Pathogenesis of Herpes Simplex Virus Infections of the Cornea

Pathogenese van herpes simplex virus infecties van het hoornvlies

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 19 december 2002 om 16.00 uur

door

Jeroen Maertzdorf
geboren te Utrecht

Promotiecommissie

Promotor:

Prof.dr. A.D.M.E. Osterhaus

Overige leden:

Prof.dr. J.J.M. van Dongen

Prof.dr. F.G.A. van der Meché

Prof.dr. G. van Rij

Co-promotor:

Dr. G.M.G.M. Verjans

The studies described in this thesis were conducted at the Institute of Virology, Erasmus University Rotterdam, The Netherlands. The work was supported by the "Dr. F.P. Fischer Stichting", Utrecht, The Netherlands.

Cover illustration: Ramon Verberne

Printed by: Drukkerij Labor, Utrecht, The Netherlands

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Chapter 1

General Introduction

1. HUMAN HERPES VIRUSES

History and Classification of Human Herpes Viruses

The identification of human herpes virus (HHV) infections can be traced back to ancient Greece where Herpes simplex virus (HSV) infections in humans were first documented. Hippocrates used the word "herpes", meaning to creep or crawl, to describe spreading skin lesions. Although the vesicular nature of lesions associated with HSV infections had been well documented in the late eighteenth century, person-to-person transmission of HSV was only first recognised by Vidal in 1893.[1] HSV was first isolated in 1919,[2] but decades passed before it was demonstrated that there were in fact two serotypes of HSV, HSV-1 and HSV-2.[3] Over time, several other members of the HHV family have been identified. The latest member, HHV-8, was only identified last decade.[4] The major laboratory advances of the past 20 years have enabled a torrent of new insights into the biological properties of HHV.

Herpes viruses infect members of all groups of vertebrates, and the same host can be infected with multiple distinct and unique types. Initially classified on the basis of tissue tropism and more recently based on DNA sequence homology, the herpes viruses have been typically grouped into three subfamilies:[1]

(1) The alpha-herpes viruses generally have a rapid replication cycle and a broad host cell range. Alpha-herpes viruses are further characterised by their neurotropic properties and tendency to establish latent infections in neural tissue.
(2) The beta-herpes viruses, which differ in genome size and structure, have a much more restricted range of host cells and replicate more slowly in culture.
(3) The gamma-herpes viruses are lymphotropic, infecting predominantly T- or B-lymphocytes, and causing either lytic or latent infection. Herpes viruses in this group are furthermore characterised by their oncogenic properties.

To date, eight distinct HHV have been described, each causing a characteristic disease.[1] Among the alpha-herpes viruses, HSV-1 and -2 are the primary agents of recurrent facial and genital herpetic lesions, respectively, while varicella zoster virus (VZV) is the causative agent of chicken pox and shingles. The prototypic member of the group of beta-herpes viruses is human cytomegalovirus (HCMV), which is linked to congenital infections of the nervous system. In contrast, infections with two other lymphotropic beta-herpes viruses, human herpes viruses -6 and -7 (HHV-6 and –7), generally cause mild early childhood diseases. Infections with two human gamma-herpes viruses, Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8) are associated with human cancers. The most common disease caused by infection with EBV is generally known as "infectious mononucleosis", while HHV-8 is believed to play a causative role in Karposi's sarcoma.

Herpes Simplex Virus Morphology and Genomic Organisation

Herpes Simplex viruses were the first of the HHV to be discovered and are among the most intensively investigated of all viruses. Their characteristic features are their biological properties and their strategies to evade the host immune system. Particularly their ability to cause a variety of infections, to remain latent in their host for life, and to be reactivated to cause new lesions have challenged many investigators.

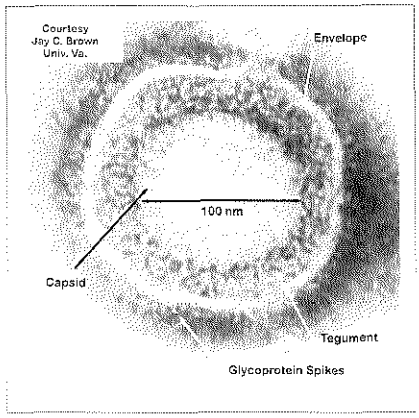

Figure 1: Electron microscopic image of the HSV-1 virion.

Herpes viruses are among the largest animal viruses known, both in structural size and genomic content.[5] The size of the HSV virion varies and its capsid is approximately 100 nm in diameter. The HSV virion consists of four elements (see Fig.1): (a) the core, containing the genome, (b) an icosadeltahedral capsid surrounding the core, (c) an amorphous and largely unstructured tegument surrounding the capsid, and (d) an outer envelope consisting of a lipid bilayer with about 12 different viral glycoproteins embedded in it.

