The role of cytokines during corneal transplantation

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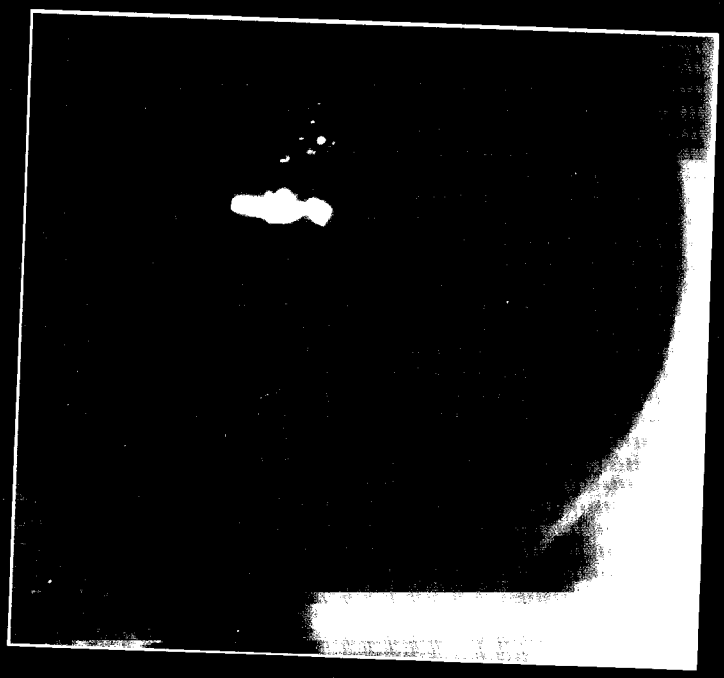

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To Luciana, Francisco and Mariana
who supported me in the preparation of this thesis.

To my Father,
who introduced me to the art of Clinical Ophthalmology.

CONTENTS

ABBREVIATIONS

ACAID	*anterior chamber immune deviation*
APC	*antigen presenting cell*
bFGF	*basic fibroblast growth factor*
C	*complement*
cDNA	*complementary deoxyribonucleic acid*
CTL	*cytotoxic T lymphocytes*
DTH	*delayed-type hypersensitivity*
EGF	*epidermal growth factor*
ELISA	*enzyme-linked immunosorbent assay*
FasL	*Fas ligand*
FGF	*fibroblast growth factor*
GM-CSF	*granulocyte macrophage colony stimulating factor*
GRO	*growth related protein*
HGF	*hepatocyte growth factor*
HLA	*human histocompatibility leukocyte antigen*
HSK	*herpetic stromal keratitis*
ICAM	*intercellular adhesion molecule*
Ig	*immunoglobulin*
IGF	*insulin-like growth factor*
IL	*interleukin*
IL-1RA	*IL-1 receptor antagonist*
IFN	*interferon*
KGF	*keratinocyte growth factor*
LC	*Langerhans cell*
LIF	*leukocyte inhibitory factor*
LIP	*liposome*
LPS	*lipopolysaccharide*
LT	*lymphotoxin*
MCP	*monocyte chemotactic protein*
MHC	*major histocompatibility complex*

MIF	*migration inhibitory factor*
MIP	*macrophage inflammatory protein*
MLN	*mesenteric lymphnode*
mAb	*monoclonal antibody*
mRNA	*messenger ribonucleic acid*
ND	*not detectable*
NK	*natural killer*
PBS	*phosphate buffered saline*
PCR	*polymerase chain reaction*
PG	*prostaglandin*
PMN	*polymorphonuclear leukocyte*
POD	*post-operative day*
RANTES	*regular upon activation normal T cell expressed and secreted*
RNA	*ribonucleic acid*
RT-PCR	*reverse transcription PCR*
SLN	*submandibular lymphnode*
TGF	*transforming growth factor*
Th	*T helper*
TNF	*tumor necrosis factor*
U	*unit*
UV	*ultraviolet*
VCAM	*vascular cell adhesion molecule*
VEGF	*vascular endothelial growth factor*
VIP	*vasoactive intestinal peptide*

Chapter 1

Introduction: Clinical and biological aspects of corneal transplantation

Corneal transplantation is the oldest and the most common and successful form of solid tissue transplantation worldwide [1]. Human corneas have been favorably transplanted since the first corneal transplantation performed by Reisinger [2], approximately 170 years ago. However, the first report was published only in 1906 [3]. Since then, the number is continuously increasing and only in the United States more than 45,000 corneal transplants are performed each year [4].

The outcome of corneal transplants depends directly on the pre-operative state of the host corneal bed before the penetrating keratoplasty. High-risk factors for corneal graft failure and rejection include among others the severity of the vascularization of the recipient cornea, history of a previous graft failure [5, 6, 7, 8], HLA class I and class II incompatibility [9], extreme dryness of the eye, chemical burns of the cornea, younger age of the host [6], previous or associated anterior segment surgery [7, 10], bilateral penetrating keratoplasty [11, 12], and size and eccentricity of the graft [8]. In non-vascularized low-risk recipients, the 5-year success rate for first corneal transplants is as high as 85 % to 90 % [7, 13, 14], while in inflamed and vascularized eye-recipients, the success rate may be as low as 20 % to 45 % [7, 15, 16]. Similar results are observed in the Cornea Unit and Eye Bank of the Hospital de Santo António in Porto, which is the major center of corneal transplantation in Portugal (Torres *et al.*, unpublished observations). Patients that are referred to us often show one or more of the high-risk factors already described above, which may contribute to an increase of the corneal failure rate in our department. Nevertheless, keratoconus, a degenerative disease of the cornea with an excellent prognosis for corneal graft survival, is still the leading cause for corneal transplantation in our department, closely followed by scarred corneas and previous graft failures [17]. The present overall 5-year success rate is 76 %, however if recipients are divided into low and high-risk patient groups, the former group shows long-term survival in 97 %, while the latter group exhibits corneal rejection in 48 % of the patients (unpublished data). Corneal graft rejection is, therefore, dependent on the studied patient population, associated risk factors and length of follow-up and may vary from center to center.

Despite the recent advances in eye banking, corneal preservation methods, surgical techniques and immunosuppression therapy, a significant number of technically successful corneal grafts will undergo failure. Immunologic rejection, undoubtedly the most important cause of late corneal failure [18], was first described by Paufique *et al.* in 1948 [19]. Clinically, corneal rejection is defined as a sudden rise of graft opacity and edema in a technically successful corneal graft that has remained transparent for at least two weeks. Early rejection should be distinguished from primary failure due to donor endothelial insufficiency, which may be the result of low endothelial cell count or undetectable endothelial disease of the donor cornea at the moment of the harvest, inadequate handling and preservation of the cornea or numerous associated surgical maneuvers, which may lead to direct trauma of the endothelium. In view of the important endothelial functions, such as the sodium pump, this corneal layer should not be damaged in any circumstance. Primary failure is then characterized by permanent edema of the graft since the first day of follow-up. Corneal rejection is more often observed during the first year after transplantation [20], however, late rejection after 20 years after transplantation has also been reported [21].

Preventing corneal allograft rejection requires the use of potent immunosuppressive drugs, such as corticosteroids, cyclosporine A or tacrolimus (FK 506). Their prolonged use can cause both ocular and systemic side effects, including cataract formation, glaucoma, hypertension, nephro- and hepatotoxicity [22, 23]. Furthermore, these drugs are not completely efficient in preventing or resolving corneal graft rejection. Thus, it is mandatory to understand the mechanisms that govern corneal rejection.

The anterior chamber of the eye is an immune privileged site that was first attributed to the fact that the normal cornea is completely avascular. Yet, there are several factors that are now known to play a role in the induction and maintenance of this immune privilege, which are fully described in chapter 2 of this thesis. Briefly, the anterior chamber associated immune deviation (ACAID) is characterized by suppression of delayed-type hypersensitivity reactions (DTH) and complement-fixing antibodies, while cytotoxic T-lymphocytes (CTL) and non-complement-fixing antibodies are enhanced [24]. This type of response is probably due to the fact that antigens entering the anterior chamber of the eye are directly transported into the venous compartment via the canal of Schlemm, bypassing the regional lymphnodes where DTH reactions are generated. Both afferent and efferent arms of the immune response are thus deviated [24, 25]. Furthermore, the aqueous humor contains various immunosuppressive cytokines, such as the transforming growth factor (TGF)-β, melanocyte stimulating hormone, and vasoactive intestinal peptide (VIP), which are capable of downregulating the cellular immune responses [26, 27]. This immune privilege can also be achieved after corneal transplantation, which may lead to a complete acceptance of the graft. However, when this immune privilege is disrupted, corneal rejection can arise.

The immunologic reaction occurring during corneal rejection was first observed and described by Maumenee [28, 29]. Subsequently, it was shown that individual layers of the cornea, such as the epithelium, stroma and endothelium, are capable of inducing immune rejection [30]. Epithelial and endothelial corneal rejection is often associated with «rejection lines» and deposition of keratic precipitates on the outer surface of the endothelium. The epithelial line is the result of necrosis of the donor epithelium and subsequent ingrowth of the host epithelium. The endothelial line, clinically known as the «Khodadoust line», usually originates at a vascularized area of the peripheral cornea [30] and, if untreated, progressively advances towards the center of the graft. This line and the keratic precipitates consist of aggregates of lymphocytes and other immune cells [31], such as neutrophils and macrophages.

The T-cell dependent immune effector mechanisms CTL and DTH are strongly implicated in corneal rejection [32, 33, 34]. Several observations showed that the massive cellular infiltration present during graft rejection reactions, consist mainly of T-lymphocytes and macrophages [35, 36]. The pivotal role of these cells in corneal graft rejection has been confirmed by showing that local administration of anti-CD4 monoclonal antibodies [37, 38] or liposomes containing clodronate (a drug that selectively depletes phagocytizing cells) [39], can enhance corneal graft survival in a rat model. However, the molecular mechanisms such as the cytokine networks that rule the different cells involved in corneal transplantation are not completely understood. Nevertheless, studies concerning allograft rejection in other organs have shown that T helper (Th) cell derived cytokines play a central role in organ rejection [40, 41, 42, 43]. Cytokines are polypeptide or glycoprotein cell-regulators, produced by white blood cells and a variety of other cells in the body after cell stimulation. Their multifunctional actions are achieved by receptor-mediated pathways, influencing the immune and inflammatory responses as well as their own release.

From the work performed in other organs and those mentioned above it is clear that cytokines may undoubtedly play a role in corneal transplantation. It can be envisaged that modulation of cytokine production or activity during corneal transplantation may contribute to the induction of corneal graft tolerance. However, since little is known about the interactions between the different cytokines during the process of corneal graft rejection, and before human intervention in clinical studies can be started, it is essential to analyze these mediators in an experimental model of allotransplantation. With this in mind, we embarked on a study to investigate cytokine gene expression in the donor and acceptor corneas of rats submitted to corneal transplantation. A better understanding of the cellular and molecular immune phenomena occurring in experimental corneal allotransplantation is likely to have profound implications for potential therapeutic strategies in humans. The four major issues that were addressed at the beginning of this study were:

a) Which cytokines does the normal cornea express?
b) Which cytokines are involved in corneal transplantation and in corneal rejection?

c) Is it possible to inhibit the development of corneal rejection by cytokine modulation? What are appropriate methods to modulate the cytokine network?

d) Which information can be gathered for a possible use in high-risk patients?

The questions raised above were investigated using human and rat corneas and a rat model of allotransplantation.

This thesis starts with a literature review on the role of cytokines in corneal immunopathology (chapter 2). Chapter 3 reports the measurements of interleukin-1 receptor antagonist (IL-1RA) protein in the normal human cornea. Chapter 4 describes the mRNA expression of several cytokines, such as IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 in recipient and donor corneas of rats submitted to auto- or allotransplantation. Different patterns of cytokines were observed in accepted and rejected corneas. Chapter 5 deals with the lack of a therapeutic effect of topical and systemic injections of IL-10 in enhancing corneal graft survival in the rat. Chapter 6 shows the corneal cytokine mRNA expression pattern for the same cytokines described in chapter 4 as well as for IL-12 and TNF-β / lymphotoxin in rats submitted to corneal allotransplantation that were treated with repeated subconjunctival injections of liposomes containing clodronate. Chapter 7 describes studies in which the effect of clodronate containing-liposomes that were injected subconjunctivaly in rats also submitted to an allotransplant, were analyzed with respect to cytotoxic T lymphocytes (CTL) responses and generation of antibodies. Finally, chapter 8 provides a discussion and conclusions of this thesis.

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Chapter 2

The role of cytokines in corneal immunopathology

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Submitted

SUMMARY

Immunological insults to the cornea may lead to tissue damage with loss of transparency and decreased visual acuity. Therefore, the normal cornea should endow mechanisms to counteract the immune-mediated responses initiated within the corneal layers. Although the accurate molecular mechanisms underlying corneal diseases and allotransplantation rejection are not completely understood, it is generally accepted that cytokines appear to be strongly involved. Furthermore, cytokines may also play a role in the maintenance of the integrity of the normal cornea. This review focuses on the effects of several cytokines in corneal immunopathology, including the type of the corneal immune response, angiogenesis, chemotaxis, apoptosis, wound healing, corneal disease and transplantation. This may provide important clues for future approach and treatment of corneal disease and corneal transplantation rejection.

INTRODUCTION

The cornea, a highly specialized tissue due to a unique arrangement of corneal stromal collagen fibers, is the most refractive structure of the eye and exhibits transparency, which allows light waves to reach the inner structures such as the retina. The absence of blood and lymphatic vessels observed in all layers of the healthy cornea confers a special status concerning corneal cell nutrition, cell response to injury and corneal graft

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acceptance. In view of its anatomical location in the eye, the cornea is constantly inflicted by exogenous stimuli from the environment such as trauma and infectious agents, that may induce inflammation with subsequent corneal neovascularization, scarring, loss of transparency and decreased visual acuity. Furthermore, the presumed autoimmune diseases of the cornea such as Mooren's ulcers, other melting diseases and collagen vascular diseases, the hyperreactivity as seen in allergy and atopic keratitis, and allograft reactions, can also produce similar sight-threatening outcomes. Therefore, the eye and the cornea itself should endow mechanisms to prevent the deleterious effects of inflammatory and / or immune-mediated responses initiated within the cornea in order to maintain transparency and best visual acuity.

In recent years it has become clear that the immunological response and the following inflammatory process occurring in the ocular surface may be mediated by a group of low molecular weight proteins called cytokines. Cytokines are hormone-like proteins produced and secreted by several types of cells and are involved in the communication between cells in an auto-, para- and endocrine pattern [1]. Cytokines play a key role in many biological processes, such as the induction of the immune response, recruitment of inflammatory cells, cytotoxicity, anti-viral activity, wound repair, angiogenesis, apoptosis, fever and synthesis of acute phase proteins. Cytokines act in a very complex network, in which they can induce the production and secretion of other cytokines, modulate the expression of cytokine receptors and are capable of having synergistic or antagonist effects on other cytokines [1]. Cytokines implicated in corneal immune and inflammatory responses can be expressed by infiltrating immunocompetent cells and / or by resident corneal cells (Table 1).

TABLE 1 – **CYTOKINE PRODUCTION BY CELLS OF THE OCULAR SURFACE**

CELL TYPE	CYTOKINE
Langerhans cells	IL-1
Macrophages	IL-1, IL-6, TNF, TGF-β
Mast cells	IL-4, IL-6
Epithelial cells	
Conjunctiva	IL-1, IL-6, IL-10,
Cornea	IL-1, IL-6, IL-10, IL-1RA
Keratocytes	IL-1, IL-3, IL-6, IL-8, IFN-γ, IL-1RA
Fibroblasts	IL-1, IL-3, IL-6, IL-8, IFN-γ
Vascular endothelial cells	IL-6, IL-8

Adapted from: Pleyer U. Corneal immunology and transplantation. General aspects. In: Keizer RJW, Jager M, Kijlstra A, editors. Handbook of Ocular Immunology. Buren: Aeolus Press, 1998; 73-94.

This review focuses on cytokines in normal and pathological corneas. The current knowledge concerning corneal cytokines is analyzed in order to provide new clues for treatment of corneal diseases and corneal grafting.

THE «PROTECTIVE» MECHANISMS OF THE CORNEA

The tear film confers the first protective barrier to the cornea due to its recognized bactericidal properties. The tear film contributes to the innate and adaptive immune response seen in the ocular surface, with the former being governed by the presence of high levels of lactoferrin and lysozyme. The adaptive immune response is ruled by the secretory IgA antibodies, which are secreted by plasma cells found in the lacrimal gland[2, 3]. Of interest is the diurnal variation of the tear film, showing that during eye closure, while sleeping, the levels of secretory IgA antibodies increase dramatically and a large influx of polymorphonuclear granulocytes can be observed[4]. The tear film is capable of eliminating nocuous agents by repeated washing and / or neutralization procedures, which prevents pathogens to bind and to invade the cornea. If attachment and invasion of the cornea do occur, other mechanisms, which we will describe below, are available within this tissue to combat infection.

In spite of the avascularity of the normal cornea, several components of the humoral immune system, namely immunoglobulins G (IgG)[5] and complement components[6], can be observed. The major route of immunoglobulins into the corneal stroma is probably via diffusion from the fenestrated limbal vessels[7], which only allows low molecular weight immunoglobulins to diffuse to the center of the cornea. The high molecular weight IgM is confined to the corneal limbal area. Interaction of antigen with its corresponding antibody leading to the formation of immune complexes can occur in the cornea and is readily diagnosed by ophthalmologists as Wessely's ring. Verhagen *et al.*[8] showed that the cornea also contains a complement-enhancing factor, which, probably, participates in the corneal defense mechanism, as less complement proteins are needed at the site of corneal inflammation. Activation of the complement system by immune complexes leads to the release of chemotactic complement activated products C3a and C5a, which cause an influx of polymorphonuclear granulocytes. Wessely's ring is therefore not only composed of precipitated immune complexes but also represents accumulated granulocytes.

The normal cornea shows a peculiar pattern concerning the distribution of other immunocompetent cells. For example, T and B lymphocytes can be seen only in the corneoscleral limbus[9], and MHC class II positive macrophage-like cells as well as Langerhans cells (LC) are observed only around the limbal vessels and in the periphery of the normal cornea[10, 11]. During corneal inflammation, the immune cells show the ability to migrate rapidly from the limbus towards the center of the cornea, without the necessity of blood vessels. This may suggest a cellular defense mechanism that is able to act without jeopardizing the visual function of the cornea.

Langerhans cells are histologically characterized by their dendritiform aspect, the expression of major histocompatibility complex (MHC) class II (HLA-DR) antigens and by ATPase / ADPase activity on the surface [12]. They are antigen-presenting cells (APC), due to their capability of processing and presenting antigens to T cells either locally (passing T helper cells) or in the regional draining lymphnode (activation of T cell subsets). Furthermore, Langerhans cells play a role in corneal transplantation by stimulating allospecific T cell responses through their capacity of membrane MHC class II antigen expression [10]. However, it was shown that at the time of transplantation, organ cultured-preserved corneas do not show HLA-DR positive Langerhans cells [13]. Nevertheless, corneal rejection is directly dependent on the number of Langerhans cells attracted into the cornea [14]. Elimination of corneal Langerhans cells by UV exposure results in a reduction of the incidence and severity of herpetic corneal disease [15], and in a prolongation of corneal allograft survival [16]. Taking into account that the center of the normal cornea is, presumably, devoid of Langerhans cells, we can speculate first that, immunologically speaking, their presence is not necessary in this part of the cornea. Due to the normal anatomical distribution of the several collagen layers of the cornea, the watery influx that is continuously occurring between collagen layers may force antigens to interact with Langerhans cells only in the periphery of the cornea. Second, this may be a physiological mechanism of the cornea to prevent the initiation of immune-mediated reactions, such as delayed-type hypersensitivity (DTH) reactions, that may inflict corneal impairment with extensive necrosis and scarring.

Moreover it is known, for more than a century, that the eye is regarded as an immune-privileged site [17]. This is due to several factors such as the presence of a blood-ocular barrier, complete lack of efferent lymphatic drainage pathways, direct drainage of tissue fluid into the blood, locally produced immunosuppressive neuropeptides, cytokines and growth factors [18, 19, 20], soluble and membrane-bound inhibitors of complement activation and fixation [21], limited MHC antigen expression [22], strategically located APC [23] and constitutive expression of Fas ligand (FasL) on epithelial and endothelial corneal cells and on iris tissues [24]. Apoptotic cell death, due to the interactions between Fas+ immunocompetent cells and FasL+ resident cells of the eye, actively participates in the induction of immune privilege [24]. One manifestation of the immune privilege in the eye is the induction of a deviant immune response when soluble antigens are injected into the anterior chamber. This deviated immune response, known as anterior chamber associated immune deviation (ACAID), is an antigen-specific systemic response and is characterized by inhibition of the DTH reaction and complement-fixing antibodies, while cytotoxic T cells and the non-complement-fixing immunoglobulin G antibodies are enhanced [25, 26]. ACAID is influenced by several cytokines. IL-1 when stromally injected [27] or when intracamerally injected [28], IL-2 (injected prior to antigen injection) [29], IFN-γ and TNF-α [28] can prevent the induction of ACAID. IL-1 and IFN-γ abrogate ACAID through a direct action on

Langerhans cells. Immune privilege in the anterior chamber is necessary, for instance, to avoid severe scarring due to herpes simplex virus induced-stromal keratitis. By means of the same mechanism, corneal allografts have an excellent opportunity to show long-term survival. Clinically, this is confirmed by the high one-year corneal survival rates approaching 90 %, together with the fact that corneal transplantation is in the majority of the cases performed without HLA tissue matching [30].

Most of the work in ACAID has shown that transforming growth factor-beta (TGF-β) is necessary to endow immune privilege in the cornea and anterior chamber. TGF-β is an immunomodulatory cytokine with immunosuppressive activities, including the inhibition of T and B lymphocyte activation and proliferation, the adherence of granulocytes and HLA gene expression [31]. The normal cornea is capable of expressing TGF-β and its two different isoforms, the TGF-β_1 and the TGF-β_2 [32], as well as the TGF-β receptor [33]. To become biologically effective, TGF-β should be proteolytically (plasmin) transformed into a mature form. Of the various types of TGF-β, the TGF-β_2 isoform is the most active and was shown to be constitutively present in the normal aqueous humor [34]. TGF-β may provide a «tolerogenic signal» to local APC that will activate certain subpopulations of T cells that can downregulate DTH [35]. De Boer *et al.* [36] showed that active inflammation in the anterior chamber was associated with low mature TGF-β_2 in aqueous humor when compared to normal controls. Furthermore, the soluble form of FasL, already described in serum [37], has also been detected in aqueous humor and was associated with suppression of interferon-gamma (IFN-γ) and IL-6 in aqueous humor [38]. This may suggest that soluble FasL may also play a protective role against immune-mediated damage.

Besides TGF-β, the cornea is able to produce several cytokines with anti-inflammatory properties. Recent findings reveal that epithelial and stromal cells constitutively express interleukin-1 receptor antagonist (IL-1RA) [39, 40]. The IL-1RA is the natural antagonist of IL-1, competing with both IL-1α and IL-1β for the binding to the IL-1 receptors. The large amounts of IL-1RA produced by corneal epithelial cells may indicate an inherent corneal defense mechanism to counteract IL-1-mediated responses. The importance of this fact is emphasized by the observation that the normal in vitro cultured cornea has the capacity to produce pro-inflammatory cytokines such as IL-1 [41, 42, 43, 44, 45], IL-6 [46, 47] and tumor necrosis factor-alpha (TNF-α) [48] as well as IL-8 [49, 50] (Table 1). IL-1 produces a wide variety of effects on differentiation and function of cells involved in the inflammatory and immune response. Cytokine release is, in many instances, dependent on the presence of IL-1, which is often the initiating factor of the inflammatory cascade. Therefore, the balance between IL-1 and IL-1RA in the cornea at the moment of the stimulus may dictate the type of corneal response, towards inflammation or abrogation of the immune response. Topical application of IL-1RA promotes corneal allograft survival in a murine model [51]. This may be due to the fact that IL-1RA, by inhibiting IL-1, is able to suppress Langerhans cell activity in the cornea and promoting

ocular immune privilege [52]. Niederkorn *et al.* [53] have shown that IL-1 is a regulator of Langerhans cell migration in the cornea, which can cause abrogation of the immune privilege. Our group also suggested [54] that resident corneal cells, such as the epithelial cells and the keratocytes, under traumatic stress, are triggered to secrete IL-10. This cytokine blocks the production of IFN-γ and IL-12 and downregulates the MHC class II expression on monocytes [55]. Local administration of low doses of IL-10 to the cornea proved to be efficient in ameliorating herpes simplex virus associated keratitis [56].

On account of the described specific ocular and corneal conditions, it is clear that immune reactions taking place within the corneal layers can be different from the rest of the body.

THE TYPE OF IMMUNE RESPONSE IN THE CORNEA

Immune responses triggered by CD4+ T Helper (Th) cells are dependent on the activation of different subpopulations of T lymphocytes with distinct patterns of cytokine release. A recent theory [57] has proposed that the immune response only starts when tissue cells sensor «danger», via, for example, TGF-β, heat shock proteins, IFN-γ, hypoxia or stress neuropeptides. Subsequently, APCs, via mediators released by the stressed tissue cell, are able to initiate an immunological response. These APCs such as the corneal Langerhans cells process and present antigens to immature Th (Th0) cells. Whether a lymphocyte response is skewed to a Th1 or Th2 response is dependent upon the microenvironment in which the antigen presenting cells acquire their antigen and the environment in which these antigen presenting cells mature. The type of cytokines released by the APC and other accessory cells (NK cells, mast cells) will influence the class of T cells responding to a certain antigen. Thus, the balance between IL-12 and IFN-γ on one side and IL-4 and IL-10 on the other side in addition to the presence or not of certain co-stimulatory molecules and / or prostaglandins (PG) play a role in Th differentiation, with the former group of cytokines inducing Th1 lymphocytes and the latter being responsible for Th2 activation and maturation. Th1 cells are recognized by their secreted cytokine pattern, IL-2, IFN-γ and TNF-β (Fig. 1), while Th2 cells secrete IL-3, IL-4, IL-5, IL-10 and IL-13 (Fig. 2). Cytokines from one Th subset can inhibit the activation of the other Th subset due to the balance between IFN-γ and IL-10. Therefore, the nature of the immunological response may dictate whether the exogenous or endogenous agents threatening the cornea will be eliminated, but may also dictate the outcome of corneal allografts, as well as the determination of the degree of corneal tissue damage. Fragile ocular tissues would theoretically tend to favour a Th2 type of immune response which is less damaging to the ocular environment. On the other hand an excessive local Th2 response against various allergens may lead to an allergic conjunctivitis, with or without associated keratitis, which is the most common inflammatory disease of the eye. As yet, little is known

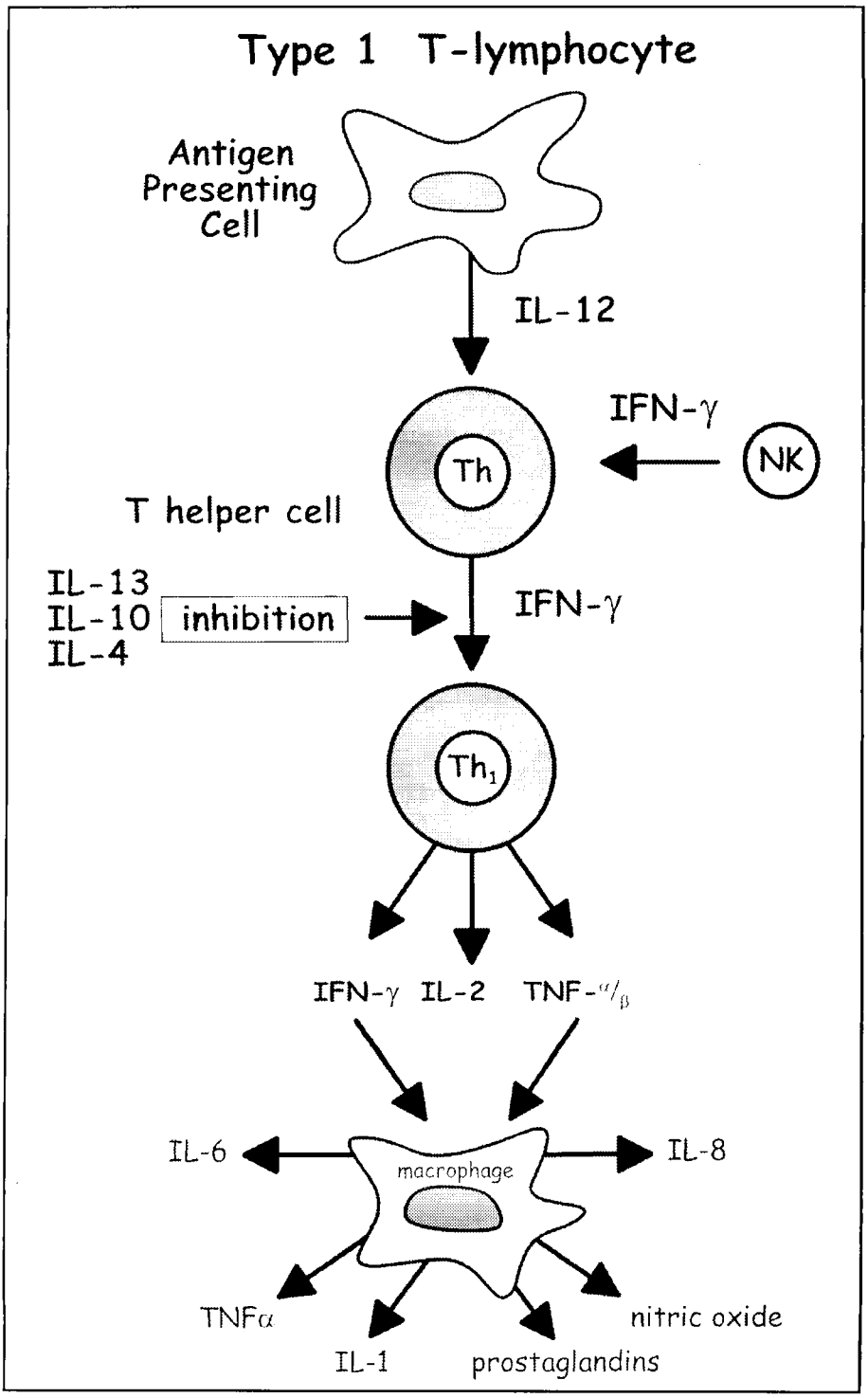

Figure 1 – The Th1 lymphocyte-derived cytokine network.

