Human Herpes Simplex Virus Keratitis:

THE PATHOGENESIS REVISITED

Herpes simplex virus keratitis bij de mens:

DE PATHOGENESE OPNIEUW BESCHOUWD

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Chapter 1

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Human herpesviruses

Morphology and classification

Virions of *Herpesviridae family* have a characteristic morphology. An icosadeltahedral capsid surrounds an electron opaque core in which double stranded DNA is located. The capsid itself is surrounded by a tegument and a viral envelope. The viral envelope is the outer surface of the virion in which virus-encoded glycoproteins, exhibited as spikes, are embedded (Fig.1).

The overall size of the virions varies from 120-300 nm. The double stranded DNA

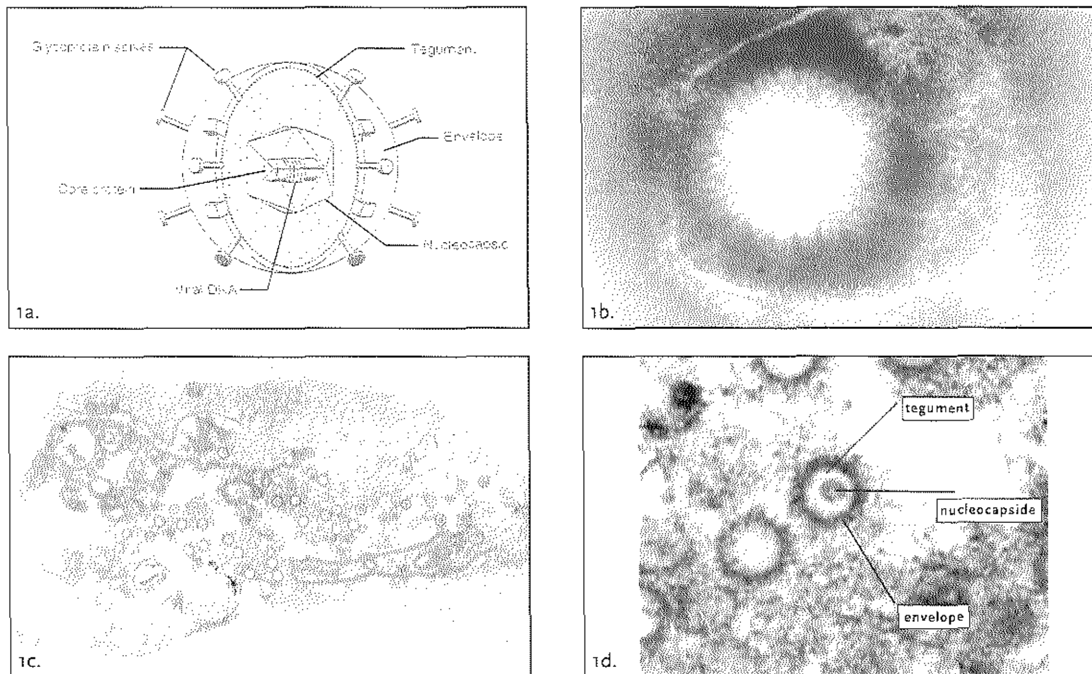

Figure 1: The morphology of herpesviruses.

a. schematic representation of the herpesvirion. Viral DNA is wrapped around a protein core that lies within a icosadeltahedal capsid . Together these form the nucleocapsid. The tegument, an amorphous protein structure, lies between the outer phospholipoprotein membrane and the nucleocapside.
reprinted with permission from Pavan-Langston, Dunkel E. Varicella Zoster virus diseases: Anterior segment of the eye. In: Pepose JS, Holland GN, Wilhelmus KR, editors. Ocular infection and immunity. St Louis: Mosby-Year Book;1996.p 934

b. Electron Micrograph (EM) of the capsid of HSV

c. EM of the nucleus of a human corneal keratocyte with multiple herpes simplex virions.

d. detail of EM c. matching the schematic representation of the virion in a.

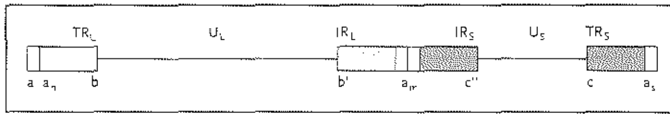

Figure 2: Schematic representation of the arrangement of DNA sequences in the HSV genome[2].

The unique sequences (thin lines) are flanked by the inverted repeats boxes

Letters below the line designate :

- a_l terminal a sequence of the unique long component.
- a_n variable number of additional a sequences
- b the b sequence
- U_L the unique sequence of the L component
- b' the repetitions of the b sequence
- a_m a variable number of a sequences
- c'' the inverted c sequence
- U_S the unique sequence of the S component
- c the repetition of the c sequence
- a_s the terminal a sequence

genome ranges from 120 to 230 kilo base pairs (kb)[1]. The genome of herpes simplex virus (HSV) or human herpesvirus type 1 (HHV-1), the prototype of the Alphaherpesvirinae (see below), has a unique organization (Fig. 2). It consists of long double stranded linear molecules with several repeated and inverted sequences. The DNA has two stretches of unique sequences, one long (unique long sequence, U_L) and one that is much shorter (unique short sequence, U_S)[2,3]. Each of these long sequences is bracketed by shorter identical DNA repeats. The viral DNA contains terminal (TR) and internal reiterated (IR) sequences. Because of the variability in the number of these reiterations, the size of individual genomes may vary by more than 10 kb[1].

The genome of herpesviruses encodes about 80 proteins. These proteins have regulatory functions, (e.g. DNA polymerase), or are structural proteins, (e.g. glycoproteins)[3]. The glycoproteins mediate attachment of the virus to cells, the capsid acts as a vector for the viral DNA[4], and several tegument proteins are involved in the initiation of viral replication[3]. Both glycoproteins and structural proteins have been shown to play a role in the adaptive host immune responses[4,5] (see below).

The family *Herpesviridae* comprises a group of ancient large DNA viruses that are widespread in the animal world, having mammals, fish, birds and reptiles as their host species. They are classified into three subfamilies α, β and γ, initially primarily on basis of tissue tropism and related pathogenesis, but to date largely on basis of DNA sequence homology.[1]

Alphaherpesvirinae

The α-herpesviruses have a relatively variable host range, a relative short replication cycle, rapid spread in culture, efficient destruction of infected cells and tendency to establish latent infections in neural tissue. They include HSV-1, HHV-1; HSV-2, HHV-2; and varicella zoster virus (VZV),HHV-3; Table 1.

Betaherpesvirinae

The β-herpesviruses have a restricted host range. The reproductive cycle is long and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalia). Latency is established in lymphoreticular cells, kidneys and secretory glands. They include cytomegalovirus (CMV), HHV-5; HHV-6,and HHV-7; Table 1.

Gammaherpesvirinae

The γ-herpesviruses have a very restricted host range. Viruses in this group infect specific T- or B-lymphocytes, causing either a lytic or latent infection. They include Epstein-Barr virus (EBV),HHV-4; and HHV-8; Table 1.

Replication and latency of herpes simplex virus (HSV).

HSV-1 is the prototype of the α-herpesviruses. The lytic or productive cycle of infection starts with the attachment of virus particles to susceptible cells. This interaction requires sequential interaction between viral membrane glycoproteins and cellular recep-

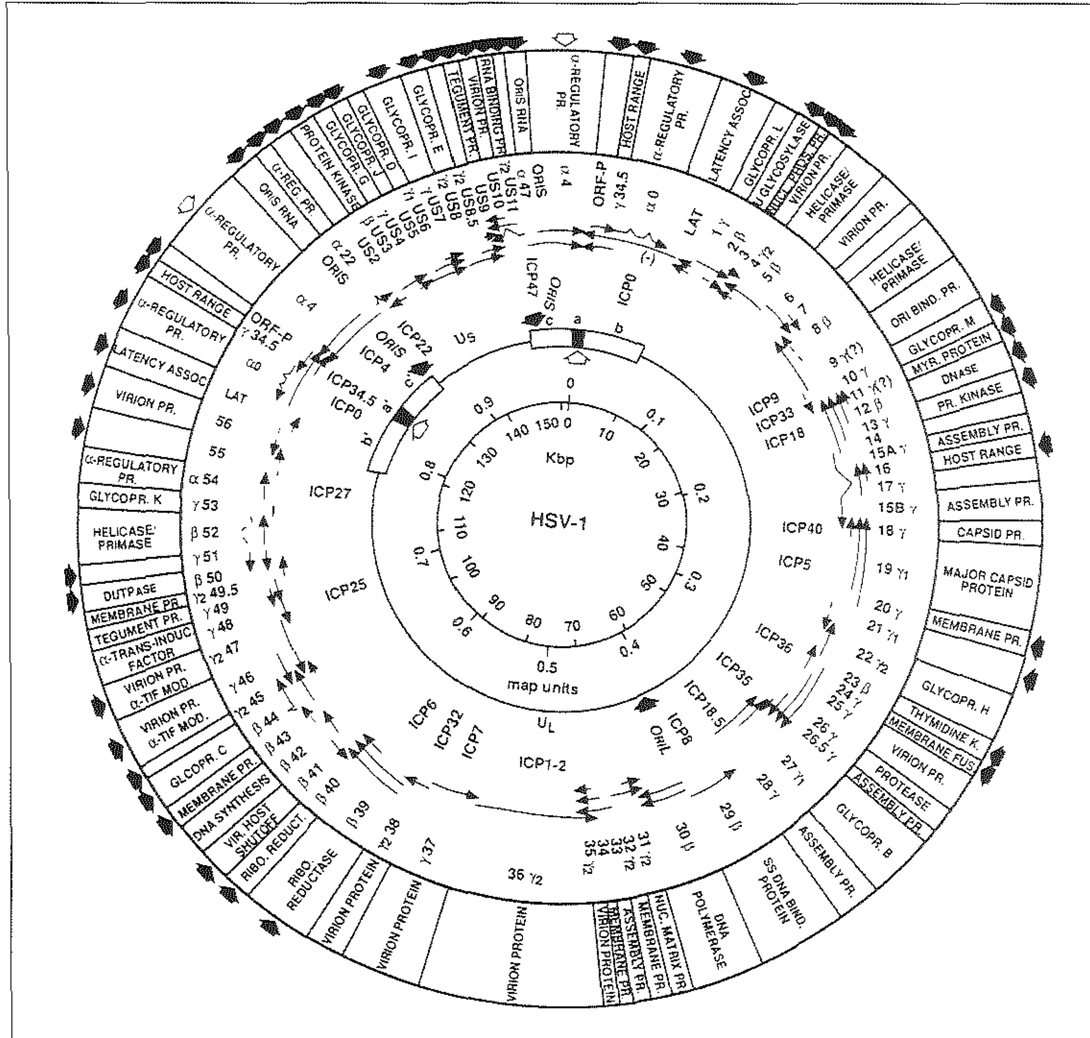

Figure 3: Functional organization of the HSV-1 genome.

The circles are described from inside out.

Circle 1: map units and kb.

Circle 2: sequence arrangement of HSV genome.

Circle 3: the transcriptional map of the HSV-1 genome. The map serves the purpose of identifying the direction of transcription, the approximate initiation and termination sites.

Circle 4: the known functions of the proteins.

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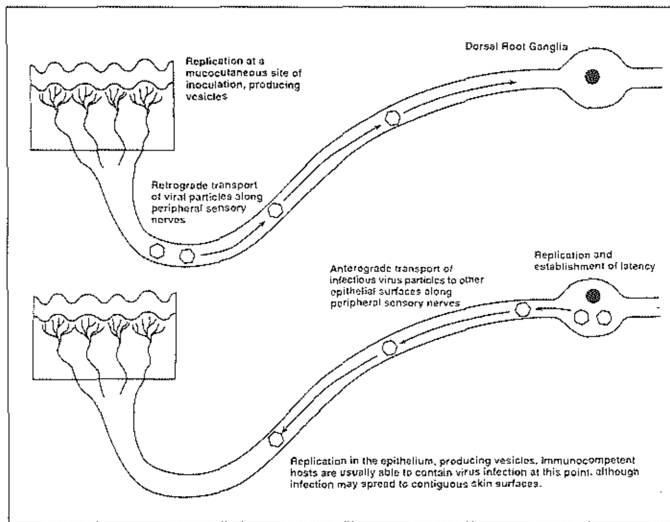

Figure 4 : Schematic diagram of HSV infection

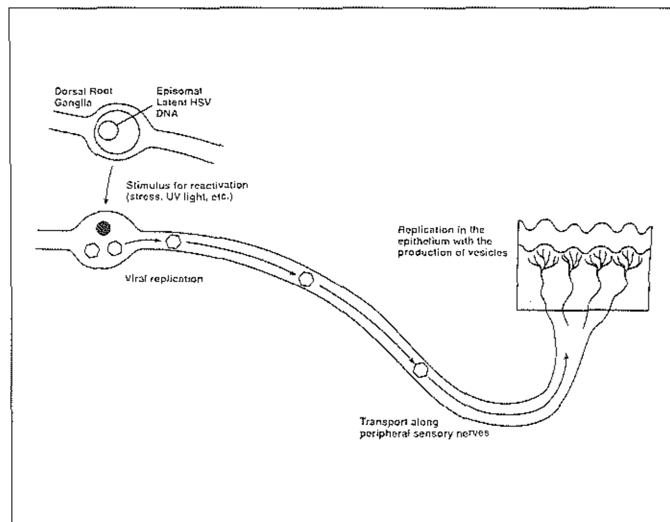

Figure 5: schematic diagram of latency and reactivation

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tors. Entry of virus mediated by fusion of the envelope and plasma membranes rapidly follows the initial attachment[3]. Upon entry into the cell, the capsids are transported to the nuclear pores where DNA is released into the nucleus. Transcription of viral DNA takes place in the nucleus in an orderly program (Fig. 3). The earliest genes expressed are the five HSV immediate-early genes (α-phase, 2-4 hours post-infection). They are important in priming the cell for further gene expression and mobilizing cellular transcription machinery. This phase is followed by the early gene expression (β-phase, 5-7 hours post-infection): the expression of a number of genes either directly or indirectly involved in genome replication. Upon genome replication viral structural proteins are expressed in high abundance during the late phase (βγ/γ phase, timing depends on viral DNA synthesis).

Viral capsids assemble in the nucleus, they bud through the host cell nuclear and cytoplasmic membranes, becoming enveloped in the process. Viral proteins are synthesized in the cytoplasm. Some of the host glycoproteins are captured during the process and end up on the outer surface of the virion. Enveloped infectious virions can either remain cell-associated and spread to other cells via virus-mediated fusion, or can be released from the cell for reinfection. "Cell-to-cell" spread has several important implications for the pathogenesis of the disease. Diseases induced by HSV are characterized by local spread and progression of lesions, thereby largely evading local immune surveillance and host mediated immune clearance[3].

One of the most striking properties of all herpesviruses is their ability to persist in an apparently inactive state for varying durations of time, referred to as latency. The cellular site of latency appears to be different from the pri-

mary tissue in which the lytic infection occurs. HSV combines this property with a tendency for neurovirulence[4]. During primary infection, neurons innervating the infected mucosa are invaded by the virus. After initial infection the HSV ascends by retrograde axonal transport in nerve axons to the innervating sensory ganglia[6,7], in which the virus replicates for several days. Subsequently, one of two mutually exclusive events can occur. Either viral replication with neuronal destruction, which can result in severe neurological devastation (myelitis or encephalitis), or establishment of a latent infection and neuronal survival (Fig. 4). Latent infection is defined as a type of persistent infection in which the viral genome is present but infectious virus is not produced except during intermittent episodes of reactivation. During latency little or no virus protein is synthesized, although an untranslated virus transcript, the so-called latency associated transcript (LAT), is produced. Occasionally, latency is interrupted, and the virus reactivates as the result of provocative stimuli. These may include physical or emotional stress, fever, exposure to ultraviolet light, immune suppression, menstruation and tissue damage. As a consequence any local trauma can reactivate the virus and cause a manifest infection[4]. Reactivated virus travels down the sensory nerve and spreads to and replicates in mucocutaneous epithelial cells, producing the characteristic symptoms (Fig. 5)[4]. Recurrent HSV infections predominantly occur at the site of primary infection.

Latent infections persist for life in the sensory ganglia[8,9]. Studies of HSV-induced skin diseases reveal that HSV-1 is isolated principally from oro-labial lesions innervated by the trigeminal ganglia, and that HSV-2 is frequently isolated from lesions in the genital areas innervated by sacral ganglia. However, in addition to these two major sites, HSV can cause latent infection in other sites of spinal ganglia[4]. In addition to ganglionic or neuronal latency, however, there is evidence for persistence of HSV DNA in both the skin[10] and the cornea[11,12,13]. LAT, however, has not been detected in these peripheral sites.

Recurrent HSV infections are thought to result mainly from reactivation of the HSV strain acquired during primary infection[14,15]. However, genotypic analysis of HSV isolates showed that a target organ is not immune to reinfection[16,17,18,19]. Yet the frequency of reinfection, with an exogenous virus, referred to as superinfection, in large scale studies seems to be low[20,21,22].

Ocular manifestations of human herpesvirus infections (Table 1)

The pattern of clinical disease resulting from primary infection with human herpesviruses is largely determined by the virus involved,the portal of entry and the immune status of the host. Generally in the fully immunecompetent host both primary and recurrent infections cause mild or no symptoms[4]. When clinical symptoms do occur, they range from the common fever blisters on the lip to more rare and severe infections of the central nervous system. An overview of clinical manifestations of HHV infections is given in tables 1 and 2 and Figure 8. Immunecompromised persons, e.g. transplant recipients, neonates and AIDS patients, are at increased risk for severe herpesvirus infections.

Of the eight human herpesviruses identified to date seven have been shown to have clinical implications for ocular infection. HSV-1 and VZV are the viruses which causes eye disease most frequently[23,24]. Not only the eye proper, may be affected, also infections of the adnexae, like the eye lid skin and lacrimal gland, as well as infections of the cranial nerves[25] are possible.

HSV-2 infections of the eye are relatively rare, with clinical manifestations identical to those of HSV-1.

Primary and recurrent HSV infections in immunecompetent patients predominantly present with vesicular or ulcerating lesions on skin or mucous membranes. The mouth and lips are the most common sites of HSV-1 infection. Reactivation of herpesviruses is common in the immunecompromised host, usually resulting in asymptomatic viral shedding or in progressive mucocutaneous infection. HSV, can disseminate and cause diffuse visceral infection[26]. The clinical presentations of ocular HSV infections are discussed below.

Varicella, caused by primary infection with VZV, is a common childhood infection. Ocular involvement in varicella frequently occurs on the eyelids, whereas corneal involvement is uncommon. Recurrent VZV infection (herpes zoster) presents as a cluster of vesicular lesions which appear unilaterally in the dermatomal distribution of one or more adjacent sensory nerves[27]. Herpes zoster ophthalmicus is a clinical syndrome involving the ophthalmic branch of the trigeminal nerve. Ocular complications occur in 50-72% of patients with herpes zoster ophthalmicus[28]. In case of VZV infections of the eye the same clinical manifestations as in HSV-1 infections are possible. In addition to these manifestations, postherpetic neuralgia and cranial nerve palsies may occur[29]. Ocular disease caused by the reactivation of latent VZV is more likely to occur in the elderly and the immunecompromised[30,31,32,33].

EBV is the most common etiologic agent of infectious mononucleosis[34]. EBV is also associated with malignant disease, including different types of lymphomas and nasopharyngeal carcinoma[34,35]. Ocular manifestations occur rarely in EBV infection. When present, they may encompass a wide range of clinical pictures, including infections of the anterior segment of the eye[36], neuro-ophthalmological syndromes[37] and intra-ocular lymphoma[38].

Congenital infections with CMV primarily affect the reticuloendothelial system and the central nervous system (CNS), whereas acquired CMV infection in immunecompetent individuals is usually asymptomatic. Some patients, however, develop a syndrome resembling infectious mononucleosis. Progression to invasive organ disease is mainly seen in immunecompromised patients[39]. Ocular manifestations are rarely seen in congenital CMV infections. Ocular involvement in CMV infections in the adult population is confined to immunecompromised patients. In this group CMV is a common cause of retinitis[40].

Of the more recently discovered human herpesviruses, HHV-6 and HHV-7 have been associated with febrile illnesses and the childhood disease, exanthema subitum[41]. In immunecompromised patients the spectrum of disease is extended to solid organ infections and bone marrow depression[42]. To date HHV-7 has not been associated with ocular disease. Clinically manifest infections with HHV-8 are predominantly seen in immunecompromised patients. This virus seems to resemble EBV in its possible oncogenic properties[43]. In isolated cases HHV-6 and HHV-8 are associated with intraocular lymphomas [38,44].

Table 1: Clinical presentations of human herpesvirus infections

human herpesvirus	general		ocular	
Class	Immune competent	Immune compromised	Immune competent	Immune compromised
herpes simplex virus type 1 HSV-1 HHV-1 α	mucocutaneous infections predominantly facial and skin pharyngitis + mononucleosis infectiosa encephalitis (rare) neonatal infections (rare) disseminated disease (rare)	 encephalitis disseminated disease	Bell's palsy blepharitis conjunctivitis epithelial keratitis immune stromal keratitis necrotizing keratitis (neurotrophic keratopathy) endotheliitis trabeculitis anterior uveitis acute retinal necrosis	frequent recurrences prolonged infections acute retinal necrosis
herpes simplex virus type 2 HSV-2 HHV-2 α	mucocutaneous infections predominantly genital neonatal infections encephalitis (rare) disseminated disease (rare)	 encephalitis disseminated disease	See HSV-1 ocular manifestations rare	
varicella zoster virus VZV HHV-3 α	chickenpox (varicella) shingles (zoster)	reinfection recurrence of infection	conjunctivitis blepharitis skin infection of rI nV HZO See HSV-1 extra-ocular muscle palsies	disseminated disease increased incidence and recurrent HZO retrobulbar neuritis acute retinal necrosis progressive outer retinal necrosis

human herpesvirus	general		ocular	
Class	**Immune competent**	**Immune compromised**	**Immune competent**	**Immune compromised**
Epstein-Barr virus EBV HHV-4 γ	infectious mononucleosis Burkitt's lymphoma Hodgkin disease T-cell lymphoma nasopharyngeal carcinoma	B-lymfoproliferative disease oral leukoplakia immunoblastic lymphoma	Parinaud oculoglandular disease cranial nerve palsies dacryoadenitits conjunctivitis episcleritis epithelial keratitis immune stromal keratitis anterior uveitis chorioretinitis papillitis primary ocular lymphoma	
cytomegalovirus CMV HHV-5 β	congenital infections infectious mononucleosis hepatitis (rare) pneumonitis (rare)	infectious mononucleosis neuropathy, meningo-encephalitis hepatitis Carditis pneumonitis GI lesions		CMV retinitis
human herpesvirus type 6 HHV-6 β	exanthem subitum febrile illness	febrile illness pneumonitis hepatitis encephalitis bone marrow depression	primary ocular lymphoma	

human herpesvirus	general		ocular	
Class	Immun competent	Immune compromised	Immune competent	Immune compromised
human herpes-virus type 7 HHV-7 β	exanthem subitum febrile illness	post-transplant multi-organ infection		
human herpes-virus type 8 HHV-8 γ		associated with: Kaposi -sarcoma angiosarcoma primary effusion B-cell lymphoma multricentric Castleman's disease multiple myeloma	intraocular lymphoma	Kaposi sarcoma of adnexa and conjunctiva intraocular lymphoma

Epidemiology of HSV-1 infections

Transmission of HSV-1

Herpesviruses do not persist well on environmental surfaces, and infection needs direct inoculation into areas where they can replicate. The first line of defense against the virus is the keratin layer of the superficial epidermis, which prevents direct access of these viruses to cell-membranes. In the absence of keratin, for instance in mucous membranes, cells are more prone to be infected[45]. HSV-1 can be transmitted following close contact with the secretions, skin, or mucous membranes of a person shedding virus. Infection occurs via the mucosal surfaces. Infections with HSV-2 are usually acquired through sexual contact.

Humans are considered to be the only natural reservoir of HSV. HSV infects its host generally in the first decades of life[4]. Primary infections with HSV manifest clinically only in 1-6% of the time[46] and most episodes of clinical disease are manifestations of reactivation of HSV infection. Because only a third of the individuals who harbor HSV recognize disease symptoms during primary or recurrent infections, serologic studies are used to defining the prevalence of HSV infections in the population. Seroprevalence rates of HSV vary with age, sex, sexual behavior, socio-economic status and geographic location[47]. In adult populations the prevalence of HSV-1 antibodies varies from 45%[48] to 88%[47, 49, 50]. Corresponding with seroprevalence 70-80% of healthy individuals have HSV- DNA in their trigeminal ganglion.

There is a trend towards a decreasing incidence of HSV-1 infections in the developed world[47, 52,53]. Antibodies to HSV-2 are rarely found before ages of onset of sexual activity. There is a trend towards an increasing incidence of HSV-2 infections in both the developed and developing countries[47,48,54]. Although most genital HSV infections are caused by HSV-2 infection, a recognizable and growing portion is attributable to HSV-1 infections[55].

Among recipients of organ transplants, transmission of viruses through donor tissues is a well recognized clinical problem. CMV is the most prominent example. Manifest infection may occur, besides from reactivation, by graft-to-host transmission of donor-derived virus as a result of transplantation. Though rare, there are reports demonstrating probable HSV transmission to seronegative organ transplant recipients[56,57].

Corneal transplantation is the most common type of tissue transplantation in The Netherlands. It is usually a safe procedure with little associated morbidity. In 5-10% of these patients herpetic keratitis is the reason for penetrating keratoplasty (PKP)[58]. Herpetic keratitis recurs relatively frequently (10-20%) after PKP. After PKP for reasons unrelated to HSV infection patients may still develop herpetic keratitis in their graft[59,60-61,62], referred to as: 'newly acquired HSV keratitis after PKP'. The origin of HSV infection in these cases is unknown.

Epidemiology of ocular HSV-1 infection

Ocular HSV infections are mainly confined to the anterior segment of the eye, including the conjunctiva and the eyelids (table 2). HSV appears to be the most common infectious cause of blindness in developed countries[63]. In developing countries, where chlamydia conjunctivitis is endemic, it is only second to trachoma as a cause of corneal blindness. In malnutrition combined with measles infection, HSV keratitis is a common cause of bilateral corneal blindness in children in the developing world[64].

Despite the common exposure to HSV, ocular manifestations are only observed in about 1% of those exposed. This may be an underes-

timation because presenting symptoms of blepharitis and conjunctivitis are mild, and the condition remains largely unrecognized.

Only 5% of ocular HSV disease represent primary infections[65]. Liesegang et al. reported an incidence of 8.4 first ocular HSV infections per 100,000 person-years[66]. The overall prevalence of ocular HSV infection has been estimated at 149 cases per 100,000 person-years[65]. Blepharitis, conjunctivitis and epithelial keratitis are the most common symptoms of primary ocular HSV infections. The predominant form of recurrent disease is epithelial and stromal keratitis (see below; Table 2, page 25). Disease is bilateral in 12-19%[67,68] of cases. A history of epithelial keratitis is not a significant risk factor for recurrent epithelial keratitis, whereas previous multiple episodes of stromal keratitis markedly increase the probability of subsequent stromal keratitis[69]. The interval between attacks shortens with time[67].

Herpetic stromal keratitis is a leading infectious cause of blindness worldwide[66].

HOST IMMUNE RESPONSE TO HERPESVIRUSES

Following a viral infection, the immune system will recognize the virus as a foreign entity and try to eliminate it from the host. At first, non-specific immune cells, including macrophages, polymorphonuclear cells (PMN) and natural killer (NK) cells, which are part of the innate immune system, are attracted to the site of infection. Secondly, a virus-specific immune response, (i.e. adaptive immunity) develops in which both B and T lymphocytes are involved. This constellation of immune responses to the virus serves to protect the infected organism from disseminated viral disease and death[70].

Innate immune response

The first line of defense against herpesviruses is formed by the innate immune system. This system consists of cellular and soluble components. Macrophages and PMN recognize viral antigens in a non-specific fashion and eliminate the virus by phagocytosis and subsequent intracellular degradation. Both cell types show a marked increase of uptake of virus when virions are opsonisized by antibody or complement[71]. The anti-viral effect of NK cells involves the lysis of viral infected cells and production of IFN-γ upon stimulation with other cytokines (e.g. IL-12). The recognition of a virus-infected cell by NK cells is mediated either by the opsonization of an infected cell by virus-specific antibody[72], or by antigen recognition independently of the histocompatibility complex (MHC)[73]. Herpesvirus infection results in down-regulation of MHC class I molecules at the surface of an infected cell, rendering the cell susceptible to NK cell killing[74]. Low NK cell activity is linked with increased human sensitivity to disseminated herpesvirus infections, including those with HSV, EBV and CMV[72,73]. Cells of the innate immune system are located in large numbers at strategic sites in and behind the physical barrier of the organism, or circulate through the body fluids awaiting attraction to the site of infection by chemotaxis.

The complement system and cytokines/chemokines secreted by virus infected cells and inflammatory cells, form the major soluble component of the innate immune response to herpesvirus infection. Viral infection directly stimulates the production of IFN α and β by infected cells. Both IFN α and β inhibit viral replication[75], enhance the ability of NK cells to kill infected target cells[70] and facilitate antigen processing and presentation by MHC[75]. The secretion of various chemokines, like interleukin 8 (IL-8), is induced at sites of viral infection providing a chemotactic signal for immune cells to infiltrate the affected tissue.

This first line of immune defense facilitates an immediate response to invading infectious agents, irrespective of the site of entry. Moreover, it will limit dissemination of the virus, while at the same time the development of a specific immune response in the draining lymphoid tissues is initiated to combat the remaining virus and to build up immunological memory.

Adaptive immune response

Specific immune responses are mediated by lymphocytes, recognizing the antigen by specific membrane-bound antigen receptors. The adaptive immune response can be divided into a humoral and a cellular immune response, mediated by B and T lymphocytes, respectively.

Humoral immunity:

Virus-specific antibodies are important in the defense early in the course of viral infection and in defense against viruses that are liberated from lysed cells. Within several days

after onset of a herpesvirus infection, antibodies to some of the viral proteins appear in the circulation. The major immunogens of HSV are the viral proteins expressed at the cell surface of infected cells: the glycoproteins[76,77]. Neutralizing virus-specific antibodies bind to envelope proteins and prevent viral attachment and entry into host cell. Opsonizing antibodies may enhance phagocytic clearance of viral particles. Secretory immunoglobulins of the IgA isotype, i.e. sIgA, may be important for neutralizing viruses that enter via the mucous membranes and trigger complement-mediated lysis of infected cells[79] Infectious cell-free virus is mainly detected during primary infection. Herpesviruses like HSV have a cell-to-cell spread and survive latently in the sensory neurons, being inaccessible to antibodies after entry in the cell. In HSV infection, antibodies do play a role in limiting dissemination the virus from the primary site of infection to the innervating sensory ganglia and other parts of the nervous system[78, 79]. Severe cases of herpesvirus infections have been described in antibody deficiency syndromes, indicating that antibodies are an important component in the immune response to HSV infection.[78]

Cellular immunity:

Severe HSV infections in patients with impaired T cell immunity, e.g. AIDS patients and transplant recipients, indicate that these cells play an important role in controlling viral infection[80]. Both virus-specific $CD4^+$ and $CD8^+$ T cells are mandatory in controlling herpesvirus infection. They distinguish virus-infected from non-infected cells by their T cell receptor, recognizing virus-derived peptides associated with MHC class II or I molecules expressed at the cell surface of infected cells, respectively[79]. Following activation, T cells can kill infected cells or secrete cytokines. Based on their cytokine production profile, T cells can be divided into two groups, T1 and T2 cells. T1 cells mainly secrete IL-2, IFN-γ and TNF-α, whereas secretion of IL-4, IL-5 and IL-10 is restricted to T2 cells. The subgroup of T cells referred to as T0 cells secrete both T1 and T2 cytokines. In general herpesvirus-specific T cells responses are both T1 and T0-like.

Immune evasion

Herpesviruses have evolved numerous strategies that favor their own survival by evading host immunity. These strategies fall into two categories: indirect immunomodulatory effect of viral proteins by their interaction with the protein machinery of the host cell and the direct inhibitory effect of viral encoded proteins.

Upon herpesvirus infection, virus-specific $CD8^+$ T cell recognition is hampered by the low level of MHC class I expression on infected cells. Several herpesvirus proteins, like infected cell protein (ICP) 47 of HSV-1, have been shown to inhibit the assembly and cell surface expression of stable MHC class I molecules[81]. Recently, ICP 22 of HSV-1 has been shown to inhibit $CD4^+$ T cell responses[82]. Additionally, herpesviruses are able to prevent apoptosis of infected cells[83]. Some herpesviruses produce molecules that directly inhibit innate and adaptive immune responses. For example, human CMV encodes a protein (UL18), that inhibits T1/0 responses and is homologous to MHC class I proteins, which acts as a decoy for NK cells[84] and an EBV protein, that shows homology to IL-10[81]. Furthermore, three glycoproteins of HSV modulate the innate immune response by binding to complement factor 3b (gC)[85] or the humoral response by binding to the Fc portion of IgG molecules (complex of gE and gI)[86].

Immunopathology

An immunopathogenic response is an immune response that causes tissue damage.

This can occur when cells are destroyed with a limited regenerative capacity, or when repair deposits impair the function[87]. Several mechanisms of immunopathology have been described. A consequence of persistent infection with some viruses is the formation of circulating immune complexes composed of viral antigens and specific antibodies. These complexes may become trapped in tissues and trigger inflammatory responses in which complement and PMNs play a major role[87].

During a immunopathogenic response the immune reaction changes focus with time, beginning with an appropriate antiviral response and spreading to encompass host antigens. Some viruses are known to contain amino-acid sequences that are also present in some self antigens. It has been postulated that because of this "molecular mimicry" antiviral immunity can lead to immune responses against self antigens. The virus antigen should be different enough from host sequences to initiate an immune response, but similar enough that the response is cross-reactive. The resulting 'anti-host' response will then be maintained even after clearance of the virus[88]. Alternatively, virus infection may induce autoimmunity in the absence of shared antigens, a mechanism known as 'bystander activation'.

Immune privileged sites

Immune responses are absent in some tissues or organs, which phenomenon is generally known as immune privilege. This phenomenon is considered to be aimed at preservation of the individual (brain, eye) on the one hand and preservation of the species on the other hand (testis, ovary, pregnant uterus).

The eye is a largely immune privileged site, which is important since minute manifestations of inflammation may interfere with vision.

A wide range of factors contribute to the immune privilege of the eye. For example the intra-ocular fluids have immunosuppressive capacities, like transforming growth factor-β (TGF-β)[89], and the central cornea lacks the key player in the immune response, the antigen presenting cell (APC)[90]. The eye constitutively expresses Fas ligand, which may kill Fas-expressing inflammatory cells that enter these tissues[89]. The implication of the immune privileged state is, that transplanted tissue, that would be rejected in most other locations of the body, is accepted when grafted into the eye: corneal transplants.

Herpesvirus Keratitis

Corneal morphology and innervation

The cornea is the transparent tissue in the front of the eye that is primarily responsible for focusing light on the retina. The central cornea is about 0.52 mm thick. The tissue comprises five layers: epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium (Fig. 6). In the normal state the cornea does not contain blood vessels[91].

The corneal *epithelium* is a stratified squamous, non-keratinizing epithelium, approximately five cell-layers thick. Epithelial cells adjacent to Bowman's layer can divide and renew the epithelial layer in two to four days. Epithelial wounds heal quickly over an intact Bowman's layer.

Bowman's layer is a very thin (8-10 μm) acellular zone beneath the epithelium. Bowman's layer is often said to be resistant to trauma, offering a barrier to corneal invasion by micro-organisms and tumor-cells, but it is not known to which extent this is true. Bowman's layer is considered to have no regenerative capacity.

The *stroma* which constitutes about 90% of the cornea consists mainly of collagen fibrils, ground substance and keratocytes. The collagen fibrils of the cornea are uniform and small, about 250-300Å in diameter. The ground substance surrounding the collagen fibrils is rich in glycosaminoglycans. The ground substance plays a role in maintaining the regular array of collagen fibrils. With stromal edema the individual collagen fibril does not change, the volume of the ground substance increases, and with this the space between collagen fibrils.

The keratocyte is the predominant cell of the stroma, but accounts only for about 5% of the dry weight of the cornea. In response to stromal injury the keratocytes migrate into the wound area and undergo transformation into fibroblasts. They contribute to the scar formation by proliferation and collagen production.

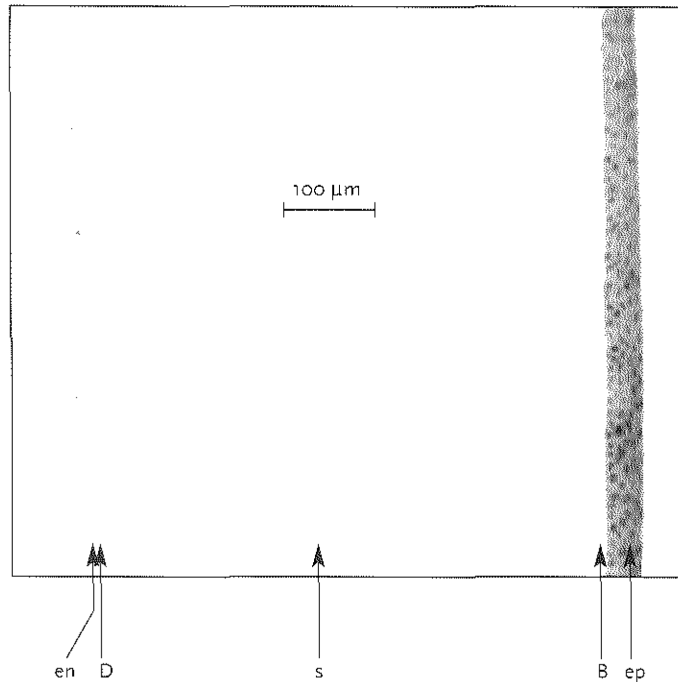

Figure 6:
Photomicrograph of a cross section of a normal human cornea:
ep the epithelium,
B Bowman's layer
s stroma,
D Descemet's membrane
en endothelium

Descemet's membrane is produced by the endothelium and approximately 10μm thick.

The *endothelium* is a monolayer of regularly shaped hexagonal cells, lying posterior on Descemet's membrane. The main function of the corneal endothelium is control of stromal hydration. This is essential for corneal transparency. Endothelial cells generally do not

show mitotic activity. Cell density below a critical number (400-600 cells/mm^2) is devastating for the transparency of the cornea[91].

