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Effects of ischaemia-reperfusion and cyclosporin-A on cardiac muscle ultrastructure

F. Jurado, J.M. Bellón, J.A. Pareja, A. Golitsin, L. Millán, G. Pascual and J. Buján

Department of Morphological Sciences and Surgery (Surgical Research Laboratory), Faculty of Medicine, University of Alcalá de Henares, Madrid, Spain

Summary. The present study investigates the effects on the cardiac muscle cell of two of the determining factors for the success of organ transplant; ischaemia-perfusion and immunosuppressive treatment with cyclosporin-A (CsA). To this end an abdominal, heterotopic heart transplant model in singenic Sprague-Dawley rats was employed.

Three study groups were established: Group I (control, n=15) animals undergoing heart transplant without treatment; Group II (n=15) animals undergoing heart transplant and subjected to a daily dose of CsA in a cremophor vehicle (Sandimun®) (5 mg/kg/sc); Group III (n=15): animals undergoing heart transplant and administered a daily dose of pure CsA (5 mg/kg/sc). Recipient animals were sacrificed 7, 14, 21, 30 and 50 days after transplant. During the post-operative period, heart function was assessed by daily abdominal palpation. Graft specimens obtained at each follow-up period were subjected to light and transmission electron microscopy. Immunohistochemical analysis of specimens was performed using the rat macrophage-specific monoclonal antibody MCA-341.

The ischaemia/reperfusion process induced considerable alteration to cardiac muscle cells of control animals. Effects, apparent after the first week of transplant, included mitochondrial swelling and loss of cristae, hypertrophy of the sarcoplasmic reticulum and structural changes to sarcomeres. Two weeks after transplant, the myocardium was infiltrated by inflammatory cells. These effects diminished 30 days post-transplant. Cardiac tissues of treated animals (groups II and III) showed similar behaviour although, in the latter group, mitochondrial damage was greater and intense myocardial fibrosis took place. Infiltration of cardiac muscle by white blood cells did not take place until 3 weeks post-implant.

These results indicate: a) The ultrastructural changes detected in cardiac fibres of animals of the three study groups were attributable to the ischaemia/reperfusion process rather than to treatment with CsA; b) CsA appears to augment mitochondrial damage and myocardial fibrosis; c) the inflammatory response was delayed and reduced by the immunosupressant; and d) the cremophor administration vehicle did not seem to exert an independent toxic effect on the myocardium.

Key words: Heart transplant, Ischaemia/reperfusion, Cyclosporin-A, Myocardium, Mitochondria

Offprint requests to: Prof. Julia Buján, Ph.D., Department of Morphological Sciences and Surgery, Faculty of Medicine, University of Alcalá de Henares, Crta. Madrid-Barcelona Km. 33,600, 28871 Alcalá de Henares (Madrid), Spain

Introduction

Organ transplant is currently widely accepted as therapeutic treatment of terminal conditions of the liver, heart, kidney and pancreas. One of the determining factors for the success of a transplant is the preservation of the organ during the period of ischaemia. Significant contributions include the development of new, improved preserving solutions (Jeevanandam et al., 1992; Stringham et al., 1994) and the use of immuno-suppressant drugs (Meiser et al., 1991). However, cardiac transplant research has shown that the reduction of the period of ischaemia and the use of new preserving solutions have failed to attenuate the adverse effects of the ischaemia-reperfusion process on the morphology and function of the myocardium (Menasché et al., 1993; Fremes et al., 1995).

Moreover, cyclosporin A (CsA), the immuno-supressant of choice for post-transplant treatment, despite its success in the control of acute rejection has been shown to induce myocardial fibrosis in the transplanted heart (Karch and Billingham, 1985) and to exert a toxic effect on myocardial cell mitochondria (Millane et al., 1994). Other authors have reported enzymatic alterations in the myocardium in the presence of CsA (Hutcheson et al., 1995). Recently, Tatou et al. (1996) have suggested that cremophor, the administration vehicle for CsA, may induce an independent toxic effect on myocardial cells.

The present investigation describes the use of an abdominal, heterotopic cardiac transplant model in the rat to evaluate the structural alterations to the myocardium provoked by the ischaemia-reperfusion process,

and the toxicity of CsA and its vehicle (cremophor) towards the cardiac muscle cell. Singenic animals were used as donors in an effort to avoid rejection and interference of immunological factors.

Materials and methods

Female Sprague-Dawley rats weighing between 250-300g were employed. Animal care and experimental protocols were conducted in compliance with EEC guidelines (EEC-28871-22A9 animal care committee).

Microsurgical technique

The microsurgical procedure was assisted by an operating microscope (Wild M-650). Experimental animals were anaesthetised by the intraperitoneal administration of a mixture of ketamine hydrochloride (0.5mg/100g body weight) and atropine (0.005mg/100g body weight).

Transplant was performed by modification (Ono and Lindsey, 1969) of the technique originally described by Abbott et al. (1964). In no case were sterile surgical technique performed or antibiotics employed.

Prior to the extirpation of the heart, 300 IU of 1% sodium heparin were introduced into the donor jugular vein to prevent the formation of thrombi. Once harvested, the heart was preserved in Ringers lactate solution at 4 °C until the moment of transplant (approximately 30 min). Aorto-aortic and pulmonary-caval vascular anastomoses were performed by interrupted suture using 8/0 nylon monofilament thread (Ethilon, Ethicon®). Vessels were joined termino-laterally. Once the abdominal cavity was closed by silk 1/0 suture, the correct functioning of the heart was ensured by abdominal palpation. Total ischaemic periods ranged from 55 to 60 min.

In this model the heart shows an anomalous function (see Fig. 1). The left ventricle is unable to perform a haemodynamic function although it maintains mechanical functionality.

