Graefe's Arch Clin Exp Ophthalmol (1994)
232:622–627

Sarah E. Coupland
Sonja Klebe
Anne-Christine Karow
Lothar Krause
Heinrich Kruse
Robert R. Bartlett
Friedrich Hoffmann

Leflunomide therapy following penetrating keratoplasty in the rat

Received: 31 December 1993
Revised version received: 21 March 1994
Accepted: 15 April 1994

Presented in abstract form at the Berlin-Brandenburg Eye Congress, 4–5 December 1993. None of the authors has any proprietary or financial interests in the compound leflunomide

S.E. Coupland (✉) · S. Klebe
A.-C. Karow · L. Krause · H. Kruse
F. Hoffmann
Augenabteilung, Klinikum Steglitz,
Hindenburgdamm 30, D-12200 Berlin,
Germany

R.R. Bartlett
Immunopharmacology, Hoechst,
Werk Kalle-Albert, Wiesbaden, Germany

Abstract • Background: The isoxal derivative, leflunomide (LF), is a new potent immunosuppressive which has been shown to be effective in preventing autoimmune disorders and reactions leading to organ transplantation rejection. LF is thought to antagonise cytokine activity and thereby to interfere with T-helper-cell-dependent B- and T-lymphocyte proliferation. • Methods: We used LF to treat corneal allograft rejection in the rat, comparing its effect with that of cyclosporin A (CSA). Corneal buttons were grafted from Lewis/Brown Norway rats to Lewis recipients. Animals were randomly assigned to the following treatment groups: I, untreated; II, CSA (10 mg/kg i.m.); III, LF (2.5 mg/kg p.o.); IV, LF (5 mg/kg p.o.); V, LF (10 mg/kg p.o.); VI, combined therapy (LF 10 mg/kg p.o. and CSA 10 mg/kg i.m). Treatment began on the first postoperative day and was continued until rejection occurred. • Results: The mean graft rejection time in the untreated allogeneic group was 12 days. A significant delay in graft rejection was observed in all treatment groups compared with group I ($P < 0.001$). Further, the delay in graft rejection resulting from combined therapy (group VI) was statistically significant compared with all other groups ($P < 0.001$). • Conclusion: These results suggest that (a) LF when used alone is as effective as CSA in treating corneal allograft rejection in the rat, and (b) when LF and CSA are combined they are more effective than either drug alone in the prolongation of allograft survival.

Introduction

Leflunomide [LF; *N*-(4-trifluro-methylphenyl)-5-methylisoxazol-4-carboxamide] formerly known as HWA-486, is an isoxal derivative which has proven effective in the prevention and treatment of various autoimmune diseases both experimentally [see review, 2] and in clinical trials [19]. Further, LF prevented skin, kidney and heart graft rejection following transplantation in rats [13, 20, 24]. Previous studies suggest that LF and its active metabolite, A771726, interfere with T-helper-cell-dependent B- and T-lymphocyte proliferation [2]. These immunosuppressive effects are thought to be mediated largely through the antagonistic activity of LF and A771726 on many cytokines, including those required for the formation of cytotoxic cells during the transplantation rejection process [2]. Although considered as effective an immunosuppressant as cyclosporin A (CSA) following organ and tissue transplantation, LF's mode of action is considered to differ to that of CSA [1, 7]. In this study, we investigated the effect of LF, alone and in combination with CSA, in a rat orthotopic penetrating keratoplasty model.

Methods

Female Lewis rats ($RT1^l$) and Lewis-Brown Norway ($RT1^{lxn}$), approximately 15–20 weeks old and weighing 200–300 g, were pur-

chased from the Møllegaard Breeding Centre, Denmark. The Lewis rats ($RT1^l$) were used as recipients in all cases; the Lewis-Brown Norway rats ($RT1^{lxn}$) served as donors. These two rat strains differ completely at the major histocompatibility complex (MHC) antigens.

Leflunomide was obtained from Hoechst (Kalle-Albert, Wiesbaden, Germany) and was prepared in a sterile 1% carboxymethylcellulose solution (CMC) each day prior to oral adminstration using a gastric tube. CSA was obtained from Sandoz (Basel, Switzerland) and administered intramuscularly.

The animals were treated according to the provisions established by the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

Surgical procedure

Anaesthesia of both the donor (Lewis-Brown Norway) and the recipient (Lewis) rat was achieved with xylazine hydrochloride (Rompun, Bayer) and ketamine hydrochloride (Ketanest, Parke-Davis). Twenty minutes prior to surgery, the Lewis rats also received 0.5 mg/kg atropine (Eifelfango) subcutaneously and phenyl-ephrinehydrochloride (Neosynephrine-POS, 5%, Ursapharm) eyedrops to aid dilation of the iris.

