* infected with the Colombian strain-intense myocarditis with focal destruction of cardiac fibers, interstitial deposits of matritial fibrilar material, presence of mononuclear inflammatory cells and fibroblasts. H & E-400X. B -- Cardiac muscle of mouse infected with the Colombian strain *treated with MK-436:* focal mononuclear cells infiltration around small capilaries. Matritial deposits are absent. H & E-250X.

1:640); no correlation was seen between parasitological cure and the titres of IIF tests.

Morphological aspects – The interstitial matrix alterations were evaluated accordingly with the degree of inflammation, fibrosis, ultrastructural identification of collagen deposits and cellular infiltration, fibronectin, laminin and collagenic deposits.

These alterations were graded on an arbitrary three point scale: + indicates mild focal or

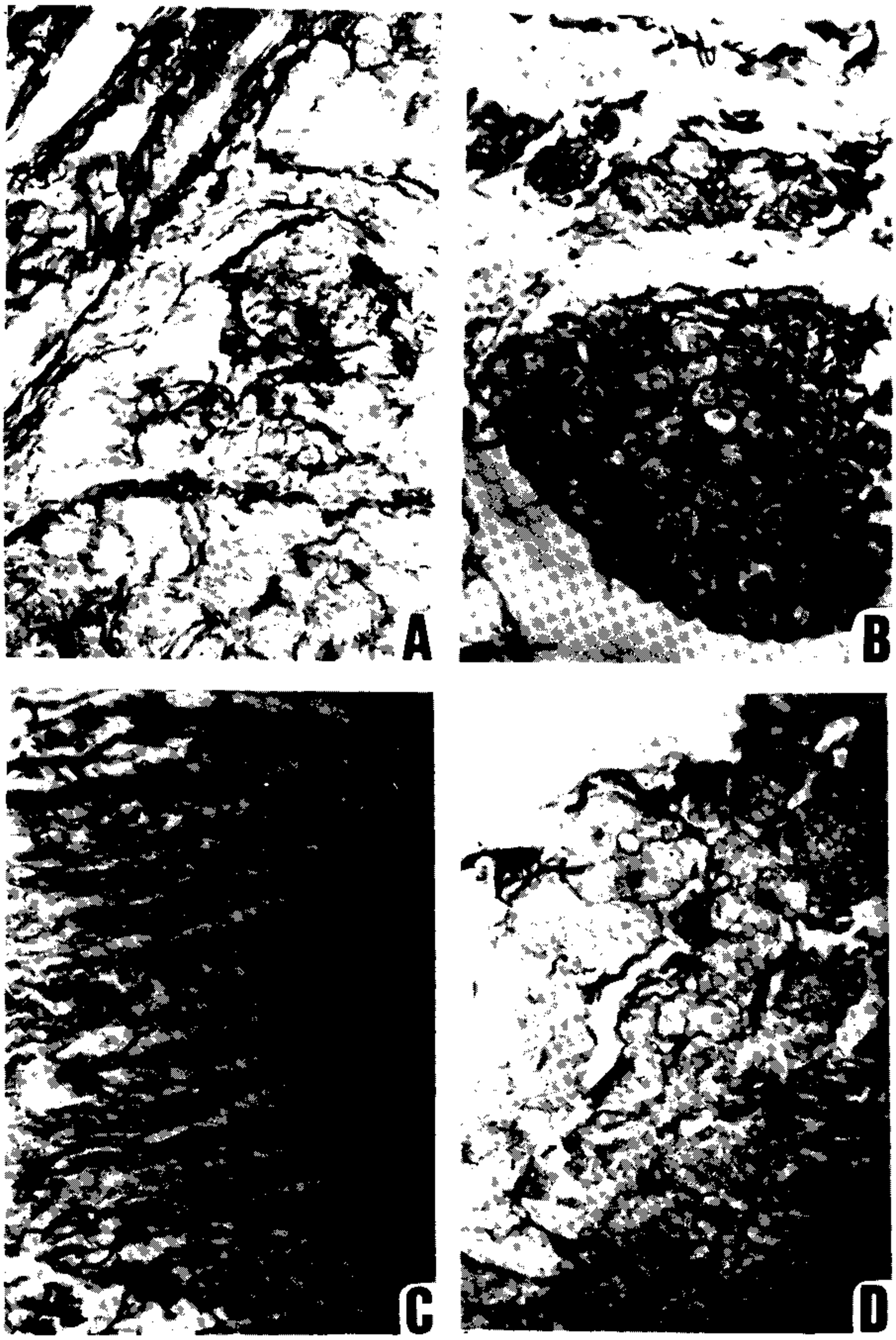

Fig. 2A, B – *Untreated control:* cardiac muscle of mouse chronically infected with *Trypanosoma cruzi* (Colombian strain) showing irregular bundles of interstitial collagen around cardiac fibers and in enlarged interstitial spaces. Picrosirius, 400X. C, D – Myocardium of mouse infected with *T. cruzi* (Colombian strain) – *treated with MK-436 and cured:* interstitial collagen deposits are less conspicuous, irregular and with fragmentation. Picrosirius, 400X.

diffuse alterations; ++ moderate focal or diffuse; +++ diffuse and severe alterations. Tables II, III and IV summarize these data for the several experimental groups.

