Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Corneal rat-to-mouse xenotransplantation and the effects of anti-CD4 or anti-CD8 treatment on cytokine and nitric oxide production

Jana Pindjáková,[1] Andrea Vítová,[1] Magdaléna Krulová,[1] Alena Zajícová,[1] Martin Filipec[2] and Vladimír Holáň[1]

1 Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic
2 The Eye Clinic, First Medical Faculty, Charles University, Prague, Czech Republic

Keywords
anti-CD4 treatment, corneal xenotransplantation, cytokines, graft survival, nitric oxide.

Correspondence
Vladimír Holáň, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 37 Prague 6, Czech Republic. Tel.: +420 220183226; fax: +420 224310955; e-mail: holan@img.cas.cz

Received: 15 July 2004
Revision requested: 15 December 2004
Accepted: 26 January 2005

doi:10.1111/j.1432-2277.2005.00112.x

Summary

Corneal xenotransplantation may be an alternative approach to overcome shortage of allografts for clinical transplantation. Orthotopic corneal rat-to-mouse xenotransplantation and syngeneic transplantation was performed and the effects of anti-CD4 and anti-CD8 treatments on corneal xenograft survival and production of cytokines, interleukin (IL)-2, IL-4, IL-10, γ-interferon (IFN-γ) and nitric oxide (NO) were evaluated. RT-PCR was used to determine the expression of genes for cytokines and inducible nitric oxide synthase (iNOS) in the grafts. The presence of iNOS protein in grafts was detected by immunofluorescent staining. We found that corneal xenotransplantation was associated with a strong upregulation of genes for both Th1 and Th2 cytokines and with NO production in the graft. Treatment of xenograft recipients with mAb anti-CD4, but not anti-CD8, resulted in a profound inhibition of IL-2, IL-4 and IL-10 production, and in a significant prolongation of corneal xenograft survival. The results show that upregulation of Th2 cytokines after corneal xenotransplantation does not correlate with xenograft rejection. Rather, corneal graft rejection is associated with the expression of genes for IFN-γ and iNOS and with NO production.

Introduction

Transplantation of cornea represents the most frequent grafting of solid tissue in the world. The success of transplantation is unusually high – over 90% of corneal allografts survive more than 1 year and over 70% of allografts survive above 7 years after grafting [1,2]. The main obstacle in corneal transplantation becomes the shortage of grafts. As corneal allografts have an unusually high success rate even with limited immunosuppression due to the special properties of the cornea and the immunological privilege of the eye, one can consider that xenografts should solve the problem of corneal allograft shortage.

The strong immune response occurring after xenotransplantation may represent the main problem in corneal xenotransplantation. Experimental models have shown that corneal xenografts are invariably rejected within 2 weeks after transplantation [3–5], while corneal allografts survive often for few weeks or indefinitely [6,7]. To manage the immune response against xenoantigens, the involvement and the role of individual cell populations and cytokines in the rejection reaction have to be recognized. However, only limited studies on the topic of corneal xenotransplantation have been published. In other models of transplantation, macrophages and $CD4^+$ T cells have been demonstrated to play an important role. It has been suggested that $CD4^+$ T cells produce, at the site of graft rejection, inflammatory cytokines and molecules activating macrophages that can mediate effector functions [8,9]. Abundant infiltration with macrophages was also demonstrated in rejected corneal allografts [9,10]. In

allograft models a significant expression of Th1 cytokine genes [interleukin (IL)-2, γ-interferon (IFN-γ)], with very low expression of genes for IL-4 and IL-10, was observed [11,12]. To study the expression of genes for relevant effector molecules at the site of corneal xenograft rejection, we established a model of orthotopic corneal xenotransplantation in a concordant species combination (rat-to-mouse). Using a semiquantitative RT-PCR method, we analyzed the expression of genes for IL-2, IFN-γ, IL-4, IL-10 and inducible nitric oxide synthase (iNOS) in corneal xenografts during acute rejection and after anti-CD4 or anti-CD8 therapy. The production of cytokines and nitric oxide (NO) by xenograft explants was analyzed in parallel and correlated with gene expression. The presence of iNOS protein in grafts was studied using fluorescent immunohistochemistry. It was found that corneal xenograft rejection is associated, on the contrary to corneal allograft rejection, with strong expression of genes for both Th1 and Th2 cytokines. The pattern of gene expression and production of cytokines were altered after the treatment of graft recipients with monoclonal antibody (mAb) anti-CD4. The results suggest that corneal xenograft rejection correlates with the production of IFN-γ and NO and can occur in the absence of detectable IL-4 and IL-10.

Materials and methods

Animals

Mice of inbred strain BALB/c of both sexes at the age of 7–10 weeks and rats of inbred strain Lewis were used in the experiments. The mice were obtained from the breeding colony of the Institute of Molecular Genetics, Prague; the rats were purchased from Charles River Deutschland (Sulzfeld, Germany). The experiments were performed in accordance with the law governing the protection of animals. The 'Principles of Laboratory Animal Care' (NIH publication no. 86-23, revised 1985) was followed. The experiments were approved by the local Animal Ethics Committee.