Sensory innervation of the cornea is supplied by the ophthalmic branch of the trigeminal nerve (ramus I, nervus V). The nerve fiber pattern of the cornea has been described as radially oriented nerve bundles entering the cornea from the sclera at the middle one third of its thickness[91]. The nerves lose their myelin sheath after traversing 0.5-2.0 mm into the cornea and then continue as transparent axon cylinders. After passing Bowman's layer, they ramify and end within the epithelium as free nerve endings[92,93]. The nerve bundles in the subbasal plexus of the human cornea form a regular dense meshwork with equal density over a large central and mid-peripheral area[94] (Fig. 7).

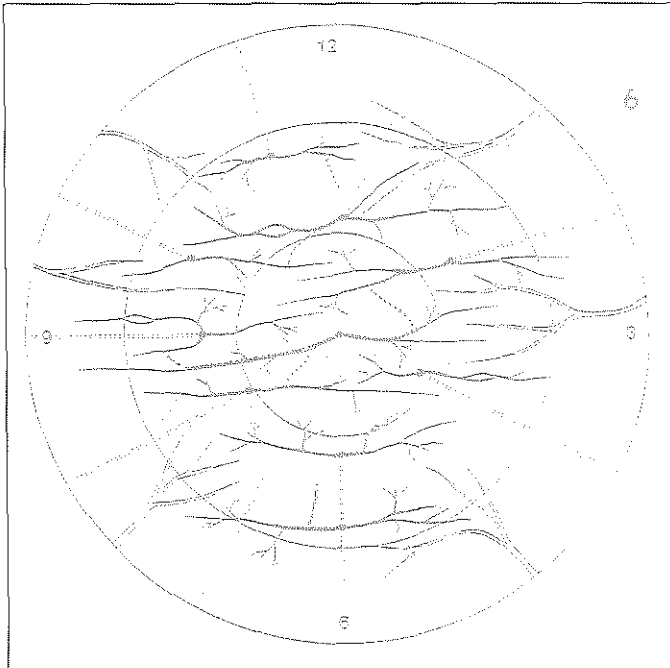

Figure 7:

Nerve fiber bundles in the subbasal plexus run first in the 9-3 hours direction. After the first bifurcation they run in the in the 12-6 hours direction and after the second bifurcation they run again in the 9-3 hours direction. The beaded fibers run singly and obliquely after branching.

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The cornea: an immune privileged site

The cornea is considered to be an immune privileged tissue, in part because it cannot directly be accessed by the immune system[95]: the cornea lacks blood and lymphatic vessels, structures that provide the conduit for transportation of immunologic components into and out of most tissues[96]. The absence of blood vessels leads to sequestration of corneal antigens from the circulation. The normal cornea also lacks professional antigen presenting cells (APCs) such as dendritic cells and macrophages, which are resident in most other tissues[97]. Factors present in the normal cornea that contribute to immune privilege are IL-1 receptor antagonist and Fas ligand. Both are constitutively expressed and promote ocular immune privilege by different pathways[98,99]. The cornea is in close contact with the fluid of the anterior chamber of the eye. Several mechanisms play a role in the immune privilege of the anterior chamber: blood-ocular barrier, the absence of lymphatic drainage from the intraocular structures, deviant host immune response after introduction of antigens (aqueous chamber associated immune deviation: ACAID) and soluble factors in the aqueous humor having a immunosuppressive effect on APC and T cells[100,101].

Although the cornea is an immune privileged site, the complete immune apparatus is present in the iris and at the limbus, a tissue that constitutes the zone between the cornea and surrounding conjunctiva. The conjunctiva has a rich lymphatic network arising approxi-

mately 1 mm peripherally from the limbus. During the course of inflammation Langerhans cells (dendritic cells) migrate from the limbus into the central cornea and the blood-ocular barrier can break down. In chronic inflammation the cornea becomes vascularized and thus the immune privileged state can be lost[96]. Moreover, early in the inflammatory process, leukocytes extravasate from blood vessels in the limbus and migrate towards the center of the cornea.

Viruses causing keratitis

Herpesvirus and adenovirus infections are the most common viral infections of the anterior segment of the eye. Clinical manifestations of herpesvirus infections are summarized in Tables 1 and 2. Infections with herpesviruses present usually in isolated cases, and are generally unilateral. The herpesviruses HSV-1 and -2, VZV and CMV may cause active disease in a wide variety of ocular structures. However, ocular disease is mainly caused by HSV-1 and VZV.

Some viruses show an increased frequency of presentation associated with immunodeficiency syndromes, including molluscum contagiosum virus, CMV,VZV and papilloma viruses. Other viruses, like adeno-, entero- and coxsackieviruses cause epidemic outbreaks of disease.

Some viral infections are becoming exceedingly rare, or have even been eradicated, because of vaccination programs (e.g. smallpox, measles, rubella, mumps), but some still cause significant morbidity in numerous developing countries. Especially the combination of malnutrition and measles, should be mentioned in this context.

HSV keratitis

HSV keratitis ranges from a superficial inflammation of the cornea to a complex ocular disease. It is usually initiated by the cytopathic effect of the virus and followed by inflammatory responses, which may affect all layers of the cornea. The ocular sequelae of HSV infections are determined by the frequency and duration of the recurrent disease episodes and the immunological response elicited. The four main categories of HSV keratitis[102,103] are infectious epithelial keratitis (IEK), neurotrophic keratopathy, herpetic stromal keratitis (HSK), and endotheliitis. Each of these is subdivided into more specific clinical presentations (Fig.8, page 25 and 73)

Infectious epithelial keratitis

The earliest epithelial lesions of HSV infection in the cornea are small vesicles in the epithelium[104], which have also been described as punctate epithelial keratopathy[105]. Vesicles coalesce and a branching, linear lesion with terminal bulbs (swollen epithelial borders) develops, leading to the most common presentation of HSV keratitis: dendritic keratitis. In the borders of the lesion infectious virus may be present. As the disease process continues the center ulcerates and extends through the basement membrane[106]. An enlarged dendritic ulcer, that is no longer linear, is referred to as a geographic ulcer. Another manifestation of IEK is the marginal ulcer. The proximity of the limbus leads to a rapid infiltration of leukocytes and neovascularization. The ulcer may lack the typical dendritic shape[107].

Neurotrophic keratitis

Multiple episodes of herpetic disease can result in structural injury to Bowman's layer and corneal stroma. Early findings include irregularity of corneal surface and lack of normal luster. Punctate epitheliopathy may progress to a persistent epithelial defect, oval shaped with smooth grayish borders[102,103]. Untreated or mistreated lesions may progress

into ulceration, opacification, vascularization, and perforation of the cornea.

Herpetic stromal keratitis

The corneal stroma may be affected as result of a corneal HSV infection through a variety of mechanisms. Stromal keratitis is a more severe and prolonged manifestation of HSV disease and occurs when the virus initiates a chronic corneal inflammation. It can be classified as immune stromal keratitis (ISK) and necrotizing stromal keratitis (NSK)[102,103]. These two manifestations are not mutually exclusive and probably a continuum.

ISK may present as superficial stromal scarring or the so called "ghost scars", deeper located infiltrative stromal keratitis. A rare form of ISK is a white ring shaped stromal lesion or the so-called immune ring. The inflammatory response results in cellular infiltrate, edema, neovascularization and corneal scarring as a result of fibrosis and tissue destruction.

The predominant feature of NSK is necrosis with tissue loss, often masked by edema in the active phase of the inflammation. The clinical picture can evolve rather quickly and is sometimes refractory to treatment. The edema and neovascularization are more pronounced compared to ISK. Corneal scarring often leads to irregular astigmatism which may strongly interfere with vision.

Corneal endotheliitis

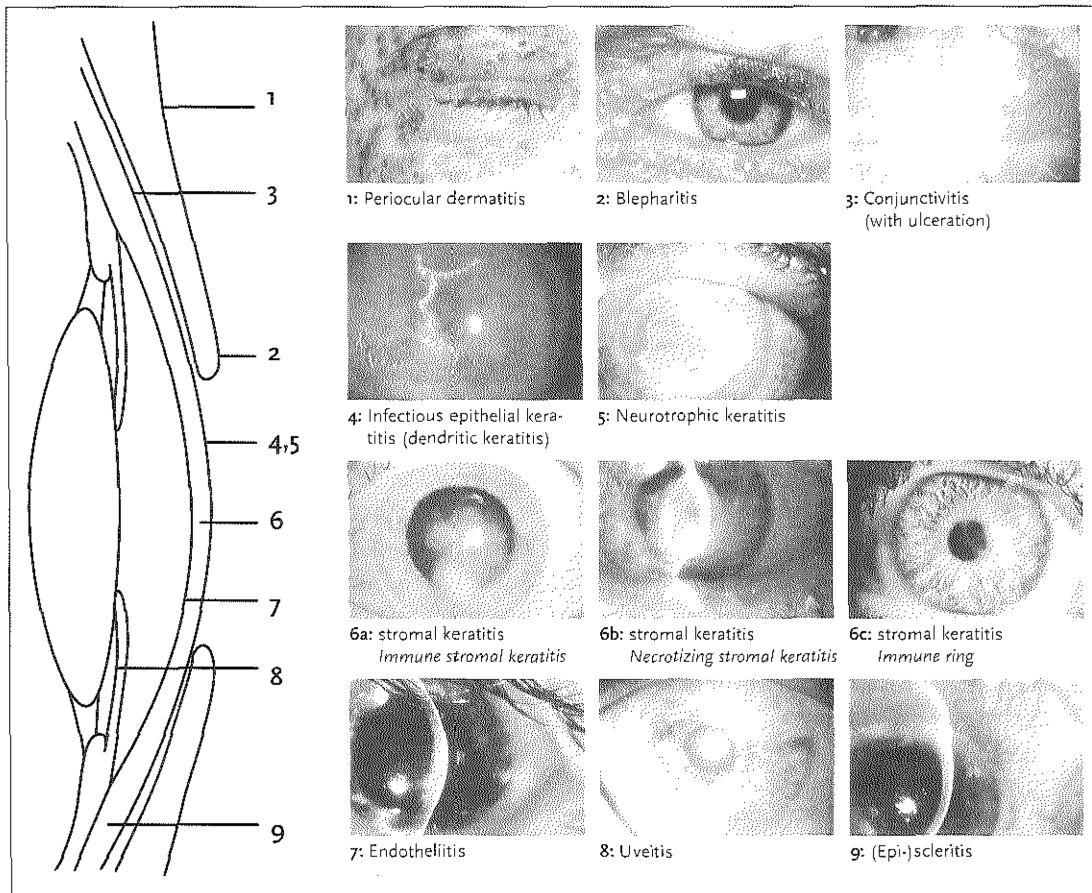
Corneal endotheliitis is an inflammatory reaction at the level of the corneal endothelium. In isolated endotheliitis stromal infiltrate and neovascularization are absent. Patients characteristically have keratic precipitates (KP), stromal and epithelial edema, and mild iritis. HSV endotheliitis can be classified based on the distribution of the KP and the configuration of the overlying stromal and epithelial edema[102]. The most common form is disciform endotheliitis. Diffuse and linear forms of endotheliitis are also observed[108,109]. When inflammation persists or is left untreated, secondary neovascularization and scarring may occur. Chronic endotheliitis may lead to endothelial decompensation.

Herpes keratitis after penetrating keratoplasty

Herpesvirus infection in a corneal graft may present with the whole range of manifestations as in non-operated eyes. Early after transplantation IEK may present as a large epithelial defect without the characteristic dendritic appearance[101]. In addition to this, herpetic keratitis might present with the manifestations of an allograft reaction: from a linear endotheliitis resembling a Khodadoust line[109] to a diffuse endotheliitis, indiscernible from a full blown graft rejection[110].

Table 2: prevalence of clinical manifestation in individuals suffering from ocular HSV infection

Clinical manifestation	Initial clinical disease %	Recurrent disease %
Blepharitis[67,68,111,112]	38-54	4-20
Conjunctivitis[67,68,111,112]	54-84	4-20
Epithelial keratitis (dendritic)[67,68,111,112]	15-63	47-60
Stromal keratitis (ISK+ NSK)[67,68,111,112,113],	2-6	20-48
(Kerato-)uveitis[67,111]	4	1
Acute retinal necrosis[114]	<0.00001	<0.00001
Others	<0.1	<0.1



Full color version: page 73

Figure 8: Presentations of herpes simplex virus infections of the anterior eye segment

Adapted from: R. Sundmacher: A clinico-virologic classification of herpetic anterior segment diseases with special reference to intraocular herpes. In Herpetische Augen Erkrankungen DOG 1980. Eds R. Sundmacher. München: JF Bergmann Verlag 1981: 206

Diagnostic criteria for HSV keratitis and differential diagnosis

Traditionally the diagnosis of HSV keratitis is based on history and clinical presentation, occasionally complemented by viral culture. Viral isolation in cell culture remains the standard for the detection of infectious virus. This technique is not always available in general ophthalmology practice. Viral culture is an insensitive technique, particularly for the isolation of HSV-1 from the deeper layers of the cornea. Cultures are only found positive in herpetic keratitis with an epithelial defect. The polymerase chain reaction (PCR) represents a sensitive research laboratory tool and has the advantage of detecting very small amounts of DNA or RNA. The detection of HSV DNA by PCR provides evidence for the presence of viral DNA, which not necessarily means the presence of infectious virus.

Many lesions may present with a dendritic or dendriform lesion, including the epitheliopathy seen in healing corneas after a dendritic ulcer, a healing corneal abrasion, and the early stages of acanthamoeba keratitis. In ISK many other causative agents of infection are possible. However, 66% of unilateral ISK is caused by HSV[115]. Other viruses causing ISK are VZV, EBV, mumpsvirus, rubellavirus, measlesvirus and influenzavirus. Tuberculosis, lues acanthamoebiasis and onchocerciasis are other infections associated with ISK. The complete differential diagnosis of stromal keratitis is extensive and beyond the scope of this thesis. In severe necrotizing disease, the clinical picture can be suggestive of bacterial keratitis, with ulceration, dense cellular infiltration and necrosis. Differential diagnosis of unilateral corneal edema with a normal endothelium in the opposite eye should bring up angle closure glaucoma and iridocorneal endothelial syndrome. When KPs are the only presenting sign other causes of anterior uveitis are possible.

HSV has been implicated in other diseases of the anterior segment (Fig 8 and table 1). HSV DNA has been detected in the iridocorneal endothelial syndrome[116,117], in the aqueous humor during a period of intra-ocular pressure elevation in Posner-Schlossman syndrome[118] and in idiopathic endotheliopathy[119,120].

Pathogenesis of corneal HSV infection and implications for therapy

Infectious Epithelial Keratitis

IEK results from viral replication in corneal epithelium and destruction of corneal epithelial cells: cytopathic effect of the virus. The duration of viral replication in the cornea depends on viral factors, including viral load, virus strain[121] and host factors like immune status.

The immune response in IEK consists of HSV-specific IgG and IgA present in the tear film and corneal stroma, antibody-dependent cell-mediated cytotoxicity and HLA class I and class II restricted immune responses[122]. In addition to the specific immune reaction, IFN-α/β production by the infected epithelial cells limits the spread of the virus and allows resolution of the lesion.

These dendritic lesions heal without scar formation, when the epithelium is the sole tissue affected. As the disease process continues, the infection can extend through the basement membrane[106], into corneal fibroblast (keratocytes) in the superficial corneal stroma underlying the site of the lesion resulting in HSK[123,124].

In IEK topical application of antivirals accelerates the elimination of replicating virus from the cornea[125]. In this phase of the disease the immune response is directed at limiting the spread of the virus. Interference with this immune response, for instance by corticosteroids, may favor the spread of the virus into the stroma[126].

Neurotrophic keratopathy

HSV-induced neurotrophic lesions are neither infectious nor immunologic in origin. Neurotrophic keratopathy is a multifactorial disease. Impaired corneal innervation in combination with decreased tear secretion produces non-healing epithelial defects. Basement membrane damage and stromal inflammation interfere with normal epithelial wound healing.

The resulting damage should determine the therapeutic approach. Artificial tears in dry eye syndromes, protection of a vulnerable basement membrane by bandage contact lens, or low strength steroids in chronic stromal inflammation. The keratopathy may be exacerbated by the chronic use of topical medications, especially antiviral treatment and toxic antibiotics.

Herpetic stromal keratitis

The permanent loss of vision associated with HSV-1 corneal infection is due to an inflammatory response in the corneal stroma. When the herpesvirus reaches the corneal stroma, viral antigens are presented on stromal cells and a cell mediated immune response is initiated.

- The experimental mouse model of HSK

Current knowledge on the immunopathogenesis of HSK is largely based on studies performed in the experimental mouse model for HSK[127,128]. The mouse develops a non-necrotizing form of HSK that resembles many characteristics of ISK in humans.

In the mouse model, HSK does not develop in the absence of T cells[129,130]. The inflammatory infiltrate in the cornea is dominated by PMN. These PMN produce proteolytic enzymes, resulting in surrounding cell lysis and are probably responsible for most of the tissue damage[124]. However, in most cases of HSV-1 infection the chronic inflammation was found to be regulated by the $CD4^+$ cells[131,132].

Other cell types involved are Langerhans cells and macrophages. Langerhans cells are capable of presenting antigens to $CD4^+$ T cells. They rapidly migrate from the limbus into the central cornea after HSV infection[133,134].

$CD4^+$ T cells mediate their immunological functions through production of interrelated cytokines.

The cytokines IL-2 and IFN-γ are produced in HSV −1 infected mouse corneas[135,136]. In the mouse model there is no IL-4 and IL-10 production during active HSK. Based on the pattern of cytokine production these $CD4^+$ T cells are referred to as Th1 cells. IFN-γ plays a pivotal role in the extravasation of PMN from blood vessels in the peripheral cornea. IL-2 enhances IFN-γ production subsequently inducing the chemotaxis of PMNs to the site of initial infection, and activates the PMN when arrived at the place of infection[137]. PMN are known to secrete several other cytokines with possible antiviral function like IFN-α, and TNF-α and nitric oxide[138]. Production of Th2 cytokines such as IL-10 and IL-4, detected in mouse corneas during the resolution phase, have been shown to inhibit HSK[139].

- Human herpetic stromal keratitis

Data on pathogenesis of human HSK, until recently, were limited to histopathologic studies of corneal specimens removed at corneal transplantation for HSK. The only human specimens of active inflammation are those from necrotizing stromal keratitis. In immune stromal disease transplantation is preferably performed after inflammation has subsided.

Immune Stromal Keratitis

The experimental mouse model shows clinically the most striking resemblance with human ISK. However, in contrast to the murine cornea, corneas of patients with non-necrotizing keratitis do exhibit intra-corneal expression of viral antigens[140,141,145]. It seems

logical that the intra-corneal immune response in human ISK is –at least in part- directed to HSV antigens, definite proof however is still lacking. Studies on T cell involvement in the immunopathogenesis of stromal keratitis are limited to immunohistologic analysis and phenotypic characterization of corneal infiltrating T cells[142-143]. Immune rings are thought to be a ring shaped antigen-antibody-complement precipitate similar to that of a Wessely ring[143]. This immune complex attracts a cellular infiltrate, clinically visible with the resulting edema.

Antiviral treatment in ISK is both directed at the presumed presence of the virus and at prevention of severe reactivation of infection during steroid therapy. Topical antivirals should be used in a therapeutic dose. It has not been established that oral antiviral therapy is effective in acute stromal disease. However, visual acuity improved over 6 months in significantly more patients[144]. The main indication for oral acyclovir in ISK is prevention of recurrent disease by a long-term low dose of acyclovir[145].

Steroid therapy in stromal keratitis reduces the risk of persistent or progressive stromal keratitis by 68%, and leads to a significant shorter time to resolution[146]. Steroid dosage depends on the severity of disease. In corneal disease it is rarely necessary to use dosages exceeding dexamethasone 0.1% six times a day. When edema and hyperemia are resolving tapering can be started slowly.

Necrotizing Stromal Keratitis

In NSK cellular destruction is the result of both active viral replication in the corneal stroma and a severe immune reaction[147]. Viral profiles (inclusion bodies, particles, DNA) and viral antigens are detected frequently (up to 91%) in corneal buttons of NSK patients[148]. Cellular infiltrate consists predominantly of PMNs and mononuclear cells, in combination with a granulomatous reaction[148].

Both the viral replication and the severe immune response should be treated vigorously. Control of HSV replication should precede attempts to modulate the immune response. Both topical and systemic antivirals should be used in high therapeutic doses, combined after two days with corticosteroid therapy[149].

■ Corneal endotheliitis.

Endotheliitis probably results from lytic viral infection of the endothelium[108, 148,150] with release of infectious virus into the aqueous[151,152,153]. In addition to lytic infection of endothelial cells, viral antigens may be expressed on the surface of infected cells. Immunecompetent cells may attack the endothelium, leading to enhanced damage. This process is probably analogous to the cellular immune response in ISK. Histopathologic studies in patients with acute endotheliitis are missing. The immune response is deleterious to the eye because the endothelium has no regenerative capacity. Endothelial dysfunction leads to stromal and epithelial edema.

Treatment is similar to immune stromal keratitis.

Scope of the thesis

The aim of this thesis is to elucidate pathogenic mechanisms of different forms of human HSV keratitis. HSV infection of the corneal epithelium causes a classical dendritic shaped lesion. Many studies could explain the development and growth in dendritic keratitis, but none of these found the anatomical substrate for the linear branching pattern. The most obvious explanation would be, that the shape of dendritic ulcers corresponds with the anatomical pattern of innervating nerves of the cornea. In *chapter 2* a relationship between the shape of dendritic ulcers in infectious epithelial keratitis and the subbasal nerve plexus of the corneal epithelium is postulated.

Recurrence of HSV keratitis is a common complication after PKP for corneal opacities resulting from HSV infection. After PKP, for reasons unrelated to HSV keratitis, epithelial defects may still be caused by HSV. In *chapter 3* the incidence of newly acquired HSV keratitis after PKP is determined and possible contributing factors are assessed. Several possibilities as to the origin of the infecting HSV exist. These include reactivation of latent virus in the trigeminal ganglion, horizontal spread, or transmission through the donor cornea. To test the assumption of graft–to-host transmission of HSV by PKP, surplus corneal material was examined for the presence of HSV DNA. Because the amount of viral DNA available could be very limited, a new method independent of viral culture, was developed to allow distinction between different virus strains. The newly developed technique was used to test our hypothesis that graft–to-host transmission of HSV is possible. This new method was used to determine the incidence of HSV-1 superinfection in patients with recurrent HSV keratitis.

Although HSK has been studied extensively in the mouse model, it is not clear what triggers the immune response and to what extent the mouse data correlate with findings in human keratitis. The most logical idea, that virus-derived proteins are the eliciting factor for the immune response, has been ruled out in the experimental HSK mouse model. Alternative sources of the keratogenic antigens, like auto-antigens, have been suggested. Data on the pathogenesis of human HSK are limited. Therefore, in *chapter 4* the antigen-specificity of corneal T cells in HSK patients was investigated. Besides this, corneas of patients with HSK were examined for the presence of corneal antigen reactive T cells (auto-reactive T cells).

Chapter 5 provides a concise summary of the data generated in the framework of this thesis, and concludes with an overall discussion of the data and their possible impact on current ophthalmologic practice.

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CHAPTER 2

The Dendritic Pattern in Epithelial Herpes Simplex Virus Keratitis

A Hypothesis for the Dendritic Pattern of Epithelial Herpes Simplex Virus Keratitis

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Submitted

Summary

Purpose:

Herpetic epithelial keratitis (HEK) is a common sequel of a corneal infection with herpes simplex virus (HSV). Classical HEK lesions have a dendritic shape.

The objective in this study was to test the hypothesis that the subbasal nerve plexus of the corneal epithelium is responsible for the shape of dendritic herpes simplex virus keratitis.

Methods:

A total of 114 patients with recurrent HEK (rHEK) were included. Eighty-five eyes developed rHEK without previous corneal surgery (group I) and 29 eyes developed rHEK after penetrating keratoplasty (PKP) performed for corneal opacities resulting from HSV keratitis.

Branching patterns and orientation characteristics of the dendritic lesion in the corneal epithelium were determined with the use of slitlamp photographs. For each dendrite, all branching angles were measured and the orientation on the cornea was determined.

Results:

In both groups a preference for branching angles near 90° and 135° is found. The orientation (main angle) of the dendritic lesion in group 1 has a preference for either a vertical or a horizontal distribution on the cornea. Group 2 does not show a preference for any particular orientation on the cornea.

Conclusions:

The branching pattern and the orientation of the dendritic lesion in rHEK in not operated eyes correlated with the anatomy of the subbasal nerve plexus in the human corneal epithelium. After PKP the branching pattern of the dendritic lesions is the same as in eyes without previous corneal surgery, however the vertical or horizontal orientation on the donor cornea is lost. This might be explained by the random placement of the donor button during transplantation.

Introduction

The neurotropic herpes simplex virus (HSV) is a frequent cause of corneal disease that may lead to corneal opacification requiring penetrating keratoplasty (PKP).[1] Herpes simplex epithelial keratitis (HEK) has a classical dendritic shape. The underlying pathogenesis of this characteristic lesion has to our knowledge never been elucidated. Knowledge of the underlying morphogenesis of the dendrite will increase understanding of the mechanisms of dendritic lesion formation and viral spread in the cornea.

The morphological features of epithelial HSV infection are – in part – due to contiguous cell-to-cell spread of the virus in the corneal epithelium restricted only by local immune surveillance[2,3]. This "contiguous cell-to-cell spread" does, however, not explain the branching pattern of the dendritic corneal lesion. One would expect a similar growth pattern in HEK as in bacterial keratitis: an almost circular corneal lesion would develop. The affinity of herpes simplex virus for neural tissues has led to the idea that the shape of the epithelial dendritic lesions might correspond with the anatomical pattern of the corneal nerves: in 1919, Vogt reported abnormally pronounced corneal nerves in dendritic keratitis[4]. However, several studies have failed to demonstrate a correlation between the anatomical pattern of the corneal nerves and the pattern of dendrites in HEK.[2,3,5,6]. The nerve fiber pattern of the mammalian cornea has been described as radially oriented nerve fibers entering the

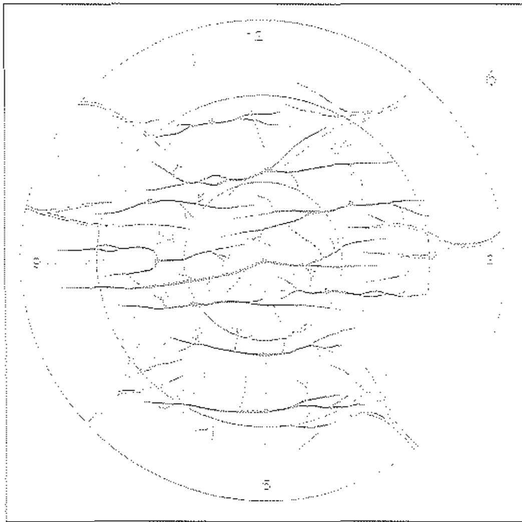

Figure 1a:
Nerve fiber bundles in the subbasal plexus run first in the 9-3 hours direction. After the first bifurcation they run in the in the 12-6 hours direction and after the second bifurcation they run again in the 9-3 hours direction. The beaded fibers run singly and obliquely after branching.

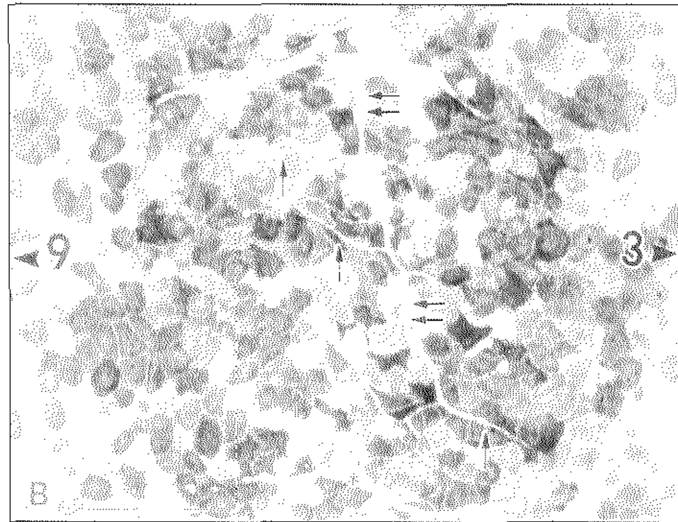

Figure 1b:
Light micrograph of nerve fiber bundles and their side branches in the subbasal plexus of a semi thin 1 μm section. Bar = 0.05mm, the numbers 9 and 3 represent the 9 and 3 o'clock position. * = Main nerve fiber bundle running horizontally oriented (Fig. 1a and 1b both reprinted with permission from: Müller LJ, Vrensen GFJM, Pels L, Nunes Cardozo B, Willekens B. Architecture of human corneal nerves. Invest Ophthalmol Vis Sci 1997;38:985-994)

corneal stroma via the sclera. After passing Bowman's layer in the periphery of the cornea they ramify in the subbasal plexus as leashes[7,8,9].

Nowadays it is known by the work of Müller et al.[10] that the nerve fiber bundles of the human cornea form a regular meshwork in the subbasal plexus, running between the corneal epithelial basal cell layer and Bowman's layer. (Fig. 1a and b). Single fibers turn upwards into the upper layers of the corneal epithelium[10].

The aim of the present study was to investigate the possible correlation between the orientation and branching pattern of human HEK dendritic lesion and the anatomy of the subbasal nerve plexus of the human corneal epithelium.

Patients and methods

Patients

To determine whether the branching pattern and the orientation of the dendritic HSV lesions are congruent we examined dendritic and geographical lesions in the corneal epithelium. One hundred fourteen patients with rHEK were included. After informed consent was obtained, photographs were taken with a Zeiss slitlampcamera (Oberkochen, Germany) and fluorescein staining with blue light and /or Rose Bengal staining with white light. Patients were divided into two groups: group 1 consisted of patients who developed rHEK without previous corneal surgery and group 2 consisted of patients with rHEK in their donor corneas after PKP.

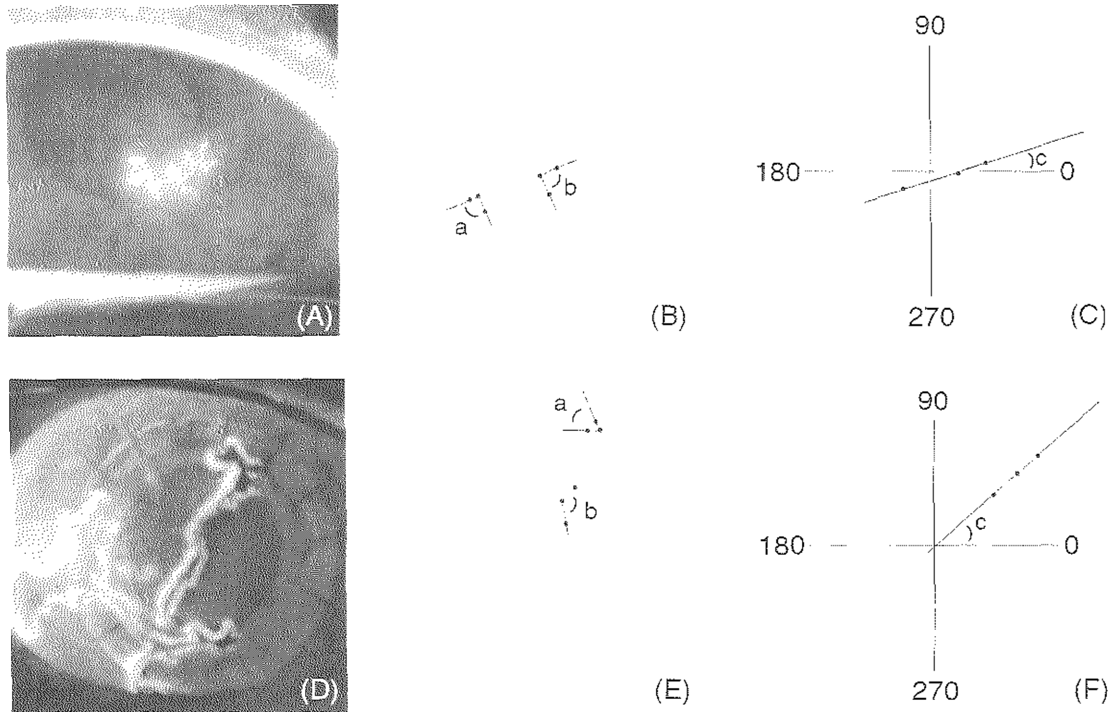

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Figure 2 Measurement of the branching angles and the main angle in a dendritic lesion.
Examples of a dendritic lesion on a non-operated cornea (2a) and on a cornea after PKP (2b).

2A + D: the original dendritic lesion.

2B + E: branching angles: points were drawn in the middle of all branches of the dendritic lesion. Lines were drawn through straight parts of the dendritic lesions using these points The angles between two consecutive parts of the dendritic lesion were measured as a branching angle (angle a and b). (Not all branching angles of this dendritic lesion are drawn in this figure for reasons of clarity)

2C + F: main angle: points (shown only in the longest branch) are drawn in the middle of all branches of the dendritic lesion. The connecting line using most points was considered the best fitting or direction line. Angle c is measured with the use of the horizontal axis.

Inclusion criteria:

Only patients with epithelial herpetic keratitis were included in this study. The diagnosis of HEK was based on ocular examination and clinical history, complemented with viral culture after photography (data not shown). Only one active HEK period per patient was allowed in this study.

Exclusion criteria:

Photographs with subepithelial fluorescein leakage, were not taken into consideration. Very small dendrites were excluded because the line of orientation and branching angles could not be determined accurately (group 1: n=9; group2: n=3). Dendritic lesions in the recipient cornea of group 2 were excluded (n=4). The present study was performed according the Declarations of Helsinki.

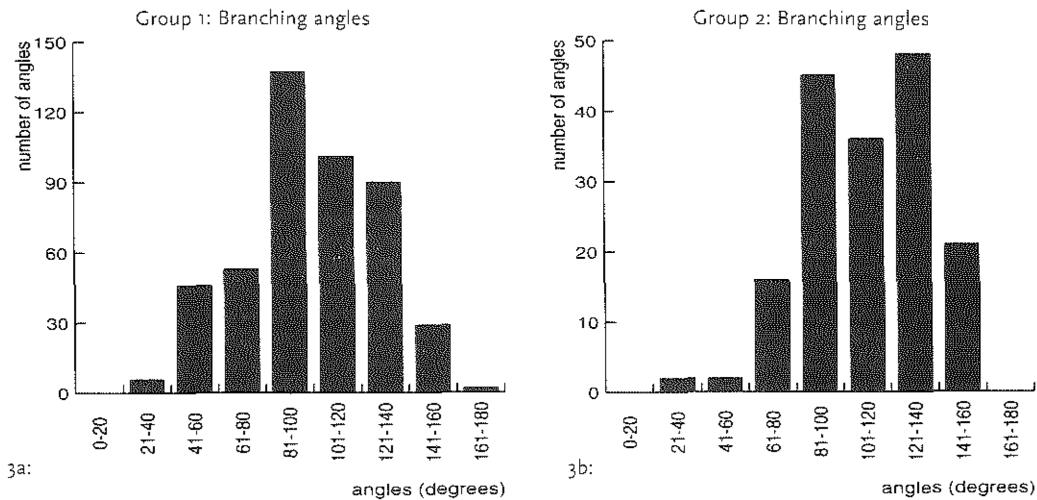

Figure 3 Frequency distribution of the branching angles.

Horizontal axes: branching angles in degrees divided in groups of 20°.

Vertical axes: number of measured branching angles.

3a: Group 1 (non-operated corneas). n=85 patients.

3b: Group 2 (after PKP). n=29 patients.

Measurement of the main and branching angles of human HEK dendrites

All color transparencies of HEK dendritic lesions were projected onto paper. The borders of the dendritic lesion, the corneal-limbal borders, the donor-recipient interface and the 12 o'clock position of the cornea were traced on the same paper. From these drawings the branching angles and the main angle were measured.

Measurement of branching angles:

To determine the branching angles, lines were drawn through at least two points placed in the middle of a straight part of distinctive and well-demarcated branches of the dendritic lesion (Fig. 2B +E, angles a and b). The angle (for instance a or b) between two consecutive direction lines was measured. All measurable branching angles of the dendritic lesion were determined.

Measurement of main angles in human HEK

The orientation of the dendritic lesion on the cornea was determined as illustrated in figure 2C+F. Points were placed in the middle of the location where a side-branch forks off. Connecting lines were drawn through these points. The straight line going through most points was accepted as best fitting line, the direction-line. The main angle is the angle between the direction line and the 0ffl line of a superimposed orthogonal grid (Fig. 2C + F, angle c). To determine whether a horizontal or vertical preference in orientation is present the relative main angle, the smallest angle between the 0-180ffl axis and the main line of orientation the dendritic lesion, was determined. In case of dendritic lesions with multiple elongated branches, the direction-line through the longest branch was used to determine the main angle.

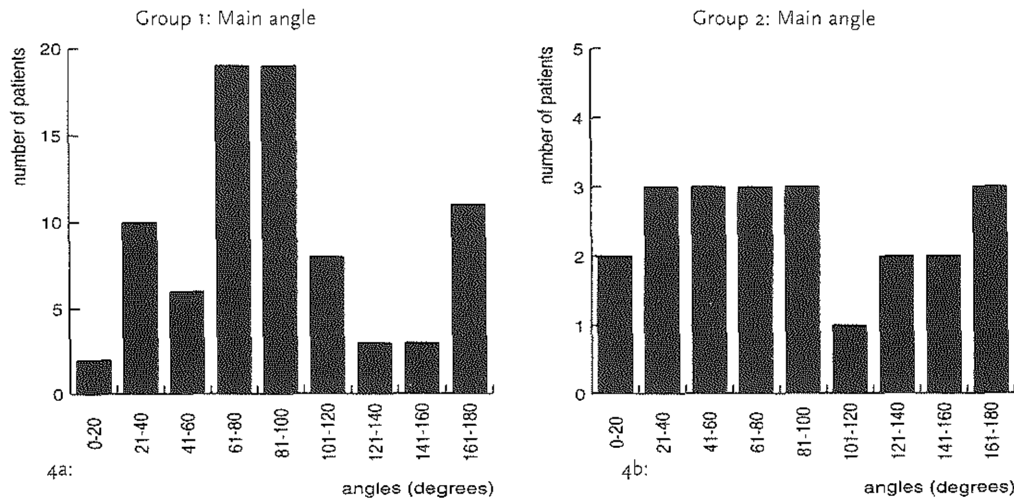

Figure 4 Frequency distribution of the main angles.