MICE INFECTED WITH THE COLOMBIAN STRAIN (TABLE I)

Untreated infected controls -- Table II –

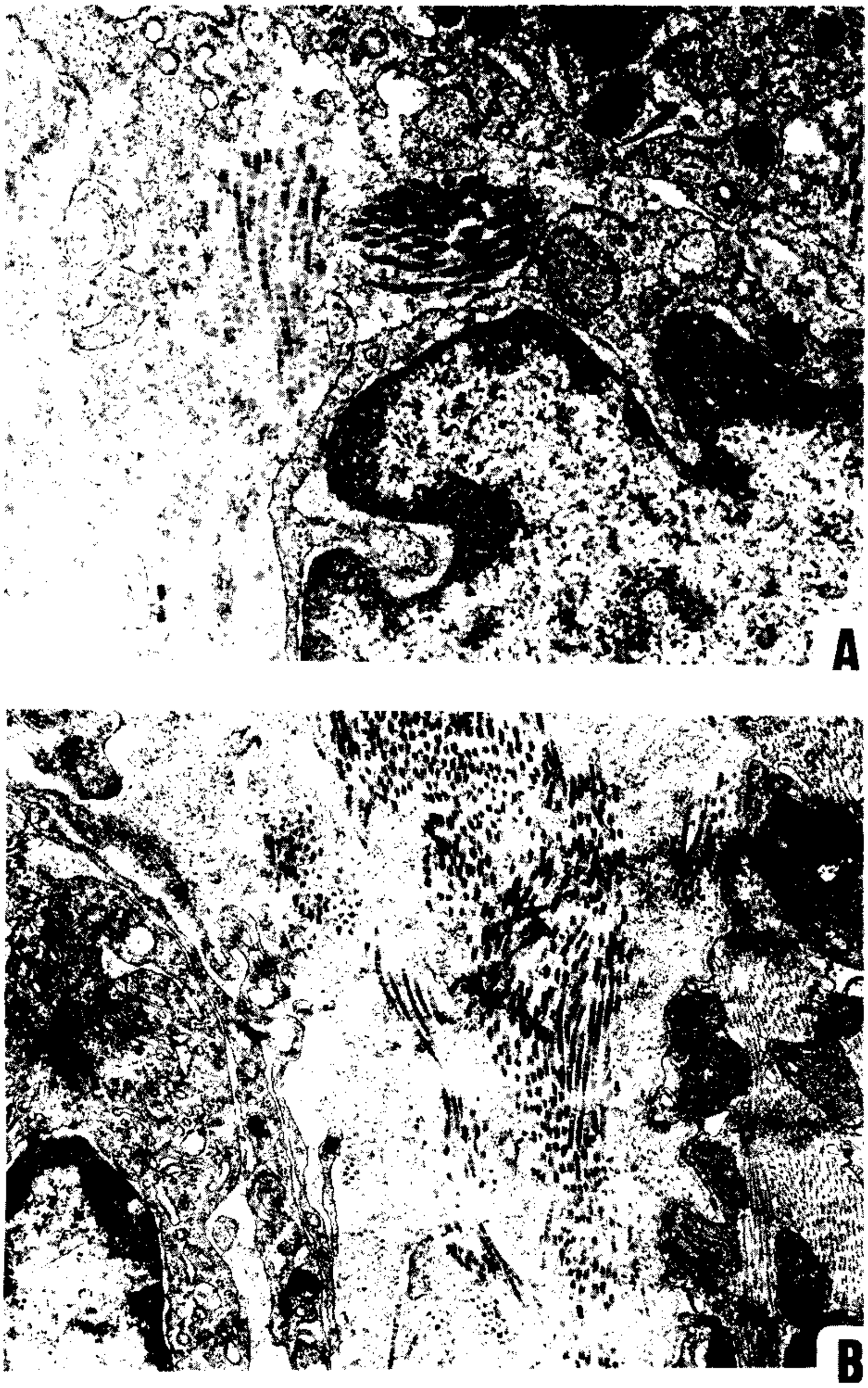

Fig. 3: cardiac muscle of mice chronically infected with the Colombian strain: *untreated controls*. A – Interstitial space of the myocardium showing collagen bundles and abundant matritial components; macrophages with idented nucleus, abundant lysosomes and cytoplasmic projections. (E. M. 6.300X). B – Collagen deposits as small bundles, isolated fibrils and abundant microfibrils; cardiac cell with normal structure and interstitial cell (E. M. 4.000X).