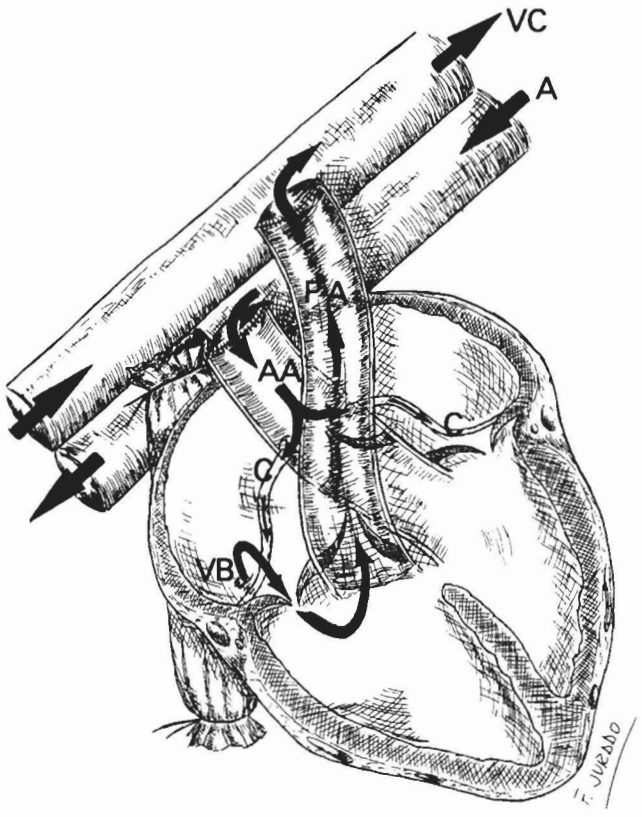

Fig. 1. Diagram of transplanted heart action. Blood fills the aortic arc (AA) of the transplanted heart and is interrupted by a functioning aortic valve. Flow is diverted through the coronary arteries (C), irrigating the entire myocardium, and drains into the coronary venous basin (VB) of the right auricula. Flow continues through the right ventricle and returns via the inferior vena cava (VC) of the recipient. A: abdominal aorta; PA: pulmonary artery.

Post-transplant assessment of cardiac function

During the post-operative period, recipient animals were subjected to daily abdominal palpation. Normal transplanted heart beat was used as an indicator of the adequacy of the experimental model (Tauber et al., 1978).

Experimental design

Ninety animals (45 donors, 45 recipients) were employed for abdominal, heterotopic cardiac transplant. Recipient organisms were divided into 3 study groups: Group I (control, n=15), animals undergoing heart transplant without treatment; Group II (n=15), animals undergoing heart transplant and administered a daily subcutaneous dose of CsA in a cremophor vehicle (Sandimun®) (5 mg/kg) from the day of transplant to sacrifice; Group III (n=15), animals undergoing heart transplant and injected daily with pure CsA at the same dose and frequency. The pure cyclosporin was administered in an ethanol-Tween solution following the procedure described by the manufacturer (Sandoz, Spain).

Three healthy animals of the same species and similar age and weight were used as controls for the morphological study. Control animals were not subjected to intervention or treatment.

Follow-up times

Animals were sacrificed at: 7, 14, 21, 30 and 50 days post-transplant. A mean number of 3 cardiac grafts were obtained at each follow-up time.

Morphological and ultrastructural analyses

Morphological and ultrastructural observation were performed using light and transmission electron

microscopy respectively. Specimens were obtained by perfusing the animals own and transplanted heart with Ringers lactate solution. A double approach method was used. First, a catheter was introduced into the right jugular vein for the administration of 1 ml sodium heparin (1%) to prevent clot formation. Following mid-laparotomy, a second catheter was introduced into the inferior vena cava. Once the two perfusion systems were opened, a incision in the abdominal aorta was performed, to let the preserving solution flow. Cardiac grafts were obtained at each follow-up time by re-intervention.

Heart specimens for optical microscopy were fixed by immersion in a 10% formol solution and embedded in paraffin to obtain 5 micron-thick transverse sections. Staining was performed using hematoxylin-eosin and Massons trichrome stains.

Recipient animals were prepared for transmission electron microscopy by perfusion at 100 mm Hg for 15 minutes with a 3% glutaraldehyde/1% paraformaldehyde (1:2) fixative solution via the inferior vena cava. Cardiac grafts were fragmented into small portions, fixed in 3% glutaraldehyde for two hours and placed in Millonig buffer (pH 7.3). For ultrastructural analysis, these specimens were postfixed in 1% osmium tetroxide, dehydrated in a graded acetone series and embedded in Araldite to obtain thin sections. Finally, the contrast of grids was enhanced with lead citrate prior to observation under a ZEISS 109 transmission electron microscope.

Immunohistochemical analysis

Specimens were fixed and embedded in parafine as in the optical microscopy studies. The technique employed was that of alkaline phosphatase with the monoclonal antibody specific for rat macrophages MCA-341 (Serotec). It was performed as referenced in an anterior study (Bellón et al., 1996).

Results

Macroscopical results

Four of the experimental animals died during surgery (three due to anaesthesia and one due to haemorrhage) and were immediately replaced. No cases of intraperitoneal infection or infection at the site of laparotomy were detected. Further macroscopical findings are listed in Table 1. In some cases defective beating of the transplanted heart was recorded (Table 2).

Histological results

Group I (Control)

One week after transplant the most significant observation was a change in the size and shape of the cardiac muscle cell. Most of these cells had lost their cylindrical morphology. Nuclei were clear with loose chromatin. Micropinocytotic vesicles were commonly found in the vicinity of the plasma membrane. At times, the basal membrane was separated from the plasma membrane by a strip of low electron density. Intercellular spaces were often widened and the rupture of longitudinal cell junctions was evident. In some areas, intercalated discs had lost their distinct morphology. Mitochondria were dilated and many lacked cristae (Fig. 2). In the cellular cytoplasm, vesicles of clear content formed by dilation of the sarcoplasmic reticulum were detected amongst myofibrils. Also observed within mitochondria, were small matrix granules containing lipofuschine. The sarcomere was disorganised; Z-lines were diffuse and myofibrils showed a random array.

Cell lesions were prominent during the first stages of the experiment. Cellular swelling was most evident 14 days after transplant. Some cells showed small oedematous areas in the cytoplasm (Fig. 3a). Transverse sectioning of the cardiac fibre revealed the abundant proliferation of the sarcoplasmic reticulum with numerous highly dilated vesicles (Fig. 3b). Also observable were secondary lysosomes associated with what seemed to be small lipid globules (Fig. 4a) and myelin figures. Mitochondria were of a considerable size showing a disorganised internal structure (some showing membranous bodies), oedema and scarce matrix granules. Some of the cells showed a lack or reduction in the number of small mitochondria and the sarcotubular system.

Alterations to myofibrils were detected in several cells. Different degrees of damage to the cardiac fibre could be distinguished ultrastructurally. Fibres mostly affected showed modification of sarcomere structure. Bands and myofibrils were rarely distinguishable. Only dark areas, possibly derived from the condensation of Z-line filaments, were observed (Fig. 4b). Fewer changes were detected in other fibres; sarcomeres were better

Table 1. Macroscopical findings.

	GROUP I (control)	GROUP II (follow-up times, days)	GROUP III
Infarcted areas	30	50	14
Aneurism*	30.50	-	-
Hypertrophy	7-14	-	-
Atrophy	21	-	-
Thrombosis**	-	-	21-50

*: arterio-arterial junction; **: arterio-venous junction.

Table 2. Evolution of heart beat in transplanted hearts.

	GROUP I (control)	GROUP II (days)	GROUP III
Bradicardia	++ 13	++ 12	++ 5 + 11 ++ 12

+: mild; ++: hard.