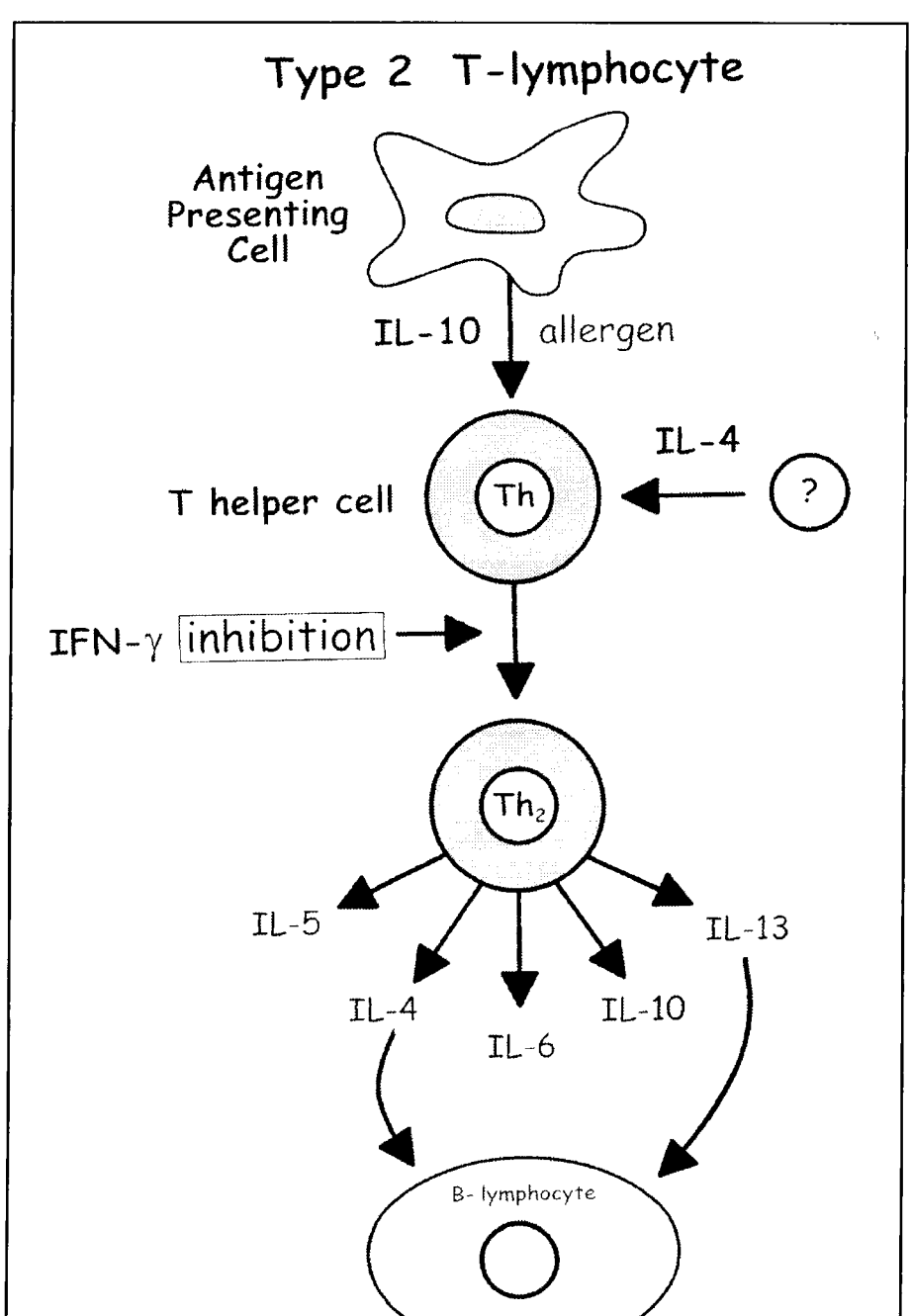

Figure 2 – The Th2 lymphocyte-derived cytokine network.

about the local factors in the corneal / limbal areas that influence the maturation of these antigen presenting cells and only scarce data are available concerning the Th1 / Th2 balance and their released cytokines in inflammatory diseases of the ocular surface. The general properties of the cytokines involved in corneal immune mediated processes are described in Table 2.

TABLE 2 – **CHARACTERISTICS OF THE CYTOKINES INVOLVED IN CORNEAL IMMUNE-MEDIATED PROCESSES**

CYTOKINE	CELL SOURCE	STIMULANT	TARGET	MAJOR FUNCTIONS
IL-1	Macrophages, APC, Corneal cells, Keratinocytes	Exogenous agents, IFN-γ, TNF-α, IL-2, IL-3, IL-1	T cells, B cells, Epithelial cells, Fibroblasts	Production and activation: IL-2, IL-2R, IL-6, IL-8, TNF-α, type IV collagen, collagenases
IL-2	Th1 cells	IL-1	T cells, B cells, NK cells	Growth factor for T, B, NK cells, Production: IL-1, Phagocytosis
IL-3	T cells, NK cells, Keratinocytes	Antigen, IgE, Parasitic infection	Mast cells, Eosinophils, Macrophages, Basophils PMN	Colony stimulating factor for hematopoietic cells
IL-4	Th2 cells, Mast cells	Antigen	Macrophages, B cells, Fibroblasts	Growth factor for T and B cells, Ab «switching», Expression MHC class II
IL-5	T cells	Antigen	Eosinophils	Colony stimulating factor for eosinophils
IL-6	T cells, Macrophages, Fibroblasts, Corneal cells, Vascular endothelium	Endotoxin, IL-1, TNF-α	B cells, Hepatocytes	B cell growth factor, Ab production, Acute phase proteins
IL-8	Macrophages, T cells, Corneal cells, Fibroblasts, PMN, Endothelium	IL-1, TNF-α, Basophils	PMN, T cells, Vascular endothelium	Chemotaxis: T cells, PMN, Angiogenesis
IL-10	Th2 cells, Macrophages, B cells, Keratinocytes	Stressed tissue, Antigen	T cells, Macrophages	Inhibition APC by down-regulating MHC class II, Th class control: promoting Th2; inhibiting Th1, Cytokine synthesis inhibition
IL-12	Macrophages, APC	Stressed tissue, Antigen	T cells	Regulator of Th1 cells
IL-13	T cells, B cells, Mast cells	Antigen	B cells, Macrophages	Growth factor for B cells and monocytes
TNF-α	Macrophages, T cells, B cells, Keratinocytes	Endotoxin, Infectious agents, IL-1	Leucocytes, Fibroblasts, Vascular endothelium	T cell activation, Macrophage and PMN activation MHC expression, IL-1 and IL-6 synthesis, Angiogenesis, Adhesion molecule upregulation
TNF-β	T cells	Antigen	T cells, Cytolytic T cells, NK cells	Cytotoxicity, MHC class I expression, B cell proliferation, Angiogenesis
IFN-γ	Th1 cells, Macrophages, NK cells	Antigen, IL-12, IL-18	T cells, Macrophages	Increased expression MHC, Th class control: promoting Th1; inhibiting Th2, Macrophage activation
TGF-β	Virtually all cells	Wounding, Inflammation	Immune cells, Epithelium, Fibroblasts	Induction of proinflammatory cytokines, Upregulation of adhesion molecules,
IL-1RA	Macrophages, Corneal cells	IL-1	Cells that produce IL-1	Inhibition of IL-1 functions by competitively binding to the IL-1R

Analysis of the cytokine profile in patients with allergic eye disease has revealed that vernal conjunctivitis and giant papillary conjunctivitis, often seen in contact lens wearers, are associated with a Th2 type response possibly caused by an up-regulation of the cytokine gene cluster on chromosome 5 which harbours the typical Th2 cytokines IL-3, IL-4, IL-5 and IL-13 [58]. On the other hand it was observed that patients with atopic keratoconjunctivitis were characterised with a shift towards a Th1 cytokine profile [58]. Onchocercal keratitis (river blindness) is also considered to be mediated via a Th2 type response since it is accompanied by an upregulated IL-4 and IL-5 expression but not IL-2 or IFN-γ [59, 60]. IL-4 knockout mice produce less severe onchocercal keratitis [59]. Herpetic stromal keratitis (HSK) is associated with an IFN-γ and IL-2 response (Th1 type response) whereas its recovery is associated with a Th2 response as evidenced by the expression of IL-10 [61]. Corneal rejection is characterised by a Th1 (IL-2 and IFN-γ) cell response, although a role for Th2 (IL-4) class of response could not be excluded [54].

EFFECTS OF CYTOKINES ON CORNEAL ANGIOGENESIS

Neovascularization is a direct consequence of inflammation and it is important for the inflammatory components of the immune response to reach the site of the insult. It is also a deleterious mechanism that induces a higher influx of inflammatory components to the site of injury and into the yet normal surrounding cornea, leading to detrimental effects such as corneal opacity, edema and scarring. Clinically, corneal neovascularization increases the risk of immunologic rejection, possibly due to the loss of the immune privilege [62]. Efforts should be taken into consideration to prevent the ingrowth of new corneal vessels.

Corneal neovascularization is a complex response to a number of stimuli and involves a sequence of events. The first stage involves the dilation of the existing limbal vessels followed by adhesion and diapedesis of leucocytes and activation of endothelial cells. Secondly, the degradation of the extracellular matrix with migration and proliferation of endothelial cells and formation of capillaries can take place [63]. This entire process is mediated by different types of cells, including macrophages, lymphocytes, mast cells, platelets, and polymorphonuclear leucocytes [64], and by the following mediators, for instance, adhesion molecules such as the soluble E-selectin and vascular cell adhesion molecule-1 (VCAM-1) [65], growth factors such as the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF) and TGF-β [63, 64]. Other cytokines such as IL-1, IL-8 and TNF-α are also thought to be involved at certain stages of neovascularization. IL-1 and IL-8 activate the vascular endothelial cells and IL-1 is known to induce corneal collagenase and metalloproteinase expression [66, 67], which play an important role in extracellular matrix degradation, allowing the ingrowth of new vessels [64].

The normal cornea is completely avascular. Trauma, hypoxia, infection or deposition of immune complexes usually precedes corneal angiogenesis.

Ingrowth of new vessels may be necessary to let both antibodies and the immunological effector cells reach the focus of inflammation. The cornea has often been used as a model to study the angiogenic activity of certain cytokines. BenEzra's group [68, 69] showed that IL-1 was a potent inducer of corneal angiogenesis after implantation of cytokine-containing pellets (Elvax-40) into the corneal stroma of rabbits. Others have shown that IL-8 can also induce neovascularization in the cornea [70, 71]. Of interest is the observation that IL-1, which can be produced by corneal cells [41, 42, 43, 44, 45], is capable of inducing stromal keratocytes to produce IL-8 [49, 50]. This indicates that IL-1, in addition to its direct involvement in the induction of corneal angiogenesis, can also have its effects exacerbated via the induction of IL-8, giving rise to the possibility of resident corneal cells to actively participate in the ingrowth of new vessels. Literature shows controversial data concerning the role of TNF-α in corneal neovascularization [72, 73, 74, 75], however a recent study revealed that IL-1β and TNF-α are able to upregulate the cell surface glycoprotein Thy-1 marker on endothelial cells of newly formed blood vessels [76]. This observation suggests a major role of proinflammatory cytokines in corneal angiogenesis. Of interest is a recent study by Volpert *et al.* [77] who showed that the Th2 cytokine IL-4, when incorporated into pellets implanted in the rat cornea, is a potent inhibitor of bFGF-induced corneal angiogenesis. As yet little is known how corneal angiogenesis is controlled and the recent finding of a corneal gene product similar in sequence and protein structure to the family of angiopoietins, may yield new clues to the mystery why the cornea is avascular [78].

CYTOKINES AND THE RECRUITMENT OF PHAGOCYTES INTO THE CORNEA

Inflammation is characterised by a massive infiltration of leucocytes into the injured tissues. Tissue distress, resulting in the release of cytokines and other mediators, initiates the migration process. Cell adhesion molecules, surface glycoproteins, play a role in the initial adhesion of inflammatory cells to the vascular endothelial cells and activate the transendothelial migration of leucocytes [79]. Neutrophils are the most prominent cell type to migrate to the cornea in the early stages of inflammation. However, the mechanisms involved in the recruitment of cells from the vascular space into the corneal avascular tissue remain to be explained. Potent chemokines such as macrophage inflammatory protein (MIP) and IL-8, two related cytokines [80], are thought to be involved.

A few hours after eye-closure a marked influx of polymorphonuclear granulocytes can be observed in the tear film of healthy volunteers [4]. Although complement activation was thought to attract these leucocytes via C5a, current ideas implicate IL-8 as the main chemoattractant. Eye closure may induce local hypoxia, which in turn may lead to IL-1 release followed by IL-8 production by ocular resident cells. Firm experimental evidence for this hypothesis is still lacking although in vitro experiments have shown

that the addition of exogenous IL-1 to corneal cell cultures stimulates the synthesis of IL-8 whereby stromal cells produce approximately 30 fold more IL-8 than epithelial cells [50]. Also conjunctival epithelial cells have been shown to secrete IL-8 upon stimulation with IL-1 [81]. Other neutrophil chemotactic factors produced by the cornea include growth-related protein--alpha (GRO-α) [82]. Chemotactic factors for monocytes and lymphocytes such as monocyte chemotactic protein-1 (MCP-1) and regular upon activation normal T cell expressed and secreted (RANTES) are also readily produced by corneal keratocytes upon stimulation with either IL-1α or TNF-α [83]. The fact that generally speaking, chemokine production is more easily induced by keratocytes than epithelial cells indicates that trauma to the cornea will mainly lead to recruitment of inflammatory cells to the corneal stroma.

Yan *et al.* [84] showed that MIP-2, besides its effect on neutrophil migration to the herpetic cornea, can directly contribute to the severity of the disease. Furthermore, MIP-1α also upholds the development of the inflammatory process that leads to severe HSK. Recently, Tumpey *et al.* [85] showed that MIP-1α knockout mice had no detectable infiltrating CD4+ T cells as well as the T-derived cytokines IL-2 and IFN-γ in the cornea. It was also suggested that MIP-1α influences the expression of other chemokines.

Interleukin-1 produced in the corneal epithelial cells is believed to act in an auto- and paracrine manner to induce the synthesis of other cytokines such as IL-6, MIP, the IL-1 receptor and more IL-1. Thus, IL-1 in an indirect manner, by stimulating the production of MIP, contributes to the massive cellular infiltration within the diseased cornea.

THE EFFECT OF CYTOKINES ON CORNEAL APOPTOSIS AND WOUND HEALING

The cornea consists of three different types of cells: the epithelial, stromal and endothelial cells. The properties of these different cell types are of crucial importance for the integrity of the cornea. The limbal epithelium contains the stem cells that can either differentiate into conjunctival or corneal epithelial cells. Cytokines derived from the underlying corneal or conjunctival stroma play an important role in dictating the differentiation pathway of these limbal stem cells [86]. Growth factors released into the tear film by the lacrimal gland are also considered to be involved in the later steps of corneal epithelial maturation [87].

Cytokines participate in corneal wound healing. Together with the numerously known growth factors, they give a very complex network of signals to the different types of cells involved in the healing process. IL-1 initiates repair of the wounded cornea by activating various repair genes and turning a quiescent stromal keratocyte into a repair type fibroblast [88]. Corneal epithelial wounding induces the production of IL-1 by these cells and in turn the released IL-1 promotes the production of keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) by corneal

stromal cells [89, 90, 91]. Together with epidermal growth factor (EGF), exuberantly present in the normal tear film [92, 93], they play an important role on corneal epithelial and stromal cell proliferation and wound healing. A recent study by Kovalchuck *et al.* [94] showed that local application of a complex of cytokines extracted from peripheral leucocytes, including IL-1, IL-6, TNF, migration inhibitory factor (MIF) and leucocyte inhibitory factor (LIF), is effective in promoting corneal healing and regeneration in an experimental model of corneal post-traumatic perforation. TGF-β is also a promoting factor in the wound-healing process and fibrogenesis [95, 96]. It is thought that the ability of TGF-β, present in the cornea [32] and aqueous humor [34], to stimulate fibroblasts and to suppress T and B cells may induce protection to the cornea from prolonged immune processes and activation of the tissue repair mechanisms. Wounding of the cornea has been associated with loss of stromal keratocytes and evidence has been provided that this is due to apoptosis of these cells. The exact triggers leading to keratocyte apoptosis are not yet known and both IL-1 and the Fas-Fas ligand system have been implicated to be involved [97]. Moreover, IL-1 released by corneal epithelial cells is a stimulant for collagenase expression that may also be involved in stromal development and repair [98].

Fas is a protein expressed on the surface of several types of immunocompetent and non-immunocompetent cells, while FasL is the natural ligand for Fas. The Fas+ cells undergo apoptosis when they bind to a cell expressing the FasL. In the cornea, FasL is constitutively expressed in epithelium and endothelium [24]. Apoptotic cell death minimises the release of cell components thereby avoiding immunological reactions against self-antigens. Apoptotic cell death of T lymphocytes entering the anterior chamber is thought to result in the release of IL-10, which may in turn play a role in the ACAID phenomenon [99]. It also confers protection to the cornea against inflammatory cells entering through the conjunctiva and limbal vessels and the anterior chamber. Therefore, FasL besides a role in the wound healing process also plays a role in immune privilege induction and in corneal allograft acceptance [100].

CYTOKINES DURING CORNEAL DISEASE AND CORNEAL TRANSPLANTATION

Cytokines are implicated directly in corneal inflammation and disease due to the capacity of corneal cells to synthesise a variety of cytokines and growth factors [41, 42, 101, 102]. Cytokines are strongly involved in the pathogenesis of herpetic stromal keratitis (HSK) [15]. The pro-inflammatory cytokines IL-1α and IL-6 may play a role in initiating HSK [48]. HSK is driven by T-cells, CD4+ [103] and CD8+ T-cells [104], which is evidenced by the fact that athymic mice do not develop HSK [105]. IL-1α, IL-6, IL-4, IL-10, TNF-α [48], MIP-2 [84] as well as IL-2 and IFN-γ [61] are upregulated during HSK. It was shown that IL-2 mediates corneal inflammation and the severity of the disease by upregulating local production of IFN-γ and contributing to

the PMN chemotactic gradient and viability in the cornea [106]. Thus, treating rats with anti-IL-2 monoclonal antibody results in a significant improvement of the herpetic disease [107]. Pseudomona aeruginosa keratitis that might be seen in contact lens wearers is also associated with high levels of IL-1, IL-6 and TNF-α [108]. The same pro-inflammatory profile in addition to IL-10 is detected in alkali-burned corneas [109, 110]. Analysis of corneal mRNA expression for various cytokines showed an increased expression for IL-1α and IL-8 in pseudophakic bullous keratopathy as compared to normal controls or Fuch's dystrophy [102]. Analysis of corneal explants obtained from recipients after keratoplasty revealed that IL-1 and IL-6 protein levels were increased in patients with inflammatory signs as compared to previously non-inflamed corneas obtained from cases with scars, corneal dystrophies or keratoconus [111]. However, Fabre *et al.* [112] showed that keratoconus corneas present four fold more IL-1 binding sites when compared to normal corneas. It was suggested that IL-1 could be directly involved in the pathogenesis of this corneal dystrophy, due to the ability of IL-1 to trigger keratocytes to produce collagenase and PGE_2 [113, 114]. Corneal UV exposure-mediated inflammation is also associated with the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α by stromal cells [115]. IL-1 is associated with corneal injury in vitamin A-deficiency [116]. Analysis of cytokine expression in primary Sjögren's syndrome has been performed by analysing tissue obtained by impression cytology or direct biopsy [117]. IL-6 expression was significantly elevated in Sjögren versus non-dry eye patients whereas IL-1 and IL-8 expression were detectable at a similar frequency in both groups.

Cytokine analysis of biopsies obtained from various patient groups with chronic allergic eye disease has revealed that vernal conjunctivitis and giant papillary conjunctivitis patients express a Th2 array of cytokines whereas atopic keratitis is associated with a shift towards a Th1 cytokine profile [58].

Cytokines also play an important role in corneal transplantation, by influencing the immune and inflammatory response towards acceptance or rejection of corneal allografts. Earlier, we showed that corneal graft rejection in a rat model of allotransplantation [54] is mainly associated with the expression of the Th1 cytokines IL-2 and IFN-γ. Surgical trauma due to the corneal transplantation induced transient expression of IL-1, IL-6, IL-10, and the chemokines MCP-1 and MIP-2. Dana *et al.* [52] have demonstrated the importance of IL-1 in corneal allograft rejection via its effect on Langerhans cell migration. Besides the immune reaction locally present to corneal allografts, a systemic cytokine immunoreactivity may be found in the host. Serum levels of IL-2 receptor [118] and TNF-α [119] were shown to be raised during corneal allograft rejection.

From the above described effects of cytokines, IL-1 is the most multifunctional cytokine acting in the cornea (Table 3). IL-1 initiates the inflammatory cascade and the immunological response [113] and, eventually, induces corneal tissue damage. Although IL-1 is a key player in the inflammatory response in the cornea, it also contributes to the tissue repair process [120].

TABLE 3 – **PRIMARY AND SECONDARY CORNEAL EFFECTS OF IL-1**

Angiogenesis
Langerhans cell migration
Collagenase and metalloproteinase expression
Injury in vitamin A-deficiency
Scarring in herpetic stromal keratitis
Allograft rejection

FUTURE PROSPECTS

It is commonly accepted that cytokines can be directly involved in the pathogenesis of several corneal diseases. The key for corneal inflammation is located in the keratocyte. This cell is able, upon several stimuli, to produce various cytokines involved in proteolytic processes, activation of resident and immunocompetent cells (auto and paracrine effects) in order to produce other cytokines and stimulate an immune response (Fig. 3). Targeting one or more cytokines, for example with monoclonal antibodies, or administering new cytokines, may show beneficial results in abrogating corneal disease or in increasing corneal graft acceptance. However, this type of treatment may influence aberrant expression of certain cytokine-mediated responses. The cytokine network is extremely complex due to the fact that one single cytokine may have many functional targets, which are also shared by other cytokines. Moreover, cytokines can have synergistic and / or antagonist effects on other cytokines. These general properties indicate that targeting individual cytokines may not always be successful. IL-10 administration alone, via various routes, did not prolong corneal graft survival in a rat model of allotransplantation [121]. Animals that were injected subconjunctivally with the highest dose of IL-10 even showed a trend towards earlier rejection when compared to controls [121]. However, it is known that cytokines can show paradoxical effects [122], suggesting that further investigation is required in order to determine a role for IL-10 in corneal acceptance. Nevertheless, it was reported that treatment with anti-IFN-γ or anti-IL-2 [107], and plasmid DNA encoding IL-10 [123] significantly reduced the incidence and the severity of herpes simplex virus-1-induced corneal inflammation.

Corticosteroids, cyclosporine A and tacrolimus (FK 506) are the most potent inhibitors of inflammatory cytokine production available for the clinical practice. Besides the fact that these drugs are not always efficient enough to control inflammation, they are often associated with serious ocular and systemic side effects such as cataract formation, glaucoma, nephrotoxicity, hepatotoxicity and hypertension [124, 125], which limits their prolonged use. Therefore, other therapeutical strategies are now being conceived to act more specifically on determined individual

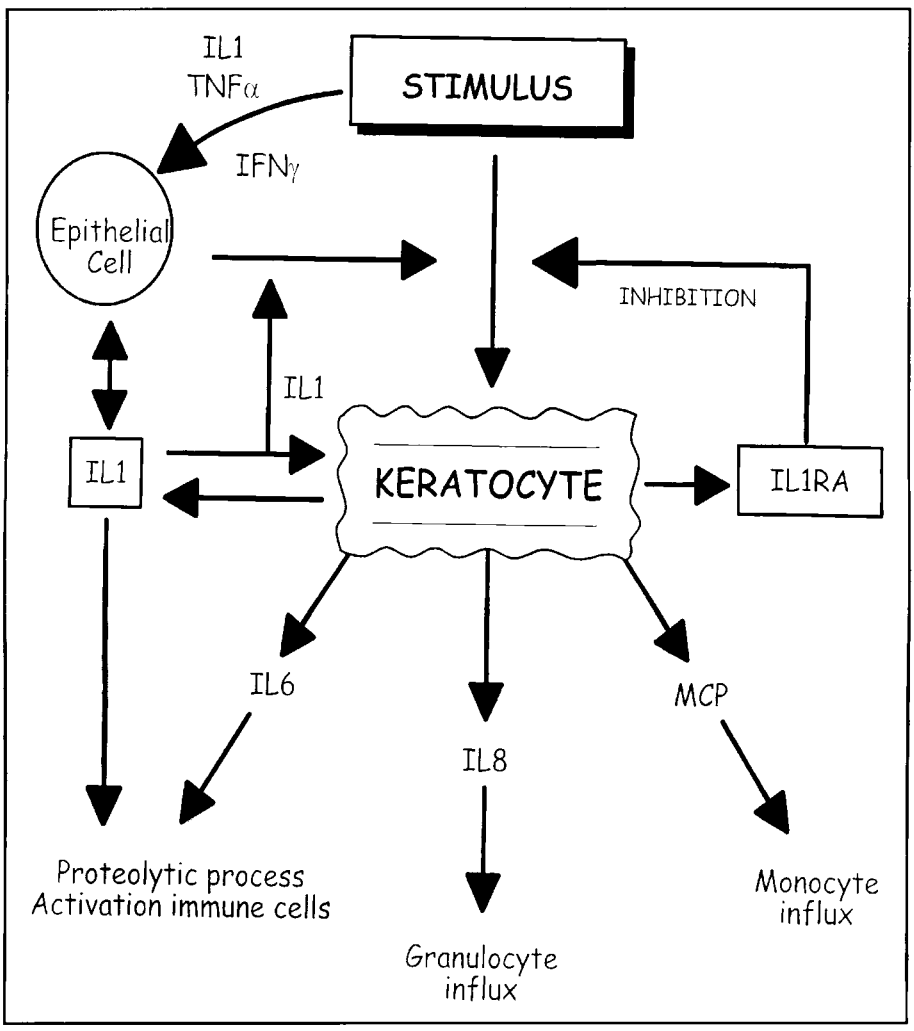

Figure 3 — The role of keratocyte in corneal inflammation.

members of the cytokine network. Nowadays, it is already possible to make peptides or recombinant proteins in large quantities, which allows the use of naturally occurring regulatory proteins such as the IL-1 receptor antagonist, anti-inflammatory cytokines (IL-4, IL-10, IL-13), anti-viral interferons, soluble cytokine receptors and growth factors. They will probably contribute to a better treatment of various inflammatory eye diseases such as keratitis, scleritis, dry eye disease, persistent corneal epithelial defects and corneal allograft rejection. Concerning corneal transplantation in animal models, there are several successful therapeutical methods to enhance allograft acceptance, including the use of slow releasing drug devices [126], the administration of monoclonal antibodies against adhesion molecules [127, 128, 129] and CD4+ T-cells [130, 131], liposomes containing drugs that

deplete macrophages [132], gene transfer with or without promoters [133, 134] and oral immunization [135, 136].

Transferring the acquired knowledge learned from animal experiments into the prospect of treating human corneal immune-mediated diseases and promoting allograft survival, we can speculate that, in the near future, oral antigen-immunization or gene therapy will challenge the current therapies. Oral immunization or oral administration of antigens is an effective method for downregulating the immune response of the host to several antigens, including the alloantigens. Orally induced corneal graft enhancement is alloantigen specific and can be induced after providing the host with corneal or epidermal cells extracted from the donor. Oral immunization may be helpful in diminishing corneal rejection due to its capacity of downregulating DTH, cytotoxic T cells and mixed lymphocyte responses to specific alloantigens in the animal model [137]. Theoretically, such methods appear simple and harmless but several practical questions should be raised concerning the absorption of the cell inoculum, the amount of cells necessary to produce clinical effects and the number of required doses before surgery. Donor corneas can be maintained in standard culture conditions for periods up to 1 month, which is rather short to prepare cell cultures and feed the patient. If these difficulties can be overcome then, a giant step has been taken towards corneal graft acceptance in high-risk patients.

Gene therapy, i.e. genetic manipulation by introducing novel genes into the donor cornea prior to transplantation in order to induce corneal allograft acceptance is another exciting challenge for the near future. In animal models, this technique proved to be efficient in modulating the immune response to alloantigens and ameliorating the endothelial function [138]. Several methods to deliver the genes into a target cell have been used such as liposomes [134, 139], molecular conjugates and recombinant viruses [138]. Viral vectors mainly from adenovirus are so far the most used in animal models. Although these viruses do not replicate, viral proteins can be immunogenic and subsequently inflammation may occur. However, due to the immune privilege of the cornea and the anterior chamber of the eye, these viral particles might be associated with a diminished recognition by the immunocompetent cells. Adenovirus-gene transfer into the corneal tissues does not induce long-term gene expression, since the virus is not incorporated into the host cell genome, but only remains episomal [138]. Gene therapy could also be used to induce ex-vivo short-term endothelial cell replication in the donor cornea to counteract the loss of endothelial cells due to the managing of the cornea during surgery [140], which is the second cause of corneal allograft failure [141]. Gene therapy techniques also allow the possibility to include inducible promoters that can be turned on or off when desired. Based on the properties of immunosuppressive cytokines such as IL-4, IL-10 and IL-13, the Th2 cytokines are perfect candidates for such therapy. Other strategies include regulation of cytokine production by interfering with the promotor genes, using «antisense oligo nucleotides» to block translation of RNA and inhibiting enzymes (metalloproteinases) involved in secretion of certain cytokines.

Gene manipulation introduces a number of ethical problems that should be solved. Therefore precise protocols should be elaborated to prevent gene manipulation without therapeutical purposes. In addition, further investigations are now required to get a better understanding of these potential therapeutical tools concerning their application in humans.

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Chapter 3

Expression of the interleukin-1 receptor antagonist in the normal human cornea

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SUMMARY

The human cornea has been shown to express a number of inflammatory cytokines including IL-1, IL-6 and IL-8. In view of the potent proinflammatory activities of interleukin-1 (IL-1), regulatory mechanisms should be present in the human cornea to control IL-1 mediated inflammatory and immune responses. This is important for the maintenance of the integrity and transparency of the cornea. To test this hypothesis, the authors determined the presence of IL-1 receptor antagonist (IL-1RA) in the normal human cornea using an enzyme-linked immunosorbent assay (ELISA). IL-1RA is a natural antagonist of IL-1 and competes with IL-1 for the binding to its receptors thereby blocking the inflammatory response. Corneas were either tested immediately or after a 24-hour culture period. Furthermore, the authors separately analyzed the three layers of the cornea. Their results present evidence for the constitutive expression of the IL-1RA protein in the normal human cornea and show that both epithelial and stromal cells produce IL-1RA. The epithelial cells are the major source of corneal IL-1RA immunoreactivity, and secrete IL-1RA during culture. Stromal cells contain detectable, albeit low amounts of cell associated IL-1RA. No IL-1RA was detected in the endothelial cell layer. A more accurate understanding of the balance between IL-1 and IL-1RA in ocular tissues and the role of the IL-1RA under physiologic and pathophysiologic conditions will be necessary for an eventual use of IL-1 receptor antagonist as a therapeutical tool.

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INTRODUCTION

To maintain its integrity and transparency, the cornea should be endowed with mechanisms that regulate both immune and inflammatory responses. Vascularization, fibrosis and scarring are normal features of severe inflammation elsewhere in the body, but will lead to decreased visual acuity and even to blindness when they occur in the cornea [1].

A group of multifunctional proteins, the cytokines, including interleukin 1 (IL-1), tumor necrosis factor (TNF) alpha, IL-6 and IL-8, have been implicated as important mediators of the inflammatory responses [2]. IL-1, one of the mediators first to appear during inflammation, is produced and secreted by a variety of different cell types. Interleukin-1 refers to two related proteins, IL-1 alpha and IL-1 beta [3]. Both forms are produced by different genes but bind to the same receptors and induce similar biologic responses. IL-1 initiates various inflammatory responses and induces the synthesis of other cytokines, leading to a complex cascade of events in the involved tissue or organ. It has also been implicated as a mediator of tissue destruction in many human diseases and may contribute to maintenance of chronic inflammation [3, 4, 5].

All mayor cell types of the human cornea have been reported to express IL-1 [6, 7, 8]. Furthermore, it has been shown that the exogenous addition of IL-1 can stimulate the synthesis of IL-6 [9] and IL-8 [10, 11] by human corneal epithelial, stromal and endothelial cells. The observation that cytokines such as IL-1 and IL-8 can induce neovascularization [12, 13], when applied within the corneal stroma, underlines the importance of these mediators.

In view of the potent proinflammatory activities of IL-1, regulatory mechanisms should be present to control IL-1 initiated corneal inflammatory sequelae. To test this hypothesis we determined the presence of IL-1 receptor antagonist (IL-1RA) in the normal human cornea. IL-1RA is a natural antagonist of IL-1, that shares aminoacid sequence homology to both IL-1 alpha and beta [14] and competes with IL-1 for the binding to type I and type II IL-1 receptors [15, 16].

We report here that both epithelial and stromal cells of normal human corneas contain IL-1RA protein. Only epithelial cells, however, secrete a soluble form of IL-1RA (sIL-1RA) during tissue culture.

MATERIAL AND METHODS

Human Corneal Tissue

Human corneas were obtained from the Eye Bank of The Netherlands Ophthalmic Research Institute (NORI, Amsterdam). The corneas were of good quality but were excluded from clinical use since they did not satisfy the inclusion criteria for transplantation. Donors age varied from 60 to 79 years (mean age: 73). Corneas were placed in Dexsol (Chiron, Irvine, USA) at 4°C and used within 72 hours of enucleation. The corneoscleral rims

were removed using a 10-mm trephine. In total, 12 corneas were used. Initially, five human corneas were used in pilot experiments to test the proper IL-1RA ELISA conditions. Subsequently, seven corneas were used for the quantification of IL-1RA; four were used to prepare total corneal extracts and three corneas were tested to analyse separate corneal layers.