Horizontal axes: main angles of the dendritic lesions in degrees divided in groups of 20°.

Vertical axes: number of patients.

4a: Group 1 (non-operated corneas). n=85 patients.

4b: Group 2 (after PKP). n=29 patients.

Statistical Analyses:

The Mann-Whitney test was used to test against a difference in the prevalence rate of branching angles between the two groups studied. Because in group 2 the donor corneal button is placed randomly rotated in the recipient, it is hypothesized that all angles are equally likely (i.e. no preference for certain angles). The exact Pearson chi-square test is used to test the null hypothesis that the two angle distributions (without specifying these distributions) are the same in both groups. The chi-square goodness-of-fit test was used to test how well a hypothesized uniform distribution of the main angle fits to the observed data.

Results

In total 114 patients with recurrent HEK were included of which 85 had not undergone surgery (group 1) and 29 had undergone a PKP procedure (group 2).

Branching angles of human HEK dendritic lesions

In both groups there was a preference for branching angles near 90° and 135° (Fig. 3a and b).

In group 1 the 90° and 135° bars account for 88% (56% and 32% respectively) of all measured angles. In group 2 this is 94% (42% and 52% respectively). There is a significant difference in preference of group 1 for the angles near 90° and of group 2 for the angles near 135° (Mann-Whitney test: p= 0.028 and p= 0.003 respectively, Fig. 3A and B).

Main angles of human HEK dendritic lesions

In group 1 the angles of orientation are not distributed uniformly. There is a preference for main angles around 90ffl and 180ffl (chi-square goodness-of-fit test: p<0.0005. Fig. 4a) Group 2 does not show a preference for any particular main angle as can be deduced from the uniform distribution of the orientation of the main angles (chi-square goodness-of-fit test: p=0.989, Fig. 4b). A comparison of the angle distributions (without specifying these distributions) in both groups using the ordinary exact Pearson chi-square test yields a p-value of 0.285, Indicating the significant difference in orientation of the dendritic lesion between the two groups.

For a direct comparison of the two groups in order to test the latter hypothesis, group 2 is too small to have sufficient power.

Discussion

HSV-1 needs a neuronal substrate to travel from the trigeminal ganglion to the cornea and cause rHEK[11,12]. To our knowledge, this is the first study to show a positive congruency between the anatomy of the subbasal nerve plexus of the corneal epithelium and the pattern of the dendritic lesions in patients with HSV-1-induced rHEK.

The current model for rHEK is: virus particles reach the cornea by the sensory nerve fibers and the dendritic lesions develops within the corneal epithelium by contiguous cell-to-cell spread[2,3]. It is suggested that the initial virus targets are the deeper epithelial layers[13]. Disruption of the epithelium through this mechanism alone, would lead to a situation similar to bacterial keratitis, leading to the same round or oval shaped lesions. The previous studies, however, do not explain the branching pattern of the dendritic lesion adequately. Mechanical factors as blinking or pooling of infectious virus in the tear meniscus could be responsible for vertical or horizontal lesions on the cornea. However in other infectious processes of the cornea linear branching lesions are not found, indicating a different underlying pathogenesis in epithelial HSV keratitis.

Until recently the corneal epithelial innervation have been described as radial oriented nerve bundles, ramifying as leashes in the subbasal epithelial nerve plexus[7,8,9]. In 1997 Müller et al. described the subbasal nerve plexus of the corneal epithelium as a regular meshwork with mainly perpendicular and oblique angles (45° or 135°).[10].The epithelial subbasal nerve plexus of the human cornea is unique in the animal world.[10,14].

Our results show that the branching angles of dendritic lesions have a preference for angles near 90° and 135° in both groups (Fig 3). Thus, the subbasal plexus of the corneal epithelium might indeed be the anatomic structure responsible for the characteristic form of the dendritic lesion in recurrent HEK.

Our hypothesis of distribution of reactivated HSV to the epithelial cells is illustrated in figure 5: The reactivated HSV travels from the ganglion along the sensory nerves, through the epithelial subbasal nerve plexus, to the end fibers that reach up into the corneal epithelial wing cells. As a result of this, only wing cells above a nerve fiber will become infected. By contiguous cell-to-cell spread HSV will extend to the surface epithelium. When the spheres of infected cells around the tip of a nerve touch each other, they fuse and form a linear lesion, branching off dependent on the pattern of the underlying subbasal nerve plexus.

Consequently, the spheres around the last nerve endings also explain the phenomenon of end bulbs in dendritic keratitis. Our hypothesis of distribution is confirmed by the clinical description of the evolution of a dendritic lesion [13,15].

The fractal properties attributed to HEK are, to our opinion, not caused by the growth pattern of the virus or the corneal epithelium, but by the underlying subbasal nerve plexus[16,17]. The differences found in fractal properties between early dendritic lesions and amoeboid shaped dendritic lesions can be explained by the cell-to-cell viral spread, leading to a more geographical or amoeboid shaped corneal lesion. When the size of the outlines of a dendritic lesion increases or in other words the longer the corneal epithelium is involved, the fractal properties of the dendritic lesion will diminish[17].

The results of main angle measurements show a discrepancy between the transplanted and non-transplanted eyes (Fig 4). Group 2 (post-PKP in donor cornea) does not have a preference for any main angle, indicating that the orientation of dendritic lesion can be found in every direction on a donor cornea after PKP. This can be explained by the fact that the original 12 o'clock position of the donor corneal button has been lost when it is placed randomly in the recipient during the PKP procedure. The finding, that dendritic lesions are similar in form after PKP, but merely rotated, suggests the same grid-like anatomy of the subbasal plexus in the donor cornea after PKP. This could have implications for concepts about reinnervation after PKP. After enucleation nerve fibers in the cornea degenerate within 10 hrs[10]. Degenerated nerves or other (glia) tissue in the subbasal plexus may remain at their location and are perhaps able to facilitate the ingrowth of new nerves along the previous paths of the old subbasal plexus. Baum[5] has shown that a few days after PKP a dendritic lesion can be seen on a rabbit cornea. It is important to note that this study used a method of external inoculation of HSV-1 after PKP and did not induce reactivation of herpetic keratitis.

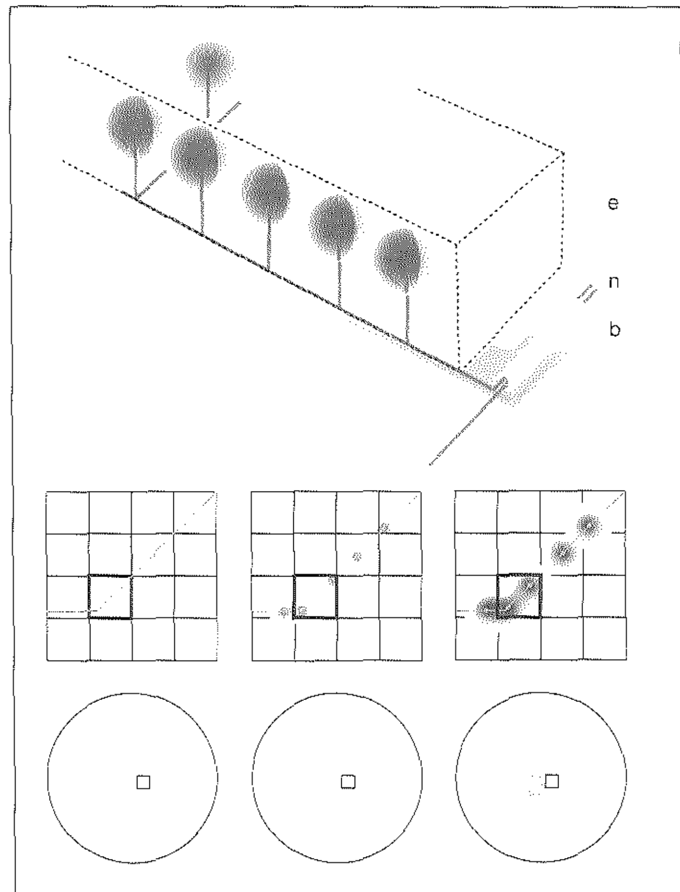

Figure 5: Hypothesis on the spread of reactivated HSV into epithelial cells:

Top: schematic three-dimensional drawing of corneal epithelium and subbasal plexus. The herpes simplex virus reaches the corneal epithelium through the subbasal plexus with its terminals at the location of the corneal epithelial wing cells. Spheres are drawn as a gradient to show the spread of herpes simplex virus into the corneal epithelium from cell to cell from the tip of the nerve fiber.

e= epithelium, n= nerve fibers, b= Bowmans' layer

Middle: frontal view: schematic drawing of the distribution of the herpes simplex virus in the corneal epithelium. Grey oblique line: nerve fiber of subbasal plexus underlying the corneal epithelial cells. The dots represent the nerve fiber endings in the wing cells of the epithelium.

Middle and bottom: From left to right: 3 consecutive stages in the development of a dendritic lesion. Left: no pathology. Middle: early dendritic lesion (dark cells). Right: complete dendritic lesion.

Confocal microscopy indicates that reinnervation of the central basal epithelium occurs within 2 years after PKP.[18] We observed HSV dendrites in the central part of the donor button after a mean time of 31 months, but also within the first year after PKP (range: 1-192 months, data not shown). At this moment, however, little is known about the reinnervation of the corneal epithelium shortly after PKP[18,19,20].

The difference in branching angles between non-transplanted (preference 90°) and transplanted eyes (preference 135°) may be caused by specific preservation of parts of the subbasal plexus after transplantation.

This study is the first to provide indirect evidence that the subbasal nerve plexus of the corneal epithelium is responsible for the dendritic pattern in HSV keratitis. The relation between the HEK and the subbasal nerve plexus explains the morphological features, the viral spread and the fractal properties, and is confirmed by the clinical description of the evolution of a dendritic lesion.

In vivo confocal microscopy might substantiate our hypothesis on the relation between the herpetic dendritic tree and the subbasal nerve plexus. Yet, there might be problems visualizing the subbasal nerve plexus by confocal microscopy in active HEK. Because it is known that sensitivity decreases during each episode of herpetic keratitis, structural damage of the subbasal nerve plexus might occur during or after infection. The original nerve plexus might be changed after an infection episode.

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Chapter 3

Pathogenetic Mechanisms of Herpetic Keratitis occurring after Penetrating Keratoplasty

Newly Acquired Herpes Simplex Virus Keratitis after Penetrating Keratoplasty

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Summary

Background:

After penetrating keratoplasty for reasons unrelated to herpes simplex virus (HSV) keratitis, any nonspecific epithelial defect may still be caused by HSV. The purpose of this study is to determine the incidence of *newly acquired herpetic keratitis* and to assess contributing factors.

Methods:

The authors retrospectively studied the results of 2398 penetrating keratoplasties performed between 1980 and 1995. Three typical case histories are discussed.

Results:

Of 2112 patients in whom the primary diagnosis was not related to HSV keratitis, 18 presented with epithelial herpetic keratitis in their corneal graft. The incidence of newly acquired herpetic keratitis after penetrating keratoplasty was 1.2 per 1000 person-years. In most cases, the infection occurred in the first 2 years after the transplantation. Most often, well-known reactivating stimuli could have caused the HSV infection.

Conclusions:

Herpes simplex virus keratitis may develop after penetrating keratoplasty even without a clinical history of HSV in the host. Thus, HSV should be considered in the differential diagnosis of a postpenetrating keratoplasty epithelial defect. The high incidence of this infection in the first 2 years after such surgery suggests a causal relation between corneal transplantation and the HSV infection.

Introduction

Herpetic keratitis may recur after penetrating keratoplasty (PKP) performed for herpes simplex virus (HSV)-related corneal disease. After PKP for reasons unrelated to HSV, however, herpetic keratitis may not be readily suspected.

The HSV keratitis after PKP performed for any reason may present as a classic dendritic keratitis (Fig 1) or as nonspecific large epithelial defects (Fig 2).[1,2] Either of these may occur together with an allograft reaction.[3] To our knowledge, only seven patients with *newly acquired HSV keratitis after PKP* have been described,[1-4] which may suggest that its incidence rate is as low as in the normal population. Nevertheless, as much as 95% of the population older than 60 years of age is seropositive for HSV type 1 (HSV-1)[5] and carries the virus in a latent phase in the sensory ganglia. A wide variety of stimuli have been found to be able to stimulate recurrence, including febrile illnesses, stress, menstruation, sunlight, heat, and trauma. Any local trauma can reactivate the virus and cause a manifest infection, including penetrating keratoplasty combined with postoperative topical steroid medication and suture removal[6,7]. Immune reactions also are considered as reactivating stimuli.[3] We would therefore expect the incidence of newly acquired HSV keratitis after PKP to be higher than in the normal population. The incidence of new cases of HSV keratitis in the normal population is 8.4 per 100,000 person-years.[8] Because there are, to our knowledge, no data on the actual incidence of newly acquired HSV keratitis after PKP, we currently have tried to assess it by reviewing the medical records of our patients who had transplantations between January 1980 and January 1995. We also have tried to distinguish any possible reactivating stimuli.

Patients and Methods

We retrospectively examined the medical records of 2398 patients who had transplantations performed between January 1980 and January 1995. Of these, 2112 transplantations (88.1%) had been performed for various corneal diseases unrelated to HSV. Of this group, 18 patients later presented with an HSV keratitis, all of which were epithelial. The clinical data were scored and are presented in Table l. Only one patient (Table 1, patient 10) had a history of herpetic infections, which, in her case, were fever blisters.

In the total group of 2112 transplants, 229 whole globes stored in a moist chamber at 4°C were used for lamellar keratoplasty; for PKP, 359 corneas had been in short-term storage in McCarey-Kaufman medium and another 1524 corneas stored in organ culture at 34°C in minimal essential medium. Of the 18 newly acquired HSV after PKP, 3 corneas were stored in McCarey-Kaufman medium, 14 in minimum essential medium, and 1 in a moist chamber. Possible causes of endogenous reactivation in these cases have been listed in Table 2. Three cases are discussed to show how varied the signs of newly acquired HSV keratitis after PKP may be.

Case Reports

Case l.

A 53-year-old woman (Table 1, patient 10) with bilateral keratoconus underwent PKP in her right eye in April 1988. Her corneal epithelium was intact for the first 4 postoperative weeks. She then presented with a large epithelial defect and a small infiltrate (Fig 3). Viral cultures from the cornea turned out to be positive for HSV-1. She was using topical dexamethasone 0.1% six times daily. As a result of frequently recurrent episodes of herpetic keratitis with stromal loss, 3 years later a conjunctival flap was deemed necessary to prevent perforation. Almost 2 years later, a repeat PKP was performed.

Case 2.

A 73-year-old man (Table 1, patient 13) underwent a PKP in June 1991 for corneal scarring secondary to a penetrating trauma in his right eye. Five months after surgery, while the patient was receiving topical dexamethasone 0.1% twice daily, an epithelial defect developed with marked stromal infiltration and localized endothelial precipitates (Fig 4). Topical treatment with tobramycin and atropine 1% was unsuccessful. We increased the dexamethasone 0.1% to six times daily to prevent an allograft reaction. Bacterial cultures were negative. Five months later he underwent a repeat PKP. Histologic examination results of the removed corneal button showed an active inflammatory infiltrate, predominantly lymphocytic. No micro-organisms could be shown on light microscopy. Three months later he returned with localized stromal edema, an endothelial *Khodadoust* line, and a central dendriform herpetic keratitis. The topical medication used at that time was topical dexamethasone 0.1% twice daily.

Case 3.

A 28-year-old man (Table 1, patient 16) with congenital glaucoma underwent a third PKP in 1992 in his right eye. Immediately after surgery, the epithelium was absent and showed no recovery despite additional treatment with bandage contact lenses and frequent lubrication with autologous serum.[9] He was using prednisolone 0.5% eye-drops eight times daily. Three months after surgery, viral cultures finally were positive for HSV-1.

Results

Of the 2112 corneal transplants carried out for reasons unrelated to HSV keratitis, herpetic keratitis all of which were epithelial, developed in the graft of 18. Their clinical data are presented in Table l. The interval between the

transplantation and the onset of the keratitis varied from 1 day to 13 years. However, as many as 11 patients (61%) 1 had their onset within 1 year and another 4 patients (22%) within the next year. Taken together, the incidence of newly acquired HSV keratitis after PKP was 1.2 per 1000 person-years.

In 13 patients, the infection was characterized by a dendritic keratitis, 8 of which had positive cultures for HSV-1. In another 5 patients, the keratitis presented as a non-healing epithelial defect or as a non-specific ulcer. All these cases were HSV-1 culture proven. In three patients, the epithelial keratitis was associated with a graft rejection: the eye was red, there was localized stromal edema, and there were endothelial precipitates predominantly on the graft. We could not relate either the primary diagnosis calling for a PKP or the method the donor corneas had been stored to the occurrence of the newly acquired HSV keratitis. The newly acquired HSV keratitis occurred in 14 cases during an episode of infection while the patient used dexamethasone 0.1% twice daily or less. In another seven cases, doses of four to six times daily of dexamethasone 0.1% were used. So in most cases, steroids could have been the reactivating stimulus.

In this patient group, the surgical trauma, suture removal, steroids, and immune reactions could have been the reactivating stimuli (Table 2). In 16 of 18 patients, 1 of the mentioned stimuli could play a role in the occurrence of the HSV infection. Thus, in 16 of 18 cases (88.9%), the presentation could be the result of endogenous reactivation.

Discussion

In this retrospective study (1980 to 1995) of 2112 corneal transplantations for reasons unrelated to HSV, we identified 18 cases of newly acquired epithelial herpetic keratitis after PKP. In the literature, only seven similar cases have been reported, which may suggest that its incidence rate is as low as that in the normal population.

We found an incidence of 1.2 per 1000 person-years, which is 14.2 times the incidence of herpetic keratitis in the normal population (95% confidence interval, 8.2 to 21.07).[8] We may have underestimated the actual incidence of newly acquired HSV keratitis after PKP, because we focused primarily on epithelial defects, whereas a herpes infection in a corneal graft may also present as a keratouveitis, resembling an allograft reaction.[1-4,10,11]. In addition, an herpetic endotheliitis resembling the Khodadoust line in allograft reactions has been recognized in normal corneas.[12]

Interestingly, the highest incidence of newly acquired HSV keratitis after PKP occurred in the first 2 years after the surgery, which suggests a relation between the corneal transplantation and the presentation of the infection. It remains unclear, however, what actually triggers the reactivation of the infection. Also, it remains unsettled where, the HSV originates. Insight into these two questions might lead to better prevention of the infection. Several possibilities as to the origin of the virus exist, such as reactivation of the latent virus in the trigeminal ganglia, viral transmission through tear-shedding, viral transmission through donor corneas, and viral growth in the storage medium.

Endogenous reactivation of latent virus in the trigeminal ganglion is the most important cause of active herpetic keratitis.[13-15] Probably, reactivating stimuli related to the corneal transplantation played a role in our patients.

In this patient group, the surgical trauma, suture removal, steroids, and immune reactions could have been the reactivating stimuli (Table 2). Ocular surgery, per se, appears to be a fairly weak stimulus to reactivate HSV,

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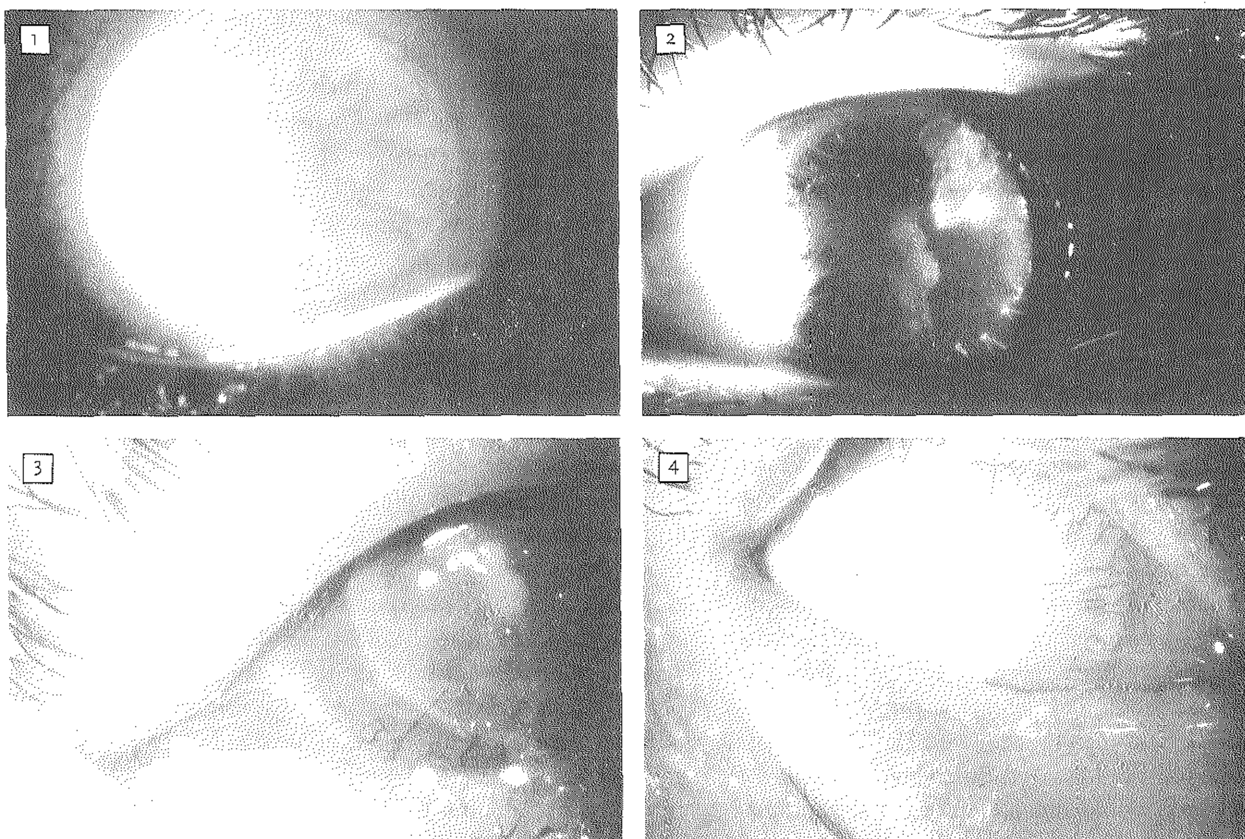

Figure 1
Classic herpes simplex viral keratitis in donor cornea (Table 1, patient 2).

Figure 2
Nonspecific epithelial defects and superficial stromal scarring in a patient who had transplantation for keratoconus (Table 1, patient 15).

Figure 3
Large epithelial defects small stromal infiltrate with positive culture for HVS type 1, moth after PKP for keratoconus (Table 1, patient 10).

Figure 4
Stromal infiltrate (Table 1, patient 13) with negative bacterial cultures. Three months after repeat PKP, the patient showed a beginning allograft rejection and a classic central dendriform herpetic keratitis.

Table 1
HSV = herpes simplex virus; TBC = tuberculosis; assoc = associated; PKP = penetrating keratoplasty; dex= dexamethasone phosphate 0.1%; fml = fluorometholone 0.1%; predn= prednisolone sodium phosphate 0.5%; pf = prednisolone acetate 1%; albicort pb= triamcinolone acetonide 40mg peribulbar; LKP = lamellar keratoplasty; nd= not done

* Storage method: MK= McCarey-Kaufman medium; MCh= moist chamber 4C; MEM= minimal essential medium
** Presentation time until: a, first PKP; or b, second PKP

Table 1. Clinical Data of Patients with Newly Acquired Herpes Simplex Virus Keratitis

Case No. Gender Age (yrs)	Year of Operation	Storage*	Primary Diagnosis	Time until presentation of HSV (mos)**	Graft Rejection	Use of Steroids	First Signs	Culture
1, F, 59	1980	MK	Lattice dystrophy	156	–	none	Recurrent dendrtic ulcer on donor	+
2, F, 74	1980	MK	Reis Bueckler dystrophy interstial keratitis	9	–	fml 3 albicort pb	Central dendrites on donor	nd
3, M, 70	1984	MK	Pseudophakic bullous keratopathy	a, 0 RePKP	–	dex 6	Central infiltrate in donor	–
				b,18	–	dex 1	Large ulcer on donor	+
4, M, 39	1985	MCh	Lattice dystrophy	99	–	none	Recurrent dendrites on LKP	–
5, F, 53	1985	MEM	Interstitial keratitis assoc TBC	14	–	dex 1	Recurrent dendrite on interface	nd
6, M, 75	1986	MEM	Interstitial keratitis assoc measles	51	–	dex 1	Dendrite on donor	+
7, M, 73	1986	MEM	Pseudophakic bullous keratopathy	2	–	dex 4	Recurrent dendrites on donor and recipient	+
8, F, 48	1987	MEM	Chemical burn	5	+	dex 2	Central epithelial defect	+
9, M, 74	1987	MEM	Interstitial keratitis assoc TBC	11	–	dex 1	dendrite on interface	nd
10, F, 53	1988	MEM	Keratoconus	1	–	dex 6	Recurrent dendrite on interface	+
11, F, 59	1989	MEM	Chemical burn	9	–	dex 2	Atypical geographic ulcer in donor	+
12, F, 82	1989	MEM	Interstitial keratitis assoc TBC	21	–	dex 1, oral prednisone 5mg/d	Dendrite on donor ulcer in recipient	–
13, M, 73	1991	MEM	Penetrating trauma	a, 5 RePKP	–	dex 2	1, nonspecific ulcer interface	+
				b, 3	+	dex 2	2, dendtrites on donor	+
14, F, 82	1992	MEM	Pseudophakic bullous keratopathy	3	–	predn 6	Geographic ulcer on donor	+
15, F, 21	1992	MEM	Keratoconus	1	–	dex 4	Recurrent dendrites on interface	–
16, M, 28	1992	MEM	Buphthalmos third PKP	0 or 3	–	predn 8	Nonhealing epithelial defect after 3 mos	+
17, F, 72	1992	MEM	Fuchs endothelial dystrophy	23	–	fml 3	Multiple dendrites on donor, pressure rise	+
18, F, 69	1992	MEM	Fuchs endothelial dystrophy	a, 22	+	dex 1	Small dendriform lesions, pressure rise	nd
				b, 25	–	pf 8	Multiple large dendrites on donor	+

Table 2: Positive Causes of the Endogenous Teactivation in the 18 Patients

Unknown trauma	2
Corneal trephination	2
Suture removal	4
High steroids	7*
Immune reaction	4*

* In one patient, both factors could have played a role.

because the incidence of HSV keratitis thereafter remains comparatively low compared to the high prevalence of latent virus in the general population. Moreover, HSV keratitis after cataract surgery appears to be rather rare. Another possibility could be transmission of HSV by way of tear shedding. In 1967, Kaufman et al[16] found asymptomatic ocular shedding in 1.8% of specimens taken from patients without a history of herpetic eye disease. This finding could not be confirmed, however, by studies from Kaye et al.[17]

Yet another possibility might be the transmission of virus through the donor cornea. Rabies, Creutzfeldt-Jakob disease, and hepatitis B virus are proved to be transmitted by corneal transplantation.[18,19] Recently, extraneuronal localization of HSV-DNA was reported in the corneas of patients with no history of herpetic eye disease.[20-23] Transmission of this DNA by corneal transplantation could be possible when this DNA is viable. In the group of patients who had transplantation for diagnoses not related to HSV, 5% to 10% of them will be seronegative for HSV. These patients might be at risk of developing an HSV infection after transplantation with an HSV-positive donor cornea. This might lead to a primary infection presenting directly after surgery as a slow or nonhealing defect or lead to primary graft failure or to colonization of the trigeminal ganglion with subsequent reactivation leading to a manifest infection. Openshaw et al[24] could not show seroconversion after transplantation of corneas containing viral sequences in HSV-1 naive recipient rabbits in the 5 months thereafter. To prove transmission from donor cornea to recipient, the isolation of the HSV-DNA from the donor and the comparison of this to the HSV -DNA from the virus isolated from the herpetic keratitis should be performed involving several hypervariable segments of virus DNA.[25]

Complete identity of the viral genome of donor and recipient probably will be hard to prove. The viability of the HSV-DNA detected with the polymerase chain reaction remains a controversial issue.

Cleator et al[20] showed viral growth in organ culture storage, although its occurrence is rather rare. We could not find a relation between this clinical picture and the corneal storage technique.

We believe that, in most cases, the presentation of the infection will result from endogenous reactivation of the trigeminal ganglion by transplantation-related reactivating stimuli. We want to emphasize the need to consider HSV keratitis in the differential diagnosis of a post-PKP epithelial defect in a patient without a history of HSV infection.

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Amplification of Reiterated Sequences of Herpes Simplex Virus Type 1 (HSV-1) Genome To Discriminate between Clinical HSV-1 Isolates

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Summary

Herpes simplex virus type 1 (HSV-1)-related disease ranges from a localized, self-limiting illness to fatal disease in immunocompromised individuals. The corneal disease herpetic keratitis may develop after reactivation of a latent virus or reinfection with an exogenous herpesvirus. Molecular analysis of the virus involved may allow distinction between these two options. The HSV-1 genome contains several hypervariable regions that vary in numbers of reiterating regions (reiterations I to VIII [ReI to ReVIII]) between individual strains. Twenty-four HSV-1 clones, derived by subcloning of HSV-1 (strain F) twice in limiting dilutions, were tested in a PCR-based assay to analyze the stabilities of ReI, ReIII, ReIV, and ReVII. ReI and ReIII proved to vary in size upon subcloning, whereas ReIV and ReVII were stable. Subsequently, 37 unrelated isolates and 10 sequential isolates from five patients, all with HSV-1-induced keratitis, were genotyped for ReIV and ReVII. Of the 37 unrelated samples, 34 (92%) could be discriminated, while the genotypes of the viruses in sequential samples were identical for each individual. Conclusively, the data show that the approach presented allows the rapid and accurate discrimination of HSV-1 strains in studies that address the transmission and pathogenesis of HSV-1 infections.

Introduction

Herpes simplex virus (HSV) type 1 (HSV-1) infections are widespread in the human population and may cause a variety of disease symptoms, including localized recurrent ocular lesions like uveitis and keratitis[16]. Clinical manifestations associated with herpetic corneal infections are herpetic epithelial keratitis and the development of the potentially corneablinding disease herpetic stromal keratitis. It may be of clinical importance to know whether recurrent corneal HSV-1 infections are caused by reactivation of a latent virus or reinfection with an exogenous virus. Genetically different HSV-1 strains can induce different types of ocular lesions[8, 35]. Intratypic differences between HSV strains have been demonstrated by plaque morphology, serology, and DNA restriction analysis[5, 15, 22]. The method generally used to discriminate HSV-1 strains is restriction fragment length polymorphism (RFLP) analysis[7, 15, 17–19, 24, 29–31]. Since this technique depends on virus culture to obtain sufficient quantities of viral DNA, it is unsuitable for rapid diagnosis or when no virus can be isolated. Vogel et al.[31] reported on an alternative method for clinical HSV strain differentiation that uses PCR amplification and subsequent RFLP analysis. We have chosen to develop a different strategy, based on the variability of reiterated sequences within the HSV-1 genome. The genome of HSV-1 consists of a unique long (U_L) and a unique short (U_S) sequence, each of which is flanked by inverted repeat sequences[14, 32]. Several hypervariable regions, designated reiterations I to VIII (ReI to ReVIII), have been identified within the HSV-1 genome (Fig. 1). These regions contain multiple repeating sequences, which vary in numbers between unrelated HSV-1 strains[3, 9, 10, 13, 24, 25, 28, 34, 35]. The stability of these regions varies. ReI, ReIII, ReIV, and ReVII have been demonstrated to be relatively stable during a short period of viral replication, and it has been suggested that several of these hypervariable regions could be used as markers to discriminate HSV-1 strains[27, 28]. ReI and ReIII are located within the "a" sequence of the repeat regions that flank the unique short sequence. ReIV is present twice within the HSV-1 genome and is located within introns of both the genes US1 and US12, whereas ReVII is located within the protein-coding region of US10 and US11[3, 12, 13]. This report describes the development of a PCR method

that is used to discriminate HSV-1 strains and that is based on the variability of reiterated sequences within the HSV-1 genome. This approach was successfully used to discriminate 37 unrelated corneal HSV-1 isolates obtained from patients with herpetic corneal disease. Additionally, sequential HSV-1 isolates from five herpetic keratitis patients were compared.

Materials and Methods

Clinical samples and viruses.

Corneal swab specimens were obtained from 37 patients with herpetic keratitis at the Rotterdam Eye Hospital (Rotterdam, The Netherlands) for diagnostic purposes. Sequential samples ($n = 2$) were obtained (mean time interval, 19 months; range, 9 to 38 months) from 5 patients: from the same eye for four patients and from different eyes for one patient. Virus was grown on human embryonic lung fibroblasts and was harvested when approximately 75% of the monolayer displayed a cytopathic effect. All culture samples were confirmed to be HSV-1 positive by PCR (data not shown). To determine the stability of the hypervariable regions, 24 subclones were generated from HSV-1 F (ATCC VR-733) by subcloning twice in limiting dilution as described before[26].

Nucleic acid extraction.

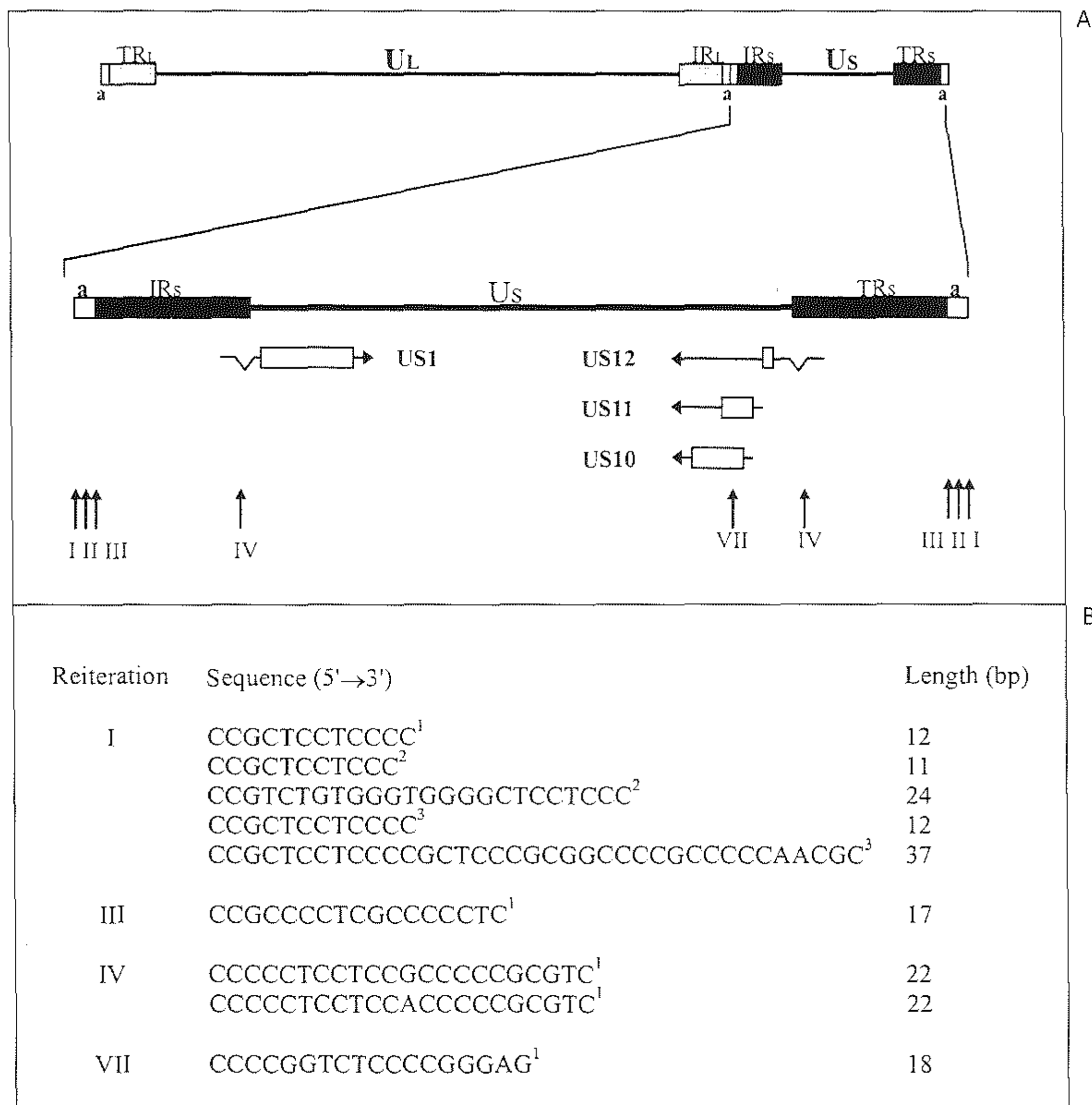
DNA was extracted from 100 µl of virus culture samples by a guanidinium thiocyanate-Celite binding method, as described before[1]. Briefly, a sample was added to a tube containing 1 ml of lysis buffer and 40 µl of Celite suspension (Fischer Scientific, Den Bosch, The Netherlands), mixed, and incubated for 10 min at room temperature. The Celite-bound DNA was washed twice with wash buffer, twice with 70% (vol/vol) ethanol, and once with acetone and was subsequently dried. DNA was extracted by resuspending the pellet in 150 µl of water at 56°C for 10 min. A volume of 5 µl of the resulting DNA suspension was used per PCR mixture.

PCR amplification.

Primers were designed to amplify distinct regions in the HSV-1 genome that contained ReI, ReIII, ReIV, or ReVII. PCR amplification was performed with several combinations of primers (Table 1). The PCRs were performed in 50-µl volumes. The reaction mixture contained 1.25 U of cloned *Pfu* DNA polymerase (Stratagene Europe, Amsterdam, The Netherlands), corresponding buffer supplemented with 5% (vol/vol) dimethyl sulfoxide (DMSO), each of the primers at a concentration of 1 µM, and each deoxynucleoside triphosphate, including equimolar amounts of dGTP and 7-deaza-2'-dGTP (Boehringer Mannheim, Mannheim, Germany), at a concentration of 200 µM. A 5-µl sample of the DNA suspension was added, and the reaction mixtures were overlaid with 50 µl of mineral oil. PCR amplification was carried out as follows: an initial denaturation step of 95°C for 5 min, followed by 45 cycles of alternating denaturation (1 min, 95°C), primer annealing (1 min at the appropriate temperature; Table 1), and primer extension (1 min, 72°C). A final extension step of 7 min at 72°C was included. For negative control samples, the DNA suspension was replaced by water. All PCRs were performed in a Perkin-Elmer 480 thermocycler (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Detection of amplified products.

