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Function of inflammatory cells and neoral cyclosporin-A in heart transplant-associated coronary vasculopathy

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Summary. The role of Sandimmun Neoral® (S-n) and the immune response in transplant-associated coronary vasculopathy (TACV) was evaluated in a Lewis (Lew)-to-Fischer-344 (F344) rat abdominal heterotopic heart transplant model. Some of the transplant recipients were treated with S-n (5mg/kg/day) for 14 days post-transplant, or until sacrifice. Grafts were subjected to immunohistochemical (ED1, CD4, CD8 and α-actin+ cells) analysis from day 7 to 100 post-transplant. Singenic controls did not develop TACV, irrespective of whether they had received the drug or not. TACV was detected in Lew-F344 transplants regardless of S-n administration with participation of ED1+, CD8+ and α-actin+ cells, although its incidence was lower in animals receiving prolonged S-n treatment. In this model, accelerated arteriosclerosis of the graft appeared to be related more to the rejection effect than to the action of the immunosuppressive agent.

Key words: Heart transplant, Coronary vasculopathy, Sandimmun Neoral, Inflammatory cells

Introduction

Coronary vasculopathy, or accelerated arteriosclerosis, is currently the major impediment for the long-term survival of a heart transplant recipient.

This process is considered by many to be a complication of chronic rejection mediated by the local immune response and to require the active participation of the cells of the donor vessel wall and recipient mononuclear cells, particularly T lymphocytes, macrophages and neutrophils which infiltrate the graft (Hruban et al., 1990; Young-Ramsaran et al., 1993; Deng et al., 1998). In contrast, other authors suggest that coronary disease is mainly attributable to the immunosuppressive agent used in post-transplant therapy. Accordingly, a considerable amount of data relates cyclosporin-A (CsA) to the development of transplant-associated coronary vasculopathy (TACV) (Uretsky et al., 1987; Stamler et al., 1988; Paul et al., 1994).

In an attempt to elucidate both the role of the immunosuppressive agent and the participation of the immune response in the development of TACV, an abdominal heterotopic cardiac transplant model in the rat was used. The immunosuppressive agent used was Sandimmun Neoral® (S-n), a water-free microemulsion of CsA. To our knowledge, the effects of this new agent on the coronary circulation of the transplanted graft have not yet been investigated.

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Materials and methods

Male Lewis (Lew) and Fischer-344 (F344) rats weighing 250-300g were employed. Animal care and experimental protocols were conducted in compliance with EU (European Union) guidelines (EEC-28871-22A9 animal care committee).

Allogenic Lew-to-F344 transplants were performed by the Ono and Lindsay technique (Ono and Lindsay, 1969). The microsurgical procedure and the assessment of heterotopic cardiac graft function during the post-operative period were conducted as previously described (Jurado et al., 1998). Singenic transplants served as controls for each strain.

Experimental design

The recipient animals were divided into two study groups (see Table 1) according to whether or not they were administered S-n (5 mg oral dose/Kg/day). With the aim of evaluating any possible long-term (100 days) differences attributable to the interruption of treatment, group II (treated with S-n) was subdivided into: A) animals which received the immunosuppressive agent from the day of surgery until sacrifice; and B) animals administered S-n only during the first 14 days after transplant.

The follow-up times were based on those used in

previous investigations (Jurado et al., 1998) which showed them to be the most adequate for the short, mid and long-term evaluation of the graft. A mean number of 3 cardiac grafts were obtained at each follow-up time.

Morphological and ultrastructural analyses

Morphological and ultrastructural studies were performed on specimens of both transplanted and native hearts 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 flows. 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 to observation under a ZEISS light microscope.

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 paraffin as for the optical microscopy studies. The alkaline phosphatase-labeled avidin-biotin technique was employed using monoclonal antibodies specific for rat monocytes/macrophages ED1 (MCA-341, Serotec, Oxford, England), human smooth muscle-specific α-actin (1A4, A-2547, Sigma, St. Louis, USA) and rat lymphocytes CD4, W3/25 (CTS 515G, Labgen, Barcelona, Spain) and CD8, MRC OX-8 (CTS 418G, Labgen, Barcelona, Spain). The protocol consisted of incubation with the primary antibody (1:100 in Tris-buffered saline; TBS, pH 7.6) for 1 hour at 37 °C, 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 °C. Nuclei were counterstained for 5 min with acid hematoxylin.