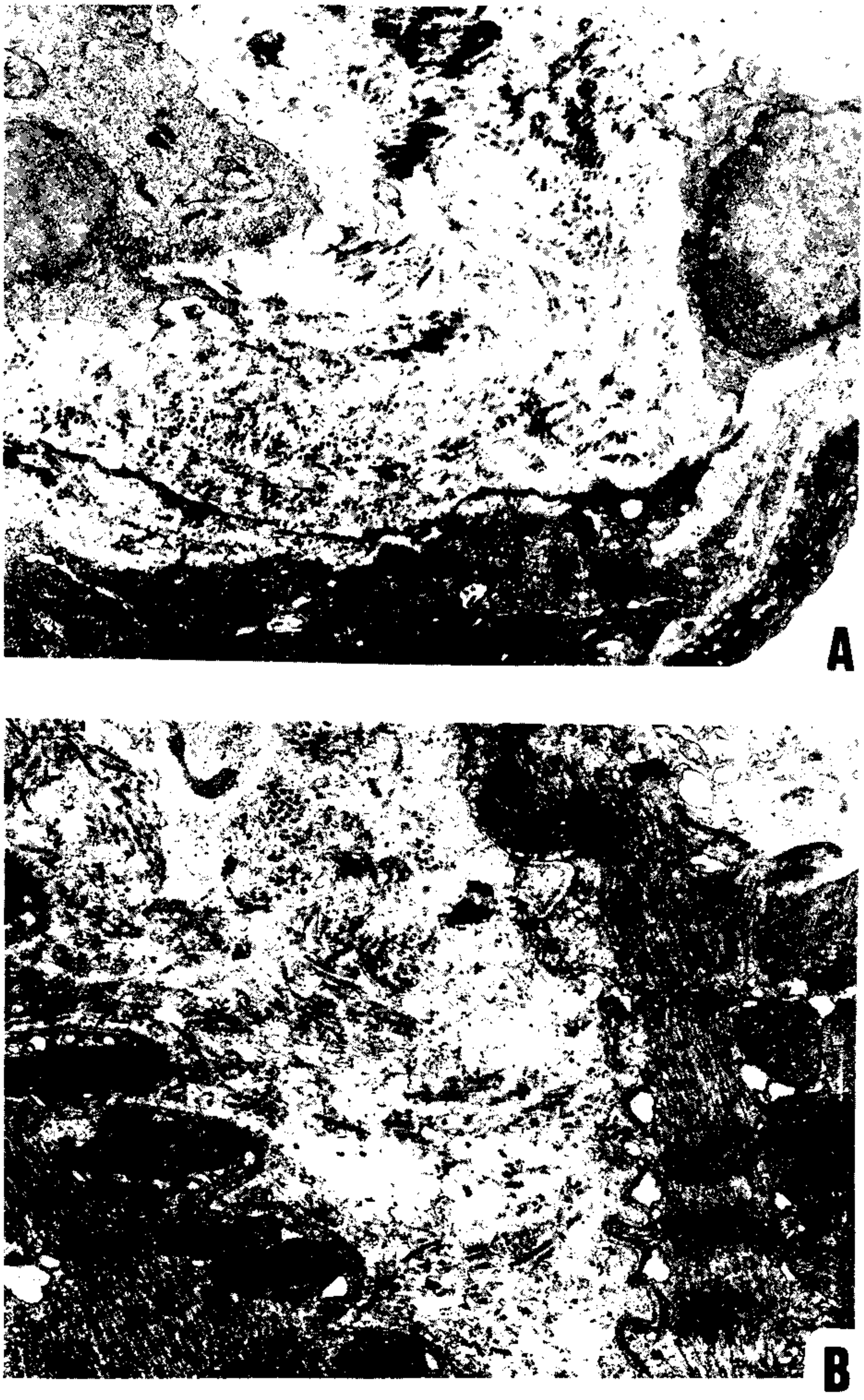

Fig. 4: myocardium of mice chronically infected with the Colombian strain – treated with the MK-436: A – *uncured*. B – *cured*. The interstitial space shows in both cases collagens deposits with disorganization of the fibrils, electrodense deposits (arrows) and lytic alterations of the matritial components (E. M. 3.250X, 4.000X).

