Institut für Medizinische Immunologie

an der Martin-Luther-Universität Halle-Wittenberg

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Establishment of an ELISA-based crossmatch procedure for the detection of donor-specific anti-HLA antibodies in patients with corneal grafts

Dissertation

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Referat und bibliographische Beschreibung

Das Ziel der vorliegenden Arbeit war (i) eine routinetaugliche Methode zum Nachweis von Spender-spezifischen anti-HLA Antikörper (Kreuztest) zu etablieren, wenn vitale Lymphozyten des Spenders nicht zur Verfügung stehen, und (ii) der Nachweis, dass Spender-spezifische anti-HLA Antikörper bei der Corneatransplantation (CTX) als Kontraindikation zu betrachten sind, trotzt des immunologisch-privilegierten Zustandes im Auge.

Bei der Corneatransplantation konnte bisher auf Grund der längeren Lagerfähigkeit des Transplantats (bis zu 4 Wochen), die für einen Kreuztest notwendigen vitalen Lymphozyten nicht aasserviert werden. Für die Etablierung des neuen ELISA-basierenden Antibody Monitoring System HLA Klasse I und Klasse II (Micro-AMS ELISA) wurde als Ausgangsmaterial für die Isolierung von HLA Klasse I und Klasse II Molekülen ein Teil der explantierten Cornea von 19 Transplantatempfängern eingesetzt. Des weiteren wurden 45 Patienten auf das Vorhandensein von Spender-spezifischen anti-HLA Antikörpern vor CTX untersucht und mit dem Auftreten von immunologischen Komplikationen nach der CTX und dem Transplantatverlust korreliert. Die Resultate des Micro-AMS ELISA wurden durch den Nachweis der entsprechenden anti-HLA Antikörperspezifitäten mittels Quik-ID® Klasse I und Klasse II ELISA im Empfängerserum und der korrespondieren HLA Antigene beim Spender mittels HLA Genotypisierung verifiziert.

In 8 von 19 der Patienten (42 %) konnten Spender-spezifische Antikörper gegen die HLA Moleküle der explantierten Cornea nachgewiesen werden. In 75 % konnten entsprechende anti-HLA Antikörper im Quik-ID® Klasse I und Klasse II ELISA nachgewiesen werden.

In der Gruppe mit Spender-spezifischen anti-HLA Antikörpern vor der CTX wurden bei 79 % der Patienten kurz nach der CTX akute und chronische Rejektionen beobachtet. Bei einem Patienten erfolgte eine akute Abstoßung des Transplantats innerhalb von 5 Tagen, drei Patienten verloren ihr Transplantat innerhalb weniger Wochen. In 75 % konnten die entsprechende anti-HLA Antikörper im Quik-ID® Klasse I / Klasse II ELISA und in 69 % die korrespondierenden HLA Antigene beim Spender nachgewiesen werden.

In der Gruppe ohne nachweisbare Spender-spezifische anti-HLA Antikörper vor der CTX wurden bei 77 % der Patienten innerhalb des Beobachtungszeitraums von 10 Monaten (SD = ± 6) keine immunologischen Komplikationen beobachtet. Bei vier Patienten dieser Gruppe wurden nach der CTX immunologische Abstoßungsreaktionen beobachtet, welche bei zwei Patienten nach 17 bzw. 23 Monaten zum Transplantatverlust führten.

Die Ergebnisse zeigen eine eindeutige Assoziation zwischen dem Vorhandensein von Spender-spezifischen anti-HLA Antikörpern vor CTX und dem Auftreten von hyper-akuten und akuten immunologischen Komplikationen nach CTX bis zum Transplantatverlust auf. Die immunologischen Komplikationen nach CTX inklusive Transplantatverlust bei 4 Patienten ohne nachweisbare Spender-spezifische anti-HLA Antikörper vor CTX zeigen die Notwendigkeit eines Testes zum Nachweis von *de-novo* anti-HLA Antikörpern im post-CTX Verlauf.

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Abbreviations

Ab	antibody
ACAID	anterior chamber-associated immune deviation
ASHI	American Society for Histocompatibility and Immunogenetics
APC	antigen-presenting cell
CD	cluster of differentiation
CM	crossmatch
CTX	corneal transplantation
DAF	decay accelerating factor
DSO	German Foundation of Organ Transplantation
DTH	delayed-type hypersensitivity
EFI	European Federation for Immunogenetics
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	fluorescent-activated cell sorting
HLA	human leukocyte antigen
IL	interleukin
LCT-CM	lymphocyte cytotoxicity crossmatch assay
mAb	monoclonal antibody
MHC	major histocompatibility complex
MIC	MHC class I-associated genes
NK cell	natural killer cell
PCR	polymerase chain reaction
RT	room temperature
SSP-PCR	sequence-specific primer based polymerase chain reaction
TCR	T cell receptor

1. Introduction

In humans the eye represents the most important sense organ. Approximately one fifth of all inhabitants in the Western countries exhibit a reduced vision caused by different reasons. The acute or chronic reduction of the vision is the most important reason for patients' examinations by ophthalmologists. In contrast patients with acute pain, perforation or accidents with aggressive chemicals of the eyes are generally sent to the emergency departments of local hospitals or medical departments of universities.

Glasses or contact lenses mainly correct the reduced vision based on changes in the optical way of the eye, although in same cases corrections of the lens or cornea have to be performed by refractive surgery. Organic changes in the *Nervus opticus* or in the *retina* are irreversible in nearly all cases and are not yet curable such as glaucoma or diabetic retinopathy. Due to the modern technology in microsurgery such as cataract extraction and cornea transplantation, pathological alterations of the cornea, the lens or the vitreus humour (*Corpus vitreum*) are curable.

However, there exist some drawbacks and major limitations concerning allogeneic cornea transplantation (CTX) which are (i) the availability of high quality corneas from post-mortem donors and (ii) the loss of transplanted corneas due to immunological rejections despite the "immune privileged" status of the eye. In all Western countries the cornea allografting is the most frequent transplantation with > 40.000 cases / year in the USA (113), > 3.000 / year in Germany (Seitz, 2005) and > 2.200 / year in the United Kingdom (George and Larkin, 2004).

1.1. History of corneal transplantation

The cornea transplantation from post-mortem donors represents the oldest successful grafting of a solid tissue. The first manuscript describing the idea to replace destroyed eyes or corneas was already found in Egypt about 4.000 years ago. Blindness from infections, injuries and corneal scars was known since the earliest times, but not curable in a "medical system" based on religious omens, spirits and healing rituals.

At the beginning of the 19th century the introduction of new microsurgical instruments led to the first successful surgery of corneal grafting in animals and humans but all transplanted corneas were opaque. In 1824 Franz Riesinger replaced human opaque cornea with animals´ grafts and termed this technology "keratoplasty". The first successful transplantation in animals was performed by Samual Bigger in 1837 on a gazelle by Bedouins in Africa.

During the late 19th century new microsurgical techniques were introduced such as the laminar technique from Philipp Franz von Walther (1782-1849) as well as new instruments such as the clockwork trephine developed by Arthur von Hippel in 1886. Despite this technical development, most of the graftings were not successful due to the limited knowledge of the medical background concerning anaesthesia, aseptic surgery and

preventative measures to avoid graft rejections. Therefore the first successful human corneal grafting was performed by E. Zirm in Olmutz near Prague on December 7th 1905 (Moffatt et al, 2005) (Figure 1).

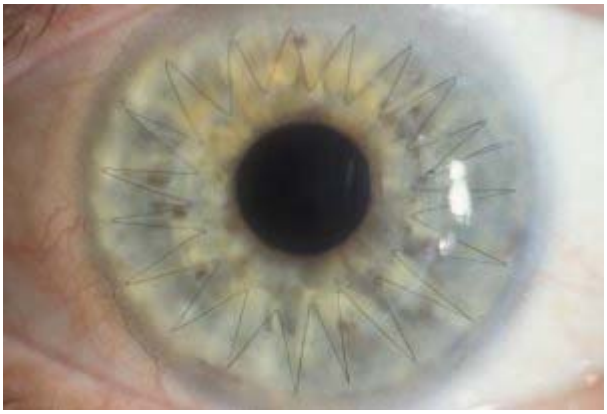

Figure 1. Photo of a successful keratoplasty with suture in zigzag look (6 months after CTX)

1.2. Medical background

1.2.1. Overview of anatomy of the eye

The complete visiual system consists of the eye as the optical part and different protecting and accessory structures as the eyelid, lacrimall glands und muscles. The eye has an apple-like shape with the clear cornea at the surface in the front of the eye and the sclera as the capsule surrounding the whole eye. The optic nerve and the vessels, the Anterior and Ventral centralis retinae form the “stalk of the apple” (Figure 2). Together with the eyelid and the tears the cornea protects the inner parts of the eye from germs, dust and other particles.

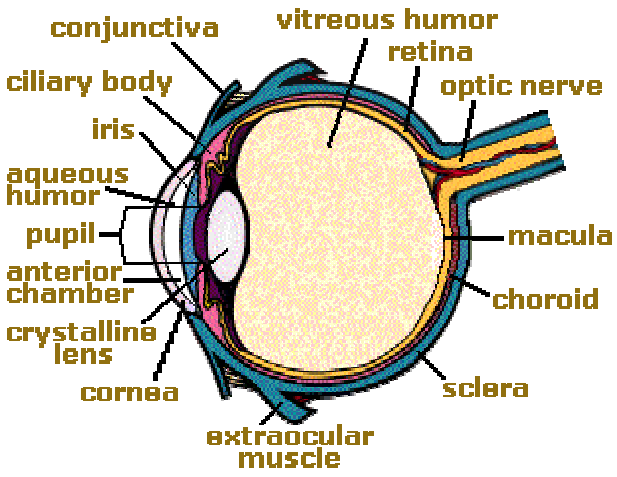

Figure 2. Schematic diagram of the anatomy of the human eye (www. Tedmontgomery.com /the_eye)

The cornea is clear as glass, but also durable, and acts as the eye's outermost lens. It contributes to about 65 - 75 % of the total focusing capacity. It bends the incoming light onto the lens, which refocuses the light onto the retina, a layer of light sensing cells in the background of the eye (Figure 3).

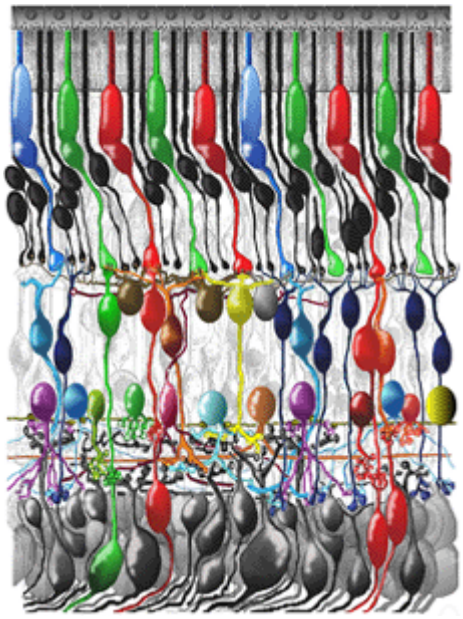

Figure 3. Schematic diagram of the human retina. Each of the coloured cones (blue, green, red) is only sensitive for light with a specific wave length, whereas the rods (black) are the most light-sensitive cells (http://webvision.med.utah.edu/anatomy.html).

For clear vision the light must be focussed by the cornea and the lens to exactly hit the retina. The cells in the retina convert the light into nervous impulses, which are sent to the brain via the optic nerve. The brain interprets the information from different light sensitive cells in the retina (rods and cones) as an image (Figure 4).

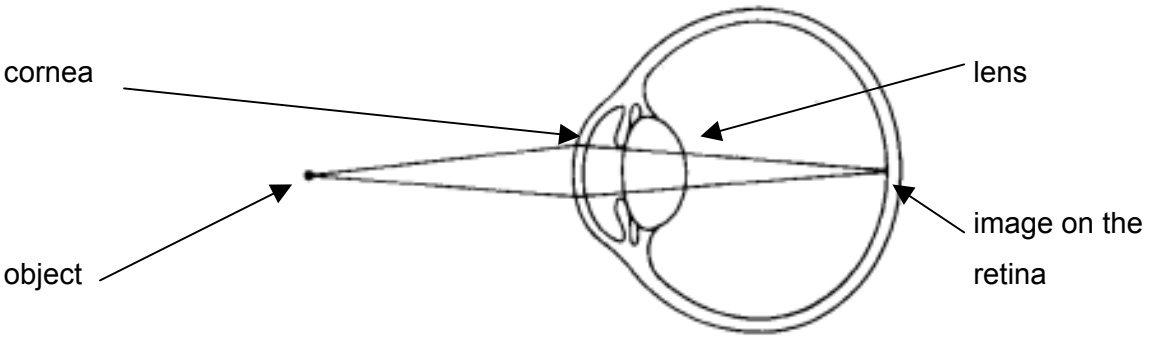

Figure 4. Accommodation of an object near to the eye. Cornea and lens focus the light onto the retina, where it is transformed into an image.

The cornea is an oval with a size of 11 to 12 mm exhibiting a thickness in the centre of about 500 µm. The thin epithelial cell layer on the outer surface represents about 90 % of all cells (Figure 5). The compact Bowman's lamella (Bowman's layer) is located between the upper parts of the stroma under the epithelial cells. The stroma consists of collagen fibres, which surround vacuoles responsible for about 90 % of the cornea's thickness. It is mainly composed of water (nearly 80 %) and of collagen (16 %), but also contains some keratinocytes. The inner part of the stroma, the thin Descemet's membrane is the most solid part of the cornea. When compared to the upper stroma layer the collagen fibres in the Descemet's membrane exhibit morphologic differences by presenting a more compact phenotype.

Furthermore, the inner part of the cornea consists of a monolayer of hexagonal endothelial cells with direct contact to the anterior chamber (*Camera anterior bulbi)*. These cells are characterized by a high metabolic activity and are involved in the stromal dehydration. In contrast to the other layers in the cornea, the endothelial cells have no regenerative potential and upon their destruction are lost forever.

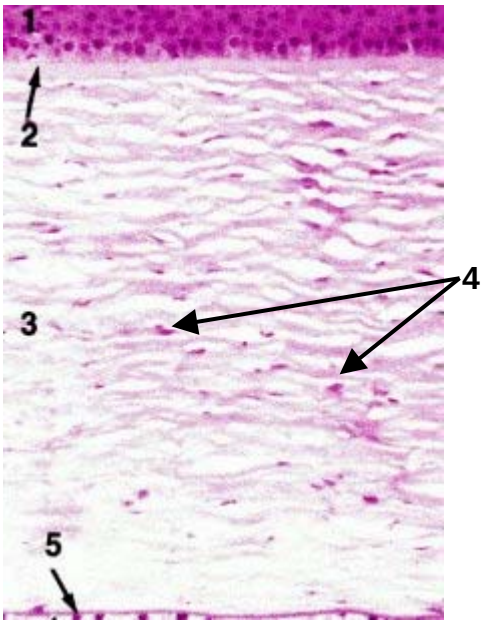

Figure 5. Histology of the cornea. 1 - multilayer epithelial cells, 2 - Bowman´s layer, 3 - stroma, 4 - keratinocytes, 5 - Descemet´s membrane with a monolayer of endothelial cells (taken from: Weather et al., Histologie fonctionnelle Manuel et Atlas, 1979).

It is generally accepted that the cornea is free of blood vessels since the capillary network ends at the border between cornea and sclera. The eye has been postulated as an "immune privileged" organ due to (i) the avascular nature of the cornea and anterior chamber and (ii) the presence of only few immune competent cells in this compartment such as T lymphocytes, NK cells, macrophages or dendritic cells (DC). These features are regarded as the main reasons contributing to the prolonged survival of allografts.

Tears ensure the nutrition of the epithelial cells on the outer surface, whereas the liquid present in the anterior chamber mediates that of the inner surface. Other functions of the tears are the avoidance of the corneal desiccation, the cleaning of the cornea from allergens, foreign bodies or inflammatory cytokines, and the protection of the surface from bacterial infections. The pumping function of the inner endothelial cells is essential to keep the cornea clear. The fluid from the anterior chamber leaks slowly into the corneal stroma and may lead to the reduction of the vision by the corneal swelling (oedema). To avoid this oedema the endothelial cells pump this excessive fluid out of the stroma.

Consequently the clinical outcome of the cornea transplantation is based on some anatomic features of the eye. For transplantation only the central part of the cornea is generally employed.

1.1.2. Indication for corneal grafting

The cornea can be affected by different diseases, which can be either conservatively treated with (i) drugs, (ii) glasses or soft lenses, and in later stages (iii) by surgery or (iv) cornea grafting.

The most common eye diseases are allergies from pollen, drugs, animal hair, certain cosmetics and cleaning solutions of contact lens. These symptoms are temporary and can be eliminated when patients avoid further contacts with the allergic agents. In addition some kinds of conjunctivitis (Pink Eye) caused by bacterial or viral infections can spread from one person to another, thereby leading to chronic corneal infections in some families. The “Dry Eye” syndrome is a result of a decreased production of tears associated with reduced moisture of the eye, wound healing and protection against infections, although this syndrome is often found in patients with rheumatoid arthritis or Sjoegren’s syndrome. In worse cases these disorders of distinct origin could result in a destruction of the cornea and therefore could subsequently lead to cornea grafting.

Keratoconus

Keratoconus is a bilateral, non-inflammatory and progredient disorder of the cornea which is not associated with pain. It occurs with an incidence of 1 per 2.000 inhabitants and mainly affects younger adults than older persons depending on the different kinds of dystrophies (Gruenauer-Kloevekorn and Duncker, 2006). The frequency of this disease is independent of sex and ethnic groups. Its progression occurs slowly over a period of 10 to 20 years.

During the course of disease the cornea becomes thin and changes to a more conical shape than its normal gradual curve. In some cases the deformation of the cornea results in a localized rupture of the inner layers of the cornea (Figure 6). Although keratoconus can affect

only one eye, this disease mainly involves both eyes resulting in asymmetric distortion of vision.

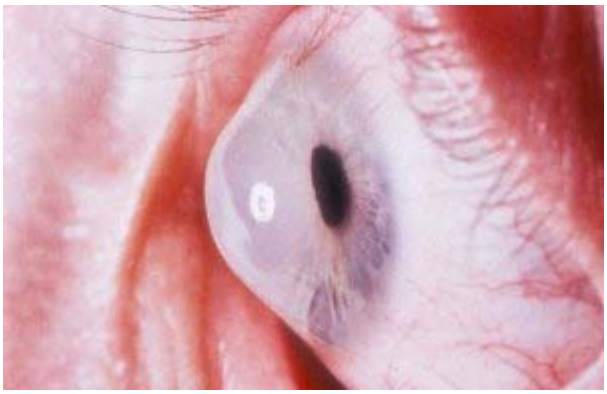

Figure 6. Extended stage of keratoconus

The first symptoms are slight blurring and distortion of vision, mostly together with sensitivity to glare and light. The classic symptom is the occurrence of multiple “ghost” images, in particular with highly contrasted objects as a light point on a dark background, e.g. candlelight in a dark room. Moisture from the eyes seeps into the stroma (corneal hydrops) and the swollen cornea leads to a significant decrease in vision. If both eyes are affected the deterioration in vision affects the patient’s ability to drive a car or read normal sized letters. Eyeglasses or contact lenses can be used to correct the nearsightedness in the early stages of keratoconus. However, in 10 to 25 % of patients with keratoconus, corneal grafting or penetrating keratoplasty is necessary when the disorder progresses and the cornea becomes too thin for correcting the vision by glasses or lenses.

Fuchs´ dystrophy

The Fuchs´ endothelial dystrophy or late hereditary endothelial dystrophy is an eye disease occurring in the fifth or later decade of life and cannot be cured. This disorder generally affecting both eyes is more prominent in women than in men. The Fuchs´ dystrophy exhibits dominant familial inherent patterns and shows no correlation with environmental factors. However, some links to systemic diseases such as mutations in collagen VIII have been described (Zhang et al., 2006).

In adults the endothelium lacks mitotic activity. Thus upon trauma or injury the surrounding endothelial cells only slide over the destroyed area. The malfunction of the corneal endothelium pump system leads to the disruption of the corneal dehydration system resulting in microscopic alterations of the complete cornea. These include (i) focal thickening of the Descement’s membrane, (ii) corneal stromal oedema, (iii) secondary folds in the Descement’s membrane, and (iv) fine pigment-dusting in/on the corneal endothelium.