The genome of HSV consists of double-stranded DNA containing 152 kilo-base pairs (kb) for HSV-1 and 154 kbp for HSV-2, with a G+C content of 68% and 69% for HSV-1 and HSV-2 respectively.[6] The HSV-1 genome can be viewed as consisting of two covalently linked components, designated as long (L) and short (S) unique sequences, flanked by inverted repeats (see Fig. 2A). These flanking repeats enable the inversion of the L and S components, yielding four linear isomers, which are found in equal concentrations in populations of wild-type virus-infected cells.[7,8] The HSV-1 genome is a linear, double stranded DNA duplex that circularises upon release from the capsid into the nucleus of the host cell. HSV-1 encodes at least 84 different polypeptides[9] (see Fig. 2B). Most proteins have multiple properties; hence HSV genes can encode several hundred different functions. Of the 84 known polypeptides, at least 47 are not essential for viral replication in cultured cells,[10] although they are not completely dispensable. Some execute essential functions in differentiated cells or alter cellular metabolism to ensure high yields of progeny virus. Mutant viruses lacking these genes thus can only survive in fully permissive cultured cells but not in their natural host. The tegument proteins and the glycoproteins are amongst the major targets for the host defence system. For example, viral surface glycoproteins are a major target for virus neutralising antibodies, while cellular immunity is often targeted to epitopes within both glycoproteins and tegument proteins.[5]

Next to the intertypic variation between HSV-1 and -2, intratypic variation exists within each sero-type. At the DNA level, differences between HSV-1 strains appear to result from base substitutions and variability in the number of repeated sequences present in a number of regions of the genome. Variations in the number of these reiterated sequences have been used to distinguish between different HSV-1 strains in epidemiological studies.[11,12] Hayward *et al.* were the first to find that restriction endonuclease patterns could

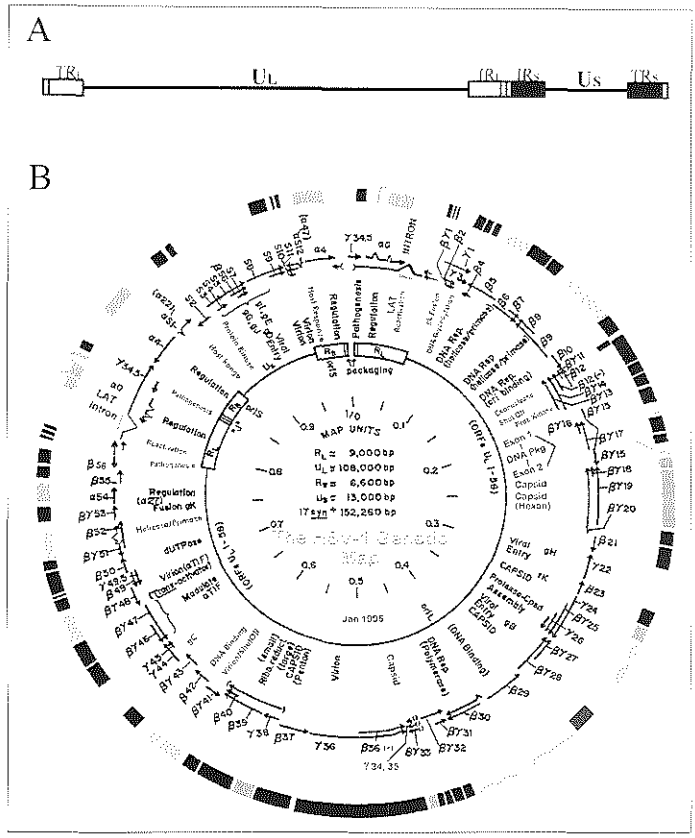

Figure 2:
(A) Schematic representation of the HSV genome with its unique long (UL) and short (US) regions, which are flanked by inverted repeats (IR and TR). (B) Genetic map of HSV-1, indicating the locations of genes and the proteins they encode.

be used to genetically differentiate unrelated HSV strains.[13] With this technique, known as restriction fragment length polymorphism (RFLP) analysis, it has been shown that individuals can be infected with multiple different strains of HSV at the same anatomical site.[14] This demonstration that a target organ is not immune to reinfection is an important issue from a public health viewpoint and provides major difficulties for vaccine development. Although probably suitable for most clinically related studies on HSV reinfection, RFLP has as a major disadvantage that it is very laborious and depends on the isolation of viable virus. To overcome these shortcomings we developed a PCR-based technique for the differentiation of HSV-1 strains, as described in chapter 1 of this thesis. In the following chapters (chapter 2 and 3), this technique was employed to investigate the transmission of HSV-1 in humans.

Herpes Simplex Virus Replication

Entry of HSV into a host cell involves the attachment of the virion to the surface of the cell, mediated through viral glycoproteins C (gC) and gB, and interaction of gD with cellular receptors. This interaction results in the subsequent fusion of the viral envelope and the plasma membrane to release the capsid-tegument structure into the cytoplasm (see Fig. 3). From here, the capsid is transported to the nucleus where it releases the viral DNA. Upon entry of the host cell, the tegument proteins are released into the cytoplasm and execute some crucial functions for the initiation of viral replication. The VP16 tegument protein (also known as αTIF) induces the gene transcription of the earliest set of viral genes. The virion

host shutoff (vhs) protein, which is also embedded in the tegument, causes non-specific degradation of mRNA and shutoff of host protein synthesis.[5] Transcription of the viral genome, replication of viral DNA, and assembly of new capsids take place in the nucleus of the host cell.

