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# Role of macrophages in myocardial apoptosis following cardiac transplant. Influence of immunosuppressive treatment

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**Summary.** Cytotoxic T cells may induce myocardial apoptosis by histiocyte activation during rejection following allogenic heart transplant. The aim of the present investigation was to evaluate the macrophage response and its relationship to the programmed death of cardiomyocytes in rejection and during cyclosporin-A (CsA) treatment.

An abdominal, heterotopic heart transplant rat model was used establishing two groups: singenic (ST) and allogenic (AL) transplant. 5mg/kg/day (s.c.) CsA (Sandimun®) was administered to half of the animals in each group. Morphological and structural analysis was performed 7, 14, 21, 30, 50 and 100 days post-transplant. Macrophages were detected using the monoclonal antibody (ED1). The TUNEL method was used to visualise apoptotic cells.

Two weeks after ST in animals without immunosuppressive treatment, the transplanted myocardium had been extensively infiltrated by inflammatory cells, many of which were ED1-positive. At 21 days follow-up, the number of labelled cells had fallen. In animals treated with CsA the amount of ED1-positive cells was lower than that seen in the anterior group. Only a few isolated cells of the infiltrate were TUNEL-positive. In the AT group, rejection took place between 9-15 days in the untreated animals. The myocardium was highly infiltrated by mononuclear cells. Some were ED1-positive. Small groups of apoptotic cells were visible in the infiltrate and in some vessel lumens. Rejection was resolved in animals treated with CsA. The macrophage response diminished during follow-up in a similar way to that occurring in the ST. Few cells showed TUNEL positivity. It may be concluded that: a) CsA treatment diminishes the amount of infiltrated macrophages; b) animals receiving ST or AT, show a low level of apoptosis; c) in the present model, the apoptosis of cardiomyocytes does not appear to be induced by macrophages; and d) in this model it is not possible to relate apoptosis and rejection.

**Key words:** Apoptosis, Macrophages, Allogenic transplant, Acute rejection, Cyclosporin

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## Introduction

Recent studies have shown that during rejection following allogenic transplant in the rat, myocardial cells may undergo apoptosis (Szabolcs et al., 1996). Cytotoxic T lymphocytes and delayed-type hypersensitivity effector cells (macrophages) play a major role in the immune mechanism of rejection in allogenic cardiac transplant (Tilney et al., 1984; Hall, 1991; Barry, 1994). The Cytotoxic T cells involved in the acute rejection process are able to lyse target cells by several mechanisms (Griffiths and Mueller, 1991; Higuchi and Aggarwal, 1994; Ju et al., 1994) inducing apoptosis either directly or by macrophage activation. Although such mechanisms of action are unknown, it has been suggested that under certain circumstances, macrophages may induce necrosis and apoptosis on neighbour cells (Cohen et al., 1992; Thompson, 1995; Iwanaga et al., 1994; Maino and Joris, 1995). The aim of this investigation was to evaluate the macrophage response and its implication in the programmed cell death of cardiomyocytes during rejection and after immunosuppressive treatment with CsA. This agent inhibits the production and proliferation of cytotoxic T lymphocytes (Cohen et al., 1984; Wish, 1986).

## Materials and methods

Male Sprague-Dawley (recipients, donors) and Lewis (donors) rats weighing 250-300g were employed. Animal care and experimental protocols were conducted in compliance with EEC guidelines (EEC-28871-22A9

animal care committee).

Transplant was performed by the Ono and Lindsay (1969) technique. The microsurgical procedure and heterotopic cardiac graft function during the post-operative period was performed as previously described (Jurado et al., 1998).

Two models of abdominal, heterotopic cardiac transplant were used: singenic (Sprague to Sprague, n=36) and allogenic (Lewis to Sprague, n=36). In each implant model, a daily dose of 5 mg/kg de CsA (Sandimun®) was administered subcutaneously to half the transplant recipients while the remaining animals received no treatment.

Follow-up study periods were established at 7, 14, 21, 30, 50 and 100 days post-transplant. A mean number of 3 cardiac grafts were obtained at each follow-up time.

Morphological and ultrastructural observation were performed using light and transmission electron microscopy respectively. The collection and preparation of specimens was performed according to a methodology described elsewhere by the present authors (Jurado et al., 1998).

### *Immunohistochemical analysis*

The monoclonal antibody ED1, specific for rat monocytes/macrophages MCA-341 (Serotec), was used for paraffin-embedded slices. The alkaline phosphatase-labeled avidin-biotin method was employed. The protocol consisted of incubation with the primary antibody (1:100 in Tris-buffered saline; TBS, pH 7.6) for 1 hour at 37 ºC, incubation with IgG-biotin (1:50 in TBS) for 45 min, and labelling with avidin coupled with alkaline phosphatase (1:200 in TBS) for 20 min. These steps took place at room temperature. The images were developed with a chromogenic substrate containing α-naphtol phosphate and Fast Red. This step took place at 37 ºC. Nuclei were contrasted for 5 min with acid hematoxylin.