Assessment of coronary damage

A morphometric study was performed on 30 transverse 5-micron-thick histological sections (including sections from the apex to the upper part of the heart) using a computerized image analyser (MICRON). Estimation was made of the proportion of coronary arteries showing TACV and the degree of participation of inflammatory cells (CD4, CD8 and ED1) and smooth muscle cells (α-actin+) in the vascular lesion. The degree of arterial intimal thickening was scored using a scoring system previously described (Adams et al., 1992), (scale 0-5, 0: normal, to 5: ocluded). All arteries seen in each histological section were examined and scored.

Measurements were evaluated by means of a descriptive statitistical method to calculate the arithmetic mean and standard deviation. The Student-Newman-Keuls test for comparison of means was also applied to determine the significance of the results obtained at the different stages.

Results

Histological results

Group I (untreated transplant recipients)

A substantial inflammatory response was observed

Table 1. Study groups.

	GROUP I (untreated transplant recipients)		GROUP II (transplant recipients treated with S-n)	
			Continuous treatment[a]	Interrupted treatment[b]
Singenic Transplants (control)	-------- --------	Lew-Lew (n=12) F344-F344 (n=12)	Lew-Lew (n=12) F344-F344 (n=12)	Lew-Lew (n=3) F344-F344 (n=3)
Allogenic Transplants	Lew-F344 (n=8)	---------	Lew-F344 (n=12)	Lew-F344 (n=3)
Study Times	failure of the graft	7, 14, 50 and 100 days	7, 14, 50 and 100 days	100 days

[a]: from transplant to sacrifice; [b]: from transplant to day 14 after transplant.

in the singenic transplanted hearts at 14 days. There was an abundance of ED1+ cells and a smaller proportion of CD8+ cells in the endomyocardium. In contrast, the pericardium only harboured weakly stained CD4+ cells and a majority of CD8+ lymphocytes. TACV was not detected in the vessels of these grafts (Fig. 1a), although in the lumen of some coronary arteries adhered mononuclear cells were seen at 14 days post-transplant. The inflammatory infiltrate diminished after 50 days.

The untreated F344 recipients of Lew grafts survived 50.4±18 days post-transplant. The grafts were infiltrated by ED1+, CD4+ and CD8+ cells in the myocardium which also showed intense fibrosis. TACV was detected in 54.31% of the vessels (Fig. 1b). Intimal hyperplasia was concentric and semi-occlusive (large arteries) or occlusive (small vessels).

The native hearts of singenic/allogenic transplant recipients showed no vascular changes.