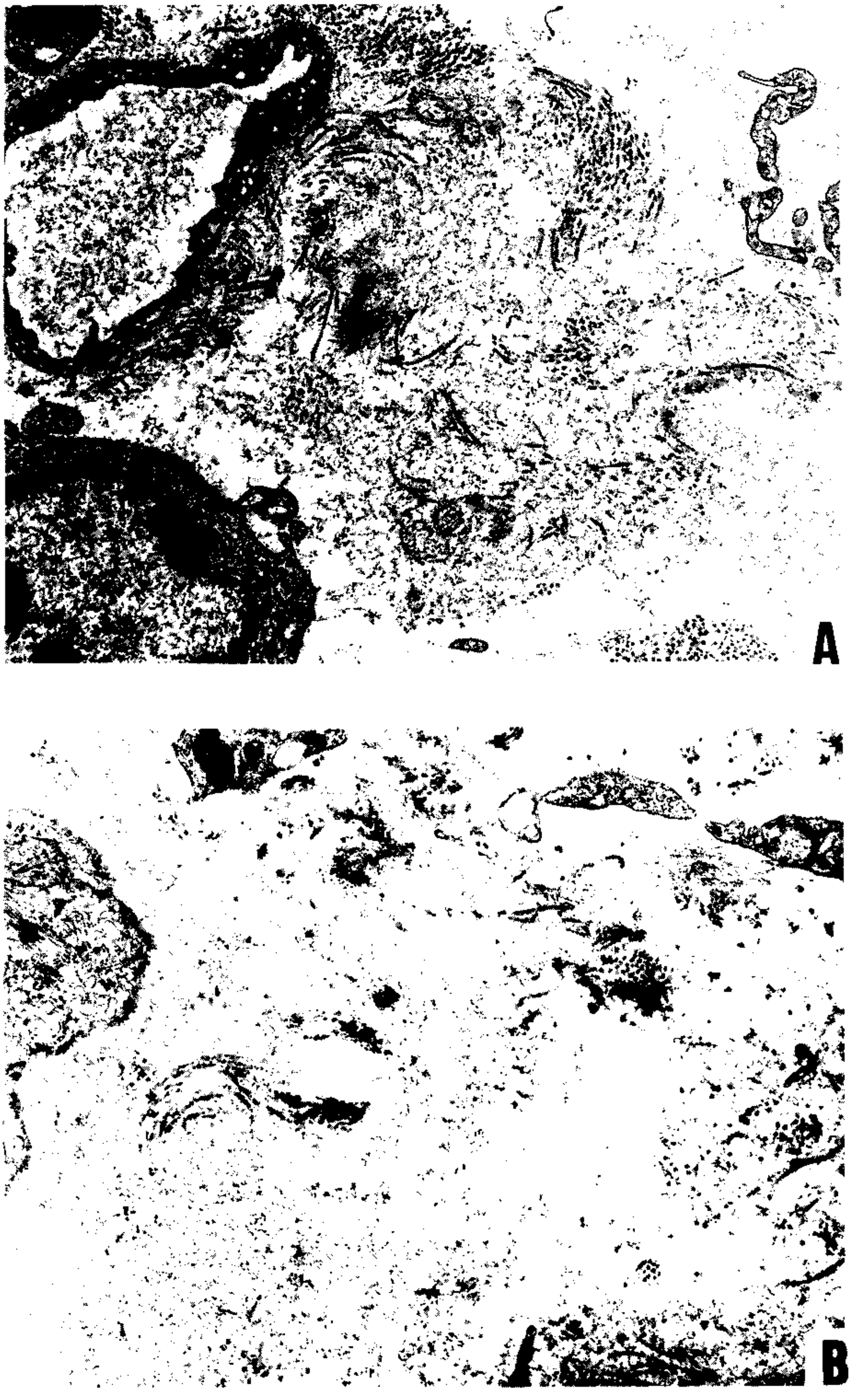

Fig. 5: *infected mice treated and cured* – A – Cardiac interstitium with irregular and scarce collagen deposits, showing regressive alterations, lytic destruction and granular electrodense transformation. (E. M. 1.300X). B – Lytic alterations of the matrix and electrodense deposits (arrow) (E. M. 7,000X).