### *In situ apoptotic cell labelling*

Recognition of apoptotic nuclei was performed by modification of the TUNEL method (Negoescu et al., 1996). TUNEL is based on the in situ detection of nucleosomal DNA fragmentation characteristic of apoptosis. In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Specimens were deparaffinized, hydrated and equilibrated in TBS buffer. They were then subjected to microwave irradiation (SANYO EM-704T) for 5 min (350W) in 0.01M citrate buffer (pH 6). The detection of DNA fragmentation was performed using a TdT Fragment End Labeling kit (TdT FragEL™, Calbiochem, CN Biosciences INC., USA). Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. The images were developed with a chromogenic substrate containing Diaminobenzidine (DAB). The specimens were contrasted for 5 min with methyl green. Morphological evaluation and characterization of normal and apoptotic cells were performed under a light microscope (Zeiss, Jena, Germany).

### *Cell counting and statistics*

Labelled cells with the monoclonal antibody ED1 of the myocardial infiltrate were counted in 30 photographic fields (x 400) under a Zeiss light microscope, using samples taken at each study period (7, 14, 21, 30, 50 and 100 days after surgery) in both transplant groups (treated and untreated).

The results obtained from cell counting were subjected to a descriptive statistical method to determine the arithmetic mean and the standard deviation. Student's 't' test was applied to the mean values obtained. Analysis of variance was then used for paired data. Finally, the mean values were compared by applying the Student-Newman-Keuls test.

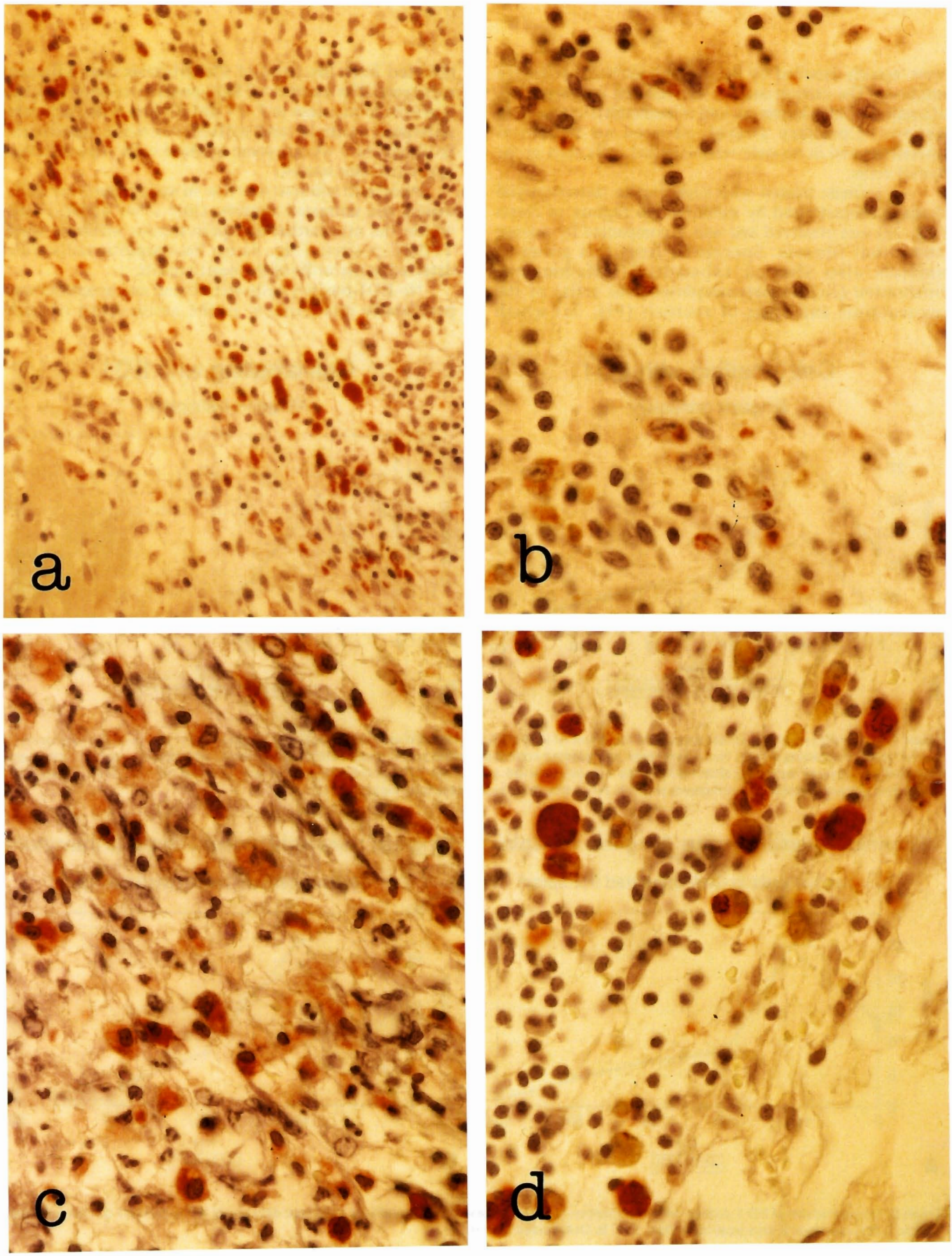
## Results

### *Singenic transplant group*

No treated or untreated transplanted singenic heart was rejected. Some animals suffered episodes of incidental bradycardia which were later resolved and in no case led to the death of the animal.