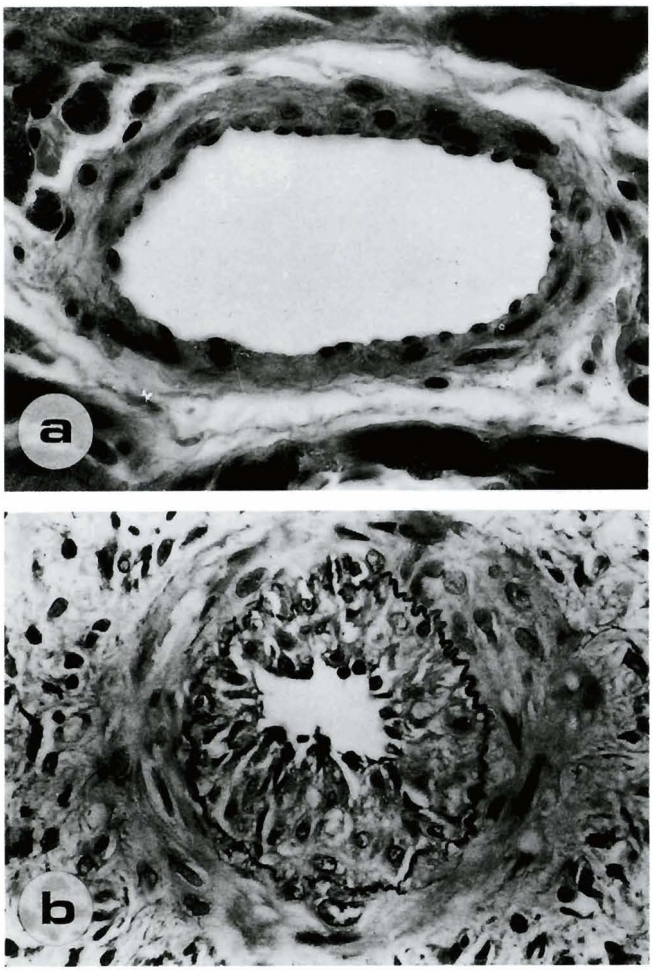

Fig. 1. Morphology of the coronary artery in the transplanted heart (Group I). **a.** Normal artery in a Lew-to-Lew singenic graft 50 days after transplant. x 500. **b.** TACV in the vessel of a Lew-to-F344 graft around 50 days post-transplant. x 630

Group II (transplant recipients treated with S-n)

Singenic transplant recipients treated with S-n showed an increase in interstitial fibrosis and a decrease in each of the inflammatory cells under study with respect to similar untreated grafts.

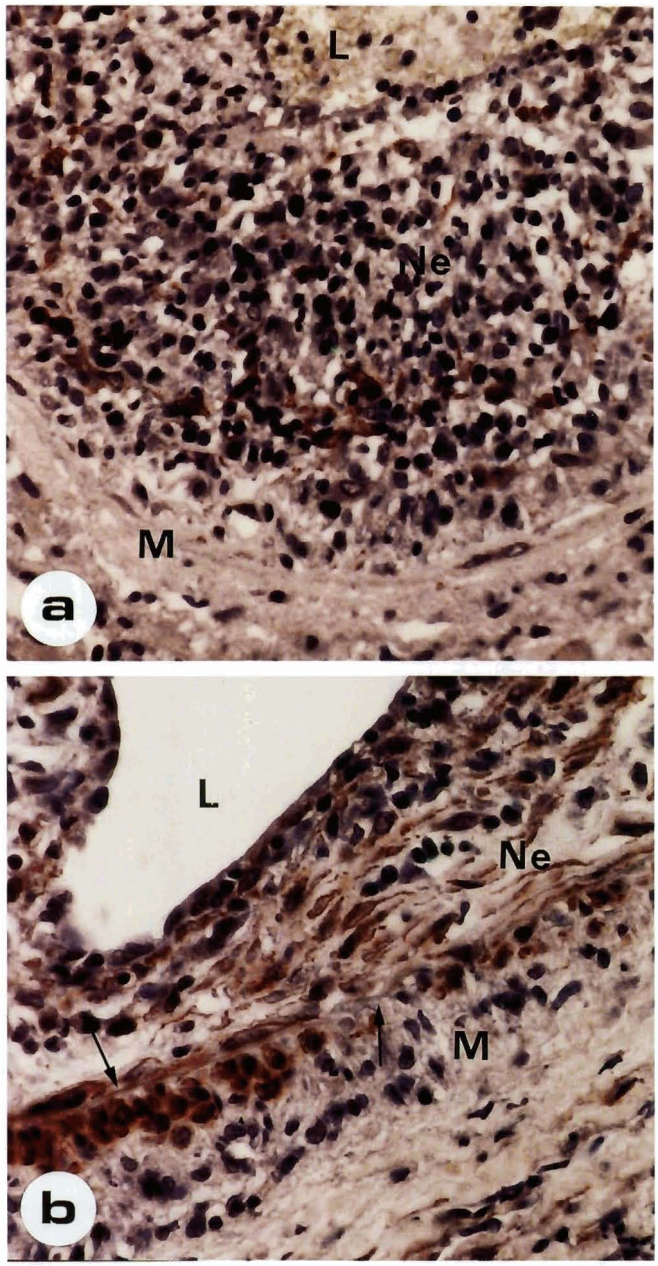

Fig. 2. a. α-actin+ cells (red colour) in the neointima (Ne) of a Lew-to-F344 graft coronary artery at 100 days post-transplant (interrupted treatment). M: medial layer; L: arterial lumen. x 400. **b.** Uneven distribution of α-actin+ cells (red colour) in the medial layer (M) and neointima (Ne) of a Lew-to-F344 graft coronary artery at 100 days post-transplant (continuous S-n treatment). Arrows: internal elastic lamina; L:

When the immunosuppressive treatment was interrupted at 14 days, observations made in the Lew-to-F344 grafts were similar to those recorded in group I. Transplanted hearts showed necrosis, tissue destruction and intense myocardial fibrosis at 100 days. The

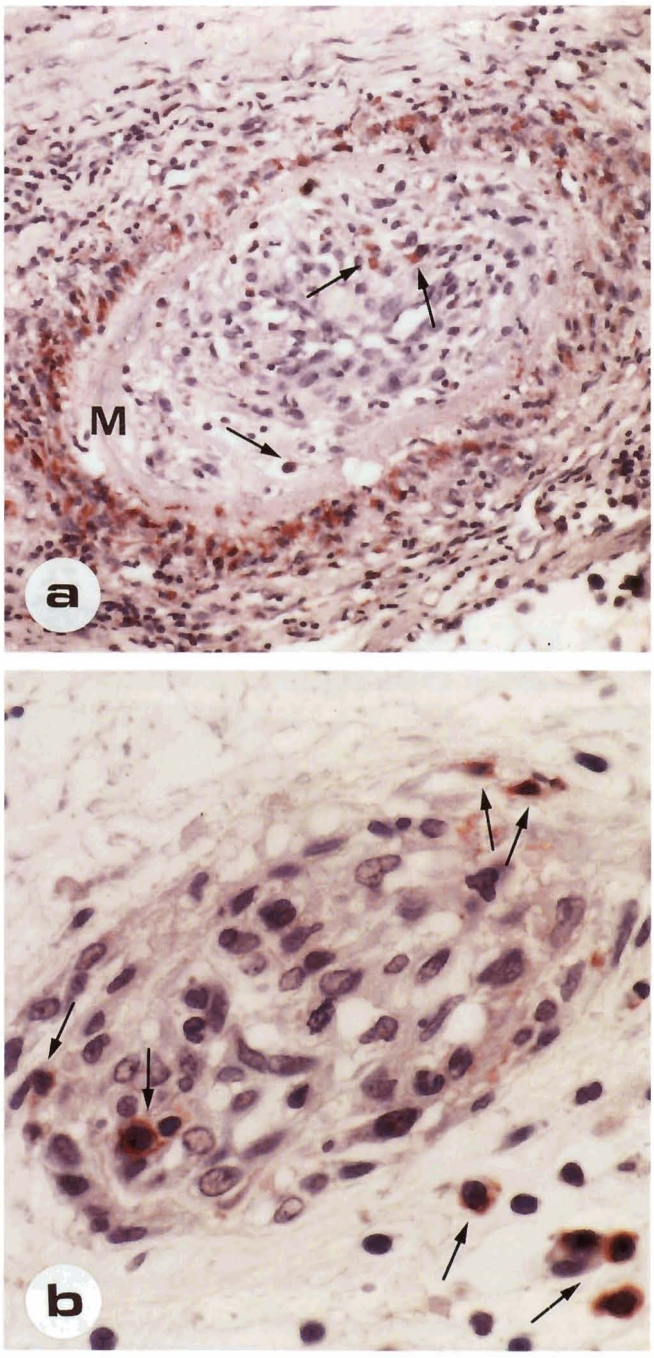

Fig. 3. Lew-to-F344 grafts (interrupted treatment). **a.** An epicardiac vessel with stenosis surrounded by ED1+ cells (red colour) may be observed. A few of these cells may also be seen in the lumen cell mass (arrows). M: medial layer. x 200. **b.** CD8+ lymphocytes (arrows) inside/outside a small occluded vessel. x 630