Preparation of Whole Corneas

Corneal buttons were washed in fresh serum-free Basal Medium Eagle (BME) (Gibco BRL, Life Technologies, UK). Each button was then divided into two equal portions and weighed separately. One half was tested immediately and the other part was incubated in 450 µl BME containing 100 IU of penicillin and 100 µg of streptomycin for 24 hours at 37°C in a humidified environment with 5 % of CO_2. After the incubation, the supernatant was harvested. The corneal specimens were then washed in phosphate buffered saline (PBS) containing 0.5 M NaCl and placed in 450 µl of this buffer containing 0.1 % polysorbatum 20 (tween), for one hour at room temperature. Both cultured and uncultured corneal pieces were cut into approximately 2 mm^3 portions and placed in 450 µl PBS and lysates were obtained by four cycles of freezing (– 70°C) and thawing. All samples were stored at – 20°C until use. The protein concentration in supernatants and lysates was measured using the Bradford [17] method with human serum albumin as standard.

Preparation of Corneal Cells

Three corneas were separated into the three major layers. First the endothelial cell layer was peeled off with a fine corneal forceps. Then, the epithelial cell layer was separated with a solution of 16.8 mM ethylene-dinitrilotetraacetic acid tetrasodium salt (tetrahydrate) (EDTA) (Merck, Germany) in Puck's saline A (Gibco BRL). After one hour incubation in a humidified environment at 37°C, the epithelial cell layer was lifted from the stroma with a pair of fine corneal forceps. All three cell layers were placed separately in 450 µl BME. After 24 hours incubation at 37 °C, the supernatants were harvested and stored at – 20°C until use. After culture the stromas, now lacking both the epithelial and endothelial cell layers, were cut into approximately 2 mm^3 pieces and were subjected to the freeze and thaw procedures described above.

IL-1 Receptor Antagonist Assay

Immunoreactive IL-1RA was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R & D Systems, Minneapolis, USA). The

sensitivity of the assay was 6.5 pg/ml. The specificity is high as the assay recognises only IL-1RA and not IL-1α or IL-1β. The assay is based upon a quantitative enzyme immunoassay technique and employs a monoclonal antibody specific for IL-1RA to coat microtiter wells. After incubating the wells with recombinant IL-1RA standard (0 - 2000 pg/ml) or corneal samples, the bound IL-1RA was detected with an enzyme-linked polyclonal anti--IL-1RA antibody. After the addition of substrate the results were measured with an ELISA reader at 450 nm. The concentration of IL-1RA in the corneal samples was determined by comparing their readings with those of the standard curve. Samples with a high concentration of IL-1RA were diluted several fold (1/20, 1/40, 1/80) and data were calculated from those dilutions which gave readings in the linear part of the standard curve. Negative controls included culture medium (BME) and PBS alone. IL-1RA was expressed as nanograms per mg of total protein present in the tested samples.

RESULTS

To assess whether the normal human cornea expresses the IL-1RA protein, whole corneas were used to obtain both culture supernatants and cell lysates. After 24 hours incubation in BME, the amount of corneal IL-1RA protein detected in the culture supernatant by the IL-1RA ELISA, averaged 148 ng per mg of total protein (Table 1). The protein tested in culture supernatants represents the released IL-1RA *i.e.*, the soluble form of IL-1RA (sIL-1RA). To detect intracellular IL-1RA, whole corneas were tested after repeated freezing and thawing cycles. The amount of cell associated IL-1RA protein detected in uncultured corneas was 81 ng per mg of total protein and after 24 hours culture it was 152 ng per mg of protein.

TABLE 1

CULTURE PERIOD	ng CORNEAL IL-1RA / mg TOTAL PROTEIN	
	RELEASED	CELL ASSOCIATED
0 hrs	0 *	81 ± 32
24 hrs	148 ± 35	152 ± 89

Interleukin-1 receptor antagonist protein in supernatants and lysates of normal human corneas. Data represent the mean ± standard deviation of the mean of four corneas.

* medium alone did not contain detectable IL-1RA.

To determine which cell layer was responsible for the production of the corneal IL-1RA, the three major cell layers were separated and cultured in BME. After 24 hours incubation, only the epithelial cell layer

TABLE 2

ng CORNEAL IL-1RA / mg TOTAL PROTEIN	
Epithelium	187 ± 21
Stroma	0.2 ± 0.09
Endothelium	ND *

Interleukin-1 receptor antagonist protein in supernatants after separate culture of the three layers of the normal human cornea. Separate layers of the cornea were cultured for 24 hours at 37°C. Data represent the mean ± standard deviation of the mean of three corneas.

* ND: not detectable.

showed a significant release of IL-1RA. The amount of the IL-1RA protein produced by the epithelium averaged 187 ng per mg of total protein (Table 2). Low levels were detected in the medium after culture of the stroma (mean: 0.232 ng per mg of total protein). No IL-1RA immunoreactivity was found in the supernatant of the endothelial cell layer. To investigate whether the stroma contained cell associated IL-1RA after 24 hours culture, it was subjected to cycles of freezing and thawing. This revealed a low level of IL-1RA protein (mean: 15 ng per mg of total protein).

DISCUSSION

This paper shows the expression of the IL-1RA protein in the normal human cornea. To our knowledge, the production of IL-1RA within the normal human cornea has not yet been published. For the human eye, the presence of IL-1RA was reported earlier within cultured retinal pigment epithelial cells [18]. Our study shows that corneal epithelial and stromal cells express the IL-1RA protein. The epithelial cell layer is the major source of IL-1RA immunoreactivity in the cornea. No IL-1RA was detected in the endothelial cell layer and only low levels were present in the stroma.

IL-1RA represents the first known naturally occurring protein that functions as a specific receptor antagonist of any cytokine or hormone-like molecule [19]. IL-1RA is a member of the IL-1 family of cytokines [14, 20] and is a polypeptide that specifically blocks IL-1 activity. It was recently identified and cloned [21, 22, 23]. IL-1RA shares amino-acid sequence homology with both IL-1 alpha and IL-1 beta and the genes for all three cytokines are located on the human chromosome 2q14 [24]. IL-1RA binds to both type I and II IL-1 receptors and specifically inhibits binding of both IL-1 alpha and beta without stimulating target cells [15, 16] (Fig. 1). There are two known forms of IL-1RA: the secreted and intracellular IL-1RA. Both forms are derived from the same gene but are different due to the use of alternative first exons and RNA splicing [23]. Both forms possess an identical specific activity; glycosylation appears to be a feature of secretion [23].

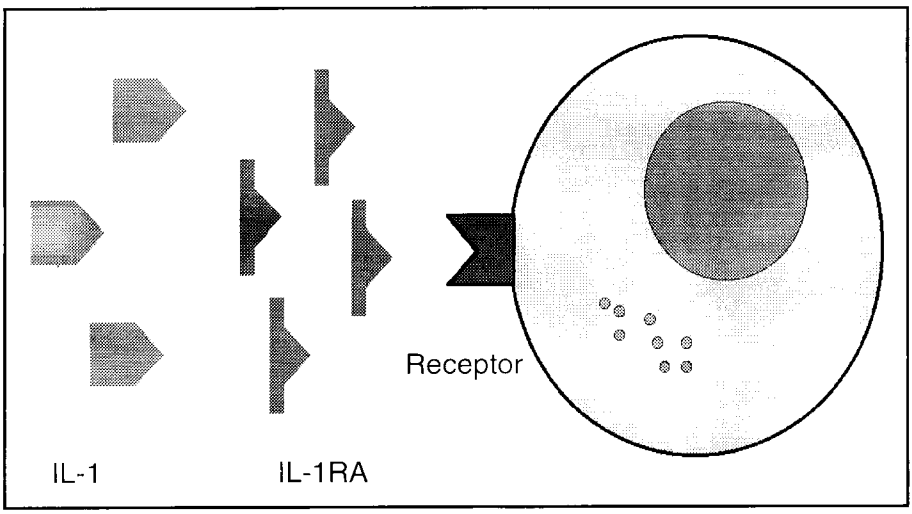

Figure 1 – The interleukin-1 receptor antagonist (IL-1RA) competitively inhibits the binding of interleukin-1 (IL-1) to the IL-1 receptors.

Although the IL-1RA ELISA assay used in our study does not discriminate between secreted IL-1RA and intracellular IL-1RA, the methodology we used does allow the conclusion that the epithelium produced a secreted form while the stromal cells may produce the intracellular form of the corneal IL-1RA. Further evidence concerning the presence of intracellular IL-1RA or secreted IL-1RA in the various corneal layers requires mRNA analysis. Whether «*de novo*» synthesis of IL-1RA protein occurs in the human cornea may not be concluded from the experiments described in this report, but the fact that we recently observed mRNA expression for IL-1RA in the cornea of normal rats, strongly points to a local constitutive synthesis of this regulatory cytokine within the cornea [25].

IL-1 is a proinflammatory cytokine primarily produced by mononuclear phagocytes but also by a number of other cell types [3] including corneal cells [6, 7, 8]. IL-1 produces a wide variety of effects on differentiation and function of cells involved in the inflammatory and immune response. The release of other cytokines is, in many instances, IL-1 related. Within the human cornea, IL-1 can induce the synthesis of IL-6 [9] and IL-8 [10, 11]. IL-1 and IL-8 are known to be mediators of corneal neovascularization in animal models [12, 13]. The corneal cells can produce IL-1 receptor mRNA [26], indicating that endogenous IL-1 may have autocrine functions in these cells. The human cornea can thus actively participate and propagate the inflammatory and immune response within its layers.

Although IL-1 is important to initiate the host response to injury and infection, increased and prolonged production of this cytokine is implicated in several diseases [3, 4, 5]. Obviously, the maintenance of IL-1 related inflammatory and immune response within the cornea can cause deleterious effects to this tissue with consequent decrease in visual acuity. Because IL-1 is one of the early appearing mediators it is interesting to speculate

that IL-1RA constitutively expressed in the corneal cells may contribute to an inherent corneal «defence» mechanism for IL-1 mediated responses. The high concentrations of IL-1RA present in the epithelium may have a protective function as these cells are continuously exposed to inflammatory and environmental stimuli [23] and may reflect the natural antagonism of the IL-1 produced in these cells [6, 23].

IL-1RA modulates IL-1 mediated inflammatory effects both in vitro and in vivo [19, 27]. Several studies performed in experimental animal models have shown that IL-1RA can reduce the severity of diseases [28, 29, 30]. In the eye, a reduction of the ocular inflammation after intravitreal injection of IL-1RA in a rabbit model of uveitis has been reported [31]. The data presented in this paper indicate that the level of IL-1RA in the corneal epithelium may be in the range of approximately 200 nanograms per milligram of soluble corneal protein. As yet quantitative data are not available for the corneal expression of IL-1 protein. In vitro experiments have shown that up to 1000-fold greater amounts of IL-1RA are needed to counteract IL-1 activity [27].

A more accurate understanding of the balance between IL-1 and IL-1RA in tissues and the role of the IL-1RA under physiologic and pathophysiologic conditions will be necessary for an eventual use of IL-1RA as a therapeutical tool.

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Chapter 4

Cytokine mRNA expression during experimental corneal allograft rejection

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SUMMARY

Allograft rejection is the main cause of corneal graft failure. T lymphocytes and macrophages have been implied to be involved in corneal rejection, but little is known about the molecular mechanisms in this process. In this study, cytokine mRNA expression in the cornea was analyzed during experimental corneal transplantation. The donor and acceptor corneas of two groups of rats were studied after receiving an allo- (PVG to AO rat) or autograft (AO rat). For controls, central buttons and peripheral corneal rings of non-transplanted contralateral eyes were used. At different post-operative days (1, 3, 7, 12 and 19), the corneas were removed and subjected to mRNA isolation. All corneal samples underwent semi-quantitative reverse transcriptase-polymerase chain reaction analysis for interleukin-1 beta, interleukin-1 receptor antagonist, interleukin-2, interleukin-4, interleukin-6, interleukin-10, tumor necrosis factor-alpha, interferon-gamma, monocyte chemotactic protein-1 and macrophage inflammatory protein-2 mRNA expression. Corneal rejection, characterized by opaque corneas with prominent neovascularization, was always diagnosed around day 12. Contralateral non-grafted corneas showed constitutive mRNA expression for interleukin-1 receptor antagonist and in a few samples also monocyte chemotactic protein-1 and macrophage inflammatory protein-2 mRNA was found. Both allo- and autografts expressed mRNA for the cytokines found in contralateral non-grafted tissue, as well as

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for interleukin-1 beta, interleukin-6, interleukin-10 and tumor necrosis factor-alpha. In allografts, the mRNA levels for these cytokines remained constant throughout all post-operative days, with increased interleukin-6 mRNA expression after post-operative day 12. The analysis of the autografts revealed high cytokine mRNA levels until post-operative day 3 or 7, which decreased from then on, except for interleukin-1 receptor antagonist. mRNA for interleukin-2, interleukin-4 and interferon--gamma was not observed in autografts at any time point and in allografts, until post-operative day 12. Interleukin-2 and interferon-gamma mRNA showed maximal expression on POD 12 in allografts. IL-10 mRNA levels decreased immediately after POD 1 in autografted eyes. For TNF-α, an increased mRNA expression starting on POD 7 was found in recipient rings of allografted eyes, while in autografts a weak expression was seen in some samples. MIP-2 transcription increased on POD 12, while in autografts, its expression was not markedly different from that detected in the contralateral non-grafted peripheral cornea.

INTRODUCTION

Corneal transplantation is one of the most successful grafting procedures performed worldwide in man. The pre-operatory corneal state however, such as vascularization of the acceptor cornea and corneal disease that necessitated surgery, may influence the prognosis of the graft. Literature reports success rates up to 90 % for first grafts placed in non-vascularized corneas [1, 2], while high failure rates are expected for repeated transplants or when grafting is performed in vascularized corneas [2, 3]. The success of keratoplasty, compared to other tissue transplantations in the body, may be due to the avascularity of the grafted corneal tissues, the easy topical application of immunosuppressive treatment and the immuno-privileged microenviroment of the anterior segment of the eye [4, 5]. Structures of the anterior chamber are able to down-regulate antigen--presentation and lymphocyte activation, contributing to the development of an anterior chamber associated immune deviation (ACAID) [6]. Corneal allograft rejection is however, still the main cause of graft failure [7, 8].

Allograft rejection is histologically characterized by massive infiltration of T-cells and macrophages. T-cells are the predominant cell-population, whereby CD4+ cells have an important role in the response to allogeneic corneal cells [9, 10, 11], leading to a delayed type hypersensitivity response. Little is known about the molecular mechanisms governing the interactions between the immunocompetent cells involved in allograft rejection. A number of

studies concerning allograft rejection of other organs [12, 13, 14, 15, 16, 17, 18] implicated cytokines in the pathogenesis and maintenance of graft inflammation, tissue destruction and rejection. Cytokines are low molecular weight polypeptides that coordinate cellular responses. They are produced and secreted by various cell types and act via cell-surface receptors in an auto, para or endocrine manner [19, 20]. Important functions of cytokines are the regulation of inflammatory and immune responses of organs and tissues. They may induce proliferation, differentiation, secretion or migration, depending on their target cell. Allograft reactions in heart and kidney [14, 15] were associated with upregulated mRNA expression of acute proinflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6, and expression of T-lymphocyte-derived cytokines such as IL-2, IL-4 and interferon-gamma (IFN-γ). Activated, infiltrating cells and resident cells in the graft were found to produce and secrete several cytokines [16]. The identification of intragraft cytokine patterns may be important, as it is known that cytokines such as IL-2 and IFN-γ induce cellular immunity mediated by T-cells of the Th1 class, whereas IL-4 and IL-10, are involved in the Th2 mediated immune response. The balance between released cytokines may determine the outcome of the immune mechanism towards graft acceptance or graft rejection.

In order to assess the involvement of different cytokines in experimental corneal allograft rejection in the rat, we determined the temporal transcription patterns of IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) in the cornea by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We report here that corneal transplantation induced mRNA expression of the described cytokines. The most remarkable finding was that mRNA for IL-2, IL-4 and IFN-γ was expressed only when corneal rejection was clinically evident. This strongly suggests that T-cell mediated responses are involved in corneal rejection.

MATERIALS AND METHODS

Experimental animals

Inbred male AO and PVG rats (Harlan Sprague Dawley, Indianapolis, IN, USA), weighing 200-250 g (10-12 weeks old) were used in the corneal allograft rejection experiments. Animals were treated according to the ARVO Resolution on the use of animals in research. The two strains of rats differ at the Major Histocompability Complex (AO rat: RT1u, RT6b, RT7a; PVG rat: RT1c, RT2b, RT3a, RT6a, RT7a) and corneal allograft transplantation from PVG to AO rats (described below) had a very protracted rejection time.

Corneal surgery

Recipients rats were anesthetized by intramuscular injection of 0.5 ml kg^{-1} of fluanison and phentanylcitrate (Hypnorm; Janssen Pharmaceutica, Tilburg, The Netherlands) and intraperitoneal (IP) injection of 0.15 ml kg^{-1} diazepam solution (5 mg ml^{-1}). Topical anesthesia was implemented with a single drop of oxybuprocaine hydrochloride (0.2 %) administered just before surgery. Maximal midriasis was achieved by a subcutaneous injection of 0.25 ml kg^{-1} atropine sulphate (0.5 mg ml^{-1}). Donors for corneal allograft transplantation were killed by intracardial injection of 0.2 ml of sodium pentobarbital (200 mg ml^{-1}; Nembutal; Algin, Maassluis, The Netherlands). Corneas from donors and recipients were both trephanized with a 3-mm trepane. Donor corneas were kept in corneal preservation medium at room temperature until use, for a maximum of 1 hr. Surgery was performed under an operating microscope and in all cases only one eye (OD) was operated. Buttons were adjusted into the acceptor cornea and sutured using a 10/0 monofilament nylon (2 stitches per quadrant), according to the technique previously described [21]. The eye was continuously irrigated with PBS and touching of the iris and lens was avoided; viscoelastic substances were not used in spite of the flatness of the anterior chamber, nor was any effort made to refill the anterior chamber. Immediately after surgery, one or two drops of chloramphenicol without steroids were given.

Experimental design

A total of 36 orthotopic corneal transplants were performed in AO recipient rats. PVG rats were used as corneal donors for allogeneic grafting (PVG > AO) (19 transplants). Of the 15 rats transplanted initially, four rats had to be excluded for post-surgical problems and were replaced. As a control for cytokine release due to surgical trauma only, autotransplantation (AO > AO) was used, the trepanized 3-mm corneal button was rotated 180° and sewn back into the corneal rim (17 rats). In this group two rats were excluded for post-surgical problems and replaced. Transplanted animals were evaluated clinically, under general anesthesia, by slitlamp and operating microscope examination on the first post-operative day (POD) and then every three days. Six animals were excluded from the study because of technical failures such as extensive synechias to the graft (n = 4) and large hyphemas (n = 2). Edema of the graft, opacity of the graft and neovascularization of the graft and the recipient cornea were each graded from 0 to 4 as previously described [22]. Complete corneal rejection was diagnosed by severe opacity and ingrowth of vessels in the graft [23].

At different PODs *i.e.*: 1, 3, 7, 12 and 19 days after penetrating keratoplasty, corneas were obtained from three rats at each time point. During anesthesia by IP injection of pentobarbital, heparin was administered intravenously and the animals were perfused with 200 ml sterile pyrogen-free saline. Corneas of the transplanted and the contralateral

non-transplanted eye were immediately removed with a fine curved corneal scissors and divided into corneal buttons (3 mm) and corneal rings. All samples were frozen in liquid nitrogen and stored at –70°C until required for RNA isolation and cDNA synthesis.

RNA isolation and cDNA synthesis

Total RNA was isolated separately from all corneal samples by a single-step extraction method [24], using 0.4 ml RNAzol (Cinna Biotecx Laboratories Inc, Houston, Tx, USA). RNA was dissolved in 10 µl of DEPC-treated water; 9 µl of the RNA solution was used for reverse transcription and the remaining 1 µl served as a control for genomic DNA contamination, in the β-actin PCR. cDNA was synthesized in a 20 µl volume containing 25 ng μl^{-1} oligo (dT)12-18 primer, 10 U μl^{-1} Superscript II RNaseH reverse transcriptase, 10 mM DTT (Gibco-BRL, Eggenstein, Germany) and 0.5 mM dNTP's (Pharmacia, Woerden, The Netherlands) according to the manufacturers' instructions. After 1.5 hr incubation at 42°C, the enzymatic reaction was inactivated by heating the mixture at 68°C for 5 min.

PCR primers and internal control probes

The sense and anti-sense PCR primers as well as the internal control probes for rat β-actin, IL-1β, IL-1RA, IL-6, IL-10, TNF-α, IFN-γ, MCP-1 and MIP-2 were described previously [25]. Sequences for IL-2 and IL-4 primers and probes are shown in table I.

TABLE I – **DNA SEQUENCES OF OLIGONUCLEOTIDE PCR PRIMERS AND CONTROL PROBES**

CYTOKINES	PRIMER	SEQUENCE
IL-2	sense	CATGTACAGCATGCAGCTCGCA
	anti-sense	ACCACAGTTGCTGGCTCATCAT
	control	TCCCCATGATGCTCACGTTTAAATTTTACT
IL-4	sense	AGCCCCCACCTTGCTGTCAC
	anti-sense	CTTTCAGTGTTGTGAGCGTGGA
	control	ACGGCAACAAGACACCACGGAGAACGAGA

The sequences of the oligonucleotides are shown in 5' to 3' direction.

Polymerase chain reaction

Semi-quantitative RT-PCR was performed as previously described [25]. The cDNA samples of each individual RNA extraction were first normalized to yield equal amounts of β-actin cDNA. Samples with a low β-actin cDNA concentration were excluded from the study. Subsequently, 5 µl of normalized cDNA from each separate corneal sample was subjected to several PCR cycles with the specific cytokine primers. The PCR reaction mixture (50 µl) contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM sense and anti-sense oligonucleotide primers and 1 U Taq DNA polymerase (Promega, Madison, WI, USA). The number of PCR cycles for the different cytokines was 30 cycles for IL-1β, IL-6, IL-10, TNF-α, IFN-γ, MCP-1 and MIP-2; 35 cycles for IL-2 and 40 cycles for IL-1RA and IL-4. The conditions of each cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, except for IL-2 (61°C), IL-4 (59 °C) and TNF-α (50°C) and elongation for 1 min at 72°C. A titration (1/20, 1/40, 1/80) of positive control cDNA from LPS- or PMA-stimulated rat spleen cells and a negative control, which consisted of all reagents without template, were always included in each PCR.

Analysis of the PCR products

PCR products were analyzed by 2 % agarose gel electrophoresis. Visualization was achieved by ethidium bromide staining. Subsequently, gels were submitted to Southern blotting using Genescreen-plus membranes (NEN-Du Pont's Hertogenbosch, The Netherlands) and the filters were hybridized with specific oligonucleotide probes, complimentary to sequences within the region flanked by the PCR primers. These internal control probes were labeled at the 5' end with (γ-32P)ATP, using T4 polynucleotide Kinase (New England Biolabs, Beverly, MA, USA). Pre-hybridization was carried out in 6 × SSC, 1 % SDS, 5 × Denhardt's and 100 µg ml^{-1} sheared herring sperm DNA for 1 hr at 65°C. Hybridization was carried out overnight with 20 pmol of labeled probe. Hybridized membranes were washed with 2 × SSC, 0.1 % SDS at 5°C below the melting temperature of the used internal oligo and exposed to X-ray film (X-Omat, Kodak, Rochester, NY, USA) for several hours. Semi-quantitative RT-PCR analysis was used to determine mRNA expression of multiple cytokines in corneal buttons and adjacent corneal rings after corneal transplantation in rats. As controls, central buttons and peripheral corneal rings of contralateral, non-transplanted eyes were used. Analysis was performed on corneal samples isolated at different PODs after corneal allo- or autotransplantation. All samples were normalized for β-actin mRNA expression. The RT-PCR method used was able to demonstrate semi-quantitative differences in the amount of cytokine mRNA [25]. Samples were obtained from three rats for each POD, except for day 19 of contralateral, non-grafted central buttons; one sample was excluded due to a low amount of β-actin mRNA.

RESULTS

Clinical features

Corneal grafting induced a mild immediate postoperative inflammation in all cases, evidenced by edema and opacity of the graft and the ingrowth of new vessels in adjacent corneal rings. Corneal edema paralleled corneal opacity on grading for the first PODs of both types of grafts, but rats that received allogeneic transplantation always had higher grades for opacity on the last days, when compared to corneal edema. Autografts and allografts showed mild to moderate edema and opacity (grade 1-2) until POD 3, with the former becoming transparent afterwards (Fig. 1). Increased edema and opacity (grade 3) was detected in allografts around POD 12 with opacity reaching its maximum (grade 4) on POD 19. Ingrowth of new vessels started on POD 1 in both auto and allografts but remained limited to the acceptor cornea in the former (grade 1-2). In allografts, large vessels grew into the graft (grade 3-4) around POD 12. Regression of corneal vascularization was seen in autotransplants only between POD 12 and POD 19. In this model, rejection was diagnosed when the three factors described rated a grade of 3 or more and when vessels reached the graft (Fig. 2). Due to the design of the experiment, 9 rats were killed prior to the onset of rejection. All of the remaining animals started rejecting their corneas, according to the criteria mentioned above, on day 12.

Figure 1 — Accepted corneal autograft.

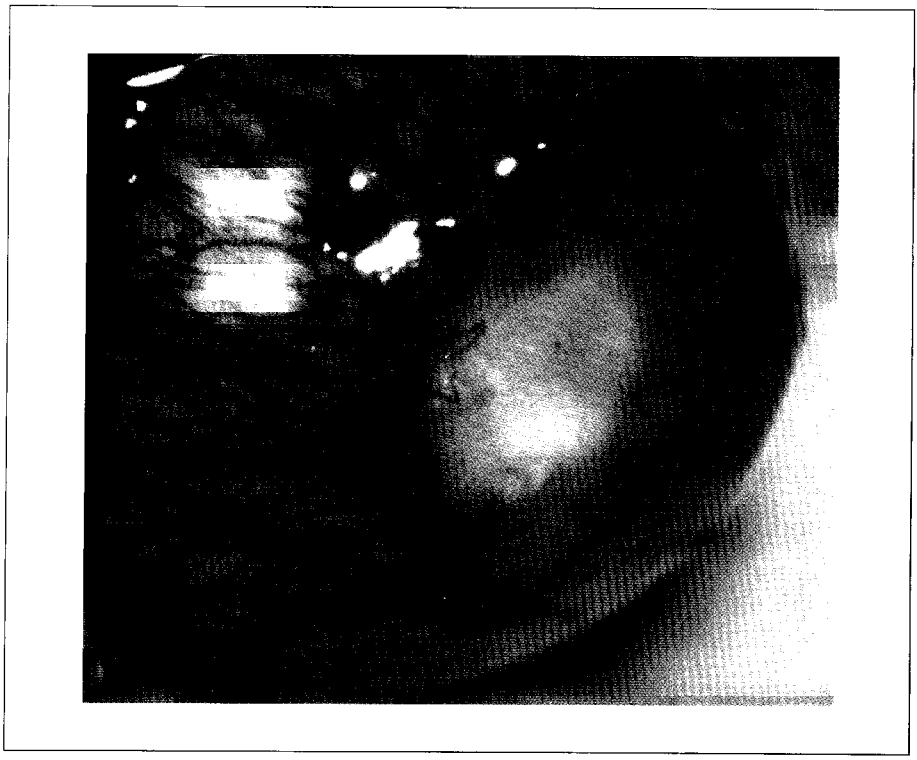
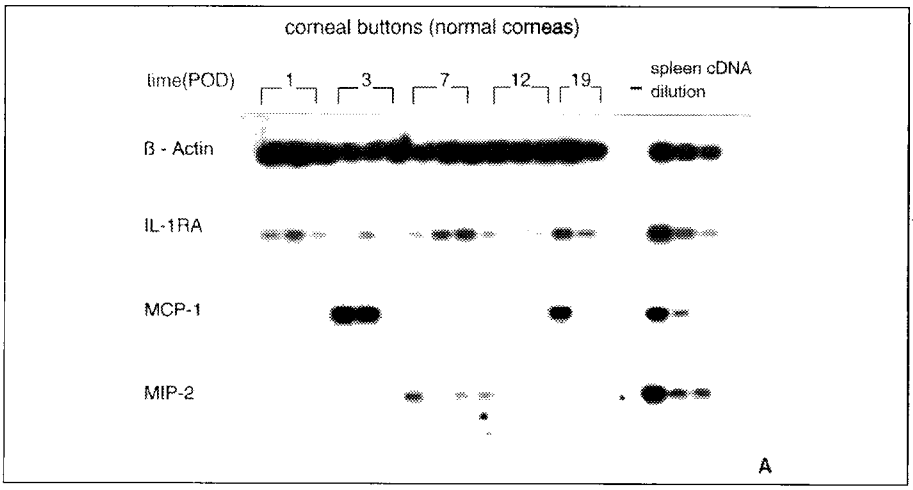
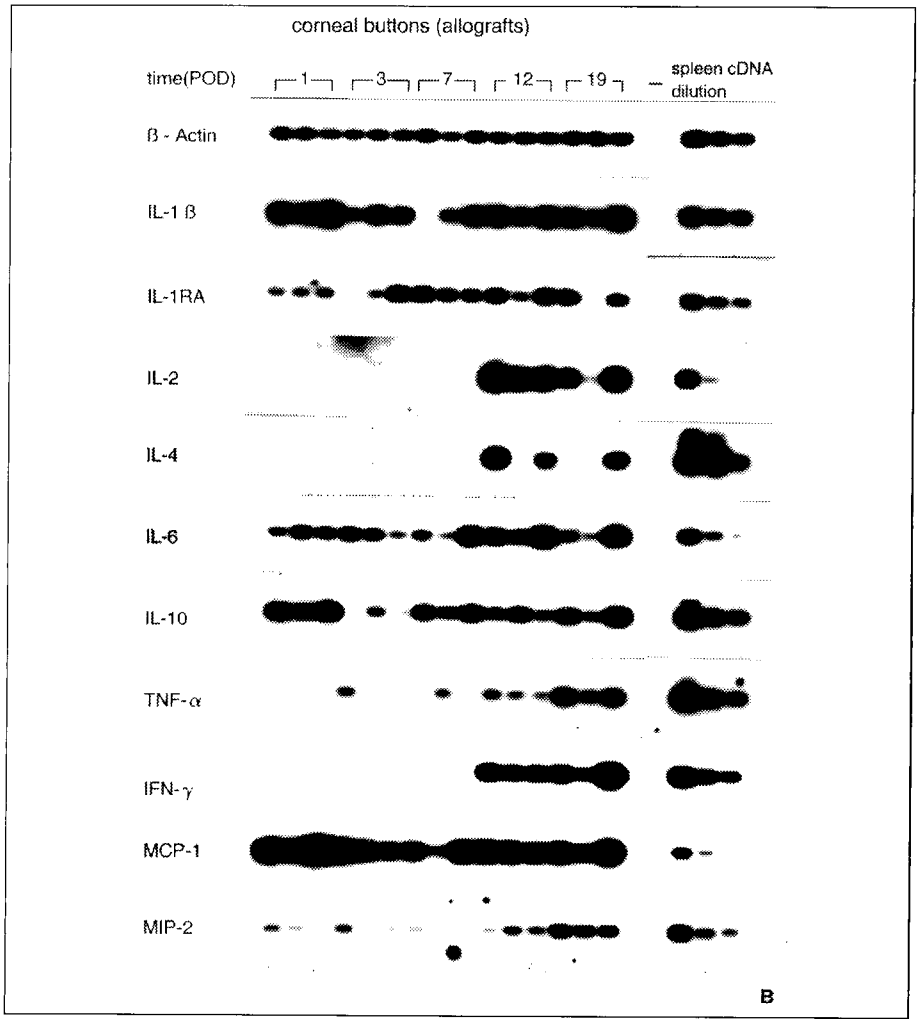


Figure 2 — Rejected corneal allograft. Graft shows opacity grade 3 and ingrowth of new corneal vessels.