Technique of corneal transplantation

The procedure was adapted from the technique described previously by She *et al.* [13] and modified by the authors [7]. In brief, donor corneas 2 mm in diameter were removed and placed in a balanced salt solution. The recipient mice were anesthetized by intramuscular injection of a mixture of ketamine (Calypsol 5%) and xylazine (Rometar 2%) diluted in saline. The recipient right cornea was marked with 1.5 mm trephine and excised using Vannas scissors after penetrating the anterior chamber with a sharp needle. The graft was sutured into the recipient bed using a running suture (11-0 Ethilon; Ethicon, St-Stevens-Woluwe, Belgium). After transplantation antibiotic ointment (Ophthalmo-Framykoin; Zentiva, Prague, Czech Republic) was applied on the eye. The suture was not removed. The grafts were examined every day using the operating microscope. Mice with complications such as cataracts, prolapse of the iris, inflammation, or hemorrhage were excluded from the study. The opacity of the grafted cornea was used as the indicator of graft rejection [14]. The scale of opacity was 0–4. Grade 2 or more was considered as rejection.

Treatment

Monoclonal antibodies anti-CD4 (clone GK1.5) [15] and anti-CD8 (clone TIB150) [16] were prepared in the form of ascites in nu/nu mice and were injected intraperitoneally, every other day beginning the day of grafting, at a dose of 200 μg/mouse.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of genes for cytokines and iNOS was quantified using semiquantitative RT-PCR. Total RNA was isolated from corneal grafts by means of TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Two μg of total RNA was reverse transcribed into cDNA in 20 μl reaction mixture, as described previously [17]. The cDNA samples of each individual RNA extraction were first normalized to yield equal amounts of β-actin. Subsequently, equal amounts of cDNA from individual corneal samples were amplified by several PCR cycles with the specific cytokine primers (Table 1). The number of PCR cycles for the different primers was 28 cycles for β-actin; 35 cycles for iNOS, IL-2, and IFN-γ; 40 cycles for IL-4

Table 1. Oligonucleotide sequences of mouse primers used for RT-PCR.

Primers	Sense (5′–3′)	Antisense (5′–3′)
β-actin	TGTGATGGTGGGAATGGGTCAG	TTTGATGTCACGCACGATTTC
IL-2	GCTTGTTGAGATGATGCTTTGACA	GTCAAAGCGCACCCACTTCAAGC
IL-4	ACGGAGATGGATGTGCCAAACGTC	CGAGTAATCCATTTGCATGATGC
IL-10	CCAGTTTTACCTGGTAGAAGTGAT	TGTCTAGGTCCTGGAGTCCAGACTC
IFN-γ	TACTGCCACGGCACAGTCATTGAA	GCAGCGACTCCTTTTCCGCTTCCT
iNOS	TGGGAATGGAGACTGTCCCAG	GGGATCTGAATGTGATGTTTG

and 45 cycles for IL-10. The conditions of each cycle consisted of denaturation for 2 min at 94 °C, annealing for 35 s at 58 °C for iNOS, 60 °C for IL-4 and IFN-γ and 63 °C for β-actin and IL-2, and elongation for 45 s at 72 °C. PCR products were analyzed by 2% agarose gel electrophoresis. Visualization was achieved by ethidium bromide staining.

Cytokine determination

Corneal graft explants were removed from recipient eyes and were cultivated in the wells of 96-well tissue culture plates (Nunc, Roskilde, Denmark) in 200 μl of RPMI 1640 medium (Sigma Chemical Corp., St Louis, MO, USA) containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 5×10^{-5} M 2-mercaptoethanol and 10 mM HEPES buffer. Samples were incubated at 37 °C in an atmosphere of 5% CO_2. Supernatants were harvested after 24 h for IL-2; 48 h for IFN-γ and 72 h for IL-4 and IL-10. The production of cytokines in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA) using sets of cytokine-specific capture and detection mAb purchased from PharMingen (San Diego, CA, USA) [18]. For quantification of cytokine levels, standards for IL-2, IL-4, IL-10 and IFN-γ (Genzyme, Boston, MA, USA) were included in all ELISA determinations.

Production and determination of NO

Individual corneal grafts were removed and cultivated in the wells of 96-well tissue culture plates (Nunc) in 100 μl of complete RPMI 1640 medium. Supernatants were harvested after 48-h incubation and samples were frozen at −20 °C until further use.

Nitrite was measured using the Griess reaction [19]. In brief, 80 μl of the tested supernatants was incubated with 40 μl of 1% sulfanilamide (in 2.5% H_2SO_4) and 40 μl of 0.3% *N*-1-napthyletylenediamine dihydrochloride (in 2.5% H_2SO_4) at room temperature for 5 min. Nitrite was quantified by spectrophotometry at 550 nm using sodium nitrite as a standard.

Immunofluorescent staining

The whole eyeballs were removed at the time of killing and frozen in OCT compound embedding medium (Miles Diagnostic Division, Elkhart, IN, USA) at −70 °C until processing. Corneal tissues were stained for the presence of iNOS with a primary rabbit polyclonal antibody against the peptide of mouse origin NOS2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Non-immune rabbit IgG was used for negative control staining. Tissue sections (7 μm thickness) were fixed in acetone, washed and rehydrated in phosphate-buffered saline (PBS). Fifty μl of primary antibody (diluted 1:200 in PBS/2.5% bovine serum albumin) was added to each section on the slide. The slides were then incubated for 1 h, washed and incubated for another 1 h with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson-Immuno-Research Lab, West Grove, PA, USA). After washing, the slides were mounted in vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and viewed under the microscope with the appropriate excitation filter.