From the first week onwards, the transplanted heart of animals not subjected to immunosuppression showed infiltration of the myocardium by inflammatory cells. Some of these cells were labelled with the ED1 antibody and the maximum labelling was observed at 2 weeks post-transplant (graph 1a and Fig. 1a). At this stage, many of the infiltrated mononuclear cells showed a granular cytoplasm which was of an intense red/purple colour (Fig. 1a). A few labelled cells were also found in the pericardium. From 21 days post-transplant, the number of labelled cells decreased progressively and the intensity of labelling was reduced (Fig. 1b and graph 1a) in myocardial infiltrated areas, although a few groups of highly stained, ED1-positive cells could still be seen in the pericardium. In specimens from animals sacrificed 100 days after transplant, a few isolated interstitial cells

**Fig. 1.** Cell labelling with the anti-monocyte/macrophage monoclonal antibody ED1. **a.** A positive ED1 antibody reaction in the inflammatory infiltrate at 14 days post-singenic transplant in animals not subjected to immunosuppression. x 200. **b.** At 50 days post singenic/allogenic transplant, only a few cells are marked with the ED1 antibody. x 400. **c.** Cell labelling at 14 days post-allogenic transplant with no immunosuppression. x 400. **d.** In both types of transplant the number of labelled cells in CsA-treated animals is lower with respect to untreated animals at 14 days post-transplant. x 400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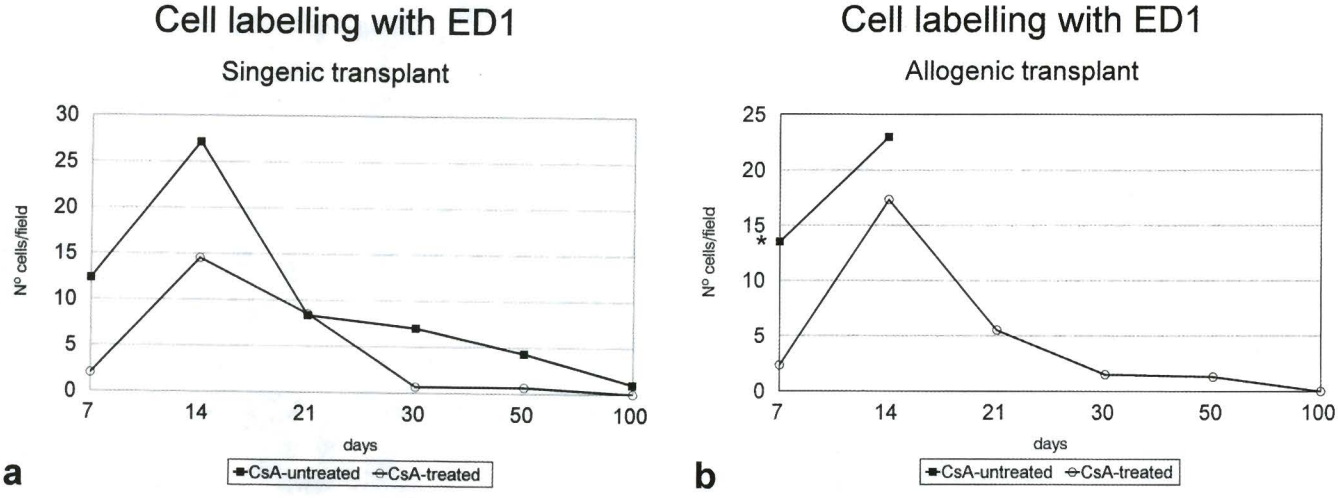
were weakly labelled by the anti monocyte/macrophage antibody. The cardiac muscle showed cell inflammation, alteration of the sarcomere and mitochondrial damage in general. Coronary arteries showed no structural alterations and in no case were labelled cells seen in the vessel walls.

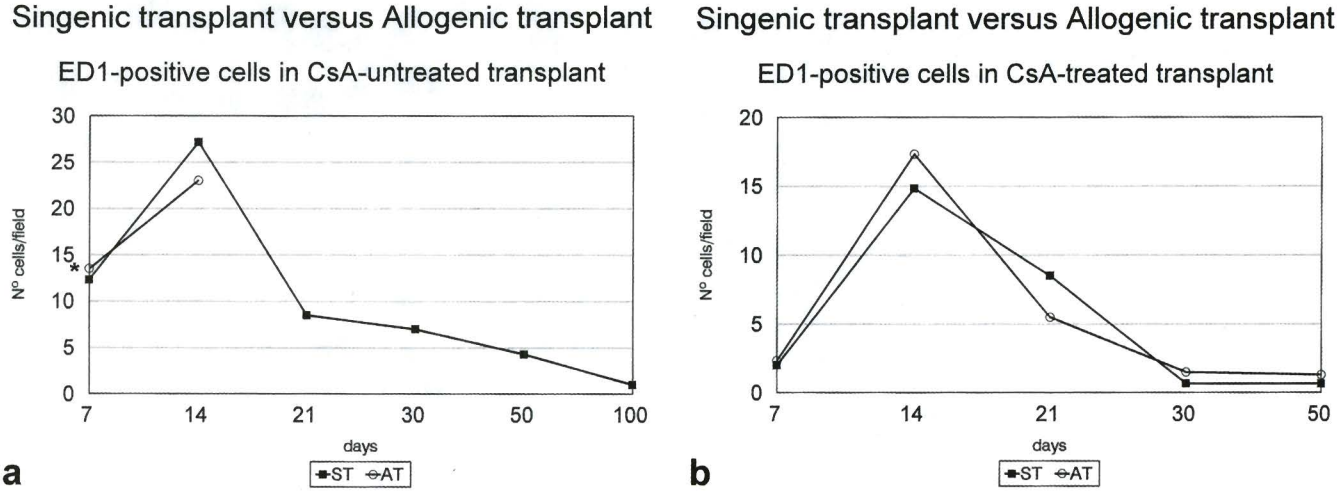
In animals treated with CsA greater mitochondrial damage characterised by swelling and rupture of crests (Fig. 2a) was observed and was accompanied by intense interstitial fibrosis. Specimens from this group of animals also showed substantial inflammatory infiltration (rich in polymorphonuclear cells) of the myocardium and areas of necrosis (Fig. 3a) which affected both the cells of the infiltrate and adjacent myocytes. The number of ED1-positive cells (graph 1a) was seen to decrease at 7, 14 and 30 days with respect to untreated animals, and differences were significant ($p<0.05$). At 3 weeks, some of these cells were detected in perivascular spaces.