The productive replication cycle of HSV gene expression is tightly regulated and ordered in a cascade fashion of increasing complexity (see Fig. 3).[15] The earliest genes to be expressed in the replication cycle of HSV are the immediate early- or α-genes, which act to activate subsequent viral gene expression. Alpha genes appear to have solely regulatory functions and are responsible for the initiation of viral replication. Two of these immediate-early genes, US1 and US12, encoding for proteins ICP22 and ICP47, have been reported to be involved in evasion of immune recognition by both $CD4^-$ and $CD8^+$ T cells, respectively.[16,17]

This first phase of gene transcription is followed by the expression of the early- or β-genes that are either directly or indirectly involved in viral genome replication. Finally, upon genome replication, viral structural proteins are expressed in high abundance during the late- or γ- phase. These proteins are involved in assembling the capsid in the nucleus and modifying the membranes for virion formation.[5]

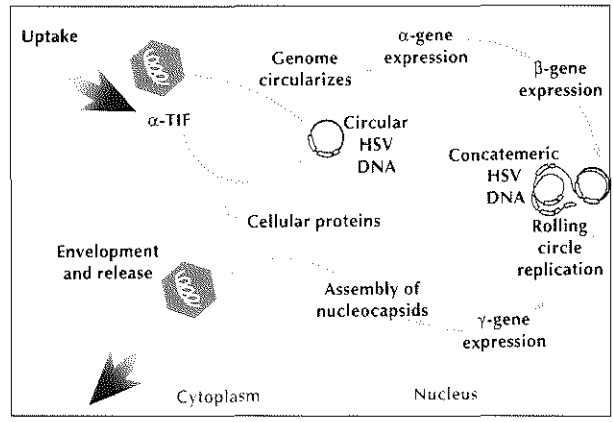

Figure 3: Replication cycle of HSV.

Viral capsids assemble in the nucleus, and bud through the nuclear membrane, which contain the viral glycoproteins. During the maturation process, the capsids are surrounded by tegument proteins that may functionally interact to aid in envelopment. Enveloped infectious virions can either remain cell associated and spread directly from cell-to-cell, or can be released from the cell for infection of other host cells.

Latency and Reactivation of HSV

The ability of HSV to persist lifelong in the human host is one of the most striking aspects of HSV infection. This ability of HSV to persist in its host is perhaps best illustrated by the fact that in up to 80% of healthy adult individuals HSV-1 DNA can be detected in the trigeminal ganglia, the main site of HSV-1 neuronal latency.[5] After a primary productive infection in epithelial cells, the virus spreads through the tissue and some virions enter sensory neuron axons. The virions are then translocated by retrograde axonal transport to the cell body in the innervating sensory ganglion. In the ganglion, virus either replicates productively or establishes a latent infection in the neuronal cell nucleus. During this latent phase, viral gene expressing is shut down with the exception of expression of the latency-associated transcripts (LATs). Although the function of these transcripts is not well known,

they are believed to play a role in down-regulation of lytic gene expression, thus promoting latency. Because of this relative lack of viral gene expression and the target cell of latency (i.e. neuronal cells), HSV is able to evade eradication by the immune system. In humans, latent virus can reactivate after local stimuli such as local trauma and tissue damage, or by systemic stimuli like stress, hyperthermia, exposure to ultraviolet light, hormonal imbalance and so forth. When the virus reactivates it restarts viral replication after which progeny viruses travel back through the neurons, usually to the site of initial infection. The frequency of reactivation has been related to the severity of the primary infection and the number of neural cells harbouring latent virus.[18]

2. HSV INFECTIONS

Epidemiology and Disease Manifestations

The spectrum of disease caused by HSV infections includes a wide variety of primary and recurrent infections in mucous membranes, skin tissue and the central nervous system (CNS). HSV-1 and HSV-2 are usually acquired by different routes and affect different areas of the body, but the signs and symptoms that they cause overlap.[19] While HSV-2 is more frequently associated with genital infections, HSV-1 infections are best known for causing herpes labialis. Intra-oral lesions are indicative for primary HSV-1 infection, lip lesions are more characteristic for recurrent infection. Although most genital HSV infections are caused by HSV-2, an increasing proportion is attributable to HSV-1.[10] Most individuals contract HSV infection during the first decades of life. The prevalence of HSV-1 antibodies varies from 45% to 88% in adult populations and is influenced by sex, geographic location, socio-economic status and age.[10] Antibodies to HSV-2 are rarely found before the age of onset of sexual activity, but HSV-2 seroprevalence increases to 35-60% by age 60.[20,21] This prevalence has increased dramatically since the late 70's and is highest among prostitutes (75%) and male homosexuals (83%).[20]
Apart from these most common orofacial and genital infections, HSV can also cause a variety of other diseases. Other cutaneous HSV-1 lesions generally manifest as eczema herpeticum, but can also be more disseminated. Viral infections of the eye are usually caused by HSV-1, most commonly presenting as conjunctivitis, blepharitis or herpetic epithelial keratitis (HEK).[22,23] More severe cases of corneal HSV infections can also lead to potentially blinding diseases like herpetic stromal keratitis (HSK). A more detailed description of ocular HSV infections is given in the section below. Infection of the CNS can result in herpetic encephalitis, which is one of the most devastating manifestations of HSV infections. HSV encephalitis causes case fatality rates that exceed 70% among older children and adults if left untreated, and only a small minority regain normal neurological function.[9] HSV infections can also cause serious complications in neonates, frequently associated with encephalitis or disseminated infection and a lethal outcome. The incidence of neonatal HSV seems to be directed related to seroprevalence of HSV-2 and is most commonly acquired when the mother experiences a primary genital infection during child birth.[10] Finally, another group of individuals at risk for severe HSV infections are immunocompromised patients like organ transplant recipients and AIDS patients.[24] Because of their inability to mount an adequate

