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ORIGINAL ARTICLE

FK778, a novel immunosuppressive agent, reduces early adhesion molecule up-regulation and prolongs cardiac allograft survival*

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Summary

The adhesion molecules, P-selectin, ICAM-1, and VCAM-1 are important mediators of T-cell adhesion and T-cell co-stimulation. We investigated the effect of the malononitrilamide FK778 on cardiac allograft survival, acute allograft rejection, and adhesion molecule up-regulation in a heterotopic, cardiac transplantation model. Rats received low- or high-dose FK778 or no treatment. Grafts were harvested on the fifth postoperative day for histologic examinations. To assess allograft survival, recipients were treated for a maximum of 10 days and grafts were harvested after cessation of the contractile activity. FK778 low dose showed a mild but significant decrease in mononuclear infiltration but failed to markedly reduce histologic rejection, adhesion molecule up-regulation, or to prolong allograft survival. However, high-dose FK778 treatment significantly reduced early up-regulation of P-selectin, ICAM-1, and VCAM-1, abolished infiltration, reduced histologic rejection and resulted in prolonged cardiac allograft survival. Therefore, FK778 is a novel, highly desirable immunosuppressive drug for transplantation medicine.

Introduction

Leflunomide and its active metabolite, A771726, belong to a new group of immunosuppressive isoxazol derivates. Their action is exerted by binding specifically to dihydroorotate dehydrogenase and inhibiting the *de novo* pyrimidine biosynthesis [1]. In addition, they inhibit the cytokine- and growth factor receptor-associated tyrosine kinase activity [2]. Both, T- and B-cell function are directly inhibited [3]. Prolongation of graft survival of the skin, heart, kidney, and liver has been proven in various allotransplantation models [4]. However, because of the long plasma half-life of A771726 (11–16 days), its practical use in organ transplant recipients is questionable [2]. FK778, a synthetic malononitrilamide (MNA) derived from A771726 has very good bioavailability and a much shorter half-life than the parent drug and would make dose adjustment in patients easier [5]. The few studies with MNAs in experimental transplantation have been performed with encouraging results [5,6].

Selectins and adhesion molecules have been demonstrated to be critically involved in leukocyte recruitment during cardiac allograft rejection [7–10]. After activation of the endothelium, a transient and reversible intermittent adhesion results in 'rolling' of unactivated leukocytes along the endothelial surface. The selectins, consisting of three different molecules (L-, E-, and P-selectin) participate in this initial low-affinity process. Leukocytes become activated by various chemotactic factors and a stronger interaction between integrins expressed on leukocytes and intercellular adhesion molecules on the activated endothelium results [11]. This adhesion process

allows leukocytes to migrate through the endothelial cell lining and to gain access to the surrounding tissues. In patients after cardiac transplantation, an up-regulation of ICAM-1 and P-selectin has been shown in endomyocardial biopsies [12,13]. Serum levels of sICAM-1 and sVCAM-1, the soluble forms of ICAM-1 and VCAM-1, were found significantly elevated a few days before a rejection episode and thus exert a predictive value [14].

The goal of the present study was to evaluate the immunosuppressive efficacy of FK778 in a stringent rat heart transplantation model and its effect on early adhesion molecule up-regulation.

Materials and methods

Animals

Male Brown-Norway (BN) rats (200–300 g) were used as donors, male Lewis (Lew) rats (200–300 g) were used as recipients. Animals received humane care in compliance with the 'Principles of Laboratory Animal Care' (NIH Publication No. 86–23, revised 1985) and the German Law on the Protection of Animals was followed. All animals were obtained from Charles River Laboratories (Sulzfeld, Germany) and were maintained in the local animal care facilities. They were housed in rooms with 12 h light/dark cycles, and were provided water and dry food *ad libitum*.

Cardiac grafting

Heterotopic cardiac transplantation was performed as described by Ono and Lindsey [15]. Briefly, rats were anesthetized using a combination of ketamine hydrochloride (100 mg/kg, i.m.) and xylazine hydrochloride (10 mg/kg, i.m.). The donor received a bolus of 500 IU heparine i.v. Cardiac arrest was achieved using 20 ml Bretschneider solution (Custodiol®, Köhler Chemie, Alsbach-Hahnlein, Germany). The hearts were excised after ligation of the venae cavae and the pulmonary veins and stored in cold lactated Ringers solution. The aorta and the pulmonary artery of the donor heart was anastomosed to the abdominal aorta and vena cava inferior of the recipient, respectively. Transplantations were performed within 30 min and the grafts were kept cool most of the time. Graft survival was monitored by daily palpation of the beating graft through the abdominal wall. The time of rejection was defined as the last day of palpable cardiac contractions, and the rejection was confirmed histologically.