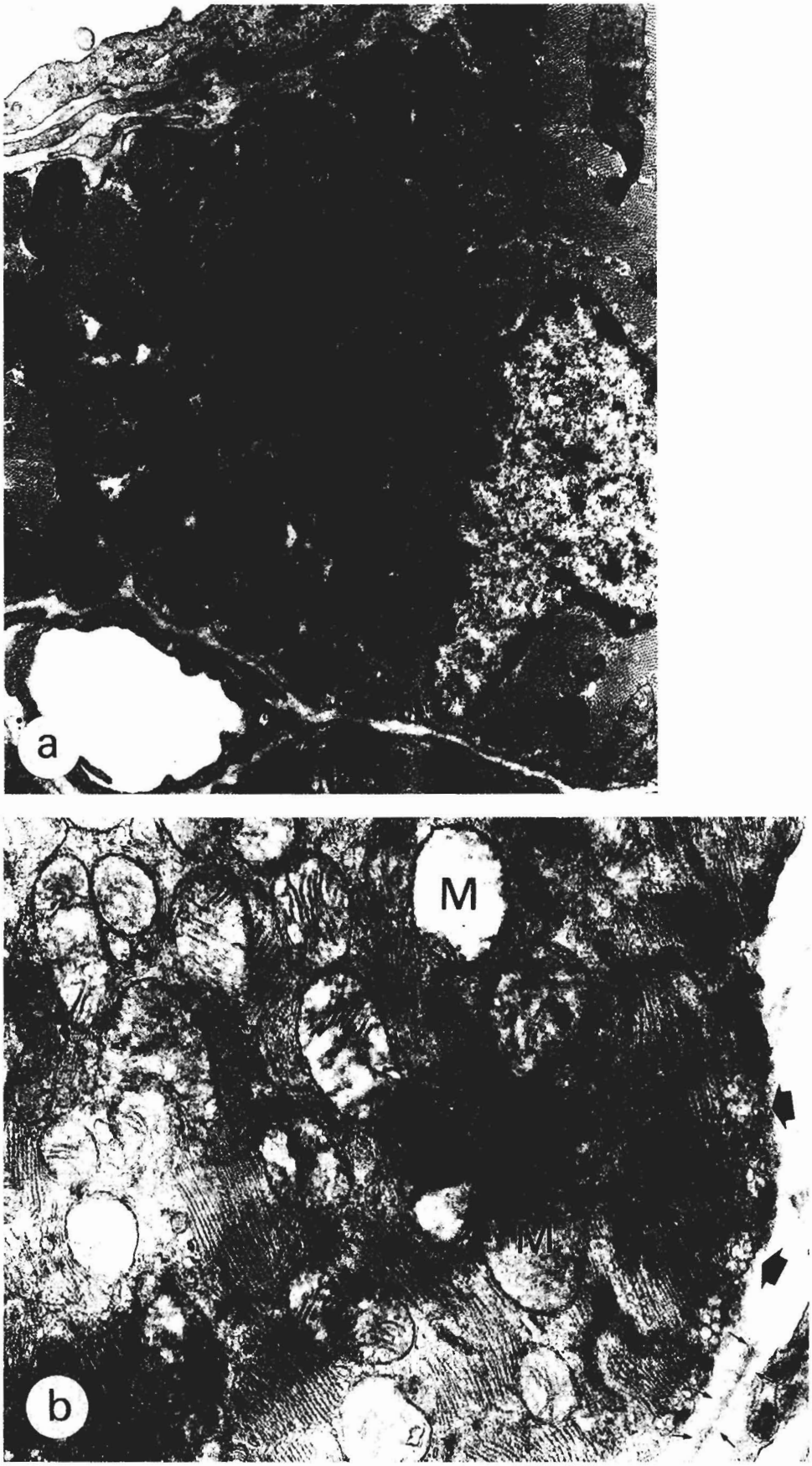

Fig. 2. a. Normal ultrastructural appearance of the myocardium. Transverse section of cardiac fibre showing mitochondria (M) with numerous parallel cristae and myofilaments (F). TEM, x 7000.
b. Ultrastructural appearance of control group transplanted heart myocardium at 7 days post-implant. Affected mitochondria (M), numerous micropinocytotic vesicles close to the plasma membrane (large arrow) and alterations to the basal lamina (thin arrow). TEM, x 12,000