Under sterile conditions and using an operating microscope, two donor corneal buttons (3.5 mm) were harvested from the Lewis-Brown Norway rats using a trephine and curved Castroviejo scissors. The donor animals were then killed by ether inhalation. The left eyes of the recipient Lewis rat were prepared by removing a central 3.0-mm button using a trephine and Castroviejo scissors. A drop of sterile methylcellulose (1%) was placed over the 3.0-mm corneal opening before the donor cornea was fixed with ten interrupted sutures (11–0 nylon, Ethicon). The anterior chamber was not re-established following surgery. Prior to closure of the eyelids with three or four interrupted sutures (7–0 nylon, Ethicon), Polyspectran (Alcon Thilo) eyelid gel was placed over the operated eye.

The animals were randomly sorted into groups as follows: I, untreated; II, CSA 10 mg/kg; III, LF 2.5 mg/kg; IV, LF 5 mg/kg; V, LF 10 mg/kg; VI, combined therapy, LF 10 mg/kg and CSA 10 mg/kg. The postoperative treatment began 24 h following surgery and continued until the appearance of allograft rejection. A total of 75 penetrating keratoplasties were performed in the allogeneic group; a control group of 10 Lewis rats received syngeneic grafts. The corneal sutures were not removed.

Postoperative care

Forty-eight hours following surgery, the eyelid suture was removed, allowing for the first assessment of the cornea on the slit-lamp microscope by a masked observer. Slit-lamp evaluations were performed every 2–3 days under intramuscular anaesthesia using ketamine hydrochloride, with assessment of the corneas by scoring of graft opacity, oedema and vascularisation [9]. Graft opacity was scored from 0 to 4: 0 = clear cornea; 1 = slight haze; 2 = increased haze with view of anterior chamber; 3 = increased haze without view of anterior chamber; 4 = opaque cornea. Graft oedema was scored from 0 to 4: 0 = no corneal thickening; 1 = slight corneal thickening; 2 = diffuse oedema; 3 = diffuse oedema with microcystic oedema of epithelium; 4 = diffuse oedema with bullous keratopathy. Graft neovessels were scored 0 to 4: 0 = no vascularisation; 1 = peripheral cornea; 2 = neovessel growth to wound edge; 3 = vessels on graft; 4 = total vascularisation. Photographs were also taken for documentation (Fig. 1).

Upon occurrence of allograft rejection (cumulative rejection rating >6), the animal was anaesthetised and whole blood was collected via cardiac puncture. In the leflunomide-treated animals, the plasma levels of leflunomide's primary metabolite, A771726, were determined using HPLC. The cardiac puncture was performed on average 24 h after the last dose of leflunomide.

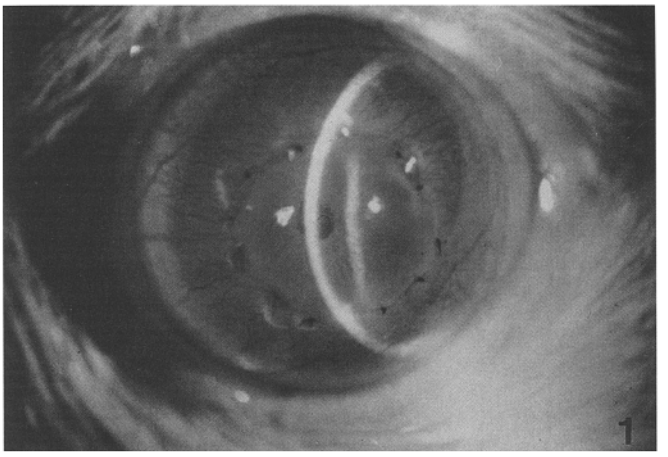

Fig. 1 Slit-lamp photograph of a graft from a rat in group III (LF 10 mg/kg), day 10 ($\times 40$). This eye was scored as corneal clarity 1, corneal oedema 1, vascularisation 2, yielding a rejection score of 4

Results

Clinical evaluation

On removal of eyelid sutures in both the syngeneic and allogeneic groups, the grafts were clear and characterised by very mild postoperative oedema and no vascularisation. Weekly slit-lamp examination of the syngeneic group until the 6th postoperative week revealed clear grafts with minimal oedema and vascularisation of the peripheral cornea only.