Immunosuppression

FK778 was provided by Fujisawa GmbH (Munich, Germany). It was freshly dissolved in 1% carboxymethyl cellulose and applied orally by daily gavage from the day of transplantation.

Experimental groups

Six groups were studied with six rats in each group (see Table 1). Groups 1 and 4 received no treatment. Groups 2 and 5 received high-dose FK778 (20 mg/kg/day), groups 3 and 6 low-dose FK778 (5 mg/kg/day) once daily. Groups 5 and 6 received their treatment for 5 days, groups 2 and 3 for 10 days post-transplantation. Hearts were harvested after 5 days in groups 4, 5 and 6 and after cessation of graft activity in groups 1, 2 and 3.

Preparation of the hearts

After the recipient was killed, both the native and the heterotopic hearts were removed and perfused with saline through the aorta. The hearts were divided into two parts. One half was fixed in 4% formalin and embedded in paraffin, the other half was embedded in Tissue Tek® (Sakura, Germany), rapidly frozen in liquid nitrogen and stored at −70 °C.

Histologic and immunocytochemical examinations

The 2 μm paraffin sections were stained with hematoxylin-eosin and examined by standard light microscopy.

Table 1. Experimental groups.

Group	*n*	Treatment	Graft survival (days)	MST (days)	ISHLT
1	6	–	6, 6, 6, 6, 6, 7	6.2 ± 0.4	–
2	6	FK778 (20 mg/kg/day) for 10 days	13, 15, 16, 18, 20, 20	17.0 ± 2.8*	–
3	6	FK778 (5 mg/kg/day) for 10 days	6, 6, 6, 7, 7, 8	6.7 ± 0.8	–
4	6	–	Graft harvest on POD 5	–	3B, 3B, 3B, 4, 4, 4
5	6	FK778 (20 mg/kg/day) for 5 days	Graft harvest on POD 5	–	1A, 1A, 1B, 2, 2, 2
6	6	FK778 (5 mg/kg/day) for 5 days	Graft harvest on POD 5	–	3A, 3A, 3A, 3B, 3B, 3B

ISHLT, International Society for Heart and Lung Transplantation classification; MST, mean survival time; POD, postoperative day.
*Only high-dose FK778 significantly prolonged allograft MST compared with untreated controls.

The degree of parenchymal rejection was evaluated using the working formulation of the International Society for Heart and Lung Transplantation (ISHLT) as follows: 0 = no evidence of acute rejection; Ia = focal infiltrate; Ib = diffuse but sparse infiltrate; II = one focus only with aggressive infiltration and/or focal myocyte damage; IIIa = multifocal aggressive infiltrates; IIIb = diffuse inflammatory process; IV = diffuse, aggressive polymorphous infiltration and/or edema and/or hemorrhage and/or vasculitis [16].

Frozen specimens were cut into 6 μm sections and placed onto poly-L-lysine-precoated slides (Sigma Diagnostics, Sigma-Aldrich, Munich, Germany). Mononuclear infiltration cells were identified using monoclonal antibodies against CD4 (T-helper cells, OX38; Serotec, Düsseldorf, Germany), CD8 (cytotoxic/suppressor T cells, OX8; Serotec), and ED1 (macrophages, ED1; Serotec). Infiltration density of cardiac tissue was defined as number of cells per high power field (magnification 400×) and assessed by computerized analyses (Leica, Bensheim, Germany). A total of nine high power fields per section and three sections per heart were examined.

For detection of vascular adhesion molecule up-regulation, antibodies against ICAM-1 (1A29; Biozol, Eching, Germany), VCAM-1 (5F10; Covance, Richmond, CA, USA), and P-selectin (CD62Pabr; Research Diagnostics Flanders, NJ, USA) were used for peroxidase staining. Molecule expression was investigated using computerized analyses (Leica Bensheim) and defined as the area of the vessel wall that stained positive for the specific molecule. In addition, immunohistochemical analysis was done in a blind review by two observers. The score assigned was determined by consensus of the two observers. The intensity of the staining was scored from 0 to 4 as follows: 0 = no visible staining; 1 = few cells with faint staining; 2 = moderate intensity with multifocal staining; 3 = concentric staining; 4 = intense, concentric staining.