immune response to the virus, frequently reactivating HSV can cause severe local or systemic infections in these individuals.

Nowadays, most HSV infections can be efficiently counteracted by treatment with aciclovir, a synthetic purine-nucleoside analogue that disrupts viral DNA replication. It is the most effective clinically used antiviral drug to date and has become the standard of therapy for HSV infections.[10]

Transmission of HSV

Transmission of HSV usually occurs by close contact via the mucosal surfaces. The type and severity of HSV infection that results depends on the host's immune status. Primary HSV infections are commonly subclinical. Most episodes of clinical disease are manifestations of recurrent HSV infections. Although many HSV infections are asymptomatic, around one-third of the world's population have recurrent HSV infections, capable of transmitting HSV during episodes of productive viral replication. Viral shedding and thus transmission can however also occur without clinical symptoms, which particularly holds for genital infections.

Transmission of herpes viruses through organ transplantation poses a serious health problem. The most frequent cause of herpes virus transmission through organ transplantation involves CMV. Although probably rare, transmission of HSV through organ transplantation may also occur.[25-27] Corneal transplantation, in medical practice referred to as penetrating keratoplasty (PKPL), is the most common type of tissue transplantation in the Netherlands, with usually successful outcome and few side effects. In few cases however, patients may develop corneal HSV infections after corneal transplantation performed for reasons unrelated to HSV. Such cases are referred to as newly acquired herpetic keratitis.[28-30] Although the origin of HSV infection in these cases is unknown, data from animal studies have suggested that transmission of replication-competent HSV through corneal transplantation is possible.[31] Although HSV latency in sensory ganglion neurons is well documented, studies by the group of Dunkel *et al.* have also suggested the existence of extraneuronal latency in the cornea.[32,33] In these studies, HSV gene expression was detected in corneas of latently infected rabbits and human quiescent herpetic corneas. Especially the detection of LAT expression in quiescent human HSK corneas[33] implicates the cornea as an extraneuronal site of HSV latency and reactivation. If corneal latency is for real, it probably reflects a low level of HSV replication causing a quiescent HSV infection rather than representing the "classical" conditions of reactivation of latent HSV residing in ganglion neuronal cells.

The data presented in chapter 4 indicate that PKPL may be a risk factor for the development of HSK. In the same chapter, we also provide direct proof that donor to recipient transmission of HSV and subsequent replication in the patient can occur in human corneal graft recipients.

Epidemiology of Ocular HSV infections

HSV appears to be the most common infectious cause of blindness in developed countries,[23] largely because of its recurrent nature. Ocular HSV infections are mainly confined to the anterior segment of the eye, including the conjunctiva and the eyelids. The

overall prevalence of ocular HSV infections has been estimated at 149 cases per 100.000 person-years.[23] Ocular manifestations are only observed in about 1% of exposed individuals, and only 5% of ocular HSV disease represent primary infections.[23] Despite the relative rarity of ocular HSV disease, its outcome can have a rather profound morbidity. Primary ocular HSV infections usually result in mild-to-moderate diseases like conjunctivitis, blepharitis and HEK. However, recurrent manifestations of ocular infections can result in more serious disease like herpetic stromal keratitis (HSK). Recurrence rates of ocular HSV infections have been recorded in one-third up to 63% of patients and were more frequent in children and young adults.[23,34,35] Half of these patients had more than one recurrence over a follow-up period of 15 years, with 10% of the patients experiencing 6-15 recurrences during this time period. Studies on visual outcome following recurrences have indicated that more than 90% of patients maintain good visual acuity despite prolonged disease.[36] For a significant minority of patients with recurrent herpetic episodes however, the resulting corneal scarring may require corneal transplantation to regain sight. The pathologic characteristics of HSK and the development of corneal scarring resulting in blindness are dealt with in the next section.

Although the risk factors for ocular HSV disease are largely ill defined, a large-scale study in 326 patients showed that the risk of recurrent HEK was not affected by previous episodes of HEK. In contrast, a previous episode of HSK increased the risk of a recurrence of HSK by 10-fold, with a strong relation between risk and the number of previous episodes.[37] Furthermore, the interval between recurrences appears to shorten with time.[22]
One of the more severe ocular infections caused by α-herpes viruses is acute retinal necrosis (ARN). Although this disease is rare, retinal detachment or destruction due to a localised immune response can leave patients blinded for the rest of their lives. Most cases of ARN are caused by VZV, while in younger patients HSV is often the causative agent.[38,39] An association between ARN and a past or concurrent history of herpes simplex encephalitis has been reported by several authors.[40-42]

In chapter 3 we describe the analysis and relation of HSV-1 strains found in both the brain and eye of two patients with ARN and a previous episode of herpes simplex encephalitis.