The first symptoms of this disorder are glare and light sensitivity and in advanced stages the patients have a blurred vision in the morning, but a sharper vision during in the day. This alteration is caused by the higher evaporation when the eyes are open. At later stages a hazy vision occurs during the whole day and in addition, corneal pannus and often a bullous keratopathy are observed. The three stages span usually 10 to 20 years. The degradation of the endothelial cells and the deposition of abnormal Descement's membrane material during the first stages are detectable using the slit lamp microscopy. Sometimes a diffuse pigmentation of the central posterior surface is found. Due to the progressive stromal and epithelia oedema the main symptom in the second stage is the glare and hazy vision. In the last stage a piece of avascular sub-epithelial connective tissue appears in the central cornea, an irregular, grey, swirling sheet of scar tissue. Treatment for Fuchs´ dystrophy depends on the stage and severity of the disease. Patients with early forms may be conservatively treated with sodium chloride drops to control the corneal swelling, and in late stages with therapeutic soft lens. Approximately 10 % of all corneal grafts account for patients with Fuchs´ dystrophy which is functional for more than 2 years in nearly 80 % of patients.

Corneal ulcer and keratitis

A corneal ulcer can occur after the damage or compression of the corneal surface. An ulcer may be sterile, but in most cases includes an infection by a pathogen. Since an ulcer can lead either to a permanently reduced or complete loss of vision or to a perforation of the cornea, it must be treated as an emergency case. Most bacterial ulcers develop after damage of the corneal epithelium by small or large traumatic episodes, by malproduction of tears or by wearing contact lenses. A popular complication of rheumatoid arthritis is peripheral ulcerative keratitis with corneal destruction leading to the complete loss of vision in the end stages of disease.

Keratitis represents an inflammation or infection of the cornea, which is mainly due to:

(i) bacterial infections with e.g. *Staphylococcus aureus* or *Pseudomonas aeruginosa* (contact lens)
(ii) primary and secondary vitamin A deficiencies
(iii) viral infections with e.g. Varicella zoster virus, Herpes simplex virus or adenoviruses
(iv) traumatic corneal injury
(v) contact lenses, in particular of soft lenses and contact with cleaning solution
(vi) chronic topical steroid use
(vii) fungal infections, with e.g. *Candida*, *Fusarium*, and several *Aspergillus* species

Varicella zoster virus causes one of the most serious corneal infections. After the first contact the virus may migrate to the head and neck nerve involving an eye, part of the nose, cheek

and forehead. The ocular Herpes caused by Herpes simplex virus is the most common infectious disease leading to corneal blindness. Patients with ocular Herpes show a very high recurrence of this infection (Prabriputaloong et al., 2006).

Other sources for corneal infections are due to the penetration of the cornea by foreign objects or due to contact lens contaminated with bacteria or fungi. These infections can reduce vision and erode the cornea, leading to scars, which in the end may require a corneal graft. The corneal infection is the most serious complication due to contamination of contact lenses (Lyer et al., 2006).

Another disease leading to corneal dysfunction is the primary vitamin A deficiency detectable in patients with insufficient diet, in particular in young children or pregnant women. Secondary vitamin A deficiency syndromes can occur in patients affected by celiac disease, cystic fibrosis, pancreas diseases, cirrhosis, or after bowel and gastric resections (Mihora et al., 2004).

The emergency care depends on whether the ulcer is sterile or not. Treatment of bacterial ulcer (keratitis) includes broad-spectrum topical antibiotic and cycloplegic eye drops until hospitalization with intravenous injection of antibiotics. Sterile ulcers are treated with steroid and anti-inflammatory eye drops together with antibiotics. Viral corneal ulceration caused by herpes virus may be cured with antiviral drugs, e.g. Acyclovir®. Deep ulcers and ulcers extending through the stroma (descemetoceles) with significantly reduced or complete loss of vision in addition to conventional therapies require the corneal grafting (Miserocchi et al., 2007).

1.3. Human Leukocyte Antigen system

1.3.1. Definition and polymorphism of the Human Leukocyte Antigens

The major histocompatibility complex (MHC) was first discovered as a genetic locus responsible for rapid graft rejection between inbred strains of mice. However, the identification of genes controlling the rejection process in humans was required and a prerequisite for the development of blood transfusions and organ transplantations as treatment modalities for various diseases. Jean Dausset and coworkers (1958) described for the first time circulating antibodies reactive with antigens on human white blood cells. Since these alloantigens are expressed on human leukocytes they were named human leukocyte antigens (HLA). MHC genes are the most polymorphic genes present in the genome and are located in humans on the short arm of chromosome 6 (6p21). In humans the MHC encodes the polymorphic MHC class I, class II and class III locus referring to HLA class I and class I cell surface proteins, but also other molecules such as antigen processing components, cytokines and complement factors (Janeway et al., 2005; Figure 7).

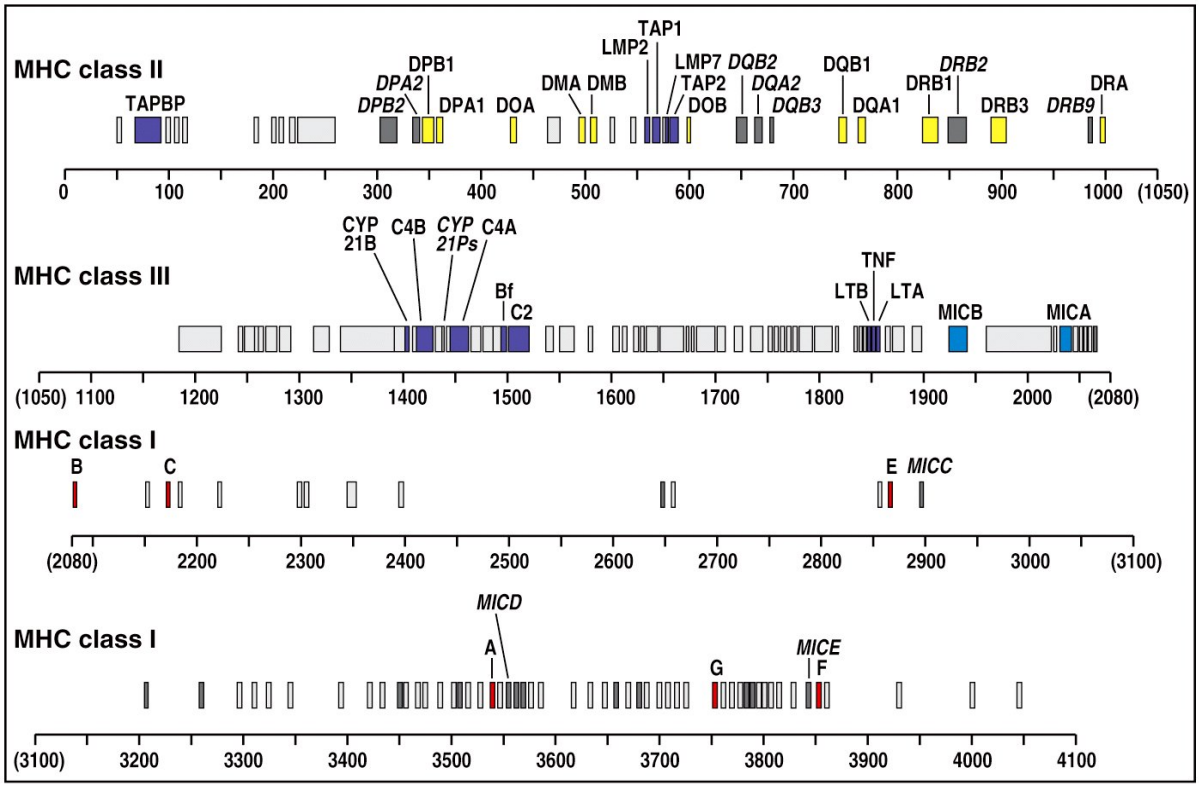

Figure 7. Detailed map of the human Major Histocompatibility Complex. The MHC class I genes are marked in red, the MHC class II genes in yellow colour. The lower scale bar indicates the approximate genetic distances in thousands of base pairs (kbp).

The human MHC consists of (i) the three classical HLA class I gene loci named HLA-A, HLA-B and HLA-Cw, which exhibit an extreme polymorphism, (ii) the HLA class II gene loci including the highly polymorphic genes HLA-DRB1, HLA-DRB3-5, HLA-DQB1, HLA-DQA1 and HLA-DPB1, (iii) the non-classical HLA genes e.g. HLA-G, HLA-E and HLA-F with a reduced polymorphism, and (iv) a group of polymorphic MHC class I-associated genes (MIC-A and MIC-B). These HLA loci are the genes of the highest allelic variability (polymorphisms) in humans with hundreds of alleles (Table 1).

Table 1. Number of serologically defined HLA antigens (allele-groups) and of HLA alleles defined by sequencing (October 2007)

HLA-	A	B	Cw	DR	DQ
Antigens	21	43	9	14	7
HLA-	A	B	Cw	DRB1	DQB1
Alleles	526	846	270	463	79

The high level of polymorphic alleles in each generation combined with the co-dominant inheritance of HLA loci from both parents leads to a number of combinations in the HLA system which is higher than the whole human population. The number of HLA phenotypes theoretically calculated on the basis of known alleles is about 4.4 x 10^{12} HLA combinations in comparison to 6.6 x 10^{9} worldwide inhabitants in November 2007.

1.3.2. Structure of HLA class I and class II antigens

The HLA class I molecule consists of the polymorphic α chain with 3 extracellular domains (α1, α2 and α3), a transmembrane region and a short intracellular part. This α chain is non-covalently linked to the non-polymorphic β_2-microglobulin (β_2-m) located on chromosome 15 (Figure 8a). The peptide binding cleft is generated by the α1 and α2 domains. The HLA class I molecule peptide binding cleft consists of a β sheet-folded basis which is flanked by α helixes at both sites leading to a closed peptide presenting structure (Figure 8b). In general HLA class I molecules present peptide fragments with a length of 8 to 11 amino acids, which directly fit into the peptide binding cleft.

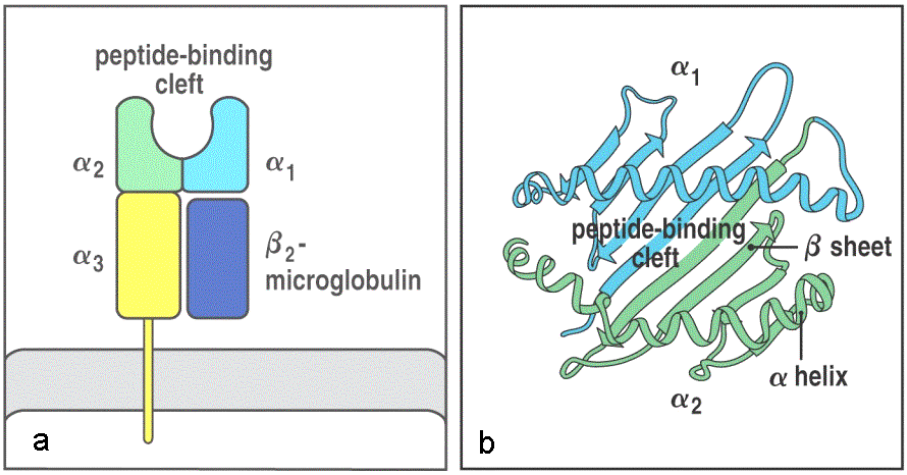

Figure 8. Schematic structure of the HLA class I molecule. The HLA class I molecules are formed by the membrane-spanning α chain (consisting of the domains α1, α2 and α3) which is non-covalently bound to the β_2-m (a). The folding of the α1 and α2 domains leads to a closed cleft in which peptide fragments bind to the HLA molecules for their presentation to cytotoxic T-cells (b).

In contrast the MHC class II molecules represent heterodimers consisting of the α and β chain with two extracellular domains (α1/α2 and β1/β2), a transmembrane region and a short intracellular part (Figure 9a). The peptide binding cleft of MHC class II molecules is generated by the α1 domain of the α chain and the β1 domain of the β chain. Generally its conformation is not as closed as the peptide binding cleft of MHC class I molecules (Figure 9b).

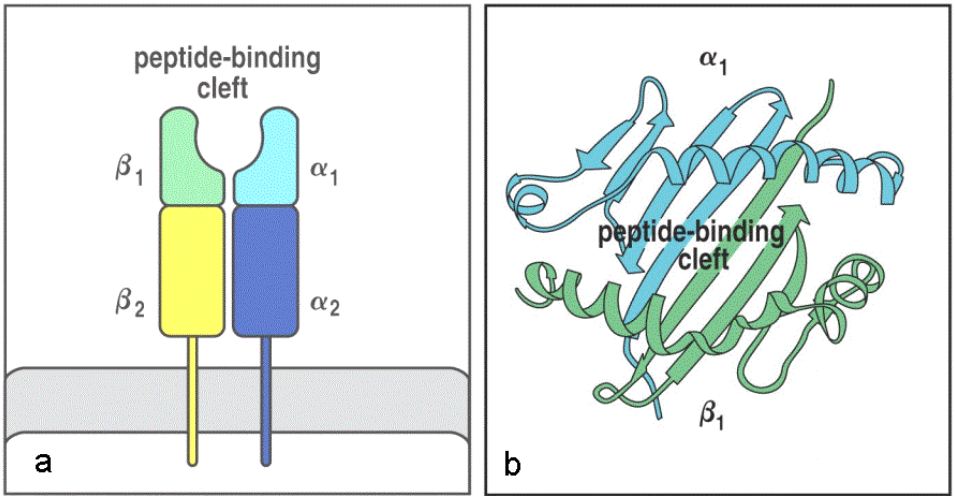

Figure 9. Schematic structure of the HLA class II molecule. The HLA class II molecules is formed by two membrane-spanning chains (α and β chain) each consisting of two domains, which are non-covalently bound (a). The folding of the α1 and β1 domains leads to an open cleft in which peptide fragments bind to the HLA molecules for their presentation to $CD4^+$ helper T-cells (b).

1.3.3. Expression and function of HLA molecules

Although HLA class I and class II antigens are located in vicinity and are involved in the T-cell-mediated immune response they exhibit a number of distinct features, which are summarized in Table 2.

The HLA class I antigens are expressed on the surface of thrombocytes and all nucleated cells with the exception of ovarial cells and sperms. In contrast, the expression of the HLA class II antigens is restricted to professional antigen presenting cells such as B lymphocytes, dendritic cells and macrophages. The main function of HLA class I surface molecules is the presentation of peptide fragments from endogenously synthesized proteins to $CD8^+$ cytotoxic T-lymphocytes (CTL). The interaction between the T-cell receptor (TCR) of the T-lymphocytes and the HLA class I/peptide complex of the nucleated cells in all tissues allows the T-lymphocytes to distinguish between “self” and “non-self”.

In contrast HLA class II antigens present peptide fragments between 13 to 20 amino acids derived from exogenous antigens internalised and processed by professional antigen presenting cells (APC) to $CD4^+$ T-lymphocytes. This process is involved in the transformation of B-cells to antibody producing plasma cells as part of the humoral immune response.

Apart from these functional aspects leading to an effective T-cell-mediated cellular immune response HLA molecules are the main targets of the alloresponse against transplanted solid organs and tissues. This alloresponse depends on anti-HLA antibodies as well as on the existence of T-cells recognizing these polymorphic structures.

Table 2. Differences between HLA class I and HLA class II antigens

Features	HLA class I molecules	HLA class II molecules
	HLA-A, -B and -Cw	HLA-DR, -DQ and -DP
Constitutive expression on tissue cells	Platelets and all nucleated cells without sperms	B cells, monocytes, DC, Langerhans' cells and epithelial cells of the thymus
Source of peptides presented	Intracellular host peptides or viral peptides	Extracellular peptides from pathogenic microorganisms
Length of oligopeptide fragments presented to T cells	8-10 amino acids	13-20 amino acids
MHC interaction with TCR of	$CD8^+$ cytotoxic T-lymphocytes	$CD4^+$ helper T-lymphocytes

1.4. The dogma of "immune privilege" of corneal allografts

Sir Peter Medawar introduced the term "immune privilege" for the phenomenon that foreign tumour cells grafted into the anterior chamber of the eye exhibited a longer survival time in comparison to tumour cells, which had been injected into the muscles, under the skin or into other organs. In contrast to other organs characterised by a high degree of vascularization foreign antigens in the anterior chamber of the eye could be isolated from the host's immune system but failed to induce an alloreactive immune response. Based on these experiments it was postulated that the absence of blood and lymph vessels in the cornea protects the graft from immune competent APCs and thus prevents an alloresponse against the donor's cornea. Generally soluble and membrane-bound factors (FasL) were found on the corneal surface and in the anterior chamber, which induce apoptosis of immune cells leading to the suppression of alloreactive processes (Chong and Dana, 2007).

The injection of allograft cells into the anterior chamber can induce anterior chamber-associated immune deviation thus inducing tolerance specific for those antigens that have been injected. In particular the Th1 immune responses, such as delayed-type hypersensitivity (DTH), are actively suppressed in an antigen-specific manner (Niederkorn et al., 2004). This phenomenon named anterior chamber-associated immune deviation (ACAID) is a unique form of immune regulation only induced by the injection of alloantigens into the anterior chamber.

The "Pros" and "Cons" concerning the "immune privilege" of cornea have been summarized by Jerry Niederkorn using the image of a "three-legged stool". According to this image each stool leg symbolises one part contributing to the maintenance of the immune privilege. The afferent part of the "immune privileged" status in the eye comprises (i) the lack of blood and

lymph vessels, (ii) lymphocytes, (iii) low expression of HLA molecules, (iv) a decreased number of APCs which in addition are immature, and (v) active immunosuppressive molecules (Niederkorn et al., 2004). The central part includes processes such as clonal deletion and suppression leading to anergy of immunocompetent cells. The efferent part of the immune response is blocked by different molecules such as (i) membrane-bound FasL on endothelial cells and soluble FasL in the anterior chamber thereby promoting the apoptosis of T-cells or (ii) by the decay accelerating factor (DAF) suppressing the antibody-mediated activation of both complement pathways (Niederkorn, 2001; Hegde et al., 2005).

It is noteworthy that the “immune privilege” has not only been found in the eye, but also in other organs and tissues such as (i) the brain which is isolated from immune cells of the circulation by the brain-blood-barrier, (ii) the pregnant uterus containing the foetus which is protected by the immunosuppressive HLA-G molecules from a maternal alloresponse and (iii) certain solid tumours which exhibit a self-protecting mechanism by the up-regulation of the HLA-G antigen and B7-H molecule expression (Bukur et al., 2003; Mahnke et al., 2007).

The immune privileged status of the cornea and of the anterior chamber is in accordance with the fact that about 82 - 90 % of primary grafts are successfully accepted without any HLA matching and/or systemic application of immunosuppressive drugs after grafting. Due to their immune privileged status transplanted corneas have been regarded as “forgotten graft” for a long time generally leading to low interest of physicians and immunologists to investigate the reasons for corneal graft losses (George and Larkin, 2004). However, it has been demonstrated that in about 10 - 18 % of primary grafts and in nearly 60 % of the regrafts an alloresponse occurred after transplantation (Thompson at el., 2003).

Until the end of last century only a few scientific groups studied the mechanisms of corneal graft loss or the immunological processes involved in the rejection or acceptance of these grafts. The corneal transplant represents a simple graft model as only epithelial and endothelial cells on a stroma with inert collagen lamillars are involved. Because of this tissue-depending simplicity it may be an adequate model to investigate rejection processes, metabolic changes during transplantations without and with complications and pharmacokinetic effects of immunosuppressive drugs. In accordance with the alloresponse against transplanted solid organs such as e.g. kidney and heart, the inner endothelial cell layer of the cornea represents the main target for the immune response of the recipient against the graft (Janeway et al., 2005).

1.5. Clinical observations correlating with corneal graft rejection

Starting with the explantation and continued by the storage and implantation of the cornea processes may be induced which finally lead to the loss of endothelial cells. This loss can occur immediately after transplantation (early rejection) or after several years (delayed type of rejection).

1.5.1 Primary and secondary graft failure and early graft rejection

The primary graft failure is defined by the following symptoms: (i) a diffuse oedematous graft on the first postoperative day, (ii) no up-clearing of the graft at any postoperative time, and (iii) lack of any identifiable cause of graft failure. The only known reasons leading to an increased risk of primary graft failure are the prolonged storage of the graft (> 7 days at 4 - 8°C) and the increased age of older donors (> 70 years) (Boehringer et al., 2002; Claerhout et al., 2007).

The reasons described for secondary graft failures are (i) surgical complications, (ii) non-immunological reasons such as persistent epithelial defects, infections or glaucomas, and (iii) graft rejections for immunological reasons. The rejections were either reversible or irreversible. They occurred after 1 to 2 weeks in initially clear and in the technical way successfully transplanted corneal grafts. The specific host's immune response attacks the donor's corneal endothelium and downregulates the draining capacity of the endothelial cells finally leading to an oedematous stroma. This process may occur during the first five years after grafting.