Within the allogeneic group, two animals were excluded from the study due to premature death and cataract formation, leaving a total of 73 allogeneic rats in the study. The allogeneic untreated group (I, $n=15$) had a mean ($\pm$SD) rejection time of 12 ± 1.4 days (Fig. 2). A significant delay in graft rejection was observed in *all* treatment groups when compared with group I ($P<0.05$, Mann-Whitney U-test) (Table 1). The mean rejection time in the CSA-treated rats (group II, $n=11$) was 16 ± 1.9 days (Table 1). This compared with mean rejection times in the LF-treated rats of 15, 16 and 17 days in groups III, IV and V respectively (Fig. 2). Although the delay in the allograft rejection between the LF-treated groups appeared to be dose-dependent, the mean rejection times in the leflunomide-treated animals did not significantly differ from one another (Table 2). Animals treated with the highest dose of LF (10 mg/kg, group V) showed a delay in the onset of an allograft rejection similar to that in those treated with CSA (group II; Fig. 2). Further, the delay in the allograft re-

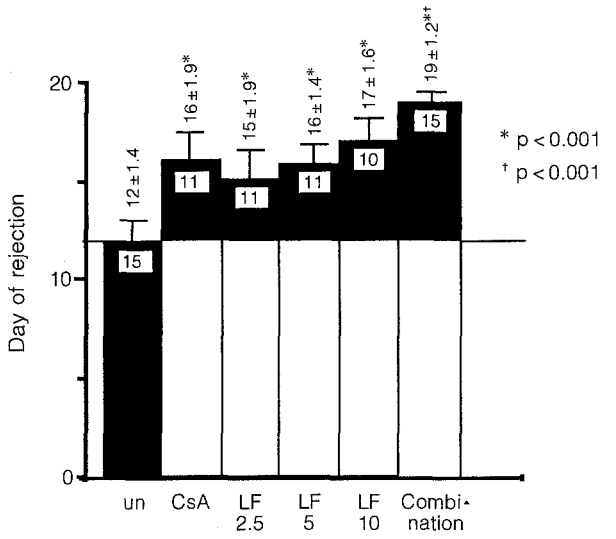

Fig. 2 Mean rejection time (days) in treated and untreated rats. Number of rats per group is shown within the columns. *un* Untreated, *LF* 10 LF (10 mg/kg), *LF* 5 LF (5 mg/kg), *LF* 2.5 LF (2.5 mg/kg), *Combination* LF (10 mg/kg) and CSA (10 mg/kg). All treated groups showed a significant delay in corneal rejection compared with the controls (* $P<0.001$). The animals treated with both medications showed a further significant delay compared with all groups († $P<0.001$)

action in group VI, where the animals were treated with both CSA and LF (10 mg/kg), was significantly greater than in all other groups ($P<0.001$) (Table 2).

When the individual rejection categories for each treatment group were evaluated, all medications delayed the attainment of a score greater than 2 in the categories of opacity and oedema compared with the control group (Mann-Whitney *U*-test, $P<0.05$) (Table 3). With respect to vascularisation, no significant delay in attainment of a score greater than 2 was achieved with any medication. Those animals treated with LF (10 mg/kg) and those with the combined regimen of LF (10 mg/kg) and CSA attained scores of >2 in the categories of opacity and oedema significantly later than the animals in groups II, III and IV (Mann-Whitney *U*-test, $P<0.05$). However, comparison between groups V and VI did not reveal a statistically significant difference the attainment of a rejection score >2 for opacity and oedema.

Table 2 Mean postoperative day when individual category scores first exceeded

Category	Group					
	I	II	III	IV	V	VI
Opacity	11	15	15	15	17	19
Oedema	12	16	15	16	18	19
Neo-vascularization	12	16	15	16	17	19

A771726 plasma levels

All doses of LF were well tolerated by the Lewis rats, with very few side effects being observed. Further, no technical problems regarding the administration of the LF-CMC solution per os (e.g. regurgitation or inhalation) were encountered. Despite this, the 24-h plasma levels of A771726 of those animals treated with leflunomide alone were low in comparison to the 24-h levels in Sprague-Dawley rats following single oral doses of 5 and 10 mg/kg leflunomide (Bartlett, personal communication). Interestingly, however, in those rats treated with CSA and LF (10 mg/kg), the leflunomide levels tended to be higher and within the range of the above-mentioned study (Fig. 3).