3. IMMUNITY TO HSV INFECTIONS

Host Immune Responses

Many viruses and their hosts have coexisted for millennia during which both have been constantly adapting. Despite effective and rapid responses of the immune system, herpesviruses are still common infectious agents in all groups of vertebrates. Acute and chronic viral infections are a constant battle between viral replication and the immune response of the host. If the immune system reacts too slow or in an inadequate manner, the host may suffer severe morbidity or die. On the other hand, an effective immune response will ultimately eradicate the virus or at least limit its replication. Despite the development of a usually highly effective immune system, some tissues of the host like the eye are poorly accessible to the immune system. These tissues are termed immune privileged sites. Moreover, throughout evolution herpes viruses have adapted several mechanisms to escape from immune detection. Nevertheless, effective immune responses do develop upon viral

infection of the eye. The main elements and cell types involved in ocular immunology are depicted in figure 4.

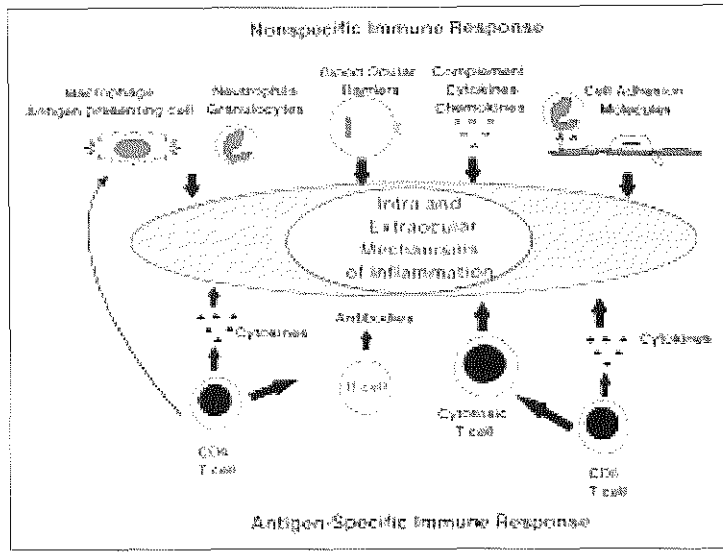

Figure 4: *Mechanisms and cellular events involved in inflammatory responses to viral infections of the eye. (reprinted with permission from: Streilein JW (ed.): Immune response and the eye. Chem. Immunol. 1999;73:90-119. Karger, Basel).*

Following HSV infection, the host's immune system will basically combat the virus by two mechanisms. At first, a non-specific immune response (i.e. innate immunity) develops, orchestrated by macrophages, natural killer (NK) cells and polymorph mononuclear cells (PMN) which are directed towards the site of infection. Innate responses start almost immediately after viral infection and provide a crucial first line of defence, especially in infection of naive hosts. Clearing of the virus however depends on a virus-specific immune response (i.e. adaptive immunity) in which both T- and B-lymphocytes are involved and takes several days to develop.

Innate Immunity

Leukocyte populations, excluding the classical T- and B-lymphocytes, as well as soluble factors, contribute to the innate immune response to viral infections. These cell types are widely distributed in the body and are critical in controlling the overall extent of viral replication and to limit the spread of virus immediately after infection. Macrophages exert their antiviral function directly through phagocytosis of viral particles and subsequent degradation, and through the secretion of anti-viral cytokines like TNF-α and IFN-α. In an indirect manner, macrophages can mediate a number of important immune-regulatory effects through the secretion IL-12, TNF-α and IL-1α.[43] The importance of NK cells in early defence against HSV infection has been clearly established.[44] Severe herpesvirus infections occur in individuals with genetic defects in NK function and in immunocompromised individuals.[45,46] In HSV infection, NK cell-derived IFN-γ exerts an anti-viral effect through inhibition of viral replication.[47] Virus-infected cells can be recognised and actively killed by NK cells through opsonization by virus-specific antibodies following interaction with Fc receptors, and through a MHC-independent recognition of viral antigen.[48] Infection with HSV also results in a downregulation of MHC class I expression,[17] making infected cells more susceptible for killing by NK cells.[49]

Soluble factors like cytokines and chemokines are the non-cellular components of the innate immune system. All cell types of both the innate and adaptive immune system contribute to the production of these factors, as well as some virus-infected or activated non-immune cells such as fibroblasts. For example, type 1 IFNs (IFN-α and IFN-β) are produced by the infected cell itself to induce protection against infection in neighbouring cells. The functions of these cytokines in anti-viral immunity include inhibition of viral replication, activation of NK cell cytotoxic activity and induction of MHC class I expression.[43,50] Besides cytokines, molecular chemotactic factors known as chemokines are produced upon viral infection by a variety of both immune and non-immune cells. These chemokines have important pro-inflammatory functions and contribute to innate immunity by attracting leukocytes to infected tissue.[51,52] Overall, innate immunity functions not only to protect the host in the early stages of infection, but also to direct the qualitative and quantitative nature of adaptive immunity.