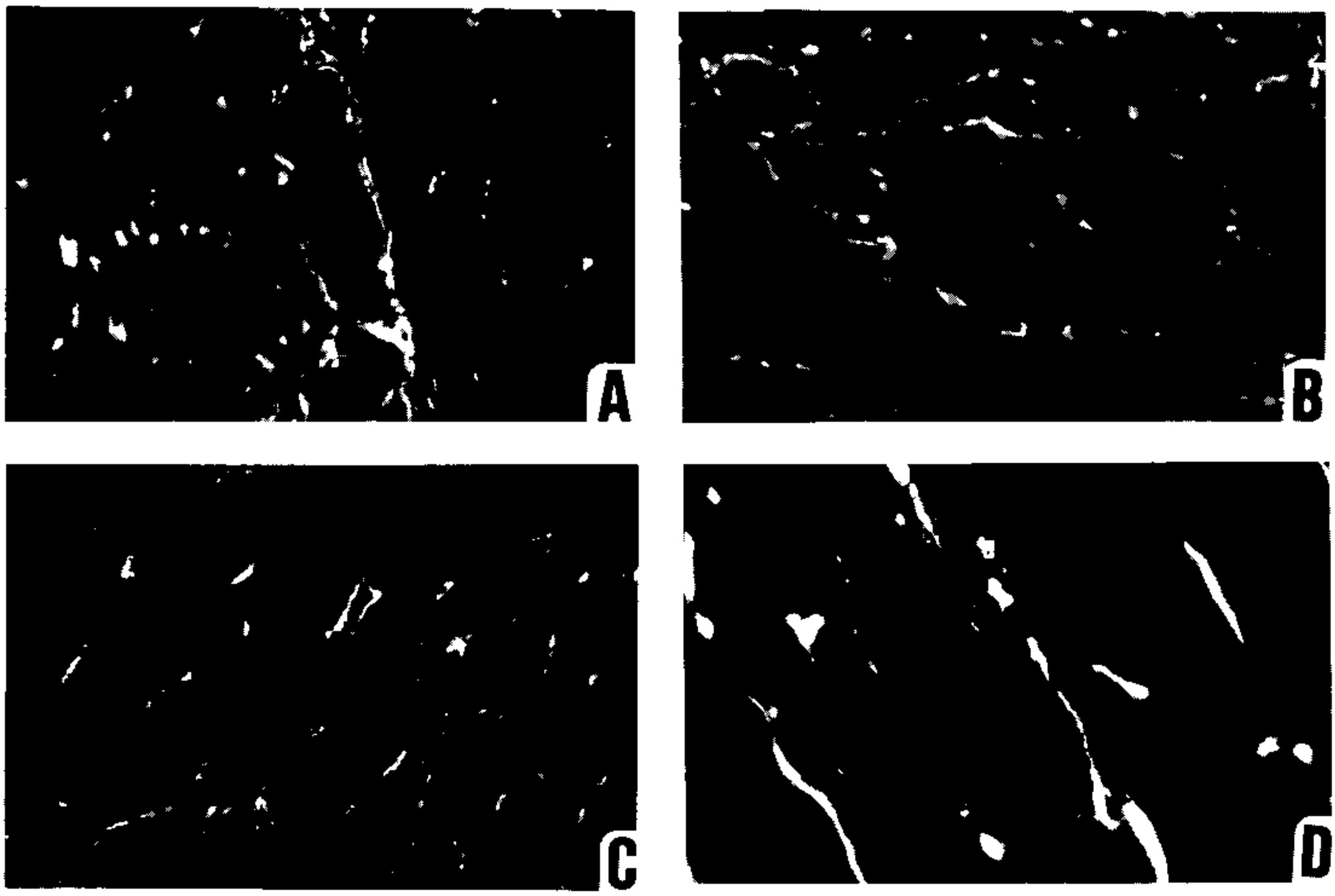

Fig. 6: *intact control mice* – Immunofluorescence test in cryostat sections of the myocardium using specific antibodies. A – Fibronectin. B – Laminin. C – Type I collagen. D – Type III collagen. E – Pro-III collagen. F – Type IV collagen. X400.

Mice with 150 and 240 days of infection showed moderate to intense inflammatory and fibrotic alterations of the myocardium, by light microscopy as diffuse and focal mononuclear inflammatory infiltration (Fig. 1a). Slight (+) to dense (+++) fibrous thickening of the interstitial space was observed, predominantly in the atria and more densely in the subepicardium and around blood vassels. The picrosirius staining showed irregular bundles of interstitial collagen around cardiac fibers and in the perivascular and subepicardial space (Fig. 2a, b) with polarized light examination.

Electronmicroscopy study – Showed moderate to intense collagen deposits as isolated fibrils or small bundles and abundant microfibrils (Figs. 3a, b) inflammatory cell infiltration was variable from case to case, from mild (+) to intense (+++). Fibroblasts were seen in small number, with dilated plasmic reticulum.