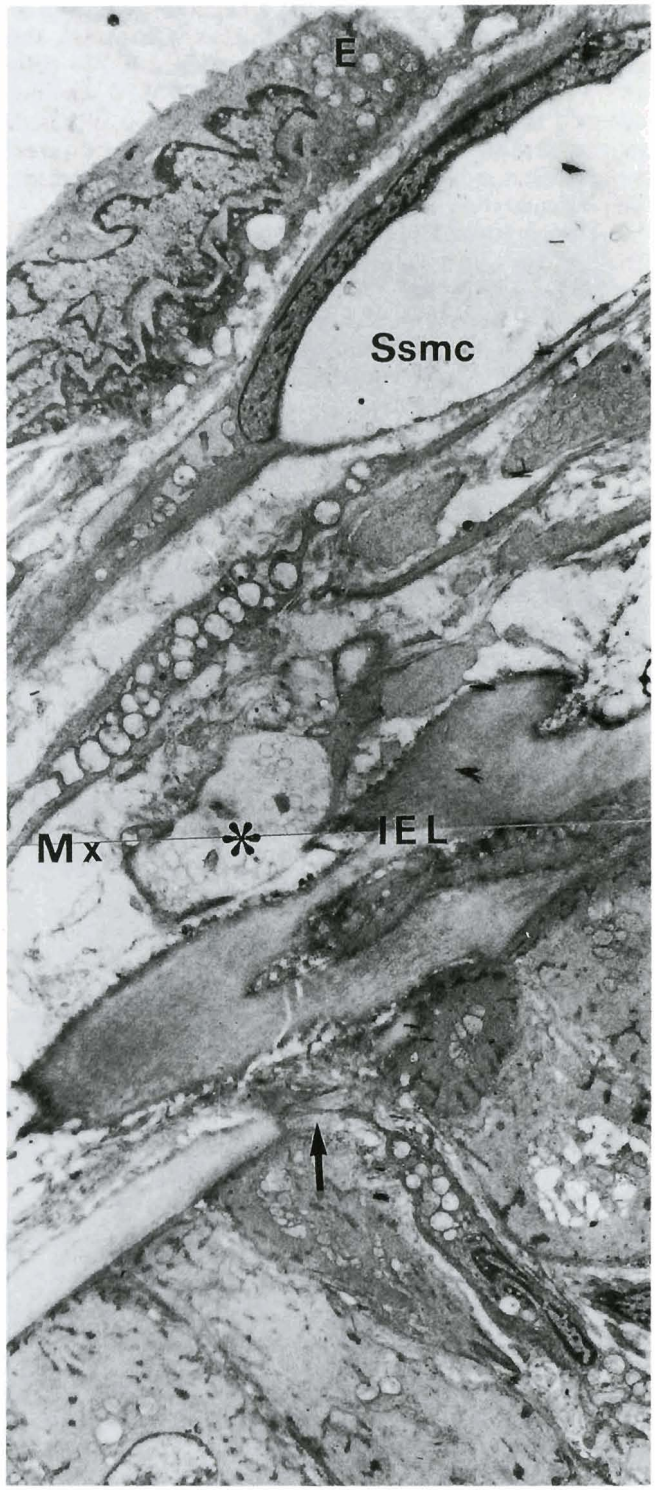

Fig. 4. Coronary artery of a Lew-to-F344 graft at 100 days post-implant (continuous S-n treatment) showing several layers of both secretory smooth muscle cells (Ssmc) and other cell types (*) over the internal elastic lamina (IEL). Mx: extracellular matrix; E: endothelium. A few Ssmc migrate towards the neointima through a fenestration (arrow) in the IEL. x 3,000

endocardial inflammatory infiltrate was rich in, ED1+ and CD8+ cells. Some CD4+ lymphocytes were seen to infiltrate via small capillaries and appeared as weakly stained cells in the pericardium. Several arteries examined in the transplanted heart developed TACV, with the participation of α-actin+ (Fig. 2a), ED1+ and a scarce number of CD8+ cells (Fig. 3). An ED1+ macrophage periadventitial barrier was detected around affected coronary arteries (Fig. 3a).

The allogenic Lew-to-F344 grafts in the continuous S-n treatment group showed intense adhesion and infiltration of ED1+ cells at 14 days both in the arterial wall and in the myocardium. CD8+ and scarce CD4+ cells were detected between cardiac fibres and in the pericardium. Some of the arteries examined in the transplanted heart developed TACV. From 50-100 days post-transplant, the number of affected vessels increased, although large arteries showed non-occlusive intimal thickening (TACV) in the shape of a half-moon comprised of several cell layers. Many of these cells showed secretory characteristics and were α-actin+ (Figs. 2b, 4). At 100 days, only a few of the pericardial cells were CD8+ and CD4+ and a few labelled macrophages could still be seen in the endocardium.