Adaptive Immunity

The humoral and cellular adaptive immune responses mediated by antibodies and T cells, respectively, are the two main antigen-specific effector systems for resolving viral infections.[43] Antibodies control virus replication by neutralising free viral particles or killing virus-infected cells through complement-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity (ADCC). This mechanism of ADCC has been validated as a critical component of antiviral defence against HSV infections, especially in immunocompromised individuals and neonates.[53]

The major targets for antibodies are the surface glycoproteins and outer capsid proteins of HSV.[54,55] Binding of neutralising antibodies to glycoproteins prevents viral attachment to and infection of host cells. Although the protective role of antibodies in HSV infection is not completely clear, they seem to exert a major effect during primary infection. Especially secretory immunoglobulins of the IgA isotype may be important in neutralising HSV particles that enter via the mucous membranes. However, high titres of neutralising antibodies do not always prevent or diminish recurrent HSV infections.

Antibodies serve as a major defence against free viral particles, while the main function of T cells is the recognition and elimination of virus-infected cells. T cells can be subdivided into two subsets, $CD4^+$ and $CD8^+$ T cells, which recognise viral antigen in association with host major histocompatibility complex (MHC) molecules II and I, respectively. In HSV -infected cells, viral proteins are degraded into short peptides that subsequently bind to MHC molecules. These MHC/peptide-complexes are then translocated to the cell surface for recognition by antigen-specific T cells.[43] Both $CD4^+$ and $CD8^+$ T cells play a central role in antiviral immunity either directly by their cytolytic properties or indirectly by their secretion of cytokines upon activation or in case of $CD4^+$ T cells by the induction of a specific humoral immune response. Remarkably, in HSV infections in humans, $CD4^+$ T cells have emerged as the predominant killer cell phenotype.[56]

Severe HSV infections are common in patients with impaired T cell immunity (e.g. AIDS patients or transplant recipients), indicating that T cells play an essential role in controlling HSV infections.[57] For HSV infections, both clinical observations in humans and studies performed in experimental animal models have clearly indicated the importance of $CD8^+$ cytotoxic T cells (CTL) in the resolution of HSV disease. Likewise, $CD4^+$ T cells probably play an equally critical role in the resolution of HSV, given the importance of this cell type

in mediating delayed type hypersensitivity responses and clearing local infections.[56] Similarly, in several mouse models of HSV infections, both $CD4^+$ and $CD8^+$ subsets of T lymphocytes have been shown to be important in protection against lethal infection and to enhance viral clearance.[58]

Immune Evasive Mechanisms of HSV

HSV has developed various strategies to evade host immune surveillance and persist *in vivo*. This capability of HSV to circumvent defensive mechanisms of the immune system enables it to avoid detection and elimination by the immune system. One key adaptation of the virus to do this is to infect the CNS, a tissue that is not readily accessible to the immune system, and to establish latency within ganglion cells. The fact that during a latent infection no or very limited viral protein expression takes place and because of a lack of MHC class I expression on neural cells makes the virus impassive for T cell mediated recognition. During active viral replication however, HSV also has several "tricks" to evade the immune system. During lytic infection the HSV ICP47 protein, encoded by the US12 gene, interferes with the transporter associated with antigen presentation (TAP).[17] Through this action peptide translocation into the ER is blocked resulting in inhibition of viral antigen presentation through MHC class I and recognition by $CD8^+$ T cells. Additionally, HSV has recently also been shown to inhibit recognition of infected cells by $CD4^+$ T cells. Barcy *et al.* showed that HSV -infected B cells exhibited a strongly reduced capacity to stimulate antigen-specific $CD4^+$ T cells.[16] Although its mode of action is not yet known, this inhibitory effect on APC function is most likely mediated by the HSV ICP22 protein, encoded by the US1 gene.

Another strategy of HSV to evade immune responses involves interference with complement-mediated virus neutralisation and lysis of infected cells through binding of complement component C3 by gC.[59]

When a host cell gets infected by a virus it will usually go into apoptosis. Alternatively, infected cells can be induced by T cells to enter the apoptotic phase. To be able to complete viral replication and produce progeny viral particles, HSV like many other viruses has adapted methods to inhibit this suicidal tendency of cells.[60] On the other hand, HSV infection of T cells results in elimination of virus-specific T cells by inducing apoptosis of neighbouring T cells in a Fas-dependent fashion. This of immune evasion is referred to as fratricide.[61]

Ocular Immune Privilege

The immunological consequences of viral infections of the eye usually differ from those in other tissues because the eye is an immune-privileged site. The eye has acquired this immune-privilege status through evolution in order to allow local immune mediators to provide protection without interfering with tissue function. Especially for the eye this is an important issue since its precise microanatomy can be disturbed as a result of local inflammation. Even minor tissue destruction -as a consequence of inflammation- might interfere with the transmission of visual images, resulting in visual impairment or complete blindness.