Cytokine mRNA expression in central corneal buttons

Forty-four corneal buttons were analyzed; 30 from transplanted eyes (15 allografts and 15 autografts) and 14 samples from contralateral, non-transplanted eyes. In non-grafted corneas (Fig. 3A), mRNA for IL-1RA was detected in all tested samples while mRNA for MCP-1 and MIP-2 was observed only in a few of the corneas. None of the other cytokines tested (IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ) were found in non-grafted corneas. Transplantation induced a different cytokine mRNA expression pattern in allografts (Fig. 3B), as compared to autografts (Fig. 3C and Table II). After allogeneic corneal transplantation, IL-1β mRNA was highly expressed from POD 1 through to POD 19, while after autotransplantation, its expression was high until POD 7 and decreased from then on. IL-1RA mRNA was highly transcribed at all time points both in allo and autografted corneal buttons. mRNA coding for IL-2, IL-4 (in three out of six samples) and IFN-γ were expressed only after POD 12 when rejection was clinically evident. IL-6 mRNA expression increased after POD 12 in allografts, while in autografts it decreased around POD 7. IL-10 mRNA was constantly expressed at all the time points in allografts, while in autografts a decrease after POD 3 was observed. In allografts,


corneal buttons (normal corneas)
time(POD)
1
3
7
12
19
spleen cDNA dilution
ß - Actin
IL-1RA
MCP-1
MIP-2
A


corneal buttons (allografts)
time(POD)
1
3
7
12
19
spleen cDNA dilution
ß - Actin
IL-1 ß
IL-1RA
IL-2
IL-4
IL-6
IL-10
TNF-α
IFN-γ
MCP-1
MIP-2
B

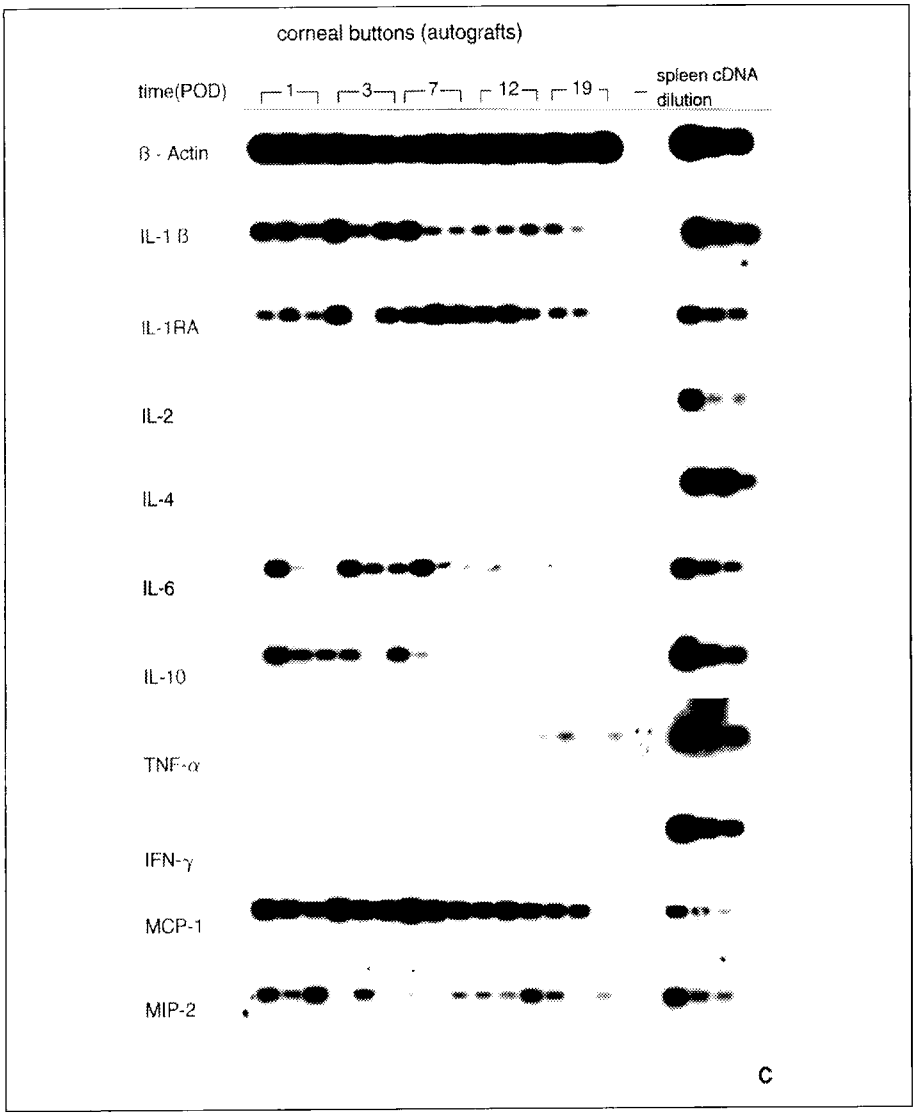
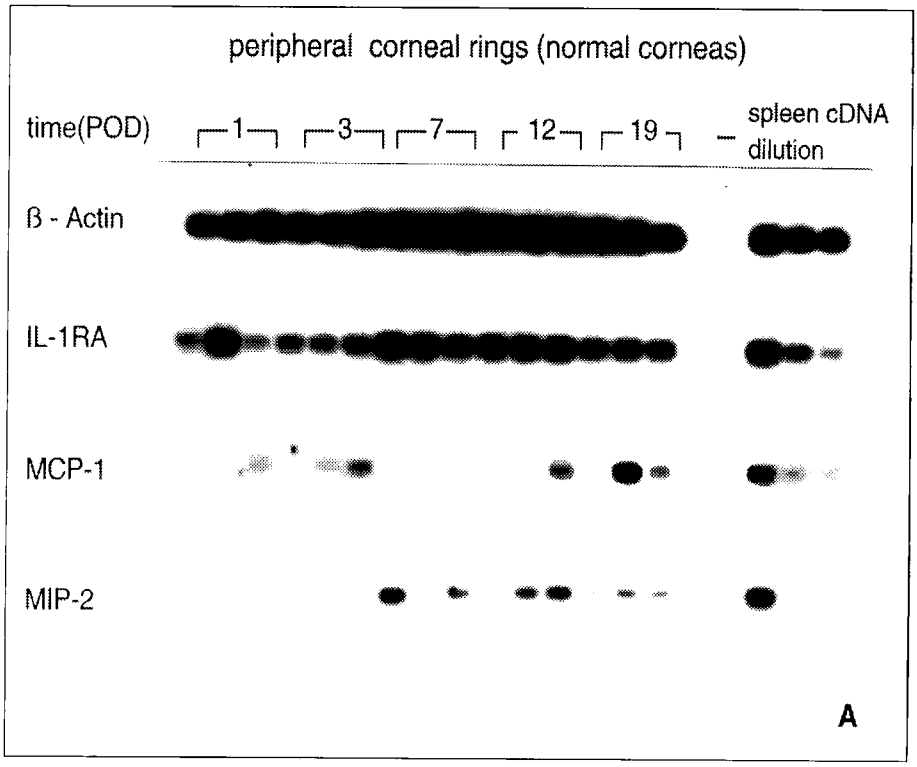


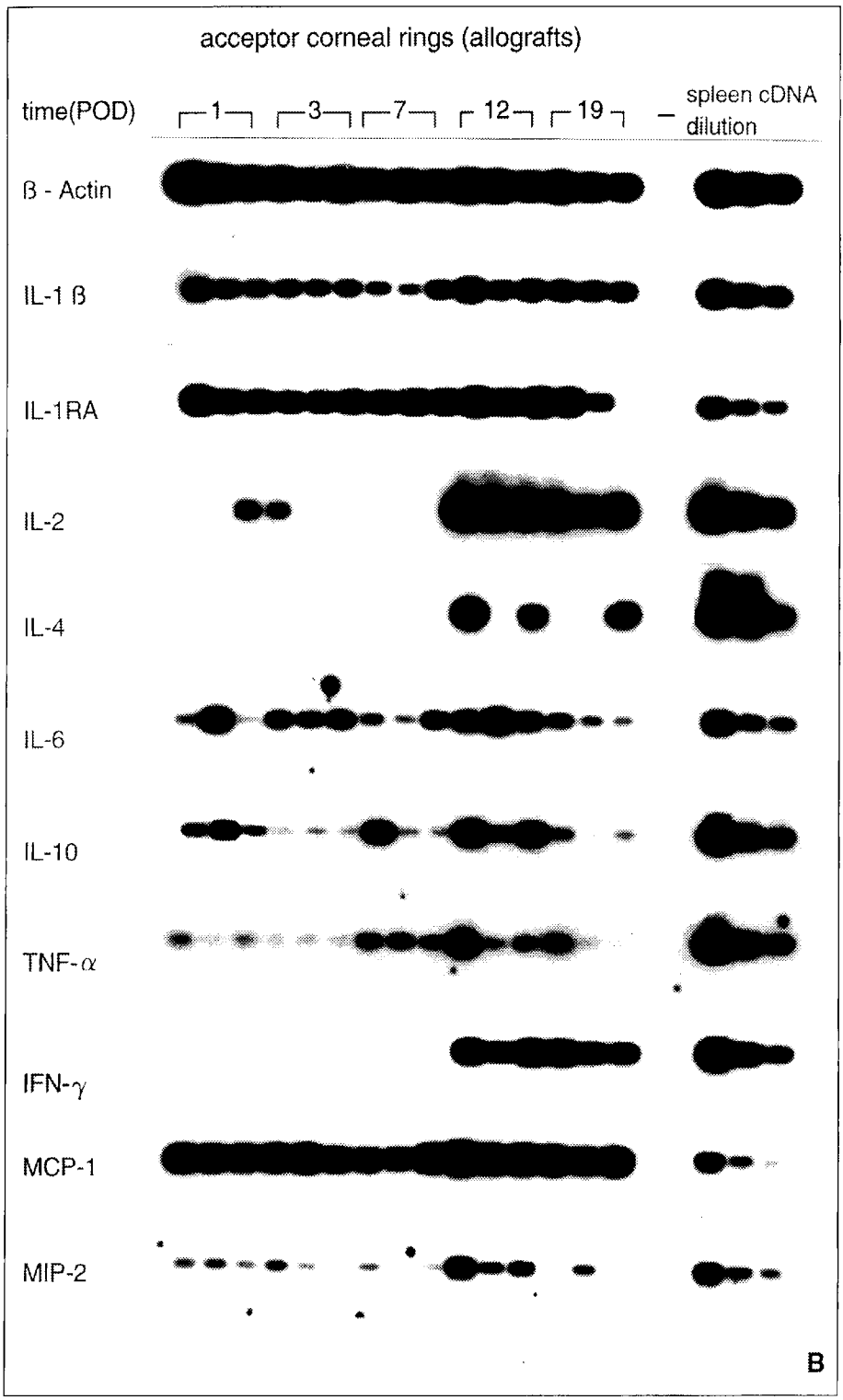
Figure 3 — Cytokine mRNA expression in central corneal buttons. RT-PCR analysis was performed on contralateral, non-grafted corneas (A), allografts (B) and autografts (C), harvested at different points in time (indicated on top) after corneal transplantation. Blots show the results from three individual rats for each POD. In addition, the negative control (–) (all reagents without cDNA template) and positive controls (a 1/20, 1/40, 1/80 dilution of spleen cDNA) are shown.

mRNA for TNF-α and MIP-2 was slightly expressed from POD 1 but an increase of their transcription levels was noticed on POD 19. In autografts, a weak TNF-α mRNA expression was found in some samples. MIP-2 mRNA expression decreased after POD 1 in autografts. Finally, there were no differences in allo- or autograft corneal buttons concerning MCP-1 mRNA transcripts levels throughout all PODs.

Cytokine mRNA expression in acceptor corneal rings

Analysis was also performed in corneal rings to determine differences between both acceptor and donor corneas concerning the cytokine transcription patterns. Acceptor corneal rings from both transplanted and non-transplanted eyes were subjected to RT-PCR analysis for cytokine mRNA expression. Normal corneal rings (Fig. 4A) showed mRNA for the same cytokines as in contralateral, non-grafted corneal buttons (Fig. 3A). Transplantation induced a similar cytokine mRNA expression pattern of IL-1RA (stronger expression in allografts than in autografts), IL-2, IL-4, IFN-γ and MCP-1 in rings (Table II and Fig. 4B and 4C), as compared to corneal buttons. IL-1β mRNA expression was slightly increased after POD 7 in allogeneic transplantation while after autotransplantation there was a decreased expression after POD 3. In recipient rings of allograft eyes, IL-6 mRNA showed maximal expression on POD 12, while in autografts, a marked decrease was observed after POD 3. IL-10 mRNA levels decreased immediately after POD 1 in autografted eyes. For TNF-α, an increased mRNA expression starting on POD 7 was found in allografts, while in autografts a weak expression was seen in some samples. MIP-2 transcription increased on POD 12, while in autografts, its expression was not markedly different from that detected in the contralateral, non-grafted peripheral cornea.




acceptor corneal rings (allografts)
time(POD)
1
3
7
12
19
–
spleen cDNA dilution
ß - Actin
IL-1 ß
IL-1RA
IL-2
IL-4
IL-6
IL-10
TNF-α
IFN-γ
MCP-1
MIP-2
B

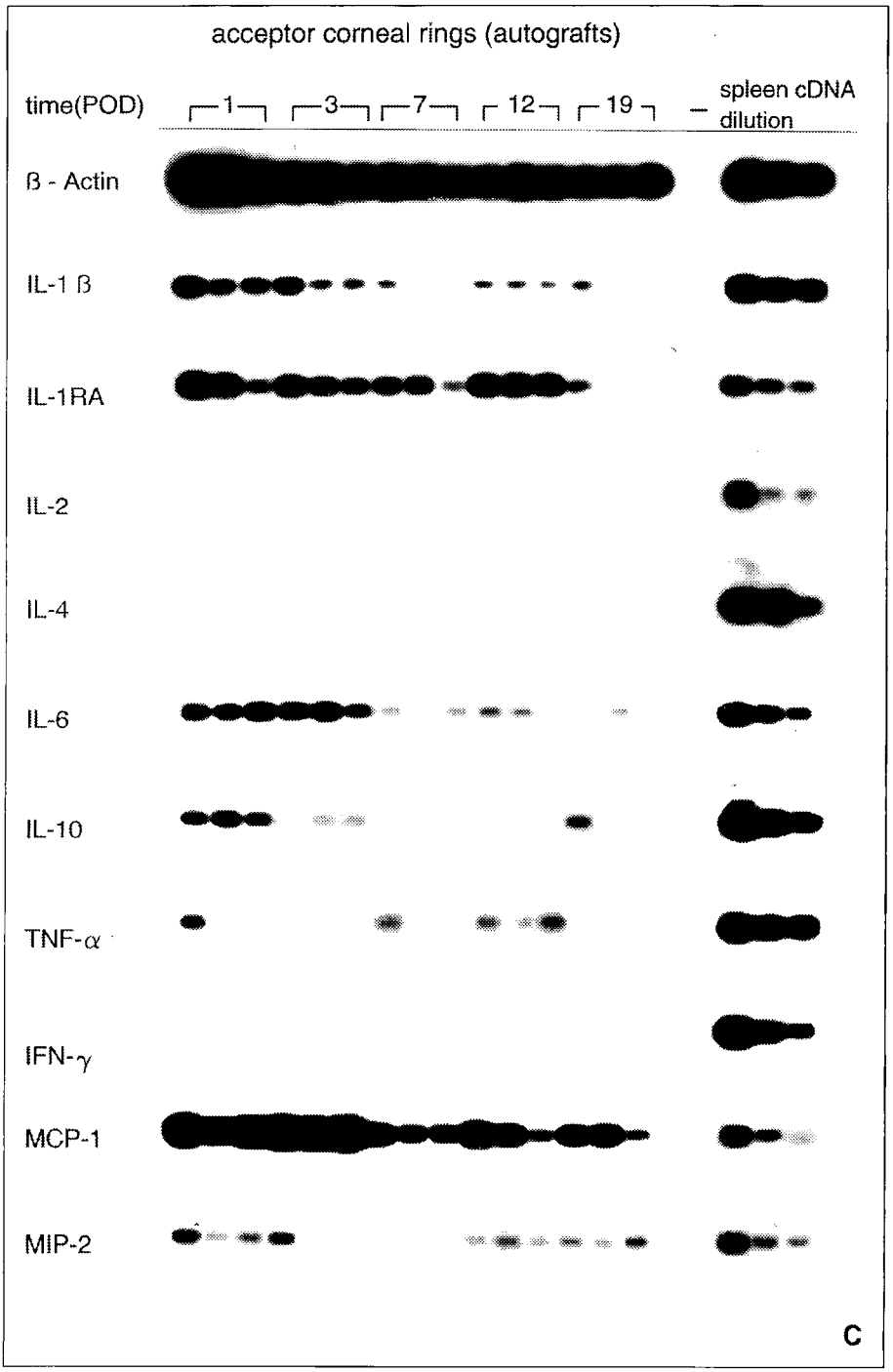


Figure 4 — Peripheral corneal rings from contralateral, non-grafted corneas (A), allografts (B) and autografts (C) were submitted to RT-PCR analysis. Samples were isolated at different points in time. Blots show the results from three rat experiments for each POD. The negative control (–) and the titration of positive control are also shown.

TABLE II — **ANALYSIS OF CYTOKINE mRNA EXPRESSION AFTER CORNEAL TRANSPLANTATION BY SEMI-QUANTITATIVE RT-PCR. COMPARISON OF ALLO- AND AUTOGRAFTS AND CONTRALATERAL, NON-GRAFTED CORNEAS**

CYTOKINES	ALLOGRAFTS			AUTOGRAFTS			NON-GRAFTED EYES
POD	(1-3)	(7)	(12-19)	(1-3)	(7)	(12-19)	
IL-1β	+++	+++	+++	++	+	+	—
IL-1RA	++	+++	+++	++	++	++	++
IL-2	—	—	+++	—	—	—	—
IL-4	—	—	++	—	—	—	—
IL-6	++	++	+++	++	++	+	—
IL-10	+	+	++	+	±	±	—
TNF-α	±	+	++	±	±	±	—
IFN-γ	—	—	+++	—	—	—	—
MCP-1	+++	+++	+++	+++	+++	++	+*
MIP-2	+	+	++	+	+	+	+*

— no detectable product; ±, +, ++, +++: increasing amount of detectable product
POD: postoperative day; * present only in a few corneal samples

DISCUSSION

This paper documents the mRNA expression patterns of several cytokines, including IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, MCP-1 and MIP-2 in the whole cornea after corneal transplantation in the rat, by RT-PCR analysis. To our knowledge, this is the first study that characterized different temporal cytokine mRNA expression patterns for corneal allo- and autografts. Semi-quantitative RT-PCR is a sensitive and reproducible method for mRNA expression analysis [16, 26] and allowed discrimination between cytokine transcription levels in the contralateral, non-grafted corneas and in allo- and autografts. No major differences were observed between corneal buttons and corneal peripheral rings, suggesting that inducers of corneal inflammation were present at the same time both in the acceptor and donor corneas (Table II). The present results imply that the cytokines studied are involved in the pathogenesis of corneal graft rejection. As the methodology used here only provides information concerning RNA at the tissue level, further investigations which may include in situ hybridization are required to determine the RNA cellular source, and immunohistochemistry and ELISA studies are needed to demonstrate whether the increased mRNA expression in the cornea results in increased cytokine protein levels.

A prominent place of IL-2 and IFN-γ, noted as T-cell derived cytokines, in allograft rejection has already been described earlier in other transplantation models [12, 13, 14, 15, 16, 17, 18]. Corneal rejection in the rat was also associated with high mRNA expression for IL-2, IL-4 and IFN-γ, which underlines the central role of T-cells in corneal allograft rejection [10, 11]. Since IL-4 mRNA was not present in all rejected corneas, IL-2 and IFN-γ were considered immunological «markers» for corneal rejection in this model.

Although both CD4+ and CD8+ lymphocyte subpopulations can be found within the graft, it has been suggested that CD4+ T-cells may have the most important role in corneal graft rejection [9, 11]. Activated naive CD4+ T-helper (Th0) cells can differentiate into Th1 or Th2 cell subsets, depending on the cytokines within the microenvironment of the graft. The Th phenotype dictates the type of immune response occurring in the graft. Th1 cells, characterized by their IL-2 and IFN-γ production, are responsible for the delayed-type hypersensitivity responses seen in allograft reactions [27]. Th2 cells secrete IL-4 and IL-10 and are associated with a more benign immune response and have been reported to be correlated with graft tolerance and humoral immunity [28]. The observed cytokine mRNA pattern in grafted corneas does not demonstrate a specific Th1 or Th2 cell profile. However, the present finding of high IL-2 and IFN-γ mRNA levels in all rejected graft samples and the importance of CD4+ T-cells in corneal rejection noted by others [9, 10, 11], may suggest that Th1 mediated responses play a role in corneal rejection in this model. IL-2 propagates the graft immune response by stimulating the proliferation and activation of CD4+ T-cells. IFN-γ may be involved in activating the recruited macrophages in the graft tissue and also induces MHC class I and II antigen expression on graft cells [29].

Macrophages and Langerhans cells (LC) also have an indisputable place in corneal allograft rejection. Van der Veen *et al.* [23] managed to prevent experimental corneal allograft rejection with subconjunctival injections of liposomes containing a drug that depleted phagocyting cells. Macrophages and neutrophils are probably the cell types first to appear in the graft during the inflammatory process and to contribute to the initial cellular infiltrate seen in both acceptor and donor corneas [30]. Recruitment of macrophages and neutrophils into the graft tissue, is likely to be triggered by potent chemotactic cytokines such as MCP-1 and MIP-2 respectively. In intragraft analysis, both cytokines were expressed at POD 1 both in allo- and autografts, suggesting that these mediators contribute for the aspecific inflammatory response to surgery and later for the rejection process. Whether the presence of MCP-1 and MIP-2 in some contralateral, non-grafted corneas, is due to a constitutive expression or is related to cornea handling remains to be determined.

IL-1β, IL-6 and TNF-α are the mediators first to appear during inflammation [31] and are involved in several inflammatory responses, including the synthesis of other cytokines, activation of T-cells and migration of monocytes, macrophages and LC. The early detection of mRNA for these proinflammatory cytokines with increasing expression over time in

allografts, underlines the importance of these mediators in the initiation and effector phases of corneal rejection. Because mRNA expression of these cytokines was already detected on POD 1, it is plausible that corneal cells are also contributing to the intragraft cytokine mRNA expression and actively participate in the process of graft inflammation and rejection. After transplantation, it is likely that released IL-1 or TNF-α can trigger resident corneal cells, such as epithelial cells and keratocytes, to produce other cytokines, including IL-1β, IL-6, IL-10, MCP-1 and MIP-2. Cultured corneal cells have been shown to produce a number of inflammatory mediators, including cytokines such as IL-1 [32, 33, 34], IL-6 [35], IL-8 [36, 37] and IL-1RA [38, 39].

Autografts also induced cytokine mRNA upregulation and inflammation. The inflammatory process was transient, clinically evidenced by mild edema and opacity and served to repair damaged tissue secondary to corneal surgery. Clinically, corneal rejection was found only after the new vessels reached the graft tissue. This suggests that neovascularization is a critical step in corneal rejection, allowing T-cells to reach the graft within the vessels rather than by infiltration and passage through the acceptor corneal stroma. The observation that cytokines such as IL-1 [40, 41] and IL-8 [42], which is related to rat MIP-2 [43], can induce neovascularization when applied within the corneal stroma, underlines the importance of these mediators in the pathogenesis of corneal rejection.

IL-1RA, IL-4 and IL-10 are cytokines associated with anti-inflammatory properties or graft tolerance. IL-1RA is the natural antagonist of IL-1, recently described [44] and has been implicated in reducing the severity of many diseases [45, 46]. The presence of IL-1RA in all tested samples of the contralateral, non-grafted corneas, confirms the constitutive expression of this cytokine, suggesting the existence of an inherent corneal «defense» mechanism for IL-1 mediated responses. However, the difficulty of using this antagonist to inhibit IL-1 is evidenced in in-vivo experiments, in which up to a 1000 fold greater amounts of IL-1RA were needed to counteract IL-1 activity [47]. Further investigations are required to determine the potential role of this cytokine in preventing corneal diseases and rejection. IL-4 and IL-10 are closely associated with the induction of graft tolerance. The role of IL-4 in tolerance is manifested by the induction of the Th2 phenotype. It is an immunomodulatory cytokine, probably also acting in activated macrophage inhibition [28]. IL-10 is secreted by a variety of cell types, including CD4+ Th2 subsets and monocytes / macrophages, and is a potent inhibitor of Th1 cells. Our observations that corneal buttons obtained one day following surgery already showed IL-10 mRNA expression, suggest that resident corneal cells, such as epithelial cells and keratocytes, may be triggered to produce IL-10. This cytokine is capable of blocking the production of IFN-γ and IL-12 and downregulating the MHC class II expression on monocytes [27]. In the cornea, IL-10 has been found to suppress the development of herpes simplex virus type 1 stromal keratitis [48].

Further knowledge of the molecular mechanisms during corneal transplantation may lead to a better understanding of the rejection process.

Corneal rejection is still the major obstacle to successful transplantation in high-risk corneas. In summary, corneal transplantation induces different temporal patterns of cytokine mRNA expression in allo- and autograft, with the most remarkable finding of high mRNA levels for IL-2, IFN-γ and IL-4, noted as T-cell derived cytokines, during the rejection process.

ACKNOWLEDGMENTS

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Chapter 5

Interleukin-10 treatment does not prolong experimental corneal allograft survival

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SUMMARY

In view of the known anti-inflammatory activities of interleukin (IL)-10, we investigated whether the administration of recombinant murine IL-10 prolonged corneal graft survival. A major histocompatibility complex (MHC) mismatched rat model with AO rats as recipients of PVG donor corneas was used. A total of 39 corneal allografts was included in this study and divided into 7 groups for different treatment. Groups I (n = 6), II (n = 8), III (n = 6) and IV (n = 7) were injected subconjunctivally (SC) with saline (control), 0.5 ng, 5 ng or 50 ng of IL-10, respectively, on the day of transplantation and then on post-operative days (POD) 2, 4, 6, 8 and 10. Group V (n = 4) and group VI (n = 4) were injected intraperitoneally (IP) with saline (control) or 1 μg of IL-10, respectively, on the day before surgery, the day of grafting and then on POD 2, 4 and 6. Finally, group VII (n = 4) was injected with both SC 5 ng of IL-10 and IP 1 μg of IL-10 on the same days as the previous groups. The median days for corneal rejection in the various groups were: group I: 11.3 ± 0.9; group II: 11.5 ± 0.9; group III: 11.6 ± 0.8 and group IV: 10 ± 1.0. Statistical analysis revealed a trend towards rejection (p = 0.08) in group IV (compared to group I). In groups V and VI, corneal rejection was evident on day 12 and in group VII the median time for rejection was 10.5 ± 0.8. These results indicate that IL-10 treatment does not prolong corneal allograft survival, and may even accelerate rejection.

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INTRODUCTION

Immunological rejection is still the main cause of corneal graft failure [1, 2] despite the worldwide reported high successful 5 year-survival rates for corneal transplants. In high-risk patients with highly vascularized corneas, chemical burns or history of previous corneal graft rejections, immunosuppression induced by administration of corticosteroids or cyclosporin A regimens may not be sufficient to prevent corneal rejection. Furthermore, systemic administration of these drugs may cause multiple and significant side effects, which may require dosage reductions or even a complete cessation of the given treatment. Moreover, true tolerance is probably never achieved with pharmacological immunosuppression. Therefore, new therapeutic strategies have to be developed which interfere in the immune mechanisms that underlie corneal rejection.

Cytokines have been implicated to play an important role in corneal rejection by stimulating inflammatory and T helper (Th) cell responses. Recently, we showed that corneal allograft rejection in a rat model was associated with the expression of interleukin (IL)-2, IL-4 and interferon (IFN)-gamma [3], which indicates that both Th1 and Th2 cellular immune responses are involved in this process. Manipulation of the cytokine network has had a beneficial effect on herpes keratitis. Local administration of IL-10 [4] or IL-10 DNA [5] to the cornea ameliorated herpes simplex virus-associated keratitis. IL-10, originally known as cytokine synthesis inhibitory factor (CSIF) [6], is an 18.5 kDa protein and is produced by a variety of cell types, such as Th2 cells, mast cells [6] and monocytes / macrophages [7]. Previously, we found that IL-10 mRNA is expressed in the cornea during graft rejection and surgical trauma [3]. IL-10 has pleiotropic effects and has the ability to interfere in various functions of different cell types. For example, IL-10 is capable of reducing antigen-specific T-cell proliferation by downregulating the major histocompatibility complex (MHC) class II expression [8], which is particularly important for antigen presentation. IL-10 also inhibits production of several cytokines, such as IL-2 and IFN-gamma by T-cells and pro-inflammatory cytokines IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-alpha by macrophages [7, 9, 10]. Moreover, IL-10 induces expression of IL-1 receptor antagonist (IL-1RA) [11], the natural antagonist of the proinflammatory cytokine IL-1. Recent studies suggest that IL-10 inhibits monocyte production of IL-12, a pivotal cytokine for Th1 cell differentiation and cell-mediated immunity [12]. In vivo, a high dose of IL-10 partially suppressed the sensitisation and expression of a delayed-type hypersensitivity (DTH) response [13]. Furthermore, IL-10 inhibits macrophage and NK-cell activity [10, 14] and stimulates Th2 cells and B-cells [15].

Based on the properties of IL-10 described above, we raised the hypothesis that the administration of IL-10 would induce long-term corneal graft acceptance. In this study, we analysed the role of subconjunctival (SC) and / or intraperitoneal (IP) injections of murine recombinant IL-10 on corneal graft survival in a rat model of allogeneic corneal transplantation. The obtained results reveal that IL-10 does not promote corneal graft survival and that the highest dose of IL-10 injected SC may even accelerate rejection.

MATERIALS AND METHODS

Experimental animals

Inbred male AO and PVG rats (Harlan Sprague-Dawley, Indianapolis, IN, USA), 12-15 weeks old and weighing 240-290 g, were housed and treated according to the ARVO resolution on the use of animals in research. In all experiments, AO rats were used as recipients of PVG corneal grafts.

Interleukin-10

Escherichia-coli-derived recombinant murine IL-10 was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). According to the manufacturer IL-10 had a purity of more than 98 %, was biological active *in vitro* and had an ED50 of 2 ng/ml. Prior to the injection, IL-10 was diluted with phosphate buffered saline in order to obtain the various concentrations used.

Corneal Surgery

Corneal transplantation was performed as described earlier[16]. Briefly, the cornea of one eye of each receptor rats and corneas of donor rats were trepanned with a 3-mm trepane prior to corneal grafting. Due to the shallow anterior chamber of acceptor rats, maximal midriasis was induced to avoid touching of the iris during surgery and subsequent formation of synechia. Midriasis was achieved by SC injection of atropine sulphate and by topical application of atropine eye drops. Donor corneal buttons were placed into the host corneal rim with a 10/0 monofilament nylon running suture. Occasionally, a small air bubble was injected into the anterior chamber at the end of surgery to increase its depth. Topical chloramphenicol and atropine ointments were applied to the eye immediately after surgery.

Experimental design

A total of 44 corneal allografts were performed. Five animals were excluded from this study due to large anterior synechia. The remaining 39 grafted rats were divided into 7 groups (Table 1) for different treatments. Rats of group I (n = 6) were injected subconjunctivally with 50 µl of saline on the day of corneal transplantation and then on postoperative days (POD) 2, 4, 6, 8 and 10. Groups II (n = 8), group III (n = 6) and group IV (n = 7) received 0.5 ng, 5 ng or 50 ng IL-10, respectively, of recombinant murine IL-10 (Pepro Tech Inc., Rocky Hill, NJ, USA), in a total volume of 50 µl on the same days as group I. Group V (n = 4) and group VI (n = 4) were injected intraperitoneally with 1ml of saline or 1 µg of IL-10, respectively, on the day before surgery, the day of corneal grafting and on POD 2, 4 and 6.

Rats of group VII (n = 4) were injected both with 5ng subconjunctival IL-10 on the day of transplantation and on POD 2, 4, 6 and 8, and 1 µg intraperitoneal IL-10 on the day before surgery, on the day of corneal grafting and on POD 2, 4 and 6. Rats were clinically evaluated every two days after transplantation and kept alive until 1 week after the diagnosis of corneal rejection.