Discussion

Since immunologic rejection remains the leading cause of graft failure in human corneal transplants [11], various animal models have been used to study the mechanisms of the rejection process. The rabbit model, used by earlier investigators [3, 4, 10, 12, 14, 18], allowed the

Table 1 Comparison of the corneal rejection times of the individual groups (Mann-Whitney *U*-test; error probability $P=0.05$). Group I, untreated allogenic controls; group II, CSA (10 mg/kg); group III, LF (10 mg/kg); group IV, LF (5 mg/kg); group V, LF (2.5 mg/kg); group VI, combined therapy (LF 10 mg/kg and CSA 10 mg/kg); n.s., not significant

Groups	I	II	III	IV	V	VI
I		I<II $P<0.001$	I<III $P<0.001$	I<IV $P<0.001$	I<V $P<0.001$	I<VI $P<0.001$
II			n.s.	n.s.	n.s.	II<VI $P<0.001$
III				n.s.	n.s.	III<VI $P<0.01$
IV					n.s.	IV<VI $P<0.001$
V						V<VI $P<0.001$
VI						

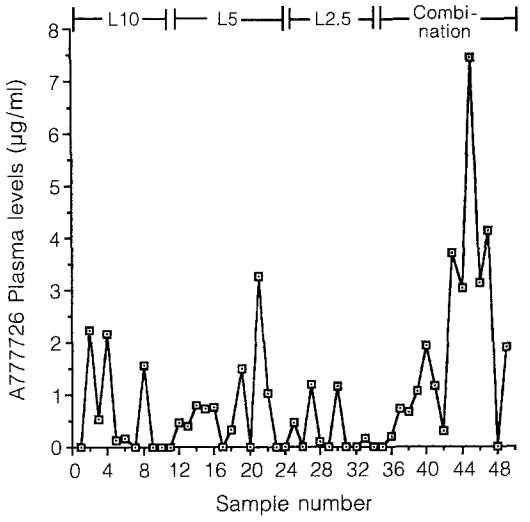

Fig. 3 A771726 plasma levels in groups III–VI as determined by HPLC. *L*10 LF (10 mg/kg), *L*5 LF (5 mg/kg), *L*2.5 LF (2.5 mg/kg), *Combination* LF (10 mg/kg) and CSA (10 mg/kg). Detection limit of HPLC 0.1 µg/ml

examination of rejection in an orthotopic graft. A disadvantage of the rabbit model was the inability to control the histocompatibility differences between donor and recipient [25]. Although the well-defined immunogenics of inbred mice offered a solution to this problem, orthotopic grafts were very difficult due to the relatively small mouse eye. Heterotopic corneal grafts to the thoracic cage were, therefore, used to study the immunobiology of corneal allografts in the mouse [22]. In 1985, Williams and Coster developed a rat model of penetrating orthotopic keratoplasty which combined the advantages of the rabbit and mouse models [25]. The similarity of the distribution of class I and class II MHC antigens in the rat and human corneas [23] furthered the acceptance of this model in the study of anterior segment immunopharmacology. Using two strains of rats which are MHC incompatible, we examined the efficacy of LF, alone and in combination with CSA, in the rat penetrating keratoplasty model.

Leflunomide, is a novel immunosuppressant thought to antagonise cytokine activity and thereby to disrupt T-helper-cell-dependent B- and T-lymphocyte cooperation and proliferation [2]. Initial studies showed that LF was as effective as conventional medications in the treatment of autoimmune diseases in animals [see review, 2]. Smith Lang and co-workers also found that the administration of LF (10 mg/kg) prevented the development of disease in an experimental model of autoimmune uveitis [21]. Further, preliminary results from patients suffering from severe rheumatoid arthritis suggest that both clinical and immunological parameters of the disease could be improved with LF [19].

Although earmarked, as a result of the above studies, as a strong inhibitor of B-cell activation, LF showed many characteristics in common with – and was considered to be as proficient as – CSA in the treatment of autoimmune diseases [1, 2]. Consequently, it was not surprising when LF, like CSA, was observed to prevent skin, heart and kidney graft rejection in animal studies [13, 20, 24], suggesting that LF also inhibited disorders requiring T-cell – T-cell cooperation, necessary in transplantation rejection. Mode of action studies suggest that the immunosuppressive effects of LF and its active metabolite, A771726, could be mediated to a large extent through its antagonistic activity on various cytokines, e.g. interleukin-2 [7]. LF also inhibits tyrosine kinase, an important mediator in the proliferation of various cell types [2].