Immune privilege in the eye is accomplished by structural features, soluble immunosuppressive factors and cell surface modifiers of immune function.[62] Next to a tight

blood-ocular barrier to prevent entrance of virus into the eye, the cornea of the eye lacks blood and lymph vessels that usually provide the main route of accessibility of inflammatory cells or immunological factors to other tissues. The aqueous humour in the anterior chamber of the eye, which is in close contact with the cornea, also contains several immunomodulatory factors.[63] Their functions include suppression of T cell activation and their differentiation towards the Th1 phenotype, modulation of T cells to suppress reactivation of bystander T cells as well as the suppression of macrophage and neutrophil function to further limit potential destructive inflammatory reactions.[64,65] Finally, expression of Fas ligand by ocular cells can trigger apoptosis of infiltrating Fas -expressing inflammatory cells.[66,67]

4. PATHOLOGY OF CORNEAL HSV INFECTIONS

Corneal HSV Infections

Primary ocular HSV infections most likely result from direct infection of the corneal surface. The majority of these cases involve lesions in the superficial epithelial layer of the cornea (see Fig. 5). These lesions result from the direct cytopathic effect of the virus, and typically heal without permanent loss of vision. Recurrent corneal HSV infections are generally confined to the epithelial layer. However, about 20% of individuals with recurrent cornea HSV infections go on to develop disease in the deeper stromal layer of the cornea. Involvement of the stromal layer of the cornea probably follows loss of integrity of Bowman's membrane resulting from recurrent infections. This membrane forms a tight and strong physical barrier situated between the epithelial and stromal layer of the cornea. One type of stromal disease is a necrotising form of herpes stromal keratitis (HSK), which leads to scarring of the cornea and progressive loss of vision with each recurrence. Corneal scarring and oedema formation associated with HSK is a frequent reason for corneal transplantation.

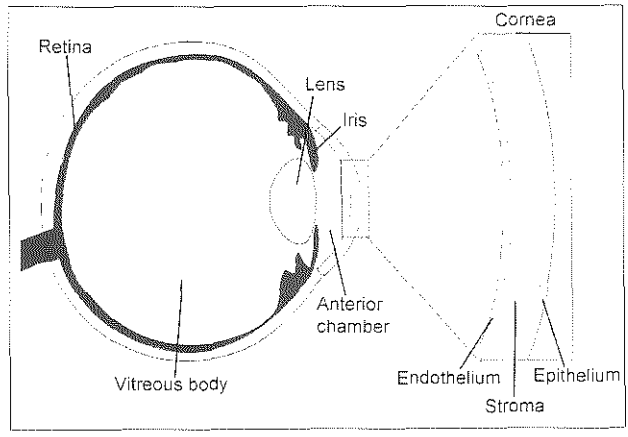

Figure 5: *Cross-sectional view of the human eye. Enlargement shows the anatomical make-up of the cornea with three distinctive layers of cells.*

Pathology of Herpetic Stromal Keratitis

HSV-1 corneal infections are a leading cause of non-traumatic blindness in developed countries.[68] The virus can replicate and cause lesions in the epithelial layer of the cornea (HEK), but permanent loss of vision is associated with a cell-mediated inflammatory response in the corneal stroma known as HSK. This immunopathologic process is the cause of the corneal scarring seen in HSK and involves both innate and adaptive immune responses to the infecting virus.[69]

Studies on HSK have greatly benefited from the availability of several animal models of the disease. The most extensively used models involve HSV-1 infection of the mouse cornea. Following inoculation of HSV-1 on the scarified cornea of a susceptible mouse, the animal develops a delayed onset of corneal inflammation that resembles many of the characteristics of recurrent HSK in humans. The main events in the development of murine HSK are schematically reflected in figure 6.

HSK in the mouse model is characterised by a bi-phasic cellular infiltrate of mainly neutrophils into the stromal layer (see Fig. 6).[70] The first wave of neutrophils invading the cornea is only transient and is involved in viral clearance,[71,72] while the second wave of neutrophil infiltration coincides with clinical manifestations of the disease. Essential for the development of HSK is the involvement of $CD4^+$ T cells, orchestrating the extravasation and activation of these neutrophils, considered to be directly involved in corneal destruction.[73-75]

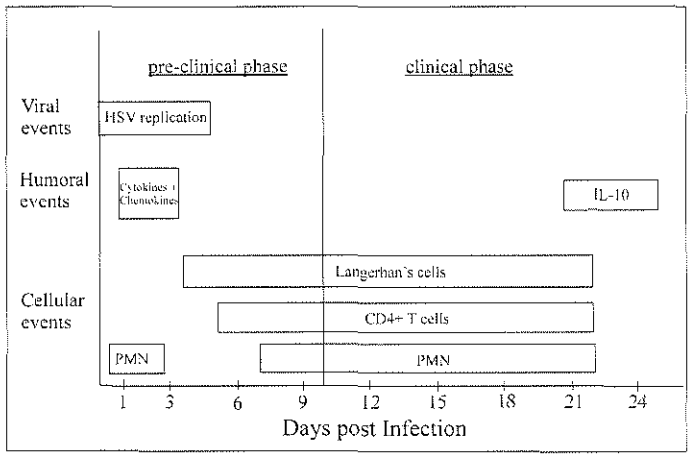

Figure 6: *Key events in the development of HSK as observed in the experimental mouse model. (adapted from ref.70)*