The number of animals in some of the groups was small in view of the «pilot» nature of these experiments. Local subconjunctival injections were performed at this stage of the study instead of topical application to ensure high concentrations of IL-10 in the limbal area.

TABLE I — **RATS WERE DIVIDED INTO GROUPS FOR DIFFERENT TREATMENTS**

	N° OF RATS	ROUTE	PRODUCT INJECTED	AMOUNT INJECTED	DAYS OF INJECTION
Group I	6	SC	Saline	50 µl	0,2,4,6,8,10
Group II	8	SC	IL-10	0.5 ng	0,2,4,6,8,10
Group III	6	SC	IL-10	5.0 ng	0,2,4,6,8,10
Group IV	7	SC	IL-10	50 ng	0,2,4,6,8,10
Group V	4	IP	Saline	1 ml	-1,0,2,4,6
Group VI	4	IP	IL-10	1 µg	-1,0,2,4,6
Group VII	4	SC / IP	IL-10	5 ng / 1 µg	-1,0,2,4,6,8

SC: subconjunctival, IP: intraperitoneal; -1: day before surgery; 0: day of grafting; 2-10: postoperative days.

Clinical evaluation

Grafted animals were examined by slit-lamp for clinical evaluation. Opacity and edema of the graft were graded, as described earlier by others [17], ranging from 0 (clear cornea; no edema) to 4 (opaque cornea, allowing no identification of anterior chamber structures; epithelial and stromal bullous keratopathy). Neovascularization was graded in four separate quadrants of the cornea with a score ranging from 0 (no vessels in both receptor and acceptor corneas) to 4 (neovascularization reaching the center of the graft in all quadrants) as described earlier by van der Veen *et al.* [16]. Adding the score of all quadrants lead to a maximally possible score of 16. Corneal rejection was diagnosed after a sudden increase of graft opacity and edema.

Injections

Subconjunctival injections were given with a «Hamilton» syringe after adequate systemic and topical anaesthesia [16]. To obtain an equal distribution

around the limbus, the total amount injected was divided over four different sites near the peripheral cornea. This resulted in a circular subconjunctival bleb. Intraperitoneal injections were given with a 27 gauge needle syringe.

Statistical analysis

Groups were compared using Fischer's exact test. Differences were considered statistically significant when the P value was < 0.05.

RESULTS

A mild edema was observed on the wound borders and graft on the first day after surgery. After POD 2, rats of all groups showed clear corneal grafts until the appearance of the first signs of rejection. The results presented here are based on slit-lamp observation of corneal rejection (Table 2), according to the criteria mentioned above. In control group I, rats injected with saline subconjunctivally, corneal rejection was observed on POD 10 in 2 rats (33 %) and on POD 12 in 4 rats (67 %), with a median rejection time of 11.3 ± 0.9 days. In all rats, opacity and edema of the rejected corneal grafts had a maximum score of 3. New corneal vessels grew through the receptor cornea but stopped at the border of the wound (maximum score 8). No major differences concerning graft opacity, graft edema and corneal neovascularization were observed between rats of the control group and groups II or III. Rats showed similar evaluation scores as seen in group I. In group II, corneal rejection was clinically detected on POD 10 in 2 rats (25 %) and on POD 12 in 6 rats (75 %) with a median rejection time of 11.5 ± 0.9 days. In group III, rejection was noticed on POD 10 in 1 rat (17 %) and on POD 12 in 5 rats (83 %) with a median time of 11.6 ± 0.8 days. Corneal graft rejection in groups II and III was not statistically different from group I. A more severe clinical picture was noticed in rats of group IV, with higher grades of corneal opacity (29 % had score 4), corneal edema (57 % had bullous keratopathy) and intense neovascularization towards the center of the graft (observed in 43 %) (Fig. 1). Rejection was diagnosed on POD 8 in 1 rat (14 %), on POD 10 in 5 rats (72 %) and on POD 12 in one rat (14 %), with a median rejection time of 10.0 ± 1.0 days. Statistical analysis revealed a trend towards earlier corneal rejection ($p = 0.08$) when group IV was compared to controls (group I). Animals of group V and group VI, which received saline or IL-10 intraperitoneally respectively, showed no clinical differences. In both groups, maximal score for corneal opacity and edema was 3 and new vessels reached the borders of the graft. Corneal rejection was diagnosed on POD 12 in all cases of both groups. Finally, 3 rats of group VII (SC and IP injections of IL-10) developed corneal rejection on POD 10 (75 %), while 1 rat did so on POD 12 (25 %), with a median rejection time of 10.5 ± 0.8 days. A trend towards earlier corneal rejection was observed when group VII was compared to group I or to group III ($p = 0.09$).

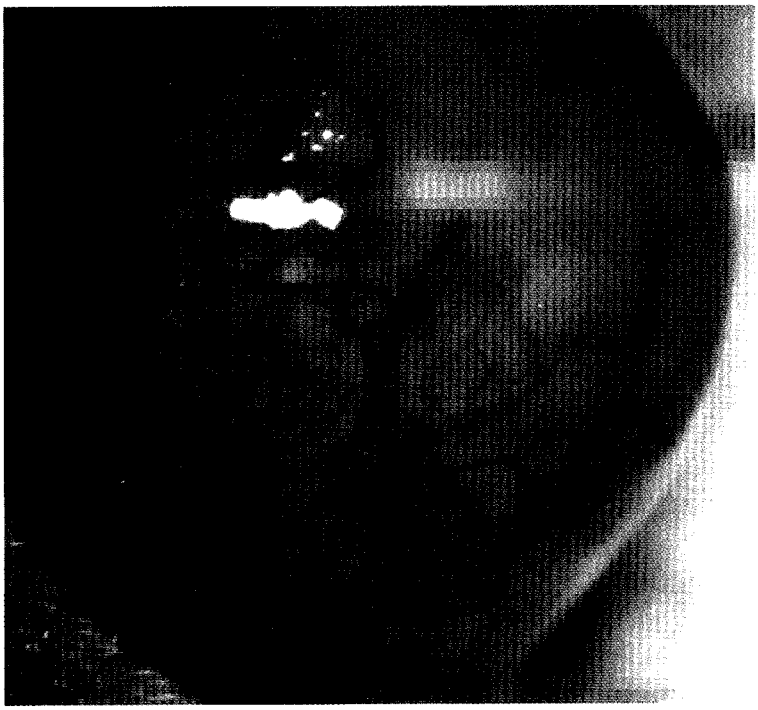

Figure 1 – Severe corneal allograft rejection with graft opacity grade 4 and ingrowth of large corneal vessels towards the center of the graft.

No attempts were made to investigate a possible difference in the number or phenotype of effector cells infiltrating the corneas of IL-10 treated rats as compared to controls.

TABLE 2 — **RAT DISTRIBUTION PER POSTOPERATIVE DAYS OF CORNEAL REJECTION; SLIT-LAMP OBSERVATION**

	POD 8 (%)	POD 10 (%)	POD 12 (%)	MEAN ± SD
Group I	0 (0)	2 (33 %)	4 (67 %)	11.3 ± 0.9
Group II	0 (0)	2 (25 %)	6 (75 %)	11.5 ± 0.9
Group III	0 (0)	1 (17 %)	5 (83 %)	11.6 ± 0.8
Group IV	1 (14 %)	5 (71 %)	1 (14 %)	10.0 ± 1.0
Group V	0 (0)	0 (0)	4 (100 %)	12.0
Group VI	0 (0)	0 (0)	4 (100 %)	12.0
Group VII	0 (0)	3 (75 %)	1 (25 %)	10.5 ± 0.8

POD: postoperative day; SD: standard deviation.

DISCUSSION

This paper documents that local or systemic application of recombinant murine IL-10 does not enhance corneal allograft survival in the rat. The lack of therapeutic effect may be due to the fact that optimal doses or routes of administration for IL-10 might not have been achieved in our study. A dose-dependent effect was observed for IL-10 subconjunctival injections, whereby the highest dose of IL-10 resulted in worse graft outcome. In these rats, the presence of bullous keratopathy and deep corneal vascularization towards the center of the graft indicates that IL-10 induced a severe and deleterious corneal response. These reactions were possibly due to an unknown local immunogenic effect of murine IL-10. A trend towards accelerated corneal rejection was also observed in group VII whereby animals received combined intraperitoneal and subconjunctival IL-10 injections. The SC route was chosen as the best potential route for IL-10 administration, since IL-10 is able to suppress the activation of antigen--presenting cells (APC) [8].

Macrophages and Langerhans cells (LC), which are present in the limbus and in the peripheral normal cornea [18], are known to actively participate in corneal graft rejection [16]. The IP route was used based on its reported benefit in a mice model of stromal herpetic keratitis [4]. However, in our model, the IP route alone or in association with the SC route did not result in prolonged corneal graft acceptance. The lack of efficacy in our model of allotransplantation was probably not due to species incompatibilities for the IL-10 used (murine IL-10 in a rat corneal allograft model) since others have shown the effectiveness of murine IL-10 in various models of inflammation in the rat [19, 20]. These earlier observations suggested that IL-10 could play a regulatory role in the immune response and could possibly also enhance graft survival.

Several studies have shown that IL-10 may indeed play a beneficial role in graft acceptance. Cardiac graft tolerance was associated with high expression of IL-4 and IL-10 [21, 22]. Postoperative IP administration of IL-10 has been shown to significantly accelerate heart graft rejection in mice and was associated with both an increased circulating complement dependent cytotoxic antibody titer and anti-donor cytotoxic T cell activity [23]. Bacchetta *et al.* [24] showed a correlation between IL-10 production and the induction of tolerance in patients transplanted with HLA mismatched haematopoietic stem cells. Blocking IL-10 using monoclonal antibodies resulted in accelerated skin graft rejection [25]. On the other hand, a number of studies have indicated that IL-10 might promote graft rejection. Several studies documented a correlation between intragraft expression of IL-10 and graft rejection [26, 27, 28, 29]. Acute kidney rejection was associated with the appearance of IL-10 and did not prevent in vivo expression of the Th1-derived cytokines such as IL-2, IFN-gamma as well as TGF-beta-1 and IL-7 [26, 27]. Furthermore, transgenic expression of IL-10 in pancreatic islet beta cells has been shown to be associated with prominent inflammatory cell infiltration and high levels of intragraft IL-10 mRNA expression was correlated with

rejection of murine pancreatic islet allografts [30]. Moreover, IL-10 exhibits specific chemotactic activity for CD8+ T cytotoxic / suppressor lymphocytes [31]. This may suggest that IL-10 is capable of exerting immunostimulatory activities towards corneal rejection and that Th2 cytokines might play a role in graft rejection.

Based on our results, it is not expected that IL-10 will be a useful tool in the prevention of corneal graft rejection. However, it is known that cytokines can show paradoxical effects [32]. Thus, further investigations are required to determine if IL-10 is beneficial or not in corneal transplantation and to get a more accurate understanding of this cytokine under physiological and pathophysiological conditions. Based on our previous work [3] which showed higher IL-10 mRNA expression in rejected than in accepted corneas, another possibility for future experiments is to investigate the effects of IL-10 inhibition in a model of corneal allotransplantation.

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Chapter 6

Changes in cytokine mRNA levels in experimental corneal allografts after local clodronate-liposome treatment

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SUMMARY

Corneal allograft rejection in rats can be prevented by subconjunctival injections of liposomes containing dichloromethylene diphosphonate (clodronate-LIP), which selectively eliminate macrophages. In this study, the effect of clodronate-LIP treatment on cytokine mRNA levels in corneal allografts was examined. AO rats received corneal grafts of PVG rats. Rats were either not treated or injected subconjunctivally with clodronate-LIP on the day of transplantation and on post-operative days (POD) 2, 4, 6 and 8. RNA was isolated from the graft and rim of corneas at different time points after transplantation and from normal controls. IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IL-12p40, TNF-α, TNF-β/LT, IFN-γ, MCP-1 and MIP-2 mRNA levels were analysed by semi-quantitative RT-PCR. Corneal rejection, observed in all untreated rats by POD 12, was associated with increased mRNA levels of all cytokines investigated in grafts and rims. Clodronate-LIP treatment prevented allograft rejection and strongly decreased the levels of IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-β/LT, MCP-1 and MIP-2 mRNA in grafts and IL-1β, IL-2, IL-4, IL-6 and IFN-γ mRNA in rims. IL-12p40 mRNA levels were unaltered

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in clodronate-treated rats, except for a transient increase in grafts at POD 3. TNF-α mRNA levels were increased by clodronate-LIP in grafts and rims early after transplantation (POD 3 and 7). Despite a normal appearance, long-term accepted corneal grafts (POD 100) contained mRNA for IL-10, IL-12p40, TNF-α, MCP-1 and MIP-2. Clodronate-liposome treatment markedly altered the mRNA levels of all cytokines investigated in corneal allografts. Our results may explain in part the mechanism by which clodronate-LIP treatment prevents corneal allograft rejection.

INTRODUCTION

Despite advances in surgical techniques and immunosuppressive drug treatment, allograft rejection still occurs in 10 to 30 % of the cases of penetrating keratoplasty and is the major cause of corneal graft failure [1,2]. Corneal allograft rejection is characterised by infiltration of mainly T cells and macrophages into the donor tissue [3,4]. Depletion of CD4+ T cells by systemic administration of specific monoclonal antibodies markedly reduced corneal graft rejection [5,6]. Topical application of such anti-CD4 antibodies also reduced allograft rejection [7]. Using a technique to eliminate macrophages, we previously showed that these cells are also crucial for the rejection process [4]. Macrophages can be eliminated in a selective manner, due to their phagocytic activity, by liposomes containing the drug dichloromethylene diphosphonate (clodronate-LIP) [8]. After uptake by macrophages, the phospholipid bilayers of the liposomes are degraded by lysosomal phospholipases and the released drug causes cell death [9]. In our study, five consecutive injections of clodronate-LIP into the subconjunctiva of rats early after transplantation produced 100 % survival of corneal allografts for up to 100 days. This treatment was characterised by reduced cellular infiltration and reduced neovascularization of the grafted cornea [4]. Recently, we found that clodronate-LIP prevented the induction of cytotoxic T cells and cytolytic antibodies directed against the donor tissue [10,11], which indicates that clodronate affects antigen presentation.

Although the molecular mechanisms underlying corneal graft rejection are still not completely understood, cytokines released at the grafted site are thought to be involved in the attraction, activation and proliferation of T cells and macrophages [12,13,14]. Recently, we have reported that corneal allograft rejection in rats was associated with increased mRNA levels of multiple cytokines, including interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1RA), IL-6, IL-10, tumor necrosis factor-alpha (TNF-α), monocyte chemo-tactic protein 1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2), which were detected immediately after transplantation and during

rejection [12]. The expression of known T cell-derived cytokines, including IL-2, IL-4 and interferon-γ (IFN-γ), was detected after rejection occurred, underlining the central role of T cells in this process. Recently, others also demonstrated expression of IL-2 and IFN-γ during corneal allograft rejection in mice [13, 14]. These results imply that cytokines contribute to corneal graft rejection. Expression of IL-1β, IL-1RA, IL-6, IL-10, MCP-1 and MIP-2 was also detected after transplantation of corneal autografts that were not rejected [12], indicating that some of these factors are already triggered by traumatic events alone.

In the present study we have expanded our analysis of cytokine expression in corneal allografts. We examined cytokine mRNA expression patterns in rejected corneal allografts and in accepted allografts of rats treated subconjunctivally with clodronate-LIP using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Our results show that clodronate-LIP treatment markedly decreased the mRNA expression of most of the cytokines investigated in the cornea after allograft transplantation.

MATERIAL AND METHODS

Animals

Inbred male AO and PVG rats (Harlan Sprague Dawley, Bicester, England), 10 to 12 weeks of age at the time of transplantation, were used in this study. These strains differ in their MHC antigens [15]. Rats were treated according to the ARVO Resolution on the Use of Animals in Research and the guidelines of the Animal Care and Ethics Committee of the Royal Netherlands Academy of Sciences. Rats were housed under standard conditions and were given food and water ad libitum.

Corneal surgery and clinical evaluation

Orthotopic corneal transplantations were performed on one eye of AO rats as described earlier [4, 12, 16]. Briefly, prior to corneal surgery, maximal mydriasis was induced by subcutaneous injection of atropine sulphate and topical atropine and phenylephrine drops, in order to prevent trauma to the iris and anterior synechiae formation. A 3.0 mm diameter trephine was used to mark both recipient and donor corneas and curved fine scissors were used to remove the buttons. Donor corneas were kept in corneal preservation medium (Eagle's modified essential medium with 2 % foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin) until use. The donor button was sewn into the recipient cornea using eight stitches of a continuous 10/0 monofilament nylon suture. Sutures were left in place for the duration of the experiments (up to POD 100).

Transplanted rats were examined using an operating microscope every other day until POD 17 and weekly until POD 100. Opacity and neovascularization of transplanted corneas was graded using a scoring system described previously [4]. Maximal opacity was graded 4 and maximal neovascularization was graded 16 (score 4 for every quadrant of the cornea). Corneal rejection was defined as a graft opacity score of three or higher in a previously clear graft.

Liposome preparation and subconjunctival injections

Multilamellar liposomes, composed of phospholipid bilayers, containing dichloromethylene diphosphonate (clodronate; kindly provided by Boehringer Mannheim, Mannheim, Germany) were prepared as described elsewhere [17]. Clodronate-LIP were suspended in PBS and stored at 4°C until use. A total volume of 100 µl of clodronate-LIP was injected into the subconjunctiva near the limbal area using a 50 µl Hamilton syringe with a 30 gauge needle. Four injections of 25 µl each were given, in four different quadrants, to produce a circular bleb (Fig. 1).

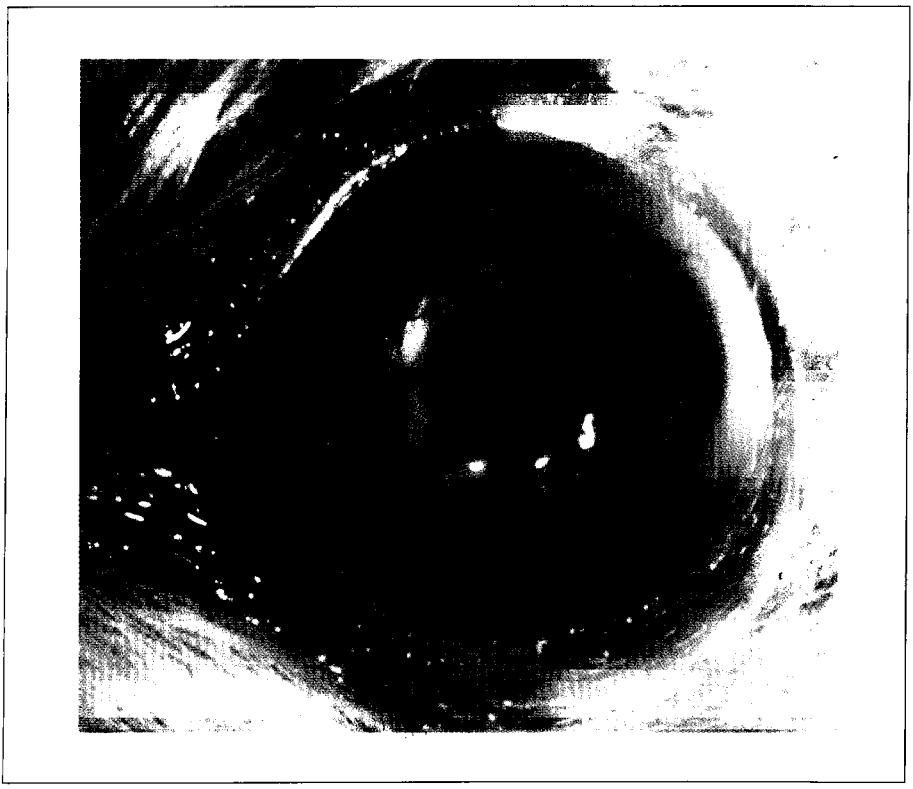

Figure 1 — A subconjunctival circular bleb is formed after the liposome injections.

Experimental design

Rats with technical transplantation or liposome injection failures (5 in total) were excluded from the study. Rats with successful corneal grafts (n = 42) were divided into two groups: (A) control rats (n = 20) that received no treatment. Of this group, five rats each were killed on PODs 3, 7, 12 or 17 by intravenous injection of a lethal dose of pentobarbital. (B) Clodronate-LIP-treated rats (n = 22) that received subconjunctival injections of liposomes at the time of corneal transplantation and on PODs 2, 4, 6 and 8. Five rats each were sacrificed on PODs 3, 7, 12 or 17 and two rats on POD 100. In addition, 4 AO rats that did not undergo surgery were used as normal controls.

After termination, rats were perfused through the left ventricle of the heart with 500 ml sterile pyrogen-free saline in order to remove cells from blood vessels. Subsequently, the graft (*i.e.* the central corneal button) and the rim (*i.e.* the adjacent peripheral ring) of the recipient cornea were removed, immediately frozen in liquid nitrogen and stored at – 70°C until RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from all samples by a single-step extraction method [18]. Corneal tissues were homogenized by vigorously vortexing in RNAzol (Cinna Biotecx Laboratories Inc, Houston, Texas) and the extracted RNA was dissolved in 10 µl sterile water. For cDNA synthesis, 5 µl of total RNA was incubated with 2.5 µg oligo(dT) 12-18 primer, 10 mM dNTPs (Pharmacia, Woerden, The Netherlands) and 200 U Superscript RNaseH reverse transcriptase (Gibco-BRL, Eggenstein, Germany) according to the manufacturer's instructions. After incubation for 1 hour at 37°C, the reaction was terminated at 65°C for 5 minutes. cDNA was stored at – 70°C until use.

PCR primers and internal control probes

The sense and anti-sense PCR primers and internal control probes for β-actin, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, MCP-1 and MIP-2 have been described [12, 19]. Primers and probes for the inducible p40 subunit of IL-12 (IL-12p40) and LT/TNF-β were designed using sequences obtained from the Genbank database and are shown in Table I.

PCR and Southern blot analysis

Semi-quantitative PCR amplification and analysis of PCR products was performed as previously described [12, 19]. For PCR amplification, cDNA

was added to a reaction mixture consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 % Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM sense and anti-sense oligonucleotide primers and 1 U Taq DNA polymerase (Promega, Madison, WI) in a volume of 50 µl. The number of PCR cycles and annealing conditions for the primers have been described [8, 19], except for IL-12p40 (38 cycles, annealing at 62°C) and TNF-β/LT (35 cycles, annealing at 63°C). Prior to cytokine PCR analysis, the cDNA concentration of all samples was normalised to yield equivalent amounts of β-actin PCR product. Differences in the yield of β-actin PCR product were determined using an ImageMaster (Pharmacia Biotech, Uppsala, Sweden). A total of 28 samples, consisting of 1 normal button, 6 grafts of untreated- and 6 grafts of clodronate-LIP-treated-rats, as well as 1 normal rim, 7 rims of untreated- and 7 rims of clodronate-LIP-treated-rats, with a low β-actin cDNA concentration were not used for further cytokine analysis. Subsequently, each cytokine PCR included all normalised samples of each group, as well as a titration (1/20, 1/100, 1/500) of positive control cDNA from lipopolysaccharide-, phorbol myristate acetate- or concanavalin A/IL-2-stimulated rat spleen cells and a negative control, which consisted of all reagents, but without template DNA.

TABLE I — **DNA SEQUENCES OF IL-12P40 AND TNF-β/LT OLIGONUCLEOTIDE PCR PRIMERS AND CONTROL PROBES**

CYTOKINE	PRIMER	SEQUENCE (IN 5' TO 3' DIRECTION)
IL-12p40	Sense	GCCAATACACCTGCCACAGA
	Anti-sense	GGGTCCGGTTTGATGATGTC
	Internal	CCCAACTGCCGAGGAGACCCTGCCCATTGA
TNF-β/LT	Sense	CTGCTGCTCACCTTGTTGG
	Anti-sense	TGTGCGCTGAGGAGAGGC
	Internal	TTGAACAACAACTCTCTCCTGATCCCCACC

PCR products were analyzed by 2 % agarose gel electrophoresis and ethidium bromide staining. For verification of their identity, PCR products were transferred to Genescreen-plus membranes (NEN-Du Pont, 's Hertogenbosch, Netherlands) and the filters were hybridized with specific oligonucleotide probes. Internal control probes were labelled at the 5' end with [γ-^{32}P]ATP, using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Hybridization was carried out in 6x SSC, 0.1 % SDS, 5x Denhardt's and 100 µg/ml sheared denatured herring sperm DNA and 20 pmol of labelled probe overnight at 65°C. Hybridized membranes were washed with 2x SSC, 0.1 % SDS at 65°C and exposed to X-ray film.

RESULTS

Clinical features of corneal grafting

Corneal transplantation caused mild opacity (grade 1-2) in some grafts of both groups on POD 2, probably due to an acute inflammatory reaction by surgical trauma. In the untreated group, opacity decreased after POD 2 and corneas remained clear until the time of rejection. In the clodronate-LIP-treated group, mild opacity (grade 1-2) was observed during the period of subconjunctival injections, but corneas regained transparency after POD 8. Corneal rejection was observed in all untreated rats within 12 days. A mean graft survival time of 10.9 days was calculated using the Kaplan-Meier test. Maximal opacity (grades 3-4) was observed at the onset of rejection. In contrast, graft rejection was not detected in the clodronate-LIP-treated group up to POD 100. In untreated rats, neovascularization progressed in time and reached grade 10 at POD 17. In most clodronate-LIP-treated rats, ingrowth of a few vessels in the recipient cornea up to the sutures was observed.

Cytokine expression in corneas of normal and grafted rats

Cytokine mRNA levels in corneal tissues of normal and transplanted rats were analyzed using RT-PCR. This method is able to demonstrate differences in mRNA levels in a semi-quantitative manner [19]. All samples were normalized for β-actin mRNA content. Southern blots with cytokines expressed in corneal grafts and rims are shown in figures 2 and 3, respectively. The cytokine expression pattern in normal and untreated rats was very similar to the results of our previous study [12]. Corneas of normal rats constitutively expressed IL-1RA and some animals (1 out of 3) showed a strong signal for MIP-2, whereas all other cytokines investigated were undetectable.

IL-1β mRNA expression

Albeit weakly, IL-1β was detected from POD 3 to 17 in 9 of 14 grafts and from POD 3 to 12 in 6 of 10 rims of untreated rats. IL-1β was not detected in grafts of liposome-treated rats, but was found in rims of these animals at POD 3 (in 2 of 3 samples) and POD 100 (in 1 of 2 samples).

IL-1RA mRNA expression

Similarly to our previous study [12], IL-1RA mRNA was detected in normal corneas. After transplantation, IL-1RA mRNA was detected in almost all samples (13 of 14 grafts and 13 of 13 rims) of untreated rats.

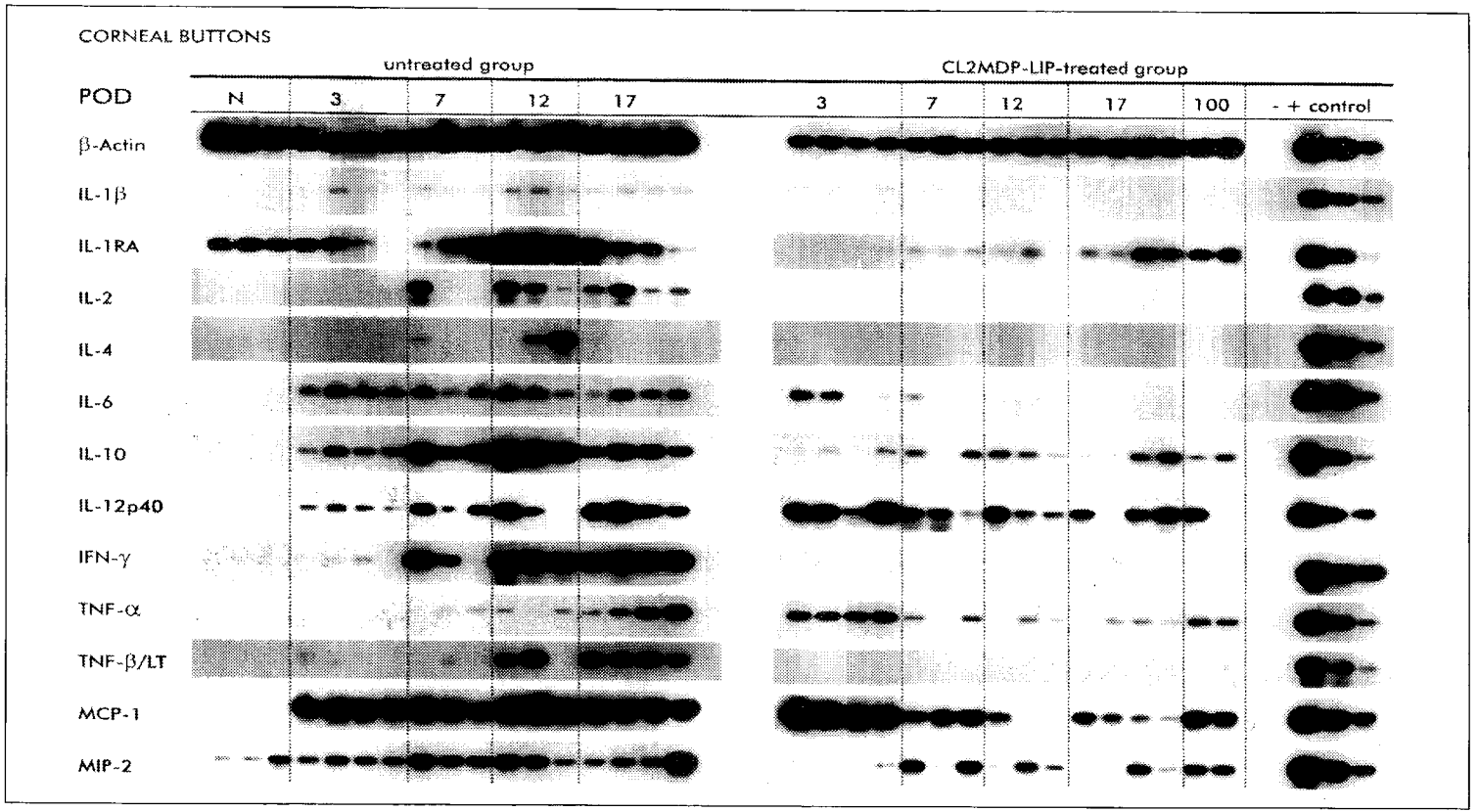

Figure 2 — Cytokine mRNA expression in central corneal buttons of normal rats (N) and grafts of untreated and clodronate-liposome-treated rats after transplantation. RT-PCR analysis was performed on corneal tissue harvested at different post-operative days (POD) after surgery. Each PCR included a negative control (all reagents, but without cDNA template) and positive controls (a 1/20, 1/100, 1/500 dilution of spleen cDNA).