In addition to the reasons for graft failure mentioned above other risk factors leading to the loss or failure of keratoplasty may occur. These include for example the size of the graft, which turned out to be dominating factor for rejection. Graft sizes larger than 8.25 mm were accompanied by a six-fold increased risk for rejection as demonstrated by Epstein et al. (2006) and Wagoner and co-authors (2007). Additional risk factors for graft failures are an increased donor and patient's age, respectively, historical rejection episodes, deep vascularization of the cornea in the host eye, previous surgery in the anterior segment and regrafting (Dua and Azuara-Blanco, 1999; Sangwan et al., 2005; Wagoner et al., 2007). This is in contrast to the studies correlating eleven factors (age, gender, atopic dermatitis, dry eye symptom, surgeon, graft size, post-mortem time, storage time, graft preservation and duration of post-operative epithelial defects) with graft rejection (Graupner et al., 2000; Kuechle et al., 2002). A correlation with early graft rejections was only observed for atopic dermatitis, tear insufficiency (dry eye) and the duration of the graft storage but not with the other parameters described as relevant in the previous studies (Naacke et al., 2001; Boehringer et al., 2002; Epstein et al., 2006; Wagoner et al., 2007; Claerhout et al., 2007).

1.5.2. Search for biomarkers indicating a rejection/graft loss

In the last decade several studies have been initiated to analyse the mechanisms leading to the rejection of corneal grafts or endothelial cell loss. In this context several groups tried to identify biomarkers, which are of prognostic relevance for an upcoming rejection. Some interleukins (IL) and other biomarkers obtained from the aqueous humour were investigated for a possible correlation to the outcome of corneal grafting.

Increased concentrations of the cytokine IL-6 produced by different cells such as T lymphocytes, macrophages and the corneal endothelial cells and known to exhibit pro- and anti-inflammatory activity were shown to be involved in rejections and thus to be a potential biomarker (Funding et al., 2005). In addition it is noteworthy that monocytes and macrophages play a crucial role in the antigen-presenting pathway during graft rejections. The surface marker CD163, a member of the B scavenger receptors, is exclusively expressed on monocytes and macrophages. Furthermore soluble CD163 molecules are significantly correlated with the number of local monocytes and macrophages. The levels of both IL-6 and soluble CD163 are increased in the aqueous humours of patients suffering from rejection episodes, although the concentrations of both molecules did not correlate with the outcome of corneal transplantations (Funding et al., 2005).

1.6. Standard procedures for solid organ transplantation

An impact of the foreign donor HLA molecules on graft rejection was already demonstrated by Khodadoust and Silverstein (1969) nearly 40 years ago. The standards of all international and national organ allocation organisations e.g. American Society for Histocompatibility and Immunogenetics (ASHI), European Federation for Immunogenetics (EFI) recommend a pre-transplant crossmatch with serum samples obtained from the recipient against vital lymphocytes from the donor for the transplantation of solid organs. This crossmatch procedure allows the detection of pre-existing donor-specific cytotoxic anti-HLA class I antibodies and/or anti-HLA class II antibodies in order to exclude a hyper-acute or an acute humoral rejection of the graft. In general the standards for solid organ transplantation recommend a regular control of the anti-HLA antibody status of all recipients on the waiting list in particular in cases of graft loss. The antibody specificities detected represent a contraindication for a following graft (Taylor et al., 1989; Takeda et al., 2000; Sumitran-Holgersson, 2001; Piazza et al., 2001).

In contrast to the transplantation of solid organs there exist so far no defined standards or recommendations for corneal grafting due to the immune privileged situation in the eye. In general keratoplasties were performed without prior tissue typing or systemic treatment with immunosuppressive drugs. Despite this situation, approximately 82 - 90 % of the first-time corneal allografts are not rejected and succeed.

A special problem arising from corneal transplantations is the fact that this tissue is stored up to four weeks from the date of explantation. This length of storage time leads to the impossibility of the conventional crossmatch procedure (CDC-CM), which is based on using freshly isolated lymphocytes of a given donor. Since this crossmatch procedure depends on the principle of complement-mediated cytotoxicity it requires the harvest vital lymphocytes from the donor, their prompt storage in liquid nitrogen (-196 °C) and their consecutive transport on dry ice (-70 °C) to the recipient's centre. This procedure is not practicable and extremely expensive in comparison to the handling of the corneal graft, which can be stored in an aseptic storage-solution in an incubator up to one month and may be transported by standard mail. The novel ELISA-based Micro-AMS HLA class I and class II crossmatch procedure for the first time allows using corneal material for the detection of donor-specific anti-HLA antibodies. In the future it may be adapted for the use of small pieces from the explanted eye e.g. parts of the retina or eye vessels as donor material which may be stored in parallel with the cornea to be transplanted.

1.7. Aim of the study

Although keratoplasties represent the most successful form of solid tissue transplantations due to the significant degree of immune privileged corneal graft rejection is the major cause of keratoplasty failure as shown by 10 – 20 % of graft losses after the first grafting and about 60 % after regraftings. Indeed a number of distinct immune mechanisms have been suggested to be involved in corneal allograft rejections. Therefore the proper selection of patients with high risk for graft loss and early detection of host alloresponse might improve the graft survival. Based on this assumption, the aim of this study was to develop a tool for monitoring immune processes which might lead to keratoplasty failure. In addition, the general lack of donors' lymphocytes due to the extended storage of the corneal transplants has to be overcome. It was investigated whether the implementation of the ELISA-based AMS crossmatch procedure prior to and after corneal grafting may be an adequate method to overcome this drawback. This strategy would allow to determine whether donor-specific anti-HLA antibodies after transplantation might give reliable prognostic data to predict and/or confirm a corneal graft rejection. Furthermore, the existence of donor-specific anti-HLA antibodies was correlated with the clinical outcome of keratoplasty.

Thus, evidence should be provided that at least under certain circumstances i.e. after destruction of the "immune privileged" status in the eye recipients´ anti-HLA antibodies directly contribute to the destruction of donors´ corneal cells.

2. Materials and methods

2.1. Materials

2.1.1. Patients and corneal tissue samples

The patient group analysed is comprised of 45 cornea recipients from the Clinic and Dispensary of Ophthalmology of the University Hospital Kroellwitz at the Martin Luther University Halle-Wittenberg.

The study was approved by the local ethics committee of the Martin Luther University and by Prof. Kirste, Head of the German Foundation of Organ Transplantation (DSO). It followed the guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients prior to this study.

The outer rims of grafted corneas were kindly provided by Dr. T. Bredehorn from the Cornea bank Halle - region East-Germany of the DSO - Department of Tissues (DSO-G). The explanted corneal material was kindly provided by Prof. G. Duncker and Dr. S. Sel, Clinic and Dispensary of Ophthalmology.

2.1.2. Ophthalmologic parameters defining visual acuity

Visual acuity is the most common clinical measurement of vision and defined the acuteness or clearness of vision, which depends on the sharpness of the retinal focus within the eye, the sensitivity of the light sensitive nervous cells in the retina, and the interpretative faculty of the brain. Visual acuity is often measured according to the size of letters viewed on a Snellen chart or the size of other symbols. The letters in each line have half of the size compared to letters in the upper line. The ability to identify the smallest black letters on a Snellen chart at the standard distance (6 metres) defines the visual acuity of a patient. Visual acuity measures the ability of the visual system to separately identify two objects with the smallest size.

In German speaking countries, visual acuity measurements are usually given in decimal number (the higher the number the better is the visual acuity of the patient). If the visual acuity is below the largest letter on the chart (first line) the patient has to move closer to the chart until she/he can read the letters. Letter size and the test distance are noted. When the patient is unable to read the chart at any distance the physician tests the remaining visual acuity as follows:

(i) counting fingers = ability to count fingers at a given distance

(ii) hand motion = ability to distinguish if a hand is moving or not in front of a patient's face

(iii) light perception = ability to distinguish if the eye can perceive any light

(iv) no light perception = inability to see any light

Besides the determination of the visual acuity another diagnostic standard approach is the measurement of the intraocular pressure (mmHg) which depends on the rate of aqueous production, facility of outflow and episcleral venous pressure. It can be measured either by palpation or by using a tonometer. This diagnosis is performed to exclude an increased intraocular pressure as origin of corneal deformation or reduced visual acuity.

2.1.3. Specific equipment

Table 3. Technical hardware and producers

Instruments	Manufacturer
Thermal shaker	Uniequip, Munich, Germany
ELISA reader MRX II	Chantilly, VA, USA
PCR thermocycler GeneAmp 9700	Applied Biosystem, Forster City, USA
Horizontal gel electrophoresis system	Geno Vision, Vienna, Austria
Power supply Consort E815	Consort, Turnhout, Belgium
Gel documentation system	LTF Labortechnik GmbH, Wasserburg, Germany
Centrifuge “mini spin”	Eppendorf GmbH, Hamburg, Germany
Research Pipettes (0.5 - 1.000 µl)	Eppendorf GmbH, Hamburg, Germany

2.1.4. Stock solutions, specific sets and reagents

All plastic materials which are not part of the commercial sets such as pipette tips, reaction tubes etc. were purchased from Greiner GmbH (Frickenhausen, Germany) if not stated otherwise. The two following stock solutions (Table 4) were prepared using the single components listed in Table 6.

Table 4. In-house prepared stock solutions

Stock solutions	Single components
TRIS-borate-EDTA buffer (20x conc. TBE)	545 g TRIS 47 g EDTA 279 g H_3BO_3 in 2,5 L dist. water
ethidium bromide solution	0,5 g ethidium bromide in 50 ml dist. water

All buffers and solutions mentioned in the following section generally represented components of the respective commercial sets (Table 5). The detailed composition of these reagents was often not available. Therefore the instructions of the manuals were cited.

Table 5. Commercial kits employed in this study and their manufacturers

Kits	Manufacturer
Antibody Monitoring System (Micro-AMS) HLA Class I and II ELISA	GTI Diagnostics, Waukesha, WI, USA
Quik-ID® Class I and Quik-ID® Class II ELISA	GTI Diagnostics, Waukesha, WI, USA
Invisorb® Spin Tissue Mini Kit	Invitek Gesellschaft fuer Biotechnik & Biodesign mbH, Berlin, Germany
GenomiPhi DNA amplification Kit	General Healthcare Europe, Munich, Germany
HLA-A*/B*/Cw* Protrans Cyclerplate system	Protrans GmbH, Ketsch, Germany
HLA-A, HLA-B, HLA-Cw, HLA-DR and HLA-DQ low resolution SSP kits	Olerup SSP AB, Saltsjoebaden, Sweden
HLA-DRB*/-DQB1* typing CTS-PCR-SSP Tray	CTS, Heidelberg, Germany

Table 6. Additional reagents required

Reagents	Manufacturer
Alkaline phosphatase-conjugated anti-human IgA/M/G antibody	GTI Diagnostics, Waukesha, WI, USA
RPMI 1640	Gibco, Invitrogen, Karlsruhe, Germany
Isotonic NaCl solution (PBS)	Lonza corp., Braine-l´Allend, Belgium
Lymphocyte Density Separation Media (LSM 1077)	PAA Laboratories GmbH, Pasching, Austria
Ethanol (96 %)	Sigma-Aldrich GmbH, Deisenhofen, Germany
Ethidium bromide	Serva Feinbiochemica GmbH, Heidelberg, Germany

Table 6 continued. Additional reagents required

Agarose	InnoTrain Diagnostics GmbH, Kronberg, Germany
AXITAQ-DNA polymerase	InnoTrain Diagnostics GmbH, Kronberg, Germany
Tris-(hydromethyl)-aminomethan (TRIS), Ethylenediamine-tetraacetic acid (EDTA), Boric acid (H_3BO_3)	Carl Roth GmbH, Karlsruhe, Germany
Distilled water, PCR grade	Fresenius Kabi GmbH, Bad Homburg, Germany

2.2. Methods

2.2.1. DNA extraction

The Invisorb® Spin Tissue Mini Kit was used for the DNA extraction of 1/3 to 1/4 from the whole outer rims of the transplanted donor cornea as well as from half of the explanted corneal materials of the recipients according to the manufacturer's instructions. The material was transferred into a 1.5 ml reaction tube and incubated with 400 µl of lysis buffer G and 40 µl proteinase K solution at 52 °C on a thermal shaker (Uniequip) for maximal 20 min. In order to lyse only the epithelial and endothelial cells from the surface of the cornea and to avoid the lysis of the whole collageneous protein of the stroma the recommended incubation time using the lysis buffer was reduced from 40 to 15 min. After a centrifugation step at 12.000 rpm for 2 min, the supernatant was transferred into a new 1.5 ml reaction tube. After adding 200 µl binding buffer T to the supernatant, the mixture was vortexed and than directly added onto the spin column containing the DNA binding membrane previously placed into a 2.0 ml collection tube and incubated for 1 min at room temperature (RT). This was followed by a centrifugation step at 12.000 rpm for 2 min. The resulting flow through fraction was discarded and the column was again placed into the collection tube. For further purification of the bound DNA from contaminating proteins and lipids the spin column was washed twice with 550 µl washing buffer by two centrifugation steps at 12.000 rpm for 1 min each. Then, the residual ethanol from the washing buffer was removed from the column by a final centrifugation step of the column into an empty collection tube for 2 min at 12.000 rpm. For the elution step the spin column was placed in a new 1.5 ml reaction tube before 50 µl pre-warmed elution buffer D (60 °C) was added to the column followed by an incubation step for 3 min at RT. In order to completely recover the DNA the column was centrifuged twice upon the addition of 50 µl elution buffer D with consecutive centrifugation steps at 10.000 rpm for

2 min, respectively. The eluted DNA was either immediately used or stored for further analyses at -20 °C.

2.2.2. Whole genome DNA amplification

Due to the low amount of extracted DNA available from the corneal material whole genome DNA amplification was performed using the GenomiPhi DNA Amplification Kit, which is based on the strand displacement amplification method (Blanco et al., 1989). This kit utilizes the bacteriophage Phi 29 DNA polymerase enzyme to amplify human genomic DNA. From 10 ng of purified human DNA each reaction generates 4 - 7 µg of DNA in 16 - 18 hours. Nine µl of cold sample buffer (4 - 8 °C) was mixed with 1 µl DNA solution (> 10 ng/µl) in a 200 µl PCR tube and incubated at 95 °C for 3 min to denature the double-stranded genomic DNA. Following this denaturation step the reaction mix was immediately cooled down to 4 °C to avoid the re-annealing of the denatured DNA strands. Then 9 µl of cold reaction buffer (4 - 8 °C) and 1 µl enzyme mix (-20 °C) were added to the solution, carefully mixed and incubated over night (16 - 18 hours) at 30 °C in a thermocycler. During this incubation the Phi 29 DNA polymerase initiates replication steps at multiple binding sites of random hexamer primers annealing to the single-stranded DNA thereby generating new copies of the DNA strands. Upon overnight incubation the reaction mixture was heated up to 65 °C for 10 min to stop the amplification process. Then 80 - 100 µl of distilled water (PCR grade) was added and the amount of amplified DNA was estimated by gel electrophoresis in a 2% ethidium bromide-containing agarose gel in comparison to samples with defined DNA concentrations.

2.2.3. HLA typing by SSP-PCR

The polymerase chain reaction (PCR) is one of the standard techniques used for the amplification of *in vitro* selected sequences/regions of genomic DNA (Olerup and Zetterquist, 1992). The technique allows both the identification of known DNA sequence motifs as well as the generation of identical copies of known/unknown DNA strands for further analyses. The common PCR technique consists of repetitive steps of (i) DNA denaturation, (ii) primer annealing and (iii) primer extension/elongation using up to 30 cycles per run. Theoretically more than one million copies can be generated from one single DNA template within 30 cycles. PCR runs using sequence-specific primers (SSP-PCR) were employed for the molecular typing of HLA class I and HLA class II genes, respectively.

The central component of an SSP-PCR typing kit comprises a set of oligonucleotide primer pairs, in which each primer pair is targeting a given HLA allele group-specific sequence motif. Only if the selected primer pairs attach to their complementary DNA strands a PCR product can be generated. In addition to the HLA allele group-specific primer pairs an internal control primer pair of the human growth factor gene was employed as a control. Subsequently the

amplification products were separated using a horizontal gel electrophoresis system and visualised using a 1.5 % ethidium bromide stained agarose gel. Thus, in a positive reaction both the HLA allele group-specific and the control amplification product will occur, whereas in the absence of an HLA-specific reaction, only the internal control amplificate is detectable (see reaction protocol as attachment 1).

For HLA class I genotyping either the commercially available HLA-A*/B*/Cw* Protrans Cyclerplate system (Protrans) or, alternatively, the single locus kits HLA-A, HLA-B and HLA-Cw low resolution SSP (Olerup) were used, whereas for the HLA class II genotyping either the commercially available HLA-DRB*/-DQB1* typing CTS-PCR-SSP Tray kits (CTS) or the single locus kits HLA-DR and HLA-DQ low resolution SSP (Olerup) were employed.

2.2.4. Detection of anti-HLA antibodies

The anti-HLA class I and class II antibodies were detected using the Quik-ID® Class I and Class II solid phase ELISA systems (GTI Diagnostics), respectively. The Quik-ID® Class I ELISA kit provides HLA class I glycoproteins from platelets of 40 donors each immobilized in different micro-wells by means of an anti-HLA class I monoclonal capture antibody (see reaction protocol as attachment 2). Each kit includes a separate 8-well strip containing four wells of negative controls (NC), one well of positive control (PC), one well without any HLA molecules (no antigen, NA) and two blank wells (Blank).

The Quik-ID® Class II ELISA kit provides HLA class II glycoproteins from 30 EBV-transformed cell lines of B lymphocyte origin each immobilized in different micro wells by means of an anti-HLA class II-monoclonal capture antibody (see reaction protocol as attachment 3). The controls of the Quik-ID® Class II ELISA kit include the “no antigen control” (NA) and the monoclonal antibody (mAb) control (MO, only consisting of the capture anti-HLA class II antibody without any HLA molecules fixed in the well), a separate 8-well strip containing four wells of negative controls, two wells of positive controls, and two blank wells. Both kits include negative and positive anti-HLA class I antibody or class II antibody control serum samples, respectively.

For the detection of anti-HLA class I antibodies a set of six 8-well strips coated with HLA class I molecules or controls and labelled with different colours was employed (Table 7A) whereas for the determination of anti-HLA class II antibodies a set of five coloured 8-well-strips coated with HLA class II molecules or controls was used (Table 7B). All strips were fixed in a micro well frame according to code of colour labelling at the top.

Table 7. Application schemes of the Quik-ID® Class I (A) and Quik-ID® Class II (B) antibody detection ELISA kits

A) Quik-ID® Class I

	Y	G	B	P	R	O
A	1	9	17	25	33	N
B	2	10	18	26	34	N
C	3	11	19	27	35	N
D	4	12	20	28	36	N
E	5	13	21	29	37	P
F	6	14	22	30	38	NA
G	7	15	23	31	39	B
H	8	16	24	32	40	B
	1	*2*	*3*	*4*	*5*	*6*

B) Quik-ID® Class II

	P	F	W	V	B
A	1	9	17	25	N
B	2	10	18	26	N
C	3	11	19	27	N
D	4	12	20	28	N
E	5	13	21	29	P
F	6	14	22	30	P
G	7	15	23	MO	B
H	8	16	24	NA	B
	1	*2*	*3*	*4*	*5*

(A) Letter codes for HLA class I colours: Y = yellow, G = green, B = blue, P = purple, R = red, O = orange; (B) Letter codes for HLA class II colours: P = pink, F = fuchsia, W = white, V = violet, B = black; Letter codes for the controls: N = negative control, P = positive control, NA = no antigen control, B = Blank; MO = mAb only

Test procedure

Prior to the experiment all reagents had to be adjusted to room temperature (22 - 25 °C). First the positive serum control (PC), negative serum control (NC) and the recipient's serum sample were diluted 1:4 with specimen diluent solution (SD). Thereafter 250 µl of washing solution was added into each well and the plate was incubated for 5 min at room temperature before the washing solution was aspirated.

In the first step the following reagents were added into the respective reaction wells:

(i) 50 µl positive serum control into the positive control well

(ii) 50 µl negative serum control into the negative control wells

(iii) 50 µl recipient's serum sample into all numbered reaction wells as well as into MO control and the "no antigen control" well

The strips were sealed with a plate foil and incubated at 37 °C on a rocking platform. After 50 min incubation the content of the wells was decanted, lashed out onto an absorbent paper towel before the plate was rinsed with 250 µl washing solution/well, which was again discarded. This washing procedure was repeated at least four times.

In the second step 50 µl of the ready-to-use alkaline phosphatase-conjugated anti-human IgM/IgG antibody was added to all wells except the Blank well. The strips were closed with a plate sealer foil and again incubated at 37 °C on a rocking platform for 50 min. Then the washing procedure of the plate followed as described above.

In the third step 100 µl of the ready-to-use PNPP (p-nitrophenyl phosphate) substrate solution was added into all wells except for the Blank wells. The strips were again sealed

with foil and incubated at room temperature in the dark for 20 min. Finally the reactions were stopped by adding 100 µl of stop solution (3 M NaOH)/well, whereas 200 µl of stop solution was added into the Blank wells.