The native hearts of singenic transplant recipients showed no vascular changes. The arteries of the native hearts (in both treatment groups) showed (at 100 days) an intact endothelium under which the elastic lamina was of a fibrous appearance with no loss in thickness. An enlarged extracellular matrix was seen in the medial layer.

Histopathological analysis

The results of the morphometric study are shown in Tables 2 and 3. TACV was shown by 54.31% of the arteries in Lew grafts transplanted into F344 recipients receiving no immunosuppressive treatment and by 61.63% of the arteries in Lew-F344 grafts receiving interrupted treatment. However, when the treatment received by the Lew graft recipients was prolonged, the incidence of TACV fell by 12.63% at 100 days with respect to the interrupted treatment ($p>0.01$, no statistical significance), and by 26.96% at around 50 days with respect to the untreated recipients (Student-Newman-Keuls test $p<0.01$). A predominance of macrophages (ED1+) was observed before 50 days in the neointima while in the long-term (100 days), α-actin+ cells were the major cell type in this layer (Student-Newman-Keuls test $p\leq.01$). A low proportion of CD8+ lymphocytes was detected in affected vessels at each follow-up time and in each group. CD4+ cells were only seen in the myocardial inflammatory infiltrate. A continuous decrease in the number of α-actin+ cells in

Table 2. Evaluation of coronary damage (TACV) in Lew-to-F344 grafts.

	Study times (days)	Affected arteries[a] (%)	Luminal occlusion[b] (%)	Average lesion grade
Without treatment	50.4±18	54.31	24.57	2.83
Continuous treatment	7	4.38 +	2.5 +	0.48 +
	14	14.40 +	6.06	0.88 +
	50	27.35 *, +	14.07	1.06 *, +
	100	49.00	19.65	1.86
Interrupted treatment	100	61.63	21.12	2.16

[a]: ffected arteries; [b]: occluded arteries with respect to the total arteries seen. The Student-Newman-Keuls test showed statistical significance ($p\leq 0.01$) in theses cases: *, vs "without treatment"; +, vs "100 days in the same group (continuous treatment)". The rest of the comparisons did not show statistical significance ($p>0.01$).

Table 3. Immunohistochemical analysis of affected arteries by TACV in Lew-to-F344 allografts.

	Study times (days)	α-actin (%)		ED1 (%)		CD8 (%)	
		Medial layer	Intimal layer	Medial layer	Intimal layer	Medial layer	Intimal layer
Without treatment	50.4±18	56.13±7.03	15.94±5.06	10.07±3.54	21.35±5.00	3.33±1.00	7.79±2.47
Continuous treatment	7	100.00±0.00	0.25±0.05	3.06±1.07	85.40±3.41**	0.40±0.02	7.42±1.87
	14	95.30±2.05	2.30±0.25	12.00±3.45	79.90±2.06**	0.28±0.01	5.23±2.05
	50	70.00±5.45	20.06±7.02	5.00±2.50	30.05±1.76	0.50±0.52	6.35±3.62
	100	53.82±3.80+	40.30±3.95*, $	6.38±2.38	12.70±2.40&	0.52±0.07	6.07±4.00
Interrupted treatment	100	43.59±7.08	42.44±3.09*	6.21±1.06	9.92±2.05	0.96±0.30	11.16±6.65

The Student-Newman-Keuls test showed statistical significance ($p\leq 0.01$) in theses cases: +, vs "α-actin+ cells in media at 7, 14 and 50 days in continuous treatment"; $, vs "α-actin+ cells in hyperplasia at 7, 14 and 50 days in continuous treatment"; *, vs "ED1+ cells in hyperplasia in the same group and stage"; **, vs "α-actin+ cells in hyperplasia in the same group and stage", and &,vs "ED1+ cells in hyperplasia at 7 and 14 days in continuous treatment".

arteries showing TACV was recorded in the medial layer (Student-Newman-Keuls test $p \leq 0.01$).