Statistical analysis

Data are expressed as mean ± SD. Comparisons among groups were made using the ANOVA test with the LSD *post hoc* test. Probability values (P) of <0.05 were considered significant. Statistical analysis was performed using the SPSS statistical software package 10.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Graft survival

During graft rejection, contractility progressively weakened until no contractions could be recognized. We did never see sudden loss of graft function. The individual allograft survival data for control as well as treatment groups are summarized in Table 1. Graft survival in untreated animals (group 1) was 6.2 ± 0.4 days. Low-dose FK778 (5 mg/kg/day) administration for 10 days (group 3) did not result in a significantly prolonged graft survival (6.7 ± 0.8 days; $P = 0.62$). However, a treatment with high-dose FK778 (20 mg/kg/day) for 10 days (group 2) significantly prolonged allograft survival (17.0 ± 2.8 days; $P < 0.01$).

Mononuclear graft infiltration

The inflammatory cell subsets were studied by immunhistochemical staining with a panel of monoclonal antibodies 5 days after cardiac transplantation (Fig. 1). In untreated animals (group 4), massive infiltration of CD4-positive T-helper cells, CD8-positive cytotoxic/suppressor T cells and ED1-positive macrophages was observed in the graft. Immunosuppressive treatment with high-dose FK778 (20 mg/kg/day) significantly reduced inflammatory cell subsets. Low-dose FK778 (5 mg/kg/day) resulted in a minor decrease of mononuclear cells, but the differences

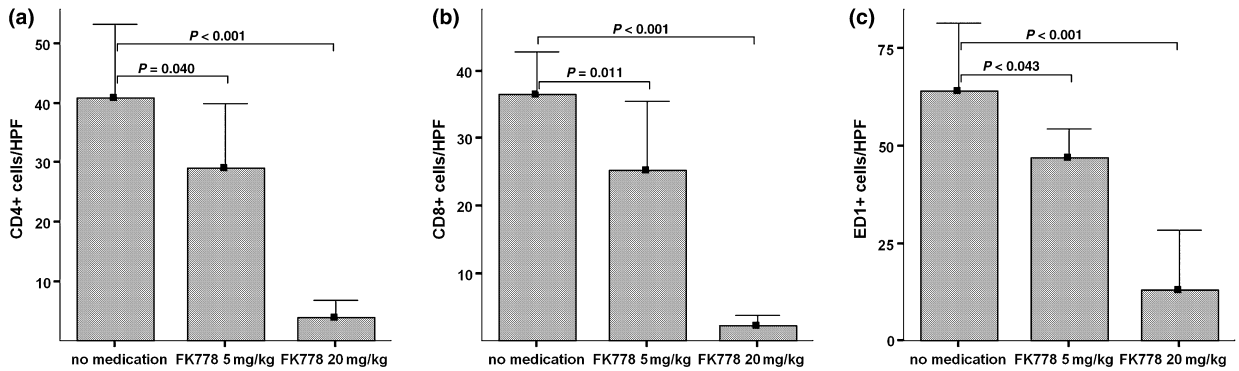

Figure 1 Inflammatory cell populations of CD4+ (a), CD8+ (b), and ED1+ cells (c) in the myocardium of Brown-Norway–Lewis (BN–Lew) cardiac allografts on the fifth postoperative day.

did reach significance as well, when compared with untreated controls.

Although a significant reduction for all three inflammatory cell subsets was shown, no changes in the composition of mononuclear infiltration was observed. The majority of inflammation cells was ED1-positive in all groups with a slight increase in the treatment groups lacking significance. Epicardial infiltration was irregularly noticed in animals of all groups and was thought to be due to mechanical graft manipulation during transplantation. This inflammation cell population was excluded from computer-assisted mononuclear cell quantification.

Grading of acute rejection

In the control group (group 4) receiving no medication, heavy mononuclear infiltration was histologically evident with extensive myocyte necroses and hemorrhages. Mononuclear cells were diffusely infiltrating the interstitial and perivascular tissues. Vasculitis with transmural arterial wall mononuclear infiltration and endothelitis as signs of microvascular rejection were apparently consistent with ISHLT grading 3B–4 in this group. After low-dose FK778 treatment (group 6), less vasculitis was noticed, and in some grafts myocyte necrosis was rather multifocal than diffuse. ISHLT grading in this group ranged from 3A to 3B, but histologic rejection changes were not markedly reduced. However, a 5-day course of high-dose FK778 (group 5) markedly reduced mononuclear infiltration and myocyte necrosis and completely abolished vasculitis. ISHLT grading ranged from 1A to 2, consistent with rather mild rejection. Although no clear vasculitis could be revealed in both treatment groups, perivascular infiltration was dramatically reduced with high-dose treatment.