Statistics

Statistical significance of differences between the means of experimental and control groups was calculated using the Mann–Whitney test.

Results

Graft survival

Syngeneic corneal grafts in BALB/c mice survived permanently. Rat corneal xenografts were rejected by untreated BALB/c mice in 7.8 ± 1.2 (mean ± SD) days after grafting (Fig. 1). Treatment of xenograft recipients with mAb anti-CD4 significantly prolonged corneal xenograft survival (16.9 ± 1.1 day, $P < 0.001$), while treatment with mAb anti-CD8 did not enhance graft survival significantly (8.9 ± 1.8 days, $P > 0.05$) (Fig. 1).

The FACS analysis was performed on the cells of the draining lymph nodes from untreated control mice and

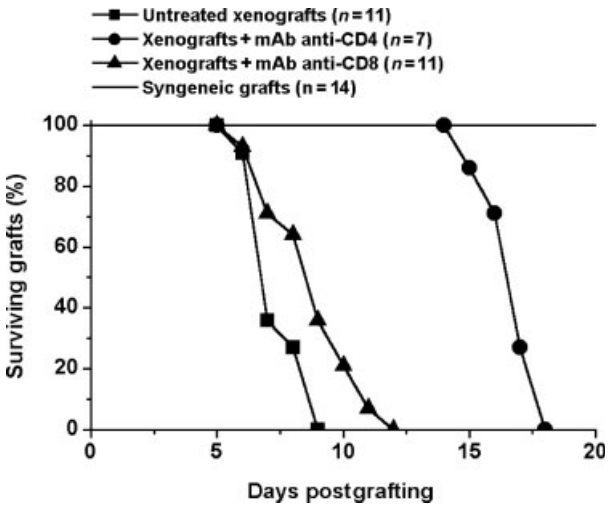

Figure 1 Survival of syngeneic and xenogeneic corneal grafts. BALB/c mice were grafted orthotopically with corneal grafts from syngeneic BALB/c donors or from Lewis rats. The recipients of xenografts were either untreated or were treated with mAb anti-CD4 or anti-CD8. Survival of the grafts was evaluated according to opacity.

mice treated with mAb anti-CD4 or anti-CD8 to determine the effectiveness of the therapy. The treatment with mAb reduced number of $CD4^+$ or $CD8^+$ cells to <2% of the control level (data not shown).

Expression of genes for iNOS and cytokines and production of cytokines in corneal graft explants

To test the expression of effector molecules in rejected corneal xenografts, the grafts were removed at the time of rejection (corneal opacity grade was >2) and were subjected to RT-PCR analysis. Xenografts and control syngeneic grafts were removed on day 8 after grafting. At this time point, the expression of genes for IL-2, IFN-γ, IL-4, IL-10, and iNOS was detected in all untreated xenografts (Fig. 2).

To determine whether the presence of cytokine mRNA in xenografts undergoing acute rejection was $CD4^+$ or $CD8^+$ T cell-dependent, we analyzed the cytokine profile in corneal xenografts from recipients treated with mAb anti-CD4 or anti-CD8. Mice depleted of $CD4^+$ T cells were divided into two groups. The xenografts in the first group were removed on day 8 after transplantation (the time of graft rejection in untreated recipients). At that time, the xenografts in anti-CD4 treated recipients had no signs of rejection (corneal opacity grade was <1) and there was no detectable expression of the genes for IL-2, IFN-γ, IL-4, IL-10, and iNOS (Fig. 2). The grafts in the second group of anti-CD4 treated recipients were analyzed at the time of graft rejection on days 16 and 17 after transplantation. These xenografts did not express the genes for cytokines IL-2, IL-4, and IL-10, but they did express mRNA for IFN-γ and iNOS. Treatment with mAb anti-CD8 did not prolong xenograft significantly survival and the expression of genes for IL-2, IFN-γ, IL-4, IL-10, and iNOS was not inhibited. In non-grafted corneas and syngeneic grafts no detectable expression of genes for cytokines and iNOS was observed (Fig. 2).

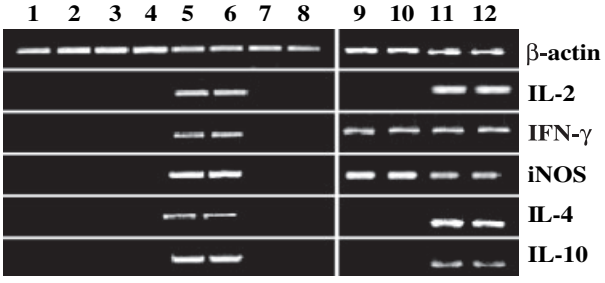

Figure 2 Expression of genes for cytokines and iNOS in corneal grafts. Expression of genes for IL-2, IL-4, IL-10, IFN-γ, and iNOS was determined by RT-PCR in two control non-grafted BALB/c corneas (lanes 1 and 2), syngeneic grafts (lanes 3 and 4), xenografts from untreated recipients (lanes 5 and 6), xenografts from recipients treated with mAb anti-CD4 removed on day 8 (lanes 7 and 8), xenografts from recipients treated with mAb anti-CD4 and removed at the time of rejection on days 16 and 17 (lanes 9 and 10) and from recipients treated with mAb anti-CD8 and removed at the time of rejection on days 8–10 (lanes 11 and 12). Each line represents an individual mouse. Xenografts in untreated recipients and syngeneic grafts were tested on day 8 after grafting (the day of xenograft rejection).