The resulting absorbance (optical density, OD) of all wells was monitored at 405 nm for the reactions and at 605 nm for the background values, respectively. The cut-off for the reaction in each well was calculated using an interpretation software based on the absorbance in the blank and the background adjustment factor, which is specific for each well. Test results with an OD value greater than the cut-off value were regarded as positive results. The criteria for a valid test are defined by (i) a negative control, which is characterised by a mean OD value ≤ 0.225, and (ii) a positive control OD value ≥ 1.000.

As readout the anti-HLA antibody specificity was defined by positive reactions that occurred only against several HLA antigens of the cells fixed in the different wells (see attachments 2 and 3).

Table 8. Flow diagram of the Quik-ID® Class II ELISA

Reagent added to the						
HLA class II	mAb	No antigen	Positive control	Negative control	Blank	Test regime
250 µl washing solution						5 min at RT
50 µl ReS			50 µl PC	50 µl NC	---	50 min at 37 °C, 100 rpm
250 µl washing solution						4-times
50 µl AGM					---	50 min at 37 °C, 100 rpm
250 µl washing solution						4-times
100 µl PN					---	30 min at RT in the dark
100 µl SS					200 µl SS	
Determination of absorbance (OD) at 405 nm						

ReS = diluted **Re**cipient **S**erum, PC = diluted **P**ositive Serum **C**ontrol, NC = diluted **N**egative Serum **C**ontrol, AGM = ready-to-use **A**nti-human Ig**G**/**M** conjugate, PN = **PN**PP substrate diluted in substrate buffer, SS = **S**top **S**olution

2.2.5. Antibody Monitoring System HLA class I and class II ELISA

2.2.5.1. Principle of the test system

The Antibody Monitoring System (AMS) HLA class I and class II ELISA kit manufactured by GTI® Diagnostics (Waukesha, WI, USA) and distributed by Diagast GmbH (Aachen, Germany) was the first commercially available solid phase ELISA kit for the direct detection of donor-specific anti-HLA class I and/or II antibodies in sera from humans. The first design of the kit used standard ELISA microtiter plates with a minimal reaction volume of 50 µl. Due to the limited donor material the AMS ELISA kit was adapted to ELISA plates with a reduced volume (15 µl) and designated as Micro-AMS HLA class I and class II ELISA.

The Antibody Monitoring System is based on five steps:

(i) preparation of a “complete protein fraction” including HLA molecules by lysis of donor cells
(ii) immobilization of the donor’s HLA molecules with monoclonal capture antibodies
(iii) incubation with serum of a given recipient to bind the donor’s HLA molecules
(iv) adding of alkaline phosphatase-conjugated anti-human IgM/G/A antibodies recognising the bound recipient’s anti-HLA antibodies
(v) visualization of the donor-specific reaction using the PNPP substrate

2.2.5.2. Controls of the AMS ELISA

In addition to the standard negative control (NC) and background wells (blank) the Micro-AMS ELISA includes two controls, which are specific for the test system: the AMS-specific control also named (i) reagent control and (ii) the lysate control.

Reagent control

Each kit includes a dried lymphocyte control pellet (DLC), a positive control serum (PC) and the components of the reagent control (RC). Briefly the dried lymphocyte control pellet was rehydrated using 500 µl complete cell culture media, e.g. RPMI 1640, and one-hour incubation time at RT. The cell pellet was resuspended by vortexing to obtain a homogenous cell suspension. The supernatant was removed after centrifugation at 4.000 rpm for 5 min in an Eppendorf micro-centrifuge and the control cells were lysed in 100 µl ready-to-use lymphocyte lysis buffer. After centrifugation of the lysate at 4.000 rpm for 5 min to remove debris and cell membranes the supernatant was transferred into a new 1.5 ml reaction tube. Afterwards the lysate was either stored on ice for a short time (maximum 4 hours) or in 20 µl aliquots at -70 °C for up to several months. It is noteworthy that the control lysate (DLC) is always used in combination with the lot-specific positive control serum (PC) instead of the donor’s cell lysate, which is used in combination with the recipient’s serum sample.

Lysate control HLA class I and class II antigens

The second AMS ELISA-specific control is necessary for the detection of a sufficient amount of bound HLA class I or class II molecules of the donor's material. After transferring the ready-to-use donor cell lysate into the wells for the lysate control, the wells were sealed and incubated for 30 min at 37°C in a water bath. In order to prevent the wells from drying the lysate and conjugate diluent was added to these wells. For the detection of the bound donor-specific HLA class I molecules in the lysate control class I wells a second mAb specific for HLA class I molecules labelled with alkaline phosphatase (LCR 1) was used before the PNPP substrate solution was added and the optical density (OD)/well was determined (Figure 10). The lysate control class II was carried out using a second mAb specific for HLA class II molecules and labelled with alkaline phosphatase (LCR 2).

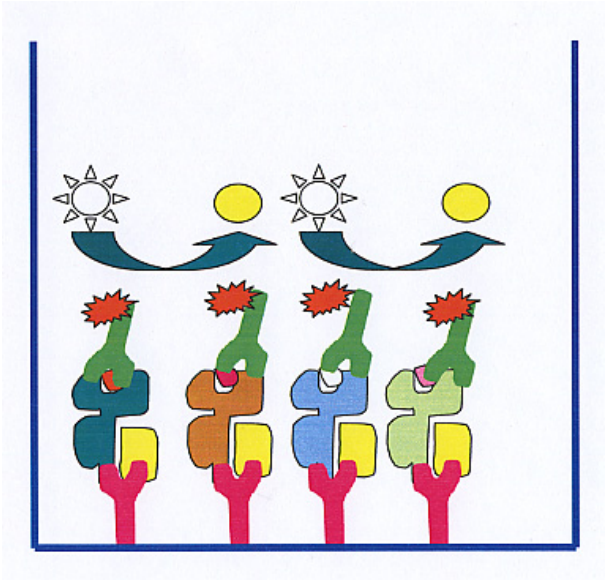

Figure 10. Schematic diagram of the lysate control. The monoclonal capture antibody (carmine) immobilized the different native HLA class I molecules derived from the donor cells (differentially coloured α chains with yellow marked β_2-m). Then, the enzyme-labelled anti-HLA antibody for the detection (green with red star) binds to a second monomorphic epitope on the HLA molecules. The binding of lysed HLA molecules from the donor tissue is visualised by the change of a colourless substrate into to a yellow reaction product, which is measured by absorbance (OD) at 405 nm.

2.2.5.3. Preparation of the donor lysate

Separation of lymphocytes

The Micro-AMS ELISA HLA class I and II kits are recommended for the use of blood lymphocytes as donor material after their isolation by density gradient separation. Briefly, 10 ml whole blood collected in sodium heparin or citrate monovettes were diluted with 20 ml isotonic phosphate-buffered saline (PBS) and then carefully layered onto lymphocyte density separation media (LSM 1077). After centrifugation at 3.200 rpm for 20 min the lymphocyte

cell rich interphase was collected and then transferred into a new 12 ml glass tube. For the elimination of the thrombocytes and remaining lymphocyte density separation media the cell suspension was washed twice with PBS by centrifugation at 900 rpm for 7 min. The resulting supernatant was discarded, the cell pellet subsequently re-suspended in 500 µl serum-free RPMI 1640 and the cell suspension was then transferred into a 1.5 ml reaction tube. The lymphocytes were washed three times by centrifugation steps at 3.000 rpm for 3 min in 500 µl RPMI 1640. After the final washing step the supernatant was completely removed and the volume of the cell pellet estimated by comparison with 1.5 ml reaction tubes containing defined volumes of PBS (20 µl, 30 µl, 40 µl, 50 µl and 70 µl).

Lysis of lymphocytes

Depending on the obtained cell pellet size the volume of the ready-to-use lymphocyte lysis buffer (1 x LLB) was calculated. Per 100 µl of the highly viscous 10 x LLB concentrate 900 µl of distilled water was added, the mixture carefully vortexed and stored on ice for up to 4 hours. For the lyses of any 10 µl of packed donor lymphocytes 100 µl of ready-to-use LLB was required. The cell pellet was re-suspended using a pipette and vortexed to achieve complete homogenisation. After incubation at 4 - 7 °C for 20 min the lysate was centrifuged at 13.000 rpm for 10 min to sediment the cell membranes und nuclei. The supernatant was then transferred into a new 1.5 ml reaction tube. The undiluted donor lysate was stored overnight in a refrigerator or up to several months at -70 °C.

Preparation of donor lysate from the outer scleral rim

The preparation of the HLA molecules (donor lysate) has to be performed within 72 hours after the cornea transplantation. One third of the outer scleral rim of the cornea was cut with a scalpel into pieces and subsequently homogenized in 150 - 350 µl freshly prepared 1x LLB depending on the volume of the tissue using a mortar, with pestle and glass powder. Then the suspension was transferred into a 1.5 ml reaction tube, vortexed and incubated at 4 - 8 °C for 1 hour. During the incubation time the suspension was mixed several times. Then, the solution was centrifuged at 13.000 rpm for 10 min before the supernatant was carefully transferred into a novel 1.5 ml reaction tube. The undiluted donor lysate was either stored overnight in a refrigerator or for several months at -70 °C. As demonstrated in Figure 11 an immunoassay must be used, which selectively recognizes HLA class I and HLA class II molecules of the donor.

A prerequisite for the assays is that other membrane-bound molecules do not interfere with the detection of donor-specific anti-HLA antibodies in order to immobilise only the HLA molecules of the donor and consecutively donor-specific anti-HLA antibodies of a given recipient.

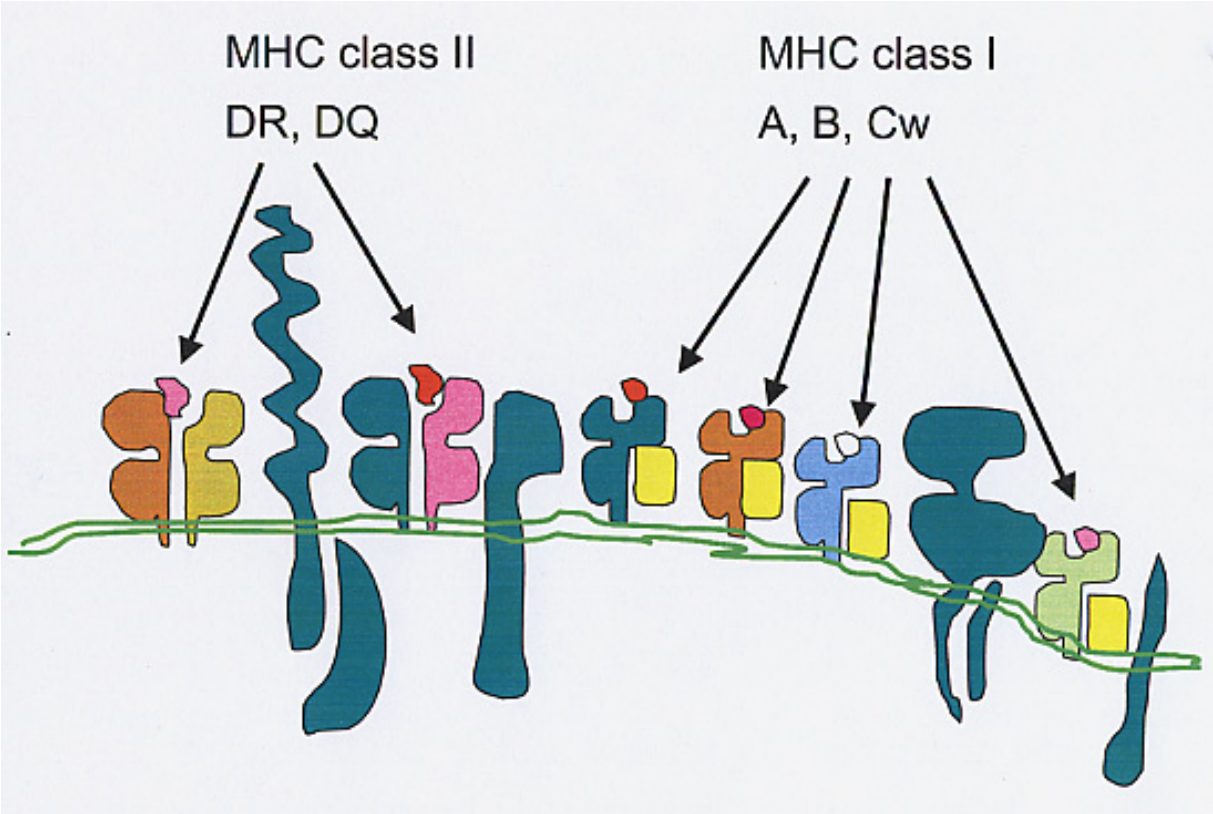

Figure 11. Schematic diagram HLA class I and class II surface antigens. HLA class I molecules (differentially coloured α chains with yellow marked β_2-m and peptide fragments in the binding grooves) and HLA class II molecules (differentially coloured heterodimers with peptide fragments in the binding grooves) are flanked by residual membrane-bound molecules such as e.g. different receptors and transport molecules (dark green).

2.2.5.4. Test procedure for scleral donor lysate

Prior to the experiments all reagents had to be adjusted to room temperature (22 - 25 °C). In parallel, the 1 x washing solution was prepared by diluting 1 volume 10 x concentrated stock solution (TCW) with 9 volumes of distilled water. The washing solution could be stored up to one week at 4 - 8 °C. Due to the complete lyses of the outer epithelial und the inner endothelial cells from a given cornea rim it is not possible to define the cell pellet size for the preparation of the lymphocyte-derived donor lysate (s. 2.2.5.3).

Thus, the dilution for the donor lysate was defined by means of a theoretical calculation of the cell number on the surface of the outer scleral rim. For analyses of both HLA class I and II, one volume of donor lysate was diluted three-fold with lysate and conjugate diluent (LCD). The positive control (PC), negative control (NC) and the recipient serum samples were diluted 1:4 with the specimen diluent (SD). For the detection of donor-specific anti-HLA class I and II antibodies one strip coated with a capture mAb specific for HLA class I molecules (blue label, MS1) and one strip coated with mAb specific for HLA class II molecules (purple label, MS2) was employed, respectively. Both strips were fixed in a micro-well frame with the coloured label codes marked at the top. The positions of the controls and the recipient-donor reactions of both class I and II strips are schematically shown in Table 9.

Table 9. Positions for the different reaction wells on the HLA class I and class II strips

blue	Colour code of the strip	purple
O	Reagent Control	O
O	Negative Control	O
O	Lysate Control LCR1 | Lysate Control LCR2	O
O	Recipient-donor reaction	O
O	Recipient-donor reaction	O
O	Recipient-donor reaction	O
O	Recipient-donor reaction	O
O	Blank well	O
MS1		MS2
HLA class I		HLA class II

All reagents or solutions applied for the test procedure were either used at a ready-to-use concentration or diluted to reach the working concentrations as outlined in the flow sheet of the Micro-AMS-ELISA kit.

In the first step the following reagents were added into the different reaction wells:

(i) 15 µl dried lymphocyte control into the positive control well

(ii) 15 µl lysate and conjugate diluent into the blank well

(iii) 15 µl donor lysate into both negative control wells, the lysate control wells (LCR1 and LCR2) and into the wells for the recipient-donor reaction

Instead of a water bath an incubator was used and the incubation times were extended from 30 min to 50 min to immobilise even low concentrations of HLA molecules from a given donor lysate. The strips were sealed with a plate foil and incubated at 37 °C on a rocking platform. After 50 min the contents of the wells were decanted, lashed out onto an absorbent paper towel before the plate was rinsed with 140 µl washing solution/well and the fluid again decanted. The washing procedure was repeated at least 4-times.

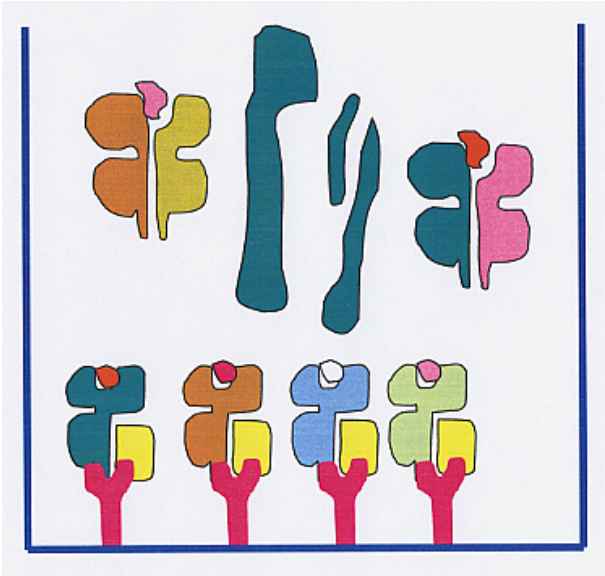

Figure 12. Schematic diagram of specific capturing of native HLA class I molecules by the capture antibody. HLA class I molecules (differentially coloured α chains with yellow marked β_2-m and peptide fragments in the binding grooves) derived from the donor cells were immobilized by an anti-pan HLA class I-specific monoclonal capture antibody (carmine), whereas residual proteins (dark green) or the HLA class II molecules (differentially coloured heterodimers with peptide fragments in the binding grooves) were not immobilized by the capture antibody.

In the second step the following reagents were transferred into the different reaction wells:

(i) 15 µl positive serum control into the positive control well

(ii) 15 µl negative serum control into both negative control wells

(iii) 15 µl recipient serum into the recipient-donor-reaction wells

(iv) 15 µl lysate and conjugate diluent into the blank well and into the lysate control wells, respectively.

The strips were sealed with a foil and incubated at 37 °C for 50 min on a rocking platform.

During the incubation time the ready-to-use conjugate solution and both lysate control reagents were prepared.

(i) 10 µl conjugate stock solution (anti-IgG/M/A) were diluted 1:100 with lysate and conjugate diluent (LCD)

(ii) 2 µl lysate control reagent for HLA class I (LCR1) were diluted with 198 µl lysate and conjugate diluent (LCD)

(iii) 2 µl lysate control reagent for HLA class II (LCR2) were diluted with 198 µl lysate and conjugate diluent (LCD).

After the incubation all wells were again washed for four times with washing solution as previously indicated.

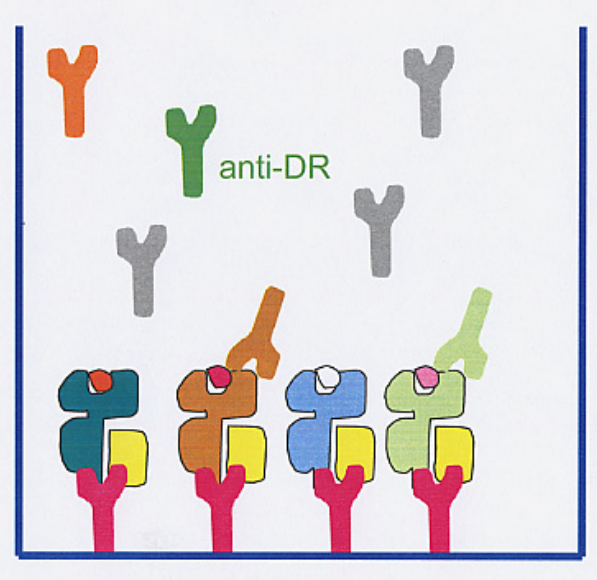

Figure 13. Schematic diagram of the antibody reaction. Donor-specific anti-HLA class I antibodies (lime and brown coloured) from the recipient´s serum specifically bind to the corresponding HLA molecules of the donor. Non-donor specific anti-HLA class I Ab (ochre), anti-HLA class II Ab (green) and among them irrelevant antibodies in the serum of the recipient do not bind to the donor´s HLA class I molecules.

In the third step the following reagents was added into the different reaction wells:

(i) 15 µl lysate control reagent for HLA class I (LCR1) into the lysate control well of the MS1-strip

(ii) 15 µl lysate control reagent for HLA class II (LCR2) into the lysate control well of the MS2-strip

(iii) 15 µl conjugate solution in the positive control well, the negative control well and all possible recipient-donor reaction wells

(iv) 15 µl lysate and conjugate diluent into the blank well.

The strips were sealed with a foil and incubated at 37 °C for 50 min on the rocking platform. During the incubation the ready-to-use substrate solution was freshly prepared. 500 µl distilled water were added to the tube with the PNPP substrate (PN) and carefully mixed. The PNPP stock solution (PN) was diluted 100-fold with substrate buffer (SB). After incubation all wells were again washed four-times with washing solution as previously indicated.

In the fourth step 50 µl substrate solution (PN) was added into all wells except for the blank wells. The strips were afterwards incubated at room temperature for 20 min in the dark without shaking.