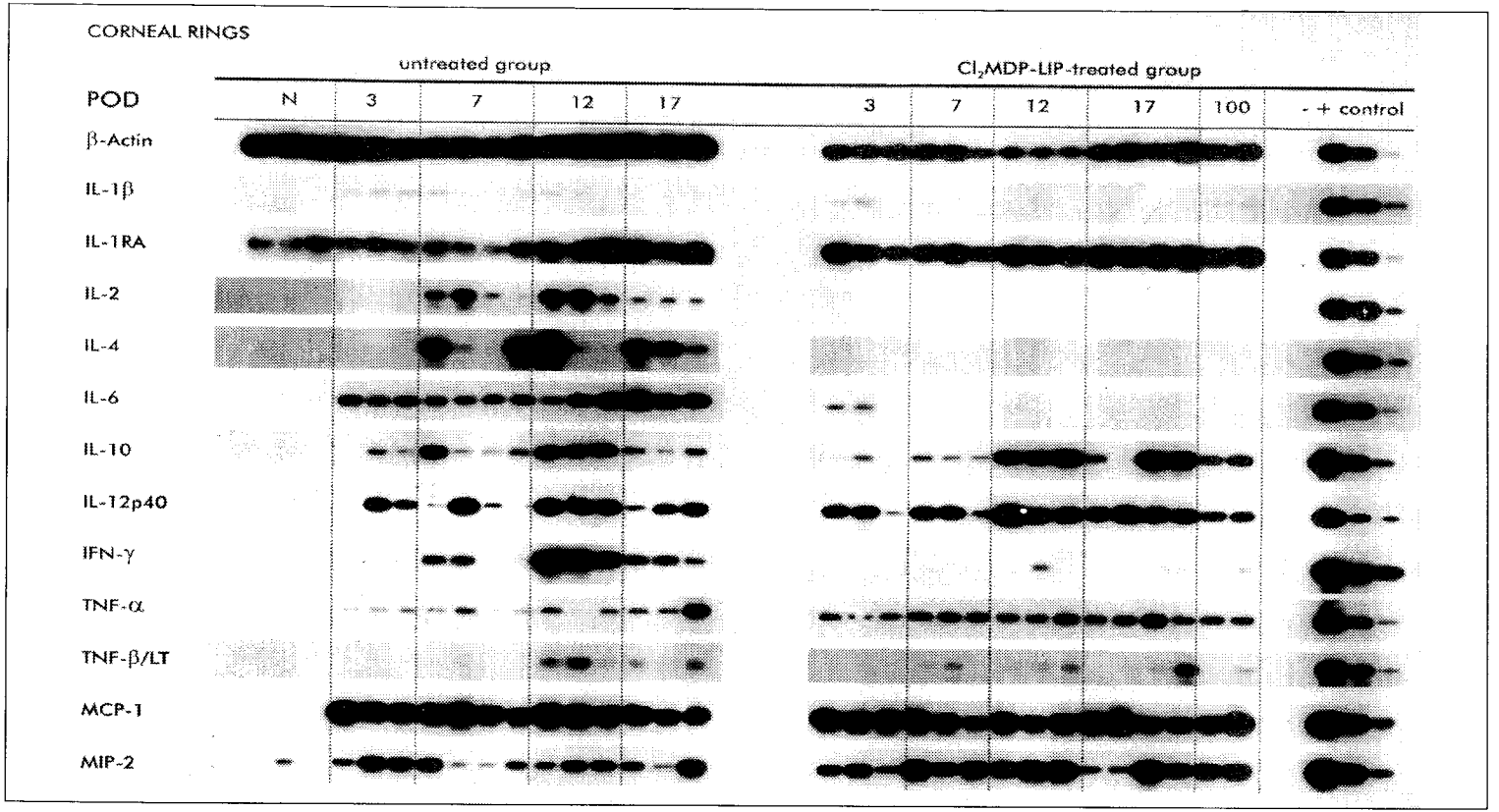

Figure 3 — Cytokine mRNA expression in peripheral corneal rims of normal rats (N) and untreated and clodronate-liposome-treated rats after allograft transplantation. RT-PCR analysis was performed on corneal tissue harvested at different post-operative days (POD) after surgery. Each PCR included a negative control (all reagents without cDNA template) and positive controls (a 1/20, 1/100, 1/500 dilution of spleen cDNA).

IL-1RA mRNA levels were slightly increased at POD 12 in both grafts and rims. Despite constitutive expression, clodronate-LIP treatment strongly decreased IL-1RA mRNA levels in grafts, but not in rims, early after transplantation. In grafts, IL-1RA mRNA levels returned to normal at POD 17 (in 2 of 4 samples) and POD 100 (in 2 of 2 samples).

IL-2 mRNA expression

IL-2 mRNA was detected from POD 7 to 17 in 8 of 10 grafts and 9 of 10 rims of untreated rats. Clodronate-LIP treatment completely abrogated the induced expression of IL-2 in both grafts and rims.

IL-4 mRNA expression

IL-4 mRNA was observed at POD 7 and 12 in 3 of 6 grafts and from POD 7 to 17 in 8 of 10 rims of untreated rats. Like for IL-2, IL-4 mRNA was absent in either grafts or rims of clodronate-LIP-treated rats.

IL-6 mRNA expression

IL-6 mRNA was detected from POD 3 to 17 after transplantation in all 14 grafts and 13 rims of untreated rats. IL-6 mRNA levels in grafts and corneal rims were steady over time. The induction of IL-6 expression was strongly reduced in clodronate-treated rats from POD 7 to 17; IL-6 was found only in grafts at POD 3 (in 2 of 4 samples) and POD 7 (in 1 of 3 samples) and in rims at POD 3 (in 2 of 3 samples). IL-6 mRNA was not detected in long-term grafts (POD 100).

IL-10 mRNA expression

Similar to IL-6, IL-10 mRNA was detected from POD 3 to 17 after transplantation in all fourteen grafts and in 12 of 13 rims of untreated rats. The highest level of IL-10 mRNA was detected on POD 12. Clodronate-LIP treatment decreased IL-10 mRNA expression in grafts, but IL-10 was detected from POD 3 to 100 in 10 of 16 samples. Clodronate-LIP did not affect IL-10 mRNA expression in peripheral rims; it was detected from POD 3 to 17 in 10 of 13 samples as well as at POD 100 (in 2 of 2 samples).

IL-12p40 mRNA expression

Like IL-6 and IL-10, IL-12p40 mRNA was present from POD 3 to 17 in 13 of 14 grafts and 11 of 13 corneal rims of untreated rats. Clodronate-LIP treatment caused a transient increase in IL-12p40 expression in grafts at POD 3, but did not affect mRNA levels at later time points (POD 7 to 17). IL-12p40 mRNA was detected from POD 3 to 17 in 12 of 14 grafts, as

well as in the two long-term grafts (POD 100). IL-12p40 mRNA was detected in all 15 rims of clodronate-treated at similar levels as compared to untreated rats.

IFN-γ mRNA expression

In grafts of untreated rats, a minor expression of IFN-γ mRNA was detected at POD 3 in 1 of 4 samples. A strong IFN-γ signal was detected from POD 7 to 17 in 9 of 10 grafts and in 8 of 10 corneal rims. Clodronate-LIP treatment markedly decreased IFN-γ expression in both grafts and rims; mRNA was detected in none of the grafts and only at POD 12 in 1 of 3 rims tested.

TNF-α mRNA expression

In untreated rats, TNF-α mRNA was detected from POD 7 to 17 in 7 of 10 grafts and from POD 3 to 17 in 10 of 13 rims. The highest mRNA levels were found on POD 17. Clodronate-LIP induced an increase in TNF-α mRNA levels in both grafts and rims early (POD 3 and 7) after allograft transplantation. At POD 100, all animals showed a strong signal for TNF-α mRNA.

TNF-β/LT mRNA expression

TNF-β/LT mRNA was detected at POD 12 and 17 in 6 of 7 grafts and, at slightly lower levels, in all 6 rims of untreated rats. Similar to IL-2, IL-4 and IFN-γ, liposome treatment completely blocked TNF-β/LT mRNA expression in grafts. In rims of clodronate-LIP-treated rats, however, TNF-β/LT mRNA was readily detected from POD 7 to 17 in 3 of 10 samples. At POD 100, a faint signal for TNF-β/LT was found in one of two rims of clodronate-treated rats.

MCP-1 mRNA expression

Like IL-6, MCP-1 mRNA was detected at similar levels from POD 3 to 17 in all 14 grafts and 13 rims of untreated rats. Expression of MCP-1 was decreased in grafts from POD 7 to 17, but not in rims of clodronate--LIP-treated rats. In these rats, MCP-1 was found from POD 3 to 17 in 12 of 14 grafts and in all 13 rims, as well as on POD 100 in all samples.

MIP-2 mRNA expression

In agreement with our previous studies [12, 19], a variable expression of MIP-2 mRNA was found in normal corneas. After transplantation, MIP-2 mRNA was detected from POD 3 to 17 in all 14 grafts and 13 rims of

untreated rats. Clodronate-LIP completely blocked the induced MIP-2 mRNA expression in 8 of 14 grafts from POD 3 to 17, but did not alter the expression in the grafts of the other 6 rats. MIP-2 mRNA was detected from POD 3 to 17 in all 13 rims of liposome-treated rats, at levels slightly above those of untreated rats. A strong signal for MIP-2 was also found in the two long-term accepted grafts (at POD 100).

DISCUSSION

The results presented here demonstrate that prevention of corneal allograft rejection by subconjunctival clodronate-LIP treatment is associated with a marked alteration of the cytokine mRNA expression profile in the allograft and surrounding recipient cornea. mRNA levels of all cytokines investigated were increased during allograft rejection as compared to levels in normal controls. Clodronate-LIP treatment caused a strong decrease of IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-β/LT, MCP-1 and MIP-2 mRNA levels in grafts and IL-1β, IL-2, IL-4, IL-6 and IFN-γ mRNA levels in corneal rims. In contrast, TNF-α mRNA levels in both grafts and rims were increased by clodronate-LIP early after transplantation. Clodronate-LIP did not alter IL-12p40 mRNA levels, except for a transient increase in grafts at POD 3. Despite a normal appearance, on clinical grounds, long-term corneal grafts still showed detectable mRNA for IL-10, IL-12p40, TNF-α and MCP-1, which were not detected in any of the normal control corneas. MIP-2 mRNA, which was occasionally present in normal corneas, also showed a strong signal in long-term accepted grafts. The prolonged cytokine expression in these corneas may be triggered by the graft, by the sutures that were still present or by a long-lasting effect of the clodronate injections. Experiments evaluating cytokine expression in normal rats treated subconjunctivally with clodronate and longitudinal studies in rats receiving an autograft could resolve this issue.

The pattern of cytokine expression in corneas of untreated rats, which rejected the allograft by POD 12, was largely consistent with the results of our previous study [12], except that expression of IL-2, IL-4 and IFN-γ was detected at an earlier time point in the present study. This suggests that the rejection process was slightly accelerated in the present study. The observed expression of IL-2, IL-4, IFN-γ and TNF-β/LT during allograft rejection is compatible with the cytokine expression pattern of Th0 cells, or of a combination of Th1 cells which produce IL-2, IFN-γ and TNF-β/LT, and Th2 cells which produce IL-4 [20]. This suggests that corneal graft rejection in this model is driven either by Th0 cells or by a mixture of Th1 and Th2 cells. Our results are in agreement with a recent study of Sano *et al.* [14], who demonstrated by ELISA and immunohistochemistry that IL-1α, IL-2, TNF-α and IFN-γ protein levels in the cornea were increased during graft rejection. The latter results further imply that increased cytokine mRNA expression in the cornea, as we have found, results in increased cytokine protein levels.

Van der Veen *et al.* [4] showed that local administration of clodronate-LIP strongly reduced the number of macrophages and T cells in the graft and recipient cornea. The almost complete abrogation of IL-1β, IL-2, IL-4, IL-6, IFN-γ and TNF-β/LT mRNA expression in allografts by clodronate-LIP suggests that during rejection these cytokines are derived from macrophages and T cells. The presence of IFN-γ and TNF-β/LT mRNA in a number of rims, but not grafts, of clodronate-treated rats can be explained by differences in T cell infiltration of these sites [4]. Macrophages are able to produce a variety of cytokines and are likely a source of IL-1β and IL-6 in the cornea in this model. A marked reduction of IL-1β and IL-6 expression by clodronate-LIP-mediated depletion of macrophages has been found in several studies [21, 22, 23]. The fact that mRNA levels of other macrophage-derived cytokines (TNF-α, IL-10, IL-12, MCP-1, MIP-2) were not completely reduced may be related to higher mRNA levels for these cytokines or to a higher sensitivity of the PCR for these factors. Alternatively, these cytokines could be produced by clodronate-LIP-resistant macrophages [23], or by other (resident) cells in the cornea [12, 24]. The reduction in the mRNA levels of these cytokines after clodronate treatment may result indirectly from the lack of IL-1β and IL-6.

Surprisingly, clodronate-LIP induced a transient increase in the levels of TNF-α and IL-12p40 mRNA in grafts, but not of other macrophage--derived cytokines, early after transplantation. Moreover, TNF-α and IL-12p40 mRNA levels in rims from POD 3 to 100 were steady over time and not markedly altered by clodronate, which was injected in the adjacent conjunctiva. Together, these results suggest that TNF-α and IL-12p40 were not produced by macrophages. Activated dendritic cells are another source of TNF-α and IL-12 [25, 26]. Dendritic cells (*i.e.* Langerhans cells), which are present in low numbers in the peripheral cornea normally and in the central cornea after transplantation [27, 28, 29], may be triggered to produce TNF-α and IL-12p40 after activation by sutures or by the massive destruction of macrophages in clodronate-LIP treated rats. Due to their limited phagocytic activity [30], dendritic cells are presumably not eliminated by clodronate-liposomes. Currently, we are investigating the fate of local dendritic cells in this model.

IL-1RA is constitutively produced in rat and human corneas [12, 19, 31, 32]. In the latter, IL-1RA is expressed mainly by epithelial cells [31, 32]. The presence of IL-1RA in the cornea implies the existence of an inherent control mechanism for IL-1 mediated responses. Recently, Dana *et al.* [33] found that IL-1RA strongly reduced centripetal Langerhans cells migration in the cornea. IL-1RA mRNA expression was not markedly decreased in both untreated corneal allografts and autografts [12]. However, IL-1RA mRNA expression in grafts, but not in the recipient corneas, of clodronate-treated rats was completely abolished immediately after transplantation. IL-1RA mRNA levels returned to normal by POD 17. Although the epithelial cell layer on the graft of these rats appeared normal early after transplantation [4] (P.F.Torres, personal observation), this finding suggests that clodronate may affect the normal activity of epithelial cells on the graft.

Our study clearly reveals that prolonged expression of IL-10, IL-12p40, TNF-α, MCP-1 and MIP-2 in corneal grafts is not correlated with rejection. This is consistent with our previous study, in which we showed IL-10, MCP-1 and MIP-2 mRNA expression in non-rejected autografts [12]. It has been hypothesised that IL-10 may contribute to graft survival by blocking cytokine production and downregulating MHC class II expression [34]. Several studies showed that topical treatment with IL-10 reduced corneal inflammation after herpes simplex virus (HSV) infection [35, 36]. Local treatment with IL-10 during corneal transplantation, however, did not prolong allograft survival [37]. Although TNF-α, MCP-1 and MIP-2 are considered as inflammatory mediators [19], and IL-12 as a pivotal factor in the development of Th1 responses [38], their role in graft rejection remains largely unknown. A number of studies have indicated that IL-12 and TNF-α may have a beneficial role in graft acceptance. IL-12 expression was found in long term cardiac allografts after rapamycin treatment [39]. Moreover, IL-12 antagonism, by antibodies or an IL-12p40 homodimer receptor antagonist, exacerbated cardiac allograft rejection [40]. Interestingly, Voest *et al.* [41] have described that IL-12 is capable of blocking corneal neovascularization. In view of this activity, expression of IL-12 in the cornea after transplantation may serve to limit angiogenesis. With regard to TNF, it has been shown that systemic administration of this cytokine decreased skin allograft rejection [42]. This tolerizing effect may result from a strong inhibition of the antigen presenting capacity of dendritic cells by TNF [43].

In summary, clodronate-LIP treatment markedly altered the cytokine mRNA expression profile in corneal allografts of rats. Whether the absence or the long-term presence of certain cytokines in the cornea is necessary to acquire definite graft acceptance remains to be clarified.

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Chapter 7

Effect of macrophage depletion on immune effector mechanisms during corneal allograft rejection in rats

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Submitted

SUMMARY

In rats, corneal allograft rejection is delayed for at least 100 days by clodronate liposomes. These liposomes selectively deplete macrophages. To investigate the immunological basis for absence of graft rejection in treated rats, the effect of these liposomes on the generation of cytotoxic T lymphocytes (CTL) and antibody production after orthotopic corneal allotransplantation was determined. Corneal transplantations were performed in AO rats, receiving corneal buttons from PVG rats. Post-operatively, one group received clodronate liposomes subconjunctivally at five time points, whereas the other group remained untreated. On post-operative days (POD) 3, 7, 12 or 17 rats were sacrificed and the presence of CTL's was investigated at three different anatomic locations and antibodies against donor antigens were tested. No significant differences were found between the groups tested 3 and 7 days post-operatively. But on POD 12 (the time of onset of rejection in the untreated group) and on POD 17, the CTL activities detected in the submandibular lymphnodes ($p \leq 0.008$) and the spleen ($p \leq 0.009$) were significantly less in the treated groups compared to the untreated groups. In the untreated groups complement-independent antibodies were present only on POD 17, whereas no antibodies were found in the treated rats. Local treatment with clodronate liposomes was shown to downregulate local and systemic CTL responses and to prevent the generation of antibodies. Local depletion of macrophages in the initiation phase of the immune response appears to lead to a less vigorous attack on the grafted tissue and therefore to promote graft survival.

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INTRODUCTION

As remedy for corneal defects, corneal transplantation appears to be very effective. Therefore, each year a large number of corneal transplantations is performed with an estimated 3-years failure rate at 10-40 %[1,2,3]. The major cause for corneal graft failure is immunological rejection of foreign tissue. The aim of topical or systemic administration of corticosteroids is to suppress this immune response, but this is not always efficient. To improve the outcome of high-risk corneal grafts in particular, research is performed in order to find new therapies which will be more effective than those currently used. Promising studies have been performed using oral immunization [4], blocking particularly cytokines or adhesion molecules [5,6,7] or local blocking with anti-CD4 [8].

Histopathological studies of corneal graft rejection show an influx of not only T lymphocytes but also many macrophages [9]. Although the current opinion is that rejection is mediated by T lymphocytes, Van der Veen *et al.* [10] performed a study to determine the contribution of the macrophage to immunological corneal graft rejection in high-risk rats. Using dichloromethylene diphosphonate (clodronate) containing liposomes, macrophages could be selectively depleted. Subconjunctival administration of these liposomes after orthotopic corneal transplantation to recipient rats with a history of corneal inflammation, surprisingly resulted in complete graft survival in all treated rats for a follow-up period of 100 days, whereas the control group rejected their grafts between 12 and 17 days post-operatively. Therefore macrophages also seem to play an important role in corneal graft rejection.

It remains to be determined how the clodronate liposomes interfered in the rejection process. With regard to the various functions of macrophages, they can be involved in both the afferent and the efferent arc of the immune response leading to graft rejection. Via processing and presentation of foreign antigen to T-lymphocytes they can participate in the afferent arc, but they are also able to play the role of effector cells and destroy the graft [11,12]. In this study we used a different rat strain combination without prior induction of keratitis to validate the clodronate-liposome treatment. To unravel the immunological basis for the absence of graft rejection in clodronate treated rats we used this strain combination to measure the CTL and antibody responses from POD 3 to 17.

MATERIALS AND METHODS

Experimental animals

Inbred male AO/OlaHsd (RT1u) and PVG/OlaHsd (RT1c) rats were obtained from Harlan UK Ltd., Bicester, England. At time of transplantation, AO recipients and donor PVG rats were 10-12 weeks of age. The animal studies were approved by the Animal Care Committee of the Royal

Netherlands Academy of Arts and Sciences and conformed to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

Donor rats were killed by an intracardial injection of pentobarbital (0.5 ml; Euthesate®, Apharmo, Arnhem, the Netherlands). Recipients were anesthetized by an intramuscular injection of a mixture of fluanisone and fentanylcitrate (0.5 ml/kg; Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and an intraperitoneal injection of diazepam (2.5 mg/kg). Oxybuprocaïne hydrochloride (0.4 %) was used for topical anesthesia of the cornea.

Orthotopic Corneal Transplantation

Full-thickness corneal transplantations were performed in the right eye of the recipient rat using the technique previously described [10]. Briefly, prior to surgery dilation of the iris was obtained via a subcutaneous injection of atropine sulphate (0.15 mg/kg) and topical administration of atropine sulphate 1 % and phenylephrinehydrochloride 10 %. Using a 3-mm trephine and curved Vannas scissors the corneal button was removed from the donor and the recipient rat. The donor cornea was stored in Eagle's modified essential medium with 2 % foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) until use. Eight stitches were made using a continuous 10/0 monofilament nylon suture (Alcon laboratories, Ft. Worth, TX) to secure the donor button into the recipient graft bed. Post-operatively no attempt was made to reform the anterior chamber or to remove the suture.

Clinical Evaluation

Grafts were observed for occurrence of graft rejection using an operating microscope, on each alternate day till post-operative day (POD) 14 and once a week thereafter. Rejection was diagnosed as an opacity score of 3 or higher in a previously clear graft. Opacity is scored from 0-4, having a score of three means that the iris vessels are not visible but one can still distinguish the pupil margins [9]. Corneal transplantations that were complicated by technical failures were excluded from the study, as well as grafts which developed an opacity score of 3 or higher during clodronate liposome injections (see experimental design).

Preparation of Clodronate Liposomes

Preparation of clodronate liposomes was performed as described before [13]. In short, 75 mg of phosphatidylcholine and 11 mg of cholesterol

were dissolved in chloroform and by low vacuum rotary evaporation a thin lipid film was produced. This film was dispersed in 10 ml phosphate buffered saline (PBS) solution in which 1.8-1.9 ml dichloromethylene diphosphonate (Clodronate®, Boehringer Mannheim, Mannheim, Germany) was dissolved. The suspension was maintained at room temperature for 2 hours followed by sonication for 3 minutes in a waterbath sonicator. After another 2 hours at room temperature, the dichloromethylene diphosphonate liposome suspension was centrifuged at 100 000 g for 30 minutes to remove free dichloromethylene diphosphonate. The liposomes were resuspended in 4 ml PBS and stored at 4°C until use.

Experimental Design

Ninety-one transplantations were performed of which 5 had to be excluded due to technical failures of the transplantation, such as extensive synechiae and hyphema, and one because of an anesthesia problem. Four of the transplantations were autografts. The allografted animals were divided into two experimental groups; an untreated and a clodronate liposome treated group. The latter received subconjunctival injections of clodronate liposomes immediately after transplantation and on POD 2, 4, 6 and 8. At each time point a total of 100 µl of these liposomes was injected near the limbus, dispensed over four locations (± 25 µl each), to achieve an equal distribution around the cornea. Previously, it was demonstrated that during clodronate liposome injections the graft became slightly more opaque than the untreated grafts [10]. It was found that an opacity score of three or higher was not acceptable, because the graft will not regain clarity after POD 8. For this reason 3 of the 41 clodronate liposome treated animals had to be excluded from the study because the graft rejection time could not be determined. In these rats failure is thought to be due to mechanical reasons, such as liposome clotting or to increased pressure around the eyeball. An immunological cause for rejection seems improbable so early after grafting in an avascular cornea and never occurred this early in the untreated group.

Experiment 1

Twenty-one grafts were followed for the appearance of graft rejection for 100 days: the four autografts in AO recipients were used to test for the correct transplantation technique and the 9 untreated allografts to determine the rejection time. Eight allogeneic grafts were treated with clodronate liposomes to check whether this treatment also prevented graft rejection in the PVG / AO rat strain combination.

Experiment 2

To determine the immunological basis for the absence of graft rejection after clodronate liposome treatment, rats were sacrificed at four different time points. Two time points were chosen before rejection occurs

in the untreated allogeneic group (POD 3 and POD 7), one at the onset of graft rejection in the untreated allogeneic group (POD 12) and approximately one five days after rejection (POD 17). Lymphoid cells were isolated from three locations to determine the presence of CTL's: the submandibular lymphnodes (SLN), the mesenteric lymphnodes (MLN) and the spleen. To analyze for the presence of alloantibodies, serum was obtained at these same time points. All serum samples were heat-inactivated and stored at – 20°C in small aliquots until use.

Experiment 3

To exclude the possibility that local administration of clodronate liposomes suppresses the whole immune system, five AO-rats were immunized intraperitonealy with PVG-lymphocytes. Three of them were treated with subconjunctival injections of clodronate liposomes on the day of immunization and 2, 4, 6, and 8 days later, the other two rats remained untreated. Ten days after immunization, rats were sacrificed and the presence of CTL's and antibodies directed towards PVG-lymphocytes was tested in a chromium release assay and immunofluorescence assay.

Cell-mediated Cytotoxicity Assay

The specific cytotoxic activity of lymphoid cells derived from grafted rats was determined via a ^{51}Chromium Release Assay. Cells isolated from the SLN, MLN and spleen were measured separately. Single-cell suspensions were prepared by gently teasing the tissue through a nylon mesh strainer (70 µm Cell Strainer, Falcon, Becton Dickinson, Franklin Lakes, NJ). The cell suspensions were washed twice and resuspended in culture medium [RPMI 1640 containing 2.0 mM L-glutamine, 10 mM HEPES buffer (Life Technologies), 100 IU penicillin/ml, 100 µg streptomycin/ml, 10 % heat-inactivated fetal calf serum (Life Technologies) and 3×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO)] at a concentration of 5×10^6 ml. Thymocytes derived from a young naive PVG rat were prepared as described above and irradiated (30 Gy) with a ^{137} Cesium source. These irradiated stimulator cells were adjusted to a cell concentration of 5×10^6 ml, 0.5 ml of this suspension was mixed with 1 ml effector cells in wells of 24-well trays (Costar, Cambridge, MA, USA), total volume 1.5 ml, and incubated at 37°C in a humidified 5 % CO_2 atmosphere. After 5 days the effector cells from 6 identical wells were pooled and cell viability was determined by trypan blue exclusion. Cells were centrifuged at 200 g for ten minutes and resuspended in culture medium at the following concentrations (10×10^6/ml; 5×10^6/ml; 2.5×10^6/ml; 1.25×10^6/ml).

Submandibular and mesenteric lymphnodes from a naive PVG rat served as target cells. Target cells were cultured for five days in culture

medium at a concentration of 5×10^6 cells/ml, after two days in culture Concanavalin A (2 µg/ml, Life Technologies) was added. On the day of the assay, target cells were washed and labeled with 100 µCi $Na_2{}^{51}CrO_4$ (Amersham, Little Chalfont, UK) at 37°C for one hour. Labeled cells were washed three times with culture medium, incubated for 30 minutes in 20 ml culture medium to remove spontaneously released ^{51}Cr, centrifuged (10 minutes, 200 g) and resuspended at a concentration of 2×10^5 cells/ml. Fifty µl of labeled target cells (10^4 cells) were plated into wells of round-bottom 96-well microtiter plates (Costar). 100 µl of effector cells in different concentrations were added in triplicate to the target cells, effector to target ratios ranged from 100:1 to 12.5:1. Plates were centrifuged at 100 g for 3 minutes, followed by a 4-hour incubation at 37°C in a humidified 5 % CO_2 atmosphere. After 4 hours the plates were centrifuged for 10 minutes at 500 g and 100 µl supernatant was removed from each well for counting of radioactivity in a Packard Auto-gamma spectrometer. The cytotoxicity of each sample was determined as follows:

$$\% \text{ Specific } {}^{51}Cr \text{ release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Spontaneous release was determined by counting supernatants from wells containing target cells and culture medium only. The maximum release was determined by counting the supernatant of wells containing target cells and 1 % Triton X-100 (Sigma). The CTL-assays were tested each time for reproducibility using cell suspensions derived from submandibular lymphnodes, mesenteric lymphnodes and the spleen of naive AO rats and of AO rats which had been immunized against alloantigens by repeated intraperitoneal injections with PVG lymphocytes and splenocytes.

Assay of Alloantibodies in serum

The presence of specific antibodies against PVG cells was tested via an indirect immunofluorescence assay. PVG lymphocytes were isolated and cultured for 3 days in the presence of Concanavalin A (2 µg/ml) to increase their size and enhance their MHC expression. After three days, the cells were washed and fixed with 2 % paraformaldehyde. Subsequently, these fixed cells were washed twice in washing medium [PBS, 1 % Bovine Serum Albumin (Sigma) and 0.2 % sodium azide], centrifuged for 10 minutes at 200 g and resuspended at 5×10^6 cells/ml. 10 µl serum was added to 90 µl of these cells. After an incubation for one hour, cells were washed and incubated for another hour at 4°C with a 1/50 dilution of rabbit anti-rat Ig antibody labeled with fluorescein isothiocynate (Rara-Ig-FITC, Nordic, Tilburg, the Netherlands). Cells were washed once and one drop of Evans blue was added to the pellet and cells were washed again. Finally, they were suspended in PBS-glycerol, a drop of Vectashield was added and mounted on a glassslide with a coverslip. The cells were scored for the

presence of immunofluorescence positive cells by a masked observer. The % of positive cells was calculated. In each assay sera from grafted rats and samples from a pool of naive AO-rats or from a pool of AO-rats immunized against PVG antigens were included.

Assay of Complement Dependent Alloantibody in serum

To test the specific cytotoxic activity of the serum samples a chromium release assay was used. The ^{51}Cr labeled target cells were prepared as described above. Fifty µl of these cells (5×10^6 cells/ml) were plated into wells of a round bottom 96-well plate and incubated with 50 µl serum dilution (1/10, 1/20, 1/40 and 1/80) obtained from grafted rats, at 37°C for 60 minutes. Subsequently 50 µl of fresh normal rat serum was added, as source of complement, and the incubation continued for 60 minutes. The plates were then centrifuged at 500 g for 10 minutes and 100 µl of the supernatant was removed and counted in a gamma counter. The % specific release of serum samples was determined as described above. In each assay, a serum sample from a pool of naive AO-rats and a serum sample from a pool of AO rats which have been immunized against PVG antigens were tested as a standard.

Statistical Analysis

The graft survival curves were compared using the Log Rank test. Statistical analysis of the cytotoxic and immunofluorescence assays was performed using the Mann-Whitney U test. The two groups were compared at the effector / target ratio 100:1 and serum dilution 1/10. The statistical analysis were corrected for multiple comparisons; $P < 0.03$ was considered statistically significant.

RESULTS

Fate of orthotopic corneal grafts

Eighty-two orthotopic corneal grafts were observed for occurrence of graft rejection (Fig. 1). The 21 rats of experiment 1 were evaluated for the follow-up period of 100 days, whereas the 61 rats used in experiment 2 were evaluated till they were sacrificed at either POD 3, 7, 12 or 17. All four autografts survived for at least 100 days. Of the 40 untreated rats with a graft, no graft survival was observed beyond twelve days (mean survival time of 10.9 days). Of the 38 allogeneic corneal grafts that received clodronate liposome treatment not a single graft rejection was observed. In the eight clodronate treated rats of experiment 1 graft survival was prolonged beyond 100 days. Comparing the survival times of the two groups a significant difference of $P < 0.001$ was found.

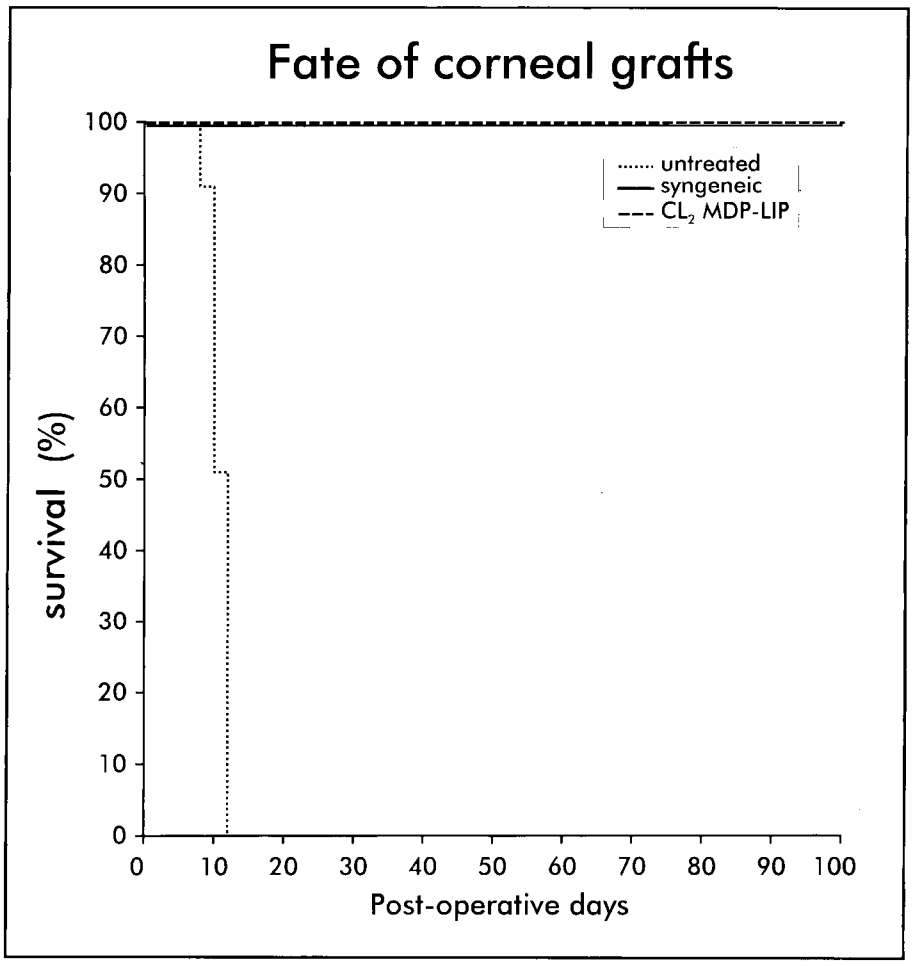

Figure 1 – Survival curves of 82 orthotopic corneal grafts in AO-rats. Three experimental groups were distinguished; AO-rats receiving autografts (----); untreated allografted AO-rats (-..-..); and clodronate-LIP treated allografted AO-rats ⟵). A significant difference was found ($p < 0.001$) comparing the clodronate-LIP treated allografts with the untreated allografts.