Figure 3 shows the results of cytokine detection in supernatants of cultivated corneal graft explants. The presence of cytokines IL-2, IFN-γ, IL-10, and IL-4 was detected in supernatants from cultivated rejected xenografts. Depletion of $CD8^+$ T cells in graft recipients did not cause any inhibition of cytokine production, except of IL-10, the production of which was markedly decreased. Anti-CD4 mAb therapy resulted in inhibition of production of all tested cytokines by rejected corneal xenografts. Only low IFN-γ production in supernatants was detected during graft rejection. Undetectable levels of cytokines were found in supernatants from non-grafted corneas, syngeneic grafts and non-rejected xenografts after anti-CD4 therapy (Fig. 3).

Presence of iNOS protein and NO production in corneal graft explants

The production of NO by corneal xenograft explants was compared on the day of rejection in untreated mice and mice treated with mAb anti-CD4 and anti-CD8. Corneal graft explants from untreated mice at the time of rejection produced a higher level of NO than grafts from mice treated with mAb anti-CD4 or anti-CD8. Xenografts in the group of mice treated with mAb anti-CD4 and removed on day 8 after grafting (without signs of rejection) produced levels of NO comparable to non-grafted or syngeneic corneal grafts used as controls (Fig. 4). For the demonstration of iNOS protein in corneal grafts we used an immunohistochemistry analysis. The results confirmed the findings obtained on the level of expression of mRNA for iNOS in all tested samples and the production of NO by xenograft explants. Immunostaining analysis showed positivity for iNOS protein in xenografts removed from untreated mice and mice treated with mAb anti-CD4 or anti-CD8 at the time of rejection (Fig. 5). Accumulation of cells producing iNOS molecules was revealed in the corneal stroma of the graft. Control non-grafted corneas, syngeneic grafts, and corneal xenografts from mice treated with mAb anti-CD4 removed on day 8 after grafting did not show any presence of iNOS molecules in grafted tissue (Fig. 5).

Discussion

The role of individual cell subpopulations and their cytokines in the rejection of xenografts is still a matter of

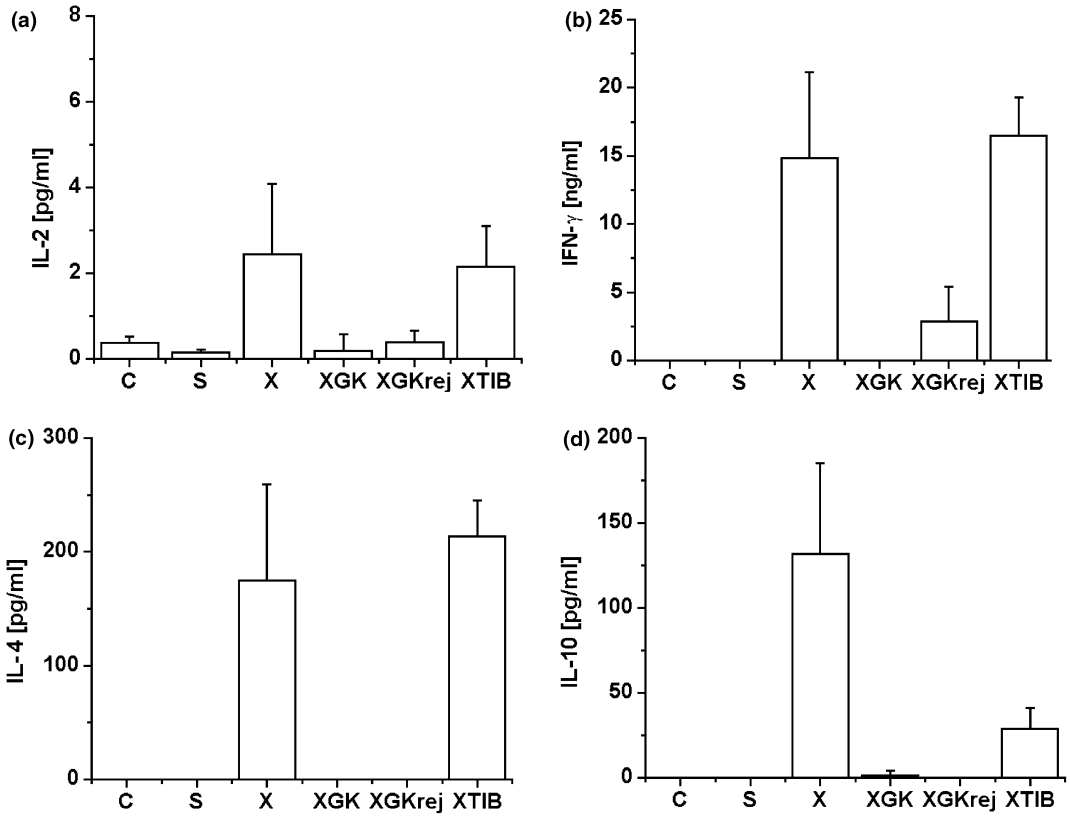

Figure 3 Production of cytokines IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d) measured by ELISA in supernatants after cultivation of corneal graft explants. C – control non-grafted corneas; S – syngeneic grafts; X – xenografts; XGK – xenografts from mice treated with mAb anti-CD4 and removed on day 8 after grafting; XGKrej – xenografts from mice treated with mAb anti-CD4 and removed at the time of rejection (days 16–17); XTIB – xenografts from mice treated with mAb anti-CD8 and removed at the time of rejection (days 8–10). Each bar represents the mean ± SD of four to six mice.

debate, because conclusive data is missing. Using a model of corneal rat-to-mouse xenotransplantation we demonstrated a pattern of the expression of genes for cytokines IL-2, IFN-γ, IL-4, IL-10, and for iNOS molecule in rejected grafts and we compared the expression of these genes in xenografts from untreated mice and mice treated with mAb anti-CD4 or anti-CD8.