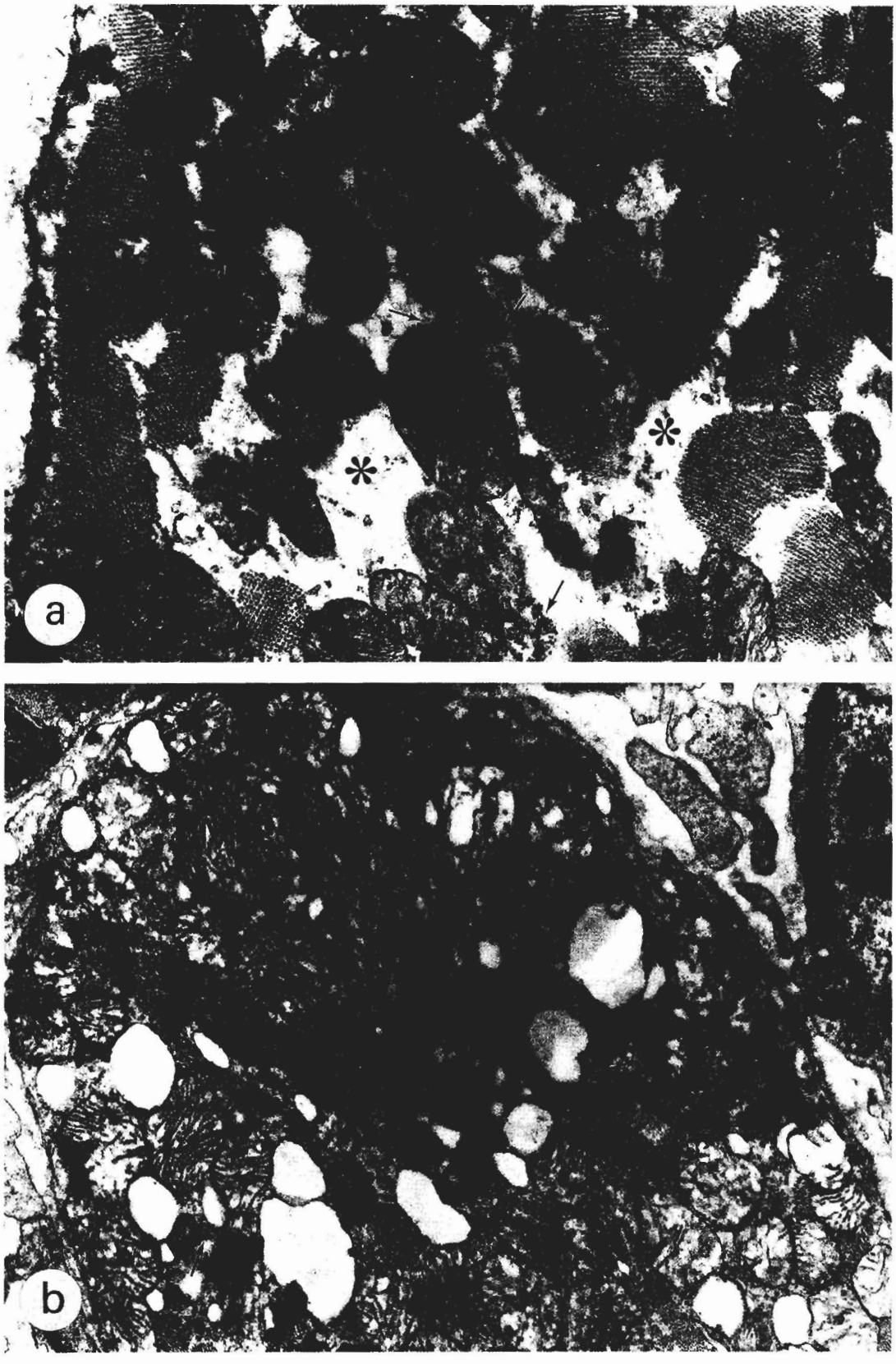

Fig. 3. Untreated transplanted heart at 14 days post-implant. **a.** Intracytoplasmic areas of oedema (*) within the cardiac fibre. TEM, x 12,000. **b.** Dilation and proliferation of the sarcoplasmic reticulum (thick arrows). (M: mitochondrion; small arrows: glycogen). TEM, x 7,000

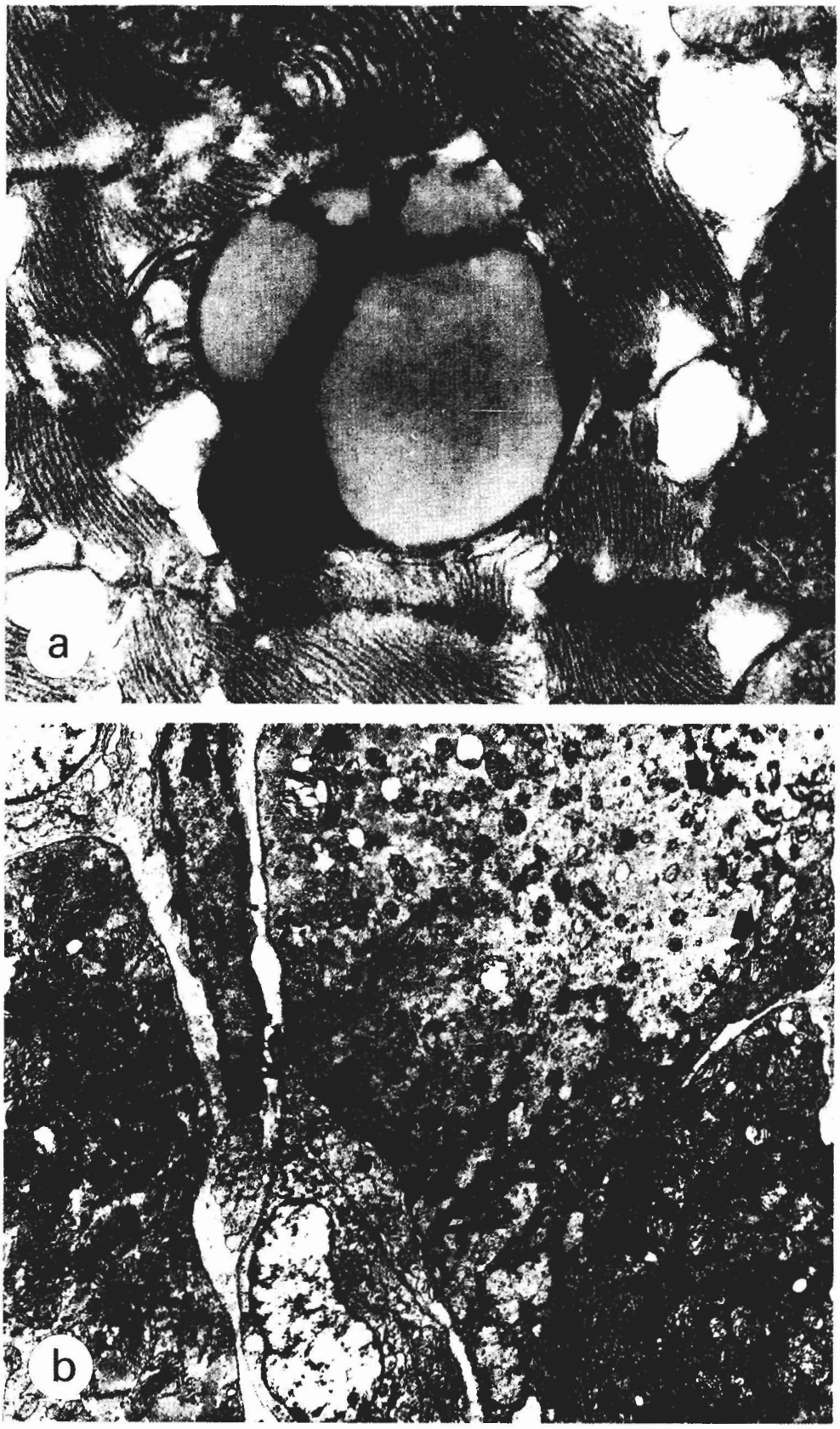

Fig 4. Untreated transplanted heart at 3 weeks post-implant. **a.** Secondary lysosomes in the cardiac fibre. TEM, x 20,000. **b.** Myofibrillar alterations to cardiac fibres. Condensation of Z-line filaments (arrows) TEM, x 3,000

preserved although myofilament loss in the I-band was apparent. This was accompanied by shortening of the band and alteration of the form and configuration of the muscular fibre and presence of sarcoplasmic masses. Many of these fibres presented changes in Z-lines. The latter were of a zig-zag appearance, a phenomenon