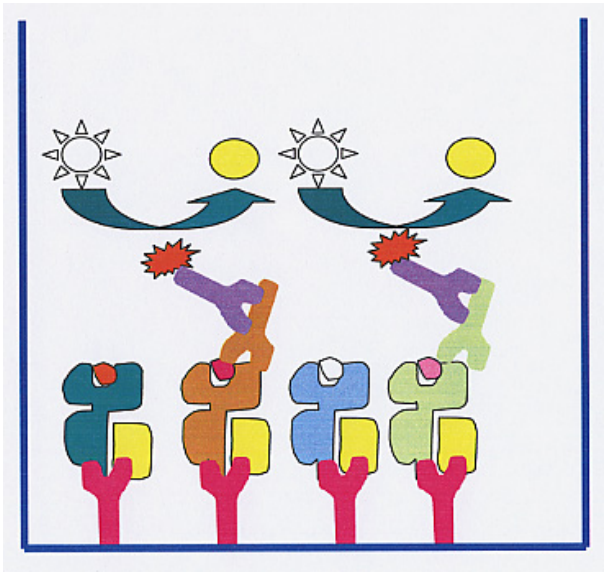

Figure 14. Schematic diagram of visualisation of the donor-specific anti-HLA antibodies. A secondary polyclonal anti-human antibody (purple) labelled with alkaline-phosphatase (red star) serves as a conjugate and binds to the donor-specific anti-HLA class I antibodies. The donor-specific antigen-antibody reaction is visualised by a change of the colourless substrate (PN) to a yellow reaction product.

In the last step the reactions were stopped with Stop Solution (3M NaOH):

(i) 50 µl stop solution (SS) were added into all wells except the blank wells

(ii) 100 µl stop solution (SS) were added into the blank wells

The absorbance (OD)/well was determined at 405 nm for the reactions and at 605 nm for the background.

Table 10. Flow diagram of the Micro-AMS HLA class I & class II ELISA

Reagent adding to the					
Blank	Reagent control	Negative control	Lysate control	Donor-recipient reaction	Test regime
15 µl LCD	15 µl DLC	15 µl DoL			50 min at 37 °C, 100 rpm
140 µl washing solution					4-times
15 µl LCD	15 µl PC	15 µl NC	15 µl LCD	15 µl ReS	50 min at 37 °C, 100 rpm
140 µl washing solution					4-times
15 µl LCD	15 µl AGM	15 µl AGM	15 µl LCR	15 µl AGM	50 min at 37 °C, 100 rpm
140 µl washing solution					4-times
---	50 µl PN				30 min at RT in the dark

Table 10 continued. Flow diagram of the Micro-AMS HLA class I and class II ELISA

100 µl SS	50 µl SS	
Determination of the absorbance (OD) at 405 nm		

LCD = **L**ysate and **C**onjugate **D**iluent, DLC = ready-to-use **D**ried **L**ymphocyte **C**ontrol lysate, DoL = ready-to-use **Do**nor **L**ysate, PC = diluted **P**ositive Serum **C**ontrol, NC = diluted **N**egative Serum **C**ontrol, ReS = diluted **Re**cipient **S**erum, AGM = ready-to-use **A**nti-human Ig**G**/**M** conjugate, LCR = diluted **L**ysate **C**ontrol **R**eagent, PN = **PN**PP substrate diluted in substrate buffer, SS = **S**top **S**olution

Quality controls and result interpretation

The absorbance (OD) of the lysate controls (LCR1 and 2) directly depend on the amount of bound donor HLA class I or II molecules, respectively, and thus represent the effective lysis of the donor material. The reagent control using freeze-dried lymphocytes as an antigen source, which is provided by the manufacturer, represents a lot-specific recipient-donor reaction with an obligatory strong positive result. The negative control defines a clearly negative result obtained with a negative human serum against a given donor lysate. The manufacturer provides the negative control serum. The criteria for a valid test are defined by (i) a negative control which is characterised by a mean OD value ≤ 0.300, (ii) a reagent control OD value ≥ 1.000 and (iii) a lysate control OD value ≥ 0.900. The reagent control and the lysate control both represent assay-specific positive controls described above (2.2.5.2.).

The difference in the OD between duplicate wells used for the same reaction should be below 20 %. Results outside of this limit should be retested. Results in the recipient-donor reaction wells with a 2-fold higher absorbance (OD) than the mean OD of the negative controls were regarded as positive demonstrating the presence of donor-specific anti-HLA antibodies in the recipient's serum sample.

3. Results

The study was performed to investigate (i) whether donor-specific anti-HLA antibodies can be detected after explantation of corneal grafts and (ii) whether corneal rejections are associated with anti-HLA antibodies. The existence of such a correlation between donor-specific anti-HLA antibodies and corneal rejections would limit the immune privileged status of this tissue generally postulated. Consequently an adequate selection of patients characterised by a high risk for the graft loss could considerably improve the outcome of corneal grafting. A prerequisite for this study was the development of an experimental strategy, which allows the detection of donor-specific anti-HLA antibodies in the recipient's serum. Therefore, the Antibody Monitoring System (AMS) HLA class I and class II ELISA was modified using material from the outer rim of a grafted cornea since isolated lymphocytes from the donor were not available.

3.1. Clinical data of patients

Between March 2005 and January 2007 after corneal grafting, the outer scleral rim of the newly transplanted cornea and 5 - 10 ml serum samples were collected from 45 recipients. From all recipients half of the outer rims of the graft and in addition the explanted central parts from 19 recipients of the own cornea or of a former corneal graft were collected.

The serum samples were stored at -20 °C until further investigation, whereas the outer scleral rims and the explanted corneal materials were stored at -70 °C to avoid degradation of HLA molecules by proteinase activity in the tissue. Twenty-one out of 45 recipients were women (47 %) and 24 out of 45 men (53 %), with an age ranging between 13 and 87 years. The mean age of women was 67.2 years (± 17.6 years), whereas the mean age of men was 54.6 years (± 17.1 years). The individuals received between one to five corneal transplants, respectively (Table 11). However, most of the recipients received only 1 transplant (44 %) and 6 out of 45 between 4 and 5 transplants.

Table 11. Number of corneal transplantations per individual

Recipients	No. of corneal transplantation				
	1	2	3	4	5
Total	20	8	11	3	3
Female	12	3	5	2	1
Male	8	5	6	1	2

From the 45 recipients 8 patients (18 %) exhibited an infection of the cornea as the main indication for corneal grafting, 8 patients (18 %) had an ulcer, 5 patients (12 %) suffered from keratitis, 3 patients (7 %) suffered from keratopathy and 4 patients (9 %) from keratoconus, whereas 9 patients (20 %) had Fuchs´ dystrophy or dystrophy of unknown origin. In 7 cases (16 %) accidents with perforation or with chemicals were the main indications.

A significant percentage of recipients had an underlying disease, which was primarily not associated with an eye disease and was due to secondary disorders or accidents. The underlying disease of 3 recipients (7 %) were systemic rheumatic diseases, and in 2 patients (4 %) the corneal transplantation was necessary as a consequence of side effects mediated by cancer therapy.

For the 25 recipients (56 %) with repeated corneal transplantations the most common indication for the current grafting in 16 cases (64 %) was the loss of graft function due to immunological reasons such as chronic rejection or vascularisation with and without clouding. Furthermore, repeated keratoconus and graft ulcer was found in 5 recipients (20 %). Protrusion of Descemet's membrane (descemetocele) was in 3 cases (12 %) and the lesion of the outer epithelial cell layer (erosio) was in 4 recipients (16 %) the indication for a current corneal transplantation. The underlying disease of all recipients, the indication for and the number of the current corneal grafting are summarized in Table 12.

Table 12. Patients´ features, diagnoses, indications, numbers and date of the last corneal grafting

ID	Sex	Age	Diagnosis	Indication for CTX	No. of CTX	Date of CTX
1	f	66	Herpes infection	Herpes infection	1	22.11.06
2	f	70	bullous keratopathy, Aphakia operata, Morbus Rieger	chronic CTX rejection	3	9.11.06
3	m	49	bullous keratopathy	bullous keratopathy	1	1.12.06
4	f	76	Fuchs´ dystrophy, Herpes infection	chronic CTX rejection	3	17.5.06
5	m	67	ulcer with perforation at rheumatism	corneal graft ulcer	4	11.1.07
6	f	53	ulcer after accident with perforation	ulcer	1	8.6.06
7	m	67	ulcer with perforation after Facialis paresis (carcinoma of middle ear)	ulcer with perforation	1	20.10.06
8	m	42	accident with perforation	vascularisation and clouding	3	18.10.06
9	f	77	keratoconus	keratoconus recidive	4	6.12.05
10	m	59	ulcer with perforation	ulcer with perforation	1	31.1.07
11	f	42	keratoglobus	ulcer with perforation	1	12.1.07
12	f	72	ulcer with perforation	descemetocele	3	19.9.06
13	f	53	Herpes-induced keratitis	ulcer with perforation	1	9.6.06
14	m	54	Herpes-induced keratitis	vascularisation and scars	1	16.5.06
15	m	38	keratoconus, Down syndrome	graft ulcer with perforation	2	6.11.06

Table 12 continued. Patients´ features, diagnoses, indications, numbers and date of the last corneal grafting

ID	Sex	Age	Diagnosis	Indication for CTX	No. of CTX	Date of CTX
16	f	87	repeated erosio after cornea surgery, decompensation and pseudophakia	Pseudophakia, keratopathy	1	23.5.06
17	m	64	clouding of cornea for unknown reason	clouding of cornea with unknown reason	1	15.11.06
18	m	53	accident with chemicals grade 3./4.	loss of graft and vascularisation	5	27.7.05
19	f	78	Fuchs´ dystrophy	Fuchs´ dystrophy	1	10.8.06
20	f	82	rheumatism, Herpes infection and repeated ulcer	graft ulcer, chronic CTX rejection	5	22.6.05
21	m	60	keratitis for unknown reason (uveitis)	clouding and vascularisation of graft	3	8.11.05
22	m	63	keratitis for unknown reason	central scar	1	8.8.06
23	f	36	macular dystrophy	macular dystrophy	1	23.11.06
24	m	43	keratitis, facial neural paralysis (accident)	descemetocele with perforation	2	17.1.07
25	m	54	keratoconus	chronic CTX rejection	5	18.5.06
26	f	80	Herpes-induced keratitis	graft ulcer and descemetocele	2	18.5.06
27	m	56	Herpes-induced keratitis	chronic CTX rejection	3	7.3.06
28	m	82	keratitis for unknown reason	scar and vascularisation	1	28.11.06
29	m	73	keratopathy	clouding of cornea	3	8.12.05
30	f	41	keratoconus	keratoconus	1	30.5.06

Table 12 continued. Patients´ features, diagnoses, indications, numbers and date of the last corneal grafting

ID	Sex	Age	Diagnosis	Indication for CTX	No. of CTX	Date of CTX
31	f	69	Fuchs´ dystrophy	Fuchs´ dystrophy	1	23.5.06
32	f	77	rheumatoid arthritis	graft erosio	4	12.3.05
33	f	32	stem cell-TX after CML	chronic CTX rejection	2	8.12.05
34	f	82	Fuchs´ dystrophy	Fuchs´ dystrophy	1	1.11.06
35	m	64	accident with chemicals	chronic CTX rejection	2	28.4.05
36	m	13	keratitis, vascularization, corneal decompensation	graft erosio	3	12.4.05
37	m	25	glaucoma, bullous keratopathy	clouding and oedema of the graft	2	30.8.05
38	f	79	accident with perforation, ulcer	accident and ulcer	1	23.3.05
39	m	62	scars, endothelial cell dystrophy, keratitis,	scar	2	16.5.06
40	m	64	ulcer with perforation	chronic CTX rejection	2	7.4.05
41	m	20	accident with perforation, scars	graft loss	3	25.8.05
42	f	86	corneal dystrophy	corneal dystrophy	1	6.6.06
43	f	86	Fuchs´ dystrophy, scars	CTX rejection, erosio	3	2.8.05
44	m	70	Fuchs´ dystrophy, glaucoma, cataract	Fuchs´ dystrophy, cataract	1	17.3.05
45	m	69	Herpes infection, cataract, ulcer	CTX rejection, erosio	3	12.11.04

Prior to and after grafting different ophthalmologic parameters were checked to define the success of the corneal transplantation.

In addition, in the first year after grafting the transplants should be checked quarterly and thereafter at minimum once per year for the following ophthalmologic parameters:

(i) the visual acuity as note for the success of grafting

(ii) the intraocular pressure, which depends on the rate of aqueous production, the facility of outflow and episcleral venous pressure

(iii) vascularization, scars and clouding as signs of recurrence of the eye disease

(iv) ulcer and erosio as complications after grafting or recurrence of the eye disease

The ophthalmologic parameters of all recipients prior to transplantation and at the last examination date after transplantation are listed in Table 13.

Table 13. Ophthalmologic parameters of the recipient's eye and cornea before grafting and of the transplanted donor cornea at the time of the last post-transplant examination date

No.	Ophthalmologic parameters before grafting				Ophthalmologic parameters at the last examination date					
	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Ulcer/ Erosio	Last check-up
1	light perception	norm	yes	yes	0,4	17	no	no	no	6 m
2	hand motion	9	yes	yes	0,05	4	no	no	no	6 m
3	counting fingers	15	yes	yes	0,4	17	no	no	no	6 m
4	0,17	6	no	yes	0,8	13	no	no	no	1 y
5	0,025	soft	no	yes	0,05	10	no	no	no	3 m
6	light perception	norm	yes	yes	hand motion	46	no	no	no	9 m
7	light perception	soft	no	no	counting fingers	norm	no	no	no	6 m
8	hand motion	norm	yes	yes	0,05	15	no	no	no	3 m
9	0,1	19	no	no	0,1	20	no	no	no	6 m

Table 13 continued. Ophthalmologic parameters of the recipient's eye and cornea before grafting and of the transplanted donor cornea at the time of the last post-transplant examination date

No.	Ophthalmologic parameters before grafting				Ophthalmologic parameters at the last examination date					
	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Ulcer/ Erosio	Last check-up
10	counting fingers	soft	no	yes	hand motion	norm	yes	no	yes	9 m
11	0,05	soft	no	no	0,2	20	no	no	no	6 m
12	counting fingers	--	no	no	--	--	--	--	--	4 w
13	0,025	norm	yes	yes	0,1	11	no	no	no	6 m
14	hand motion	15	yes	yes	--	--				
15	0,04	soft	yes	no	0,07	18	no	no	no	3 m
16	hand motion	norm	no	yes	0,3	16	no	no	no	6 m
17	0,3	15	yes	yes	--	--	--	--	--	< 2 w
18	hand motion	20	yes	yes	hand motion	15	yes	yes	no	1 y
19	0,03	19	no	yes	0,3	16	no	no	no	6 m
20	0,03	norm	no	yes	0,1	17	yes	yes	no	1 y

Table 13 continued. Ophthalmologic parameters of the recipient's eye and cornea before grafting and of the transplanted donor cornea at the time of the last post-transplant examination date

No.	Ophthalmologic parameters before grafting				Ophthalmologic parameters at the last examination date					
	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Ulcer/ Erosio	Last check-up
21	0,03	12	yes	yes	counting fingers	norm	no	yes	yes	1 y
22	hand motion	16	no	yes	0,05, cataract	--	no	no	no	9 m
23	0,2	12	no	yes	0,3	--	no	yes	no	6 m
24	light perception	soft	no	no	light perception	norm	no	no	no	3 m
25	0,1	7	yes	yes	0,3, cataract	16	no	no	no	1 y
26	hand motion	soft	no	yes	0,3	16	no	no	no	1 y
27	hand motion	soft	yes	yes	hand motion	19	no	yes	no	1 y
28	0,2	--	yes	yes	0,2, cataract	17	no	yes	no	9 m
29	hand motion	11	no	yes	0,5	24	no	yes	no	6 m

Table 13 continued. Ophthalmologic parameters of the recipient's eye and cornea before grafting and of the transplanted donor cornea at the time of the last post-transplant examination date

No.	Ophthalmologic parameters before grafting				Ophthalmologic parameters at the last examination date					
	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Ulcer/ Erosio	Last check-up
30	0,028	16	no	no	0,5	15	yes	no	no	6 m
31	0,1	16	no	no	0,6	12	no	no	no	1 y
32	hand motion	higher	no	yes	--	--	--	--	--	2 m
33	hand motion	--	yes	yes	0,2	--	no	no	yes	1 y
34	0,05	norm	--	--	--	--	--	--	--	n.a.
35	0,05	--	--	--		--	--	--	--	n.a.
36	hand motion	norm	yes	yes	hand motion	norm	yes	yes	yes	11 m
37	light perception	10	no	yes	0,03	11	no	no	no	16 m
38	0,1	norm	no	yes	hand motion	norm	no	no	yes	2 m
39	0,3	14	no	yes	0,2	19	no	yes	yes	17 m

Table 13 continued. Ophthalmologic parameters of the recipient's eye and cornea before grafting and of the transplanted donor cornea at the time of the last post-transplant examination date

No.	Ophthalmologic parameters before grafting				Ophthalmologic parameters at the last examination date					
	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Visual acuity	Intraocular pressure	Vasculari-sation	Scars/ Clouding	Ulcer/ Erosio	Last check-up
40	hand motion	13	--	yes	hand motion	--	yes	yes	--	2 y
41	light perception	8	--	yes	0,6	9	no	yes	no	2 m
42	light perception	18	no	no	0,07	--	--	--	--	2 w
43	0,3	16	yes	no	0,6	14	yes	no	no	15 m
44	0,3	18	no	yes	0,3	12	no	no	no	2 y
45	light perception	norm	no	yes	0,05	norm	yes	yes	no	23 m

norm = palpatoric normotensive intraocular pressure, soft = palpatoric low intraocular pressure, higher = palpatoric lightly higher intraocular pressure, w = week, m = month, y = year, n.a. = follow-up not available, -- = not determined during examination or loss of graft before first examination date

In addition to the ophthalmologic parameters the immune reactions were checked at each examination date after grafting:

(i) hyper-acute rejection episodes of the corneal graft in the first two months

(ii) changes of the endothelial cells as sign of a weaker immunological reaction against the transplant

(iii) rejection episodes later than 2 months after grafting

(iv) loss of corneal graft

As demonstrated in Table 14 the immunological features significantly differ between all recipients after grafting from courses without complication up to graft loss. Four out of 45 recipients (9 %) exhibited hyper-acute rejections leading in 3 cases to graft loss within one week, one and two months, respectively. Changes in the endothelial cells such as a reduced number of cells or small keratic precipitates were detected in 4 out of 45 graft recipients (9 %). In 3 cases the morphological alterations of endothelial cells were associated with reversible rejection episodes without graft loss, whereas in one case the rejection was irreversible leading to graft loss after 23 months. In addition, 3 out of 45 recipients (7 %) exhibited reversible rejection episodes without detectable alterations of endothelial cells.

Table 14. Immunological parameters of the corneal transplant at the last examination date and presence of rejection parameters/graft losses

No.	Hyper-acute rejection	Changes of endothelial cells	Last examination date (§)	Rejection episodes	Loss of graft
1	no	no	6 m	no	no
2	no	no	6 m	no	no
3	no	yes	6 m	yes	no
4	no	yes	1 y	yes	no
5	no	no	3 m	no	no
6	no	no	9 m	no	no
7	no	no	6 m	no	no
8	no	no	3 m	no	no
9	no	no	6 m	no	no
10	yes	no	9 m	yes	no

Table 14 continued. Immunological parameters of the corneal transplant at the last examination date and presence of rejection parameters/graft losses

No.	Hyper-acute rejection	Changes of endothelial cells	Last examination date (§)	Rejection episodes	Loss of graft
11	no	no	6 m	no	no
12	yes	--	4 w	--	4 w
13	no	no	6 m	no	no
14	no	--	n.a.	--	--
15	no	no	3 m	no	no
16	no	no	6 m	no	no
17	yes	--	1 w	--	1 w
18	no	no	17 m	--	17 m
19	no	no	6 m	no	no
20	no	no	1 y	no	no
21	no	no	1 y	no	no
22	no	no	9 m	no	no
23	no	no	6 m	no	no
24	no	no	3 m	no	no
25	no	no	1 y	no	no
26	no	no	1 y	no	no
27	no	no	1 y	yes	no
28	no	no	9 m	no	no
29	no	no	6 m	no	no
30	no	no	6 m	no	no
31	no	no	1 y	no	no
32	yes	--	2 m	--	2 m
33	no	no	1 y	no	no
34	--	--	n.a.	--	--

Table 14 continued. Immunological parameters of the corneal transplant at the last examination date and presence of rejection parameters/graft losses

No.	Hyper-acute rejection	Changes of endothelial cells	Last examination date (§)	Rejection episodes	Loss of graft
35	--	--	n.a.	--	--
36	no	--	11 m	yes	11 m
37	no	no	16 m	no	no
38	no	no	2 m	no	no
39	no	--	17 m	yes	no
40	no	no	2 y	no	no
41	no	yes	2 m	yes	no
42	--	--	2 w	--	--
43	no	no	15 m	no	no
44	no	no	2 y	no	no
45	no	yes	23 m	--	23 m

The occurrence of rejection episodes, changes of endothelial cells and graft losses is highlighted in red letters. w = week, m = month, y = year, n.a. = follow-up not available or only for the first post-transplant week, -- = not defined due to an oedema of the stroma of the transplant or unavailable follow-up data, § = last examination date after corneal transplantation.