In the present study, LF was observed to be effective in delaying the onset of the allograft reaction in rats following penetrating keratoplasty. All doses of LF used were as effective as CSA in delaying the onset of the allograft reaction in the cornea. When the highest dose of LF (10 mg/kg) was combined with CSA, a further delay in the allograft reaction in comparison with all groups was apparent, suggesting a synergistic effect of LF and CSA. Such a synergistic effect has also been recently observed in skin and heart transplantation in the rat model [20, 24].

When the rejection categories were assessed individually, LF appeared to have the greatest effect on reduction of corneal opacity and oedema. Groups in which the highest dosage of LF was used (V and VI) displayed a significantly delayed increase in graft oedema and opacity compared with all other allogeneic transplant groups. Such a sustained reduction in oedema by LF was observed during the treatment of adjuvant-induced polyarthritis in Lewis rats [17]. The effect of LF on vascularisation was not significant when compared with CSA-treated and control animals (Table 3, Fig. 1). Neovessel growth (probably stimulated by the retained sutures) would not, however, be expected to be delayed by LF with its specific T- and B-cell immunosuppression.

That LF promotes a significant prolongation of corneal allograft survival has also been observed recently by Niederkorn et al., who compared LF (10 mg/kg) and its active metabolite, A771726, with oral CSA following corneal transplantation in rats [15]. These authors achieved a mean rejection time of 29.7- and 32.4 days in those rats treated with LF and A771726, respectively. Further, almost one third of LF-treated cases maintained clear grafts 3 weeks after discontinuation of the medication. This prolonged graft survival, compared with the current study, may be explained by: (a) the removal of corneal sutures in the early postoperative period, as described by Callanan and co-workers [6]; (b) the "pretreatment" of the animals for 2 days prior to the operation; and (c) the surprisingly low plasma levels of A771726 achieved in our Lewis rats (Fig. 3). In humans it is known that the late removal of keratoplasty sutures

is associated with corneal neovascularisation and an increased risk of graft failure [16]. Further, in the rat penetrating keratoplasty model, removal of corneal sutures results in the regression of neovessels [6], which could promote host sensitisation to donor alloantigens.

The efficacy of CSA is associated with a time lag and is consequentially reduced if therapeutic tissue levels are not achieved during the early stages of antigen recognition [5, 8]. Surprisingly, (pre)treatment with CSA by Niederkorn and co-workers did not result in a prolongation of corneal allograft survival [15]. The effect of "pretreatment" with LF in transplantation studies has not been sufficiently investigated. In contrast, intentional *delay* of LF and CSA treatment following skin and heart transplantation has been examined [20, 24]. In animals allowed to undergo acute rejection crisis, only LF was able to reverse the rejection reaction and produce significant delay in allograft survival. These results confirm previous suggestions that LF and CSA have differing mechanisms of immunosuppression [1, 7].

With respect to the plasma levels of A771726 achieved in the present study, investigations are in progress to determine whether Lewis rats may have pharmacokinetics differing from those in the Sprague-Dawley strain, in which previous LF studies have been conducted.

In conclusion, LF when used alone is as effective as CSA in delaying corneal allograft rejection in the rat. Further, when LF and CSA are combined they are more effective than either drug alone in the prolongation of allograft survival. By virtue of its minimal side effects [19], LF is a promising new immunosuppressive which may prove of value in preventing graft failure in human corneal transplants.

Acknowledgements The technical assistance of Mrs Karin Oberländer and Mrs Helga Höffken-Zimmermann is sincerely appreciated. The authors also thank Dr Hopfenmüller, MD, PhD of the Statistical Department, Klinikum Steglitz, for his advice in data interpretation, and Mr Michael Steffens, Hoechst AG, Werk Kalle-Albert, for the HPLC determination of the A771726 plasma levels. The first author was supported by a Sydney Eye Hospital Foundation Scholarship and by the Deutsche Forschungsgemeinschaft (Ho 674/4–2).