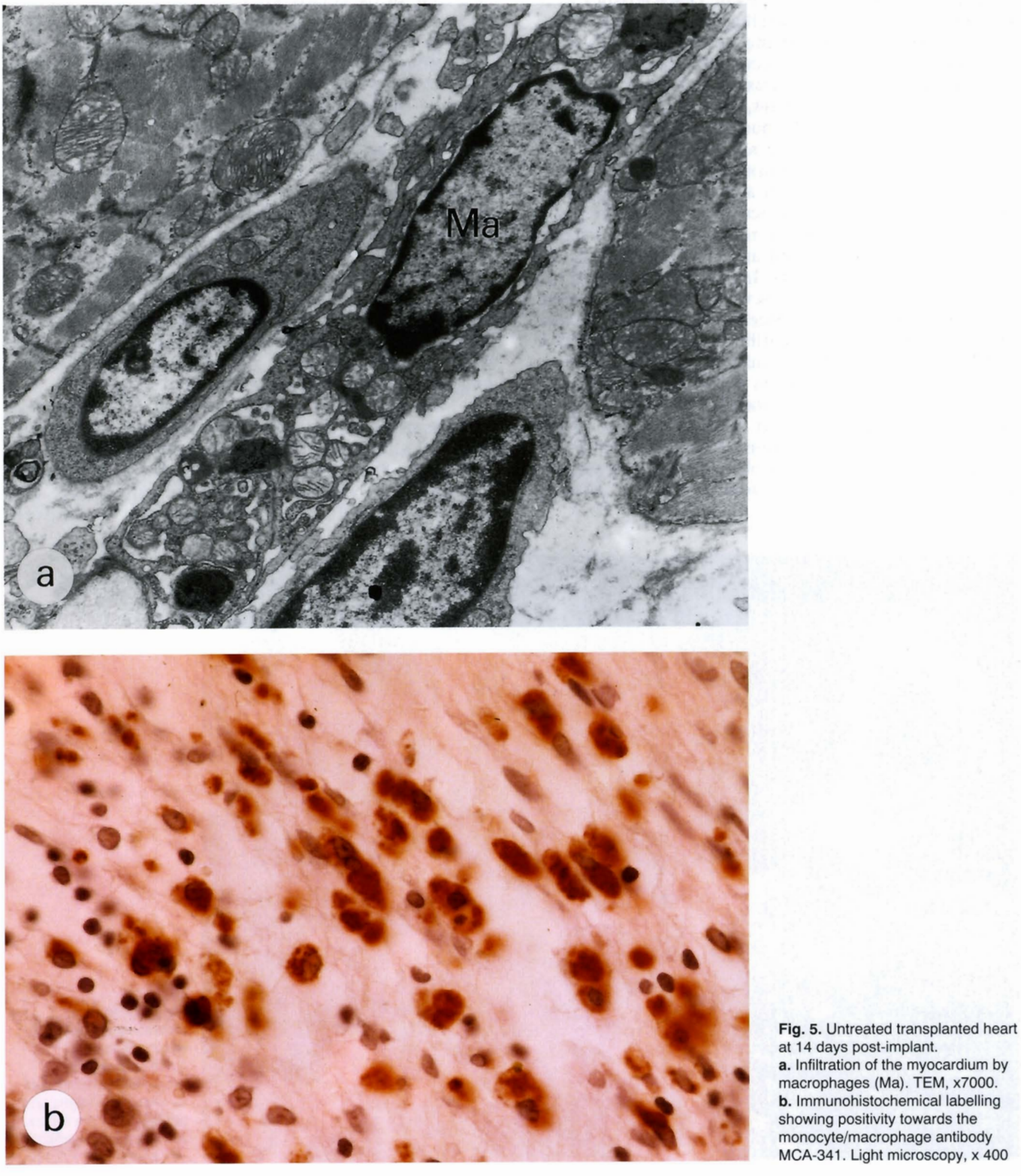

Fig. 5. Untreated transplanted heart at 14 days post-implant.
a. Infiltration of the myocardium by macrophages (Ma). TEM, x7000.
b. Immunohistochemical labelling showing positivity towards the monocyte/macrophage antibody MCA-341. Light microscopy, x 400

termed "streaming" of the Z-line. At times, the Z-lines were distorted or fragmented.

At this stage (14 days post-transplant), the myocardium was highly infiltrated by several types of white blood cell. Those with macrophagic characteristics were predominant as shown by labelling with the monoclonal antibody specific for rat macrophages MCA-341. Macrophages were seen to accumulate between cardiac fibres and in perivascular spaces (Fig. 5a,b).

Thirty days after transplant, the recovery of cell size and shape commenced. The nucleus presented a normal morphology with a visible nucleolus. Cellular and mitochondrial oedema diminished. Reticulo-sarcoplasmic vesicles were smaller and many had fused with the plasma membrane. In myocardial intermediate areas, contraction of sarcomeres resulting in the virtual disappearance of the I-band and shortening of the A-band was observed (Fig. 6). In these cells, myofibrils were randomly disposed. Several atrophied cardiac muscle cells were found adjacent to pericardiac vessels. These cells had scarce myofibrils, clear nuclei, small and scarce mitochondria and sarcoplasmic bodies. There was also an increase in the intercellular fibrous component. At times, intercalated discs were irregular in structure and dilated at several points of the longitudinal nexus-type junctions. An abundance of non-degranulated mastocytes was observed close to vessels. Tissue infiltration by white blood cells was notably inferior.

Group II

In this group the changes involving the size and shape of the cardiac muscle cell were similar to those observed for the control group at each particular follow-up time. Similar modifications to the basal lamina and nucleus were apparent. However, mitochondria were more affected than those of control specimens. These were of great size and often showed dilation of the internal membrane. This rendered a small bubble appearance to the cristae. On occasion, rupture of cristae and oedema were detected in addition to the presence of intramitochondrial lamellar bodies. An increase in granules was observed in the mitochondrial matrix at 7 days post-transplant. This increase diminished with time and reappeared 50 days post-transplant. Certain cardiac muscle cells showed sarcoplasmic bodies between myofibrils. The sarcoplasmic reticulum was scarcely dilated. The anomalous disposition of myofilaments comprising myofibrils was occasionally observed. Z-lines formed a zig-zag pattern (Fig. 7a). In certain areas of the ventricular wall, alterations to intercellular, longitudinal cardiac fibre junctions were detected.