3.2. Development and implementation of the Micro-AMS HLA class I and class II ELISA for corneal material

The Micro-AMS class I and class II ELISA was originally established for the use of HLA molecules isolated from single lymphocytes from blood or spleen. In case of a corneal grafting both sources of single cells are not available due to (i) the long storage time of corneas from post-mortem organ donors (HLA typed corneas in national or international Eyebanks) and (ii) post-mortem donation of corneas without other organs or blood samples available for HLA typing and crossmatching. In order to adapt the Micro-AMS class I and class II ELISA the explanted inner part of the cornea obtained from 19 recipients was used. The control group included 12 recipients undergoing the first corneal grafting, thus the explanted material was the original own cornea. Concerning the seven recipients, who had

already obtained several corneal transplantations the old donor corneal material was explanted due to loss of function (Table 15).

For the isolation of HLA class I and class II molecules half of the explanted cornea was used, whereas the other half of the cornea was stored in the Clinic and Dispensary of Ophthalmology. In all cases sufficient HLA molecules could be isolated from the inner part of the cornea resulting in sufficient signals as lysate controls for HLA class I as well as for HLA class II reactions (OD > 0.900). The data of the HLA class I and class II crossmatches are shown in Table 7 (Micro-AMS ELISA). After a positive reaction of the Micro-AMS ELISA the data received were further validated by defining the respective anti-HLA antibodies using the Quik-ID® Class I and/or II ELISA (Table 15).

In 8 out of 19 patients (42 %) donor-specific anti-HLA class I and/or class II antibodies were detectable. In 6 of these 8 patients exhibiting donor-specific antibodies these results were confirmed using the Quik-ID® ELISA clearly validating the existence of anti-HLA antibodies. In one case anti-HLA antibodies were not detected with the Quik-ID® Class I or Class II ELISA. This might be due to a restricted HLA antigen pattern since this assay does not comprise all known, but only the most common HLA phenotypes. In one case the serum available was only sufficient to perform the Micro-AMS ELISA but not the Quik-ID® ELISA which requires about 3 ml of the recipient's serum.

Table 15. Detection of anti-HLA antibodies using the Micro-AMS ELISA. The results of the Micro-AMS-ELISA HLA class I and class II assays are summarised using recipient's serum against the material from the explanted corneal allograft of the last donor or the recipient's original explanted cornea. The recipient's anti-HLA antibody status was defined using the Quik-ID® Class I and/or II ELISA.

No.	Micro-AMS ELISA		anti-HLA antibody status		No. of CTX
	Class I	Class II	Class I	Class II	
1	Ø	Ø	Ø	Ø	1
2	positive	Ø	Ø	Ø	3
3	Ø	Ø	Ø	Ø	1
5	Ø	Ø	Ø	Ø	4
7	positive	Ø	n.d.	n.d.	1
8	positive	positive	positive	DR 15, DR 51, DQ 06	3
10	Ø	Ø	Ø	Ø	1

Table 15 continued. Detection of anti-HLA antibodies using the Micro-AMS ELISA

No.	Micro-AMS ELISA		anti-HLA antibody status		No. of CTX
	Class I	Class II	Class I	Class II	
11	Ø	Ø	Ø	Ø	1
12	positive	Ø	A 26, A 33	n.d.	3
15	positive	Ø	B 07, Cw 02	Ø	2
17	Ø	Ø	Ø	Ø	1
19	positive	Ø	B 07	Ø	1
22	positive	Ø	positive	positive	1
23	Ø	Ø	n.d.	n.d.	1
24	Ø	Ø	n.d.	n.d.	2
28	Ø	Ø	Ø	Ø	1
34	positive	Ø	B 48, Cw 03	n.d.	1
44	Ø	Ø	Ø	Ø	1
45	Ø	Ø	Ø	Ø	3

The positive reactions of the AMS ELISA and antibody specificities/reactions of the Quik-ID® ELISA are highlighted in red letters. n.d. = not done.

3.3. Retrospective detection of donor-specific anti-HLA antibodies

After the successful implementation of the Micro-AMS HLA class I and class II ELISA for the detection of donor-specific anti-HLA antibodies using small pieces of corneal tissue instead of enriched lymphocytes 45 patients were retrospectively analysed for their antibody status prior to grafting. From all 45 patients material of the outer corneal rim was available from the donor's cornea. From these outer corneal rims enough HLA molecules were isolated for both HLA class I and class II ELISA as demonstrated by the positive controls (LCR1 and LCR2, respectively). For the isolation of HLA class I and II molecules one third of the outer rim of the donor cornea was used. About one sixth was stored for the DNA isolation and the other half of the outer rims was stored in the Clinic and Dispensary of Ophthalmology. The data of the HLA class I and class II crossmatches with novel grafts are shown in the column named Micro-AMS ELISA in Table 16.

In case of a positive crossmatch result in the Micro-AMS ELISA the anti-HLA antibody status of the recipient as well as the genetic HLA typing of the corresponding donor cornea were investigated. In 11 of 19 cases (58 %) anti-HLA antibody specificities were found against one

ore more HLA antigens in the donor cornea. In addition in 3 cases (16 %) donor-specific anti-HLA antibodies were found but due to the lack of donor material (2 cases) for the HLA typing or the lack of recipient's serum (1 case) for the anti-HLA antibody detection it was not possible to define the corresponding anti-HLA antibody specificity. In one case (11 %) anti-HLA antibody specificities were found which were not directed against the HLA antigens in the donor cornea. In 4 cases (21 %) anti-HLA antibodies were not detected with the Quik-ID® Class I or Class II ELISA. This might be due a restricted HLA antigen pattern since this assay does not comprise all known, but only the most common HLA phenotypes.

Table 16. Detection of donor-specific HLA antibodies by the Micro-AMS ELISA using recipient's serum against material from the outer rims of the current corneal allograft. The corresponding recipient's anti-HLA antibody status and the donor's HLA genotype are listed additionally.

No	Micro-AMS ELISA		Anti-HLA Ab status		HLA typing of class I and/or class II	No. of CTX
	Class I	Class II	Class I	Class II		
1	Ø	Ø	Ø	Ø	n.d.	1
2	positive	Ø	Ø	Ø	A*01, *68; B*51,* 56; Cw*01, *15	3
3	Ø	Ø	Ø	Ø	n.d.	1
4	positive	positive	Ø	Ø	A*03; B*07, *35; Cw*04, *07	3
5	Ø	Ø	Ø	Ø	n.d.	4
6	Ø	Ø	n.d.	n.d.	n.d.	1
7	positive	Ø	n.d.#	n.d.#	A*02, *25; B*13, *18; Cw*06, *12	1
8	positive	Ø	positive	DR 15, DR 51, DQ 06	A*02; B*44, 57; Cw*05, *06; DRB1*04, *07; DRB4*; DQB1*03 (7), *03 (9)	3
9	Ø	Ø	n.d.	n.d.	n.d.	4
10	Ø	Ø	Ø	Ø	n.d.	1
11	Ø	Ø	Ø	Ø	n.d.	1

Table 16 continued. Detection of donor-specific HLA antibodies by the Micro-AMS ELISA using recipient's serum against material from the outer rims of the current corneal allograft

No	Micro-AMS ELISA		Anti-HLA Ab status		HLA typing of class I and/or class II	No. of CTX
	Class I	Class II	Class I	Class II		
12	positive	Ø	A 26, A 33	n.d.	A*01, ***26**; B*27, *25; Cw*01, *04	3
13	Ø	Ø	n.d.	n.d.	n.d.	1
14	positive	Ø	A 24	n.d.	A*01, ***24**; B*07, *45; Cw*07	1
15	positive	Ø	B 07, Cw 02	Ø	A*01, *31; B***07**, *52; Cw*07, *12	2
16	Ø	Ø	Ø	Ø	n.d.	1
17	positive	Ø	Ø	Ø	A*02, *23; B*07, *52; Cw*07, *12	1
18	Ø	Ø	n.d.	n.d.	n.d.	5
19	positive	Ø	B 07	Ø	n. d.#	1
20	positive	positive	A 02, B 27	DR 12	A***02**; B*14, ***27**; Cw*01, *08; DRB1***12**, *15;	5
21	Ø	Ø	n.d.	n.d.	n.d.	3
22	positive	Ø	positive	positive	n. d.#	1
23	Ø	Ø	n.d.	n.d.	n.d.	1
24	Ø	Ø	n.d.	n.d.	n.d.	2
25	Ø	Ø	n.d.	n.d.	n.d.	5
26	Ø	positive	Ø	DR 15	DRB1*01, ***15**; DQB1*05, *06	2
27	Ø	Ø	n.d.	n.d.	n.d.	3
28	Ø	Ø	Ø	Ø	n.d.	1
29	Ø	Ø	n.d.	n.d.	n.d.	3
30	Ø	Ø	n.d.	n.d.	n.d.	1

Table 16 continued. Detection of donor-specific HLA antibodies by the Micro-AMS ELISA using recipient's serum against material from the outer rims of the current corneal allograft

No	Micro-AMS ELISA		Anti-HLA Ab status		HLA typing of class I and/or class II	No. of CTX
	Class I	Class II	Class I	Class II		
31	Ø	Ø	n.d.	n.d.	n.d.	1
32	positive	Ø	Ø	n.d.	A*23, *33; B*27, *51; Cw*02, *05	4
33	positive	positive	A 02, Cw 07, Cw 06	DQ 06	A***02**, *31; B*07, *51; Cw***06**, ***07**; DRB1*03, *15; DQB1*03, ***06**	2
34	positive	Ø	B 48, Cw 03	n.d.	A*02; B*27, *55; Cw*02, *04	1
35	positive	Ø	A 02; B 15	positive	A***02**; B***15**; Cw*03	2
36	positive	Ø	B 13	n.d.	A*29, *30; B***13**, *44; Cw*06	3
37	Ø	Ø	n.d.	n.d.	n.d.	2
38	Ø	Ø	n.d.	n.d.	n.d.	1
39	positive	Ø	A 02, 03	n.d.	A***02**, ***03**; B*35; Cw*03, *04	2
40	Ø	Ø	n.d.	n.d.	n.d.	2
41	Ø	Ø	n.d.	n.d.	n.d.	3
42	positive	Ø	A 01, 02, Cw 07	n.d.	A***01**, ***02**; B*08, *40; Cw*02, ***07**	1
43	Ø	Ø	n.d.	n.d.	n.d.	3
44	Ø	Ø	Ø	Ø	n.d.	1
45	Ø	Ø	Ø	Ø	n.d.	3

HLA donors' antigens which were recovered as anti-HLA antibody specificities in the recipients' sera are highlighted using bold and underlined numbers. n.d. = not done, # = not done as no corneal material was available for HLA genotyping or due to insufficient amounts of recipient's serum for the anti-HLA antibody detection.

3.4. Correlation of early graft survival of recipients with the Micro-AMS ELISA results

All recipients with the transplanted corneas were divided into two groups depending on the crossmatch Micro-AMS ELISA results. The first group included all recipients with positive results in the Micro-AMS ELISA Class I and/or Class II, whereas the second group classifies all recipients with negative results in both Class I and Class II Micro-AMS ELISA. However, there exist no significant differences for the main indications for corneal grafting between patients with or without donor-specific anti-HLA antibodies as demonstrated in Table 17.

Table 17. Main indications for corneal allografting in recipients with and without donor-specific anti-HLA antibodies

Main diagnosis	All recipients * N=45 (100%)	Recipients with positive crossmatch results * N = 19 (100%)	Recipients with negative crossmatch results * N = 26 (100%)
Herpes infection or keratitis	13 (29%)	6 (32%)	7 (27%)
Keratoconus	4 (9%)	1 (5%)	3 (12%)
Keratopathy	3 (7%)	1 (5%)	2 (8%)
Fuchs´ dystrophy or macular dystrophy	9 (20%)	5 (26%)	4 (15%)
Ulcer	8 (18%)	3 (16%)	5 (19%)
Accident	7 (16%)	2 (11%)	5 (19%)
Other indication	9 (20%)	5 (26%)	4 (15%)

* Several recipients show two or more indications for corneal grafting which leads to a higher value than 100 % in the collective of patients.

Interestingly, no significant differences concerning the number of transplantations were detected between both groups (Table 18).

Table 18. Distribution of the number of corneal transplantations of all recipients. Group I represents recipients with positive crossmatch results, group II recipients without donor-specific anti-HLA antibodies.

Number of actual CTX	1	2	3	4	5
All recipients (N=45)	20	8	11	3	3
Group I (N=19) Positive CM results	7	5	5	1	1
Group II (N=26) Negative CM results	13	3	6	2	2

In 15 of 19 patients (79 %) rejection episodes leading to special anti-rejection treatment or graft loss was observed (Table 19). In 4 cases (21 %) no clear results could be obtained due to different reasons: Concerning the post-transplantation examinations one patient appeared only once within the first quarter after the grafting, whereas three patients never appeared after their grafting at the Hospital. Therefore, the post-transplant parameters of these three patients were only available for the two first weeks after transplantation.

As shown in Table 19 only donor-specific anti-HLA class I antibodies were detectable in 15 out of 19 patients (79 %). In 3 patients (16 %) both anti-HLA class I and class II antibodies were identified. Furthermore in one recipient (no. 26) who had received an HLA class I-matched corneal graft anti-HLA class II antibodies against the novel transplant were additionally found.

Table 19. Occurrence of an alloresponse/graft loss observed in 19 recipients **with** donor-specific anti-HLA antibodies against the last corneal allograft

No.	Micro-AMS ELISA		Occurrence of rejection episodes, morphological changes of endothelial cells and/or graft loss	No. of CTX
	Class I	Class II		
2	positive	Ø	special anti-rejection treatment	3
4	positive	positive	rejection, changes of endothelial cells	3
7	positive	Ø	carcinoma patient	1
8	positive	Ø	additional anti-rejection treatment	3
12	positive	Ø	acute rejection after 4 weeks	3
14	positive	Ø	matched graft, only 2 months follow-up	1
15	positive	Ø	only 3 months follow-up	2
17	positive	Ø	hyper-acute rejection after 5 days	1
19	positive	Ø	Ø	1
20	positive	positive	Ø	5
22	positive	Ø	cataract	1
26	Ø	positive	matched graft, Ø	2
32	positive	Ø	graft loss after 2 months	4
33	positive	positive	ulcer	2
34	positive	Ø	follow-up not available	1
35	positive	Ø	follow-up not available	2
36	positive	Ø	complete graft erosio	3
39	positive	Ø	additional anti-rejection treatment	2
42	positive	Ø	follow-up not available	1

As demonstrated in Table 20 in 20 out of 26 recipients (77 %) without donor-specific anti-HLA antibodies prior to grafting the post-transplantation course proceeded without any complication such as rejection episodes or morphological alterations in the endothelial cells. In 4 out of 26 patients (15 %) reversible rejection episodes were observed despite negative results in the Micro-AMS ELISA using serum samples which were collected at the day of

transplantation. In two patients a graft loss even after 17 months (no. 18) and 23 months (no. 45) was described, as summarised in Table 20.

Table 20. Occurrence of an alloresponse/graft loss observed in 26 recipients **without** donor-specific anti-HLA antibodies against the last corneal allograft

No.	Micro-AMS ELISA		Occurrence of rejection episodes, morphological changes of endothelial cells and/or graft loss	No. of CTX
	Class I	Class II		
1	Ø	Ø	Ø	1
3	Ø	Ø	changes of endothelial cells	1
5	Ø	Ø	Ø	4
6	Ø	Ø	Ø	1
9	Ø	Ø	Ø	4
10	Ø	Ø	1 rejection episode	1
11	Ø	Ø	Ø	1
13	Ø	Ø	Ø	1
16	Ø	Ø	Ø	1
18	Ø	Ø	graft loss after 17 months	5
21	Ø	Ø	Ø	3
23	Ø	Ø	Ø	1
24	Ø	Ø	Ø	2
25	Ø	Ø	Ø	5
27	Ø	Ø	1 rejection episode	3
28	Ø	Ø	Ø	1
29	Ø	Ø	Ø	3
30	Ø	Ø	Ø	1
31	Ø	Ø	Ø	1
37	Ø	Ø	Ø	2
38	Ø	Ø	Ø	1
40	Ø	Ø	Ø	2

Table 20 continued. Occurrence of an alloresponse/graft loss observed in 26 recipients **without** donor-specific anti-HLA antibodies against the last corneal allograft

No.	Micro-AMS ELISA		Occurrence of rejection episodes, morphological changes of endothelial cells and/or graft loss	No. of CTX
	Class I	Class II		
41	Ø	Ø	changes of endothelial cells	3
43	Ø	Ø	Ø	3
44	Ø	Ø	Ø	1
45	Ø	Ø	graft loss after 23 months	3

4. Discussion

The aim of the study was to develop a tool for monitoring immune processes which might lead to keratoplasty failure since the proper selection of patients with a high risk for graft loss and early detection of host alloresponses might improve the graft survival. An ELISA-based AMS crossmatch employed prior to and after corneal grafting might be an adequate procedure to be implemented in this context. Indeed, using a modified ELISA-based crossmatch it could be demonstrated in this thesis that this assay could be successfully implemented for allocation of HLA typed donor corneas. In addition, there is evidence provided that the “immune privilege” status of the eye has to be revisited due to distinct immune mechanisms which appear to be involved in corneal allograft rejection.

4.1. The novel ELISA-based AMS crossmatch procedure in comparison to the established standard procedures

It has been demonstrated that antibodies directed against antigens of donor lymphocytes are a prominent cause of hyper-acute rejections in recipients of different solid organ allografts (Patel and Terasaki, 1969; Ahern et al., 1982; Chapman et al., 1986). Indeed, a negative crossmatch between the recipient’s serum and lymphocytes of the donor is hitherto regarded as the best predictor for short-term survival of renal allografts. The standard method for the detection of donor-specific antibodies directed against HLA class I and/or class II molecules is the complement-derived lymphocytotoxicity (CDC) assay, which has been developed more than thirty years ago. However, this crossmatch technique sometimes fails to identify antibodies since non-complement-fixing alloantibodies or low antibody concentrations are not detected. Therefore more sensitive assays are urgently required to identify low to marginal antibody concentrations which may be relevant for the clinical outcome. As a result the CDC-CM was modified to a variant [anti-human-globulin (AHG)-enhanced CDC-CM] using secondary anti-human immunoglobulin antibodies in addition to the primary donor-specific antibodies. This procedure results in a considerably higher sensitivity of the AHG-enhanced CDC-CM due to an increase in the antibody-mediated complement activation (Gebel and Bray, 2000; Karpinski et al., 2001). The flow cytometric (FC) crossmatch (Garovoy et al., 1983, Takeda et al., 2000) is even more sensitive and allows the detection of low titre antibodies (Bittencourt et al., 1998). However, this technology often results in false positive crossmatch data due to antibodies not directed against HLA antigens (Christiaans et al., 1996; Kerman et al., 1999). Therefore, a positive FC-CM does not necessarily correlate with a poor outcome as determined by graft rejection (Kerman et al., 1999; Lobashevski et al., 2000). In addition, non-complement-activating alloantibodies can be detected by the more sensitive FC-CM and have been reported to be associated with an increased allograft rejection despite the absence of a positive CDC crossmatch (Scornik et al., 1994; Scornik,

1995). In order to avoid these problems a solid phase immunoassay based on a flow cytometric procedure named FlowPRA^{TM} was designed which utilizes purified HLA molecules immobilized on the surfaces of microparticles (Pei et al., 1998; Gebel et al., 2001; 2002; Khan et al., 2003). The advantage of this method is beside its high sensitivity and particularly its independence from the quality of the cells. Therefore, a novel ELISA-based crossmatch technology (Antibody-Monitoring System-AMS; GTI Diagnostics, WI, USA) exhibiting a high sensitivity and an independence of the quality of donor cells was employed and optimised for pre- and post-transplant monitoring.

In the case of cornea grafts only the central part is transplanted, whereas the outer rim is stored in sterile buffer for the detection of a possible bacterial contamination. In general, a crossmatch is not performed since the cornea is regarded as an “immune privileged” tissue. However, it has been postulated by various authors that local immunoreactions may lead to the clouding of the allogenic graft due to the marginal expression of HLA antigens. Although the cell-mediated immunity has been described to be the main cause of corneal allograft rejection (Niederkorn, 2001) complement activating alloantibodies also has an impact on corneal allograft rejection in mice (Hegde et al., 2002). Furthermore, the studies of Roy and co-workers (1992) and of Boisjoly et al. (1993) strongly suggest that the occurrence of post-transplant antibodies against donor HLA represents a high risk of corneal allograft rejection in contrast to so-called panel-reactive pre-transplant antibodies (% PRA). These only show antibody specificities against HLA antigens of selected panel cells in general but not against donor antigens. In a later study donor-recipient CM has been demonstrated to be a powerful procedure for the selection of recipients for corneal transplantation in patients who were presensitised by an anterior graft or a previous corneal rejection (Des Marchais et al., 1998). In particular the AMS ELISA fulfils the donor-specificity requested (Roy et al., 1992; Boisjoly et al., 1993) as this assay exclusively detects donor-specific anti-HLA antibodies.