The interstitial matrix of the myocardium showed mild to moderate deposits of fibronectin and laminin, as compared with uninfected controls (Figs 6a, b; 7a, c). Fibronectin appeared as focal deposits in the subendocardiun and

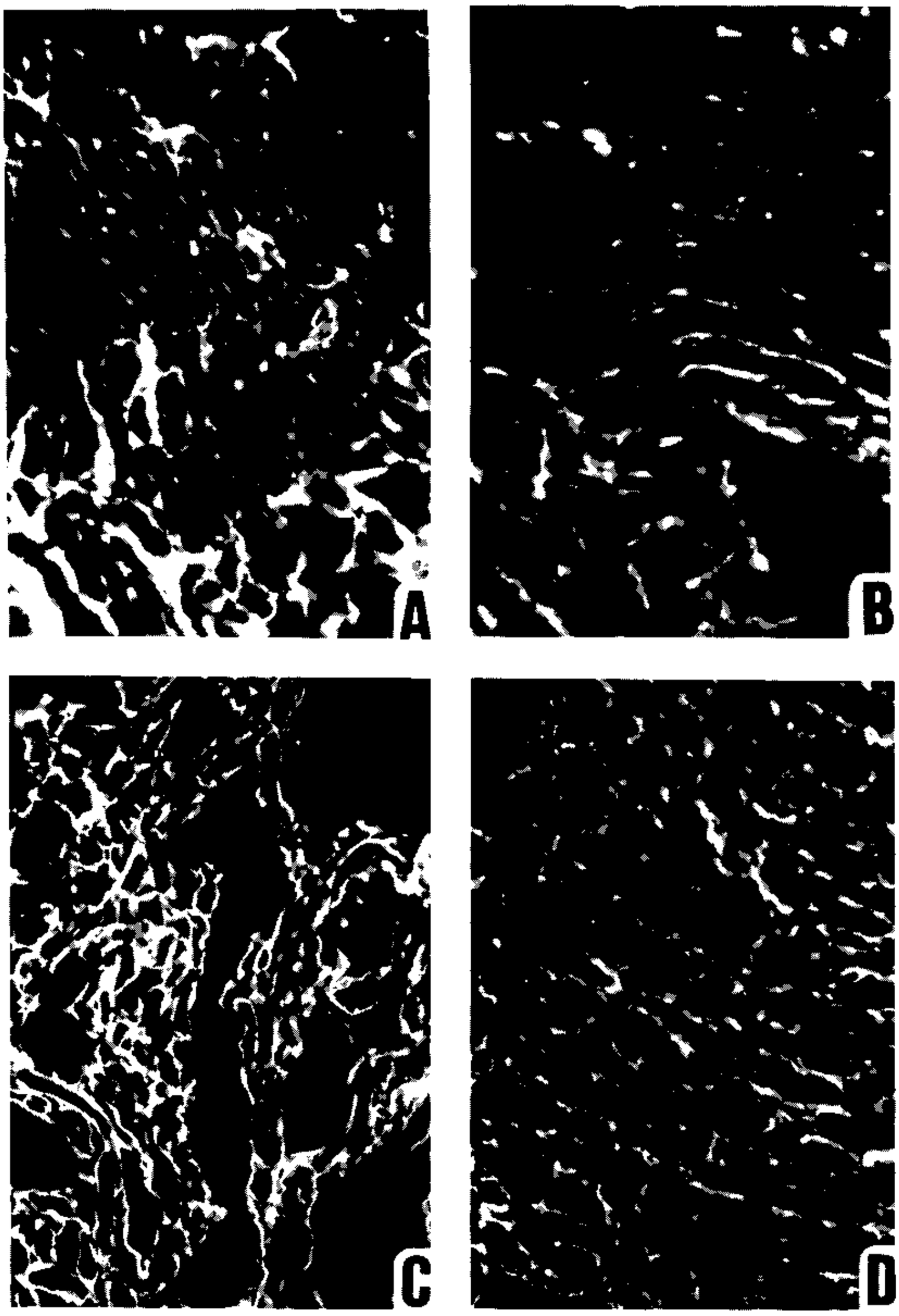

Fig. 7A, B – Fibronectin C, D – Laminin. A – *Untreated control:* interstitial deposits of fibronectin, related with inflammatory infiltration. X250. B – *Treated and cured mouse:* small and low intensity fluorescent deposits of fibronectin, as compared with the untreated control. X400. C – *Untreated control:* diffuse thickening of the sarcolema of cardiac cells, specific for laminin deposits. X250. D – Treated and cured mouse: slight deposits of laminin around fibers. X250.

interstitial spaces (Fig. 7a). Laminin appeared as diffuse deposits at the basal membrane of cardiac fibers in the enlarged interstitial space (Fig. 7c). Collagen immunotyping showed predominantly Types III, Pro-III and IV deposits (Figs 8a, c) significantly increased as compared with uninfected controls (Figs 6d, e, f). Type I collagen distribution was similar to that seen in control mice (Fig. 6c).