Amplicons were size fractionated in 2% agarose gels and were visualized by ethidium bromide staining. The specificities of the amplicons were confirmed by Southern blotting[20]. Briefly, the electrophoresed samples were transferred onto Hybond N^+ membranes (Amersham, Pharmacia Biotech).



Reiteration	Sequence (5'→3')	Length (bp)
I	CCGCTCCTCCCC[1]	12
	CCGCTCCTCCC[2]	11
	CCGTCTGTGGGTGGGGCTCCTCCC[2]	24
	CCGCTCCTCCCC[3]	12
	CCGCTCCTCCCCGCTCCCGCGGCCCCGCCCCCAACGC[3]	37
III	CCGCCCCTCGCCCCCTC[1]	17
IV	CCCCCTCCTCCGCCCCCGCGTC[1]	22
	CCCCCTCCTCCACCCCCGCGTC[1]	22
VII	CCCCGGTCTCCCCGGGAG[1]	18

FIG. 1. Map of herpes simplex virus type 1 (HSV-1) genome and location and sequences of reiterations.

A HSV-1 DNA consists of 2 covalently linked components (L and S), each of which consists of unique sequences (U_L and U_S) flanked by inverted repeat sequences (IR and TR). The short "a" sequence is located at both termini of the genome and in the inverse orientation at the L-S junction[14]. The enlargement of the S component shows the 5'—>3' orientations of mRNA species as horizontal arrows with introns as V-shaped indents. Protein coding regions are shown as open boxes. Vertical arrows indicate locations of reiterations and Roman numerals as location numbers as defined by Rixon et al.[12].

B Reiteration-specific sequences, as indicated by the superscript numbers, were derived from the following strains: 1, MP17; 2, USA-8; 3, F.

Hybridization was performed overnight at 37°C with [γ-32P]ATP-labeled Re-specific oligonucleotides (Table 1). Posthybridization washes were performed twice with 23 SSC (13 SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 37°C for 10 min. The filters were exposed with intensifying screens at −80°C. In case of small differences in length between amplicons from individual samples, the DNA fragments were electrophoresed on denaturing (8 M urea) 6% acrylamide gels[20]. The lengths of the amplicons were estimated by comparison to a 100-bp DNA ladder (Gibco BRL). To confirm differences in amplicon length, all samples tested were finally electrophoresed in order of increasing length.

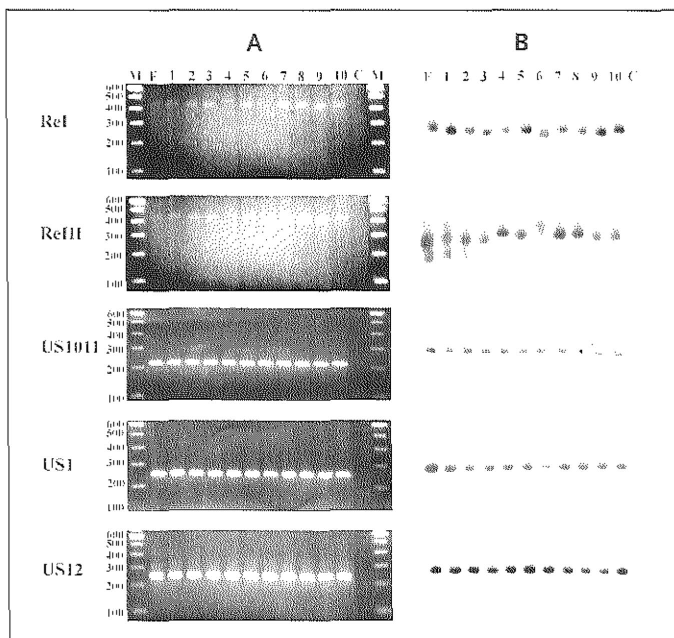


FIG. 2. Amplification of hypervariable regions within the HSV-1 genome.

A PCR amplification of regions containing ReI, ReIII, ReIV, and ReVII was performed with DNA from various HSV-1 (strain F) subclones. Amplicons were electrophoresed on a 2% agarose gel and were visualized by ethidium bromide staining. Ten representative samples from 24 subclones analyzed are shown. Lane F, parental strain HSV-1 F; lanes 1 to 10, HSV-1 F subclones; lane C, water control; lanes M, 100-bp molecular size marker. Numbers on the left are in base pairs.

B Autoradiogram of DNA in gel from panel A after Southern blot transfer and hybridization with a Re-specific probe.

Results

Amplification of hypervariable genomic HSV-1 regions containing ReI, ReIII, ReIV, and ReVII.

On the basis of documented variability and stability[27, 28], hypervariable regions containing ReI, ReIII, ReIV, and ReVII were selected as candidate templates for PCR-mediated discrimination of unrelated HSV-1 strains.

Amplification of these regions was not possible or was insufficient under standard PCR conditions (data not shown).

Alternative conditions, selected to decrease the formation of secondary structures due to the high G+C contents of these sequences, improved amplification of the target sequences and allowed direct visualization of the amplicons with ethidium bromide. The specificities of the amplicons were confirmed by hybridization with a γ-^{32}P-labeled Re-specific probe following Southern blotting (Fig. 2). Consistent results were obtained in all cases in subsequent experiments. To test the stabilities of ReI, ReIII, ReIV, and ReVII, PCR amplification of these regions was performed with 24 separate subclones of HSV-1 F, and the sequences of these regions were compared with those of the amplicons of the parental strains (Fig. 2). For the regions containing ReIV and ReVII, amplicons from all 24 subclones were identical in size to those of their parental strains, indicating the stability of ReIV and Re- VII during the two limiting dilution rounds. For the regions containing ReI and ReIII, not all separate subclones showed the same amplicon length as their parental strains, differences being greatest for ReIII (Fig. 2). Consequently, ReIV and ReVII were further used to discriminate 37 unrelated

TABLE 1. PRIMERS USED FOR AMPLIFICATION AND DETECTION OF HSV-1 REITERATIONS

Genome region	Primer[1]	Optimal annealing temp (°C)	Sequence (5' —> 9')	Position in genome[2]
"a" sequence	ReIF	72	GCCGCCACCGCTTTAAAGGGCCGC	125976–125999 and 152234–152257
	ReIR		GTGCTCTGTTGGTTTCACCTGTGGCAGC	126368–126395 and 151838–151865
"a" sequence	ReIIIF	72	TCTCTACCTCAGTGCCGCCAATCTCAGGTC	126742–126771 and 151462–151491
	ReIIIR		CGAAGACGCAATAAACGGCAACAACCTG	127171–127198 and 151035–151062
US1	ReIVUS1F	64	TCCGACGACAGAAACCCACC	132333–132352
	ReIVUS1R		GTCCCGGAGGACCACAGTGG	132615–132634
US12	ReIVUS12F	58	TTTTTGCACGCGTAAGCAC	145853–145871
	ReIVUS12R		TGGTGTCCAGGAAGGTGTCC	145535–145554
US10-US11	ReVIIUS1011F	56	AGCGTATGCTCCATGTTGTG	144697–144716
	ReVIIUS1011R		CGAGAACCTAGGGAACCCA	144928–144946
ReI	ReI probe	37	CCGCTCCTCCCC	
ReIII	ReIII probe	37	CCGCCCCTCGCCCCCTC	
ReIV	ReIV probe	37	CCCCCTCCTCCACCCCCGCGTC	
ReVII	ReVII probe	37	CCCCGGTCTCCCCGGCAG	

1 F, forward; R, reverse.

2 Positions correspond to the genomic HSV-1 sequence HE1CG (accession no. X14112).

HSV-1 isolates obtained from keratitis patients. The results of the analyses performed with all 37 clinical corneal HSV-1 isolates are summarized in Table 2. As an example, differences in amplicon lengths between unrelated clinical isolates from 10 patients are shown in Fig. 3A. The variability in the US10- US11 region (ReVII) was low, showing only three different alleles. Regions US1 and US12 (ReIV) showed a wider variety of alleles, with 14 and 15 different alleles detected among the 37 samples analyzed, respectively (Table 2). Combination of the results for the three amplified regions showed that 34 of the 37 isolates (92%) displayed unique combinations of amplicons. For some clinical samples, no PCR product could be detected by ethidium bromide staining or multiple fragments appeared. This was probably due to the poor quality of the template DNA. Hybridization with the labeled probe, however, readily enabled the detection of the Re-specific amplicon in these samples (data not shown).

Analysis of sequential corneal HSV-1 isolates. Sequential corneal HSV-1 isolates obtained from five patients with recurrent herpetic corneal infections were analyzed (Fig. 3; Table 2). The ReIV- and ReVII-specific amplicons showed interindividual variations in length. However, the amplicons from the sequential samples from each individual were identical.

TABLE 2. Length of reiteration-specific amplicons of corneal HSV-1 isolates

Isolate or sample and patient no.[1]	Estimated amplicon length (bp) Region US10-US11 (ReVII)	Region US1 (ReIV)	Region US12 (ReIV)
Unrelated HSV-1 isolates			
1	215	270	370
2	215	280	220
3	215	280	270
4	215	290	280
5	215	295	230
6	215	305	300
7	220	210	310
8	220	220	220
9	220	220	220
10	220	260	260
11	220	260	290
12	220	260	290
13	220	280	260
14	220	280	260
15	220	290	260
16	220	290	280
17	220	305	230
18	220	305	310
19	220	370	370
20	220	380	300
21	220	380	390
22	220	410	220
23	220	410	460
24	220	420	300
25	220	420	420
26	225	260	270
27	225	260	280
28	225	290	260
29	225	290	270
30	225	295	220
31	225	295	290
32	225	320	220
33	225	320	320
34	225	320	380
35	225	340	220
36	225	340	340
37	225	370	370
Sequential samples			
1a	215	280	270
1b	215	280	270
2a	215	280	220
2b	215	280	220
3a	215	295	230
3b	215	295	230
4a	215	305	300
4b	215	305	300
5a	225	220	220
5b	225	220	220

1 Three pairs of patients (patients 8 and 9, patients 11 and 12, and patients 13 and 14) were infected with unrelated clinical isolates with identical DNA patterns.

Discussion

In the present paper, we present a PCR-based approach that allows the rapid and accurate discrimination of unrelated HSV-1 strains. The method generally used to discriminate HSV-1 strains is RFLP analysis[7, 15, 17–19, 24, 29–31]. This method requires virus culture, is time-consuming, and is highly labor-intensive. Furthermore, culture requires viable virus, which is not always obtainable from certain types of clinical samples (e.g., cerebrospinal and intraocular fluids). More recently, a system that uses PCR amplification and subsequent RFLP analysis has been developed to facilitate discrimination of HSV-1 strains, eliminating the necessity of virus culture. This method, however, is not significantly less time-consuming or labor-intensive than conventional strain differentiation[31]. Conventional RFLP analysis with restriction endonucleases that recognize 6 bp (6-bp REs) is insufficient for differentiation of HSV-1 strains of a predominant genotype. The use of 4-bp REs and RFLP analyses of reiterated sequences greatly improved the differentiation rate[28]. As in our study, the RFLP analysis of reiterated sequences was based on various numbers of repeats. Use of both techniques generated similar results, verifying the applicability of either method in molecular epidemiological studies[27, 28]. Similar hypervariable regions have been used successfully to discriminate strains of other herpesviruses like Epstein-Barr virus and human cytomegalovirus[33, 36].

To be applicable in a PCR-based assay for discrimination of different HSV-1 strains, these regions should show a considerable degree of variability and should remain stable during a relatively short time of replication. We tested the suitability of several HSV-1 hypervariable regions for discrimination of unrelated HSV-1 strains.

Due to their G+C-rich sequences, standard

PCR protocols failed to reproducibly amplify the regions tested. The high G+C content increases the formation of secondary structures, preventing consistent amplification of the repeats. We tested a number of PCR conditions in order to obtain consistent DNA amplification. Addition of DMSO as a cosolvent to the reaction mixture has previously been shown to facilitate DNA amplification of G+C-rich sequences[12]. Introduction of the exonuclease activity of the *Pfu* DNA polymerase enzyme in the PCR mixture prevents "skipping" of the repeats, which could result in the formation of products smaller than the actual size of the template repeat[2]. Another modification was the introduction of 7-deaza-2'-dGTP. This analogue of dGTP is equally well incorporated into DNA but exerts a lesser binding strength to dCTP than normal dGTP[6,21]. The use of *Pfu* polymerase, 50% 7-deaza-2'-dGTP as a replacement for 100% dGTP, and 5% DMSO resulted in the most consistent ampli- fication of the large alleles. The specificities of the amplicons were confirmed by hybridization with Re-specific probes after Southern blotting.

Analysis of subclones of HSV-1 F showed that the stability of the ReI and ReIII sequences was too low to be useful for discrimination of HSV-1 strains. In contrast, ReIV and ReVII were shown to be stable during this procedure. Thus, regions US1 (ReIV), US12 (ReIV), and US10-US11 (ReVII) were chosen for use in the discrimination of unrelated corneal HSV-1 isolates.

In agreement with previous studies, the variability in the US10-US11 region was found to be relatively low[27-29]. We detected only three different alleles among 37 unrelated clinical HSV-1 isolates, which is not surprising since ReVII is located within a protein-coding region, making it a target for selective pressure. More drastic changes in the length of US10- US11 could influence the translation or function of the proteins encoded by genes US10 and US11. In contrast, the ReIV-containing sequences are located in the introns of genes US1 and US12. We found 14 and 15 different alleles for regions US1 and US12, respectively, in the 37 corneal HSV-1 isolates analyzed. Comparison of the alleles from the three regions for all 37 corneal HSV-1 isolates revealed 34 unique combinations. The isolates with identical combinations were obtained at different time points, indicating that this was most likely not due to contamination during virus isolation or culture procedures.

Sequential corneal isolates from five individuals with recurrent herpetic corneal infections were analyzed. For each individual,

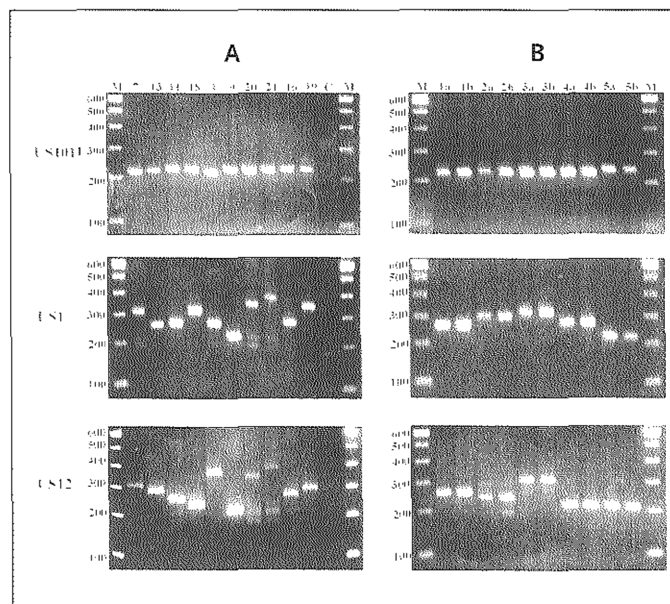


FIG. 3. Variability of Re-containing regions US1, US12, and US10-US11 between unrelated and sequential corneal HSV-1 isolates.
PCR amplification was performed with DNA from corneal HSV-1 isolates. Amplicons were analyzed as described in the legend to Fig. 2.
A Results for 10 representative samples among the 37 samples analyzed.
B Amplicons from sequential samples from five individuals. Lane C, water control; lanes M, 100-bp molecular size marker. Numbers on the left are in base pairs.

sequential samples showed identical DNA patterns, while the patterns for samples from different patients were different. These results indicate that the recurrent infections were most likely caused by the same virus. A comparative sequence database search revealed several point mutations between different HSV-1 strains, in addition to various numbers of repeats. More detailed analysis, like sequencing of the amplicons, might provide more conclusive evidence for this assumption. This also demonstrates that these hypervariable regions remain stable during reactivation and replication of latent HSV-1 in the corneas of these individuals.

Additionally, we have also analyzed clinical samples in which no viable virus can usually be detected[4]. Re sequence-specific PCR analyses were performed with DNA isolated from affected corneal buttons and rims obtained from patients with herpetic stromal keratitis during therapeutic keratoplasty. The PCR approach proved to be sensitive enough for amplification of the low levels of viral DNA present in these samples (unpublished data). The major advantage of the approach presented is that it provides the opportunity to discriminate HSV-1 strains without virus culture or RFLP analysis, making it convenient for rapid diagnostic testing. Although not suitable for classification of HSV-1 strains, it provides a powerful tool that can be used to address questions regarding reactivation and the modes of transmission of HSV-1. For example, it could be used to assess the risk of HSV-1 transmission through cornea transplantation and other manifestations of recurrent HSV-1 infections.

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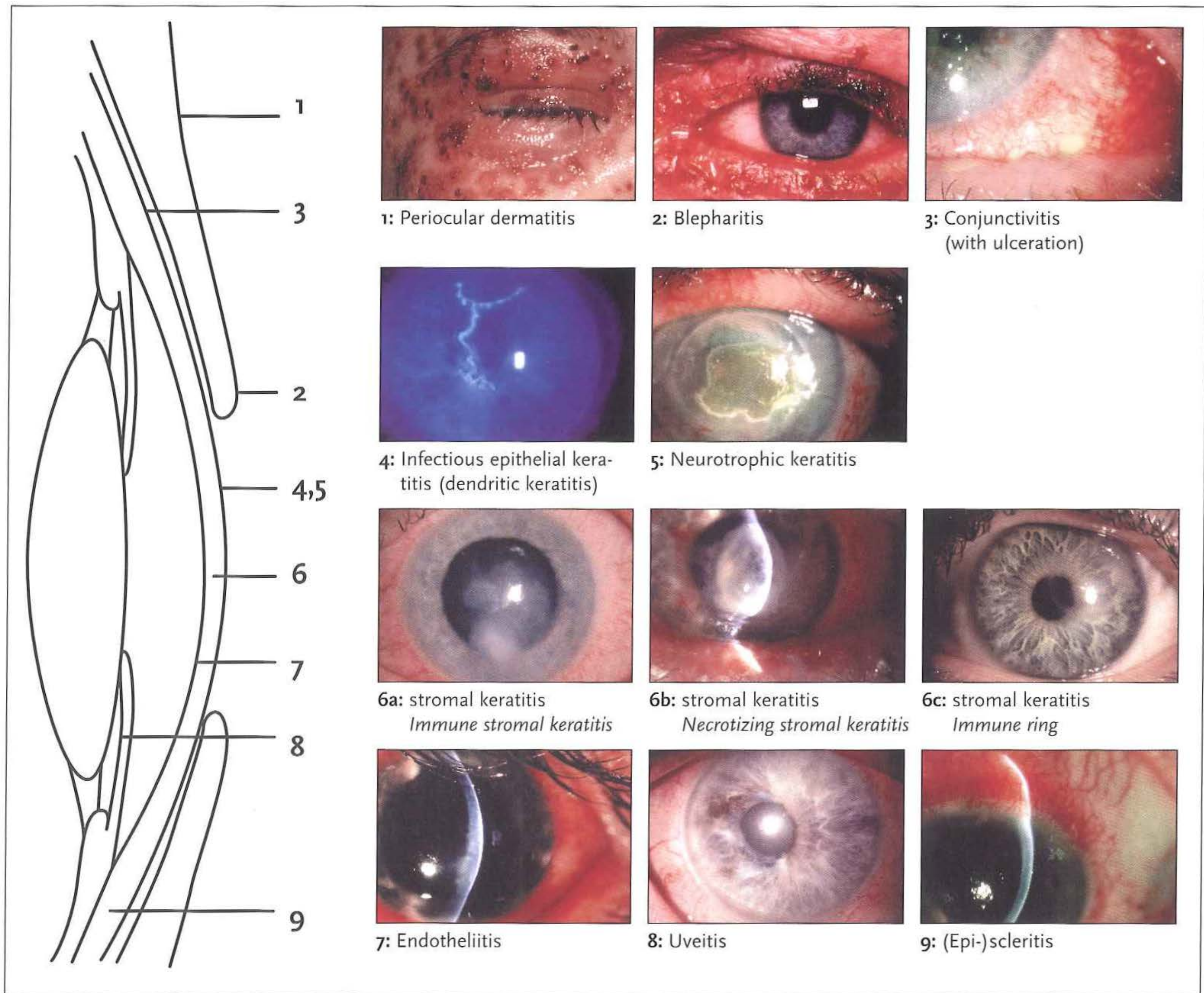


Figure 8, chapter 1: Presentations of herpes simplex virus infections of the anterior eye segment

Adapted from: R. Sundmacher: A clinico-virologic classification of herpetic anterior segment diseases with special reference to intra-ocular herpes. In Herpetische Augen Erkrankungen DOG 1980. Eds R. Sundmacher. München: JF Bergmann Verlag 1981: 206

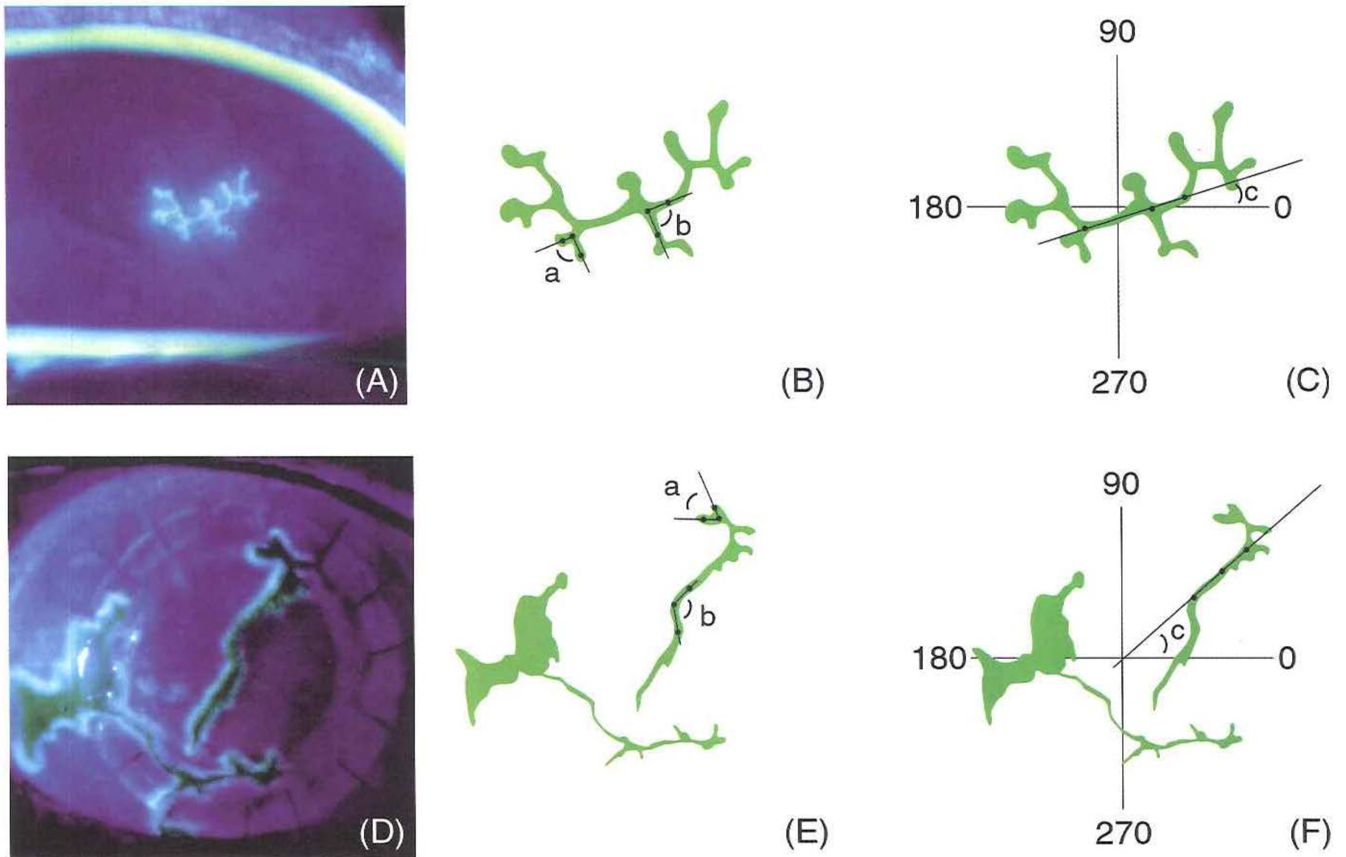


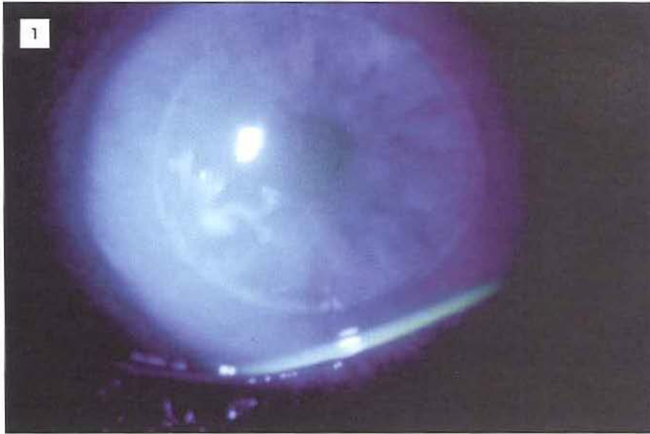
Figure 2, chapter 2: Measurement of the branching angles and the main angle in a dendritic lesion.

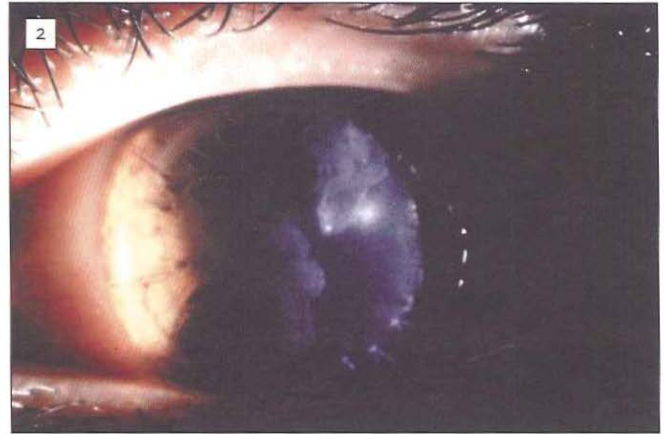
Examples of a dendritic lesion on a non-operated cornea (2a) and on a cornea after PKP (2b).

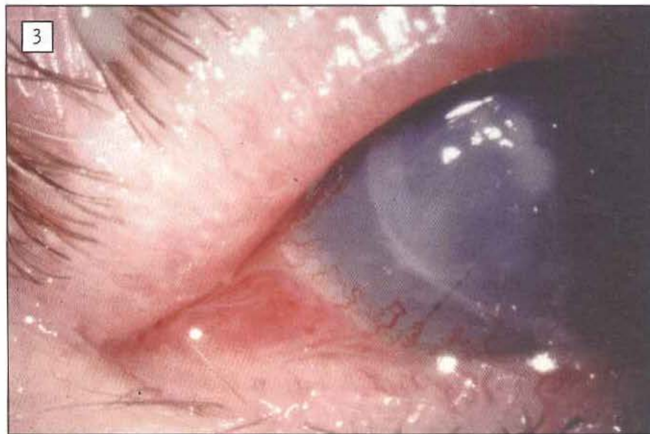
2A + D: the original dendritic lesion.

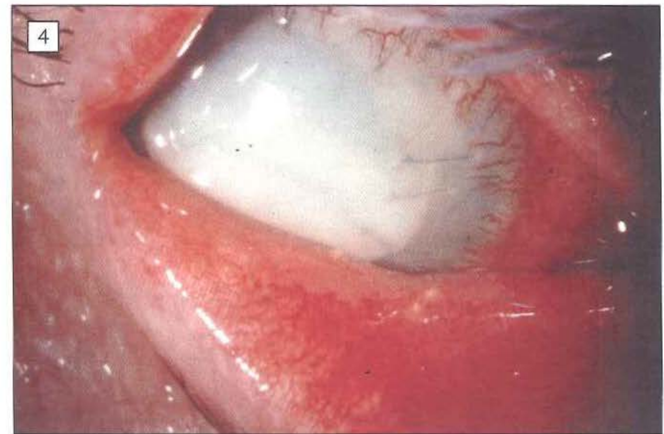
2B + E: branching angles: points were drawn in the middle of all branches of the dendritic lesion. Lines were drawn through straight parts of the dendritic lesions using these points The angles between two consecutive parts of the dendritic lesion were measured as a branching angle (angle a and b). (Not all branching angles of this dendritic lesion are drawn in this figure for reasons of clarity)

2C + F: main angle: points (shown only in the longest branch) are drawn in the middle of all branches of the dendritic lesion. The connecting line using most points was considered the best fitting or direction line. Angle c is measured with the use of the horizontal axis.









Figures 1-4, chapter 3:

Figure 1

Classic herpes simplex viral keratitis in donor cornea (Table 1, patient 2).

Figure 2

Nonspecific epithelial defects and superficial stromal scarring in a patient who had transplantation for keratoconus (Table 1, patient 15).

Figure 3

Large epithelial defects small stromal infiltrate with positive culture for HVS type 1, moth after PKP for keratoconus (Table 1, patient 10).

Figure 4

Stromal infiltrate (Table 1, patient 13) with negative bacterial cultures. Three months after repeat PKP, the patient showed a beginning allograft rejection and a classic central dendriform herpetic keratitis.

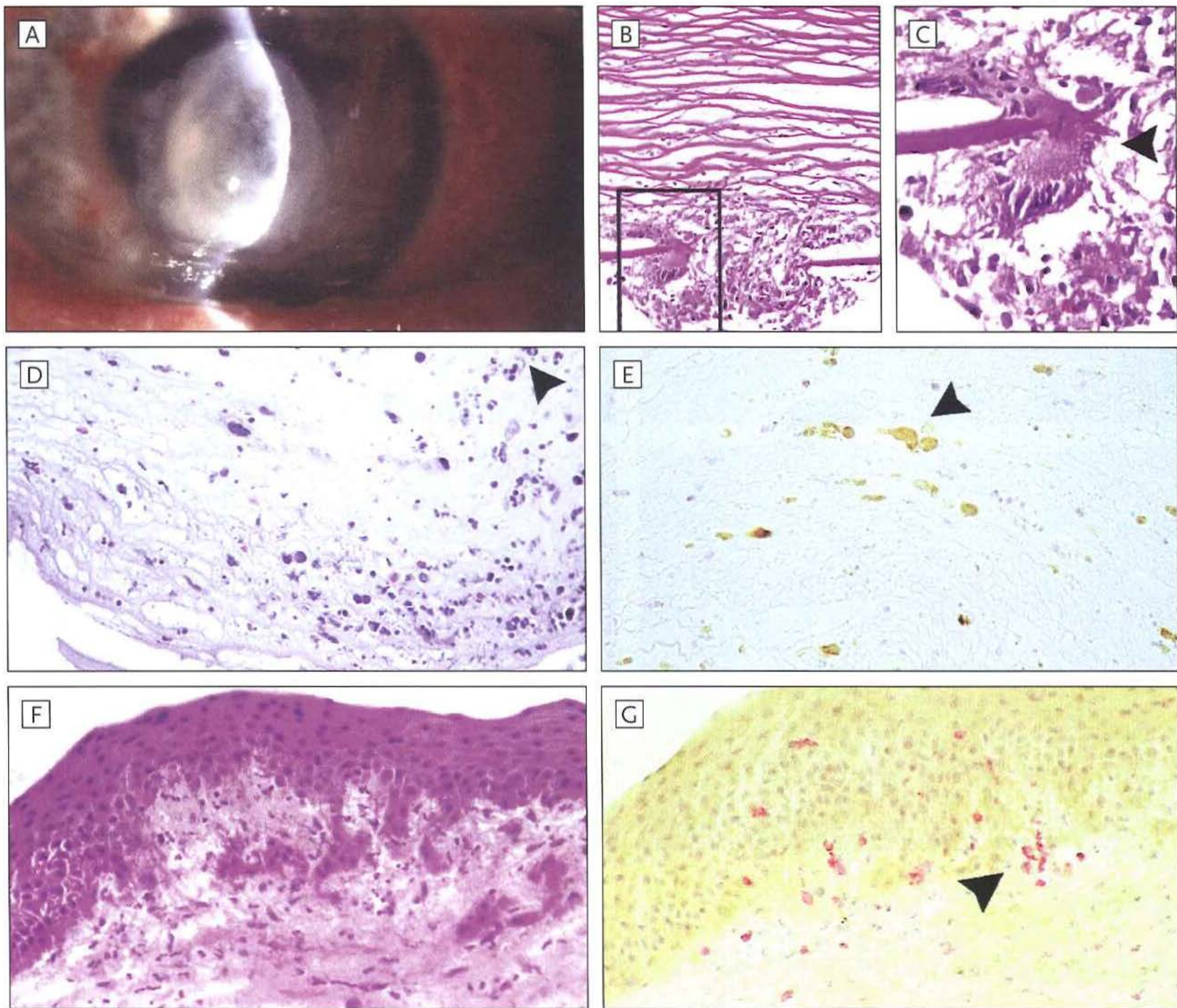


Figure 2, chapter 4:

Macroscopic, histologic and immunohistologic examination of corneas from patients with herpetic stromal keratitis. (A) Central corneal ulcer with descemetocele and impending perforation of the cornea of patient #5. (B and C) Central view of keratectomy specimen of patient #1 showing stromal infiltration of inflammatory cells and a florid granulomatous reaction with focal rupture of Descemet's membrane (Dm) (B; periodic acid - Schiff. PAS, magnification x100). Inset: higher manification of then multinucleated giant cell (arrow) protruding through this rupture (C; PAS, x400). (D). Paracentral view of keratectomy specimen of patient #5 shows (adjacent to the ulcer) a deep stromal defect with edema and infiltration of polymorphonuclear cells (arrow) reaching up to Dm (HE straining, x200). (E) Immunohistochemical staining for HSV-1 antigens demonstrating HSV antigens in stromal keratocytes (arrow) in the corresponding section of the cornea of patient #5 (hematoxylin counterstained, x400). (F) Central view of keratectomy specimen of patient #6 with reactive hyperplasia of the epithelium. The cornea is infiltrated with predominantly mononuclear cells (HE, x200). (G) Immunohistochemical staining for $CD4^{+}$ cells demonstrating predominantly subepithelial localization of $CD4^{+}$ cells (arrow) in the corresponding section of the cornea of patient #6 (hematoxylin counterstained, x200).

Corneal Herpes Simplex Virus Type 1 Superinfection in Patients with Recrudescent Herpetic Keratitis

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Summary

Purpose.

Herpetic keratitis is a common sequel of a corneal infection with herpes simplex virus (HSV)-1. Recrudescent herpetic keratitis (RHK) may result in irreversible damage to the cornea. Recurrences may be caused by reactivation of endogenous HSV-1 or reinfection with exogenous HSV-1. The objective of this study was to determine the incidence and risk factors involved of HSV-1 superinfection in patients with RHK.

Methods.

From 30 patients with RHK, sequential corneal HSV-1 isolates were genotyped by PCR amplification of the hypervariable regions located within the HSV-1 genes *US1*, *US10/11*, and *US12*. The clinical data from the patients obtained retrospectively were: ophthalmologic history, clinical picture during recurrences, number and time points of penetrating keratoplasty (PKP), and steroid or acyclovir treatment.

Results.

Whereas the sequential corneal HSV-1 isolates of 19 (63%) of 30 patients had the same genotype (designated as group 1), the sequential isolates of 11 patients (37%) were genetically different (designated as group 2). Among the clinical data analyzed, only the time point of PKP was significantly different between the patient groups. Although no patients in group 1 had undergone transplantation between samplings, 4 of 11 patients in group 2 underwent PKP during the interrecurrence period in the same eye from which the corneal HSV-1 isolates were obtained.

Conclusions.

The data demonstrate that RHK is frequently associated with corneal reinfection with a different HSV-1 strain and suggest that PKP is a risk factor for corneal HSV-1 superinfection.

Introduction

Herpes simplex virus (HSV) infections may elicit a variety of serious diseases in humans, including chronic herpetic keratitis.[1,2] A hallmark of HSV and other neurotropic herpes viruses is their ability to establish latency in sensory nerve ganglia of the host.[1] Despite the induction of an acquired state of immunity after primary HSV infection, recrudescent herpetic lesions are often observed.[1] Patients who have had corneal HSV-1 infection risk recurrent corneal disease throughout life. Particularly prolonged or recurrent episodes of herpetic keratitis can result in decreased vision or blindness due to the development of herpetic stromal keratitis (HSK).[2,3]

Recrudescent HSV infections are thought to result from reactivation of the HSV strain acquired during primary infection.[4-6] However, reinfection with a new HSV strain (i.e., superinfection) at the site of primary infection has also been documented.[6,7] The route or mode of HSV superinfection and its clinical consequences remain enigmatic. Genetically different HSV strains have been shown to induce different types of ocular lesions.[8] Furthermore, newly acquired herpetic keratitis may develop after penetrating keratoplasty (PKP) in patients who undergo transplantation for reasons unrelated to HSV infection, suggesting the possibility of HSV-1 transmission through cornea transplantation.[9] These issues underline the clinical importance of knowing whether recurrent corneal HSV-1 infections are caused by reactivation of latent virus or superinfection with a different virus strain.

Molecular analyses of corneal HSV-1 isolates may allow distinction between both options.

The genome of HSV-1 consists of a unique long (UL) and a unique short (Us) component, each of which is flanked by a pair of oppositely oriented repeat elements. Several hypervariable regions have been identified in the HSV-1 genome. These regions encompass unique tandemly repeated sequences, reiterations (Re) that vary in copy number and nucleotide sequences (Fig. 1).[1,10,11] Generally, two types of restriction fragment length polymorphism (RFLP) analyses are used to differentiate HSV-1 isolates. One type is the variation due mostly to a gain or loss of a restriction enzyme cleavage site. The other appears as variation in length of cleaved fragments derived from Re-containing genomic HSV-1 regions.[11] Among the eight Re regions described for HSV-1, ReIV and -VIII (both located within the introns of genes *US1* and *US12*) and ReVII (located within the protein coding region of genes *US10* and *US11*) have been shown to remain stable during in vitro culture and have been used as sensitive and reliable markers to differentiate HSV-1 strains.[12-15]

We have recently developed a PCR method, based on the stability and strain-to-strain differences of ReIV, -VII, and -VIII that has facilitated the differentiation of up to 92% of unrelated HSV-1 strains.[12,15] The purpose of the present study was to determine the incidence and risk factors involved in corneal HSV-1 superinfection in patients with recrudescent herpetic keratitis (RHK).