In current study it was determined whether the cornea allograft rejection is associated with the existence of donor-specific anti-HLA class I and/or class II antibodies of the recipient. Therefore, the AMS ELISA was employed with a lysate obtained from the outer cornea rim to extract HLA molecules of the donor. In the case of cornea transplants the AMS ELISA has an advantage when compared to the classical CDC crossmatch due to its increased sensitivity.

In addition, not only lysates of lymphocytes, but also of tissues such as cornea, which generally are poor of cells can be used for HLA antigen isolation of a given donor. Unexpectedly also anti-HLA class II antibodies were detectable in the recipients´ sera presented in this study. However, the lysate controls using alkaline phosphatase-conjugated mAb for detection of donor-derived HLA class I or class II molecules, respectively, which are included in the AMS ELISA confirmed the positive anti-HLA class II antibody results. These data suggest that anti-HLA class II antibodies may contribute to a corneal graft rejection

since in three out of the patients (Table 19) exhibiting complications after the transplantation anti-HLA class and anti-HLA class II antibodies have been detected.

The novel AMS ELISA was designed using the sandwich-technology with a monoclonal capture antibody directed against monomorphic epitopes of HLA class I or class II molecules, respectively. This allows in a further step to detect individual antibodies of the recipient directed against the captured HLA molecules of the donor and has the advantage that a loss of epitopes of the HLA molecules due to their solid phase-coating may be avoided.

The Micro-AMS ELISA shows non-ambiguous positive results at least using four-fold higher serum dilutions in comparison to reliable results of the CDC-CM (Altermann et al., 2006). Non-cytotoxic anti-HLA antibodies which are not detectable with the standard lymphocytotoxicity assay can be verified with the Micro-AMS ELISA Class I and/or Class II (Schlaf et al., 2005; Altermann et al., 2006; Schlaf et al. 2006).

Thus this study for the first time describes the use and optimisation of the AMS crossmatch ELISA for the identification of donor-specific antibodies suggesting that this assay is a sensitive and reliable tool with obvious advantages over the classical CDC crossmatch.

However, the general substitution of the CDC crossmatch by the AMS ELISA is not possible as this novel assay is quite time-consuming requiring about 4 to 5 hours including the lysis of the donor cells. Therefore, its use in the routine of a tissue typing lab is limited. In particular in emergency duties in which the standard CDC crossmatch is performed in about 2.5 hours it cannot be substituted by an assay which requires at least twice this time. Therefore, the standard CDC crossmatch may rather be implemented as an additional assay applied for special cases of crossmatch analyses with no limitations of time (e.g. kidney grafts of living donors). Another problem of the AMS ELISA is the amount of serum required. The standard CDC crossmatch needs only 3 µl of serum (PBL, separated T- and B-cells), whereas the AMS ELISA requires 12 µl for the detection of anti-HLA class I and class II antibodies. In this respect the Micro-AMS ELISA represents a huge advantage in comparison to the original design of the conventional AMS ELISA working with at least 50 µl of serum. In conclusion, the study nevertheless strengthens the urgent requirement for a novel monitoring procedure in addition to the conventional CDC crossmatch method routinely employed by all HLA laboratories.

It is the current challenge to proceed with the miniaturization of this procedure to the formate of Terasaki plates using the same volume of serum which is currently required for the CDC crossmatch. Furthermore, the incubation times have to be optimised to shorten the complete procedure to 3 hours. The modifications of both parameters (volume and time) would lead to a crossmatch assay valuable also during emergency duties and even for solid organ transplantation. This miniaturised variant of the AMS ELISA may have the potential to completely exchange the official CDC crossmatch methodology.

A DNA-based HLA class II typing method was developed using tissue of the explanted eye from post-mortem donors in combination with a FACS-based crossmatch procedure with cultured retinal pigment epithelial cells (Jenisch et al., 1995; Zavazava et al.,1996). In both studies retinal pigment epithelial cells were employed and cultured under sterile conditions after their isolation by tryptic digestion of the retina. For the crossmatch procedure these vital cells were additionally stimulated with recombinant IFN-γ to upregulate the HLA antigen expression. In contrast to the time consuming and technically challenging studies of the group around Jenisch and Zavazava the current thesis implements a simple method for DNA-based HLA class I and class II typing and a routine able ELISA-based crossmatch procedure using donor material which, as a general advantage, must not be vital. The method can be performed in each HLA laboratory within a time span of 4 hours.

4.2. Clinical relevance of donor-specific anti-HLA antibodies for the outcome of cornea transplantations

In a recent study 45 randomly collected patients who all underwent a corneal grafting were investigated for the existence of donor-specific anti-HLA class I and/or class II antibodies. If possible the clinical data before and after the transplantation were collected in all patients.

The whole collective was divided into two groups depending on the occurrence of donor-specific anti-HLA antibodies against the current transplant. In terms of general indications for corneal grafting there were not striking differences between both groups (Table 12 and 17). Due to the limited number of patients the correlation of donor-specific anti-HLA antibodies with the number of transplantations is difficult since four patients with 4 or 5 graftings were included in the group of patients without donor-specific antibodies. Generally, non-immunological reasons must be the cause of keratoplasty failure in this four patients. In this context keratoconus and severe chemical/alkali burns have to be mentioned.

The aim of the current study was to determine the immunological cause responsible for the rejection episodes/graft losses of the keratoplasties. In solid organ transplantation donor-specific anti-HLA antibodies represent a contraindication for a given transplantation according to the Eurotransplant (ET) guidelines and the German Transplantation Law. Therefore, the study was focused on the presence of donor-specific anti-HLA antibodies as main part of the humoral alloresponse correlated with impaired post-transplantation courses until graft loss.

For the first time the modified Micro-AMS ELISA offers a methodological approach to perform a crossmatch prior to corneal grafting thereby broaden the ET allocation guidelines to cornea transplantations.

In about 80 % of recipients without donor-specific anti-HLA antibodies (Table 20) no complications in the post-transplantation course were observed. However, a negative

crossmatch result prior to transplantation does not exclude a rejection episode/graft loss as demonstrated in 4 recipients with reversible rejections and 2 patients suffering from a graft loss.

In contrast patients with donor-specific anti-HLA antibodies prior to transplantation (Table 19) exhibit an increased level of post-transplant complications with the exception of three recipients whose post-transplant monitoring data were only available for two weeks. In 13 of the remaining 16 recipients (81 %) complicated post-transplantation courses were found which were characterised by either acute rejection episodes or chronic courses of rejections of grafts despite permanent treatment with immunosuppressive drugs. Interestingly, 2 out of 16 recipients lost their grafts within one month, and the other 2 recipients within the first two years.

To confirm the presence of anti-HLA antibodies detected by the AMS ELISA and to identify their specificities the recipients' sera were analysed using the Quik-ID® ELISA. Furthermore, the donors' corneas were genotyped to identify the corresponding HLA antigens. From the 19 recipients with donor-specific anti-HLA antibodies only 16 recipients were considered in this evaluation since in two cases no genotyping could be performed due to the lack of donor material. In one case the study was performed with a volume of recipient's serum insufficient for the Quik-ID® ELISA.

In 11 out of 16 recipients (69 %) anti-HLA class I and/or class II antibody specificities were identified which were in accordance with the donor's HLA type (Table 16). In the serum samples of 4 recipients (25 %) no anti-HLA antibodies and in one recipient (6 %) no anti-HLA antibody specificity corresponding with the donor's genotype were detectable. This phenomenon might occur when a mixture of anti-HLA antibodies with various specificities is present in the serum of the recipient. Antibodies not detectable in the Quik-ID® Class I & II ELISA which is less sensitive when compared to the AMS ELISA may be another reason. An additional explanation for different results in the Quik-ID® ELISA and the Micro-AMS ELISA could be the occurrence of anti-HLA-DP antibodies as shown after the transplantation of solid organs such as kidneys (Qiu et al., 2005). The HLA-DP molecules are not constitutively expressed on the cell surfaces, but their expression might be induced by ischemic stress during the storage and transport of the donor cornea. However, EBV-transformed B lymphocyte cell lines used in the Quik-ID® class II ELISA expressed HLA-DP molecules only at a very low level suggesting that these anti-HLA-DP antibodies were not detectable by this method.

According to current study anti-HLA antibodies play a key role in the failure or rejection of the keratoplasties. Recipients with an alloimmunisation against the donor's HLA phenotype exhibit a significantly higher incidence of acute rejection episodes, chronic rejections and graft losses.

The "immune privileged" status of the cornea, which has been described above (section 1.4., page 12) is mainly due to the fact that the central part of the cornea lacks blood vessels. Therefore, only a very low transport rate of the donor's HLA molecules through the recipient's antigen presenting cells to the local lymph nodes exists. Upon the destruction of the blood-cornea barrier due to various reasons the donor's cornea may induce a humoral immune response against the graft. Indeed, an increased rejection frequency after pre-sensitization of a recipient by blood transfusions or transplantations of other organs or tissues has been shown in many studies, which is further strengthened by the current analyses for a pre-sensitization induced by formerly grafted corneas.

During the last decade the influence of anti-HLA class I antibodies for corneal grafting has controversially been discussed. The Collaborative Corneal Transplantation study (CCTS) comprising a high number of recipients (N = 419) did not show any correlation between the degree of the HLA matches and graft survival (Collaborative Corneal Transplantation Studies Research Group, 1992). In contrast, several clinical studies presented a striking effect of HLA class I matching on the improved survival of corneal grafts (Boisjoly et al., 1990; Vail et al., 1994; Volker-Dieben et al., 2003).

The results and conclusions of the CCT study are under discussions since the HLA class I and II typings were performed in different laboratories with discrepancy rates for the HLA-DR typing (HLA class II antigen) about 45 %, for the HLA class I locus HLA-A about 12 % and for HLA-B about 21 %, respectively. Based on this significant discrepancy any conclusions from that study must be drawn very carefully as critically discussed by Hopkins et al. (1992) and Zavazava et al. (1996).

In a retrospective study with 459 recipients (Khaireddin et al., 2003) showed a significant influence of HLA-A, HLA-B and HLA-DR matches in both the low-risk and the high-risk groups, which were defined according to various criteria after cornea transplantations. The differences in graft survival between both groups at 1-, 5- and 10-years were 20 %, 45 % and 29 %, respectively. A donor-recipient match of ≥ 2 alleles in HLA-A, -B or -DR types reduced the rejection rate by about 10 % in the low-risk group after 10 years. However, in the high-risk group the same match reduced the rejection rate by about 40 % in the first three years. In this context it is noteworthy that the HLA-B mismatches were found to be the most important prognostic factors in both groups.

Several publications dealing with anti-rejection therapies clearly show the relevance of immune suppression to save the cornea by eliminating detrimental immunological processes directed against the graft. These systemic strategies limit the “immune privilege” of the eye at least under certain pathological circumstances. Most physicians used topical corticosteroids (Prednisolone) for routine post-transplantation management and treatment of graft rejection (Randleman and Stulting, 2006). For the routine management in nearly half of all recipients with high-risk corneal grafts topical Cyclosporine in addition to Prednisolone was employed.

The application of new immunosuppressive drugs well known from the field of solid organ transplantation increases the hope to extend the graft survival after high-risk corneal transplantations. In a cohort from 17 recipients with 23 grafts a reduction of irreversible graft rejections was demonstrated upon systemic treatment with a high dosage of Tacrolimus. In contrast, patients with low dosages of Tacrolimus exhibited irreversible graft rejections and most recipients with transient treatment developed rejections shortly after changing of treatment. (Sloper et al., 2001).

Nguyen and colleagues (2007) reduced the incidence of endothelial graft rejection from 9.1 % in a group of recipients with short-term topical steroid treatment (6 months) to 4.9 % in the group with long-term topical steroid treatment (1 year). Sangwan et al. (2005) recommended treatment of the first graft rejection episode with systemic application of intravenous steroids within one week of the onset of a rejection to reduce the number of recurrent rejection episodes, thus increasing the graft survival. The same intensive systemic immunsuppression with Cyclosporin A and/or Mycophenolatmophetil for recipients with emergency keratoplasty as well as after high-risk keratoplasty was recommended by Maier and co-workers (2007).

4.3. Different features of primary graft failures and early graft rejections – clinical and immunological reasons leading to the same outcome

In a large study with 440 consecutive penetrating keratoplasties the group of Naacke et al. (2001) analysed the indications for primary graft losses. In 79 recipients (18 %) graft rejections were observed within the average time span of 10.5 ± 9.3 months. Approximately 50 % of all rejections were reversible, while patients with bullous keratopathy or regrafts showed a significantly higher risk for irreversible rejections than patients with keratoconus or Fuchs´ dystrophy. The irreversibility of graft rejections correlated with the thickness (oedema) of the cornea (Naacke et al., 2001).

In addition a correlation between the presence of Herpes simplex virus type 1 DNA in bioptic samples and primary graft failures was described (Cockerham et al., 2000). In contrast to Herpes simplex type 1, DNA from Herpes simplex virus type 2 and Varicella-zoster virus was

not found in any of the bioptic samples studied by Cockerham and co-workers. The case reports of De Kesel and co-authors (2001) confirmed the influence of Herpes simplex virus type 1 upon the development of primary graft failures. The corneal graft opacification after Herpes infection or rejection episodes was accompanied by increased levels of total protein and IL-6 in aqueous humour samples collected directly after the occurrence of this alteration due to the breakdown of the blood-aqueous barrier of the eye (Van Gelderen et al., 1999). Patients with clear grafts only exhibited a minimal increase in both parameters in contrast to patients undergoing cataract surgery.

Twenty-three patients who suffering from keratoconus and additionally showing a rejection episode after the first penetrating keratoplasty were analysed for possible risk factors leading to rejections (Epstein et al., 2006). The size of the graft turned out to be the most important factor for rejections i.e. grafts with sizes ≥ 8.25 mm were accompanied by a six-fold higher risk for rejection. Indeed, the graft size as a prominent risk factor for rejection and graft failure was confirmed in a larger study by Wagoner et al. (2007) demonstrating rejection episodes in nearly 60 % of all 157 penetrating keratoplasties during the first 3 months. Furthermore, severe endothelial rejections were described as reasons for serious complications leading to an impaired post-transplantation course with a high risk of graft failure within the first 3 post-transplantation years.

In contrast to the study of Epstein and colleagues (2006) the group around Wagoner (2007) identified additional risk factors for graft failures such as increased donor age, increased patient age and historical rejection episodes of the recipients. Dua and Azuara-Blanco (1999) identified the deep vascularization of the corneal stroma in the host eye and previous surgery in the anterior segment as additional risk factors. The relevance of anterior segment surgery and regrafting as risk-factors for graft rejections was confirmed by Sangwan and colleagues (2005) in a study investigating 184 first graft rejection episodes from 1.972 consecutive penetrating keratoplasties. More than half of all first rejection episodes occurred in the first year but were reversible in 63.3 % of the cases.

Different results were provided by the studies of Graupner et al. (2000) and Kuechle et al. (2002) who analysed in total the outcome of 397 normal-risk keratoplasties with a final median follow-up of 18 months. Endothelial graft rejection episodes were detected in only 5.5% and mainly described to be of the acute type. Only four recipients exhibited chronic focal rejections, and 12 recipients showed questionable rejection episodes with isolated small keratic precipitates. Out of 11 factors (age, gender, atopic dermatitis, dry eye symptom, surgeon, graft size, post-mortem time, storage time, graft preservation and duration of post-operative epithelial defects) which were analysed by the group of Graupner, Kuechle and co-workers a correlation with graft rejection was observed only with atopic dermatitis, tear insufficiency (dry eye) and duration of graft storage, but not with the parameters relevant in

previous studies (Naacke et al., 2001; Boehringer et al., 2002; Epstein et al., 2006; Claerhout et al., 2007; Wagoner et al., 2007).

In accordance with the majority of the clinical studies this thesis demonstrates a striking influence of donor-specific anti-HLA antibodies upon the acceptance of corneal allografts. 77 % of the patients **without** donor-specific antibodies prior to grafting proceeded during the follow-up period of averagely 10 months (± 6 SD) without any complications. No graft losses, rejection episodes and/or alterations of the endothelial cells were observed. However, four patients without donor-specific anti-HLA antibodies prior to grafting exhibited rejection episodes/graft losses despite the lack of these antibodies. This may be due to a *de-novo* immune response arising after grafting and not treatable with the standard local application of immunosuppressive agents.

In our study 79 % of the patients **with** donor-specific anti-HLA antibodies prior to transplantation exhibited severe immunological complications up to the complete loss of grafts during a follow-up period of averagely 7 months (± 5 SD). This in comparison to the "negative group" shortened follow-up period strengthens the importance to detect donor-specific antibodies prior to cornea transplantations as the main reason for the shortened follow-up period. The "positive group" is characterised by the early occurrence of hyper-acute and acute rejection episodes leading to complete graft losses in 4 out of 16 patients (25 %). Based on these results is may be speculated that this high number of early graft losses can be reduced by routinely performed crossmatch assays to detect donor-specific anti-HLA antibodies prior to corneal grafting in analogy to recommendations applied to the grafting of other solid organs.

In addition, the novel ELISA-based crossmatch technique presented in the recent study for the first time offers the possibility to perform a post-transplantation monitoring if first signs of a rejection occur. The four patients of the "negative group" exhibiting rejection episodes/graft losses despite the lack of donor-specific antibodies before transplantation show the urgent requirement of the continuous post-transplantation monitoring of donor-specific anti-HLA antibodies.

5. Conclusions

- Between 10 and 20 % of patients undergoing first corneal transplantation and about 60 % of patients with retransplantations exhibit an unsuccessful outcome of the keratoplasty and thus require a retransplantation.
- The major reason of graft failure is an alteration of the “immune privileged” status in the eye caused by accidents, infectious diseases, underlying systemic diseases and/or aggressive systemic therapies (chemotherapy).
- These groups of patients urgently need an analysis of their immunological status prior to further transplantation in particular the status of donor-specific anti-HLA antibodies has to be investigated.
- For the first time the modified Micro-AMS HLA class I and class II ELISA allows the detection of donor-specific anti-HLA antibodies directed against the explanted material of rejected corneal grafts. Thus the AMS ELISA enables the analyses of underlying immunological mechanisms of corneal graft rejections induced by a donor-specific humoral immune reaction.
- Using small tissue samples from the eye (e.g. outer corneal rim) the AMS ELISA could be implemented independently from vital lymphocytes which are required for the conventional complement-dependent lymphocytotoxicity (CDC) or flow cytometry (FACS) crossmatch assays. This aspect is important in particular in the context of corneal transplantations since the graft is stored up to 1 month after the donor´s death and consequently no single cells are available.
- There is no requirement for specific and expensive technical equipment apart from an ELISA reader which belongs to the standard instrumentation of any laboratory.
- In contrast to vital lymphocytes which have to be stored in liquid nitrogen and must be transported on dry ice the handling of donor samples (e.g. pieces of the eye) for the AMS ELISA is very simple since no vital material is required. The usage of material taken from an explanted eye does not lead to additional logistical effort and costs for the Cornea banks.
- This assay offers for the first time the possibility to allocate HLA typed donor corneas including a crossmatch procedure prior to the corneal transplantation in a larger geographical surrounding (e.g. region of Eurotransplant Community) in analogy to the standards applied for the allocation of solid organs.