Adhesion molecule expression

In nontransplanted native rat hearts, neither P-selectin nor ICAM-1 or VCAM-1 was observed on heart vasculature. During acute rejection, a slight induction of P-selectin and an intense induction of ICAM-1 and VCAM-1 was observed in the no treatment group (group 4) with an observer ranking of 1.7 ± 0.5, 3.1 ± 0.7 and 3.3 ± 0.3, respectively. Computer analyses revealed a positive staining in $21.4 \pm 15.8\%$, $19.5 \pm 3.1\%$ and $24.0 \pm 5.3\%$ of the vessel wall. Low-dose FK778 treatment (group 6) did not affect adhesion molecule up-regulation. Observer rankings were 1.5 ± 0.6 (P-selectin: $P = 0.55$), 2.4 ± 1.0 (ICAM-1: $P = 0.14$) and 3.1 ± 0.2 (VCAM-1: $P = 0.28$) and showed no significant differences compared with group 4. In computer analyses, $17.5 \pm 11.6\%$ ($P = 0.56$), $21.5 \pm 23.4\%$ ($P = 0.76$) and $25.6 \pm 8.3\%$ ($P = 0.08$) of the vessel wall was positive for P-selectin, ICAM-1 and VCAM-1, respectively. No significant differences were observed for all adhesion molecules. However, high-dose FK778 nearly abolished adhesion molecule up-regulation (group 5). Observer rankings (0.1 ± 0.1, 0.3 ± 0.4, and 0.5 ± 0.6; $P < 0.01$) and computer scores ($1.1 \pm 1.8\%$, $P < 0.01$; $0.3 \pm 0.4\%$, $P = 0.01$; $3.6 \pm 3.5\%$, $P < 0.01$) for P-selectin, ICAM-1, and VCAM-1 revealed significantly less adhesion molecule expression compared with group 4.

Correlation of adhesion molecule expression and rejection grade

In many centers, steroid therapy is intensified for acute rejection if ISHLT grading is ≥3A. Therefore, we classified histologic rejection 'moderate' for ISHLT grading ≤2 and 'severe' for ISHLT ≥3A. We tested the usefulness of mean adhesion molecule expression ≥10% of the vessel wall as a marker for severe rejection. Sensitivity of P-selectin, ICAM-1 and VCAM-1 was 67%, 83% and 100%, respectively.

Discussion

In this study, FK778, a novel MNA derived from leflunomide, was found to prolong rat cardiac allograft survival. Leflunomide is an isoxazol derivate that is easily absorbed and rapidly converted to an active open ring metabolite, A771726 [4]. Although leflunomide has shown to be effective in preventing cardiac allograft rejection [17], it was not regarded suitable for clinical transplantation because of its extensive half-life of 15 days [18]. FK778 is a synthetic derivate of A771726 with a similar immunologic efficacy as leflunomide but a shorter half-life. In rodents, the half-life was found to be within several hours [19].

Single drug therapy with FK778 in a dose of 5 mg/kg/day was found to be ineffective in prolonging of cardiac allograft survival in this high-responder rat strain combination and showed only minor changes in ISHLT gradings. Furthermore, no effect on early adhesion molecule expression was observed in this dosage. The reduction of graft mononuclear infiltration on the fifth postoperative day must have been mediated by other mechanisms. FK778 is known to act by binding to and inhibiting dihydro-orotate dehydrogenase, a mitochondrial enzyme responsible for converting dihydro-orotate to orotate, a critical step in the *de novo* pyrimidine synthesis process [5]. T-cell proliferation and alloantibody synthesis by B cells is effectively inhibited [20]. Furthermore, the MNAs dose-dependently inhibit the generation of oxygen radicals *in vitro* and *in vivo* [21]. The slight but significant decrease of graft mononuclear infiltration observed in this group was insufficient, however, to markedly reduce histologic rejection or to prolong graft survival.

High-dose FK778 treatment significantly prolonged cardiac allograft survival to 17.0 ± 2.8 days. This is consistent with the observation of Qi *et al.*, who observed a mean graft survival of 14.3 days in the DA-PVG rat strain combination after 10 days of treatment [20]. In the study of D'Silva *et al.*, leflunomide in doses ranging from 5 to 20 mg/kg, likewise for 10 days was used in DA-to-PVG allografts [22]. They showed a mean graft survival of 14–17 days, suggesting a similar immunosuppressive potency for leflunomide and FK778.