Apoptotic cell rate was low in specimens from CsA-treated and untreated animals. Only a few isolated cells within the myocardial and perivascular infiltrate were TUNEL-positive (Fig. 4a,b). No apoptotic cells were detected in the walls of the heart vessels.

No ED1-positive cells were found and apoptosis was practically non-existent in the native heart of both




**Graph 1.** Arithmetic mean values obtained from the count of infiltrated-labelled-cells with the monoclonal antibody ED1. Cell counting was done in several sections obtaining cardiac tissue from heart transplanted from both treated and untreated animals. The results obtained from cell counting were subjected to statistical study. Significant differences can be observed ($p<0.05$) at 7, 14 and 30 days in ST and at 7 and 14 days in AT, when CsA-treated and untreated groups are compared. **a.** ST. **b.** AT. *: this value is at 9 days post-transplant: 13.5.




**Graph 2.** Medium values of ED-1 postive cells. Comparison between ST and AT. There are no significant differences between them. **a.** CsA-untreated groups. *: this value is at 9 days post-transplant: 13.5. **b.** CsA-treated groups.

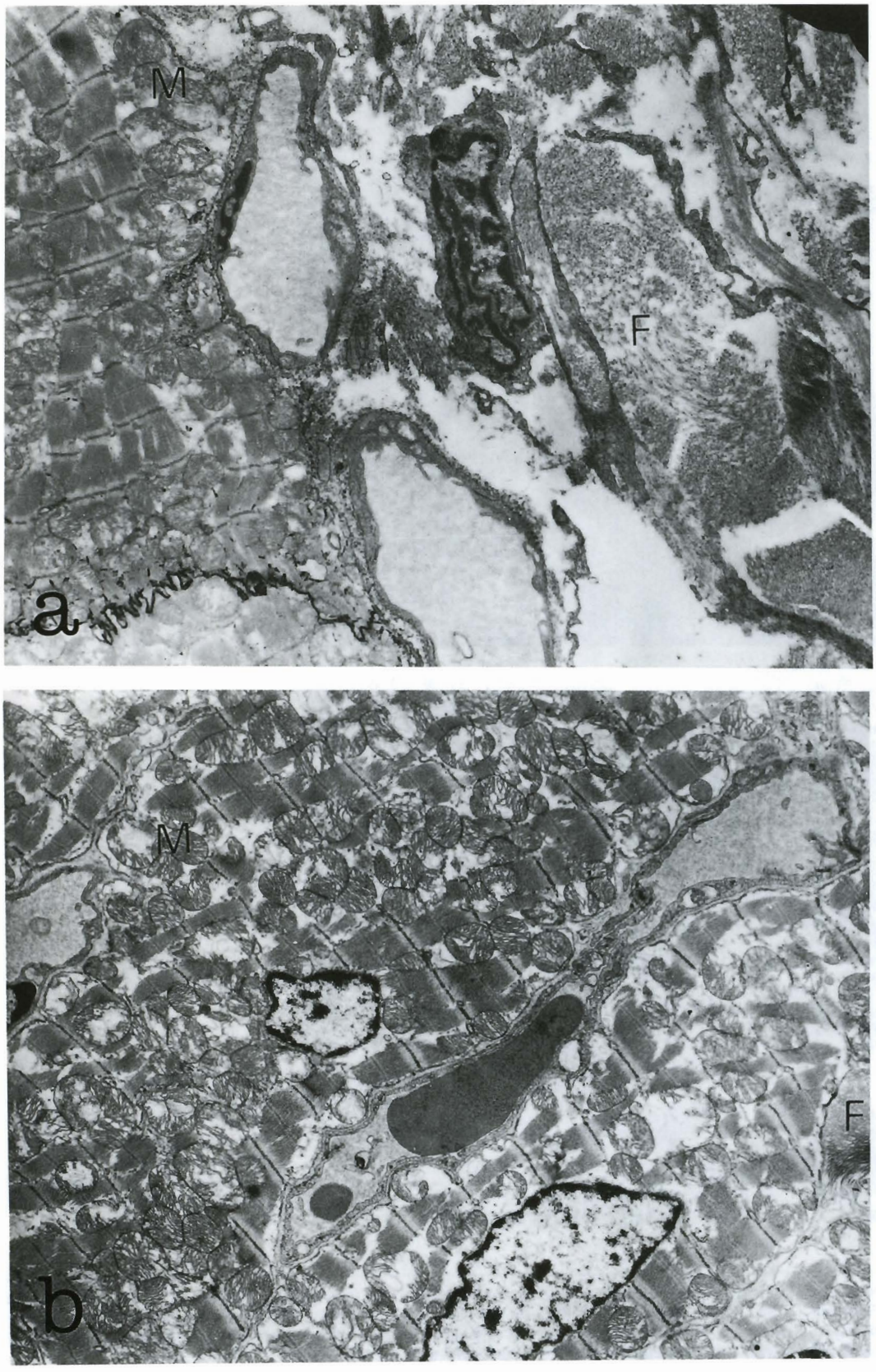



**Fig. 2.** An increase in interstitial and perivascular fibrosis (F) occurs at 30 days after the onset of immunosuppressive treatment. Persistent alterations to the sarcomere and aggravation of the mitochondrial (M) changes may be observed.
**a.** Singenic. **b.** Allogenic.
x 3,000

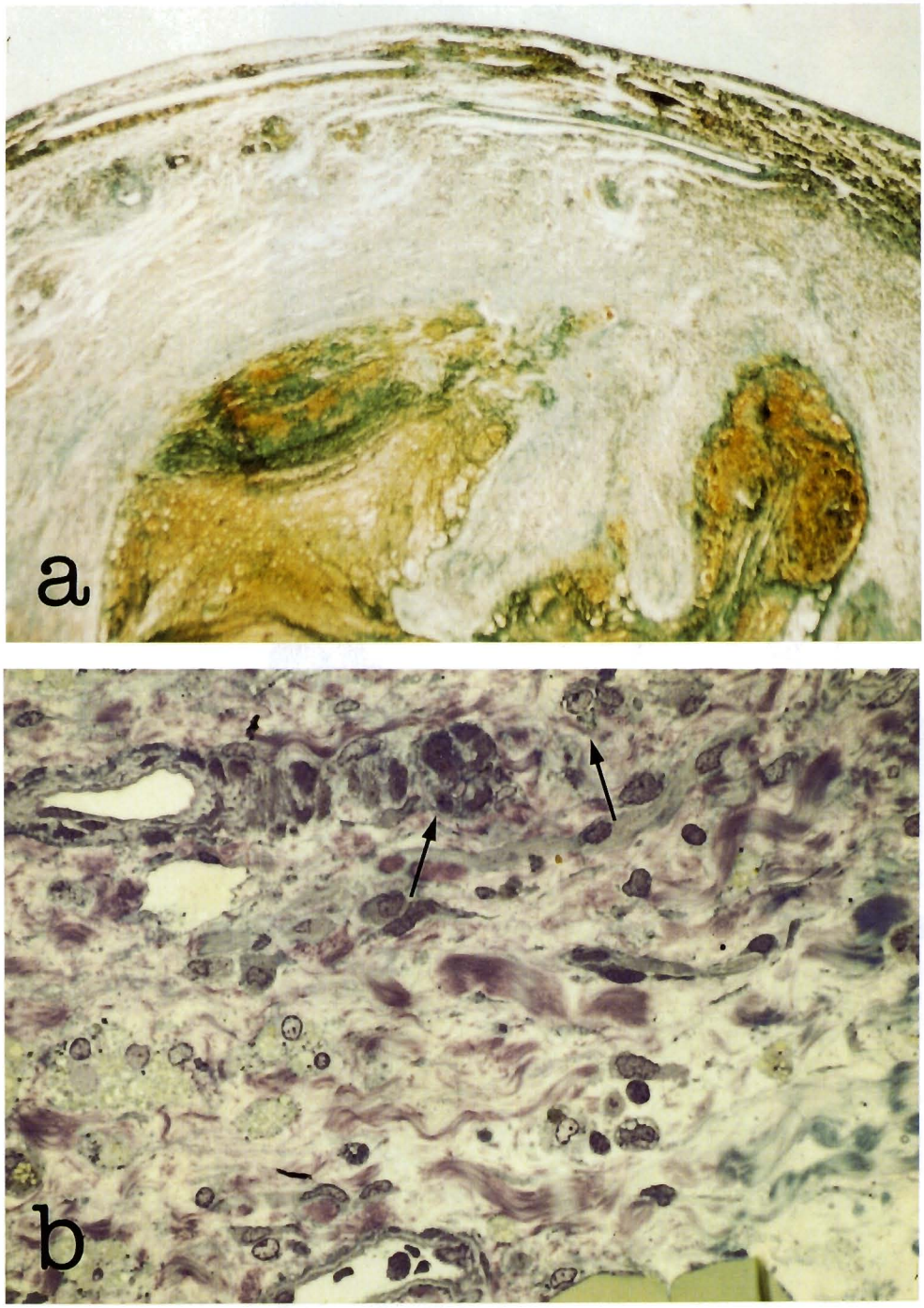
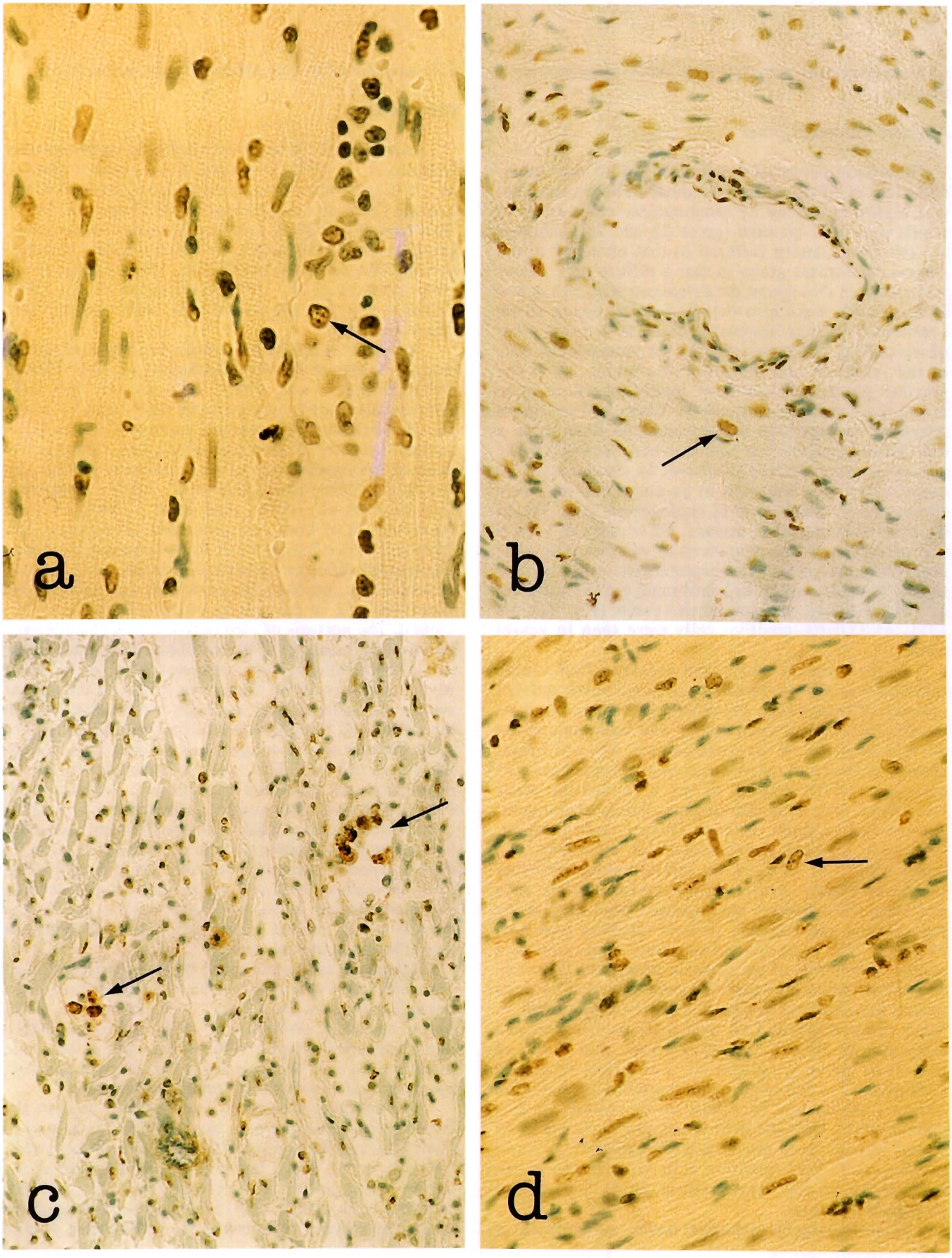