In previous studies of models of corneal allograft rejection a strong expression of genes for IL-2 and IFN-γ and a low expression of genes for IL-4 and IL-10 have been described [11–13]. The observations underlined the role of the Th1 response in corneal allograft rejection. On the contrary, the rejection of corneal xenografts in our study was accompanied by an abundant expression of genes for Th2 cytokines (IL-4 and IL-10), in addition to the expression of genes for Th1 cytokines (IL-2 and IFN-γ). Cytokines of Th1 cells are committed mainly in cell-mediated responses, primarily in activation of macrophages, whereas Th2 cytokines are more involved in the antibody response, the predominant component of hyperacute and acute vascular rejection of xenografts [20]. Moreover, Th2 cytokines have immunosuppressive properties and thus they can downregulate the cell-mediated response and production of Th1 cytokines [21]. Upregulation of the genes for IL-4 and IL-10 has been demonstrated in concordant and discordant types of xenotransplantation [22–24], where distinct roles for IL-10 have been suggested. Local delivery of IL-10 and/or TGF-β enhanced the xenogeneic immune response and resulted in shortened xenograft survival of rat islets in mouse recipients in contrast to discordant (dog-to-rat) types of combinations where application of IL-10 prolonged xenograft survival [24].

To determine whether the production of cytokines in corneal xenografts undergoing acute rejection was $CD4^+$ or $CD8^+$ T cell-dependent, we analyzed the cytokine profiles in corneal xenografts removed from recipients treated with mAb anti-CD4 or anti-CD8. We found that mice treated with mAb anti-CD8 rejected corneal xenografts with a similar kinetics as did untreated recipients and that the expression of genes for the tested cytokines IL-2, IFN-γ, IL-4, and IL-10, and the production of these cytokines in rejected tissue were maintained. In other

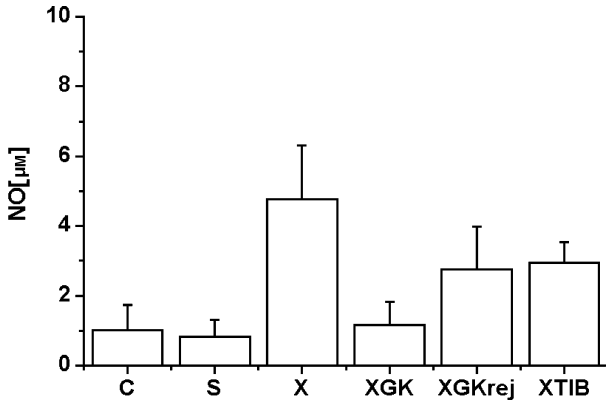

Figure 4 NO production by cultivated corneal graft explants. Supernatants from cultivated graft explants were harvested after 48 h of incubation and concentrations of NO were measured by Griess reaction. C – control non-grafted corneas; S – syngeneic grafts; X – xenografts; XGK – xenografts from mice treated with mAb anti-CD4 and harvested on day 8; XGKrej – xenografts from mice treated with mAb anti-CD4 and harvested at the time of rejection on days 16 and 17; XTIB – xenografts from mice treated with mAb anti-CD8 and removed at the time of rejection on days 8–10. Each bar represents the mean ± SD of four to six mice.

models of xenotransplantation, including discordant corneal transplantation, it has been suggested that $CD8^+$ T cells are not required for xenograft rejection and that $CD4^+$ T cells play a more important role in graft failure, and acute rejection can occur even in the absence of $CD8^+$ T cells, NK cells, and B cells [25–27].

In addition, we found that corneal xenograft survival in mice treated with mAb anti-CD4 was significantly prolonged and was associated with the absence of Th1 and Th2 cytokines. In rejected grafts removed from anti-CD4-treated mice, a low production of IFN-γ was always detected. Thus, in the absence of $CD4^+$ T cells, corneal xenografts can still be rejected by alternative mechanisms involving other cell populations, probably including $CD8^+$ T cells, NK cells, and macrophages [5]. Previous studies described the prolonged survival of xenografts in $CD4^+$ T-depleted recipients, but simultaneously they confirmed the role of $CD8^+$ T cells in corneal xenograft rejection [5]. Cytotoxic activity of $CD8^+$ T cells for xenogeneic target cells has been demonstrated in various types of xenotransplant combinations [28–30].

Graft rejection can also be mediated by cytokines released by $CD4^+$ and/or $CD8^+$ T cells stimulated at the graft site by the xenoantigen-presenting cells of recipients and donors. Stimulated T cells can provide activation signals necessary for other cell populations to release effector molecules. In our study we confirmed the importance of IFN-γ in the rejection of corneal xenograft. All rejected xenografts, from untreated mice as well as mice treated with mAb anti-CD4 or anti-CD8, expressed genes for IFN-γ concurrently with genes for iNOS. In contrast to IFN-γ, there was no detectable expression of genes for IL-2 or production of IL-2 in mAb anti-CD4-treated recipients. In untreated recipients or recipients treated with mAb anti-CD8, despite the expression of genes for IL-2 in xenograft explants, the production of IL-2 during rejection was very low. Similarly, in other studies the lack of IL-2 and small numbers of IL-2 receptor-positive T cells was observed at all time points after transplantation [23,31].