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REF 200 010 PROTRANS HLA-A*/B*/Cw*

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Mix	pos	HLA-A* specificities	Mix	pos	HLA-B* specificities	Mix	pos	HLA-B* specificities	Mix	pos	HLA-Cw* specificities
01		A*0101-04N/06-10	01		B*07p, *8101/02, *4025, *420501/0502w, *4806	25		B*45p, *82, *83, *0720, *1514/91, *3545, *4409, *5002	01		Cw*0102-*0110
02		A*3601-04, *1117	02		B*08, *0729, *3912	26		B*40p, *49p, *50, *1546/53	02		Cw*0202-*0209, Cw*0608, Cw*1701-03
03		A*02 w/o *023502/48/50/52/55/60/64/65/72	03		B*13p, *4415/20, *4902, *5607	27		B*45p, *50, *54, *55p, *56p, *82, *1309, *15p, *3917, *4048; **Cw*0312, *1215, *1507**	03		Cw*0302-*0315/17/18
04		A*0301-09/11N-14, *3204, *3602	04		B*18	28		B*07p, *42, *54, *55, *56p/w, *6701, *81, *82, *1576w, *4506	04		Cw*0302-06/08-13/16-18; *0411; *140201/0203/03/05
05		A*2301-12, *2424, *2907	05		B*18p, *35p, *78, *3919, *5606	29		B*54, *5507	05		Cw*0302/04-10/14/17, B*1596
06		A*23p, *24p, *0246/48, *3108	06		B*35p, *53p	30		B*56p, *82, *83, *0720/24, *1576, *4506, *5403, *5508	06		Cw*040101, *040102, *0403-14, Cw*1210; B*6702
07		A*24p	07		B*35p, *53, *15p, *0712/18, *3708,*5104, *5609/11; **A10p, *68p***	31		B*49p, *59, *4418, *5115	07		Cw*0415
08		A*2501-04, *3001-04/06-12, *0252	08		B*53p, *58p, *1513, *5104, *5705	32		B*49, *50, *27p, *40p, *1571, *1815/19, *3707	08		Cw*0501-10, Cw*0810
09		A*2501-04, *2601-18/20-23, *3401-06, *6601-04	09		B*27, *47	33		B*46, *5614, *6702	09		Cw*0501-10, Cw*0802/04/05/07/12
10		A*2601-23, *4301	10		B*37p, *3538, *4406, *5108/20/36	34		B*15p, *46p, *57p, *1304/10, *3546, *4408	10		Cw*0602-11
11		A*2304, *24p, *3401/05, *0217, *2907	11		B*38, *5113, *5304/07	35		B*57p	11		Cw*0701-21/22w/23-25/27-29
12		A*2502, *2613/19, *3401/05/06, *6601/02/04	12		B*39p/w, *67, *1548w/69, *3535	36		B*58p, *5705	12		Cw*0701-03/04w/05-10/11w/12w/13-19/21/22w/23-29
13		A*3402-04, *1110	13		B*6701	37		B*49p, *51p, *52, *53p, *58p, *59, *15p, *4418, *5705	13		Cw*0801-09/11/12
14		A*11 w/o *1117; *02p, *0302w/10, *24p, *6809/26	14		B*40p, *47, *4416/21/31	38		B*51p, *78p	14		Cw*1202/08/10
15		A*2901-11, *2303, *3107/08, *3203, *7410	15		B*40p, *41, *48p, *0727, *0804/17, *1533/78, *3707, *4431	39		B*51p, *78p, *1509, *5605/06	15		Cw*120301/0303/06/11/13/15
16		A*3001-04/06-11, *0310, *3202	16		B*40p (B60), *2724	40		B*52p, *150102, *4026/28, *5107w, *7805	16		Cw*0104/09; *02p; *0314; *0413; *0508; *07p; *08p; *12p; *16
17		A*310102-07/09-11	17		B*40p (B61), *41, *44p, *45, *47, *49p, *50, *15p, *35p, *3706	41		B*78, *150102/09, *4026/28, *5605/06	17		Cw*0205, Cw*0602-10, Cw*1204/05/09, Cw*1602
18		A*3201-08, *2303, *2418, *3107/08/10	18		B*47, *2718, *3702	42		B*15p, *3526, *3932, *5122, *7803	18		Cw*0202/04-09; *0508; *0602/03/07-10; *1204/05
19		A*3301/03-07	19		B*48p, *81, 0712/18, 1562, *2724, *35p, *4012/46, *5609/11	43		B*15p, *1309, *3546, *4021	19		Cw*1402-05, Cw*0415
20		A*7401-09	20		B*15p, *1310, *4408, *5704	44		B*1512/14/19, *4408, *5707	20		Cw*1502-13, Cw*0510
21		A*6801-25/27, *0261-64/69	21		B*15p, *4802, *07p, *35p, *3907, *5514, *5603/09	45		B*1516/17/67/95, *5806/08	21		Cw*1601/02/0401
22		A*6901, *0234/35/56/62	22		B*41, *4202	46		B*14p	22		Cw*1701-03
23		A*02 w/o *020109, *240206,*3007w	23		B*42, *82, *83, *0704/19/25, *4506, *5613	47		B*14p, *3526, *3805, *3904	23		Cw*0708, Cw*1801/02
24		A*4301, *8001	24		B*44p/w, *40pw, *4104w	48		B*7301	24		negative control

The specific bands of HLA-A Primer Mixes 5, 17, 20 and 23, of HLA-B Primer Mixes 5, 9, 43 and 47 and of HLA-Cw Primer Mix 13 are in the near of the internal control band

p = partly w = weak

last name, first name date of birth

date of collection DNA-no. conc. µg/ml

DNA extraction lot-no. ratio

HLA-A* -B* -Cw*

HLA-A* -B* -Cw*

operator date

doctor date

protrans
medizinische diagnostische produkte gmbh

photo

Quik-ID Recording Sheet RS

LOT 090606-QID EXP: 24 APR 08 2008-04-24

NAME _____ ID# _____ BDT _____

TECH _____ DATE _____ <NC> (A) _____ PRA _____

WELL	ETHN	GTI#	HLA	BAF	2xNC	CTF	SAMP	DIFF	INTR
				(B)	(C)	(D)	(E)	(F)	
A1	AA	135DFW	A2 A74 B44 B75 (4,6) Cw5 Cw8	0.701					
B1	C	63HC	A1 A26 B8 B47 (4,6) Cw6 Cw7	0.748					
C1	C	92DFW	A29 A31 B60 B62 (-,6) Cw3 Cw3	0.693					
D1	C	8LB	A1 A26 B7 B27 (4,6) Cw2 Cw7	0.739					
E1	As	15HC	A11 A30 B48 B56 (-,6) Cw1 Cw8	0.690					
F1	AA	46LB	A23 A28 B41 B57 (4,6) Cw7 Cw17	0.744					
G1	AA	28LB	A74 A80 B42 B44 (4,6) Cw4 Cw17	0.815					
H1	C	129DFW	A1 A2 B57 B61 (4,6) Cw2 Cw6	0.765					
A2	AA	98LB	A23 A74 B40 B51 (4,6) Cw15 Cw16	0.701					
B2	C	2BW	A2 A3 B13 B56 (4,6) Cw1 Cw6	0.666					
C2	AA	141LB	A28 A30 B42 B63 (4,6) Cw14 Cw17	0.570					
D2	C	DB	A2 A25 B8 B27 (4,6) Cw1 Cw7	0.586					
E2	AA	77LB	A3 A30 B18 B70 (-,6) Cw2 -	0.979					
F2	AA	62LB	A74 - B50 B70 (-,6) Cw2 Cw6	0.638					
G2	C	175DFW	A3 A66 B35 B49 (4,6) Cw4 Cw7	0.600					
H2	H	65HC	A1 A31 B8 B39 (-,6) Cw7 Cw7	0.622					
A3	C	12HC	A2 A24 B48 B65 (-,6) Cw8 Cw8	0.726					
B3	C	40DFW	A3 - B44 B55 (4,6) Cw3 Cw5	0.736					
C3	U	107DFW	A11 A30 B18 B62 (-,6) Cw3 Cw5	0.667					
D3	C	147CBC	A11 A24 B51 B55 (4,6) Cw2 Cw3	0.879					
E3	C	133CBC	A2 A2 B50 B60 (-,6) Cw3 Cw6	0.704					
F3	AA	18HC	A23 A26 B7 B64 (-,6) Cw7 Cw8	0.774					
G3	C	111DFW	A2 A29 B7 B38 (4,6) Cw7 Cw12	0.787					
H3	AA	66LB	A28 A30 B35 B81 (-,6) Cw4 Cw8	0.791					
A4	H	13HC	A25 A32 B27 B61 (4,6) Cw2 -	0.541					
B4	C	150DFW	A26 A30 B38 B49 (4,-) Cw7 Cw12	0.646					
C4	AA	17LB	A1 A66 B52 B58 (4,-) Cw7 Cw12	0.628					
D4	U	1PNR	A11 A32 B13 B64 (4,6) Cw4 Cw8	0.546					
E4	AA	149LB	A3 A36 B49 B52 (4,-) Cw7 Cw16	0.635					
F4	C	LA	A3 A32 B18 B37 (4,6) Cw5 Cw6	0.580					
G4	H	61HC	A28 A31 B39 B61 (-,6) Cw3 Cw7	0.568					
H4	AA	140LB	A30 A33 B51 B70 (4,6) Cw3 Cw8	0.641					
A5	AA	51LB	A25 A33 B53 B60 (4,6) Cw3 Cw4	0.576					
B5	C	162DFW	A2 A23 B45 B62 (-,6) Cw3 Cw6	0.625					
C5	AA	154LB	A28 A29 B58 B78 (4,6) Cw6 Cw16	0.604					
D5	AA	145LB	A2 A23 B53 B63 (4,-) Cw4 Cw5	0.613					
E5	H	21HC	A24 A29 B7 B37 (4,6) Cw6 Cw7	0.609					
F5	AA	80LB	A34 - B35 B42 (-,6) Cw4 Cw5	0.694					
G5	C	84CBC	A24 A33 B44 B62 (4,6) Cw3 Cw16	0.676					
H5	C	153CBC	A24 A31 B39 B57 (4,6) Cw6 Cw7	0.616					
A,B,C,D6		POOL	NC						
E6		POOL	PC						
F6		None	NAW						
			CALCS		C=2x(A)	D=Bx(C)		F=E-D	

CE

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Class II ID Recording Sheet

C2ID - RS

LOT 060606-C2ID

EXP: 02 FEB 08

2008-02-02

NAME

ID#

BDT

TECH

DATE

<NC> (A)

PRA

WELL	GTI#	HLA			BAF	2xNC	CTF	SAMP	DIFF	INTR
					(B)	(C)	(D)	(E)	(F)	
A1	113	DR1		DQ5	1.026					
B1	366	DR7 DR103	53	DQ2 DQ5	0.836					
C1	656	DR15 DR103	51	DQ6 DQ7	0.783					
D1	560	DR8 DR18	52	DQ4 DQ7	0.863					
E1	812	DR9	53	DQ2	0.824					
F1	139	DR10 DR11	52	DQ5 DQ7	0.825					
G1	209	DR7 DR16	51,53	DQ2 DQ5	1.299					
H1	822*	DR12 DR14	52	DQ7	0.828					
A2	328	DR1 DR4	53	DQ5 DQ7	1.092					
B2	804	DR15	51	DQ6	0.885					
C2	699	DR11 DR11	52	DQ7	0.720					
D2	587	DR13 DR18	52	DQ2 DQ4	0.923					
E2	532	DR12 DR17	52	DQ2 DQ7	0.881					
F2	107	DR10 DR13	52	DQ5 DQ7	1.022					
G2	372	DR4 DR14	52,53	DQ5 DQ8	0.964					
H2	333	DR7 DR17	52,53	DQ2 DQ2	1.080					
A3	938	DR8 DR13	52	DQ4 DQ6	0.784					
B3	814	DR11 DR13	52	DQ7	0.780					
C3	126	DR17	52	DQ2	0.762					
D3	663	DR7 DR15	51	DQ6 DQ9	0.692					
E3	817	DR1 DR13	52	DQ5 DQ9	0.775					
F3	205	DR11 DR12	52	DQ7	1.549					
G3	225	DR4 DR4	53	DQ7 DQ8	0.825					
H3	317	DR10 DR14	52	DQ5 DQ7	0.894					
A4	215	DR13 DR16	51,52	DQ5 DQ6	1.018					
B4	264	DR9 DR11	52,53	DQ2 DQ7	0.712					
C4	480	DR8 DR15	51	DQ4 DQ6	0.718					
D4	373	DR7	53	DQ2	0.672					
E4	461	DR1 DR7		DQ5 DQ9	1.143					
F4	412	DR1 DR9	53	DQ5 DQ9	0.850					
G4		**MoAb**								
H4		**NAW**								
A,B,C,D5	POOL	**NC**								
E,F5	POOL	**PC**								
		CALCS				C=2x(A)	D=Bx(C)		F=E-D	

*822 - Shows some reactivity with sera containing anti-DR1.

CE

Thesen

1. Die Corneatransplantation (CTX) ist mit über 3.000 Transplantationen/Jahr die häufigste Transplantation eines soliden Gewebes in Deutschland. Die Ursachen für eine CTX sind extrem vielfältig und umfassen (i) degenerative Prozesse im Auge, (ii) Infektionen des Auges, (iii) Verletzungen der Cornea, (iv) systemische Erkrankungen bzw. Mangelerscheinungen oder (v) Nebenwirkungen von aggressiven systemischen Therapien.

2. Auf eine Korrelation zwischen der Existenz von Spender-spezifischen anti-HLA Antikörpern und dem Auftreten von Rejektionen bis zum Verlust des Corneatransplantates weisen bereits vorliegende Untersuchungen hin. Die in diesen Studien eingesetzten Methoden zum Nachweis von Spender-spezifischen anti-HLA Antikörpern im Serum der Empfänger basierten auf dem Einsatz von vitalen Zellen des Spenders als Zielstruktur in der Verträglichkeitsprobe (Kreuztest, CM = crossmatch). Als vitale Spenderzellen wurden in diesen Studien entweder in flüssigem Stickstoff (-192 °C) gelagerte Blutlymphozyten oder steril kultivierte und mit Interferon-γ (IFN-γ)-stimulierte retinale Zellen aus dem Spenderauge genutzt. Beide Methoden sind auf Grund des großen technischen und logistischen Aufwandes nicht für die Routinediagnostik geeignet.

3. Erstmalig konnte mit dem Micro-AMS HLA Klasse I und Klasse II ELISA eine Kreuztestmethode etabliert werden, welche absolut unabhängig von der Vitalitat der Spenderzellen anwendbar ist. Die Verwendung eines Teils des äußeren, nicht transplantierten Ringes der Spendercornea (Rückstellprobe) als Ausgangsmaterial in dem neuen ELISA-basierenden Kreuztest (CM) zeigt die Überlegenheit dieser Methode über alle vorher beschriebenen Techniken. Dabei werden an Stelle von Zellen nur die isolierten und aufgereinigten HLA-Klasse I- und HLA Klasse II-Moleküle des Spender benötigt. Als Ausgangsmaterial können somit, unabhängig von der Vitalität, Zell- oder Gewebeproben des Spenders dienen.

4. Für die Etablierung des modifizierten Micro-AMS HLA Klasse I und Klasse II ELISA wurde in einer Pilotstudie mit 19 Patienten ein Teil der explantierten Empfängercornea als Ausgangsmaterial für die Isolierung von HLA-Klasse I und HLA-Klasse II Moleküle eingesetzt. Bei 12 Patienten handelte es sich um die eigene Cornea und bei 7 Patienten um frühere Transplantate. 42 % der Patienten zeigten Reaktionen im Empfängerserum gegen die HLA Klasse I- und/oder Klasse II-Moleküle, isoliert aus der explantierten Cornea. Zu Verifizierung dieser Resultate wurde eine anti-HLA Antikörperspezifizierung mittels des Quik-ID® Klasse I und Klasse II ELISA durchgeführt. In 75 % der Patienten konnten entsprechende anti-HLA Antikörper im Quik-ID® Klasse I und Klasse II ELISA nachgewiesen

werden. In einem Fall konnte der Quik-ID® ELISA auf Grund fehlenden Empfängerserums nicht durchgeführt werden, während in einem anderen Fall die Konzentrationen der anti-HLA Antikörper unter der Nachweisgrenze des Quik-ID® ELISA lagen.

5. Zum Nachweis von Spender-spezifischen anti-HLA Antikörpern vor CTX wurde eine randomisierte Gruppe von 45 Patienten untersucht, die sich im Zeitraum vom März 2005 bis Januar 2007 einer Corneatransplantation in der Augenklinik der Martin-Luther-Universität unterziehen mussten. Als Ausgangsmaterial für die Isolierung der HLA Klasse I- und Klasse II-Moleküle des Spenders wurde ein Teil des nicht transplantierten äußeren Ringes des Corneatransplantats eingesetzt (Rückstellprobe). Eine Serumprobe des Empfängers wurde am Tag der Transplantation entnommen und zusammen mit dem Spendermaterial unsteril bei -20 bis -70 °C gelagert.

6. Erstmalig konnten mit der in dieser Arbeit modifizierten routinetauglichen CM-Methode im Serum des Empfängers vorhandene anti-HLA Antikörper nachgewiesen werden, die direkt gegen HLA Moleküle gerichtet sind, welche von den Zellen der Spendercornea isoliert wurden. Im Serum von 42 % der untersuchten Empfänger wurden diese- Spender-spezifischen anti-HLA Klasse I- und/oder Klasse II-Antikörper detektiert. Somit bestätigt die vorliegende Arbeit die indirekten Beobachtungen und statistischen Korrelationen verschiedener Autoren. Zur Verifizierung der AMS-ELISA Resultate wurde einerseits eine anti-HLA Antikörperspezifizierung mittels des Quik-ID® Klasse I und Klasse II ELISA, andererseits eine HLA-Genotypisierung des Corneaspenders durchgeführt. In 75 % der Fälle wurden anti-HLA Antikörper im Quik-ID® Klasse I und Klasse II ELISA nachgewiesen, wobei in 69 % die exakt passenden HLA Antigene beim Spender mittels Genotypisierung zusätzlich gefunden wurden. In 3 Fällen konnten auf Grund fehlenden Materials die beiden letztgenannten Untersuchungen nicht durchgeführt werden.

7. Bei 79 % der Patienten, bei denen vor der CTX Spender-spezifische anti-HLA Antikörper nachgewiesen werden konnten, wurden immunologische Komplikationen schon kurz nach der CTX beobachtet. Bei einem Patienten erfolgte eine akute Abstoßung des Transplantates innerhalb von 5 Tagen; drei Patienten verloren ihr Transplantat innerhalb weniger Wochen. Die meisten Patienten zeigten akute Abstoßungsepisoden oder morphologische Veränderungen der endothelialen Zellen des Transplantats. Drei der Patienten erhielten eine dauerhafte Erhöhung der Immunsuppression.

8. In 77 % der Fälle, in denen vor der CTX keine Spender-spezifischen anti-HLA Antikörper nachgewiesen werden konnten, war innerhalb des Zeitraums der Nachfolgeuntersuchungen von 10 Monaten (SD = ± 6) keine immunologische Komplikation zu beobachten. Im Gegensatz dazu entwickelte sich bei vier Patienten eine immunologische Abstoßungsreaktion, welche in zwei Patienten letztlich zum Transplantatverlust nach 17 bzw. 23 Monaten führte. Diese vier Patienten verdeutlichen die Notwendigkeit, einen Test zu etablieren, der auch Monate nach einer primär erfolgreichen CTX den Nachweis von Spender-spezifischen anti-HLA Antikörpern (Antikörper-Monitoring) ermöglicht, um eine adequate Immunsuppression einleiten zu können.

9. Die Ergebnisse dieser Studie zeigen

(i) die Möglichkeit, einen Kreuztest vor der Corneatransplantation unter Ausnutzung von Gewebematerial zu etablieren, welches direkt aus dem explantierten Spenderauge gewonnen werden kann,

(ii) die Notwendigkeit, einen einfachen und routinetauglichen Kreuztest zum Nachweis von Spender-spezifischen anti-HLA Antikörpern im späteren Verlauf nach einer Corneatransplantation in die Nachfolgeuntersuchungen zu implementieren,

(iii) die Notwendigkeit, bei Risikopatienten und bei Patienten mit mehreren CTX eine adäquate Allokation bereits typisierter Corneas vorzunehmen und mittels eines Kreuztestes hyperakute oder akute Abstoßungsreaktionen auf Grund vorhandener Spender-spezifischer anti-HLA Antikörper auszuschließen.

Lebenslauf - Dipl. Med. Wolfgang W. Altermann

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Ausbildung

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1987-1990	:	Postgraduales Studium für Transfusionsmedizin
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7/1997-6/2001	:	Firmen MEDIPRO GmbH and PROTRANS GmbH, Ketsch, BRD Produkt Manager für die molekulargenetische Diagnostik und für die Ausbildung auf dem Gebiet der HLA Diagnostik
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Besondere Kenntnisse : HLA- und HPA Diagnostik, HCV-, MICA-, KIR-Genotypisierung, HLA-, MICA-Sequenzierung, CM-ELISA, sCD30

Mitgliedschaft in Berufsorganisationen : Deutsche Gesellschaft für Immungenetik (DGI),
Arbeitsgemeinschaft der Deutschen Knochenmarkspender-Dateien
European Foundation of Immunogenetics (EFI)
Gesellschaft zur Förderung der Immundiagnostik (GFID)
Deutsche Gesellschaft für Immunologie (DGfI)

Selbstständigkeitserkärung:

Hiermit erkläre ich, Wolfgang Altermann, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Weiterhin erkläre ich, dass ich diesen Antrag auf Eröffnung des Promotionsverfahrens der vorliegenden Arbeit erstmalig beim Dekan der Medizinischen Fakultät der Martin-Luther-Universität Halle-Wittenberg stelle und in der Vergangenheit auch an keiner anderen Universität gestellt habe.

Halle, den 25.02.2008

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