**Fig. 3.** Transplant plus CsA treatment. Extensive areas of necrosis and fibrosis which affect inflammatory cells and adjacent heart myocytes may be observed. **a.** Singenic. x 25. **b.** Allogenic: small areas of angiogenesis (arrows) are seen. x 400

**Fig. 4.** Labelling of apoptotic cells (arrows) by the TUNEL method. **a.** TUNEL-positive cells in the inflammatory infiltrate at 14 days post-singenic untreated transplant. x 400. **b.** Following 14 days of treatment with CsA, singenic transplant specimens show TUNEL-positive cells in the perivascular infiltrate. x 100. **c.** TUNEL-positive cells in the lumen of some small vessels in an allogenic transplant with no immunosuppressive treatment. x 100. **d.** Apoptotic cells in the inflammatory infiltrate of an allogenic transplant with CsA treatment. x 200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immumosuppressed and non-immunosuppressed animals.

*Allogenic transplant group*

Rejection occurred between 9 and 15 days post-transplant in all the untreated animals. The myocardium was highly infiltrated by mononuclear cells, some of which were ED1-positive (graph 1b and 2a), although the labelling pattern differed to that obtained in the singenic transplant group in that a diffuse pink colour was produced throughout the cytoplasm (Fig. 1c). A few labelled cells were also detected at the edges of the ventricular cavities. Extensive areas of necrosis were observed (Fig. 3b), which affected the mononuclear cells of the infiltrate and the myocytes which, as in the previous group, appeared to be highly dilated. In some areas, the destruction of cardiac fibres and extensive interstitial haemorrhage was apparent. In these areas, a few isolated mononuclear cells that were seen among the myocytes were TUNEL-positive. In these areas, the coronary arteries appeared to be congested and were surrounded by small areas of angiogenesis (Fig. 3b). In the areas of least cardiac damage, the integrity of vessels was preserved and no alterations to vascular cells were observed. Small groups of apoptotic cells could be seen among the cells which had infiltrated the myocardium and in the lumen of some small vessels (Fig. 4c). No ED1- or TUNEL-positive cells were seen in vessel walls.