To the best of our knowledge the production of NO in rejected corneal xenografts has not been described. NO is catalytically produced by different isoforms of NO synthase [32] and previous investigations showed that NO is spontaneously produced by various corneal cell types [33,34]. This finding could explain the low level of NO production found in supernatants after the cultivation of control non-grafting corneas, syngeneic grafts, and non-rejected xenografts from mice treated with mAb anti-CD4. However, the higher NO production found in supernatants from cultivated rejected xenograft explants from untreated mice and mice treated with mAb anti-CD4 or anti-CD8 correlated with the expression of genes for iNOS and with the presence of the iNOS protein in rejected corneal grafts. The presence of iNOS and NO, the main effector molecule responsible for macrophage-mediated cytotoxicity [35], can be considered as indirect evidence for the role of macrophages in corneal xenograft rejection and thus supports the findings of other studies showing macrophages as the predominant infiltrating cells in rejected xenografts [5,36–38]. The role of macrophages in corneal allograft rejection has been well documented [39] and the depletion of macrophages at the time of corneal allografting can cause graft survival 'indefinitely' without any other form of therapy [40]. The effect of macrophage depletion in xenotransplantation models was studied only in discordant animal combinations where hyperacute rejection was prevented and xenografts were rejected in delayed xenograft rejection [36]. Suppression of NO production by a selective iNOS inhibition prolonged xenograft survival [41]. Macrophages were able to reject pancreatic islet or cardiac xenografts in the absence or presence of other effector cells, and they were an equally important mechanism for the destruction of xenografts, as in the case of allografts [37,38].

In summary, this study describes patterns of cytokine gene expression in corneal (rat-to-mouse) xenografts from untreated recipients or recipients treated with mAb anti-CD4 or anti-CD8 and demonstrates the effects of depletion of T-cell subpopulation on NO production during the rejection of corneal xenografts. The results suggest that production of IFN-γ and NO play an important role

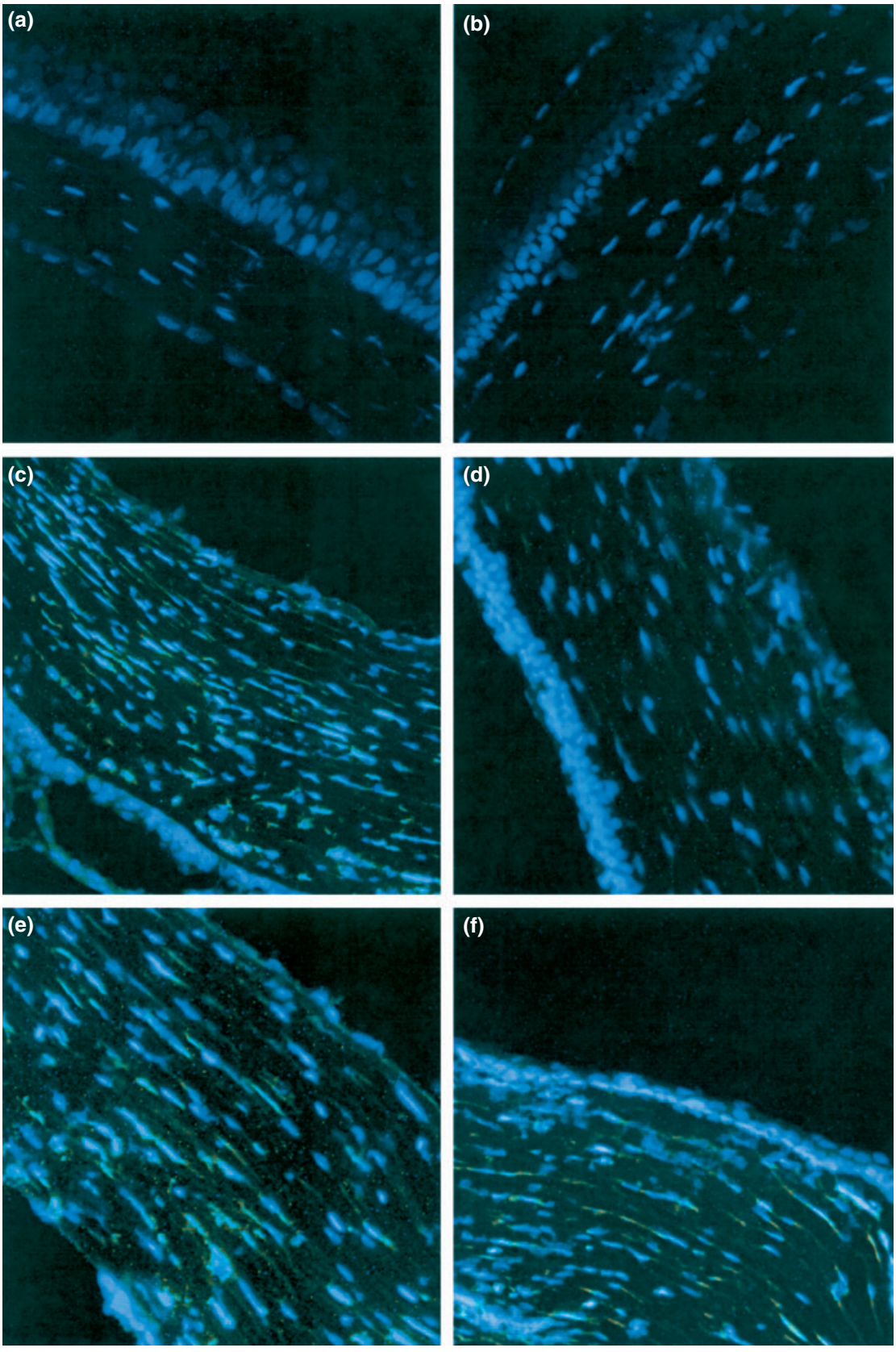
(a)
(b)
(c)
(d)
(e)
(f)