Arterial sections from singenic grafts showed minor alterations by 10% (Lew-to-Lew) and by 15% (F344-to-F344) (average lesion grade 0.12) in groups I and II.

Discussion

Cardiac transplant is presently considered the sole therapeutic measure for a series of terminal cardiopathies. The allotransplant experimental model is the closest to clinical heart transplant inducing substantial changes in the coronary circulation. In such a model, the physiological reparative process is interfered with by the immunological rejection of the grafted organ and by the immunosuppressive treatment.

Acute rejection has been largely overcome by the use of immunosuppressive agents and chronic rejection has emerged as the main cause of graft failure (Adams and Tilney, 1989). The most significant pathological finding in the latter process is the diffuse, concentric intimal proliferation incurred by large and medium calibre arteries (Billingham, 1992). Heart transplants are particularly sensitive to these vascular changes (Nitkin et al., 1985) and the appearance of TACV is the main determining factor for the survival of the heart transplant patient (Jamieson, 1992; Schroeder et al., 1992).

It has been proposed that accelerated arteriosclerosis in heart transplant recipients is a complication of rejection mediated by the immune response. The process has been associated with an accumulation of macrophages, lymphocytes and smooth muscle cells in the coronary vessel intima and with endothelial lesions provoked by cytotoxic T lymphocytes (CD8+) (Hruban et al., 1990; Young-Ramsaran et al., 1993; Deng et al., 1998).

Lew-to-F344 cardiac grafts undergo similar chronic rejection and arteriosclerotic lesioning to that produced in humans (Adams et al., 1992) and are considered a useful experimental model. TACV in Lew-to-F344 cardiac allografts develops in stages: before 30 days there is a predominance of ED1+ macrophages in the neointima accompanied by a small disperse population of T lymphocytes (CD4+ and CD8+); from 30 to 90 days, α-actin+ smooth muscle cells appear in the neointima, and from 90 days onwards these cells are the predominant type in this layer (Cramer et al., 1992; Adams et al., 1993; Russell et al., 1995).

Although the findings of several investigations confirm the existence of transplant arteriosclerosis before the introduction of CsA (Thomson, 1969; Bieber et al., 1970; Kosek et al., 1971), some authors (Paul et al., 1992, 1994) have demonstrated an atherogenic effect of the agent even in singenic transplant models. However, other authors propose that CsA has a protective effect and have shown that treatment with CsA at adequate doses can reduce intimal thickness and cellular filtration in TACV (Cramer et al., 1990; Meiser et al., 1991; Handa et al., 1993; De Caterina et al., 1995; Koskinen et al., 1995).

On the other hand, there are known side effects of S-n, such as hypercholesterolemia, hypertension and diabetes (Van Buren et al., 1998; Trull et al., 1999) which could contribute to vasculopathy. However, the direct effects of S-n on coronary vessels after transplant are unknown, although in an aortic transplant model, this agent was responsible for the same regression in intimal hyperplasia at half the dose with respect to other formulations of the drug (Little et al., 1996).

CsA could perform its protective role by interfering with the normal functioning of cooperating T cells (mainly CD4+) thus inhibiting the growth and proliferation of T lymphocytes and the activation of T cells and macrophages. It has been shown that CsA is only efficient during the initial phases of the immune response and is useful in the prophylaxis of acute rejection but not in the treatment of the immune reaction (Homan et al., 1980; Morris et al., 1985).

In the present singenic (control) recipients, TACV did not develop whether the recipients were treated with S-n or not. However, when Lew-to-F344 grafts were performed, graft vasculopathy was detected irrespective of the immunosuppressive treatment with participation of ED1+, CD8+ and α-actin+ cells. However, the incidence of TACV was somewhat lower in the case of prolonged treatment. Animals undergoing transplant develop TACV despite immunosuppressive treatment since, once sensitised by an extremely scarce number of CD4+ T cells (escaping the action of the CsA) or pre-sensitised by graft antigens, the CD8+ T cells are able to act on the target organ.

It may be concluded that in the present study, S-n reduces the severity of the lesion (TACV) when produced. Graft-accelerated arteriosclerosis, at least in this experimental model, seems to be related more to the rejection effect than to the action of the immunosuppressive drug.

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