In the animals which had received CsA, acute rejection was always overcome although some suffered episodes of bradycardia and other complications such as thrombosis, atrophy, hypertrophy and aneurisms in the vessels involved in the anastomosis. Several hearts showed evidence of chronic rejection and small areas of infarction. Cell lesions in the myocardium were maintained and, as in the singenic hearts of immuno-suppressed animals, intense fibrosis was evident. Coronary vessels preserved their structure and morphology. At 14 days, an inflammatory infiltrate composed mainly of necrotic polymorphonuclear cells and some ED1-positive macrophages were seen in the myocardium and a clearly defined labelling peak was obtained. The macrophage response decreased in a similar way to that of the singenic transplants (Fig. 1d). Significant differences ($p<0.05$) were observed between treated and untreated groups at 7 and 14 days (graph 1). When comparing both CsA treated groups (singenic and allogenic) no differences ($p>0.05$) were seen in any of the study periods (graph 2). Labelling intensity corresponded to that of allogenic transplant specimens in animals which had received no treatment. A few periadventitial cells and cells circulating in the coronary vessel lumens were also discretely labelled. A small number of TUNEL-positive cells were found in the interstices of the myocardium (Fig. 4d) and in periadventitial areas.

As in the singenic transplant model, no apoptotic or ED1-positive cells were observed in the native heart.

## Discussion

Programmed cell death occurs during embryogenesis (Abrams et al., 1993), in normal tissue turnover (Kerr et al., 1972) and also in response to several types of cell damage (Yamada and Ohyama, 1988; Thompson, 1995). This type of cell death involves the activation of genes, proteases and endonucleases which degrade chromosomal DNA (Arends and Wyllie, 1991; Cohen et al., 1992; Pietsch et al., 1993; Gagliardini et al., 1994; Núñez et al., 1994; Maino and Joris, 1995; Steller, 1995; Xia et al., 1995).

Apoptosis in the heart has been the subject of several studies (Tanaka et al., 1994; Gottlieb et al., 1994; Hamet, 1995; Hamet et al., 1995). It is known that fully differentiated heart cells preserve their capacity to die via the mechanism of apoptosis and this seems to be an intrinsic property of adult cardiomyocytes. However, the death of the myocyte may occur by necrosis (Reimer and Jennings, 1986; Benjamin et al., 1989), apoptosis (Gottlieb et al., 1994; Itoh et al., 1995) or by a combination of both cell processes (Kajstura et al., 1996a). The two forms of cell death occur independently and involve different myocyte populations. The rates of necrosis and apoptosis increase with age and play an important role in the ageing process (Kajstura et al., 1996b). The necrosis of cardiomyocytes is characterised by swelling, breakage of the membrane and lysis leading to a marked inflammatory response (Tilney et al., 1984; Maino and Joris, 1995; Thompson, 1995). In the present model, the inflammatory response was intense both in the ST and AT transplants and ultrastructural changes of this type were seen in extensive areas of the myocardium which, in rejected AT's, were associated with tissue destruction and interstitial haemorrhage. Szabolcs et al. (1996) also observed areas of eosinophylic necrosis in cardiomyocytes associated with vasculitis and interstitial haemorrhage in rejected AT's in rat. Further, Paul et al. (1992) reported necrosis, interstitial fibrosis, infiltration by mononuclear cells and myointimal proliferation in rejected cardiac allografts performed in rat. In the present model, intimal hyperplasia was not observed in any of the experimental groups.

Apoptosis is characterised by the condensation of chromatin and cell contraction with conservation of organelles (Arends and Wyllie, 1991; Cohen et al., 1992; Maino and Joris, 1995; Steller, 1995; Thompson, 1995), and in advanced stages, with nuclear and cytoplasmic vesiculation and the fragmentation of cells forming *apoptotic bodies* with no inflammatory response.

The findings of several investigations support the hypothesis that apoptosis occurs in acute rejection (Griffiths and Mueller, 1991; Higuchi and Aggarwal, 1994; Ju et al., 1994; Ito et al., 1995; Laguens et al., 1996; Narula et al., 1996; Kageyama et al., 1998). Szabolcs et al. (1996, 1998) identified several apoptotic cells (myocytes, macrophages infiltrated in the

myocardium and endothelial cells) in rat and human cardiac allograft rejection. Bergese et al. (1997) observed diffuse TUNEL-positive cells in the inflammatory infiltrate at 7 days in tolerated heart allografts in mice while after rejection, the number of apoptotic cells in the myocardial infiltrate diminished at the same time that the perivascular infiltrate augmented. Moreover, a few labelled cells were also found in the walls of the cardiac arteries. These authors suggest that apoptosis reflects a type of immunoregulation which is much more active in tolerated than rejected allografts where apoptotic cells are isolated and tissue destruction generally occurs via mechanisms other than apoptosis. In this study there was a scarce presence of TUNEL-positive cells in rejected and tolerated grafts. Labelled cells corresponded to mononuclear cells which were always present in the inflammatory infiltrate, isolated in interstitial spaces or in the perivascular infiltrate. No apoptotic cells were found in vessel walls. This seems to indicate that in the present model, the tissue destruction and heart alterations observed are the consequence of another type of mechanism and are likely to be due more to the ischaemia-reperfusion process (Jurado et al., 1998) than to apoptosis.