Figure 5 Immunofluorescent staining of iNOS molecules in (a) control non-grafted cornea, (b) syngeneic graft, (c) rejected xenograft from untreated recipient on day 8 after grafting, (d) xenograft from mouse treated with mAb anti-CD4 without signs of rejection removed on day 8 after grafting, (e) rejected xenograft from recipient treated with mAb anti-CD4 (day 17), and (f) xenograft from recipient treated with mAb anti-CD8 and removed on the day of rejection (day 8). Molecules of iNOS were stained with FITC-conjugated antibody (green) and cell nuclei were counterstained by DAPI (blue) (magnification ×200).

in corneal xenograft rejection and that the expression of genes for IL-4 and IL-10 is not required for rejection of xenografts.

Acknowledgements

This work was supported by grants NR/7816-3 and NI/7531-3 from the Grant Agency of the Ministry of Health of the Czech Republic, grants 310/02/D162 and 310/03/H147 from the Grant Agency of the Czech Republic, projects 1M6837805001 from the Ministry of Education of the Czech Republic, and by the project K5020115 awarded by the Academy of Sciences of the Czech Republic.

References

1. Williams K, Muehlberg SM, Lewis RF, Coster DJ. How successful is corneal transplantation? A report from the Australian Corneal Graft Register. *Eye* 1995; **9**: 219.
2. The collaborative corneal transplantation studies (CCTS): effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group. *Arch Ophthalmol* 1992; **110**: 1392.
3. Ross JR, Howell DN, Sanfilippo FP. Characteristics of corneal xenograft rejection in a discordant species combination. *Invest Ophthalmol Vis Sci* 1993; **34**: 2469.
4. Larkin DFP, Takano T, Stansfield SD, Williams KA. Experimental orthotopic corneal xenotransplantation in the rat. Mechanisms of graft rejection. *Transplantation* 1995; **60**: 491.
5. Higuchi R, Streilein JW. CD8+ T cell-mediated delayed rejection of orthotopic guinea pig cornea grafts in mice deficient in $CD4^+$ T cells. *Invest Ophthalmol Vis Sci* 2003; **44**: 175.
6. Sonoda Y, Streilein JW. Orthotopic corneal transplantation in mice – evidence that immunogenetic rules of rejection do not apply. *Transplantation* 1992; **54**: 694.
7. Holáň V, Hašková Z, Filipec M. Transplantation immunity and tolerance in the eye. Rejection and acceptance of orthotopic corneal allografts in mice. *Transplantation* 1996; **62**: 1050.
8. Yamamoto M, Einaga-Naito K, Kariyama M, Kawada Y, Yoshida R. Cellular basis of skin allograft rejection in mice: specific lysis of allogeneic skin components by non-T cells. *Transplantation* 1998; **65**: 818.
9. Larkin DFP, Calder VL, Lightman SL. Identification and characterization of cells infiltrating the graft and aqueous humor in rat corneal allograft rejection. *Clin Exp Immunol* 1997; **107**: 381.
10. Kuffová L, Holáň V, Lumsden L, Forrester JV, Filipec M. Cell subpopulations in failed human corneal grafts. *Br J Ophthalmol* 1999; **83**: 1364.
11. King WJ, Comer RM, Hudde T, Larkin DFP, George AJT. Cytokine and chemokine expression kinetics after corneal transplantation. *Transplantation* 2000; **70**: 1225.
12. Torres PF, de Vos AF, Van der Gaag R, Martins B, Kijlstra A. Cytokine mRNA expression during experimental corneal allograft rejection. *Exp Eye Res* 1996; **63**: 453.
13. She SC, Steahly LP, Moticka EJ. A method for performing full-thickness, orthotopic, penetrating keratoplasty in the mouse. *Ophthalmic Surg* 1990; **21**: 781.
14. Plšková J, Kuffová L, Holáň V, Filipec M, Forrester JV. Evaluation of corneal graft rejection in a mouse model. *Br J Ophthalmol* 2002; **86**: 108.
15. Dialynas DP, Wilde DB, Marrack P, *et al.* Characterization of the murine antigenic determinant designed L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T-cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol Rev* 1983; **74**: 29.
16. Gottlieb PD, Marshak-Rothstein A, Auditore-Hargreaves K, *et al.* Construction and properties of new Lyt-congeneic strains and anti-Lyt-2.2 and anti-Lyt-3.1 monoclonal antibodies. *Immunogenetics* 1980; **10**: 545.
17. Holáň V, Kuffová L, Zajícová A, *et al.* Urocanic acid enhances IL-10 production in activated $CD4^+$ T cells. *J Immunol* 1998; **161**: 3237.
18. Holáň V, Sedláčková K, Růžičková M. Production of high levels of Th1 and Th2 cytokines in mice with acquired transplantation tolerance. *Cell Immunol* 1996; **174**: 7.
19. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Anal Biochem* 1982; **126**: 131.
20. Brouard S, Gagne K, Blancho G, Soulillou JP. T cell response in xenorecognition and xenografts: a review. *Human Immunol* 1999; **60**: 455.
21. Deol HS, Tuch BE. Effect of interleukin-10 on human anti-porcine xenogeneic cellular response in vitro. *Transplantation* 2000; **69**: 112.
22. Lorant T, Krook H, Wilton J, *et al.* Intragraft cytokine mRNA expression in rejecting and non-rejecting vascularized xenografts. *Xenotransplantation* 2003; **10**: 311.