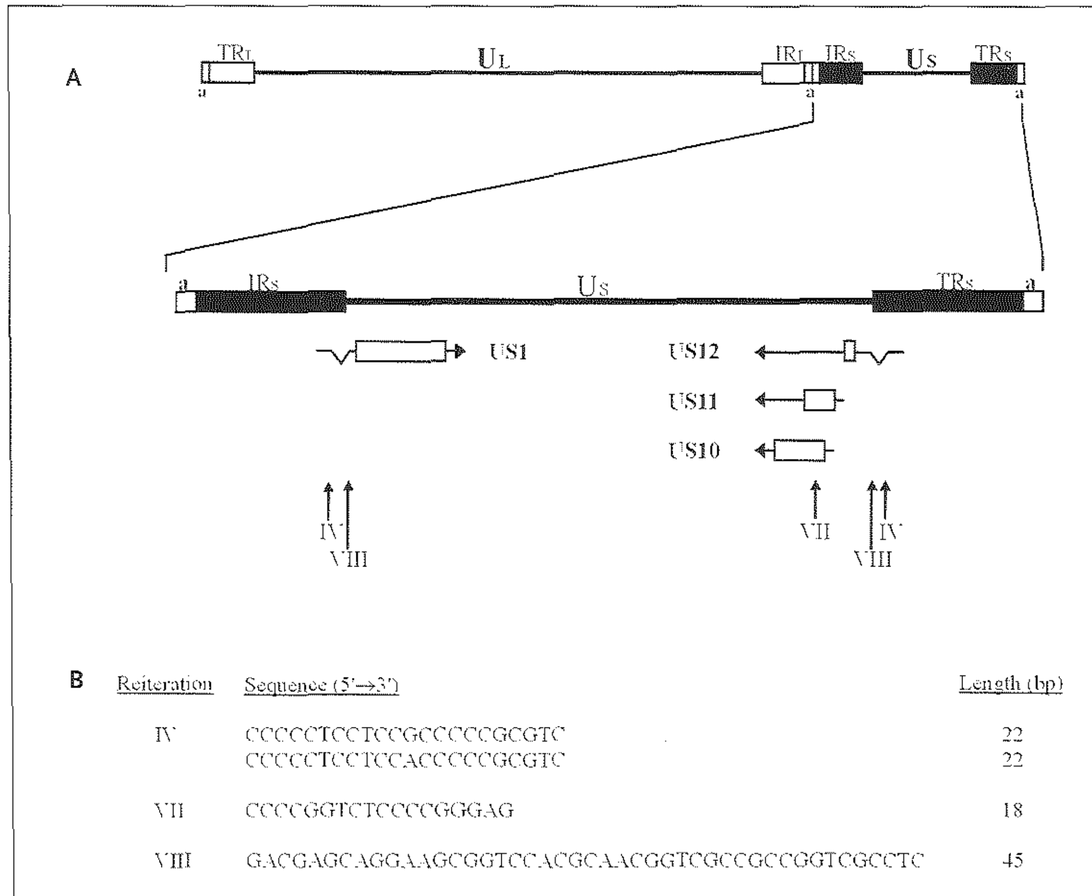
Materials and Methods

Patients and Clinical Samples

Corneal swab specimens were obtained for diagnostic reasons from suspected herpetic corneal lesions and were used to inoculate human embryonic lung fibroblasts. Virus was harvested when approximately 75% of the monolayer showed cytopathic effect and was subsequently typed for HSV-1 or -2 by immunocytology and PCR.[15] Serial samples from 30 immunocompetent patients with recurrent corneal HSV infections were found in a databank of 408 frozen corneal HSV-1 cultures collected since 1980 at the Rotterdam Eye Hospital (Rotterdam, The Netherlands). The clinical items scored retrospectively were anatomic location (i.e., left or right eye), previous history of ocular disease, clinical picture at presentation of each recurrence, therapy regimen preceding the culture dates, total number of PKPs, and PKPs between virus culture dates. The classification of herpetic keratitis was defined on clinical criteria.[2] The present study was performed according to the Declaration of Helsinki, and informed consent was obtained.

Genotypic Analyses of Corneal HSV-1 Isolates

Genotypic analyses of the viral strains were performed by amplification of the hypervariable regions within the HSV-1 genes *US1*, *US10/11*, and *US12*. This method is based on strain-to-strain differences in the number of Re and point mutations within these hypervariable genomic regions.[10,12,13,15] DNA was extracted from the primary corneal HSV-1 cultures, lysed in a guanidine isothiocyanate buffer using a silica solution (Celite; Jansen Chemika, Beers, Belgium), as described previously.[15] The PCR primers and conditions for amplifying and detecting by Southern blot analysis of the hypervariable regions of the HSV-1 genes *US1*, *US10/11*, and *US12* have been described.15 In case of small differences in length between amplicons (i.e., PCR products) from individual samples, the PCR products were run on denaturating (8 M urea) 6% acrylamide gels. The lengths of the amplicons were estimated by comparison to a 100- and 25-bp DNA ladder (Gibco BRL, Grand Island, NY). To confirm similarities or differences in amplicon length, all samples were finally electrophoresed in order of increasing length.



Reiteration	Sequence (5'→3')	Length (bp)
IV	CCCCCTCCTCCGCCCCCGCGTC	22
	CCCCCTCCTCCACCCCCGCGTC	22
VII	CCCCGGTCTCCCCGGGAG	18
VIII	GACGAGCAGGAAGCGGTCCACGCAACGGTCGCCGCCGGTCGCCTC	45

FIGURE 1. Map of the HSV-1 genome including the location and sequences of the Re regions tested.

A The prototypic HSV-1 genome encompasses the covalently linked components L and S. Each component consists of unique sequences (U_L and U_S) bracketed by inverted repeat sequences (TR_L, IR_L, IR_S, and TR_S). A short sequence, a, is repeated directly at the termini of the genome and located at the L–S junction. The enlargement of the S component shows the 5'—>3' orientations of mRNA species as *horizontal arrows* with introns shown as *V-shaped indents*. Protein-coding regions are shown as *open boxes*. Vertical arrows indicate locations of Re regions, and Roman numerals indicate their locations as defined previously.[10,12]

B Re-specific sequences have been described previously.[10,12] ReIV exists as two forms that differ from each other in a single residue.[10N]

Statistical Analyses

The statistical evaluation of the results was performed using the Fisher exact test. Results were considered statistically significant at $P < 0.05$.

Results

Patients' Characteristics and Genotypic Analyses of Sequential Corneal HSV-1 Isolates

The group of 30 patients with RHK included in this study consisted of 13 women and 17 men (mean age, 58.1 years; range, 17–78). From each patient, two ($n = 25$) or three ($n = 4$) sequential corneal HSV-1 isolates were obtained (mean time interval, 29.8 months; range, 0–170). Patient 22 had bilateral herpetic keratitis (Table 1).

To differentiate whether RHK is due to reactivation of latent HSV-1 or superinfection with another HSV-1 strain, the sequential corneal HSV-1 isolates of the patients with RHK were genotyped using a recently developed PCR-based DNA fingerprint assay.[15] The results of the PCR analyses, on the hypervariable regions of the genes *US1*, *US10/11*, and *US12*, performed on the corneal HSV-1 isolates are summarized in Table 1. As an example, the size fractionation and Southern blot analyses of the *US1*- and *US12*-specific amplicons obtained from the sequential samples of patients 1 through 5 and 20 through 24 are shown in Figure 2. The sequential corneal HSV-1 isolates of 19 (63%) of the 30 patients and 11 (37%) of the 30 patients showed either identical (patients 1–19; designated patient group 1) or different genotypes (patients 20–30; designated patient group 2), respectively (Table 1). The data suggest that more than one third of the corneas of the patients with RHK were superinfected with a different HSV-1 strain. In the case of patient 30, the newly acquired HSV-1 strain was cultured pending two post-PKP recurrences. This suggests that the newly acquired HSV-1 strain had colonized the recipient. Combining the results of the three amplified genomic regions showed that the majority of the distinguishable HSV-1 isolates displayed unique combinations of amplicons (Table 1).

In the case of patient 22, the data indicated that the bilateral herpetic keratitis was due to infections with different HSV-1 strains in either cornea. Patient 30 had two different HSV-1 strains identified. In the third episode sampled, the strain identified during the second recurrence was isolated (Table 1).

Comparison of Clinical Characteristics of Patients with RHK in Patient Groups 1 and 2

Compared with previous reports on patients with RHK,[2,9] our cohort consisted mainly of patients with severe entities of HSV-induced keratitis, such as herpetic stromal and necrotizing keratitis. This is also reflected in the high number of PKPs in the patient cohort (Table 2; mean PKPs, 1.4 per patient; range, 0–6).

The clinical characteristics of the patients in groups 1 and 2 were compared, to identify the factors predisposing for corneal HSV-1 superinfection. Overall, the immune status and ophthalmic condition did not differ significantly between both groups (data not shown). Additionally, gender, inter-recurrence period, anatomic location of the lesions (left or right eye), ocular history, and clinical picture at time of recurrences were not statistically different between both groups (Tables 1, 2).

Comparison of Therapeutic Regimen for RHK in Patient Groups 1 and 2

The clinical outcome of corticosteroid treatment before or during the convalescence period was not statistically different between both groups. The potential effects of long-term (val)acyclovir treatment were not numerous enough to be interpreted (data not shown).

Although the mean number of PKP per patient did not significantly differ between both groups, indicating that both groups were comparable in disease severity, a correlation between corneal HSV-1 superinfection and time point of PKP was observed. Whereas no

TABLE 1.

PATIENTS' CHARACTERISTICS AND GENETIC CHARACTERIZATION OF SEQUENTIAL CORNEAL HSV-1 ISOLATES FROM PATIENTS WITH RHK

				Estimated Amplicon Length (bp)[I]									Months between HSV-1 Isolates[II]		
				US10/11 Region			US1 Region			US12 Region					Eye
	Patient	Sex	Age (y)	a	b	c	a	b	c	a	b	c	a–b	b–c	Difference[III]
Group 1	1	F	17	215	215	—	295	295	—	240	240	—	11	—	+
	2	F	34	220	220	—	320	320	—	305	305	—	15	—	+
	3	M	64	215	215	—	280	280	—	220	220	—	8	—	–
	4	M	54	225	225	—	280	280	—	260	260	—	27	—	+
	5	M	44	225	225	225	420	420	420	270	270	270	56	3	+
	6	F	59	215	215	215	270	270	270	370	370	370	8	11	–
	7	M	39	215	215	—	280	280	—	270	270	—	170	—	–
	8	F	72	220	220	—	410	410	—	220	220	—	78	—	–
	9	M	74	225	225	—	320	320	—	310	310	—	22	—	–
	10	M	69	225	225	—	220	220	—	200	200	—	12	—	–
	11	F	73	—	220	—	230	230	—	240	240	—	28	—	+
	12	M	29	215	215	—	305	305	—	300	300	—	22	—	–
	13	M	60	220	220	—	280	280	—	260	260	—	21	—	–
	14	M	67	225	225	—	220	220	—	220	220	—	11	—	–
	15	F	69	225	225	—	295	295	—	290	290	—	36	—	–
	16	F	74	225	225	—	360	360	—	310	310	—	6	—	–
	17	M	52	230	230	—	260	260	—	260	260	—	77	—	–
	18	F	55	210	210	—	520	520	—	240	240	—	40	—	–
	19	F	78	230	230	230	300	300	300	260	260	260	3	4	–
Group 2	20	M	63	225	225	—	*260*	*310*	—	*250*	*300*	—	7	—	–
	21	M	46	*225*	*220*	—	*470*	*425*	—	*210*	*370*	—	22	—	–
	22	M	69	220	220	—	*290*	*360*	—	*270*	*290*	—	0	—	+
	23	F	32	225	225	—	*270*	*300*	—	270	270	—	2	—	–
	24	F	75	225	225	—	*350*	*430*	—	*370*	*320*	—	3	—	–
	25	F	38	215	215	—	*320*	*410*	—	*380*	*320*	—	85	—	–
	26	M	72	225	225	—	*370*	*300*	—	*370*	*290*	—	38	—	–
	27	M	65	220	220	—	*380*	*420*	—	*300*	*390*	—	2	—	–
	28	M	78	225	225	—	*340*	*490*	—	220	220	—	2	—	–
	29	F	77	225	225	—	*350*	*490*	—	210	210	—	28	—	–
	30	M	46	225	225	225	290	290	290	*260*	*280*	280	125	43	–

I a, b and c represent the time points at which corneal HSV-1 isolates were obtained. Data showing differences in amplicon length between sequential samples of individual patients are in italics. Based on the genotypic analyses, patients 1 through 19 were designated as group 1 (genotype sequential isolates identical) and patients 20 through 30 as group 2 (genotype sequential isolates different).

II Patient 22 had bilateral herpetic keratitis. In patient 5, the first and second isolates were obtained from the left eye, and the third isolate was obtained from the right eye.

IIII – and + indicate that corneal HSV-1 isolates obtained were from the same or contralateral cornea, respectively.

patient in group 1 received a corneal transplant between the sampled recurrences, 4 of the 11 patients in group 2 underwent a PKP during the inter-recurrence period in the same eye from which the sequential corneal HSV-1 isolates were obtained (Table 2; $P = 0.012$). Patient 30 received a corneal allograft between the first and second sampled recurrence.

Discussion

HSVs have the ability to reside in latent form within neurons of the sensory ganglia that innervate the initial site of infection. It is therefore assumed that recurrent herpetic lesions are due to reactivation of the HSV strain acquired during the primary infection.[1,4-6] In contrast, HSV superinfection in patients with recrudescent herpetic lesions has been documented.[6,7] Patients with recurrent

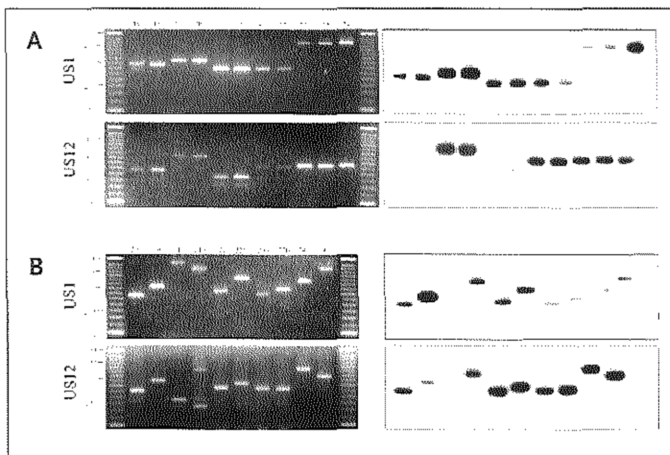


Figure 2.
Amplicons of the hypervariable regions *US1* and *US12* amplified from sequential corneal HSV-1 isolates from patients with RHK. *Left*: Amplicons were electrophoresed on 2.5% agarose gels and were visualized by ethidium bromide staining. Representative sequential samples (a, b, and c) of 10 patients are shown: patients 1 through 5; (**A**) group 1, and patients 20 through 24; (**B**) group 2. A 25-bp molecular size marker was run in parallel. Numbers on the *left* are in base pairs. *Right*: autoradiograph of DNA in gel after Southern blot hybridization with appropriate reiteration-specific probe.

herpetic keratitis risk the development of HSK, a leading cause of corneal blindness worldwide.[2,3] The objective of the present study was to examine the two types of origins and risk factors involved in corneal HSV-1 superinfection in 30 patients with HSV-1–induced RHK.

Genotypic analyses of sequential corneal HSV-1 isolates from 30 patients with RHK demonstrated that 63% of the patients (patients 1–19; designated as group 1) had evidence of reactivation of the same HSV-1 strain. From five patients in group 1, the isolates were obtained from separate eyes. HSV-1 infection of the contralateral cornea most likely occurred through the external route (cross-infection). It was interesting that sequential isolates of 37% of the patients (patients 20–30; designated as group 2) had a different genotype, suggesting corneal HSV-1 superinfection in the inter-recurrence period.

Alternatively, the instability of the analyzed hypervariable regions may account for these differences. HSVs, similar to other DNA viruses, have less genomic variability than RNA viruses and are genetically more stable after in vitro passages. [11,15] In addition to standard RFLP, several hypervariable regions within the HSV-1 genome have been used to differentiate HSV-1 isolates genetically.[11] Intratypic variation of the regions results from differences in the number of Re and point mutations.[10,12,13] The stability of the eight HSV-1–specific Re regions described varies extensively.[11] Genotypic analyses of HSV-1 single-plaque clones compared with their parental strain have shown that the hypervariable regions located within the HSV-1 genes *US1*, *US12*, and *US10/11* remain stable during in vitro culture.[13,15] Moreover, the mean inter-recurrence period of patient group 1 (30.4 months) and the proofreading activity of *Pfu* DNA polymerase, implies that the intraindividual HSV-1 genotype differences are most likely not due to a genetic alteration of the initial strain or errors in amplifying these highly GC-

Table 2.

Clinical Characteristics and Cornea Transplantations Performed on Patients with RHK

	Patient[I]	History of HSV Mediated Eye Disease[II]	Diagnosis at Time Point of Sampling[III] Time Point a	Time Point b	Time Point c	PKPs (*n*)	PKPs between Samples[IV]
Group 1	1	None	Blepharitis	Blepharitis		0	No
	2	None	IEK	IEK		0	No
	3	RecISK	IEK	IEK		0	No
	4	RecISK	IEK	IEK		0	No
	5	BilISK	IEK	IEK	IEK	0	No
	6	recISK and uveitis	IEK	NSK	NSK	3	No
	7	recISK	IEK and ISK	IEK and ISK		0	No
	8	NSK in PKP	IEK and ISK	IEK and ISK		3	No
	9	recISK	IEK and ISK	IEK		0	No
	10	Bil/recISK	IEK in PKP	IEK in PKP		1	No
	11	Bil/recISK and uveitis	IEK in PKP	IEK in PKP		2	No
	12	NewHSV in PKP	IEK in PKP	IEK in PKP		1	No
	13	NSK in PKP	IEK in PKP	NSK in PKP		2	No
	14	recISK	NSK in PKP	IEK in PKP		1	No
	15	newHSV in PKP	NewHSV in PKP	IEK in PKP		2	No
	16	NSK	IEK and uveitis	IEK		0	No
	17	recISK in PKP	IEK in PKP	IEK in PKPwith GR		2	No
	18	None	IEK in ISK	IEK and HKU		0	No
	19	NSK	IEK in PKP	IEK in PKP	IEK in PKP	1	No
Group 2	20	ISK and uveitis	blepharitis	IEK		0	No
	21	Endothelitis	IEK	IEK		0	No
	22	recIEK and ISK in PKP	IEK	IEK		2	No
	23	newHSV in PKP	IEK	IEK		1	No
	24	IEK	IEK	NSK		0	No
	25	NSK in PKP	IEK in PKP	IEK in PKP		2	*Yes*
	26	NSK	IEK in PKP	IEK in PKP		1	No
	27	NSK in PKP	IEK and ISK	IEK and ISK		3	*Yes*
	28	NSK in PKP and uveitis	IEK and uveitis	IEK and uveitis		1	No
	29	NSK in PKP	ISK in PKP	IEK in PKP		3	*Yes*
	30	newHSV in PKP	NSK in PKP	ISK in PKP	NSK in PKP	6	*Yes*

I The sequential corneal HSV-1 isolates of patients 1 through 19 (designated as group 1) and 20 through 30 (designated as group 2) hadidentical or different genotypes, respectively.

II HSV-mediated corneal diseases diagnosed were: immune stromal keratitis (ISK), necrotizing stromal keratitis (NSK), infectious epithelial keratitis (IEK) and herpetic keratouveitis (HKU). PKP, penetrating keratoplasty; LKP, laminar keratoplasty, and GR, graft rejection; rec, recurrent; bil, bilateral; new, newly acquired.

III Total number of preceding PKPs performed on each patient.

IV PKP between sample dates in the patient cohort with identical versus different HSV-1 genotypes of the sequential corneal HSV-1 isolates were statistically significant (Fisher exact test; P = 0.012). In patient 30 PKP was performed between time points a and b.

rich DNA sequences, respectively.

Analogous to our study, reinfection with new HSV-2 strains has been described in two of three patients with recurrent HSV-2 genital herpes.[7] The latter study and our data indicate that HSV superinfection is not as rare as previously suggested.[4–6] To differentiate HSV strains, most investigators have used RFLP analyses with 6-bp recognizing restriction enzymes (REs).[4–6] The lower efficacy of 6-bp RE, compared with the 4-bp RE, to differentiate HSV-1 strains may account for the different frequencies of HSV superinfection described.[11]

Generally, corneal HSV-1 infection results in the development of herpetic epithelial keratitis in approximately two thirds of patients.[2] In the present study, however, the patient cohort consisted predominantly of patients with severe entities of herpetic keratitis (Table 2). Selection of individuals with a higher susceptibility for corneal HSV-1 infection may have occurred. Alternatively, the patients in group 2 may have been superinfected with a more virulent HSV-1 strain.

Among the clinical data analyzed, only the time point of PKP was significantly different between the patient groups. Although no patients in group 1 had undergone transplantation between sampling, 4 of 11 patients in group 2 underwent PKP during the inter-recurrence period in the same eye from which the corneal HSV-1 isolates were obtained. The data suggest that PKP is a risk factor for corneal HSV-1 superinfection. Primary graft failure and endothelial abnormalities of cultured eye bank corneas have been associated with the presence of HSV-1 DNA in affected corneal allografts.[16] The high prevalence of HSV-1 DNA in eye bank corneas (~ 10%)[16] has led to the hypothesis of HSV-1 latency in corneas. Although expression of HSV-1 latency-associated transcript, a marker of latency, has been detected in latently infected rabbit corneas and human HSK corneas, corneal HSV-1 latency remains controversial.[16,17] Recently, Zheng et al.[18] have demonstrated HSV-1 transmission through PKP in an experimental rabbit model. HSV-1 DNA was detected in recipient corneal rims and the innervating trigeminal ganglion (TG) of naive rabbits that received corneal allografts from latently infected rabbits. Moreover, infectious HSV-1 was recovered from the tear film of the rabbits that had undergone transplantation.[18] Besides true ocular viral latency, putative HSV-1 transmission through PKP may be due to coincidental shedding of small amounts of infectious virus from the allograft or a low level of viral replication in corneal resident cells in the allograft at time of PKP.[18,19]

Alternatively, the TG may harbor a mixture of HSV-1 strains with which the patients were previously latently infected, before PKP. In animal model studies, corneal trauma (similar to PKP) has been shown to induce reactivation of HSV-1 causing corneal HSV-1 infection.[20,21] Assuming that the human TG can be latently infected with multiple HSV-1 strains, PKP may serve as a powerful reactivation stimulus to certain portions of the TG, allowing multiple strains to reactivate.[22]

In conclusion, this study is the first to demonstrate a high frequency of corneal HSV-1 superinfection in patients with RHK. Although we could not determine the source or mode of corneal HSV-1 superinfection in patient group 2, the data suggest that PKP may be a risk factor for transmission of HSV-1 with subsequent reactivation of the donor-derived HSV-1 strain in the corneal allograft. Recently, we have genetically characterized HSV-1 DNA isolated from a donor cornea before and after PKP in a patient with newly acquired herpetic keratitis. The DNA sequences were identical in both strains, providing conclusive evidence for graft-to-host transmission of HSV-1 through corneal allograft.[23]

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Herpes simplex virus 1 transmission through corneal transplantation

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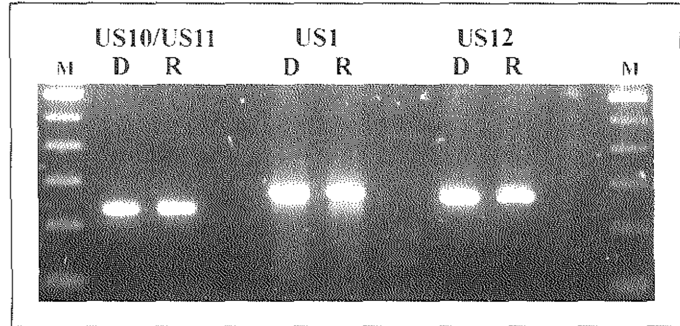
Genetic characterization of herpes simplex virus type 1 (HSV-1) DNA isolated from a donor cornea before and after corneal transplantation demonstrated the transmission of HSV-1 through transplantation. This study is the first to provide conclusive evidence for the transmission of HSV-1 by penetrating keratoplasty with subsequent reactivation of donor-derived HSV-1 in the transplanted cornea.

Herpetic keratitis is a leading cause of blindness worldwide[1]. The current therapy of choice is penetrating keratoplasty (PKP), which is performed to restore sight in eyes blinded by corneal opacity arising from infection, trauma, inherited or acquired disease. Infectious agents that have been reported to be transmitted by PKP are the Creutzfeldt-Jakob agent, hepatitis B and C virus, rabies virus, and cytomegalovirus[2]. Corneal graft donors are routinely screened for these pathogens, but not for the presence of HSV in their corneas. HSV DNA has, however, been detected in corneas with no signs of infection and eye bank donor corneas[3]. In patients without a clinical history of HSV keratitis, the emergence of herpetic keratitis has been documented after PKP, suggesting graft-to-host transmission of HSV through PKP[3,4].

A 28-year-old man with congenital glaucoma underwent a third PKP in his only functional eye. He had undergone the first PKP to treat corneal opacity from congenital glaucoma. The second and third PKP were done because the previous graft was rejected. Postoperative treatment consisted of 0.5% prednisolone eye drops eight times daily. Immediately after surgery, an epithelial defect developed for which recovery was slow despite additional treatment with bandage lenses and frequent lubrication with autologous serum. Three months post PKP, a large epithelial defect recurred from which HSV-1 was isolated (data not shown). After 3 months, the combination of uncontrollable herpetic keratitis and choroidal effusion resulted in loss of vision.



Amplification of hypervariable regions of the HSV-1 genes US10/US11, US1 and US12 obtained from the donor-derived corneoscleral rim (D) and post-PKP corneal HSV-1 isolate of the recipient (R). Sequences on Genebank; accession numbers AF324427 and AF324428 for the US1 and US12 sequences. M=100 bp molecular marker.

HSV-1 DNA was amplified by PCR from the transplanted corneoscleral rim and the patient's serum was tested retrospectively for HSV antibodies. No HSV-specific IgM or IgG were present in the patient's serum before surgery (data not shown). We hypothesized that HSV-1 was transmitted from the graft to the recipient with subsequent reactivation of donor-derived HSV-1 in the transplanted cornea. To test this assumption, the donor (D) and recipient (R) derived HSV-1 strains were genotyped using a PCR-based DNA fingerprint assay. This method is based on strain-to-strain differences in the number of DNA repeats and point mutations within the hypervariable regions of the HSV-1 genes US10/US11, US1 and US12[5]. The PCR products of the D and R derived HSV-1 strains were of the same size (Fig.). The US1 and US12 PCR products were then sequenced. These DNA sequences were identical for both

strains, with multiple unique mutations as compared to the corresponding sequences of the HSV-1 F laboratory strain (data not shown).

Donor-to-host transmission of infectious agents via corneal transplantation poses a real risk. Corneal donors are currently screened for several viruses, and bacterial and fungal cultures are done before transplantation in most eye banks. Although the prevalence of HSV-1 DNA in eye bank corneas is high (~10%),[1] only a minority of patients receiving such contaminated corneas have been shown to develop HSV-related eye disease[3,4] This may result from the antiviral immunity of the recipient following a preceding HSV-1 infection. Alternatively, it may be due to low viral loads or poor reactivation of putatively latent virus in the allograft. The latency of extraneuronal HSV-1 in the cornea is not well-characterised, but it is generally assumed that post-PKP HSV keratitis arises from reactivation of the recipient's HSV-1[1,3] However, HSV-1 related graft morbidity in patients without a previous history of HSV infection has also been described, implicating the possibility of HSV-1 transmission by PKP[3,4].

This HSV-naive patient lost his vision in his only functional eye due to graft-to-host transmission of HSV-1, which resulted in graft failure in combination with choroidal effusion after glaucoma surgery. To our knowledge, this is the first report to provide conclusive evidence for graft-to-host transmission of HSV-1 through a corneal allograft. Although apparently rare, this mode of transmission of HSV-1 should be taken seriously. Transmission of HSV-1 to a seronegative recipient may be particularly dangerous, as the virus may cause potentially blinding eye infections in patients undergoing immunosuppressive treatment. To prevent HSV-1 transmission to HSV-1 naive recipients, serological matching of donor and recipient is an option. If an HSV-seronegative patient urgently requires PKP, and no seronegative donor is available, prophylactic acyclovir should be considered, particularly during immunosuppressive therapy. Our observations stress the need for further studies on the localization and latency of HSV-1 in corneoscleral tissue.

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Screening for HSV-1 infection in corneal transplantation

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Submitted

Penetrating keratoplasty (PKP) is currently the most common form of allografting. Five to 10% of PKPs are performed for corneal opacities resulting from herpes simplex virus type-1 (HSV-1) infection. About 0.9% of PKP recipients who have not suffered from herpetic keratitis before, develop superficial epithelial defects due to HSV-1 infection[1]. An unknown number of additional cases of stromal and endothelial keratitis resembling allograft rejection, are caused by HSV-1 infection. A recent study in 25 patients with corneal HSV-1 infection after PKP, showed that the majority (n=14) developed severe ocular complications resulting in unilateral social blindness within three years (L.R. et al unpublished).

Corneal HSV-1 infection after PKP may be due to reactivation of latent virus or horizontal viral transmission. Most corneal HSV-1 infections after PKP are believed to result from virus reactivation. However, similar forms of penetrating corneal surgery do not result in a comparable incidence of herpetic keratitis. Given an annual HSV-1 infection rate among adults of 5-10% per year, with ocular involvement in about 1% the risk of ocular HSV-1 infection through horizontal spread should be less than 0.1%. Consequently, HSV-1 transmission through PKP, which we recently demonstrated to occur[2], is a real risk. The demonstration of HSV-1 DNA in 10-20% of normal eye-bank corneas[3], further highlights this risk. In HSV-1 seronegative recipients the consequences may be more serious: no pre-existing immunity against the virus is present and the local immunosuppression by the use of topical steroids aggravates this situation. In a worst case scenario, with 10 to 20% of the donor corneas indeed harbouring infectious HSV-1, and taking into account the age distribution of donors and recipients, the overall risk of HSV-1 transmission by PKP would amount to 1.8% for Western Europe, 3.0% for the USA black and 3.5% for the USA white population (Fig.).

To prevent HSV-1 transmission to seronegative patients by PKP, systemic acyclovir treatment for a period that exceeds the immunosuppressive treatment is an option. This implies testing of all recipients for HSV-1 antibodies. Matching of seronegative recipients with seronegative donors, whenever logistically possible, would be an even more attractive option, obviating the need for prophylactic antiviral treatment. The devastating consequences for especially seronegative PKP recipients, and the problems associated with prolonged systemic antiviral treatment, indicate that routine serological screening and whenever possible matching of recipients and donors for HSV-1 status should be implemented in corneal transplantation.

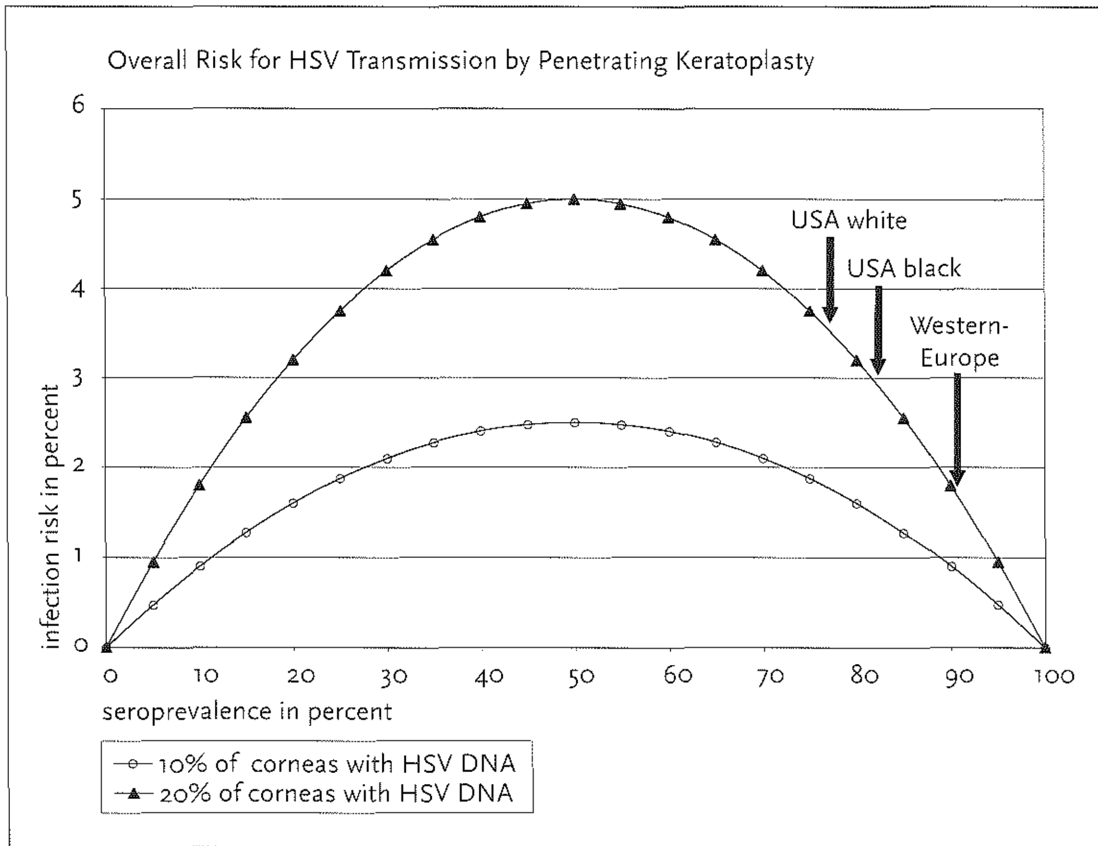

Figure: Overall risk for HSV-1 infection through PKP as a function of seroprevalence in the donor- and recipient population, with a postulated 100% transmission rate from DNA positive corneas.

White arrows indicate the calculated overall infection risk. Overall seroprevalence is corrected for age composition of donor and recipient population in the different areas[4,5].

Age distribution of corneal donors and recipients was taken in to account, using data from the Dutch Register for Corneal Transplantation.

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CHAPTER 4

Immunopathogenesis of Human Herpetic Stromal Keratitis

Identification and Characterization of Herpes Simplex Virus-Specific CD4+ T Cells in Corneas of Herpetic Stromal Keratitis Patients

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Summary

Herpetic stromal keratitis (HSK) is a corneal disease initiated by a herpes simplex virus (HSV) infection with a postulated T cell-mediated immunopathology. To study the antigen specificity of cornea-infiltrating T cells in HSK patients, T cells were isolated and expanded by mitogenic stimulation from corneas of 2 patients with HSV-1- mediated HSK. A substantial number of the T cell clones (TCCs) obtained from these T cell lines were HSV-specific. All HSV-specific TCCs were of the $CD3^-CD4^+CD8^-$ phenotype. These TCCs responded to autologous HSV-infected corneal keratocytes, which expressed HLA class II molecules following incubation with interferon-γ. Upon HSV-specific stimulation, all TCCs secreted interleukin-4, interleukin-5, and interferon-γ. The data presented suggest that HSV-specific $CD4^+$ T cells play a role in the immunopathogenesis of HSK in humans and that corneal keratocytes may act as antigen-presenting cells in this local T cell response.

Introduction

Recurrent herpes simplex virus (HSV) infections of the cornea can lead to tissue-destructive inflammation of the corneal stroma. This disease, known as herpetic stromal keratitis (HSK), is a leading infectious cause of corneal blindness world-wide. The stromal pathology seen in HSK patients is most probably not due to the direct cytopathic effect of the virus but more likely the result of a local cellular immune response (reviewed in[1]). Studies in the mouse model of HSK have shown that $CD4^-$ T cells, possibly HSV-specific, that secrete type 1 cytokines (i.e., Th1 cells) play a pivotal role in the immunopathology of this disease[1-4].

Studies on T cell involvement in the immunopathogenesis of HSK in humans is limited to immunohistologic analyses and phenotypic characterization of isolated intracorneal T cells[5,6]. In the present study, a protocol was developed that enabled the expansion and functional characterization of intracorneal T cells obtained from 2 HSK patients.

Materials and Methods

Clinical material and reagents.

Corneal buttons and peripheral blood mononuclear cells (PBMC) were obtained from 2 patients, 50- (patient 1) and 59-year-old (patient 2) men, following therapeutic penetrating keratoplasty. Although no HSV could be isolated, HSV-1- but not HSV-2- or varicella-zoster virus (VZV)-specific DNA was detected by polymerase chain reaction (PCR) in the corneal tissue of both patients (data not shown). On the basis of these observations, ocular examinations, and the clinical history of both patients, the lesions were diagnosed as HSV-1- mediated ulcerative necrotizing HSK. The patients were HLA-typed.

Isolation of PBMC and the generation of B lymphocyte cell lines (BLCLs) by transformation with Epstein-Barr virus (EBV) were performed as described[7]. Virus stocks of the MacIntyre strain of HSV-1 (ATCC VR-539) and the MS strain of HSV-2 (ATCC VR-540) were generated on Vero cells.

Generation of T cell lines (TCLs) and T cell clones (TCCs).

Intracorneal T cells were isolated from corneal buttons by treatment of disrupted corneal specimens with collagenase essentially as described previously[2]. The cells were resuspended in RPMI 1640 supplemented with antibiotics and 10% heat-inactivated pooled human serum, referred to as complete medium, which contained 50 U/mL human recombinant interleukin-2 (rIL-2; Eurocetus,

Amsterdam). The cell suspension was incubated in a 25-cm^2 flask for 1 week. The nonadherent cells were harvested, resuspended in complete medium containing rIL-2 and 1 µg/mL phytohemagglutinin-L (PHA-L; Boehringer Mannheim, Almere, The Netherlands), and divided over 20 wells of a 96-well round-bottomed microtiter plate. As feeder cell mixture, each well received 10^5 gamma-irradiated (3000 rad) allogeneic PBMC from healthy donors and 5×10^3 gamma-irradiated cells of both the BLCLs APD (International Histocompatibility Workshop, IHW 9291) and BSM (IHW 9032). In parallel, PBMC-derived TCLs were generated by the same protocol using different starting concentrations of PBMC (i.e., 5×10^5 or 1×10^5). All wells of the stimulated PBMC- and cornea-derived T cells showed T cell growth and were pooled. These cornea-derived TCLs were cloned by limiting dilution. The TCCs were restimulated every 10-14 days with PHA-L and the allogeneic feeder cell mixture.