In accordance with Szabolcs et al. (1996) and Bergese et al. (1997), no evidence of rejection or apoptosis was found in the native, and rarely found in the singenic heart.

Cytotoxic T lymphocytes are a characteristic component of the cell infiltrate in acute rejection and together with delayed-type hypersensitivity (macrophages) are the principal immune mechanisms of rejection in allogenic heart transplant (Tilney et al., 1984; Marboe et al., 1990; Hall, 1991; Barry, 1994). These cells liberate large quantities of cytokines which diffuse to areas of less damage where other mononuclear cells enter apoptosis (Jollow et al., 1997).

Bellgrau et al. (1995) proposed an apoptosis model in which interactions between the Fas ligand (Fas-L) expression by activated lymphocytes and the Fas receptors of the donated tissue surface may be responsible for triggering apoptosis of the infiltrated lymphocytes in the graft. Activated lymphocytes are more prone to undergo apoptosis (Kabelitz et al., 1993; Wesselborg et al., 1993; Pechhold et al., 1994). The T lymphocytes infiltrated in the myocardium may also lead to the apoptosis of cardiomyocytes and macrophages via Fas-Fas-L interactions (Cohen et al., 1992; Barry, 1994; Tanaka et al., 1994; Cheng et al., 1995; Nagata and Goldstein, 1995; Thompson, 1995). Myocite apoptosis through Fas-Fas-L pathway may be involved in cardiac allograft rejection in rats (Kageyama et al., 1998). In the present study, very few apoptotic cells were found (even in the rejected hearts), so we cannot relate apoptosis and rejection process in our model. When present, apoptotic cells were always observed in the inflammatory infiltrate, around the coronary vessels and even in the lumen of vessels. This leads to the suggestion that some of the TUNEL-positive cells found might correspond to activated lymphocytes and not cardiomyocytes or macrophages.

The findings of several investigations have suggested that macrophages may induce necrosis and apoptosis in the myocardium (Cohen et al., 1992; Iwanaga et al., 1994; Maino and Joris, 1995; Thompson, 1995). However, the presence of macrophages in allografts with or without clinical signs of rejection (Christmas and MacPherson, 1982; Hancock et al., 1983; Lowry et al., 1983; Mason and Morris, 1986; Gassel et al., 1990; Bohman et al., 1991) suggests that these are not relevant in immune and inflammatory events or that several subpopulations of different phenotype (Paul et al., 1992) with distinct functional characteristics invade the graft under different conditions or at different times (Gassel et al., 1990). Paul et al. (1992) observed that in singenic transplants, the proportion of ED1-positive cells increased in areas of fibrosis. The recipients of allogenic hearts sacrificed after 30 days showed a diffuse infiltration of perivascular ED1-positive cells. Szabolcs et al. (1996) found that from 4-5 days post-transplant, ED1-reactive macrophages represented more than 50% of the inflammatory cells. Most apoptotic cells were found within or close to the macrophage-rich inflammatory infiltrate. Apoptotic myocytes were found in abundance near the infiltrate and some were detected in the absence of infiltrate. In our model a high number of ED1-positive cells were found in the inflammatory infiltrate after 2 weeks of implant in both types of transplant, although number and intensity of labelled cells was lesser in rejected allogenic hearts. This seems to indicate that the population of monocytes/macrophages involved in the rejection process may differ phenotypically to that observed in the singenic transplant model. Further, the quantity and localisation of ED1-positive cells did not correspond to that of the TUNEL-positive cells, suggesting that not all of the cells suffering apoptosis are macrophages but that most of them are other types of white cells. Our results showed absence of cardiomyocytes which may have been induced to programmed death by the action of the inflammatory cells of the infiltrate found.

In the present investigation, the administration of CsA to animals undergoing transplant allowed the resolution of acute rejection in allografts. Interstitial fibrosis was detected and there was a reduction in the inflammatory response and in the number of ED1-positive cells. This was observed in the singenic transplanted hearts, too. In both present implant groups no differences were observed with respect to the number and type of cells undergoing apoptosis (with/without CsA-treatment). Other authors have reported similar results in immunosuppressed animals although, in contrast, have reported moderate myointimal hyperplasia in the coronary arteries of allografts (Paul et al., 1992; Bergese et al., 1997).

According to Dhein et al. (1995), cyclosporin inhibits the activation of programmed cell death by interference with the calcium-dependent intracellular

signals which occur in apoptosis. This inhibitory effect was not observed here, and a very low level of apoptosis was observed in the singenic and allogenic transplants, even in non-immunosuppressed animals.

It may be concluded that: a) CsA treatment diminishes the amount of infiltrated macrophages; b) a low rate of apoptosis was observed in both types of transplant so, in our model, cyclosporine had no effect on this phenomena; c) macrophages do not appear to suffer apoptosis or induce this phenomena on myocardial cells since the majority of TUNEL-positive cells found corresponded to the mononuclear cells of the inflammatory infiltrate; and d) in this model it was not possible to relate apoptosis and rejection.

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