Cytotoxic T Lymphocyte activity against donor antigens in recipients of corneal allografts

In experiment 2, at four different time points after orthotopic allotransplantation untreated AO-rats and clodronate-liposome treated AO-rats were sacrificed to determine the presence of CTL's against donor PVG cells. Cells were isolated from three locations to determine differences in local and systemic CTL activity between the two groups. Local CTL activity was determined by taking cells derived from the submandibular lymphnodes (Fig. 2). Comparing the two treatment groups at the effector to target ratio 100:1, no difference was noticed on POD 3 and 7. But at time of the onset of graft rejection in the untreated group, POD 12 and five

days later, a significant difference was noticed ($p = 0.006$, resp. $p = 0.008$). Far higher CTL activity was found in the untreated rats compared to the clodronate-liposome treated rats. The second more remote location investigated for presence of CTL's was the mesenteric lymphnodes. No difference was detected at any of the four post-operative time points (Fig. 3). The spleen was assayed for presence of «systemic» CTL activity; a similar pattern as in the local lymphnodes was found (Fig. 4). Before graft rejection in the untreated group, no activity was present, but at the time of graft destruction, significantly more CTL activity was present in the spleen of untreated rats compared to treated rats. A difference was demonstrated at both POD 12 ($p = 0.008$) and later POD 17 ($p = 0.009$).

Alloantibody response against donor antigens in recipients of allografts

The presence of antibodies directed towards donor PVG cells was tested in an indirect immunofluorescence assay. Results from sera of allografted rats are shown in Table 1. In contrast to the differences found between the two treatment groups in regard to CTL activity, a significant difference was detected only on POD 17 ($p < 0.01$). At that post-operative time point a higher percentage of alloantibodies was circulating in the blood of untreated allografted rats.

Complement dependent cytotoxic activity against donor antigens in recipients of allografts

To determine whether there is a difference in presence of complement dependent cytotoxic antibodies between the two groups, particularly on POD 17, chromium release assays were performed. Four serum dilutions at the four different time points were tested. As was to be expected no detectable cytotoxic activity was found in any of the serum samples, in clodronate-liposome treated rats, or in untreated rats on POD 3, 7, and 12. Moreover on POD 17, no cytotoxic antibodies were detectable in the serum of untreated allografted rat. The results obtained for the serum dilution 1/10 are summarized in Table 1.

Effect of subconjunctival administration of clodronate-liposomes on immune responses to intraperitonealy injected allogeneic lymphocytes

After intraperitoneal injections of PVG-lymphocytes, high CTL responses were detected in both submandibular lymphnodes and mesenteric lymphnodes in untreated rats as well as clodronate-liposome treated rats (Table 2). Also, the generation of complement dependent antibodies was not downregulated by subconjunctival injections of clodronate-liposomes in systemically immunized rats (Table 3).

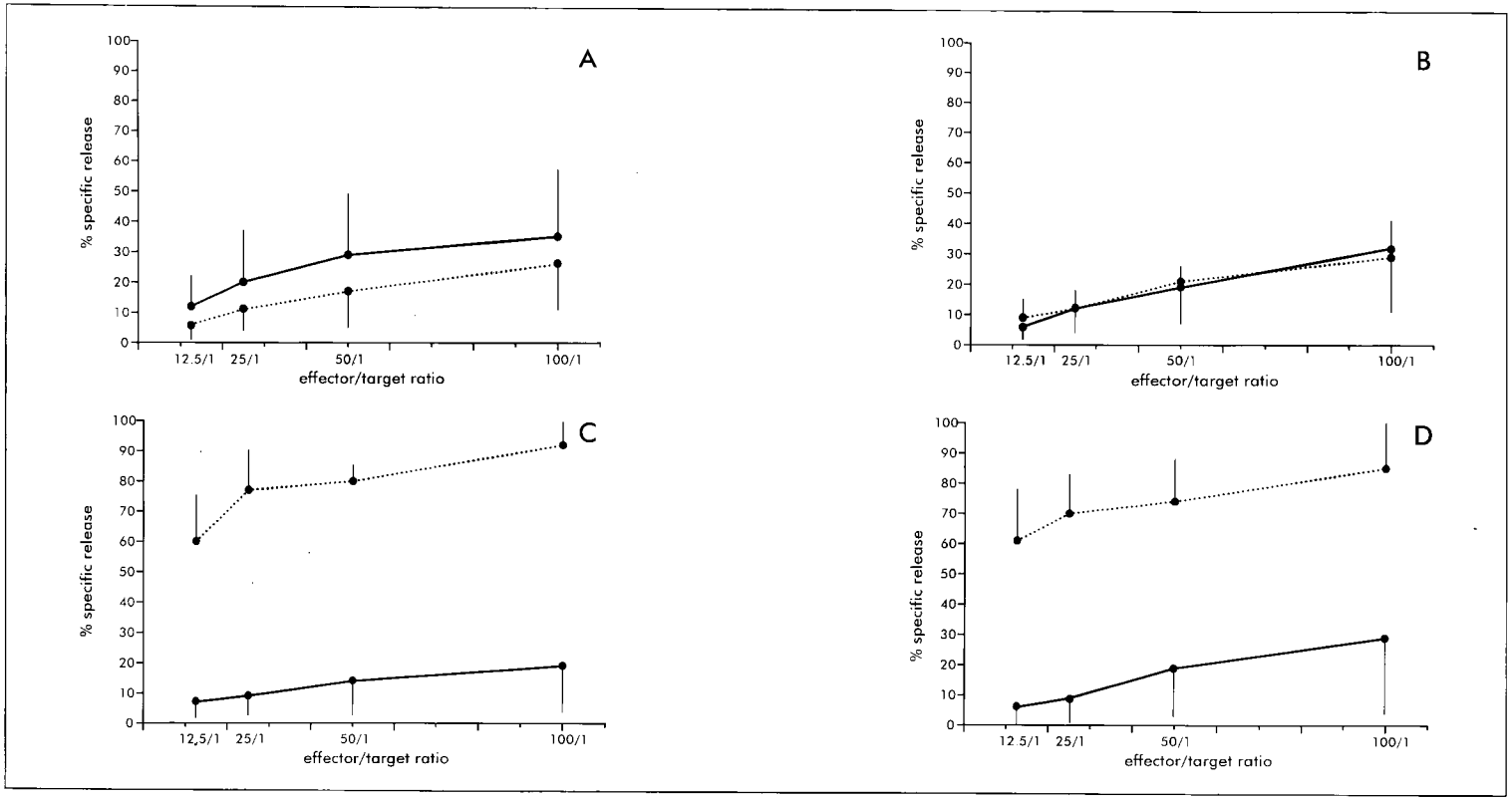

Figure 2 – Effect of clodronate-LIP treatment on CTL-responses in the submandibular lymphnodes on POD 3 (A); POD 7 (B); time of onset of graft rejection in the untreated allografts, POD 12 (C); and POD 17 (D). Values represent the mean % specific release ± SD of 5-7 rats per group. The clodronate-LIP treated group (—) was compared with the untreated allografted group (....) at the effector / target ratio 100:1 and a significant difference was observed at POD 12 ($p = 0.006$) and POD 17 ($p = 0.008$).

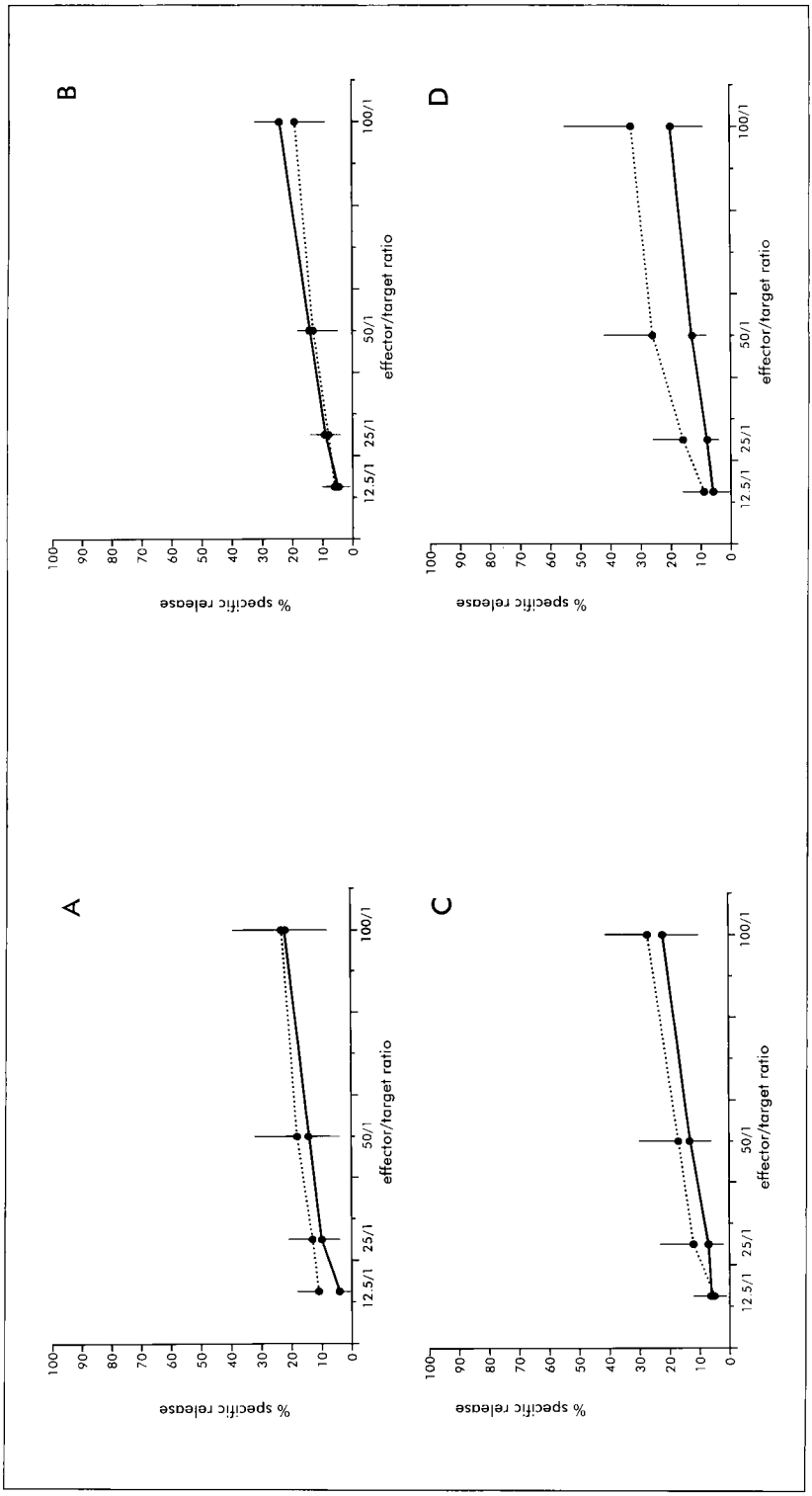

Figure 3 – Effect of clodronate-LIP treatment on CTL responses in the mesenteric lymphnodes on POD 3 (A); POD 7 (B); time of onset of graft rejection in the untreated allografts, POD 12 (C); and POD 17 (D). Values represent the mean % specific release ± SD of 5-7 rats per group. The clodronate-LIP treated group (—) was compared with the untreated allografted group (....) at the effector / target ratio 100:1 and no significant difference was observed.

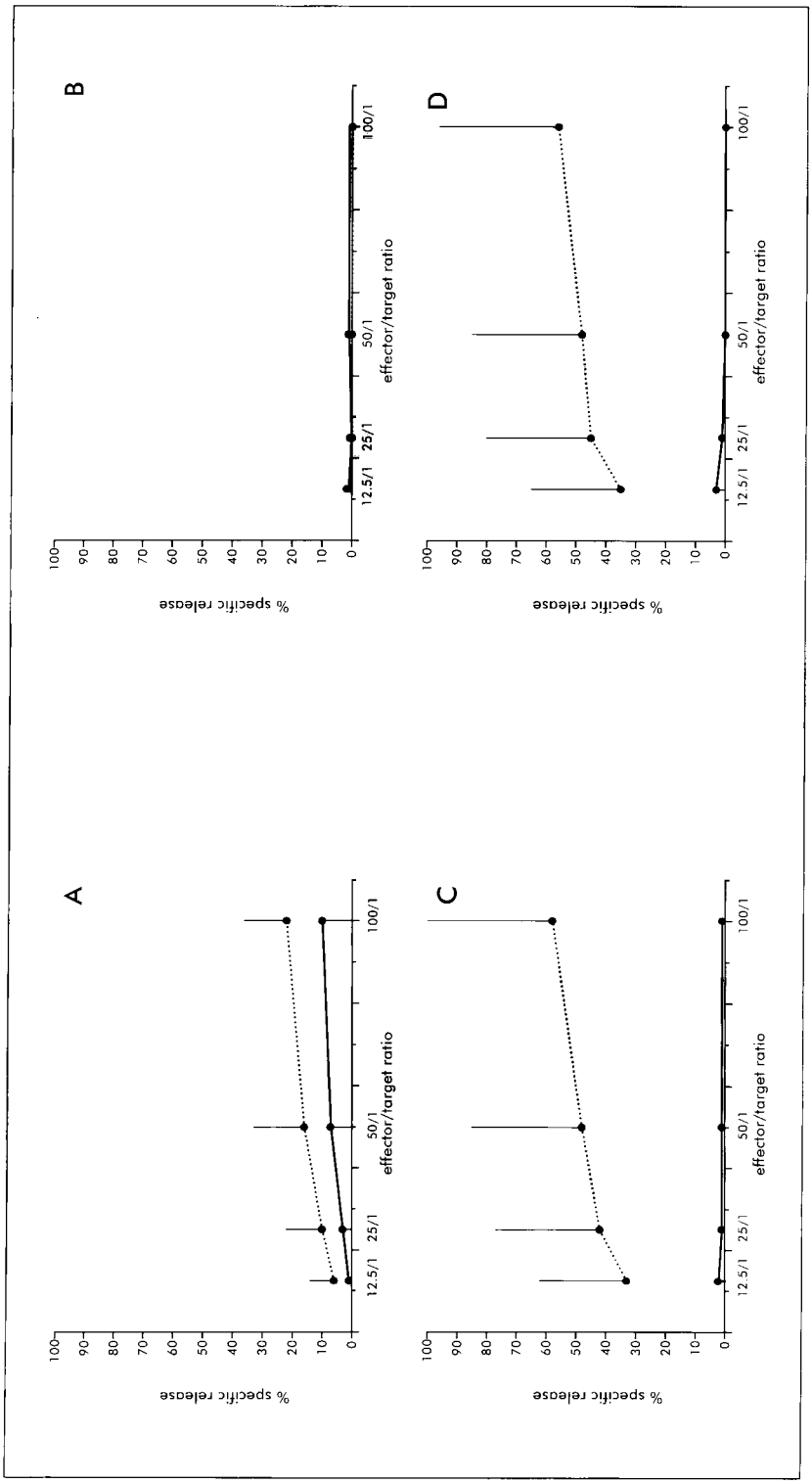

Figure 4 – Effect of clodronate-LIP treatment on CTL responses in the spleen on POD 3 (A), POD 7 (B), time of onset of graft rejection in the untreated allografts, POD 12 (C), and POD 17 (D). Values represent the mean % specific release ± SD of 5-7 rats per group. The clodronate-LIP treated group (—) was compared with the untreated allografted group (....) at the effector / target ratio 100:1 and a significant difference was observed at POD 12 ($p = 0.008$) and POD 17 ($p = 0.009$).

TABLE 1 – **EFFECT OF CLODRONATE LIPOSOMES ON HUMORAL IMMUNE RESPONSE AFTER ORTHOTOPIC TRANSPLANTATION**

	POD 3		POD 7		POD 12		POD 17	
	LIP	Untr.	LIP	Untr.	LIP	Untr.	LIP	Untr.
Antibodies *	2 ± 2	2 ± 3	2 ± 3	2 ± 2	3 ± 1	4 ± 1	3 ± 3 **	36 ± 12
Compl. dependent antibodies ***	0 ± 0	0 ± 0	1 ± 1	0 ± 0	1 ± 1	0 ± 0	2 ± 1	3 ± 5

Alloantibody response, towards PVG-lymphocytes, in AO-rats after orthotopic allotransplantation. Clodronate-liposome treated rats (LIP) are compared with untreated rats (Untr.) at different PODs for the serum dilution 1/10. Values are presented as mean ± standard deviation.

* Immunofluorescence assay (% positive staining cells)
** $p < 0.01$
*** Chromium release assay (% specific ^{51}Chromium release)

TABLE 2 – **EFFECT OF SUBCONJUNCTIVAL ADMINISTRATION OF CLODRONATE LIPOSOMES ON SYSTEMIC CELLULAR IMMUNE RESPONSES**

E/T RATIO	SLN		MLN		Spleen	
	Clodronate-LIP treated	Untreated	Clodronate-LIP treated	Untreated	Clodronate-LIP treated	untreated
12.5/1	43 ± 31	28 ± 9	6 ± 10	20 ± 20	0 ± 0	8 ± 10
25/1	67 ± 27	49 ± 4	15 ± 14	27 ± 13	2 ± 4	3 ± 4
50/1	82 ± 20	63 ± 11	31 ± 20	44 ± 10	8 ± 12	4 ± 6
100/1	100 ± 3 *	87 ± 4	52 ± 17 *	70 ± 11	14 ± 19 *	10 ± 13

Cell-mediated immune responses in AO rats immunized intraperitoneally with PVG lymphocytes. The rats were sacrificed 10 days after immunization. Values are presented as mean % specific ^{51}Chromium release ± standard deviation. The clodronate-LIP treated group was compared with the untreated group by Mann-Whitney U test for the 100/1 ratio.

* $P > 0.05$.

TABLE 3 – **EFFECT OF SUBCONJUNCTIVAL ADMINISTRATION OF CLODRONATE LIPOSOMES ON SYSTEMIC HUMORAL IMMUNE RESPONSES**

SERUM DILUTION	Clodronate-LIP TREATED	UNTREATED
1/80	38 ± 27	15 ± 21
1/40	37 ± 32	17 ± 24
1/20	49 ± 28	34 ± 25
1/10	60 ± 12 *	38 ± 2

Alloantibody response in AO rats immunized intraperitoneally with PVG lymphocytes. The rats were sacrificed 10 days after immunization. Values are presented as mean % specific ^{51}Chromium release ± standard deviation. The clodronate-LIP treated group was compared with the untreated group by Mann-Whitney U test for the 1/10 dilution.

* $P > 0.05$.

DISCUSSION

Previously it was shown that subconjunctival administration of clodronate liposomes after orthotopic corneal allotransplantation prevents graft rejection in high-risk rats beyond 100 days [10]. This in vivo depletion of macrophages after transplantation revealed that the presence of macrophages is mandatory for the rejection process. It is however unclear where in this process the clodronate-liposomes are interfering.

The current study determined the influence of macrophage depletion on the presence of CTL's and antibodies early after transplantation. In the first week after transplantation no significant difference was found between the two treatment groups. But when the onset of graft rejection occurred in the untreated rats (POD 12) and on POD 17, the CTL activity detected locally and systemically was significantly less in the treated group. Also generation of antibodies seemed to be impaired in macrophage depleted rats.

In our model, graft rejection seemed to correlate with increase of CTL activity in the local lymph nodes and the spleen on POD 12 and 17. Although corneal graft rejection is possible in the absence of CD8+-CTL's [14, 15], it has been shown that rejection can be accompanied by high CTL responses in local lymph nodes or spleen [16, 17, 18]. Van der Veen *et al.* [17] tested the same locations for presence of cytotoxic activity, directed towards donor antigens, in macrophage depleted rats after the onset of rejection had occurred. Although no difference was detected in local CTL activity, macrophage depletion downregulated CTL-activity in the mesenteric lymph nodes as well as the spleen. The results, obtained from both local and more remote lymph nodes, are in slight contrast with this study. Also the difference found between the two groups was more pronounced in this study. A possible explanation for these findings may be the use of a different rat strain combination and / or the induction of a keratitis prior to transplantation by van der Veen. The eight clodronate-liposome treated allogeneic grafts performed in experiment 1 demonstrated that administration of clodronate liposomes is also effective in prolonging graft survival in this rat strain combination for at least 100 days. Although the CTL responses were more vigorous in these untreated rats, the CTL response was still downregulated and graft rejection was prevented by macrophage depletion. Early rejection after small bowel allotransplantation is also characterized by a massive influx of macrophages [19]. In this type of solid graft rejection the macrophages seem to be mandatory as well, since intraperitoneal injection of clodronate liposomes prolonged graft survival [20]. Although the precise role of the macrophage in the graft rejection process is unknown, their presence is required for inducing donor directed cytotoxicity responses by intestinal epithelial cells [21].

Another effect of the clodronate liposomes was inhibition of antibody production. Our model showed that in untreated rats, antibodies against donor antigens were detectable 5 days after the onset of graft rejection using an immunofluorescence assay. Unfortunately it was not possible technically to perform an ADCC assay, but since the results of the

chromium release assay show that these antibodies were not complement dependent, these results indirectly suggest that they might be cell dependent cytotoxic antibodies. The study by van der Veen [17] also showed that macrophage depletion impaired generation of complement dependent antibodies after corneal transplantation. Lung allotransplantation leads to production of both types of antibodies, depleting donor macrophages prior to transplantation, by intratracheally instillation of clodronate-liposomes, does not prolong graft survival. But the antibodies involved both in ADCC and complement dependent cytotoxicity, were significantly decreased [22]. Also the presence of these antibodies is found in chronic rejection of cardiac allografts and was related to activation of macrophages [23].

Using the corneas from the rats used in this study, we also determined the effect of administration of local clodronate-liposome on cytokine mRNA expression within the cornea (IOVS, 1999, in press). In the macrophage depleted rats, decreased expression of IL-1β, IL-2, IL-4, IL-6, IFN-γ and TNF-β/LT mRNA was found. Treatment with clodronate-liposome after corneal grafting thus resulted in decreased local mRNA expression of several macrophage derived cytokines as well as T-cell derived cytokines, and reduced levels of CTL's and antibodies.

As mentioned earlier, corticosteroids have been found to be very effective in preventing graft rejection, but next to the fact that not all rejections can be inhibited, this drug also has a lot of side-effects. In attempting to find new therapeutic regimens, an important goal is the absence of systemic side-effects. A major concern for the mechanism by which clodronate-liposomes work could be that macrophage depletion was accomplished not only locally but also systemically. Experiment 3 showed that subconjunctival injection of clodronate containing liposomes, following the same procedure as after corneal transplantation, did not interfere with the cellular and humoral immune response in rats injected with allogeneic lymphocytes intraperitoneally.

At this moment a study is in process to determine the delayed type hypersensitivity (DTH) responses of clodronate treated animals after orthotopic corneal allotransplantation. Results of this study would unravel the question whether antigen presentation has occurred and the effector phase of rejection has started in these clodronate-liposome treated animals.

Our study shows that after corneal allotransplantation in untreated rats high CTL responses are detected locally and systemically, as well as complement-independent antibodies at time of rejection. We conclude that macrophage depletion, via repeated subconjunctival injections of clodronate liposomes, downregulates these high CTL responses and impairs antibody formation.

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Chapter 8

Discussion and conclusions

Allograft reactions occurring after technically successful penetrating keratoplasties influence the outcome of the grafts towards an immunologic state of enhanced corneal acceptance or corneal rejection. Despite the recent advances in eye-banking methods, surgical techniques and the use of potent immunosuppressors, corneal rejection is still the main cause of corneal failure [1]. This strongly contributes to the lack of satisfaction experienced both by patients and corneal surgeons. In spite of the extensive research performed by many groups of investigators in corneal immunopathology, the immune mechanisms that govern corneal transplantation are still poorly understood. With the results of the experiments described in this thesis we hope to have made a contribution to the knowledge on cytokine networks in corneal transplantation. Based on the analyzed cytokines, it is possible to speculate about the immune cells that may infiltrate the corneal graft and their possible role on graft outcome.

Cytokines are cell-released mediators that are implicated in cell communication and in the management of important cellular or tissue reactions, such as inflammation and the host immune defense against infection, tumor or donor antigens, the stress response, hormonal regulation, hematopoeisis and embryonic development. Cytokines can show a large range of biologic activities on disparate cells and may exhibit pleiotropic, redundant, synergistic or antagonistic effects. They play a pivotal role on the inflammatory and immune processes in organs or tissues. The chapter 2 of this thesis is dedicated to an extensive review on the role of cytokines in corneal immunopathology, including the inherent corneal defense mechanisms, the type of the immune response, angiogenesis, recruitment of phagocytes, apoptosis and wound healing. The normal cornea is devoid of blood and lymphatic vessels and constitutively expresses several mediators that are also present in the aqueous humor, which can exhibit potent anti-inflammatory properties. These facts may contribute to the maintenance of an immune privilege state observed in the anterior chamber of the eye, which has the ability to abrogate several types of immune responses. Briefly, the tissues of the eye allow alloantigens to enjoy a «peaceful» coexistence due to a specific collaboration between the immune system and the eye in order to avoid immune damage to ocular structures but giving proper immune protection at the same time. Tolerance of peripheral T

cells has been found among the mechanisms to contribute to immune privilege [2] and within the eye this occurs partly by an immune response termed anterior chamber associated immune deviation [3, 4]. This immune reaction is characterized by the fact that foreign antigens artificially introduced into the anterior chamber of the eye, generate a response which lacks CD4+T-cells that mediate the delayed type hypersensitivity and increases the production of non-complement-fixing antibodies by B-cells [5], yet allows CD8+T-cells to function as cytotoxic cells and as regulatory cells. Peripheral T-cell tolerance is also regulated by the presence of TGF-β in the aqueous humor [6]. A recent factor that is also present in the aqueous humor has been discovered and may contribute to the immune privilege by inhibiting natural killer cell functions [7]. Therefore, it is expected that the normal cornea can show unique immune reactions to infection and transplantation.

In inflammation, the cytokine cascade is probably initiated by the pro-inflammatory interleukin (IL)-1. IL-1 leads to generation of chemotactic cytokines and influx of inflammatory exsudates. IL-1 belongs to a family of mediators produced mainly by activated monocytes and macrophages. Other cell types, including the corneal cells [8, 9], are also capable of secreting IL-1. This may indicate that corneal cells actively participate in the immune processes that take place within the corneal layers. As described above and reviewed in chapter 2 of this thesis, IL-1 is involved in the induction of other pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, IL-6 [10], IL-8 [11], monocyte chemotactic protein (MCP) and regular upon activation normal T-cell expressed and secreted (RANTES) [12]. It has been observed that corneal cells upon IL-1 stimulation are also capable of producing TNF-α and IL-6 [13, 14]. Furthermore, IL-1 stimulates angiogenesis [15] as well as the expression of a variety of cell surface antigens on endothelial cell membranes, including the adhesion molecules [16]. Moreover, IL-1 endows the activation of corneal resident antigen presenting cells (APC) and also has the capacity of attracting macrophages and other APCs to the inflamed tissues [17]. These cells contribute to the anti-allograft reaction effector mechanisms by presenting the donor antigens to the resting T-lymphocytes in the regional lymphnodes.

From the observations described above, it can be concluded that IL-1 may play a central role in generating immune reactions within the cornea. Therefore, it may be hypothesized that by blocking corneal IL-1 induced responses, the inflammatory sequelae, scarring, rejection and consequently visual acuity impairment are probably prevented. This postulate is emphasized by the fact that our group was the first to report that interleukin-1 receptor antagonist (IL-1RA) is constitutively expressed by the human cornea (chapter 3), a finding that was confirmed later by others in the USA [18]. The corneal epithelial cells were found to be the major source of corneal IL-1RA. Due to their anatomical position in the cornea and to the fact that they are continuously exposed to the environment, IL-1RA production by these cells implies the existence of an inherent defense mechanism for IL-1 mediated responses. IL-1RA is the natural antagonist of IL-1, competing

with IL-1 molecules to the IL-1 receptors present on the surface of several types of cells (Fig. 1). Moreover, IL-1RA is effective in inhibiting Langerhans cells (LC) and probably other antigen presenting cell migration to the center of the cornea as well as in promoting ocular immune privilege [19]. Apparently, these may be the mechanisms by which IL-1RA enhances corneal graft survival in mice [20]. These findings together with future investigations on the effectiveness, solubility and toxicity of IL-1RA will encourage its use in penetrating keratoplasty in high-risk patients.

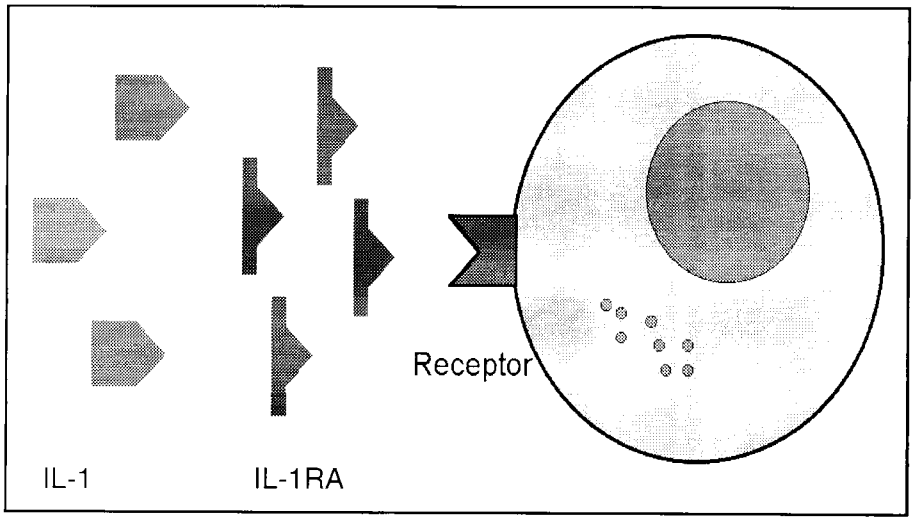


Figure 1 – Interleukin-1 receptor antagonist competes with IL-1 molecules to the IL-1 receptors on cell membranes.

In animal models it has been shown that the first inflammatory cells to infiltrate corneal allografts are detected immediately after the penetrating keratoplasty. This early infiltration consists of lymphocytes and monocytes / macrophages [21]. If a process of rejection advances, more leukocytes are triggered to infiltrate the graft. Rejected corneas show a higher degree of immune cellular infiltrate when compared to tolerant corneas. The anti-allograft immune responses are extremely complex and are not completely understood. Briefly, the immune response against the donor is induced by the presentation of major (and possibly also minor) histocompatibility complex (MHC) class antigens by antigen presenting cells to alloreactive T-cells. It is possible that, in the cornea, this recognition can also initially occur on the transplanted allogeneic APCs, in spite of the fact that the normal central cornea is usually devoid of these cells. Nevertheless, Langerhans cells, macrophages and other APC are constitutively present at the conjunctiva, limbal area and peripheral cornea [22]. These cells may migrate towards the center of the cornea upon cytokine stimulation in response to trauma, such as donor cornea handling and manipulation during preservation and preparation of the button for keratoplasty. This type of recognition is called direct recognition, and can be best demonstrated by

the observation that elimination of corneal LCs prior to transplantation by UV exposure, is followed by enhanced corneal allograft survival [23]. Corneal rejection can be observed when the amount of antigen presenting cells in the donor cornea is increased artificially. In opposition to direct recognition is the indirect recognition. This type of recognition is the normal mechanism of induction of a T-cell response. Recipient APCs present the donor antigens to T-cells in the context of self-MHC and thereby contribute to the development of corneal rejection. This results in the production of T-cell derived cytokines, which may influence the inflammatory reaction by regulating the influx of different types of cells into the graft, altering class I and class II expression, and by activating other cells like T- and B-cells, natural killer (NK) cells and macrophages as well as the activation of other T-cell related effector mechanisms [24]. When both types of antigen recognition that may take place *in vivo* are blocked, the delayed type hypersensitivity (DTH) and cytotoxic T-lymphocytes (CTL) responses to alloantigens are abolished. These findings emphasize the importance of the T-cell effector mechanisms in corneal rejection. At present, it is not clear what is the nature of an antigen presenting cell. However, it has been suggested that cells able to express MHC class II molecules at their surface under normal conditions or de novo after exposure to inflammatory cytokines such as IFN-γ, can contribute to the activation of alloreactive T-cells and therefore to the initiation of a rejection process. Activation of memory T-cells is however considered to take place after stimulation by «professional» antigen presenting cells (MHC class II positive dendritic cells) in the context of appropriate co-stimulation.