Corneal keratocyte cultures.

The adherent corneal cell cultures were maintained in 25-cm^2 flasks in keratocyte medium, consisting of SHEM (Dulbecco's MEM/F12[1:1], Gibco Laboratories, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Growing primary keratocytes, with a fibroblast-like morphology, were passaged several times and cryo-preserved. By immunofluorescence staining, all cells stained positive for vimentin, whereas almost no reactivity was found for both the acidic and basic subfamilies of cytokeratin (data not shown). This indicates that the keratocyte cultures were not contaminated with corneal epithelial or endothelial cells. All experiments were performed with keratocytes between passages 3 and 6.

HLA class II expression on keratocytes was induced by incubation with 500 U/mL recombinant human interferon-γ (rIFN-γ; Preprotech, Breda, The Netherlands) for 4 days.

T cell proliferation assays.

BLCL and rIFN-γ-pretreated keratocytes were infected with either HSV-1 or HSV-2 at an MOI of 1-3 for 20 h; >90% of these cells were shown to be infected (data not shown). The virus- and mock-infected BLCL were UV- irradiated (2.5×10^{-2} mW/mm^2). The keratocytes were fixed with 0.5% paraformaldehyde as described previously[8]. About 3×10^4 T cells, at 10-12 days after restimulation with PHA-L, were cultured in triplicate with 2×10^4 virus- or mock-infected cells in 96-well tissue culture plates in complete medium for 72 h. Over the last 18 h of culture, the cells were pulsed with 0.5 µCi of [^{3}H] thymidine. The incorporated radioactivity was determined as described[7].

^{51}Cr release cytotoxicity assay.

Cytotoxicity assays were performed in triplicate in 96 V-bottom plates, as described previously[7]. Briefly, target cells were prepared as described above. Effector cells were added to 5×10^3 target cells at an effector-to-target ratio of 30:1. Plates were incubated for 4 h at 37°C. Supernatants were harvested, and the ^{51}Cr content was measured in a gamma counter. The percentage of specific ^{51}Cr release was calculated as described previously[7].

T cell receptor (TCR) variable (V) gene α and β usage of the T cells.

The T cell repertoire and clonality of the cornea-derived TCLs or TCCs, respectively, were determined by analyzing the TCR V gene α and β usage as described previously[9]. Briefly, total RNA was isolated from ~5×10^6 T cells. cDNA was synthesized using oligo-dT and reverse transcriptase (RT) (Boehringer Mannheim) and subsequently subjected to

Table 1. Antigen specificity, T cell receptor (TCR) variable (V) gene usage, and cytokine secretion levels of T cells obtained from herpetic stromal keratitis (HSK) patients

Patno.	T cell	TCR usage		[^{3}H]-thymidine incorporation (cpm)			cytokine secretion levels (pg/ml)			
		Vα	Vβ	mock	HSV-1	HSV-2	IL-2	IL-4	IL-5	IFN-γ
1	TCL (PB)	ND	ND	1057 ± 123	1401 ± 151	ND	52	63	145	892
	TCL (cornea)	diverse		1260 ± 338	8749 ± 1040	ND	ND	ND	ND	ND
	TCC 8	2	12.3	136 ± 15	12264 ± 713	115 ± 27	<10	47	68	373
	TCC 43	2	12.3	77 ± 3	15047 ± 813	96 ± 33	24	72	280	456
	TCC 69	2	12.3	64 ± 7	8979 ± 261	70 ± 11	68	84	196	1268
	TCC 94	2	12.3	57 ± 11	10016 ± 395	67 ± 14	22	108	770	475
	TCC 101	2	12.3	50 ± 10	18760 ± 1031	82 ± 13	100	31	107	1656
	TCC 111	ND	ND	64 ± 5	16159 ± 484	64 ± 6	34	39	48	576
	TCC 114	14	3	62 ± 4	8747 ± 353	261 ± 43	ND	ND	ND	ND
	TCC 118	2	12.3	62 ± 11	7101 ± 127	74 ± 19	<10	37	52	374
	TCC 138	2	12.3	52 ± 6	20907 ± 887	54 ± 6	87	33	44	1265
	TCC 171	2	12.3	65 ± 3	20671 ± 1983	102 ± 4	<10	85	112	364
	TCC 180	7	2	36 ± 12	1512 ± 32	361 ± 44	ND	ND	ND	ND
	TCC 200	12	4	64 ± 9	15884 ± 749	83 ± 19	142	310	277	3199
2	TCL (PB)	ND	ND	1236 ± 463	1791 ± 325	ND	32	91	156	1088
	TCL (cornea)	diverse		206 ± 36	32723 ± 702	ND	ND	ND	ND	ND
	TCC 4	1	5.1	48 ± 9	12517 ± 232	15033 ± 577	20	39	124	1415
	TCC 12	1	5.1	63 ± 7	7470 ± 347	14861 ± 289	<10	788	419	978
	TCC 24	1	5.1	66 ± 17	9175 ± 121	12784 ± 316	<10	763	1109	720
	TCC 28	ND	ND	61 ± 2	1803 ± 112	2915 ± 25	14	61	27	444
	TCC 32	1	5.1	308 ± 13	12966 ± 315	19041 ± 240	48	116	215	1618
	TCC 33	1	5.1	255 ± 8	15201 ± 547	13383 ± 397	ND	ND	ND	ND
	TCC 68	1	5.1	48 ± 12	10252 ± 523	13246 ± 344	18	90	194	609
	TCC 69	1	5.1	60 ± 16	19864 ± 852	21305 ± 465	110	47	239	1446
	TCC 79	14	10	88 ± 9	2236 ± 104	701 ± 70	<10	19	52	392
	TCC 105	1	5.1	54 ± 16	10200 ± 115	10178 ± 294	26	52	150	812

NOTE:

T cells tested were peripheral blood (PB)- or cornea-derived T cell lines (TCLs) and T cell clone (TCC). TCR Vα and Vβ gene usage was determined by reverse transcriptase-polymerase chain reaction. Diverse, heterogeneous TCR Vα and Vβ gene usage; ND, not done; IL, interleukin; IFN, Interferon; T cells were incubated with mock- or HSV-1- or HSV-2-infected autologous B cells. Data are expressed as mean cpm ± SD of triplicate cultures. Cytokine secretion levels were determined in culture supernatants of T cells incubated for 40 h with autologous HSV-1-infected B cells. Cytokines were undetectable following incubation with mock-infected BLCL (data not shown)

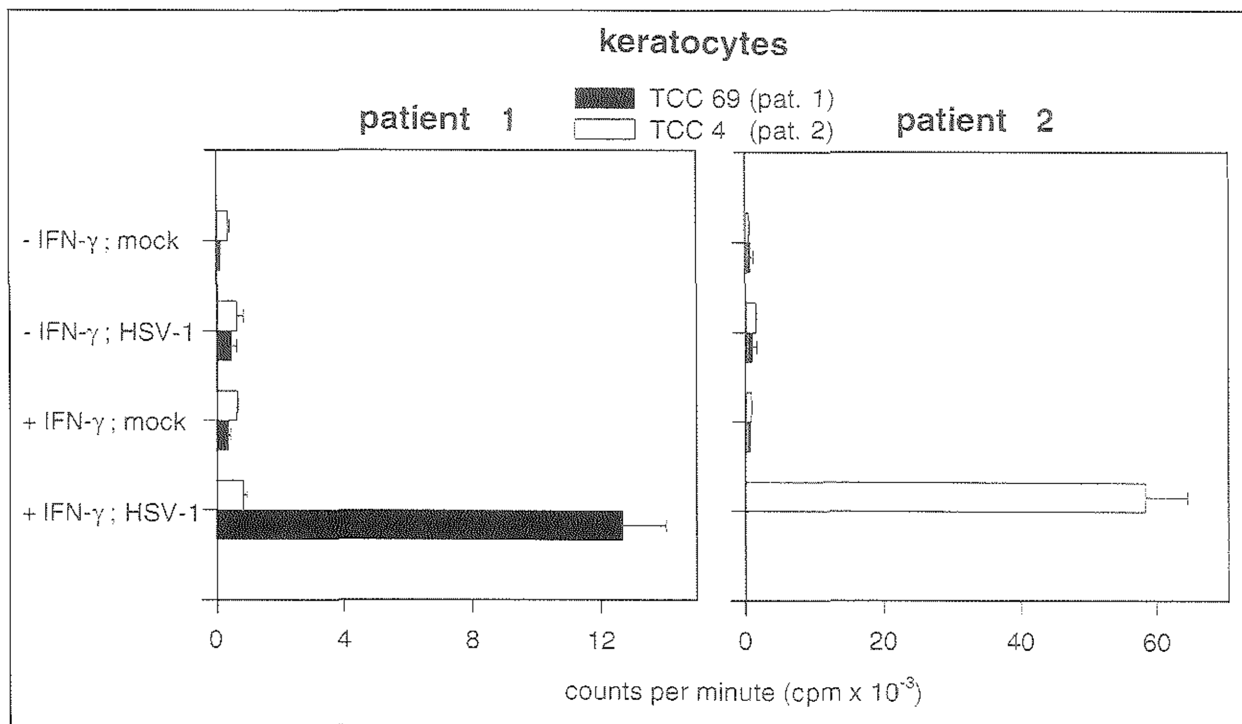

Figure 1.

Proliferative response of HLA-DR- restricted HSV-specific intracorneal CD4⁻ T cell clones (TCCs) of patient 1 and 2 to HSV-1-infected DR⁻ autologous corneal keratocytes. Adherent keratocytes of patient 1 and 2 were either left untreated (–IFN-γ) or were induced to express HLA class II (+IFN-γ) with IFN-γ. Subsequently, these cell were either mock- or HSV-1- infected, fixed with paraformaldehyde, and incubated with TCCs from patient 1 (TCC 69) or 2 (TCC 4). After incubation with [³H]thymidine was added, and [³H]thymidine incorporation by T cells was measured. Results are expressed as mean count per min (cpm) ± SD of triplicate cultures.

PCR amplification using, separately, 30 Vα or 23 Vβ gene (sub)family-specific 5' primers in combination with a 3' constant gene region α- or β- specific primer, respectively. The PCR products were characterized by Southern blot analysis. Alternatively, TCR Vβ gene usage was determined by flow cytometry using a panel of Vβ specific monoclonal antibodies that cover 60%- 70% of CD3⁻ peripheral blood T cells[10].

Cytokine secretion by TCCs.

To determine the levels of cytokines secreted by the TCCs, TCCs and autologous HSV-l- or mock-infected BLCL were incubated as described for T cell proliferation assays. Upon incubation for 40 h, cell-free culture supernatants were assayed in parallel for IL-2, IL-4, IL-5, and IFN-γ using compact ELISA kits according to procedures recommended by the manufacturer (CLB, Amsterdam, The Netherlands).

Results

To analyze the antigen specificity of T cells located in inflamed corneas of HSK patients, an experimental procedure was developed to generate TCLs from corneas. Following one round of stimulation of the nonadherent

corneal cell fraction with PHA-L and irradiated feeder cells, TCLs were obtained from 2 HSK patients. Similarly, TCLs were generated from the PBMC of these patients. Simultaneously, autologous adherent corneal keratocyte cell lines were obtained. All TCLs consisted predominantly of $CD3^+$ cells. The cornea-derived TCLs of patients 1 and 2 contained 68% and 84% $CD4^+$ and 23% and 11% $CD8^+$ T cells, respectively. The corresponding PBMC- derived TCLs consisted of 36% and 16% $CD4^+$ and 47% and 77% $CD8^+$ T cells, respectively.

In contrast to the PBMC-derived TCLs, the cornea-derived TCLs of both HSK patients demonstrated an HSV-specific proliferative response (table 1). TCCs were obtained from the cornea-derived TCLs by limiting dilution. Twelve of 115 and 10 of 58 TCCs obtained from patients 1 and 2, respectively, were HSV-specific. Whereas almost all TCCs from patient 1 were solely HSV-1-specific, the TCCs from patient 2 appeared to be HSV-1 and HSV-2-cross-reactive (table 1). All HSV-specific TCCs were $CD3^-CD4^+CD8^-$ and HLA-DR-restricted. Upon stimulation with HSV-l-infected BLCL, all TCCs tested secreted significant amounts of IL-4, IL-5, and IFN-γ. Not all TCCs secreted IL-2 (table 1).

The T cell repertoire of the cornea-derived TCLs and the clonality of the TCCs were determined by analyzing their TCR Vα and β gene usage by RT -PCR and Vβ-specific monoclonal antibodies. A heterogeneous TCR V α/β gene repertoire was observed in the TCLs of both patients (data not shown). The panel of TCCs of patients 1 and 2 consisted of 4 and 2 genetically different TCCs, respectively. The predominance of one TCC expressing TCR Vα2 and Vβ12.3 or Vα1 and Vβ5.1 was observed among the TCCs of patients 1 and 2, respectively (table 1). Sequence analysis of the TCRα and β hypervariable regions (i.e., CDR3) revealed that these TCCs were identical (data not shown).

In addition to professional APC-like Langerhans' cells and macrophages, HSV-infected HLA class II-expressing keratocytes may present antigenic peptides to cornea-infiltrating $CD4^-$ HSV-specific T cells. Although keratocytes, both in vivo and in vitro, do not normally express HLA class II molecules on the cell surface, their expression can be induced by IFN-γ[11]. The TCCs of both patients recognized only autologous HLA-DR^- HSV-l-infected keratocytes and not HLA-DR- mismatched keratocytes (i.e., keratocytes from the other patient). Results of a typical experiment are shown in figure 1.

Discussion

HSK is an HSV-incited sight-threatening chronic inflammation of the corneal stroma. Viral antigens, as well as the cellular immune response of the host, are thought to contribute to the pathogenesis of HSK[1]. Thus far, the antigen specificity of cornea-infiltrating T cells in HSK patients remained elusive. A procedure was developed that facilitated the outgrowth and subsequent characterization of in vivo-activated intracorneal T cells of 2 HSK patients. The high numbers of HSV-specific TCCs obtained from these cornea-derived TCLs indicate, as was also shown in the HSK mouse model, the infiltration of HSV-specific T cells into the cornea of HSK patients[1,2]. Although it is generally accepted that noninflamed corneas are virtually devoid of T cells, experiments using corneas from non-HSK patients would have provided additional evidence for the observed compartmentalization of HSV-specific T cells in corneas of HSK patients (however, as for HSK corneal buttons, these tissue specimens are hard to obtain). The predominance of one HSV-specific TCC in both patients is not surprising given the low numbers of T cells isolated from the corneal buttons. The number of HSV-specific T cells in the PBMC- derived TCLs of the HSK

patients was most likely too low to be detected in the proliferative assays.

Although the cornea-derived TCLs consisted of both CD4+ and CD8+ T cells, only CD4+ HSV-specific TCCs could be identified. Similar results have been reported for the HSK mouse model[1,3]. In repeated experiments, we did not observe cytolytic activity of the cornea-derived TCLs and TCCs towards autologous HSV-l-infected BLCL (data not shown). This observation is in contrast to those from the mouse model[1]. Using similar protocols, we have isolated cytolytic HSV- specific CD4+ T cells from intraocular fluid samples of patients with HSV-incited acute retinal necrosis (unpublished data). This suggests the involvement of a functionally different subset of CD4+ HSV-specific T cells in the pathology of HSK compared with acute retinal necrosis. Heterogeneity in cytolytic properties of human HSV-l-specific CD4+ TCCs has been described for PBMC-derived TCCs[12].

In the HSK mouse model, type-l cytokines such as IFN-γ predominated during the active phase of disease. Type-2 cytokines, such as IL-10 and IL-4, tend to be associated with the recovery phase of HSK[4]. This is consistent with the observation that IFN-γ contributes to corneal inflammation[13]. As described for human PBMC-derived HSV-1-specific CD4+ T cells[12], all cornea-derived HSV-specific TCCs secreted IL-4, IL-5, and IFN-γ upon antigenic stimulation, indicating their Th0 phenotype.

In contrast to findings in the HSK mouse model, HSV antigens and HLA class II-expressing keratocytes can be detected during the active phase of the disease in corneas of HSK patients[6,11]. In particular, in patients with ulcerative necrotizing HSK, HSV antigens have been detected in stromal keratocytes and the extracellular stroma[6]. Of interest, both HSK patients in the present study suffered from ulcerative necrotizing HSK. As described for human skin-derived keratinocytes[13], IFN-γ-pretreated corneal keratocytes were able to present HSV-1 antigen via HLA class II to HSV-specific CD4+ T cells.

Although viral replication is required for the induction of HSK, viral antigens and mRNA are undetectable in corneas of mice at the time of clinical manifestation[15]. The local T cell-mediated inflammatory response in HSK may therefore be directed against nonviral antigens (e.g., corneal autoantigen)[16]. A preliminary screening of the cornea-derived TCLs of both HSK patients did not reveal T cell reactivity against human corneal autoantigens (data not shown). Although this observation does not support the role of corneal autoantigen- specific T cells in the immunopathogenesis of human HSK, we cannot exclude this option.

In conclusion, this study is the first to demonstrate the presence of a T cell response specific for the inciting agent in corneas of HSK patients. In addition to T cells, neutrophils extensively infiltrate HSK corneas. The extravasation of neutrophils, which contribute to viral clearance and the subsequent destruction of corneal tissue, has been suggested to be regulated by IFN-γ[14]. We hypothesize that the immunopathogenic role of intracorneal HSV-specific CD4+ T cells in HSK is mediated by their secretion of IFN-γ upon activation by infected macrophages, Langerhans' cells, or possibly keratocytes. This may trigger neutrophil extravasation and subsequent neutrophil-mediated tissue destruction.

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Herpes Simplex Virus Specific T-Cells Infiltrate the Cornea of Patients with Herpetic Stromal Keratitis (HSK): no Evidence for Intra-Corneal Autoreactive T-Cells in Human HSK

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Summary

Purpose:

Herpetic stromal keratitis (HSK) is a T-cell-mediated inflammatory disease initiated by a herpes simplex virus (HSV) infection of the cornea. Recently, studies in the HSK mouse model have shown that the immunopathogenic T-cells are directed against the HSV protein UL6 cross-reacting with an unknown corneal autoantigen. We have analyzed whether this type of autoimmunity may play a role in human HSK.

Methods:

T-cell lines (TCL) were generated from corneal buttons of 12 patients with different clinical stages of HSV-induced necrotizing stromal keratitis (n= 9) or immune stromal keratitis (n= 3). The initiating virus was identified by PCR and immunohistology performed on the corneal buttons. Peripheral blood mononuclear cells (PBMC) were isolated and B cell lines (BLCL) were generated by transformation with Epstein Barr virus. Proliferative responses of these intra-corneal TCL were determined by culturing T-cells with autologous BLCL infected with HSV-1-, HSV-2-, wild type vaccinia virus (VV-WT) or VV expressing HSV-1 UL6 (rVV-UL6). Alternatively, T-cells were incubated with PBMC pulsed with human cornea protein extract.

Results:

Irrespective of clinical diagnosis or treatment, T-cells were recovered from the corneal buttons of all the 12 HSK patients. The intra-corneal TCL of 9 out of the 12 HSK patients showed HSV-specific T-cell reactivity. In none of the TCL, T-cell reactivity against HSV-1 UL6 or human corneal antigens was detected.

Conclusions:

The data suggest that the potentially immunopathogenic intra-corneal T-cell response in HSK patients is directed to the initiating virus and not to a human corneal autoantigen or HSV-1 UL6.

Introduction

Herpes simplex virus (HSV) infections of the cornea can elicit the development of herpetic stromal keratitis (HSK). HSK is a sight-threatening disease in which tissue destruction, edema and corneal scarring are the result of an inflammatory response in the corneal stroma. Current knowledge on the immunopathogenesis of HSK is largely based on studies performed in the experimental mouse model for HSK that closely mimics necrotizing stromal keratitis in man. In the HSK mouse model, CD4+ Th1 cells have been demonstrated to play a pivotal role in this local immunopathogenic response[1,2]. The other cell types involved are Langerhans cells (LC)[3], macrophages and notably polymorphonuclear neutrophils (PMN)[4,5]. The nature of the antigens recognized by these immunopathogenic cornea infiltrating T-cells is a matter of debate. A long-standing assumption has been that the intra-corneal T-cell response in HSK is directed to HSV-encoded antigens[6]. Recently, however, studies in the HSK mouse model have provided evidence that HSK is an HSV-induced autoimmune disease[7,8]. HSK could be induced by CD4+ T-cells directed to an epitope derived from the HSV-1 capsid protein UL6, that cross-reacts with an epitope of an antigen uniquely expressed in the murine cornea[8].

Recently, we have demonstrated the presence of HSV-specific CD4+ Th0-like cells in corneas of two patients with necrotizing ulcerative HSK. In this study no reactivity to human corneal antigens could be detected[9].

Table 1.

Characteristics of herpetic stromal keratitis patients included in the present study

Patient	Sex[a]	Age[b]	Diagnosis	Disease status	HSV-1 PCR on cornea[c]	Time since keratitis first diagnosed[d]	Time since last recurrence[c]	Topical pre-operative Steroids	Topical pre-operative Acyclovir
1	M	51	Necrotizing stromal keratitis	Active	+	352	1.5	No	Yes
2	M	60	Necrotizing stromal keratitis	Active	+	480	0	Yes	Yes
3	M	71	Necrotizing stromal keratitis	Active	+	4	0	Yes	Yes
4	M	57	Necrotizing stromal keratitis	Active	+	104	0	Yes	Yes
5	M	68	Necrotizing stromal keratitis	Active	+	240	0	No	No
6	F	90	Necrotizing stromal keratitis	Active	+	235	0	No	No
7	M	73	Necrotizing stromal keratitis	Quiescent	+	80	11	Yes	Yes
8	F	31	Necrotizing stromal keratitis	Active	-	66	1.5	Yes	No
9	F	74	Necrotizing stromal keratitis	Quiescent	ND	243	17	?	?
10	F	81	Immune stromal keratitis	Quiescent	+	309	13	Yes	No
11	M	61	Immune stromal keratitis	Active	-	50	1	No	No
12	F	64	Immune stromal keratitis	Quiescent	-	143	37	No	No

a M, male and F, female.

b Age in years.

c + or -, positive or negative HSV-1-specific PCR product detected. ND, not done.

d Time in months. Times since last recurrence means time lapse since presentation of active disease in months, whereas active disease is defined as visible stromal infiltration with edema. In the patients with a time lapse of 1- 1.5 months, patients #1, 8 and 11, the cornea was still showing evident cellular infiltration. Impending perforation made penetrating keratoplasty neccessary.

Nevertheless, autoreactive T-cells may still be involved in clinically distinct HSK entities. In the present paper we have determined the antigen-specificity of cornea infiltrating T-cells, obtained from 12 patients with different clinical forms of HSK. T cell reactivity was tested towards the HSV serotypes 1 and 2, recombinant HSV-1 UL6 and a soluble human corneal protein extract.

Materials and methods

Clinical materials and reagents

Corneal buttons and peripheral blood mononuclear cells (PBMC) were obtained from 12 patients, with HSV-induced necrotizing stromal keratitis (patients #1-9) or immune stromal keratitis (patients #10-12), following therapeutic penetrating keratoplasty. HSK classification, and quiescent disease for at least 6 months or active disease, was defined on the basis of clinical criteria[10]. The characteristics, diagnosis and pre-operative treatment of the patients studied are listed in Table 1. Patient #12 was transplanted because of a corneal graft rejection due to a necrotizing ulcerative HSK, and patients #1 and #2 have been described previously[9]. Isolation of PBMC and the generation of B cell lines (BLCL), by transformation with Epstein Barr virus, were performed as described[9]. Virus stocks of the MacIntyre strain of HSV-1 (American Type

Culture Collection; ATCC VR-539) and the MS strain of HSV-2 (ATCC VR-540) were generated and titrated in Vero cells. Recombinant vaccinia virus (VV) rVV-UL6 expressing the HSV-1 UL6 gene (strain 17) has been described and kindly provided by A.H. Patel (MRC Virology Unit, Institute of Virology, Glasgow, UK)[11]. Virus stocks of the rVV-UL6 and the wildtype strain WR (ATCC VR-1354) were generated and titered on RK13 cells (ATCC CCL-37). Protein was extracted from whole human corneas (n= 12), donated for transplantation but found unacceptable because of senile changes of the endothelium, by sonification of a Tris-buffered cornea tissue lysate as described previously for the generation of a soluble murine cornea protein extract[7,12]. The present study was performed according the Declaration of Helsinki and informed consent was obtained from all patients.

Immunohistochemistry

Corneal buttons, obtained within 1 hr after surgery, were divided in two equal parts for immunohistologic analysis and T-cell recovery. One quarter of the cornea was snap frozen in optimal cutting tissue and one quarter was fixed with formalin and embedded in paraffin. Routine histopathology (hematoxylin/ eosin and periodic acid-Schiff staining) and immunoperoxidase staining was performed on cryostat and paraffin sections as described previously[13]. Mouse anti-human monoclonal antibodies (Mabs) were used as primary antibodies. The following Mabs were used as recommended by the supplier: anti-CD3 (Dako, Glostrup, Denmark), anti-CD4 and -CD8 (Becton Dickinson, USA) and anti-HSV-1 (Dako). Peroxidase-labeled polyclonal rabbit anti-mouse IgG antibody (Dako) was used as secondary antibody and visualized using diaminobenzidine or 3-amino-9-ethyl carbazole (Sigma St. Louis, MO)[13].

DNA extraction and PCR analyses

The surplus half of the corneal specimens were minced and treated with collagenase essentially as described previously[9]. DNA was isolated from one-fourth part of the corneal cell suspension lysed in a guanidine isothiocyanate buffer using Celite solution (Jansen Chemika, Beers, Belgium) according to Boom *et al*[14]. The PCR primers and conditions for detection of HSV type 1 and 2 and varicella zoster virus specific DNA following Southern blotting have been described previously[15].

Cornea-derived T-cell lines

Corneal-derived T cell lines (TCL) were generated from the remaining corneal cell suspension as described previously[9]. After one round of mitogenic stimulation, using phytohemagglutinin-L (PHA-L; Boehringer Mannheim, Mannheim, FRG) and allogeneic feeder cells, the intra-corneal TCLs were frozen in aliquots at −135°C. Control experiments, with corneas histologically devoid of infiltrating T-cells did not result in the generation of TCLs, indicating that the method applied facilitates the recovery and outgrowth of T cells compartmentalized to the cornea (data not shown). The TCL were characterized for cell surface expression of CD3, CD4, and CD8 by triple color flowcytometry using fluorescein isothiocyanate (FITC)-, RPE- and RPE-Cy5-conjugated Mabs, respectively (Dako).

T-cell proliferation assays

Autologous BLCLs were infected with HSV-1, HSV-2 at a multiplicity of infection (MOI) of about 5 at 37oC for 20 h. The virus- and mock-infected cells were washed and UV-irradiated (2.5 x 10-2 mW/mm2). Alternatively, BLCLs were infected with the rVVs at an MOI of approximately 5 for 20 h and fixed with 1% paraformaldehyde as described previously16. The level of infection of the BLCLs with the respective viruses was determined by flowcy-

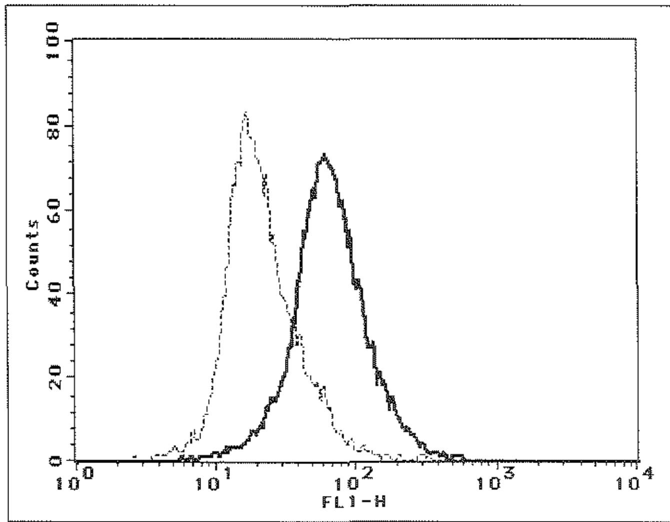

FIGURE 1.

Flowcytometric analysis of B cells infected with wildtype (dashed line) and recombinant vaccinia virus expressing HSV-1 UL6 (solid line) for the expression of HSV-1 UL6. The x-axis and the y-axis indicate the fluorescence intensity and relative cell number, respectively. For both cell populations, staining with 1% normal rabbit serum resulted in a similar histogram as for wildtype vaccinia virus infected B cells (data not shown).

tometry. The expression of HSV-1 UL6 in rVV-UL6-infected BLCLs was demonstrated using a rabbit anti-UL6 serum (2C2)11 and subsequently FITC-conjugated swine-anti-rabbit serum (Dako). As for all viruses, about 70-90% of the BLCL were shown to be infected (Fig. 1). T-cells (3 x 104/well), removed from culture at day 10-12 after one mitogenic stimulation, were cultured in triplicate together with virus- or mock-infected BLCLs (2 x 104/well) in 96-well round-bottomed plates in 150 ml complete medium at 37oC in a CO2-incubator. Complete medium consisted of RPMI-1640 (GIBCO-BRL, Breda, The Netherlands) supplemented with 10% heat-inactivated pooled human serum and antibiotics. Due to the limited ability of BLCLs to process and present exogenous antigens to T-cells, T-cell reactivity to human soluble cornea protein extract (HuSoCo; at final protein concentrations of 50 and 100 mg/ml) was performed using 105 autologous PBMC (UV-irradiated) as antigen presenting cells (APC). The cells were cultured for 72 h and pulsed with 0.5 mCi [3H]-thymidine over the last 18 h of culture. The cells were harvested and the incorporated radioactivity was determined in a beta-scintillation counter. Proliferation was considered positive when stimulation indices, counts per minute (cpm) incorporated in response to antigen / cpm incorporated in response to control), were >4. T-cell reactivity to all antigens tested for were assayed simultaneously and PHA-L (1 mg/ml) was included as positive control for T-cell proliferation. The assays were performed at least 2-times and the standard deviation was always < 30% of the mean cpm.

Results

Immunohistochemistry on corneas of HSK patients

Diagnostic analyses were performed on corneal buttons, obtained following therapeutic penetrating keratoplasty, from 12 patients with necrotizing stromal keratitis (patients #1 - #9) or immune stromal keratitis (patients #10 - 12)[10]. All patients had a history of recurrent episodes of HSV-1-induced stromal keratitis (Table 1). The corneal histopathology observed in the HSK patients (Fig 2A) included granulomatous reactions at the level of Descemet's membrane (Fig. 2B and C), suppurative keratitis with edema (Fig. 2D and E), reactive hyperplasia of the epithelium and breakdown of Bowman's layer (Fig. 2F and G). A mononuclear cell infiltrate was observed in the corneas of all patients and consisted predominantly of $CD4^+$ T cells (fig. 2G).

PCR analyses revealed the presence of HSV-1 DNA in 8 out of 11 corneas analyzed,

implicating that HSV-1 initiated the disease. HSV-1 DNA positive corneas were mainly obtained from patients with fulminant necrotizing stromal keratitis (Table 1). Evidence for an ongoing intra-corneal HSV-1 infection was only found in patient #5, demonstrated by the presence of HSV-1-infected keratocytes (Fig. 2E).

Antigen specificity of cornea-derived T cell lines from HSK patients

We have recently developed a protocol that enables the recovery and expansion of *in vivo* activated corneal infiltrating T-cells from corneal buttons of HSK patients[9]. This method facilitated the generation of intra corneal TCLs from all 12 HSK patients studied. All TCLs consisted predominantly of CD3+ T-cells and the ratio of CD4+ and CD8+ T-cells varied interindividually. Interestingly, the TCLs of two patients with quiescent necrotizing stromal keratitis consisted almost exclusively of CD4+ T-cells (patients #7 and #9; Table 1). The reactivity of the cornea-derived TCLs towards the triggering virus was analyzed in T-cell proliferation assays using mock-, HSV-1- and HSV-2-infected autologous BLCL as APC. The intra-corneal TCLs of 9 out of 12 patients showed HSV-specific T-cell reactivity (Table 2). Illustrative for the high sequence homology between the HSV serotypes, the majority of these TCLs recognized both HSV-1- and HSV-2-infected BLCLs. In case of patient #8, however, the HSV-specific intra-corneal T-cell response was restricted to HSV-2.

To test the hypothesis that an HSV-induced autoreactive intra-corneal T-cell response is involved in the immunopathogenesis of HSK in humans, the reactivity of the TCLs to recombinant HSV-1 UL6 and a HuSoCo protein extract was determined. In repeated experiments, none of the TCL showed significant responses to HSV-1 UL6 or human corneal antigens (Table 2).

Discussion

HSV infection of the cornea can result in the development of stromal keratitis, a leading infectious cause of blindness worldwide. The adult cornea is an ocular tissue without constitutive lymphoid components. Therefore, any intra-corneal T-cell found in HSK patients must have migrated into the cornea upon infection and subsequent inflammation. Experimental HSK animal models have been developed to investigate the immunopathogenesis of HSK. Based on these studies, HSK is considered to represent an immunopathological reaction in the corneal stroma coordinated by CD4+ Th1 cells[1,2].

Although the processes orchestrated by cornea infiltrating CD4+ T-cells have been studied extensively, the target antigens recognized remain unclear. Given the involvement of HSV in the etiology of HSK, HSV antigens are the most likely candidates. To address this notion we analyzed the antigen-specificity of cornea infiltrating T-cells in 12 patients with HSV-induced stromal keratitis. After one round of mitogenic stimulation, cornea-derived TCLs were successfully generated from corneas of the 12 patients studied. Intra-corneal HSV-specific T-cell reactivity, mainly HSV type-common, was observed in 9 out of 12 corneas tested. These data indicate that T-cells specific for the triggering virus infiltrate corneas of HSK patients. In patient #8, however, the HSV-specific response was solely directed to HSV-2. Possibly the determinants recognized by these T-cells are HSV type-common and are not efficiently processed and presented in HSV-1- as compared to HSV-2-infected BLCLs. Surprisingly, HSV reactive T-cells could also be detected in TCL of patients in a quiescent phase, treated with steroids and even from HSV DNA-negative corneas. These data suggest that HSV-specific T-cells can reside for longer periods of time,

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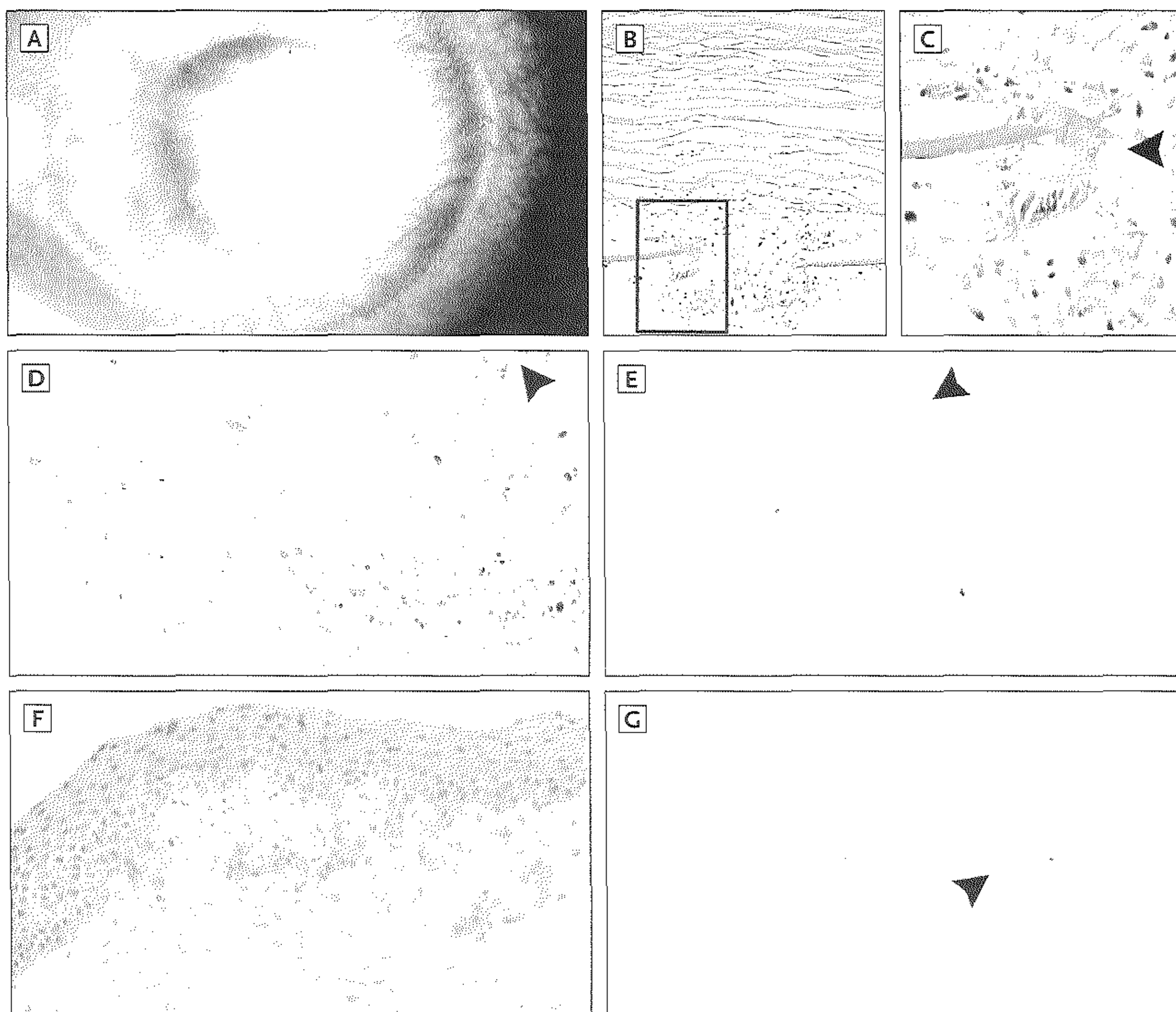

Figure 2.