Treated mice (Table III) – The cured mice, treated from the 90th or the 157th day post infection and killed 65 days after the initial dose, showed mild to moderate focal or diffuse inflammatory infiltration of the myocardium (Fig. 1b) and the fibrosis was present in a low degree (+) in 3/5 mice. The picrosirius staining showed a relative decrease of the collagen deposits and fragmentation of the collagen

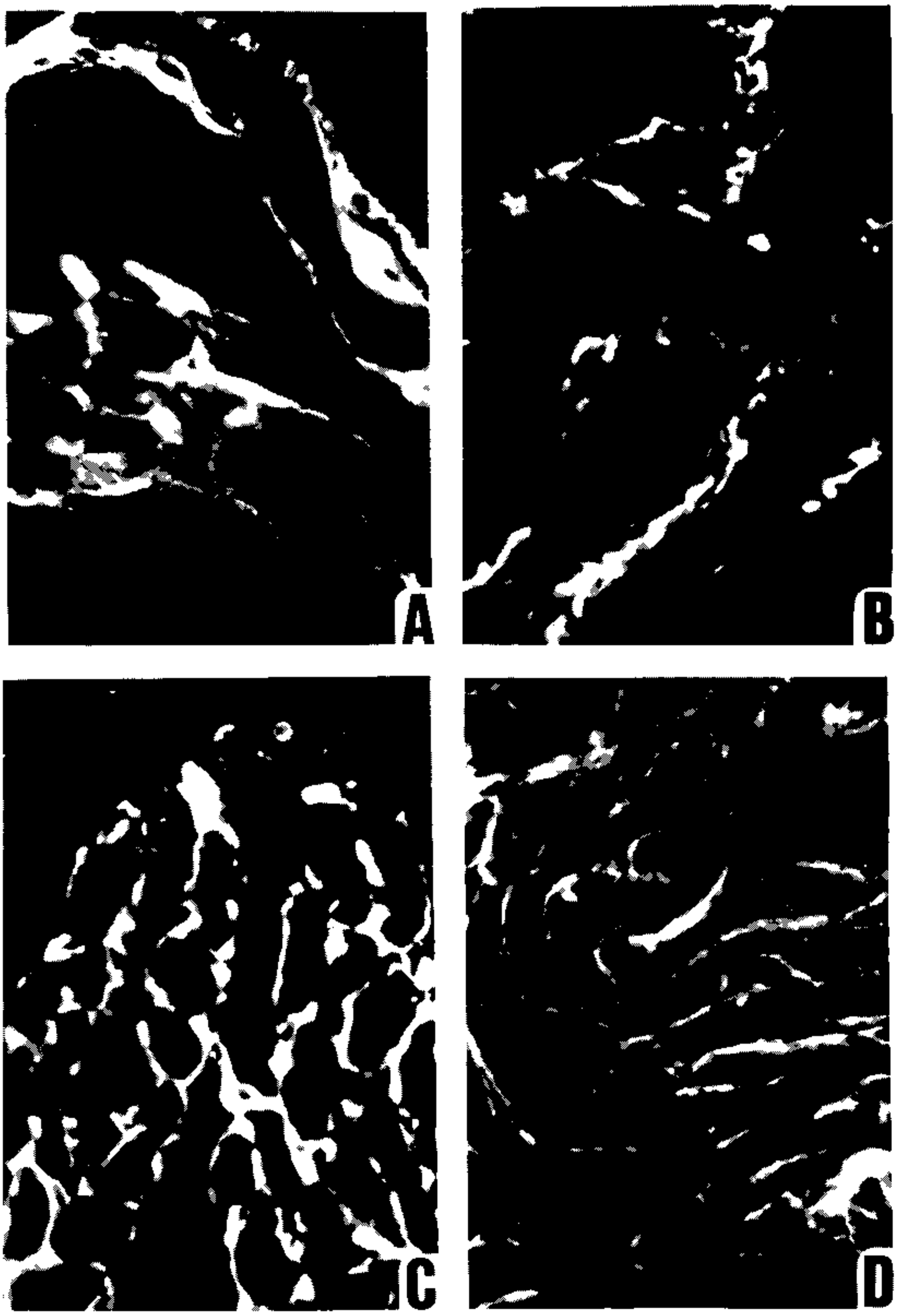

Fig. 8A, B – Pro-III collagen. C, D – Type IV collagen. A – *Untreated control:* dense interstitial deposits of Pro-III collagen, with irregular focal thickening. B – *Treated and cured:* slight and focal interstitial deposits specific for type IV collagen. C – *Untreated control:* diffuse thickening of the sarcolema of cardiac cells due to irregular deposits of type IV collagen. D – *Treated and cured mouse:* slight and focal interstitial deposits with specific fluorescence for type IV collagen. X400.

bundles (Figs 2c, d), also seen with polarized light microscopy.

By electronmicroscopy the myocardium in treated and cured mice showed enlarged interstial spaces containing scarce amorphous material with lytic alterations isolated irregular bundles of collagen with signs of collagen degradation with focal amorphous electron dense deposits (Figs 4b; 5a, b).