Two weeks following transplant, a greater disorganisation of the myofibrils and an increase in myocardial fibrosis (Fig. 7b) became apparent. There was an increase and dilation of the sarcoplasmic reticulum. The vascular surface of the cardiac fibre and endothelial cells of adjacent capillaries showed

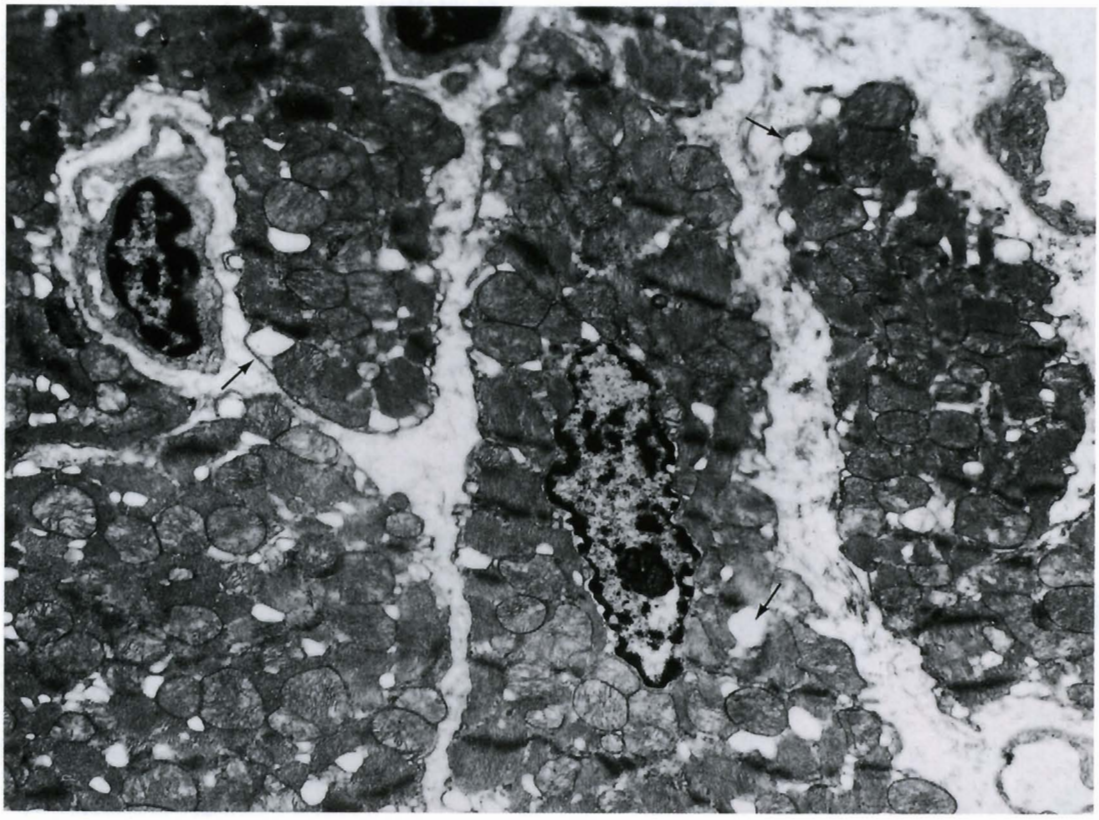

Fig. 6. Untreated transplanted heart at 50 days post-implant. Contraction of the sarcomere accompanied by loss of the I-band and shortening of the A-band Arrows: sarcoplasmic reticulum. TEM, x 3,000

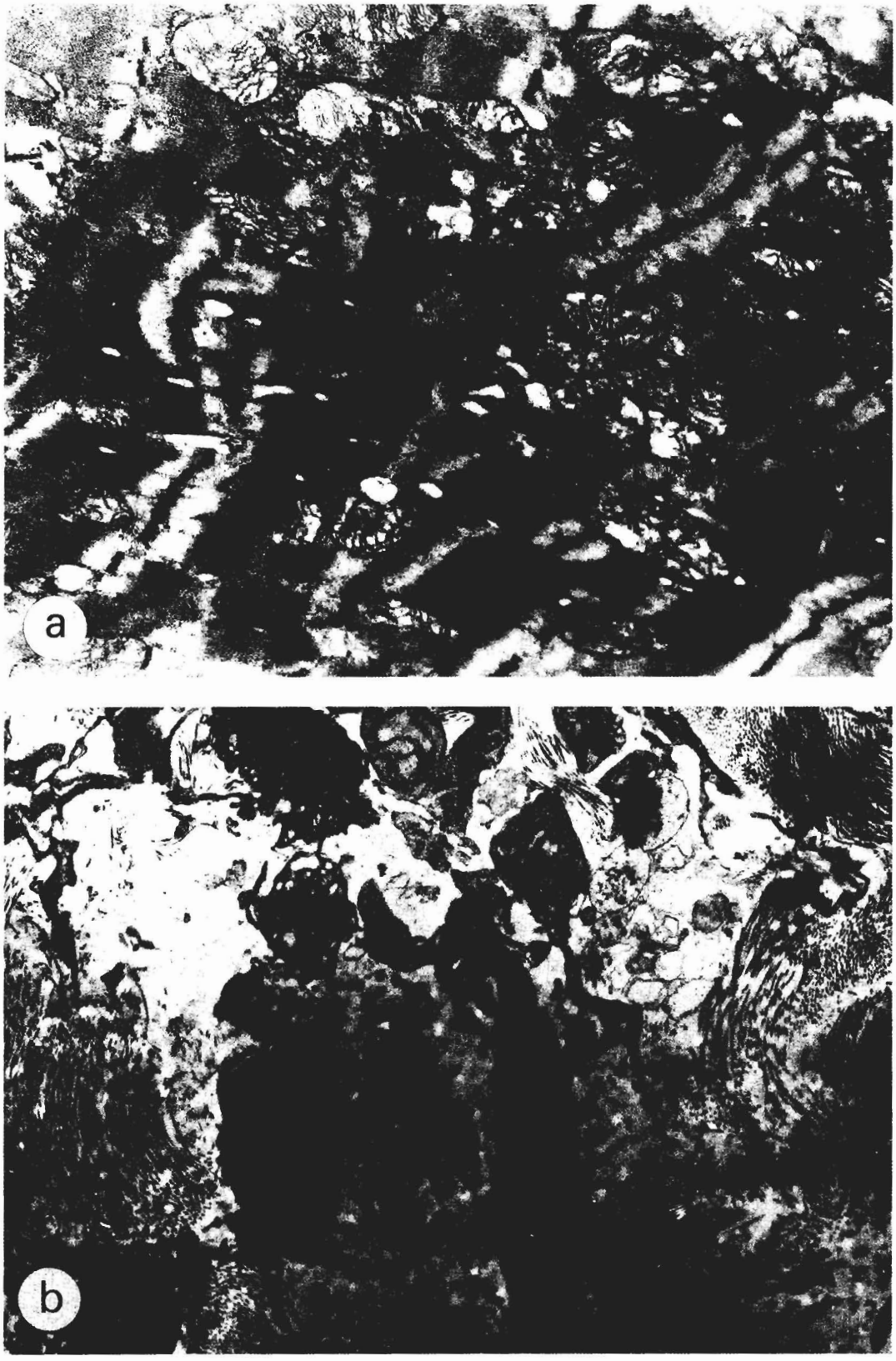

Fig. 7. Transplanted heart treated with CsA (Group II). **a.** 7 days post-implant. Large, intensely dilated, structurally altered mitochondria (M) and modification of the Z-lines. TEM, x 7,000. **b.** 21 days post-implant. Intense fibrosis of the myocardium which is infiltrated by white blood cells. TEM x 3,000