Macroscopic, histologic and immunohistologic examination of corneas from patients with herpetic stromal keratitis. (A) Central corneal ulcer with descemetocele and impending perforation of the cornea of patient #5. (B and C) Central view of keratectomy specimen of patient #1 showing stromal infiltration of inflammatory cells and a florid granulomatous reaction with focal rupture of Descemet's membrane (Dm) (B; periodic acid - Schiff. PAS, magnification x100). Inset: higher manification of then multinucleated giant cell (arrow) protruding through this rupture (C; PAS, x400). (D). Paracentral view of keratectomy specimen of patient #5 shows (adjacent to the ulcer) a deep stromal defect with edema and infiltration of polymorphonuclear cells (arrow) reaching up to Dm (HE straining, x200). (E) Immunohistochemical staining for HSV-1 antigens demonstrating HSV antigens in stromal keratocytes (arrow) in the corresponding section of the cornea of patient #5 (hematoxylin counterstained, x400). (F) Central view of keratectomy specimen of patient #6 with reactive hyperplasia of the epithelium. The cornea is infiltrated with predominantly mononuclear cells (HE, x200). (G) Immunohistochemical staining for CD4⁺ cells demonstrating predominantly subepithelial localization of CD4⁺ cells (arrow) in the corresponding section of the cornea of patient #6 (hematoxylin counterstained, x200).

Table 2.

Phenotype and antigen-specific proliferative responses of cornea-derived T-cell lines from HSK patients

Patient No.	% of cells with phenotype			[^{3}H]-thymidine incorporation (cpm)*							
	$CD3^+$	$CD4^+$	$CD8^+$	BLCL/ mock	BLCL/ HSV-1	BLCL/ HSV-2	BLCL/ VV-WT	BLCL/ rVV-UL6	PBMC/ Medium	PBMC/ HuSoCo	PHA-L
1	92	68	23	1,260 ± 122	8,749 ± 659	ND	ND	ND	131 ± 34	112 ± 52	21,026 ± 4809
2	98	84	11	19 ± 9	9,158 ± 584	7,777 ± 109	29 ± 6	24 ± 7	370 ± 54	442 ± 77	14,233 ± 374
3	97	67	29	616 ± 133	1,173 ± 116	1,576 ± 98	119 ± 15	144 ± 83	83 ± 26	155 ± 51	27,059 ± 5168
4	99	76	24	161 ± 61	1,253 ± 121	1,701 ± 97	ND	ND	292 ± 37	243 ± 13	32,448 ± 4433
5	96	29	69	67 ± 26	455 ± 69	191 ± 19	27 ± 3	48 ± 5	50 ± 7	49 ± 11	37,037 ± 559
6	96	46	49	2,085 ± 308	29,125 ± 3088	9235 ± 780	ND	ND	824 ± 55	913 ± 86	27,309 ± 1237
7	99	97	3	123 ± 33	103 ± 2	128 ± 21	131 ± 21	163 ± 32	173 ± 33	123 ± 19	15,399 ± 696
8	98	34	63	160 ± 78	332 ± 23	825 ± 39	ND	ND	131 ± 41	112 ± 42	35,183 ± 809
9	99	98	<1	109 ± 15	1,426 ± 159	4,014 ± 371	ND	ND	80 ± 58	130 ± 22	14,843 ± 1762
10	98	54	32	387 ± 72	14,298 ± 1122	1,518 ± 294	104 ± 16	113 ± 28	374 ± 103	140 ± 32	31,829 ± 968
11	96	53	43	90 ± 16	147 ± 29	170 ± 21	55 ± 15	63 ± 16	631 ± 57	290 ± 67	26,632 ± 643
12	ND	ND	ND	699 ± 172	6,718 ± 434	7,264 ± 360	857 ± 93	778 ± 73	666 ± 103	2,424 ± 232	32,449 ± 1023

* T-cells were incubated with autologous B cell lines (BLCL) infected with herpes simplex virus (HSV) type 1 or 2, vaccinia virus wild-type (VV-WT) or recombinant VV expressing HSV-1 protein UL6. Alternatively, T-cells were cultured with autologous peripheral blood mononuclear cells (PBMC) in the presence of human soluble corneal protein extract (HuSoCo), medium or PHA-L (1 μg/ml) as negative and postive control. Results using 50 μg/ml HuSoCo are presented. The data are expressed as mean cpm ± SD of triplicate cultures. ND, not done.

and even under steroid treatment, in corneas of patients with HSV-induced stromal keratitis.

In contrast to corneas of patients with necrotizing stromal keratitis[17] (Fig 2E), murine HSK corneas are devoid of HSV antigens[1,2]. Nonetheless, HSV-specific T cells have been demonstrated in whole-eye cell suspensions of mice with fulminate HSK[3]. Similarly, in 8 of the 9 HSK patients, from which intra corneal HSV-specific T-cells were recovered, the corneas were devoid of HSV antigens. On infiltration of the cornea, these HSV-specific T-cells may have been activated by viral peptides retained by corneal cells like LCs or the amount of HSV antigens in the corneas is too low to be detected by immunohistochemistry. Alternatively, potential keratogenic $CD4^+$ T-cells infiltrating HSV-infected corneas may be activated nonspecifically, (e.g. by cytokine-mediated bystander activation)[18], or activated upon recognition of sequestered corneal autoantigens unmasked or altered following HSV replication in the cornea[2]. Recently, studies performed in the murine HSK model have provided evidence for the latter assumption[7,8]. HSK could be induced in nude mice after adoptive transfer of HSV-1 UL6 peptide-specific $CD4^+$ T-cells[8] cross-reacting with an unknown corneal protein[7]. We have analyzed whether this type of autoimmunity may play a role in human HSK. In none of the TCLs generated from corneas of any of the HSK patients studied here, reactivity to HSV-1 UL6 or a HuSoCo protein extract could be demonstrat-

ed. Stimulation of the intra corneal TCLs with PHA-L resulted in high proliferative responses, indicating that this is not due to a low viability of the TCL tested (Table 2). The lack of reactivity towards HSV-1 UL6, harboring the cross-reactive epitope, is not surprising given the constraints of MHC allele-specific peptide binding[19]. In case of the HuSoCo protein extract, the negative results could be due to a true lack of autoreactive T cells or inappropriate corneal antigen preparation used. The putative corneal autoantigen could be located in the buffer-insoluble part of the human cornea extract, it may be genetically polymorph or the intra-corneal autoreactive T cell responses is mediated by CD8^{-} T-cells. Given the nature of the HuCoSo protein extract and the type of assay used, i.e. exogenous antigen preparation in a T cell proliferation assay, the potential role of CD8^{+} cornea autoantigen specific T cells in HSK could not be addressed. In the HSK mouse model, the keratogenic T-cell clone recognized an unknown Tris-buffer soluble cornea-specific antigen[7] and was able to induce the disease in HSK resistant mice arguing against genetic polymorphism of the autoantigen. The HuSoCo protein extract used here, obtained from 12 human cornea buttons and similarly generated as described in the murine HSK study[7,12], was a heterogeneous protein preparation in which the major soluble cornea protein BCP54[12] was predominantly present (sodium dodelyl sulfate – polyacrylamide gel electrophoresis analysis; data not shown). Positive peripheral blood T-cell responses, using similar concentrations of an equivalent HuSoCo protein extract or purified BCP54, have been obtained in patients with inflammatory corneal diseases[20,21]. These data suggest that the HuSoCo protein extract used in the present study may be considered immunogenic. Although not formally excluded, the lack of intra-corneal T-cell reactivity to HSV-1 UL6 and human corneal antigens does not support the hypothesis that human HSK is an HSV-induced autoimmune disease. The cloning and identification of the putative HSK related murine cornea autoantigen and its human homologue will be needed to further address the validity of the molecular mimicry hypothesis at the single antigen level.

In conclusion, the present study demonstrates T cells specific for the triggering virus in the corneas of the majority of the 12 HSK patients studied. On antigenic stimulation, the cornea-derived HSV-specific T cells from HSK patients secrete both interferon gamma (IFN-γ) and interleukin 2 (IL-2)[9] (data not shown). In the mouse HSK model, both cytokines have been shown to shown to be pathologic in the cornea of HSV-1-infected mice[4,5,22]. IFN-γ has been shown to facilitate migration of PMNs from the blood into the cornea, and upon activation by IL-2 and perhaps IFN-γ secrete proteolytic enzymes that contribute to destruction of the cornea[1,2,23]. We hypothesize that HSV-specific T-cells play an important role in the local immunopathogenesis of HSK in humans. Upon entry into the cornea they are activated by HSV-infected corneal cells or by viral peptides retained by corneal cells like LC corneal cells and subsequently initiate an cytokine-mediated immunopathogenic response in the cornea.

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Chapter 5

Summarizing Discussion/ Nederlandse samenvatting en discussie

Summarizing discussion

Infections with several members of the human herpesviruses are the cause of significant ocular morbidity. Of the human herpesviruses, HSV-1 is the most frequent cause of primary and recurrent eye disease. Despite the availability of effective antiviral treatment, recurrent HSV-1 infection continues to be the leading cause of corneal blindness in industrialized nations. This thesis describes new insights in the role of the virus and the intracorneal T cell response involved in the pathogenesis of human HSV-1-induced keratitis.

Clinical presentation of infectious epithelial keratitis: "Rocking around the clock"

HSV infection of the corneal epithelium causes a classical dendritic shaped infection, whereas HSV infection of the skin causes round vesicles. Linear branching lesions like the dendrites on the corneal surface are not found on the skin. It seems logical to postulate that HSV, with its affinity for neural tissue, causes lesions congruent with the anatomical pattern of innervating nerves of the target-organ.

Until recently, the anatomical substrate for the linear branching pattern of IEK was unknown. This was probably in part due to the absence of knowledge of the subbasal nerve plexus in the corneal epithelium. In 1997, Müller et al. [1] described the subbasal nerve plexus in the human cornea as a dense regular meshwork with bifurcations of the main nerve fiber bundles at almost right angles. The orientation of the nerve fibers in the subbasal nerve plexus showed a 9-3 hours and 12-6 hours direction[1]. In *Chapter 2* the striking congruency between the subbasal nerve plexus of the corneal epithelium and the pattern of the dendritic lesion in recurrent IEK is demonstrated. Interestingly, the branching pattern of the dendrite is the same, regardless whether the dendrite occurs in an eye with or without previous PKP. However, after PKP the orientation of dendrites looses its preference for the 9-hours and 12-6 hours direction. The finding that dendritic ulcers are merely rotated, suggests the same grid-like anatomy of the subbasal nerve plexus in the corneal epithelium after PKP. This can be explained by postulating that the original two dimensional orientation of the corneal button is lost during corneal transplantation. Reinnervation of the cornea may follow preserved parts of the subbasal plexus after transplantation. The ultimate proof, whether the subbasal nerve plexus is the structure responsible for the dendritic pattern of IEK, should be given by in vivo confocal microscopy.

HSV-1 infections after penetrating keratoplasty

PKP and HSV keratitis.

In the general population, ocular manifestations of HSV-1 infection can lead to unilateral blindness which may be an indication for PKP. Five to 10 % of PKPs are performed for corneal opacities resulting from HSV-1 infection[2]. PKP for HSV keratitis has a high rate of post-operative complications. Epithelial recurrences are a major problem after PKP. Graft rejections contribute to graft failure to a larger extent in PKP for HSV than in PKP for reasons unrelated to HSV-1 infection[3]. Recurrences of HSV keratitis after PKP and the rate of immunological graft rejection in these grafts can be reduced by antiviral prophylaxis when

steroids are employed to prevent or treat graft rejection[4]. However, the use of prophylactic antiviral therapy is largely based on experience in patients with recurrent HSV related eye disease who have not undergone PKP[5].

Newly acquired corneal HSV-1 infections after PKP

The finding, that HSV-1 keratitis occurs after PKP in patients without a previous history of HSV keratitis, raises questions about the frequency and impact of this manifestation, about the mode of transmission in these patients, and about the numbers of transplant recipients at risk to develop this inflammatory disease.

In this thesis, a retrospective analysis of the outcome of 2398 PKPs is presented[6]. Among 2112 patients transplanted for reasons unrelated to HSV infection, 18 presented with HSV-1 epithelial keratitis in their graft. The HSV-1 infection was caused either by endogenous or exogenous virus. The incidence of newly acquired HSV keratitis after PKP was calculated to be 1.2 per 1000 person years, i.e.14-fold higher than that observed in the general population[7]. A recent study conducted in 25 patients with this clinical manifestation, indicated that the majority of them (n=14) developed severe ocular complications resulting in social blindness of the infected eye within three years (unpublished observation). In most cases, the newly acquired HSV-1 infection occurs within the first two years after PKP, again suggesting a causal relationship between corneal transplantation and HSV-1 infection. In our retrospective study it remained unsettled from where the virus originated. Several possibilities exist, including reactivation of latent virus in the trigeminal ganglion and transmission through the donor cornea. Infectious agents that have been demonstrated to be transmitted following corneal transplantation including bacteria, fungi, Creutzfeldt-Jakob agent, hepatitis B- and C viruses and rabies virus[8].

To substantiate the hypothesis that transmission of HSV-1 through corneal transplantation may occur, new diagnostic tools had to be developed. A new PCR method, based on the stability and strain-to-strain differences of reiterated sequences within the HSV-1 genome, was developed[9]. The method facilitated differentiation of up to 92% of unrelated HSV-1 strains. The major advantage of the approach presented is, that it provides the opportunity to discriminate HSV-1 strains without virus culture. The method proved to be sensitive enough for amplification of the low levels of viral DNA present in corneal buttons and rims obtained from patients undergoing PKP.

It is assumed that recurrent herpetic lesions are due to reactivation of the strain acquired during primary infection. However, HSV-1 superinfection has been documented, but considered to be a relative rare event[10]. In the last part of *chapter 3*, we demonstrated a high frequency of corneal HSV-1 superinfection. Genotypic analysis, with the recently developed PCR method, demonstrated that 63% of patients with recrudescent herpetic keratitis had evidence of reactivation of the same strain (group I). Consequently 37% of the patients had a different genotype (group II), suggesting corneal HSV-1 superinfection in the inter-recurrence period. Selection of patients susceptible to HSV-1 may have occurred, because the patient cohort consisted predominantly of patients with severe herpetic keratitis. Alternatively superinfection with a more virulent strain might have occurred. Among the clinical data analyzed, only the time point of PKP was significantly different between the two groups. None of the patients of group I underwent PKP between sampling, whereas 4 out of 11 patients underwent PKP during the inter-recurrence period. The data suggest that PKP is a risk factor for corneal superinfection with HSV-1[11].

In a subsequent study the hypothesis on graft-to host transmission of HSV-1 through PKP was tested. Three months after PKP for congenital glaucoma, a young patient lost his only functional eye, as a result of uncontrollable herpetic keratitis and choroidal effusion.

Genetic characterization of the donor- and recipient derived HSV-1 strains was performed.

The DNA sequences were identical for both strains. The patient proved to be HSV-1 naïve before the transplantation. This study is the first to provide conclusive evidence for graft-to-host transmission with subsequent reactivation of donor-derived HSV-1 in a corneal allograft [12].

Prevention of newly acquired ocular HSV-1 infection after PKP

The afore mentioned data demonstrate graft-to-host transmission as one of the possible sources of HSV-1 infection after PKP.

The retrospective study on newly acquired HSV keratitis after PKP, indicates that 0.85 % of patients develop corneal epithelial HSV-1 infection[6]. The actual incidence of newly acquired HSV-1 related disease after PKP might even be higher. This study focused primarily on epithelial defects, whereas HSV-1 infections in a corneal graft may also present as stromal infiltration or endothelial infection resembling an allograft rejection[13].

Evidence is emerging that allograft rejection[14], primary graft failure[15], and endothelial cell loss of cultured eye bank corneas[16],[17] are associated with the presence of HSV-1 DNA (Table 1).

Now that we are aware of the fact that newly acquired HSV-1 infection after PKP may not be as rare as previously suggested, one could ask the following question: "Are there ways to prevent this potentially blinding infection?". This question is reminiscent of the situation with other herpesviruses in transplantation[21,22].

Corneal HSV-1 infection after PKP in seronegative recipients may be due to transmission of the virus, either community or graft acquired. The annual HSV-1 seroconversion rate among university students averages 5-10%[23], and ocular infections occur in only about 1 % of those infected[24]. Therefore, the chance of a community acquired ocular HSV-1 infection can be considered less than 0.1% per year. HSV-1 transmission with the donor cornea to seronegative patients, which was conclusively demonstrated in this thesis[12], should be considered a real risk. In HSV-1 naive recipients no pre-existing immunity against the virus is present and immunosuppression by topical steroids to prevent graft rejection aggravates this situation. The high prevalence (10-20%) of HSV-1 DNA in normal and eye bank corneas underlines the seriousness of this risk[25].

The presence of HSV-1 antibodies depends on age, socio-economic status and geographical location (Fig. 1)[26,27]. The prevalence of HSV-1 DNA in the cornea, and the risk to be an HSV-1 seronegative transplant recipient will vary accordingly. As the overall seroprevalence of HSV-1 is declining[28, 29] the risk of transmission through PKP is increasing. The actual rate of transmission of HSV-1 after PKP in immunocompromised humans, however, is unknown. In the rabbit model, in which HSV-1 naïve animals are immunosuppressed for only one week after PKP with an HSV-1 DNA containing donor cornea, a transmission rate for HSV-1 of about 18 % was found[30]. As humans are the natural reservoir for this highly infectious virus and are immunosuppressed for much longer periods of time after PKP, the transmission rate can be expected to be higher than in the animal model.

TABLE 1: COMPLICATIONS IN CORNEAL ALLOGRAFTING ASSOCIATED WITH HSV-1

Complication	data for general PKP population		HSV-1 associated graft failure		Method
	n	%	n	%	
Newly acquired HSV-1 after PKP	18/2112[6]	0.85	14/25[18]	56	viral culture
Corneal allograft rejection	291/3048[2]	9,5	8/17[14]	47	PCR
			3 /17[14]	18	GWc
Primary graft failure	215/10363[19]	2.1	2/3[20]	66	PCR
Extreme endothelial cell loss in cell culture	7/1205[2]	0.58	9/199[17]	4,5	PCR
			7/199[17]	3,5	viral culture

Determination of the overall infection risk for infection with HSV-1 after PKP.

The following variables should be included in the risk calculations: HSV-1 seroprevalence (Fig.1), geographic location of the population studied, age-distribution of the donor- and recipient-population (Fig.2), probability that the cornea of an HSV-1 seropositive patient contains virus and the transmission rate of HSV-1 after PKP (Table 2, Fig. 3 a and b):

- The study of Wutzler [27] is considered representative for the seroprevalence of HSV-1 on the Western European continent. The appropriateness of this assumption is illustrated by a pilot study on seroprevalence for HSV-1 in 290 recipients of conducted in Rotterdam between November 1998 and September 2001: 14 % of transplant recipients is HSV-1 naïve (unpublished data). The study of Nahmias[26] is considered representative for the seroprevalence of HSV-1 in the USA white (non-hispanic) population and the USA black population (Figure 1).
- Data about the age composition of corneal donor- and recipient populations in Western Europe are presented in figure 2. For the USA, the same age distribution of donors and recipients as Western Europe is assumed. Given the above-mentioned age distribution, the overall seroprevalence of HSV-1 in the donor- and recipient-population was calculated.
- Presence of HSV-1 DNA in non HSK diseased corneas varies from 10 to 21% [25,31].
- For the transmission rate of HSV-1 after PKP a sliding scale was assumed from 20-100% (fig 3b)
- A postulated negligible risk of superinfection with HSV-1 after PKP in seropositive patients.

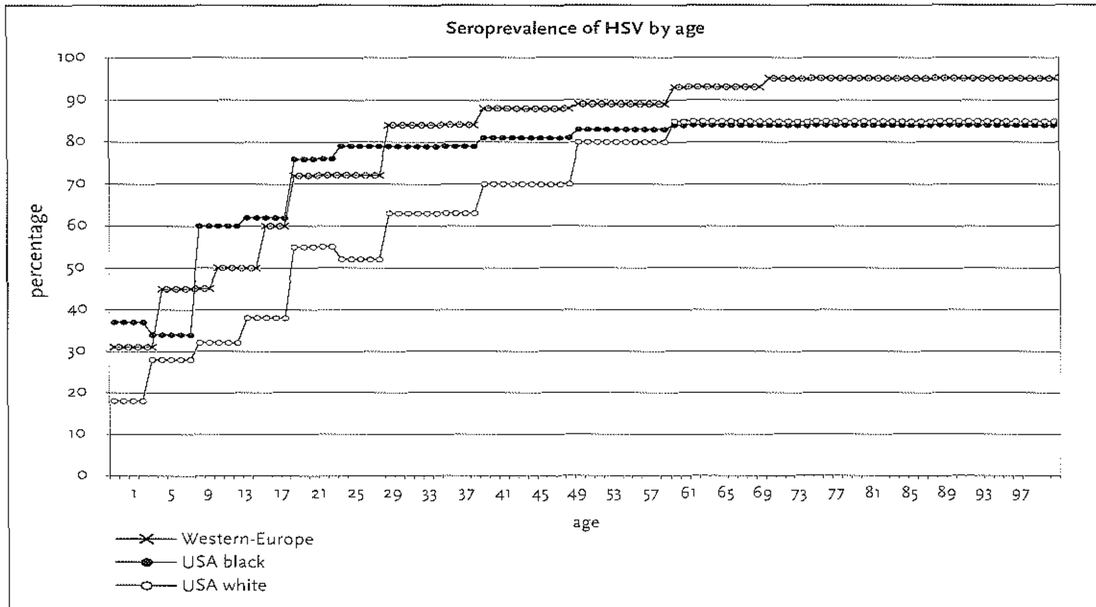


Figure 1:

Seroprevalence as function of age for Western Europe[27] and USA white- and USA black- population[26].

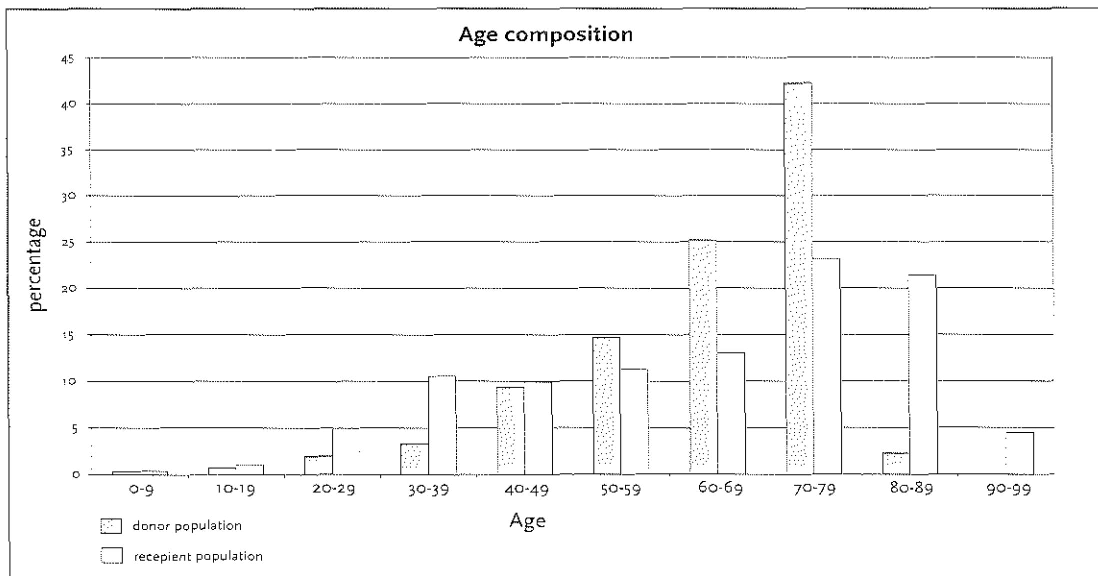


Figure 2:

The age composition of corneal donor- and recipients in the Netherlands provided by the National Register for Corneal Transplantation under the auspices of the Corneal Study group " Kok–van Alphen" of the Dutch Ophthalmologic Society. Bio Implant Services (part of the Netherlands Transplantation Foundation, Leiden) provides all corneal donor tissue for the Netherlands in conjunction with related cornea banks on the European continent.

Based on these data the overall infection risk with HSV-1 after PKP may be estimated to be:

Overall infection risk = S_D * $(1-S_R)$ * eye * Tr

S_D = overall seroprevalence in % of HSV-1 corrected for age distribution of donor population
S_R = overall seroprevalence in % of HSV-1 corrected for age distribution of recipient population
eye = proportion of donor corneas containing HSV-1 DNA
Tr = transmission rate of HSV-1 though corneal transplantation

Table 2:
Seroprevalence in donor-and recipient population with resulting infection risk for transmission rate of 100% from HSV-1 DNA+ cornea

Population	S_D in %	S_R in %	Infection risk in % when 10%-20% corneas HSV-1 DNA +
West European	92	90	0.9-1.8
USA white	81	78	1.8-3.6
USA black	83	82	1.5-3.0

Seronegative patients:

To prevent HSV-1 transmission by PKP to seronegative patients, systemic acyclovir treatment for a period that exceeds that of the immunosuppressive treatment is an option[4,5]. This implies that all recipients should be tested serologically for the presence of HSV-1 antibodies, before PKP is carried out. If in addition, also donors would be screened in the same way, matching of seronegative recipients with seronegative donors, whenever logistically possible, would be an even more attractive option. This could obviate prophylactic antiviral treatment in the matched seronegative recipients.

Seropositive patients: superinfection

Corneal HSV-1 infection after PKP in seropositive patients may be due to reactivation of latent virus in the TG, or transmission of the virus, either community acquired or through the corneal graft, resulting in superinfection. Although the data in this thesis demonstrate that corneal reinfection with a different strain is possible and suggest that PKP is a risk factor for corneal HSV-1 superinfection[11], the clinical relevance of this finding is unknown. At the time point of sampling, the clinical manifestations of HSV keratitis were comparable in the group with genetically different sequential samples. As the chance of community acquired HSV-1 ocular infection in seronegative patients should be considered less than 0.1% per year, the chance for seropositive patients to acquire a superinfection of HSV-1 through this route should be considered much less.

Seropositive patients: reactivation

Reactivation of latent virus in the trigeminal ganglion is the most important cause of active herpetic keratitis[32,33]. In seropositive patients probably reactivating stimuli related to corneal transplantation play a role in newly acquired ocular HSV-1 disease. Surgical trauma, suture removal, steroids and immune reactions can be reactivating stimuli[6]. Prevention of reactivation of HSV-1 infection after PKP in seropositive patients by prophylactic treatment with acyclovir is an option. This would mean, however, that all seropositive corneal transplant recipients should be treated during the period of immunosuppression, with all consequences of unnecessary treatment, adverse drug effects, toxicity and

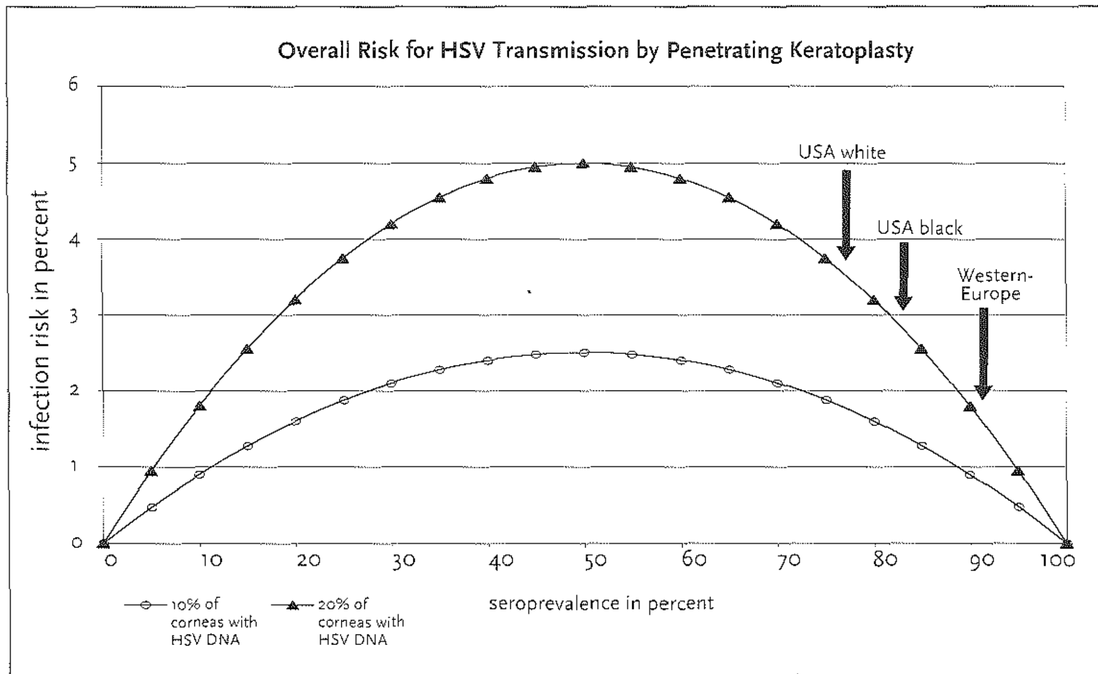


Figure 3a:

Overall infection risk for HSV-1 infection through PKP as function of seroprevalence in the donor-and recipient population. Transmission rate 10%. The white arrows indicate the calculated overall infection risk (table 2) for HSV after PKP in different geographical locations.

viral resistance. A more practical approach is, to alert ophthalmologists, about the possibility, that ocular HSV disease can develop after PKP, irrespective of the original diagnosis for transplantation. Awareness of the possible manifestations of ocular HSV disease after PKP, the use of appropriate diagnostics in these patients and the correct,high dose antiviral treatment, may prevent HSV-1 related graft failure and the possibly associated corneal blindness.

Active HSV-1 replication in corneal donor tissue:

Transplantation of donor corneas with *active* HSV-1 replication can be avoided by critical assessment of the graft prior to surgery [17]. The other cornea of the same donor should be critically examined as well, and not used for transplantation, as the explantation might have been performed with the same instruments [17].

In this way the incidence of primary graft failure associated with HSV-1 might be reduced. To date, screening of donor cornea fluids for HSV-1 DNA has no additional value[17].

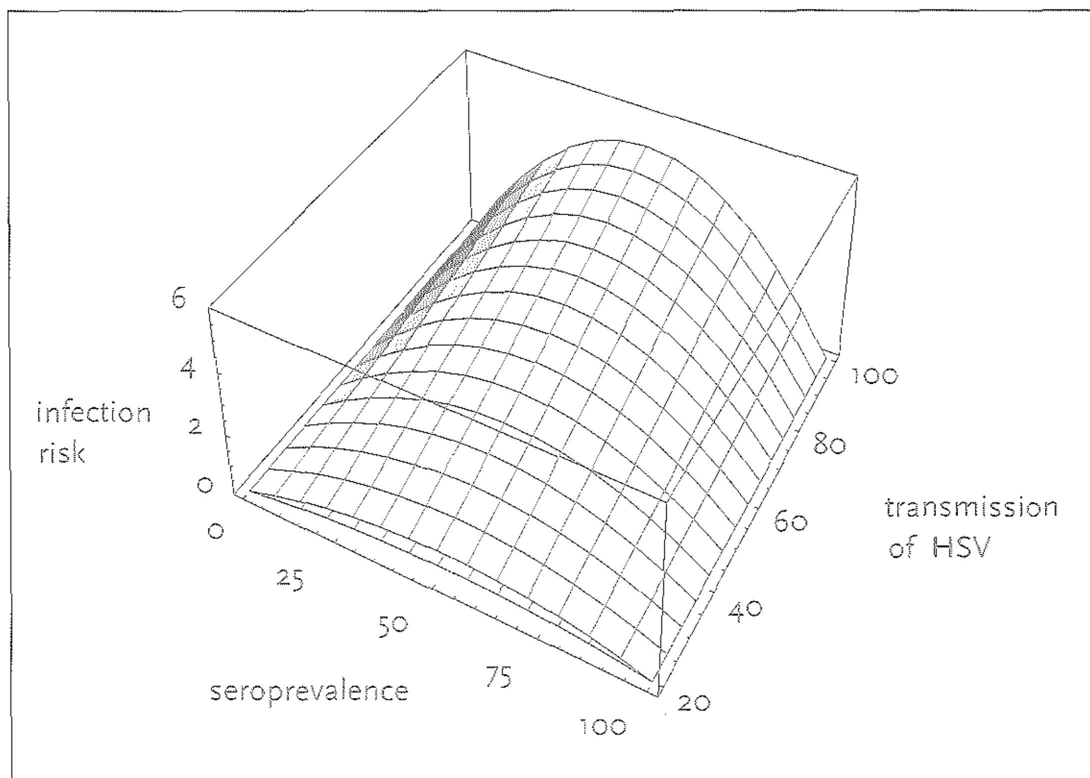


Figure 3b:

Overall risk for infection through PKP as a function of seroprevalence of donor-and recipient population and rate of HSV-1 transmission.

Collectively our data would lead to the following recommendations:

- Stimulation of awareness amongst ophthalmologists about the possible development of ocular HSV disease after PKP irrespective of the original diagnosis for transplantation.
- Serologic screening of corneal transplant recipients, and whenever possible and logistically feasible, matching of recipients and donors for HSV-1 sero-status.
- Prophylactic or early therapeutic treatment with systemic acyclovir should be considered in HSV-1 naïve corneal transplant recipients, when sero-matching for HSV-1 of corneal donor and recipients is impossible.
- Assessment of the role of HSV-1 infection in graft rejection, in a large prospective study.

Immunopathogenesis of Human HSK

The immune response following an HSV corneal infection resembles that observed following HSV infection at non-ocular sites. While the immune response elicited by the infecting virus is essential for limiting viral spread, it can have detrimental effects on the function of the cornea.

Current knowledge on the pathogenesis of HSK is primarily based on studies performed in the experimental HSK mouse model. Virus-specific cellular immune responses are central to the pathogenesis of HSK. In the mouse $CD4^+$ T helper 1 cells have been demonstrated to play a key role[34,35]. In the first part of chapter 4 we demonstrate for the first time an HSV-specific T cell response in corneas of 2 patients with active NSK. Whereas the cornea-derived T cells consist of both $CD4^-$ T cells and $CD8^+$ T cells, only $CD4^+$ HSV-specific T cells could be identified. These observations are in agreement with studies on murine HSK. In contrast, the human intra-corneal HSV-specific T cells exhibited a Th0 phenotype, rather than the Th1 phenotype shown in the mouse. This may be due to the culture conditions used to isolate and culture human intra-corneal T cells. In addition to Langerhans' cells and macrophages, HSV-infected corneal keratocytes were able to activate intra-corneal HSV-specific T cells.

The enigma in the experimental HSK mouse model remains the nature of the antigens recognized by the cornea-infiltrating T cells. During the clinical phase of HSK, the murine cornea is devoid of replicating virus, viral mRNA and viral proteins[36,37,38], suggesting that the immune response may not be primarily directed against the infectious virus. Alternative sources of antigen have been suggested. HSV replication might unmask and alter corneal tissue antigens, normally not presented to the host immune system. Recent studies have provided evidence that HSK in the mouse represents an auto-immune response to corneal tissue[39,40,41]. HSK could be induced by $CD4^+$T cells directed to an epitope derived from the HSV capsid protein UL6, that cross reacts with an epitope of an antigen uniquely expressed in the murine cornea[39,40,41], so-called molecular mimicry. Another explanation for the presence of $CD4^+$ T cells is that the T cell response results from exposure to cytokines in the infected cornea, referred to as bystander activation[42,43].

The search for auto-antigens in HSK becomes somewhat far-fetched in the human situation. In human HSK, replicating virus and viral antigens[44, 45,46] have been demonstrated in the cornea during the active as well as the inactive phase of stromal keratitis.

In the second part of *chapter 4* we demonstrate an HSV-specific immune response of $CD4^+$ T cells in nine of twelve patients. HSV-specific T cells could be detected in corneas of patients with either active or quiescent HSK, in patients treated with steroids and also in HSV DNA-negative corneas. These studies demonstrated that virus-specific T cells can reside in the cornea for prolonged periods of time[47,48,49].

The role of auto-immunity in human HSK was analyzed. In none of the T cell lines generated from corneas of HSK patients, reactivity to HSV-UL6 or a soluble protein extract of human corneas could be demonstrated. These data suggested that the T cell response in HSK patients would rather be directed to the initiating virus than to a human corneal autoantigen or HSV UL-6[48]. We hypothesize that HSV-specific T cells play an important role in the local immunopathogenesis of HSK in humans. We expect HSV-infected corneal cells or viral peptides retained in the corneal stroma or in Langerhans cells may initiate and perpetuate this local keratogenic cellular immune

response involving the interaction between infiltrating inflammatory cells and corneal resident cells by means of soluble factors like Th1 cytokines and chemokines.

Concluding remarks

The new insights described in the present thesis relate to three main areas :

- The dendritic pattern, that develops in HSV epithelial keratitis, coincides with the pattern of innervation of the subbasal nerve plexus of the cornea, again illustrating the neurotropic nature of the virus.
- PKP should be considered a risk factor for subsequent HSV infection, resulting from either direct viral transmission or reactivation.
- In herpetic stromal keratitis, HSV specific T lymphocytes infiltrate the cornea, suggesting a causative role in the pathogenesis of this inflammatory process.

Samenvatting en discussie

Verschillende leden van de familie van humane herpesvirussen kunnen ernstige oogontstekingen veroorzaken. Het humane herpesvirus type 1 (HHV-1, HSV-1), het prototype virus van deze familie, is de meest frequente oorzaak van primaire en recidiverende oogontstekingen. Ondanks de beschikbaarheid van effectieve antivirale middelen, is recidiverende HSV-1 infectie de hoofdoorzaak van unilaterale corneale blindheid in de geïndustrialiseerde wereld. Dit proefschrift beschrijft nieuwe inzichten in de rol van het virus en van de intracorneale T-cel respons in de pathogenese van HSV keratitis bij de mens.

Klinische presentatie van epitheliale HSV keratitis "Rocking around the clock".

HSV infectie van het cornea-epitheel presenteert zich over het algemeen als een klassieke vertakte laesie, die de naam "keratitis dendritica" draagt. HSV infecties van de huid daarentegen vertonen deze vertakkingen niet (Fig 8, Introduction). Men heeft tot nu toe geen verklaring waarom de laesies in de cornea zulke specifieke vertakkingen vertonen.

Vanwege de neurotrope eigenschappen van HSV heeft men al eerder gezocht naar een relatie met de anatomie van de innervatie van het eindorgaan. Tot nu toe kon deze relatie echter niet worden bevestigd.