Gastrointestinal symptoms were the most common side effects in clinical trials of leflunomide for rheumatoid arthritis [23]. Reversible body weight loss, anemia, thrombocytopenia, and liver dysfunction were occasionally found in patients exposed to leflunomide [18]. In the present study, no toxic side effects were observed in the used dosages of 5–20 mg/kg/day. In special, animals did not develop diarrhea and showed normal increase in body weight as seen in untreated animals. Thus, the used dosages were within the nontoxic range. In comparison, recent studies described FK778 to be ineffective at 2.5 mg/kg and toxic at 40 mg/kg [20].

In inflammation, as well as in acute rejection, leukocyte trafficking is crucial. Several distinct steps are involved in leukocyte recruitment. After the endothelium has become activated and expresses selectins, a transient and reversible intermittent adhesion results in the 'rolling' of unactivated leukocytes along the endothelial surface. Firm adhesion is provided by β-integrins expressed on the leukocytes and ICAM-1 or VCAM-1 on the activated endothelial cell. Finally, leukocytes migrate through the endothelial layer into the surrounding tissues. The presence of adhesion molecules in the setting of allograft rejection has been demonstrated in several organs, including the kidney [24], liver [25], and heart [26]. Both ICAM-1 and VCAM-1 as well as selectins were shown to be increased in rejected cardiac allografts and this correlated with the rejection grade [7,27]. In this study, adhesion molecule up-regulation strongly correlated with the histologic rejection grade and allograft survival. Thus, early adhesion molecule expression is regarded an important early marker for initiation of cellular rejection. Adhesion molecules may be induced by numerous cytokines including interleukin (IL)-1, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ [28]. Therefore, adhesion molecules are induced by cytokine-producing inflammatory cells and facilitate further leukocyte migration, accelerating rejection. We propose that the reduced expression of adhesion molecules on coronary endothelium is another mechanism of FK778 to inhibit transendothelial inflammatory cell migration, cytokine induction, and graft rejection.

Grafts of untreated animals showed huge interstitial infiltration of T-lymphocytes, with equal amounts of CD4- and CD8-positive cells. The latter are regarded the effector cells that ultimately mediate graft destruction, but their activation depends on CD4 cells [29]. Some cytotoxic T cells may act directly on the target cell by means of pore formation, while others trigger programmed cell death ('apoptosis'), causing target cell disintegration [30]. Thus, the effectiveness of FK778 in alleviating CD8 T-lymphocyte invasion into the graft is a crucial step in the prevention of early allograft rejection.

Macrophage infiltration is reported to begin as soon as 3 h after reperfusion of the organ, reaching peak-levels within the first week [31]. Macrophage accumulation subepicardially is regarded to be in part due to the perioperative mechanical trauma, as it can be seen in syngenic allografts either. However, interstitial infiltrations seem immune-mediated and are missing in the syngenic setting. In the treatment groups, ED1-positive macrophages were reduced in parallel to the cytokine-producing T-lymphocytes.

As shown in this study, FK778 suppresses early adhesion molecule up-regulation, mononuclear graft infiltration and has the capacity to alleviate the rejection process and to prolong cardiac allograft survival in rats. These encouraging findings warrant further evaluation in a larger animal model using an orthotopic model of transplantation and possibly in combination with other drugs.

References

1. Williamson RA, Yea CM, Robson PA, *et al.* Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range of biological effects of the immunomodulatory compound. *J Biol Chem* 1995; **270**: 22467.
2. Silva Junior HT, Morris RE. Leflunomide and malononitrilamides. *Am J Med Sci* 1997; **313**: 289.
3. Kurrle R, Bartlett R, Ruuth E, Lauffer L, Schorlemmer HU. Malononitrilamides inhibit T- and B-cell responsiveness. *Transplant Proc* 1996; **28**: 3053.
4. Waer M. The use of leflunomide in transplantation immunology. *Transpl Immunol* 1996; **4**: 181.
5. Schorlemmer H, Bartlett R, Kurrle R. Malononitrilamides: a new strategy of immunosuppression for allo- and xenotransplantation. *Transplant Proc* 1998; **30**: 884.
6. Schorlemmer HU, Kurrle R. Hyperacute skin allograft rejection in presensitized rats is abrogated by malononitrilamides. *Transplant Proc* 1998; **30**: 963.
7. Yamazaki S, Isobe M, Suzuki J, *et al.* Role of selectin-dependent adhesion in cardiac allograft rejection. *J Heart Lung Transplant* 1998; **17**: 1007.