Treated but uncured mice showed variable inflammation in the myocardium, less intense than in the untreated controls, varying from negative to moderate; on electronmicroscopy mild to moderate collagen deposits were formed by irregular bundles and signs of degradation and matritial electrodense deposits were seen (Fig. 4a).

Immunofluorescence of the interstial matrix

components and collagen immunotyping showed a clear cut decrease of these components as compared with the untreated controls. Laminin and fibronectin were present in low amounts (+) (Figs (7b, d); collagen types III, pro-III and IV were present in low level or absent (Figs 8b, d). Type I collagen was unaltered.

MICE INFECTED WITH THE 21 SF STRAIN (TABLE IV)

Untreated infected controls – On histopathological study focal or diffuse mild to moderate mononuclear infiltration of the myocardium was seen with slight fibrosis; the histopathological lesions were most intense in the animals with longest periods of infections.

Electron microscopic study, fibronectin and laminin evaluation and collagen immunotyping showed the same aspects described for the mice infected with the Colombian strain, treated and untreated. As shows in Table IV the degree of such alteration was low and varied from mild (+) to moderate (++) and was often absent (not altered).

Treated mice – Table IV – Independently of the number of days that elapsed from the first dose of treatment to the sacrifice (30, 55, 65 or 84), all the treated and cured mice showed mild focal inflammatory infiltration of the myocardium and negative to mild fibrosis. Alterations of the interstitial matrix under immunofluorescence study were mild or absent. At the ultrastructural study focal lytic alterations of the matritial components, collagen disorganization and amorphous electron dense deposits were seen.

Uninfected control mice – Histopathological and ultrastructural examination of the myocardium did not show pathological alterations. Laminin and fibronectin as well as collagen deposits showed a normal pattern as previously described (Andrade et al., 1989c) (Figs 6a, b, c, d, e, f).

DISCUSSION

Alterations of the extracellular matrix of the myocardium in mice chronically infected with *T. cruzi* reveal a progressive process with deposits of fibronectin and laminin in the early stages, followed by collagen deposits predominantly of types III and IV in the late chronic phase of infection (Andrade et al., 1989c). These collagen deposits were associated with the presence of macrophages, fibroblasts and myofibroblasts (Andrade & Grimaud, 1986). According to Perez Tamayo (1978) these are characteristically young and resorbable collagens, having sites susceptibles to the action of diverse proteases.

Treatment of chronically infected mice has proved the curative effect of MK-436 (Andrade et al., 1989a) and of Nifurtimox and Benznidazole (Andrade et al., 1989b) bringing about parasite destruction and resolution of the inflammatory myocardium and skeletal muscle alterations in high percentage of cases.

In the present study differences were detected between the experimental groups, depending on the parasite strain and the chemotherapy drug used. In mice infected with the 21 SF strain, with a low inoculum, the inflammatory and fibrotic lesions were of low intensity, even in untreated controls. The influence of parasite strain on the cardiac lesions in chronically infected mice has been previously described (Andrade, 1990) showing that they are more intense and frequent in those infected with the type III strains of *T. cruzi*. The effect of treatment with Benznidazole was better evaluated through electronmicroscopy study. In this group as well as in the animals infected with the Colombian strain, treated with Benznidazole and not cured, regressive alterations of the collagens were seen by ultrastructural study with lytic dissolution of fibrils and its transformation into a granular electrodense material as described by Andrade & Grimaud (198) for the late stage of schistosomal granuloma involution. Treatment of animals infected with the Colombian strain with MK-436 caused a clear cut regression of the inflammatory and fibrotic lesions (Andrade et al., 1989a) as was seen at histopathological study. At the present investigation these aspects were confirmed by picrosirius staining method and the electronmicroscopy study. In all the treated an cured mice, the components of the interstitial matrix showed a decrease when compared with the untreated controls. These observations may be relevant to effective treatment of chronically infected individuals with positive xenodiagnosis, in the chronic asympotomatic phase of Chagas' disease (the indeterminate form) or with mild and initial cardiac involvement taking into

account the possibility of involution of the cardiac fibrosis. The pathogenetic mechanism of chronic myocarditis in Chagas' disease is dependent on delayed hypersensitivity reactions (Andrade et al., 1987). However continued damage is apparently correlated with persistent parasitism, since parasitological cure may be followed by clearance of inflammatory lesions or their partial regression. The relationship of the fibrosis with the inflammatory process indicates that the regression of these lesions by the specific treatment could, secondarily, lend to involution of the fibrosis.

A new perspective for the clinical treatment of Chagas' disease is opened after the observation that not only the inflammatory but also the fibrotic alterations of the interstitial matrix are reversible in the dependence of parasitic clearance. Chronic asymptomatic cases in the endemic areas may benefit from curative treatment.

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