Tot voor kort was de innervatie van het cornea-epitheel niet bekend. Pas in 1997 beschrijven Müller et al[1] de subbasale zenuw plexus van het cornea-epitheel als een dicht regelmatig netwerk met vertakkingen van de zenuwvezels die vrijwel loodrecht op elkaar staan. Deze blijken georiënteerd te zijn in de 9-3 uur of de 12-6 uur richting[1]. In *hoofdstuk 2* wordt de opvallende overeenkomst in vorm aangetoond tussen het "dendritica" patroon van epitheliale HSV keratitis en de subbasale zenuwplexus van het cornea-epitheel. Opvallend is, dat het vertakkingspatroon van "keratitis dendritica" na corneatransplantatie hetzelfde blijft, maar dat de oorspronkelijke oriëntatie, in de 9-3 uur of de 12-6 uur richting, verloren gaat. De bevinding, dat herpestakken in meer of mindere mate gedraaid zijn na corneatransplantatie, suggereert dat dezelfde rasterachtige anatomie van de subbasale zenuw plexus van het cornea-epitheel aanwezig is na transplantatie. Dit kan verklaard worden door te postuleren dat de oorspronkelijke tweedimensionale oriëntatie van de donorcornea verloren gaat tijdens transplantatie, omdat de cornea donor qua oriëntatie *at random* geïmplanteerd wordt. Reinnervatie van het cornea-epitheel zou, na corneatransplantatie, overgebleven structuren van de subbasale zenuw plexus kunnen volgen.

Het uiteindelijke bewijs dat de subbasale zenuw plexus verantwoordelijk is voor het vertakkingspatroon van epitheliale herpes keratitis moet gegeven worden door *in vivo* confocale microscopie.

HSV-1 infecties na corneatransplantatie

Penetrerende keratoplastiek (PKP) en HSV keratitis.

Vijf tot 10% van alle PKPs worden verricht voor corneatroebelingen als gevolg van HSV-1 infecties van de cornea[2]. Corneatransplantatie voor de diagnose HSV keratitis heeft een hoog risico op postoperatieve complicaties. Eén van de oorzaken van postoperatieve problemen zijn recidiverende HSV infecties in het transplantaat. Bovendien treden er meer afstotings-

reacties op als HSV-keratitis de diagnose is, waarvoor getransplanteerd wordt, dan wanneer de diagnose niet gerelateerd is aan HSV[3]. Het aantal recidieven van HSV keratitis en het aantal irreversibele afstotingsreacties neemt af als er antivirale profylaxe aan de behandeling wordt toegevoegd gedurende de periode dat er immuunsuppressie gebruikt wordt[4]. Het gebruik van antivirale middelen als profylaxe is vooral gebaseerd op gegevens van patiënten met recidiverende HSV keratitis waarbij geen PKP verricht is[5].

De novo HSV-1 infectie in de cornea na PKP

Na corneatransplantatie kunnen HSV-1 infecties optreden in de cornea, dit ondanks het feit dat bij de patiënt nooit oogheelkundige afwijkingen als gevolg van HSV-1 werden gediagnostiseerd. Deze bevinding roept vragen op over de frequentie en het belang van deze infectie, over de wijze van overdracht en over de omvang van de groep, die het risico loopt deze ontstekingsverschijnselen te krijgen na transplantatie.

In dit proefschrift (hoofdstuk 3) wordt een retrospectieve studie beschreven over de resultaten van 2398 PKPs[6]. Van de 2112 patiënten, die getransplanteerd werden voor diagnosen, die niet gerelateerd zijn aan HSV-1 infectie, presenteerden 18 patiënten zich met een epitheliale keratitis in hun transplantaat. De incidentie in deze groep van *de novo* HSV-1 na PKP was 1,2 per 1000 persoon-jaren, hetgeen 14 keer zo hoog is als in de normale populatie[7]. Een recent onderzoek naar de consequenties van deze aandoening bij 25 patiënten geeft aan dat de meerderheid (n=14) ernstige oogheelkundige complicaties ontwikkelde, die uitmondden in een visus lager dan 1/60ste binnen drie jaar na transplantatie (niet gepubliceerde gegevens). In de meeste gevallen treedt *de novo* HSV-1 infectie na PKP op binnen de eerste twee jaar na operatie, hetgeen duidt op een oorzakelijke relatie tussen de corneatransplantatie en de HSV-1 infectie. In onze retrospectieve studie bleef de oorsprong van het virus onopgehelderd. Er bestaan meerdere mogelijkheden, waaronder reactivatie van latent virus in het trigeminus ganglion en transmissie van het virus door de donorcornea. Van bacteriën, schimmels, Creutzfeldt-Jakob agens, Hepatitis B -en C virussen en rabiës virus bestaat bewijs, dat deze kunnen worden overgedragen door corneatransplantatie[8].

Ter bevestiging van de hypothese, dat transmissie van HSV-1 door corneatransplantatie mogelijk is, werd een nieuwe, op PCR gebaseerde, methode ontwikkeld, die gebaseerd is op verschillen van reïtererende volgorden tussen virusstammen, welke stabiel zijn binnen het HSV-1 genoom[9].

Deze methode maakte het mogelijk bijna 92% van niet aan elkaar verwante HSV-1 stammen te onderscheiden. Een voordeel van deze benadering is dat viruskweek niet noodzakelijk is. De methode bleek gevoelig genoeg om de lage hoeveelheden virus DNA, die aanwezig zijn in corneaweefsel, dat beschikbaar komt na transplantatie, te kunnen amplificeren en karakteriseren.

In de literatuur wordt aangenomen, dat recidiverende herpes laesies veroorzaakt worden door reactivatie van de stam, die ook de primaire infectie heeft veroorzaakt. Superinfectie met HSV-1 wordt gesuggereerd als mogelijkheid, maar men gaat er vanuit dat het een zeldzaam verschijnsel is[10]. In het derde deel van hoofdstuk 3 wordt echter aangetoond, dat de frequentie van corneale superinfectie met HSV-1 hoger is dan verwacht mag worden op basis van literatuur gegevens. Met behulp van de nieuw ontwikkelde PCR methode werd viraal DNA van patiënten met recidiverende herpes keratitis geamplificeerd en geanalyseerd. Aangetoond kon worden dat in 63% reactivatie optrad van dezelfde stam (groep1), daaruit voortvloeiend had 37% van de patiën-

ten een verschillend genotype bij het recidief (groep 2). Dit duidt aan dat er een superinfectie plaatsgevonden kan hebben in de periode tussen de recidieven. De opvallende hoge frequentie, die gevonden werd, kan veroorzaakt zijn doordat er selectie heeft plaatsgevonden voor patiënten die gevoelig zijn voor herpes infecties. Het cohort bestond namelijk vooral uit patiënten, met ernstige vormen van herpetische keratitis. Een andere mogelijkheid is dat er superinfectie is opgetreden met een meer virulente stam. Van de klinische gegevens die geanalyseerd zijn, was alleen het moment waarop PKP verricht werd significant verschillend tussen de twee groepen. Geen van de patiënten van groep 1 onderging een PKP tussen de recidieven, terwijl 4 van de elf patiënten van groep 2 een PKP ondergingen tussen de gekweekte recidieven in. Deze data suggereren dat corneatransplantatie een risicofactor is voor corneale superinfectie met HSV-1[11].

In een vervolgstudie werd de hypothese getest, dat transmissie van HSV-1 door corneatransplantatie mogelijk is. Een jonge patiënt verloor zijn enige functionele oog, drie maanden na PKP voor congenitaal glaucoom, als gevolg van een onbehandelbare HSV keratitis gecombineerd met een choroidea-effusie syndroom. Analyse toonde aan, dat de DNA sequenties van de HSV-1 stammen van donor- en recipiënt identiek waren. De patiënt was seronegatief voor HSV-1 vóór de operatie. Dit onderzoek verschaft voor het eerst onomstotelijk bewijs van *"graft-to-host"* transmissie van HSV-1, met daarop volgend reactivatie in de getransplanteerde cornea[12].

Preventie van de novo corneale HSV-1 infectie na PKP

De bovengenoemde data wijzen op *"graft-to-host"* transmissie als een mogelijke oorsprong van HSV-1 infectie na PKP. Het retrospectieve onderzoek naar *de novo* HSV keratitis na PKP duidt aan dat 0,85% van de getransplanteerde patiënten een epitheliale HSV-1 keratitis ontwikkelt, terwijl de diagnose voor transplantatie niet gerelateerd is aan HSV-1 infectie[6].

De werkelijke incidentie van *de novo* HSV keratitis na PKP kan nog hoger zijn. Het huidige onderzoek was gericht op het scoren van epitheliale herpetische keratitis, terwijl een HSV-1 infectie na corneatransplantatie zich ook kan presenteren als een stromaal infiltraat of als een endotheel infectie, die sterke overeenkomst vertoont met een afstotingsreactie[13].

Uit de recente literatuur blijkt dat complicaties als afstotingsreacties[14], *"primary graft failure"*[15] en massaal endotheelcelverlies van cornea's in weefselkweek[16,17] frequent geassocieerd zijn met de aanwezigheid van HSV-1 DNA (Tabel 1).

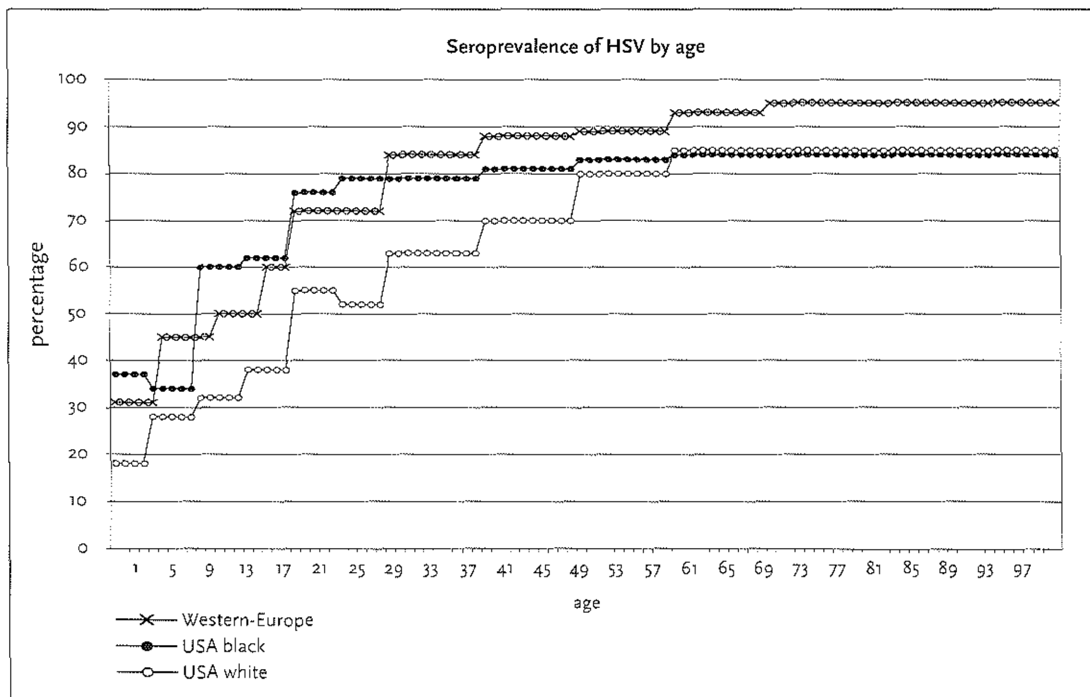
Nu dat we ons bewust zijn van het feit, dat *de novo* HSV-1 infecties na PKP niet zó zeldzaam zijn als voorheen gesuggereerd werd, kunnen we ons afvragen of er mogelijkheden zijn om deze infectie, die tot corneale blindheid kan leiden, te voorkomen. Deze vraag doet denken aan de situatie bij andere humane herpesvirussen en transplantatie[21,22].

HSV-1 keratitis na PKP in seronegatieve ontvangers kan het gevolg zijn van transmissie van virus afkomstig uit de gewone populatie of van de donor cornea. Het seroconversie percentage voor HSV-1 in de normale populatie bedraagt 5-10% per jaar[23]. Slechts 1% van deze mensen ontwikkelt ooginfecties[24]. De kans dat een HSV-1 seronegatieve patiënt na PKP het virus oploopt vanuit de gewone populatie is dus kleiner dan 0,1% per jaar.

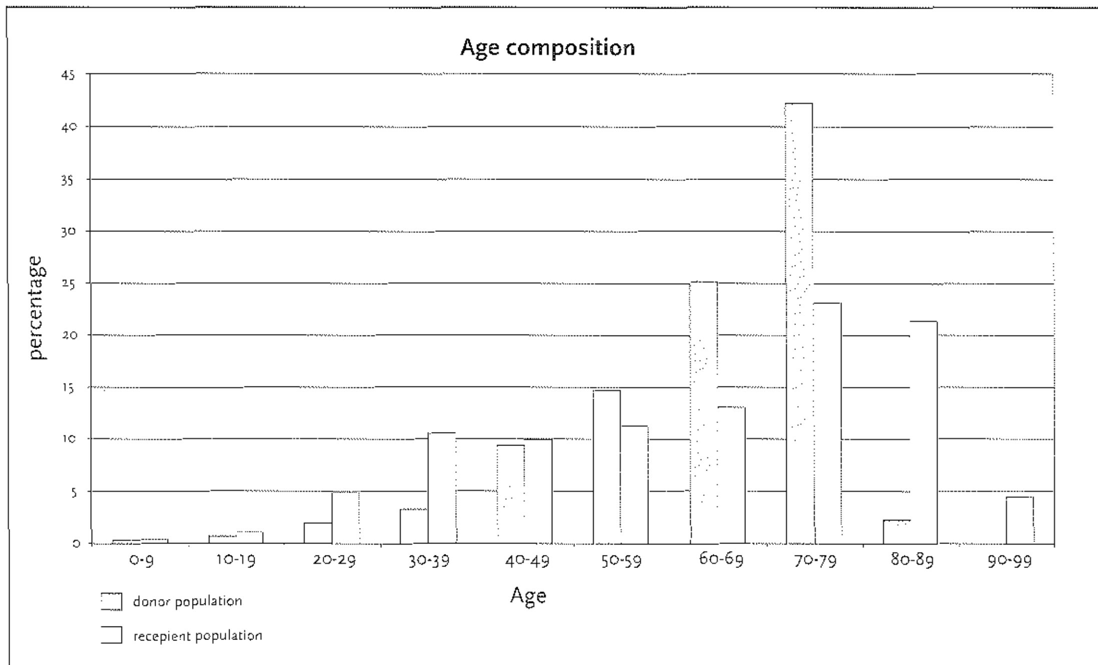
Transmissie van HSV-1 via de donor cornea naar een seronegatieve patiënt, hetgeen nu onomstotelijk bewezen is[12], moet dus als een reëel risico beschouwd worden. HSV-1 seronegatieve ontvangers hebben geen preëxistente immuniteit tegen het virus en immuunsuppressie door middel van lokaal toegediende steroïden verergert deze situatie. De hoge prevalentie

Tabel 1: Complicaties bij PKP, geassocieerd met HSV-1

Complicaties	data voor de algemene PKP populatie		verlies van T_X geassocieerd met HSV-1		Methode
	n	%	n	%	
de novo HSV-1 keratitis na PKP	18/2112[6]	0.85	14/25[18]	56	virus kweek
Afstotingsreactie van de cornea	291/3048[2]	9,5	8/17[14]	47	PCR
			3 /17[14]	18	GWc
"Primary graft failure"	215/10363[19]	2.1	2/3[20]	66	PCR
Massaal endotheelcel-verlies in weefselkweek	7/1205[2]	0.58	9/199[17]	4,5	PCR
			7/199[17]	3,5	virus kweek



Figuur 1:
Seroprevalentie als functie van leeftijd voor de West Europese[17], alsmede voor de blanke en de zwarte populatie van de Verenigde Staten van Amerika[26].



Figuur 2:

De leeftijdssamenstelling van corneadonoren en ontvangers in Nederland beschikbaar gesteld door de corneawerkgroep "Kok-van Alphen" onder de auspiciën van het Nederlands Oogheelkundig Gezelschap. Bio Implant Services (Onderdeel van de Nederlandse Transplantatie Stichting, Leiden) verzorgt al het corneadonorweefsel voor Nederland in samenwerking met corneabanken op het Europese continent.

(10-20%) van HSV-1 DNA in normale en donor cornea's onderstreept de ernst van het risico[25].

De aanwezigheid van antilichamen tegen HSV-1 is afhankelijk van leeftijd, socio-economische status en geografische locatie (Fig.1)[26,27]. De prevalentie van HSV-1 DNA in de cornea, en het risico om een seronegatieve recipiënt te zijn, zal navenant variëren. Aangezien de seroprevalentie van HSV-1 daalt[28,29], neemt het risico voor transmissie door PKP toe.

Het werkelijke transmissiepercentage van HSV-1 door PKP in immuungecompromiteerde patiënten is onbekend. In het diermodel, waar HSV-1 naïeve dieren getransplanteerd worden met een donorcornea, die HSV-1 DNA positief is, worden de dieren slechts één week na transplantatie met immuunsuppressiva behandeld. In dit onderzoek wordt een transmissie percentage van HSV-1 van ongeveer 18% gevonden. Aangezien de mens de natuurlijke gastheer is van dit zeer besmettelijke virus en patiënten na transplantatie veel langer dan een week immuunsuppressiva gebruiken, lijkt een hoger transmissiepercentage dan in het diermodel niet onwaarschijnlijk.

Bepaling van het overall infectie risico voor infectie met HSV-1 na PKP

De volgende variabelen moeten betrokken worden in de risicoberekening: Seroprevalentie van HSV-1 (Fig.1), geografische locatie van de onderzochte populatie, leeftijdssamenstelling van de donor- en recipiënt populatie

(Fig.2), de kans dat een cornea van een patiënt, die seropositief is voor HSV-1, inderdaad virus bevat en de mate van transmissie van HSV-1 na PKP. (Tabel 2, Fig. 3 a and b):

- Het onderzoek van Wützler[27] wordt representatief beschouwd voor de seroprevalentie van HSV-1 op het West-Europese continent. Dat deze aanname toegestaan is, wordt bevestigd door een pilotstudy naar de seroprevalentie van HSV-1 in Rotterdam. Van november 1998 tot september 2001 werd bij 290 patiënten, die een corneatransplantatie ondergingen, de HSV-1 infectie status bepaald: 14% van de patiënten is seronegatief voor HSV-1 (niet gepubliceerde data). Het onderzoek van Nahmias[26] wordt representatief beschouwd voor de seroprevalentie van HSV-1 voor de blanke *(non-hispanic)* bevolking en voor de zwarte bevolking van de Verenigde Staten. (Fig.1)
- Gegevens over leeftijdssamenstelling van corneadonoren en ontvangers in West Europa worden weergegeven in figuur 2. Aangenomen wordt, dat in de Verenigde Staten van Amerika, een vergelijkbare leeftijdssamenstelling bestaat als in West Europa.
  Op basis van de bovengenoemde leeftijdssamenstelling werd de *overall* seroprevalentie in de donor- en recipiëntpopulatie berekend. (tabel 2)
- 10 tot 20% van de cornea's, waarbij geen oculaire HSV-1 infectie in de voorgeschiedenis heeft gespeeld, bevat HSV-1 DNA[25,30].
- Voor de mate van transmissie van HSV-1 na PKP wordt een glijdende schaal aangenomen van 20-100% (fig 3b).
- Het risico van superinfectie met HSV-1 in seropositieve patiënten wordt in deze berekening als verwaarloosbaar beschouwd.

Gebaseerd op deze variabelen kan het *overall* infectie risico van HSV-1 na PKP geschat worden op:

Overall infectie risico= $S_D * (1-S_R) * oog * Tr$

S_D = *overall* seroprevalentie in % of HSV-1 gecorrigeerd voor leeftijdssamenstelling van de donor populatie
S_R = *overall* seroprevalentie in % van HSV-1 gecorrigeerd voor leeftijdssamenstelling van de recipiënt populatie
oog = het deel van de donor cornea's dat HSV-1 DNA bevat
Tr = mate van transmissie van HSV-1 door corneatransplantatie

Tabel 2:
Overall seroprevalentie in de donor- en recipiënt populatie met het daaruit resulterende HSV-1 infectie risico, uitgaande van 100% transmissie uit HSV-1 DNA + cornea's

Populatie	S_D in %	S_R in %	Infectie risico in % als 10%-20% cornea's HSV-1 DNA +
West Europa	92	90	0.9-1.8
VS blank	81	78	1.8-3.6
VS zwart	83	82	1.5-3.0

Seronegatieve patiënten:

Ter voorkoming van transmissie van HSV-1 door PKP naar seronegatieve patiënten, bestaat de mogelijkheid, preventief systemische therapie met acyclovir te gebruiken gedurende de periode dat immuunsuppressieve therapie toegepast wordt[4,5]. Dit houdt in dat alle ontvangers serologisch getest zouden moeten worden op de aanwezigheid van HSV-1 antilichamen, voordat een corneatransplantatie verricht wordt.

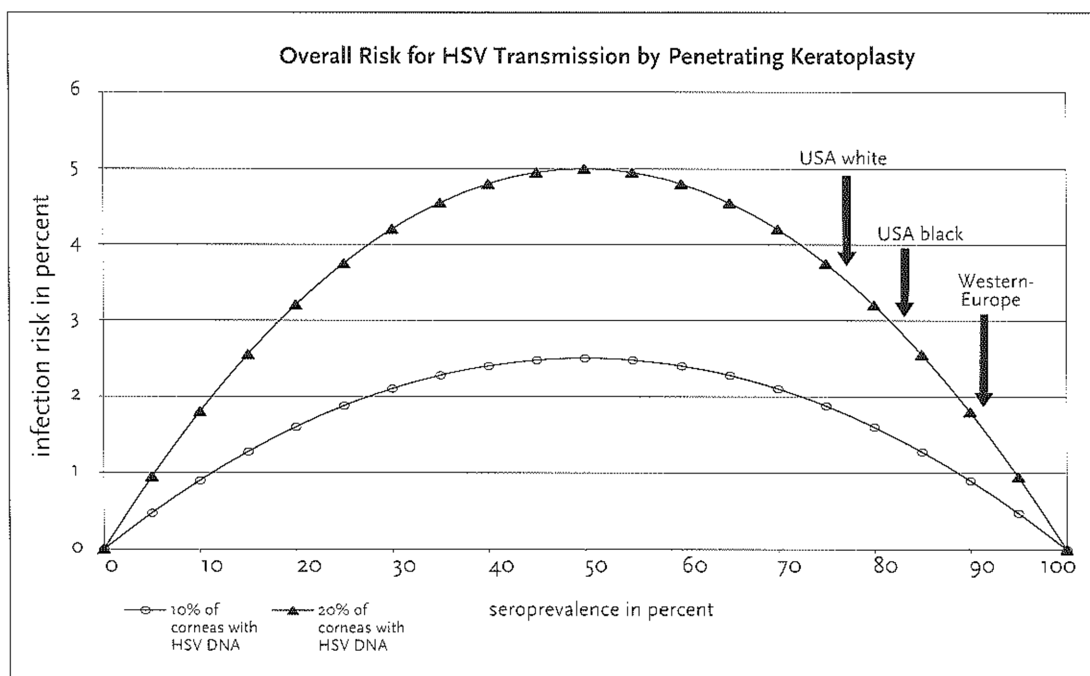


Figure 3a:

Overall infectie risico voor HSV-1 infectie na PKP als functie van seroprevalentie in de donor- en recipiënt populatie . Uitgaande van 100% transmissie. De witte pijlen geven het berekende overall infectie risico (tabel 2) aan voor HSV-1 infectie na PKP voor de verschillende geografische locaties.

Als naast het routinematig testen van de ontvanger ook de donor op dezelfde wijze getest wordt, ontstaat er een nog gunstiger situatie, omdat het dan wellicht mogelijk is seronegatieve patiënten met seronegatieve donoren te matchen. Dit zou, als matching logistiek haalbaar is, profylactische behandeling met acyclovir bij seronegatieve patiënten overbodig maken.

Seropositieve patiënten: superinfectie

Bij seropositieve patiënten kan een HSV-1 infectie van de cornea na PKP het gevolg zijn van reactivatie van latent virus in het ganglion trigeminale of van transmissie van het virus. In het laatste geval spreekt men van superinfectie. Transmissie kan zowel vanuit de bevolking, als vanuit de getransplanteerde cornea plaatsvinden. Alhoewel de resultaten in dit proefschrift aangeven dat superinfectie van de cornea mogelijk is en dat PKP een risicofactor is voor HSV-1 superinfectie van de cornea[11], is het klinisch belang van deze bevinding nog onbekend. Op het moment van afname van kweken, waren de klinische verschijnselen van de HSV keratitis vergelijkbaar in de groep met genetisch verschillende sequentiële kweken. Aangezien de kans op het verkrijgen van een HSV-1 infectie vanuit de bevolking bij seronegatieve patiënten kleiner dan 0.1% kan worden beschouwd, moet de kans voor seropositieve patiënten op superinfectie met HSV-1 nog lager worden ingeschat.

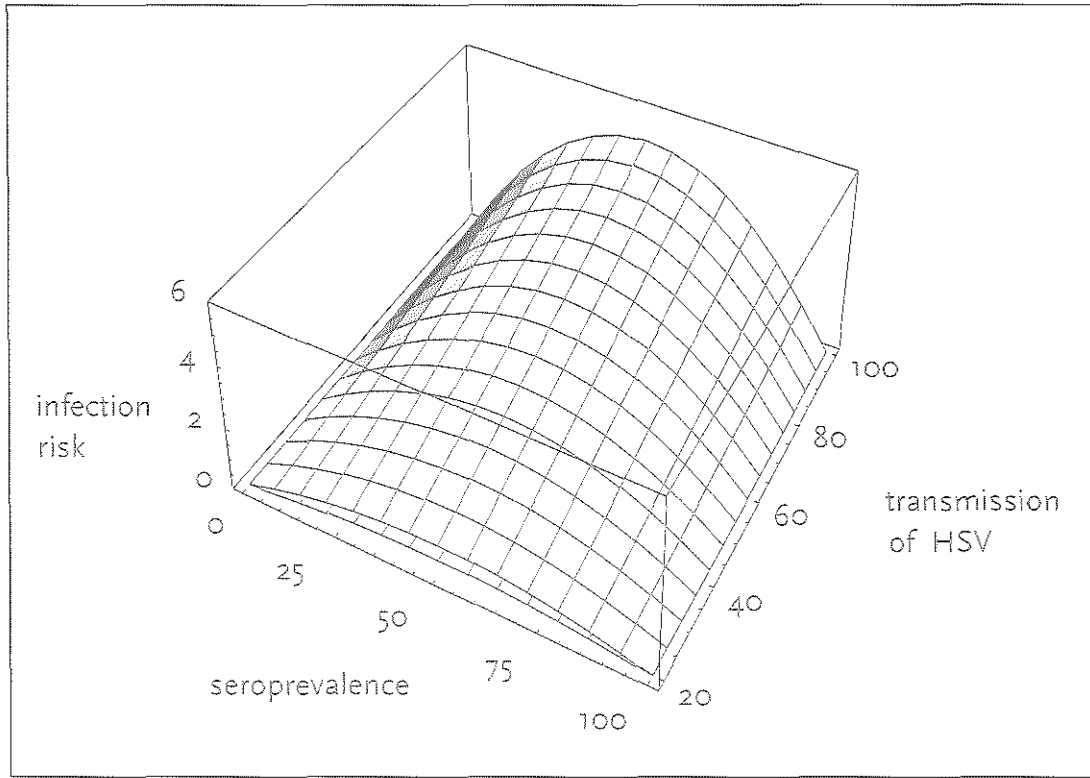


Figure 3b:

Overall risico voor infectie met HSV-1 door PKP als functie van seroprevalentie van donor- en recipiënt populatie en als functie van de mate van HSV-1 transmissie vanuit HSV-1 DNA positieve cornea's.

Seropositieve patiënten: reactivatie

De voornaamste oorzaak van actieve herpes keratitis is waarschijnlijk reactivatie van latent virus vanuit het ganglion trigeminale[31,32]. Bij seropositieve patiënten spelen waarschijnlijk reactivatie stimuli gerelateerd aan corneatransplantatie een rol in *de novo* oculaire HSV-1 infectie na PKP. Dit zouden kunnen zijn: het chirurgische trauma van de operatie of van hechtingen verwijderen, het gebruik van steroïden en afstotingsreacties[6]. Het voorkomen van reactivatie door profylactisch gebruik van acyclovir is een mogelijkheid. Dit zou betekent dat dan alle seropositieve ontvangers van een corneatransplantaat behandeld moeten worden gedurende de periode van immuunsuppressie, met alle consequenties van onnodige behandeling, bijwerkingen, toxiciteit en resistentie van het virus. Een andere, meer praktische, benadering is om oogartsen te waarschuwen, dat oculaire HSV infecties kunnen optreden na PKP, onafhankelijk van de oorspronkelijke diagnose waarvoor getransplanteerd werd.

Het vertrouwd zijn met de mogelijke presentaties van oogheelkundige HSV infecties na PKP, het gebruik van de juiste diagnostische middelen in deze patiëntengroep en de juiste, hoge dosis antivirale behandeling kan het verlies van het transplantaat en de daarmee eventueel geassocieerde corneale blindheid voorkomen.

Actieve HSV-1 replicatie in corneadonorweefsel:
Transplantatie van donor cornea's met actieve HSV-1 replicatie kan voorkomen worden door strenge criteria toe te passen bij de preoperatieve beoordeling van het transplantaat[17]. Als een actieve infectie van een cornea is aangetoond moet ook de andere cornea van dezelfde donor niet voor transplantatie gebruikt worden omdat waarschijnlijk dezelfde instrumenten gebruikt zijn bij de explantatie[17]. Op deze wijze zou de incidentie van "*primary graft failure*", geassocieerd met HSV-1, gereduceerd kunnen worden. Tot op heden heeft de screening op HSV-1 van de media, die gebruikt worden bij de weefselkweek geen aanvullende waarde[17].

Samenvattend leiden deze resultaten tot de volgende aanbevelingen:

- Het bewust maken van oogartsen dat HSV infecties zich na transplantatie kunnen ontwikkelen in het oog, onafhankelijk van de diagnose waarvoor de patiënt getransplanteerd is.
- Serologisch screenen van de ontvangers van een corneatransplantaat, en zo mogelijk en indien logistiek haalbaar, matching van ontvanger en donor voor HSV-1 sero-status.
- Als sero-matching van donor en ontvanger voor HSV-1 niet mogelijk is, moet overwogen worden profylactische of vroege therapeutische behandeling met systemisch acyclovir in te stellen bij seronegatieve ontvangers van een corneatransplantaat.
- De rol van HSV-1 infecties in afstotingsreacties moet nader worden onderzocht in een groot prospectief onderzoek.

Immunopathogenese van Humane HSK

De immuunrespons in de cornea, die volgt op een HSV infectie, komt overeen met de respons die men waarneemt bij HSV infecties op andere locaties in het lichaam. Hoewel deze immuunrespons essentieel is om de uitbreiding van het virus te beperken, kan zij de functie van de cornea nadelig beïnvloeden.

De huidige kennis over de pathogenese van HSK is vooral gebaseerd op experimenteel onderzoek in het HSK model in de muis. De virusspecifieke cellulaire immuunrespons staat centraal in de pathogenese van HSK. In het muismodel is aangetoond, dat $CD4^+$ T helper 1 cellen een hoofdrol spelen[33,34]. In het eerste deel van hoofdstuk 4 wordt voor de eerste keer in de literatuur een HSV-specifieke T cel respons aangetoond in de cornea's van twee patiënten met een actieve HSK. Hoewel uit de cornea's van deze patiënten zowel $CD4^+$ T cellen als $CD8^+$ T cellen konden worden opgekweekt, konden alleen $CD4^+$ HSV-specifieke T cellen worden aangetoond.

Deze bevindingen stemmen overeen met onderzoek in het diermodel. Niet in overeenstemming met het diermodel is het *ex vivo* cytokine profiel van de intracorneale T cellen in de mens. De humane HSV-specifieke intracorneale T cellen vertonen een Th0 fenotype, terwijl deze cellen in de muis een Th1 fenotype vertonen. Dit zou het gevolg kunnen zijn van de kweekomstandigheden gedurende kweek van humane intracorneale T cellen. Naast Langerhans' cellen en macrofagen bleken HSV-geïnfecteerde keratocyten in staat intracorneale HSV-specifieke T cellen te activeren.

De grote vraag in het dierexperimentele model van HSK blijft, welke antigenen herkend worden door de T cellen in de cornea. Gedurende de fase waarin HSK in de muis klinisch herkend wordt, kunnen er in de cornea noch replicerend virus, noch viraal mRNA, noch virale eiwitten worden aangetoond[35,36,37]. Dit suggereert, dat de immuunrespons niet direct gericht zou zijn tegen het virus. Alternatieve bronnen van antigenen worden geopperd. Zo zou HSV replicatie corneaweefselantigenen, die normaliter niet worden gepresenteerd aan het immuunsysteem van de gastheer, kunnen "ontmaskeren" en zelfs veranderen.

Recent onderzoek heeft bewijs geleverd, dat HSK bij de muis een auto-immuun respons is, die gericht is tegen corneaweefsel[38,39,40]. HSK kan veroorzaakt worden door $CD4^+$ T cellen die gericht zijn tegen een epitoop van het HSV-capside eiwit UL6, dat kruisreageert met een epitoop van een antigeen dat specifiek tot expressie komt in de cornea van de muis[39,40,41], de zo gehete "molecular mimicry". Een andere verklaring voor de aanwezigheid van $CD4^+$ T cellen is, dat de T cel respons het resultaat is van blootstelling aan cytokinen in de geïnfecteerde cornea, waar naar verwezen wordt als "bystander activation"[41,42].

De zoektocht naar auto-antigenen lijkt minder noodzakelijk als men de humane HSK pathogenese in ogenschouw neemt. Gedurende zowel de actieve als de inactieve fase HSK kunnen bij de mens replicerend virus én virale antigenen in de cornea worden aangetoond[43,44,45].

In het tweede deel van Hoofdstuk 4 wordt aangetoond, dat er een HSV-specifieke immuunrespons van $CD4^-$ T cellen aanwezig is, bij negen van de twaalf patiënten met een HSK. HSV-specifieke T cellen konden worden waargenomen zowel in de cornea van patiënten met een actief ontstekingsproces, als in de cornea gedurende een rustige fase van HSK. Bovendien konden HSV-specifieke T cellen worden aangetoond gedurende het gebruik van steroïden en in cornea's waar geen HSV DNA kon worden gedetecteerd. Meerdere onderzoeken van onszelf en anderen tonen aan, dat virus specifieke T cellen gedurende

lange periodes aanwezig kunnen zijn in de cornea[46,47,48]. De rol van auto-immuniteit in HSK bij de mens werd desalniettemin onderzocht. In geen van de T cellijnen die verkregen werden uit de cornea's van HSK patiënten kon een reactie op HSV-UL6 of op het oplosbare deel van het eiwit extract van humane cornea's worden aangetoond.

Deze voorlopige resultaten suggereren, dat de T celrespons in HSK patiënten gericht is op het verwekkende virus en niet op een cornea-autoantigen of op HSV-UL6[48]. Wij denken dat HSV-specifieke T cellen een belangrijke rol spelen in de locale immunopathogenese van HSK bij de mens. Wij verwachten dat HSV-geïnfecteerde cellen of virale peptiden, die achterblijven in het cornea stroma of in Langerhans' cellen, deze gelokaliseerde cellulaire immuunrespons initiëren en het proces onderhouden. De immuunrespons bestaat uit de interactie tussen ontstekingscellen, die de cornea infiltreren, en cellen die al aanwezig zijn in de cornea, en maakt gebruik van chemokines en Th cytokines. Het bewijs dat genoemde T cellen inderdaad bij de pathogenese betrokken zijn, is formeel echter niet geleverd.

Concluderende opmerkingen:

De nieuwe inzichten, die in dit proefschrift beschreven worden, betreffen drie onderwerpen:

- Het "dendritica" patroon, dat zo kenmerkend is voor epitheliale HSV keratitis, komt overeen met het patroon van de subbasale zenuw plexus van het cornea-epitheel, hetgeen opnieuw het neurotrope karakter van de het herpes simplex virus onderstreept.
- PKP moet als een risicofactor beschouwd worden voor een daaropvolgende HSV infectie, die ofwel het resultaat is van directe virale transmissie ofwel van reactivatie van het virus.
- Bij een HSK infiltreren HSV-specifieke T lymfocyten de cornea, hetgeen suggereert dat zij een oorzakelijke rol spelen in de pathogenese van dit ontstekingsproces.

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Abbreviations

ACAID	anterior chamber associated immune deviation
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
BLCL	B lymphocyte cell lines
CNS	Central Nervous System
cpm	counts per minute
DNA	desoxy ribonucleic acid
EBV	Epstein-Barr virus
EM	electron micrograph
gC	glycoprotein C
GI	gastro-intestinal
GWc	Goldman-Witmer coefficient
HCMV	human cytomegalovirus
HEK	herpetic epithelial keratitis
HHV	human herpes virus
HSK	herpetic stromal keratitis
HSV	herpes simplex virus
HuSoCo	human soluble cornea protein
HZO	herpes zoster ophthalmicus
ICP	infected cell protein
IEK	infectious epithelial keratitis
IFN	interferon
Ig	immunoglobuline
IL	interleukin
IR	internal reiterated sequence
ISK	immune stromal keratitis
kb	kilo base pairs
KP	keratic precipitates
LAT	latency associated transcript
LCs	Langerhans cells
MAbs	mouse anti-human monoclonal antibodies
MHC	major histocompatibility complex
MOI	multiplicity of infection
NK	natural killer cell
NSK	necrotizing stromal keratitis
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PGF	primary graft failure
PHA-L	phytohemagglutinin-L
PKP	penetrating keratoplasty
PMN	polymorphonuclear cells
r IFN	recombinant interferon
Re	reiteration
RFLP	restriction fragment length polymorphism
rHK	recrudescent herpetic keratitis
rIL	recombinant interleukin
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
rVV	recombinant vaccinia virus
SEI	subepithelial infiltrates
SPK	superficial punctate keratitis
TCC	T cell clones
TCL	T cell lines
TCR	T cell receptor
TG	trigeminal ganglion
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
TR	terminal reiterated sequence
T_x	transplantation transplantatie
U_L	unique long sequence
U_S	unique short sequence
VZV	varicella-zoster virus

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