References

1. Bartlett RR (1986) Immunopharmacological profile of HWA 486, a novel isoxazol derivative. II. In vivo immunomodulating effects differ from those of cyclophosphamide, prednisolone or cyclosporin A. Int J Immunopharmacol 8:199–204
2. Bartlett RR, Dimitrijevic M, Mattar T, Germann T, Rüde E, Thoenes GH, Küchle CCA, Schlorlemmer HU, Bremer E, Finnegan A, Schelyerbach R (1991) Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. Agents Actions 32:10–21
3. Basu PK, Hasany SM (1971) Histochemical localization of hydrolytic enzymes during corneal graft reaction. Can J Ophthalmol 10:239–247
4. Basu PK, Taichman NS (1973) Hydrolytic enzymes in corneal graft rejection. Can J Ophthal 8:577–581
5. Belin MW, Bouchard CS, Phillips TM (1990) Update on topical cyclosporin A. Background, immunology and pharmacology. Cornea 9:184–195
6. Callanan DG, Luckenbach MW, Fischer BJ, Peeler JS, Niederkorn JY (1989) Histopathology of rejected orthotopic corneal grafts in the rat. Invest Ophthalmol Vis Sci 30:413–424
7. Chong ASF, Gebel H, Finnegan A, Petraitis EE, Jiang XL, Sankary HN, Foster P, Williams JW (1993) Leflunomide, a novel immunomodulatory agent: in vitro analyses of the mechanism of immunosuppression. Transplant Proc 25:747–749
8. Faulds D, Goa KL, Benfield P (1993) Cyclosporin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in immunoregulatory disorders. Drugs 45:953–1040
9. Holland EJ, Chan CC, Wetzig RP, Palestine AG, Nussenblatt RB (1991) Clinical and immunohistologic studies of corneal rejection in the rat penetrating keratoplasty model. Cornea 10:374–380
10. Khodadoust AA (1968) Lamellar corneal transplantion in the rabbit. Am J Ophthalmol 66:1111–1117
11. Khodadoust AA (1973) The allograft rejection reaction: the leading cause of late failure of clinical corneal grafts. In: Porter R, Knight J (eds) Corneal graft failure. (Ciba Foundation Symposium) Elsevier, Amsterdam, pp 151–167
12. Khodadoust AA, Silverstein AM (1969) Transplantation and rejection of individual cell layers of the cornea. Invest Ophthalmol 8:180–195
13. Küchle CCA, Thoenes GH, Langer KH, Schlorlemmer HU, Bartlett R, Schleyerbach R (1991) Prevention of kidney and skin graft rejection in rats by leflunomide, a new immunomodulating agent. Transplant Proc 23:1083–1086
14. Maumenee AE (1951) The influence of donor-recipient sensitization on corneal grafts. Am J Ophthalmol 34:142–152
15. Niederkorn JY, Lang LS, Ross J, Mellon J, Robertson SM (1993) Promotion of corneal allograft survival with leflunomide (HWA 486), a novel immunomodulating agent. Invest Ophthalmol Vis Sci (in press)
16. Paque J, Poirier RH (1977) Corneal allograft reaction and its relationship to suture site neovascularization. Ophthalmic Surg 8:71–74
17. Pasternak RD, Wadopian NS, Wright RN, Siminoff P, Gylys JA, Buyniski JP (1987) Disease modifying activity of HWA 486 in rat adjuvant-induced arthritis. Agents Actions 21:241–243

18. Polack FM (1962) Histopathological and histochemical alterations in the early stages of corneal graft rejection. J Exp Med 116:709–717
19. Rozman B, Mladenovic V, Domlian Z, Jajic I,Popovic M, Mihailovic D, Zivkovic M, Musikic P, Campion G (1992) The effects of leflunomide (LF) in patients with rheumatoid arthritis (RA). Arthritis Rheum 35 [Suppl]: 108
20. Schorlemmer HU, Seiler FR, Bartlett RR (1993) Prolongation of allogeneic transplanted skin grafts and induction of tolerance by leflunomide, a new immunosuppressive isoxazol derivative. Transplant Proc 25:763–767
21. Smith Lang L, Glaser B, Weimer LK, Miller ST, Robertson SM, Aoki KR, Yanni JM (1992) Efficacy of novel immunomodulators leflunomide and rapamycin in autoimmune uveitis. FASEB J 6: A1048
22. Streilein JW, McCulleyJP, Niederkorn JY (1982) Heterotopic corneal grafting in mice: a new approach to the study of corneal alloimmunity. Invest Ophthalmol Vis Sci 23:489
23. Treseler PA, Sanfilippo F (1986) The expression of major histocompatibility complex and leucocyte antigens by cells in the rat cornea. Transplantation 41:248
24. Williams JW, Xiao F, Foster PF, Chong A, Sharma S, Bartlett RR, Sankary HN (1993) Immunosuppressive effects of leflunomide in a cardiac allograft model. Transplant Proc 25:745–746
25. Williams KA, Coster DJ (1985) Penetrating corneal transplantation in the inbred rat: a new model. Invest Ophthalmol Vis Sci 26:23–30