Once the allogeneic immune response is initiated, a complex mechanism consisting of inflammatory and immune cells and regulatory cytokines is immediately encountered. However, as little is known about the molecular mechanisms governing the interactions between cells, we decided to analyze the role of cytokines during corneal transplantation. A sequence of several experiments was designed using a rat model of allotransplantation developed in our laboratory. AO and PVG rats were used for that purpose. These rats differ completely in their MHC antigens. The AO rat is an albino rat (Fig. 2) whereas the PVG rat is dark with a heavily pigmented iris (Fig. 3). In all experiments, the AO rat was the recipient of a PVG cornea. The combination PVG to AO was selected due to the reliability and reproducibility of the model, which included a short period of time between corneal surgery and the appearance of the first clinical signs of corneal rejection. In all cases, rejection was evident 10 to 12 days after surgery. Technically, penetrating keratoplasty in the rat resembles the surgery performed in the human, except for several features that are described below. Large midriasis (Fig. 4) was induced in the recipient eye in order to prevent direct trauma to the iris due to the shallowness of the anterior chamber. Trauma to the albino iris may cause hyphema and anterior synechia, which may negatively influence the outcome of the graft and consequently the results of the experiments. The recipient and donor eye was proptosed and fixated with a forceps while a 3.0 diameter corneal trephine was used and twisted until the cornea was

perforated (Fig. 5). Curved fine scissors were used to complete corneal dissection. Donor corneas were then stored in Eagle's modified essential medium with 2 % fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Breda The Netherlands) until use. Due to the softness of the corneas and to the small size of corneal grafts, reference stitches at cardinal points were not given at any moment. To avoid the spinning of the graft, we developed a special method to perform the 10/0 nylon uninterrupted running suture. After each intrastromal stitch a large loop of monofilament was left behind (Fig. 6 and 7) and at the moment of the last stitch, the monofilament was pulled from 6 to 12 o'clock in both nasal and temporal quadrants. The final knot was then made and located as far as possible form the limbal area to avoid inflammation and neovascularization. One peculiar characteristic of this surgery is the fact that the rat can be rotated 360°, which avoids the necessity of the surgeon to change the position of his hands to make the stitches in opposite quadrants. No attempts were made to refill the anterior chamber of the eye at the end of the surgery (Fig. 8). The same rat model was used in all experiments performed and described in chapters 4 to 7.

Chapter 4 documents the mRNA expression of several cytokines involved in corneal transplantation. T cell derived cytokines such as IL-2, IL-4 and interferon (IFN)-γ were present only in clinically rejected corneas of rats submitted to allotransplantation. Rats submitted to autotransplantation did not show corneal rejection and did not express T-cell cytokines. These results confirm that corneal rejection in the rat model is a T cell mediated response. However, no specific T helper (Th) pattern could be defined. This cytokine pattern of expression may suggest that corneal rejection is driven either by Th0 cells alone or by a combination of Th1 cells producing IL-2 and IFN-γ and Th2 cells which secrete IL-4. It was concluded that T cell derived IL-2 and IFN-γ cytokines play the most significant role in corneal rejection. IL-2 is an autocrine growth factor for the clonal proliferation of antigen reactive T cells. This will result in the development of activated CD4+ T-lymphocytes and cytotoxic T cells expressing CD8 or CD4 antigens with restriction for class I and class II respectively. Therefore, IL-2 has the capacity to propagate and intensify the cellular effector mechanism of the immune response. The most important characteristic of IFN-γ is its ability to upregulate MHC class I and class II antigen expression on graft cells, to activate macrophages in the inflamed tissues [25] and to stimulate the differentiation of T-cells to the Th1 type. Furthermore, IFN-γ also upregulates adhesion molecule stimulation [26]. By expressing more adhesion and MHC class I molecules, graft cells will be easily recognized as targets by CD8+ immune cells. Moreover, MHC class II antigen expression recognition contributes directly to the rejection process via the antigen presentation to CD4+ T cells [27]. Two other cytokines with their mRNA expressed in corneal grafts that may regulate the ongoing rejection process are IL-6 and TNF-α. IL-6 is an acute phase mediator [10] and also plays an important role in B- and T-cell activation [28].

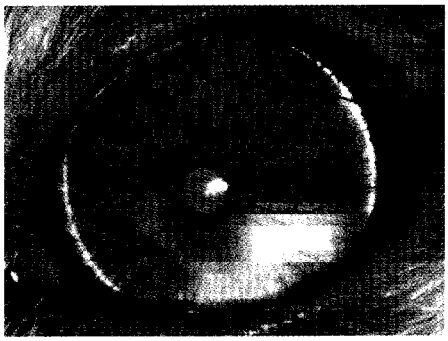
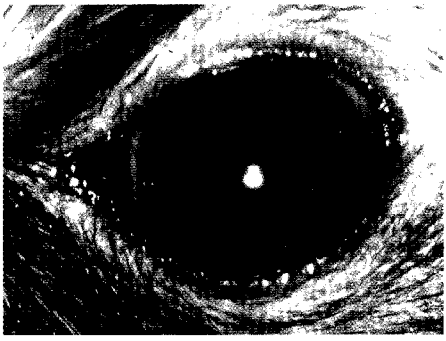


Figure 2



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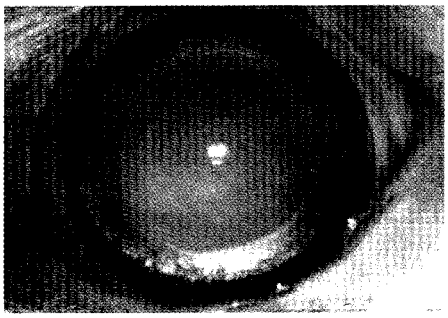
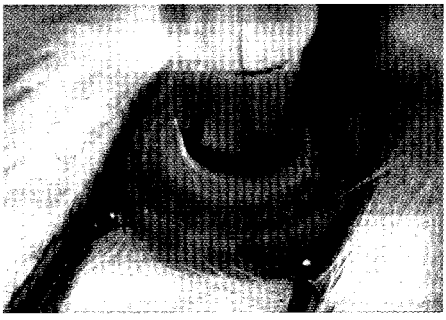


Figure 4



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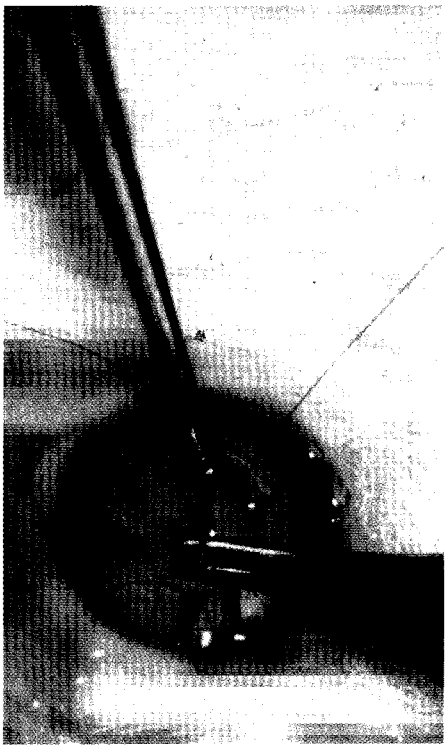

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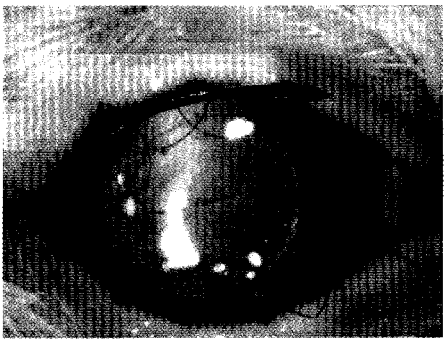

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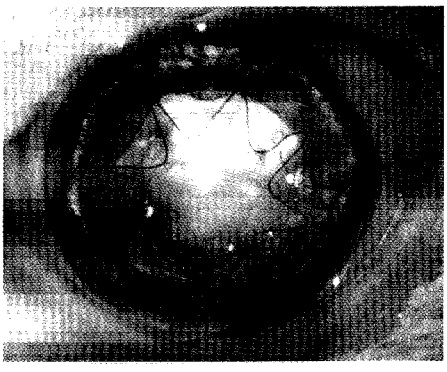

Figure 8

In addition, CTL clones can produce IL-6, which is essential for the maintenance of the cytolytic function [29]. This may suggest that IL-6 locally secreted by the infiltrated immunocompetent cells and resident corneal cells is able to govern both humoral and cellular effector mechanisms. In the kidney, IL-6 is correlated with acute rejection [30]. TNF-α is a pro-inflammatory cytokine with similar functions to IL-1. It is mainly produced by monocytes / macrophages [31], but it is also produced by corneal cells [32], and it is able to upregulate MHC class I antigens and adhesion molecules expression [33, 34] on the surface of several cell types as well as the production of corneal IL-1 [35]. Local production of TNF-α has been associated with cell destruction [36, 37]. Consequently, TNF-α may be involved in corneal allograft rejection [38, 39] and in lung graft rejection [40]. Moreover, anti-TNF-α antibodies downregulate acute episodes of lung rejection [40]. These observations confirm the complex network involved in organ rejection.

Due to the methodology used in our experiments, dealing with cytokine expression during corneal graft rejection, the cellular source of the extracted RNA was not determined. One should also keep in mind that expression of messenger RNA does not necessarily imply that the protein is also produced. Since cytokines are active at very low concentrations, detection by immunohistology can be quite difficult. Measurement of cytokine protein content by ELISA in corneal extracts is a technique that is feasible to address this problem. In agreement with our results, Sano *et al.* [41] have demonstrated by ELISA that corneal graft rejection is associated with an increase release of IL-1α, IL-2, TNF-α and IFN-γ. From our work and that of other groups, it is evident that corneal cells produce the potent chemotactic cytokines MCP-1 and macrophage inflammatory protein (MIP)-2 as well as the anti-inflammatory Th2 cytokine IL-10. By secreting MCP-1 and MIP-2, corneal cells actively participate in the inflammatory influx of cells, mainly neutrophils and macrophages, towards the center of the graft during an allograft reaction. By secreting IL-10, the cornea should be able to shift the immune response into a more benign Th2 reaction, capable of inhibiting the deleterious effects of a Th1 response. Of interest was our finding that simple surgical trauma to the cornea already resulted in the expression of IL-10 mRNA. Further studies are needed to see whether this also leads to synthesis of the protein. It can be hypothesized that transparency of the normal cornea is maintained by a specific immune microenvironment where IL-10, among other regulatory cytokines, can play an important role. The exact source of IL-10 in the cornea and its role in corneal disease and in corneal allotransplantation deserves further investigation.

In preliminary studies to address this question, we tried to modulate the corneal allograft immune response by IL-10 administration in order to achieve corneal graft acceptance (chapter 5). The wide range of *in vitro* and *in vivo* biological regulatory activities of IL-10 suggests numerous clinical applications for this cytokine [42, 43, 44]. IL-10 in the murine systems is a product of antigen-stimulated Th2 T cell clones [45], while in humans IL-10 can be secreted by both Th1 and Th2-type CD4+ T cell clones [46].

IL-10 is also secreted upon activation by a vast array of other cell types, including activated human monocytes [47], B-cells [48], and activated murine macrophages [49]. IL-10 is characterized by its major capacity of inhibiting the proliferation of Th1 subsets and the cytokine synthesis from these cells as well as from macrophages [50]. It has been hypothesized that IL-10 may contribute to graft survival by blocking cytokine production and downregulating MHC class II expression [51]. Corneal inflammation after herpes simplex virus keratitis induction is ameliorated by IL-10 application [52]. As corneal rejection is mediated by T cells, mainly from the Th1 subset, and by macrophages, at that time it seemed to us that IL-10 would be a powerful tool in preventing rejection in our rat model of allotransplantation. However, subconjunctival and / or systemic administration of IL-10 was not successful and corneal rejection was, in some cases, even accelerated. These observations are in contradiction with several studies that demonstrate the association of IL-10 with graft tolerance [53, 54]. On the other hand, IL-10 can also be associated with renal allograft rejection [55] and with exacerbation of particular diseases [56].

Van der Veen *et al.* [57] showed that subconjunctival injections of liposomes containing clodronate (clodronate-LIP) prolong corneal allograft survival indefinitely in rats. In line with the experiments described in chapter 4, we decided to analyze the messenger RNA expression of several cytokines in corneas harvested from non-treated rats and from clodronate-LIP-treated rats (chapter 6). Clodronate-LIP selectively depletes phagocytosing cells, such as macrophages, causing their cell death, but not of other immunocompetent cells [58, 59]. Macrophages can be involved in both the afferent and efferent arc of the immune response. Via processing and presenting alloantigens to T-cells, they actively participate in the afferent arc. They may render a significant contribution to the anti-allograft reaction by secreting several mediators such as IL-1β, IL-12, IFN-γ [60] and TNF-α [31], and stimulating the differentiation of T-cells. Macrophages can play the role of effector cells and therefore can cause damage to corneal graft [61, 62]. Clodronate-LIP-treated rats did not reject their corneal transplants and collected corneas did not express T-cell-derived cytokines during the entire follow-up. Almost all analyzed cytokines, except for IL-12 and TNF-α, were strongly decreased after the given treatment. Although TNF-α is considered a pro-inflammatory cytokine, capable of initiating the cytokine cascade of several cellular events, and IL-12 a cytokine that governs the differentiation of Th1 cells, they might also play a role in corneal tolerance as also described for other organs [63, 64]. Interestingly, IL-12 may also inhibit corneal neovascularization [65], a common feature of corneal inflammation that allows immune cells to easily reach the center of the graft. Non-treated rats rejected their corneal grafts after day 10 and T-cell derived cytokines such as IL-2, IL-4, IFN-γ and TNF-β / lymphotoxin (LT) were observed. This is compatible with a T-cell response such as discussed in chapter 4. Our results confirmed the importance of macrophages in corneal graft rejection. In clodronate-LIP-treated rats, macrophage depletion is not completely achieved, but only a reduction of

infiltrating macrophages along with T-cells is observed within corneal grafts and surrounding tissues [57]. Moreover, Slegers *et al.* observed a complete absence of corneal rejection in rats after a single injection of clodronate-LIP (unpublished data). Since the molecular mechanisms by which clodronate-LIP induces corneal acceptance in the animal model are still not completely elucidated, further investigations are required.

In chapter 7, we investigated the presence of cytotoxic T lymphocyte (CTL) activity in lymphoid cells isolated from submandibular and mesenteric lymphnodes, and from spleen, as well as the presence of alloantibodies in the serum, in order to seek for an immunological basis for the absence of graft rejection in clodronate-LIP treated rats. Clodronate-macrophage depleted rats show a strong downregulation of the CTL responses and an impairment of antibody generation. Once more, the importance of macrophages as effector cells in corneal rejection is pointed out. Both chapters 6 and 7 show that repeated subconjunctival injections of clodronate-LIP after corneal transplantation in the rat, are able to impair the normal function of macrophages, which is demonstrated by a decreased local mRNA expression of several macrophage-derived cytokines and by a reduced macrophage-induced T-cell activity such as the activation of CTL's and antibody production. CTL activity has been shown to be necessary for corneal rejection [66], which makes the use of clodronate-LIP a possible tool to prevent corneal transplantation in man. The major concern is that clodronate is accomplished by local and systemic effects. In attempting to find new regimens, an important goal is the absence of systemic side-effects.

The results of this thesis allowed us to speculate concerning the mechanisms that are present in corneal transplantation. Briefly, in corneal allograft rejection both non-specific and specific immunity are involved. Cells of the innate immune system, such as monocytes, macrophages and other APC cells, process and present antigens to cells of the acquired immune system in order to elicit destruction of the graft by effector mechanisms of humoral and cellular origin. Main targets are the endothelial and stromal cells but, to a lesser extent, epithelial cells can also be involved. Few data concerning the action of antibodies in allograft rejection are available. Antibody and complement deposition is responsible for rejection and chronic vascular lesions in cardiac allografts [67, 68]. Cornea-specific and donor-specific antibodies have been detected in host serum after clinical [69] and experimental [70, 71] corneal transplantation. Their role in corneal rejection is still uncertain. However, we may speculate that naturally occurring antibodies may bind to corneal graft cells, which may be then stimulated to produce and secrete mediators, such as neuropeptides and cytokines, among others. Depending on the class or sub-class of the antibody, activation of the complement system may occur with the deposition of its components on the surface of the cells. Additional leukocytes are attracted to the inflamed tissues, via C5a release. The interaction between antibodies and the infiltrated immunocompetent cells may induce a form of graft destruction via an antibody dependent cellular cytotoxicity mechanism. However, recent data shows that in a B-cell knockout mouse model of

corneal transplantation, antibodies do not play a significant role in mediating corneal rejection in low-risk eyes [72]. Regarding the cellular effector mechanisms involved in graft destruction and rejection, the allogeneic response is thought to be initiated by the activation of CD4+ and CD8+ T helper cells. CD8+ cells generate cytotoxic T cell responses. T cell activation comes forth with the production of IL-2, which is an autocrine T cell growth factor. This results in activation and proliferation of more CD4 or CD8 antigen-bearing T-cells and in production of several other cytokines as described in the different chapters of this thesis. Therefore, the type of cells and cytokines implied in the inflammatory microenvironment determines the fate of the corneal graft. This general scheme also explains the relative success of a number of immunosuppressive drugs, such as cyclosporine, which are potent inhibitors of IL-2 release.

In spite of the fact that remarkable progress in corneal transplantation has been achieved by investigations either in animal models or in clinical studies, there is no doubt that complete understanding of the immune mechanisms involved in graft rejection is still far. Therefore, further experiments are imperatively required, considering that corneal opacity is the second leading cause of worldwide blindness [73] and only penetrating keratoplasty may resolve the problem. In this thesis, we focused on the identification of different patterns of cytokines in corneal graft acceptance and rejection. The next step will be the gathering of more knowledge on experimental cytokine modulation in order to obtain long-term corneal graft survival. This may be accomplished with specific cytokine-knockout animals, cytokine delivery, such as the IL-1RA and the Th2 derived cytokines IL-4, IL-10 or IL-13 that show potent anti-inflammatory properties, or increase of endogenous cytokine synthesis. In view of the unique properties of ocular structures to have expression of FasL (see chapter 2), the artificial enhancement of FasL expression (either drug induced or via gene therapy) on corneal grafts may lead to an extra apoptosis of infiltrating T cells, during graft rejection. Targeting specific components of the immune response can also be achieved via antibody, drug or gene therapy. Immunomodulation by oral immunization is currently receiving a great deal of attention and shows promise in the treatment of autoimmune disease and allograft rejection. Niederkorn and his group [74] already showed in the animal model that corneal allograft survival could be enhanced after oral tolerance, *i.e.* after feeding the host with antigens from the donor. It is possible to feed patients with skin cells from the donor while the cornea is being preserved during a period of 3-4 weeks. Gene therapy is the most promising tool for the future. By introducing novel genes into the donor cornea by genetic manipulation, we could be able to obtain corneas that could have the capacity, for example, of secreting specific regulatory mediators, abrogating any initiative towards graft rejection by the immune system of the host. Such experiments may give conclusive answers regarding the immunopathological mechanisms underlying corneal graft rejection. Moreover, they may contribute, in a near future, to the development of new strategies to treat or prevent rejection in humans and thereby achieving longstanding acceptance of corneal grafts.

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Chapter 9

Summary

Corneal allograft rejection is currently the major cause of corneal transplant failure in humans. Although the cellular and molecular mechanisms of corneal allograft rejection are still not yet completely understood, some studies concerning allograft rejection in other organs show that cytokines can play a pivotal role in the pathogenesis of organ rejection. Cytokines are simple polypeptide or glycoprotein cell-regulators, produced by white blood cells and a variety of other cell types after cell stimulation. Their multifunctional actions are achieved by receptor-mediated pathways, influencing the immune and the inflammatory responses as well as their own production and release.

Since little is known about the role of cytokines in corneal allograft rejection, the experiments described in this thesis have the purpose to give a large contribution to the knowledge on cytokine networks in corneal transplantation.

This thesis starts with an extensive literature review on the role of cytokines in corneal immunopathology (chapter 2), including the inherent corneal defense mechanisms, the type of immune response within the major corneal layers, the factors of angiogenesis, the recruitment of phagocytes, the apoptosis mechanisms and the wound healing processes. Chapter 3 reports the constitutively expression of interleukin-1 receptor antagonist (IL-1RA), the natural antagonist of IL-1, by the epithelial and stromal cells of the normal human cornea. The production of IL-1RA protein by these cells implies an inherent corneal defense mechanism against IL-1 mediated responses. Recent studies show that IL-1RA is able to promote an ocular immune privilege state and consequently the enhancement of corneal allograft survival in an animal model. Chapter 4 describes different mRNA expression patterns of several cytokines, such as IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-2 (MCP-2), in recipient and donor corneas of rats submitted to corneal auto- or allotransplantation. Corneal rejection is characterized by the presence of T cell derived cytokines including IL-2, IL-4 and IFN-γ. Chapter 5 deals with the lack of a therapeutic effect of topical and/or systemic injections of IL-10, a well known anti-inflammatory cytokine produced mainly by the T helper 2 subset of cells, in enhancing corneal

allograft survival in the rat. Corneal rejection was even accelerated when high doses of IL-10 were used. These observations are in contradiction with some studies that demonstrate the correlation between IL-10 and allograft tolerance. However, there are also studies that show that IL-10 can be associated with allograft rejection. Thus, further investigations are required to determine the exact role of this cytokine in organ transplantation. Chapter 6 shows the importance of the administration of liposomes containing clodronate, a drug that selectively depletes macrophages, in promoting corneal allograft tolerance in rats. Corneas harvested from animals submitted to repeated subconjunctival injections of clodronate show a strong decreased expression of the analyzed cytokines, except for IL-12 and TNF-α. Accepted corneas did not express T cell derived cytokines in any studied post-operative day. The almost complete abrogation of IL-1β, IL-2, IL-4, IL-6, IFN-γ and TNF-β / lymphotoxin mRNA expression in allografts by clodronate suggests that during corneal rejection these cytokines are derived from macrophages and T cells. The cellular source of the analyzed cytokines is also discussed. In chapter 7 it was investigated the presence of cytotoxic T lymphocyte (CTL) activity in lymphoid cells isolated from submandibular and mesenteric lymphonodes, and from spleen, as well as the presence of alloantibodies in the serum, in order to seek for an immunological basis for the absence of allograft rejection in clodronate-treated rats. Clodronate induces a downregulation of the CTL activity and an inhibition of antibody production, probably due to a lack of chemical interactions between macrophages and T cells. Finally, chapter 8 provides a general discussion of this thesis.

Sumário

A rejeição imunológica é actualmente a principal causa de falência do transplante de córnea no Homem. Embora ainda não estejam completamente esclarecidos os mecanismos celulares e moleculares da rejeição do enxerto de córnea, alguns estudos efectuados em outros orgãos mostram a importância do papel das citoquinas na patogénese da rejeição. As citoquinas são polipeptídeos ou glicoproteínas que controlam a comunicação entre as diferentes células do organismo, sendo produzidas pelos glóbulos brancos e/ou outros tipos celulares. Actuam através de vias mediadas por receptores, influenciando as respostas inflamatórias e imunológicas, assim como a sua própria produção e segregação.

O papel das várias citoquinas no processo da rejeição da córnea transplantada é ainda mal compreendido. Os trabalhos descritos nesta tese têm como objectivo contribuir largamente para um melhor conhecimento da interligação das diferentes citoquinas presentes durante o transplante corneano.

Esta tese inicia-se com uma revisão bibliográfica sobre o papel das citoquinas na imunopatologia corneana (capítulo 2). São aqui discutidos os mecanismos de defesa corneana, o tipo de resposta imunológica nas principais camadas corneanas, os factores de angiogénese, o recrutamento de fagócitos, os mecanismos de apoptose e os processos de cicatrização. O capítulo 3 descreve a presença constitutiva do antagonista do receptor da interleuquina-1 (IL-1RA), o antagonista natural da IL-1, nas células epiteliais e estromais da córnea humana normal. A produção proteica do IL-1RA implica a existência de um mecanismo de defesa corneano contra as respostas mediadas pela IL-1. Estudos recentes demonstraram que o IL-1RA é capaz de promover uma situação de privilégio imunológico ocular e um aumento da sobrevida do transplante de córnea no modelo animal. O capítulo 4 descreve os padrões de expressão do RNAm de várias citoquinas, nomeadamente IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, factor de necrose tumoral-alpha (TNF-α), interferão-gamma (IFN-γ), proteína quimiotáctica dos monócitos-1 (MCP-1) e proteína inflamatória dos macrófagos-2 (MIP-2), em córneas receptoras e dadoras de ratos submetidos a auto- ou alotransplante. A rejeição corneana é caracterizada pela presença de citoquinas derivadas das células T, incluíndo a IL-2, IL-4 e o INF-γ. A falta de eficácia terapêutica da administração tópica e/ou

sistémica da IL-10 – uma citoquina anti-inflamatória bem conhecida e produzida essencialmente pelas células T «helper» do tipo 2 – no aumento da sobrevida do enxerto de córnea no rato é descrita no capítulo 5. A rejeição corneana foi mesmo acelerada quando foram utilizadas doses mais elevadas de IL-10. Estas observações contradizem alguns estudos que demonstram a correlação entre a IL-10 e a tolerância de enxertos. No entanto, alguns estudos mostram que a IL-10 pode também estar associada a rejeição de transplantes. É necessário prosseguir com esta linha de investigação para determinar o papel exacto desta citoquina na transplantação corneana. O capítulo 6 descreve a importância da administração de lipossomas contendo clodronato, uma droga que elimina selectivamente os macrófagos, no aumento da tolerância do enxerto de córnea. As córneas retiradas de animais que foram sujeitos a injecções subconjuntivais repetidas de clodronato, apresentam uma significativa diminuição das citoquinas analisadas, excepto para a IL-12 e TNF-α. As córneas que não sofreram rejeição não expressaram, em qualquer altura do design experimental, citoquinas derivadas das células T. A quase completa ausência de expressão de RNAm das IL-1β, IL-2, IL-4, IL-6, IFN-γ e TNF-β / linfotoxina provocada pelo clodronato sugere que, durante a rejeição da córnea, estas citoquinas são produzidas pelos macrófagos e células T. A origem celular das citoquinas analisadas também é aqui discutida. No capítulo 7 é descrita a investigação relativa à presença da actividade dos linfócitos T citotóxicos (CTL) nas células linfóides isoladas dos gânglios submandibulares e mesentéricos, e do baço, assim como a presença de aloanticorpos no soro, a fim de determinar a base imunológica para a ausência de rejeição corneana nos ratos tratados com clodronato. O clodronato induz uma diminuição da actividade CTL e uma inibição da produção de anticorpos, provavelmente pela ausência de interacções químicas entre os macrófagos e as células T. Finalmente, no capítulo 8 é apresentada uma discussão geral da tese.

Sommaire

La rejection du transplant cornéen est, de nos jours, la principale cause de la faillite du transplant cornéen dans l'Homme. Si bien que les mechanismes cellulaires et moleculaires de la rejection du transplant cornéen ne soient pas completement connus, certains études montrent que les interleukines (IL) ont un rôle fondamental dans la pathogénese de la rejection d'autres organes.

Les ILs sont des polypeptides ou des glycoprotéines qui controllent la communication entre les differentes céllules. Elles sont produites par les globules blancs et par d'autres types de céllules, après un stimulus. Les ILs agissent par intermède de recepteurs, en influant les réponses imunologiques et inflamatoires, aussi bien que sa propre production et segrégation.

Le rôle des ILs dans le transplant cornéen et pendant le processus de rejection cornéenne est toujours mal connu. Le propos de cette thèse est de contribuer largement pour la connaissance et mâitrise du rôle des ILs dans le transplant cornéen.

Cette thèse commence par une révision extensive de la litérature existente sur le rôle des ILs dans l'imunopathologie cornéenne (chapitre 2), où on trouve les méchanismes de défense cornéenne, le type de réponse imunologique, les facteurs d'angiogenesis, le récrutement de fagocytes, l'apoptosis et les processus de cicatrization. Le chapitre 3 montre que l'antagoniste du recepteur de l'IL-1 est présent constitutivement dans les céllules epithéliales et estromales. La production de la protéine IL-1RA implique un méchanisme de défense contre les réponses où l'IL-1 intervient. Le chapitre 4 décrit l'expression du RNAm de différentes ILs, tels que l'IL-1β, l'IL-1RA, l'IL-2, l'IL-4, l'IL-6, l'IL-10, le facteur de nécrose tumoral-alpha (TNF-α), l'interferon-gamma (IFN-γ), le MCP-1 et le MIP-2 dans des cornées réceptrices et donneuses de souris qui ont été soumises soît a un autotransplant, soît a un allotransplant. La réjection cornéenne est caracterizée par la présence des ILs derivées des céllules T, tels que l'IL-2, l'IL-4 e l'IFN-γ. Le chapitre 5 s'occupe du manque d'effet thérapeutique d'injections locales et systémiques de l'IL-10 – une IL bien connue comme agent anti-inflamatoire et qui est produite principalement par les céllules T helper 2 – dans l'augmentation de la réussite du transplant cornéen dans les souris. La réjection a même été plus rapide lorsque des

dosages plus élevées d'IL-10 ont été utilisées. Ces observations sont contradictoires avec certains études qui montraient la corrélation entre l'IL-10 et la tolérance du transplant. Le chapitre 6 montre l'importance de l'administration de liposomes contenant du clodronate, une drogue que, d'une façon sélective, élimine les macrophages dans l'augmentation du succès du transplant cornéen. Des cornées qui ont été cueillies dans des animaux qui ont été soumis a des injections subconjonctivales de clodronate montrent une diminution de les ILs analysées sauf pour les cas de l'IL-12 et de le TNF-α. Les cornées qui ont été tolerées ne révèlent pas la présence d'ILs dérivées des céllules T pendant le temps du follow-up. L'absence de l'expression du RNAm de l'IL-1β, de l'IL-2, de l'IL-4, l'IL-6, l'IFN-γ et du TNF-β provoquée par le clodronate indique que, pendant la réjection cornéenne, ces ILs sont produites par les macrophages et par les céllules T. L'origine céllulaire des ILs analysées est aussi discutée. Le chapitre 7 décrit l'investigation de la présence des linfocytes T cytotoxiques (CTL) dans les céllules isolées des ganglions submandibulaires et mésentériques et du rate, bien comme la présence d'anticorps dans le sérum, a fin de déterminer la base imunologique pour l'absence de réjection cornéenne dans les souris qui ont été soumises au clodronate. Le clodronate origine une diminution de l'activité CTL et une inibition de la production d'anticorps, probablement par l'absence d'interactions chimiques entre les macrophages et les céllules T. Enfin, le chapitre 8 fournit une discussion généralisée de la thèse.

Chapter 10

Final words

At this moment, I would like to express my most unreserved gratitude and sincerest appreciation to all who have contributed to the realization of this thesis.

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This thesis is the proof that is possible to conciliate the clinical work in a hospital with the basic research performed in a laboratory. I hope that this thesis will stimulate younger colleagues to do what I did. It is worthwhile!

Thank you all
Muito obrigado a todos