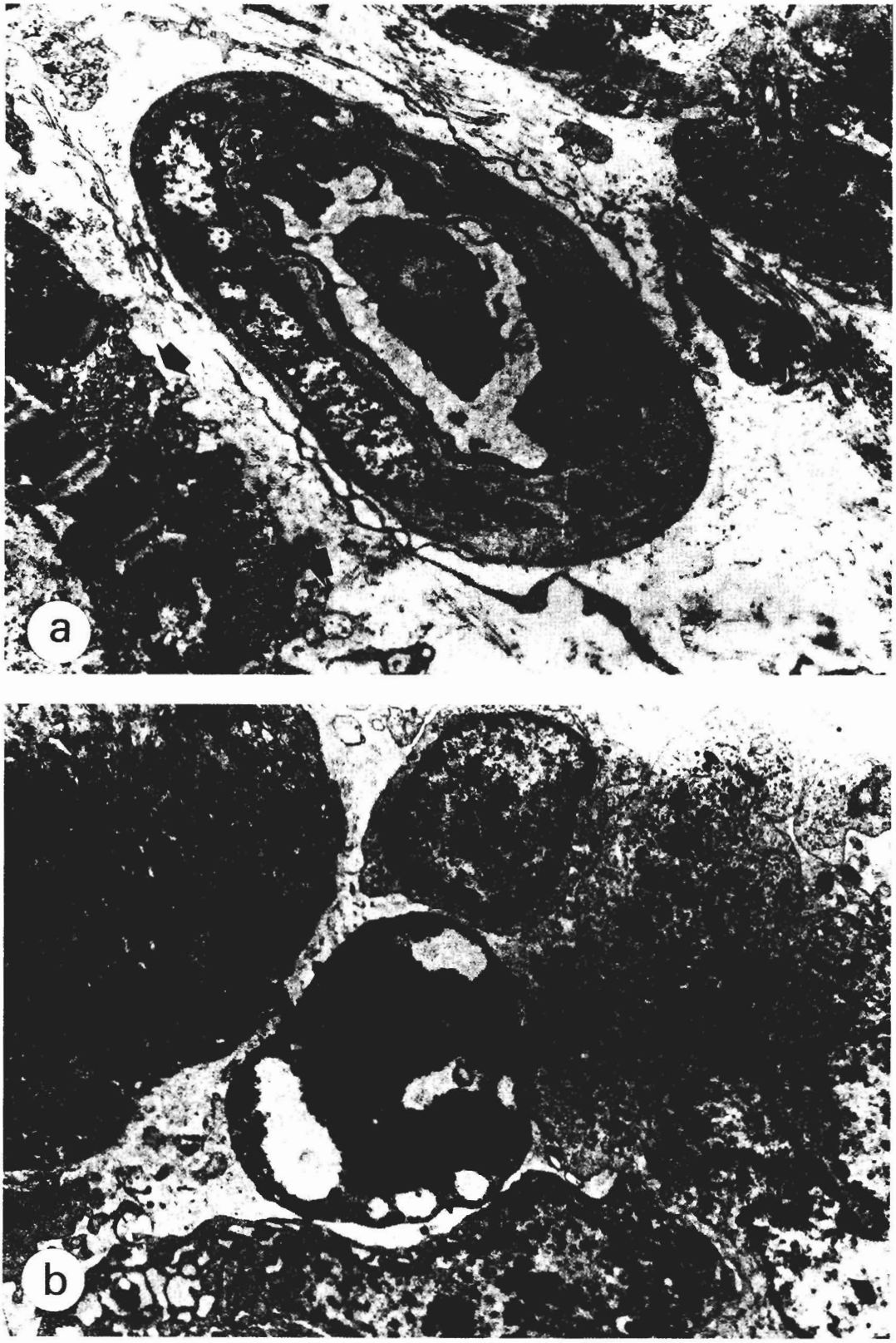

Fig. 8. Transplanted heart treated with CsA (Group III) at 21 days post-implant. **a.** Peripheral mitochondrial accumulation (arrows) showing intense perivascular fibrosis. TEM, x 4,400. **b.** Vacuolised polymorphonuclear cells (P) may be observed within the inflammatory infiltrate. TEM, x 4,400

numerous micropinocytotic vesicles. Infiltration of cardiac muscular tissue by white blood cells was less intense in this group. Maximum infiltration was recorded 21 days after transplant with a predominance of polymorphonuclear cells (PMNs).

One month after transplant, the right half of the heart showed a clear recovery. This was reflected by slight mitochondrial recovery and the recovery of the sarcomere indicated by the reappearance of all the bands. Despite the increase in fibrosis and mastocyte population, there was a decrease in the number of white blood cells infiltrating the myocardium.

Group III

In general, the same cellular effects as reported for the control group were observed. Mitochondria underwent similar changes to those of group II and were frequently seen to accumulate at the cell periphery (Fig. 8a). The inflammatory response was most intense 21 days after transplant. The myocardium was infiltrated by white blood cells including numerous PMNs (Fig. 8b). At times, the latter contained large, intracytoplasmic vesicles. The presence of granule-loaded mastocytes in perivascular spaces and, on occasion, between the actual cardiac fibres was common. As in the CsA-treated group, intense myocardial fibrosis was detected. No significant glycogen loss from cells was observed in any of the three groups.

Intense fibrosis and cell necrosis associated with areas of infarction in the left ventricle were observed in the three groups. In these areas, infiltration by inflammatory cells was observed. These findings remained constant over the entire follow-up period and at 14 days post transplant, the appearance of areas of angiogenesis followed by the formation of a new capillary network was evident. Ventricular recovery, however, did not take place. Generally, the ventricular cavity would be found to contain an organised thrombus corresponding to the donors last cardiac cycle. After 50 days of implant myocardial fibrosis continued to gain intensity.

Discussion

Recent research has demonstrated possible myocardial damage following reperfusion (Braunwald and Kloner, 1985). Reperfusion seems to induce irreversible changes in anoxic myocytes. However, it is not yet understood whether this simply corresponds to the aggravation of damage inflicted by ischaemia or if specific, additional damage is caused by the reperfusion process. Bakker et al. (1995), reported significant, structural damage to isolated rat hearts after 20 min of ischaemia. These authors observed bands of contraction, miolysis, dilated T-tubules, swelling of mitochondria and the sarcoplasmic reticulum, loss of mitochondrial granules and a reduction in mitochondrial contact sites, reduction and loss of cell glycogen and interruption of the sarcolemma. Reperfusion of these hearts caused complete structural destruction. The longer periods of ischaemia of the present investigation did not result in such severe damage following reperfusion of the transplanted organ. A manifest tissue recovery process seemed to initiate after 4 weeks of implant. Cell alterations were similar to those found by these authors following ischaemia. Despite the fact that no significant reduction in glycogen levels was detected in the present model, cristolysis in some myocardial cells and lamellar bodies in mitochondria were observed and are probably attributable to the production of free radicals during reperfusion (Bernier et al., 1986; Pesonen et al., 1995).