23. Singh NP, Guo L, Mhoyan A, Shirwan H. Predominant expression of Th2 cytokines and interferon-gamma in xenogeneic cardiac grafts undergoing acute vascular rejection. *Transplantation* 2003; **75**: 586.
24. Deng S, Ketchum RJ, Kucher M, *et al.* IL-10 and TGF-beta gene transfer for xenogeneic islet transplantation: comparison of effect in concordant vs. discordant combination. *Transplant Proc* 1997; **29**: 2204.
25. Wecker H, Winn HJ, Auchincloss H Jr. $CD4^+$ T cells, without $CD8^+$ or B lymphocytes, can reject xenogeneic skin grafts. *Xenotransplantation* 1994; **1**: 8.
26. Zhao Y, Swenson K, Wekerle T, Rodriguez_Barbosa JI, Arn JS, Sykes M. The critical role of mouse $CD4^+$ cells in the rejection of highly disparate xenogeneic pig thymus grafts. *Xenotransplantation* 2000; **7**: 129.
27. Tanaka K, Yamada J, Streilein JW. Xenoreactive CD4+ T cells and acute rejection of orthotopic guinea pig corneas in mice. *Invest Ophthalmol Vis Sci* 2000; **41**: 1827.
28. Singh NP, Guo L, Que X, Shirwan H. Blockade of indirect recognition mediated by CD4+ T cell leads to prolonged cardiac xenograft survival. *Xenotransplantation* 2004; **11**: 33.
29. Yi S, Feng X, Hawthorne W, Patel A, Walters S, O'Connell PJ. CD8+ T cells are capable of rejecting pancreatic islet xenografts. *Transplantation* 2000; **70**: 896.
30. Tanemura M, Chong AS, DiSesa VJ, Galili U. Direct killing of xenograft cells by CD8+ T cells of discordant xenograft recipients. *Transplantation* 2002; **74**: 1587.
31. Candinas D, Belliveaut S, Koyamada N, *et al.* T cell independence of macrophage and natural killer cell infiltration, cytokine production, and endothelial activation during delayed xenograft rejection. *Transplantation* 1996; **62**: 1920.
32. Brüne B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998; **351**: 261.
33. Yanagiya N, Akiba J, Kado M, Yoshida A, Kono T, Iwamoto J. Transient corneal edema induced by nitric oxide synthase inhibition. *Nitric Oxide* 1997; **1**: 397.
34. Jeng BH, Meisler DM, Hollyfield JG, Aulak KS, Stuehr DJ. Immunohistochemical localization of eNOS, iNOS, bNOS, and peroxynitrite in stored human corneas. *Invest Ophthalmol Vis Sci* 2002; **43**: 138.
35. Hibbs JBJ, Taintor RR, Vavrin Z, Rachlin M. Nitric oxide: a cytotoxic-activated macrophage effector molecule. *Biochem Biophys Res Commun* 1988; **157**: 87.
36. Wu G, Korsgren O, van Rooijen N, Wennberg L, Tibell A. The effect of macrophage depletion on delayed xenograft rejection: studies in the guinea pig-to-C6-deficient rat heart transplantation model. *Xenotransplantation* 1999; **6**: 262.
37. Wallgren AC, Karlsson-Parra A, Korsgren O. The main infiltrating cell in xenograft rejection is a CD4+ macrophage and not a T lymphocyte. *Transplantation* 1995; **60**: 594.
38. Yi S, Hawthorne WJ, Lehnert AM, *et al.* T cell-mediated macrophages are capable of both recognition and rejection of pancreatic islet xenografts. *J Immunol* 2003; **170**: 2750.
39. Streštíková P, Plšková J, Filipec M, Farghali H. FK 506 and aminoguanidine suppress iNOS induction in orthotopic corneal allografts and prolong graft survival in mice. *Nitric Oxide* 2003; **9**: 111.
40. Slegers TP, van der Gaag R, van Rooijen N, van Rij G, Streilein JW. Effect of local macrophage depletion on cellular immunity and tolerance evoked by corneal allografts. *Curr Eye Res* 2003; **26**: 73.
41. Kim JY, Kim D, Lee EM, *et al.* Inducible nitric oxide synthase inhibitors prolonged the survival of skin xenografts through selective down-regulation of pro-inflammatory cytokine and CC-chemokine expressions. *Transpl Immunol* 2003; **12**: 63.