8. Isobe M, Yagita H, Okumura K, Ihara A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 1992; **255**: 1125.
9. Kobayashi H, Miyano T, Yamataka A, *et al.* Prolongation of rat cardiac allograft survival by a monoclonal antibody: anti-rat intercellular adhesion molecule-1. *Cardiovasc Surg* 1993; **1**: 577.
10. Komori A, Nagata M, Ochiai T, *et al.* Role of ICAM-1 and LFA-1 in cardiac allograft rejection of the rat. *Transplant Proc* 1993; **25**(1 Pt 1): 831.
11. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991; **65**: 859.
12. Deng MC, Bell S, Huie P, *et al.* Cardiac allograft vascular disease. Relationship to microvascular cell surface markers and inflammatory cell phenotypes on endomyocardial biopsy. *Circulation* 1995; **91**: 1647.
13. Labarrere CA, Nelson DR, Faulk WP. Endothelial activation and development of coronary artery disease in transplanted human hearts. *JAMA* 1997; **278**: 1169.
14. Weigel G, Grimm M, Griesmacher A, *et al.* Adhesion molecule behavior during rejection and infection episodes after heart transplantation. *Clin Chem Lab Med* 2000; **38**: 403.
15. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. *J Thorac Cardiovasc Surg* 1969; **57**: 225.
16. Billingham ME, Cary NR, Hammond ME, *et al.* A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. *J Heart Transplant* 1990; **9**: 587.
17. Williams JW, Xiao F, Foster PF, *et al.* Immunosuppressive effects of leflunomide in a cardiac allograft model. *Transplant Proc* 1993; **25**: 745.
18. Mladenovic V, Domljan Z, Rozman B, *et al.* Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results of a randomized, placebo-controlled, phase II study. *Arthritis Rheum* 1995; **38**: 1595.
19. Jin MB, Nakayama M, Ogata T, *et al.* A novel leflunomide derivative, FK778, for immunosuppression after kidney transplantation in dogs. *Surgery* 2002; **132**: 72.
20. Qi Z, Simanaitis M, Ekberg H. Malononitrilamides and tacrolimus additively prevent acute rejection in rat cardiac allografts. *Transpl Immunol* 1999; **7**: 169.
21. Schorlemmer HU, Bartlett RR, Kurrle R. Malononitrilamides prevent the generation of oxygen radicals in mononuclear phagocytes and graft rejection in a rat model. *Transplant Proc* 1999; **31**: 851.
22. D'Silva M, Candinas D, Achilleos O, *et al.* The immunomodulatory effect of leflunomide in rat cardiac allotransplantation. *Transplantation* 1995; **60**: 430.
23. Smolen JS, Kalden JR, Scott DL, *et al.* Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicentre trial. European Leflunomide Study Group. *Lancet* 1999; **353**: 259.
24. Hancock WH, Whitley WD, Tullius SG, *et al.* Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. *Transplantation* 1993; **56**: 643.
25. Bacchi CE, Marsh CL, Perkins JD, *et al.* Expression of vascular cell adhesion molecule (VCAM-1) in liver and pancreas allograft rejection. *Am J Pathol* 1993; **142**: 579.
26. Allen MD, McDonald TO, Carlos T, *et al.* Endothelial adhesion molecules in heart transplantation. *J Heart Lung Transplant* 1992; **11**: S8.
27. Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober JS. Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation* 1991; **51**: 537.
28. Molossi S, Clausell N, Sett S, Rabinovitch M. ICAM-1 and VCAM-1 expression in accelerated cardiac allograft arteriopathy and myocardial rejection are influenced differently by cyclosporine A and tumour necrosis factor-alpha blockade. *J Pathol* 1995; **176**: 175.
29. Gill RG. T-cell–T-cell collaboration in allograft responses. *Curr Opin Immunol* 1993; **5**: 782.
30. Binah O. Immune effector mechanisms in heart transplant rejection. *Cardiovasc Res* 1994; **28**: 1748.
31. Dresske B, Zhu X, Herwartz C, Brotzmann K, Fandrich F. The time pattern of organ infiltration and distribution of natural killer cells and macrophages in the course of acute graft rejection after allogeneic heart transplantation in the rat. *Transplant Proc* 1997; **29**: 1715.