The mitochondrial electron transport chain is also thought to be involved in the production of free radicals (Del Maestro, 1980) although their estimation falls outside the scope of this study. Nevertheless, the notable presence of PMNs was detected. The incidence of this cell type has been reported to increase during reperfusion (Engler et al., 1986) and these cells have also been identified as a source of free radicals (Pesonen et al., 1995). It is postulated that free radicals could contribute to cell damage in the myocardium by peroxidation of the lipids in the cell membrane. This would lead to the anomalous exchange of ions between neighbouring cells and in turn cause a dysregulation of the sarcoplasmic reticulum (Forman et al., 1989). Such changes could explain the reticular alterations observed in the present study. Similarly, free radicals have been implicated in the pathogenesis of arrhythmia induced by reperfusion (Bernier et al., 1986; Bolli, 1988).

Mitochondrial contact sites were difficult to discern in the transplanted heart of the present model. Mitochondria were, generally, much altered. According to Bakker et al. (1995), the mitochondrion loses its capacity to form contact sites after periods of ischaemia in excess of 15 min rendering a low energetic state to the mitochondrion.

In animals of groups treated with CsA, there was an increased presence of mitochondrial granules during the first week after transplant. This finding is consistent with a reduced energetic state since there is an inverse relationship between the number of granules and the energetic metabolism of the mitochondrion (Bakker et al., 1995).

Yet another important consideration in transplant research is the preservation of the organ during the period of ischaemia. The different preservation solutions currently employed in heart transplant procedures have not been able to avoid the damage inflicted by ischaemia and reperfusion of the myocardium. The present results using Ringers lactate solution to preserve the heart during the phase of ischaemia were similar to those recently obtained by other authors (García-Poblete et al., 1997). These authors also described a break-down of cell junction structure. However, the animal species, model, preservation solution and periods of ischaemia-reperfusion used by these authors differed from those of the present investigation. Severs (1995) reported a

significant reduction in cell gap-junctions (present in intercalated discs) in patients suffering from chronic cardiac ischaemia. Such alterations affect the electric connection between cardiac cells which is mediated by this type of junction. Several investigations propose a possible connection between electrical communication via gap-junctions and the development of arrhythmia (Luke and Saffitz, 1991; Smith et al., 1991; Severs et al., 1992).

Research efforts concerning the use of CsA in cardiac transplants and its effects are extensive. The tissue toxicity of CsA has been clearly demonstrated in the kidney (Mason, 1984) and liver (Klintmalm et al., 1981). In both cases, dose-dependent alterations were produced. The vast majority of investigations performed on cardiac tissue evaluate the effects of CsA on the coronary arterial wall. Thus, some authors describe a beneficial effect of this drug on this vessel wall (Meiser et al., 1991; Guttmann et al., 1994) while others attribute such effects to its administration vehicle, cremophor (De Caterina et al., 1995). Authors such as Uretsky et al. (1987) and Paul et al. (1994) aim to demonstrate the implication of CsA in the pathogenesis of arteriosclerosis or vascular pathology associated with transplant. However, literature concerning the effects of this immunosuppressant on the myocardium or, more specifically, on the heart muscle cell is scarce. In 1985, Karch and Billingham reported the existence of myocardial fibrosis in the transplanted heart in one isolated case. In the present model, despite the increase in the intercellular fibrous component observed in animals of each study group, most severe myocardial fibrosis was detected in transplanted hearts treated with the CsA preparation or with pure CsA. It is consequently felt that cremophor may not be attributed to have a specific toxic effect of its own as has been suggested by others (Tatou et al., 1996).

With regards to the accumulation of white blood cells in the perivascular spaces and their infiltration in cardiac tissue, a late and less intense response was detected in animals treated with CsA. PMNs and macrophages were the predominant cell type. This suggests a delay in the inflammatory response induced by the immunosuppressant. It has been demonstrated that the action of CsA is only efficient during the initial stages of the immune response (Homan et al., 1980; Morris et al., 1983). CsA suppresses the production and proliferation of the cytotoxic T- lymphocytes but not that of the T-lymphocyte suppressers which are insensitive to its action (Cohen et al., 1984; Wish, 1986). This drug also blocks the inhibitor factor of macrophage migration, lymphocyte chemotactic and growth factors, amongst others (Thomson et al., 1983; Kahan, 1985). It is unable, however, to inhibit the chemotaxic or phagocytic action of PMNs (Wish, 1986). The accumulation of PMNs during treatment with CsA in other tissues has previously been described by the present authors (Bellón et al., 1995). Recent investigations propose a toxic effect of CsA on myocardial mitochondria. Millane et al. (1994) reported an intramitochondrial deposition of calcium associated with CsA treatment in human transplanted hearts. The present results reflected the greatest ultrastructural changes in mitochondria of animals treated with CsA. Although enzymatic or calcification investigations were not performed, greater structural loss of the mitochondrial internal membrane and increased presence of lamellar bodies were detected in these animals. Such bodies are associated with the production of free radicals in mitochondria. Subsequently, CsA could also exert an effect on myocardial lipid peroxidation contributing to cell damage (Tatou et al., 1996).

Denervation represents another important factor to be taken into account. More than 50% of denerved muscles show *target fibres* (Engel, 1961) which have been associated with alterations to the Z-lines. A similar effect was shown in the muscle wall of the right ventricle where cardiac fibres of some areas suffered modifications to sarcomere myofibrillar structure.

Structural alterations detected in the left ventricle were, in all probability, due to the absence of haemodynamic function. Although the blood supply to this area was ensured by the coronary flow, atrophy of the muscle wall was produced due to the lack of pumping action. This causes necrosis of many cells and the consequent large inflammatory response which, in part, is also provoked by the presence of the remnant thrombus.

The present findings suggest that the ultrastructural alterations detected in the cardiac fibres of the three study groups were due more to the ischaemia-reperfusion process than to treatment with CsA. This immunosuppressant seems to add to mitochondrial damage and augments myocardial fibrosis. The inflammatory response is delayed and reduced under the effect of CsA. In the present model, the administration vehicle cremophor, does not seem to exert an independent toxic effect on the myocardium.

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