In addition to T cells, infiltrating macrophages and corneal Langerhans cells have also been implicated as important mediators in the immunopathology of experimental murine HSK.[76-79] One of the key events in the immunopathogenic processes in HSK is the interaction of infiltrating T cells and macrophages with resident corneal fibroblasts. Through this interaction, the corneal fibroblasts are activated and induced to secrete multiple pro-inflammatory cytokines and chemokines. In chapter 5, this activation of resident human corneal fibroblasts was investigated by mimicking their interaction with infiltrating T cells and macrophages in an *in vitro* culture system. In addition, cytokines and chemokines can be secreted by cornea infiltrating inflammatory cells. This induction of chemokines ultimately and inevitably results in the large-scale attraction and activation of additional T cells and neutrophils. Such massive cellular infiltrate into the cornea results in substantial swelling of the stromal layer of the cornea and causes the destruction of the stromal tissue integrity (see Fig. 7).

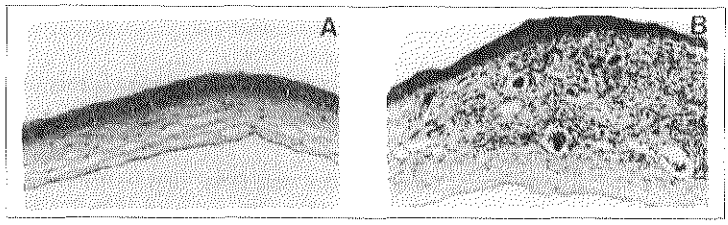

Figure 7:
Immunohistological cross-section of the mouse cornea showing (A) a healthy cornea and (B) the immunopathologic reactions in HSK. The top (dark) cell layer is the outer epithelial layer, the middle (grey) layer is the stroma and the thin lower layer represents the endothelial layer of the cornea.

The essential involvement of cornea infiltrating T cells in the development of HSK was first recognised by the observation by Metcalf *et al.* that the inflammation does not develop in T cell -deficient, athymic nude mice.[80] Subsequent studies established that the chronic inflammation in murine HSK is regulated by $CD4^+$ Th1 cells,[81-83] although the involvement of $CD8^+$ T cells has also been implicated.[84] Although little is known about the characteristics of HSK development in humans, they seem to resemble those found in the experimental mouse model. Limited studies have been performed on the characterisation of cornea infiltrating T cells in human HSK.[85-87] Results from these studies seem to relate well with the characteristic influx of $CD4^+$ T cells with a Th1 or Th0 phenotype in the murine cornea after HSV infection. However, these studies included a limited number of patients involved and few numbers of individual T cells analysed. Therefore, more detailed and broader analyses are required to get a better insight into the immunopathologic situation in human HSK.
In chapter 6 we describe a more elaborate characterisation at the single-cell level of HSV-specific T cells recovered from corneas of HSK patients.

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Chapter 2

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

Jeroen Maertzdorf, Lies Remeijer, Allegonda van der Lelij, Johannes Buitenwerf, Bert G.M. Niesters, Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

Journal of Clinical Microbiology ***1999;37:3518***

ABSTRACT

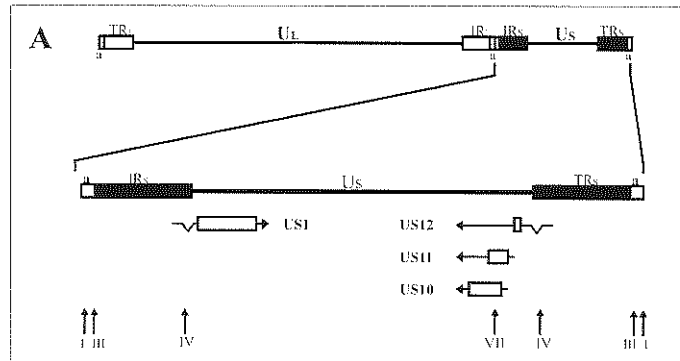
Herpes simplex virus type 1 (HSV-1) related disease ranges from localized, self-limiting illnesses to fatal disease in immunocompromised individuals. The corneal disease herpetic keratitis may develop after reactivation of a latent virus or reinfection with an exogenous herpes virus. Molecular analysis 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 (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 stability 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 ten 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 sequential samples were identical for each individual. Conclusively, the data show that the approach presented allows the rapid and accurate discrimination between HSV-1 strains, in studies addressing the transmission and pathogenesis of HSV-1 infections.

INTRODUCTION

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 (HEK) and the development of the potentially cornea blinding disease herpetic stromal keratitis (HSK).

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.[8,33] 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 between 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 an alternative method for clinical HSV strain differentiation using 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 reiteration I-VIII (ReI-VIII), 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



B

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

Figure 1.
Map of herpes simplex virus type 1 (HSV-1) genome and location and sequences of the reiterations tested. (A) The HSV-1 genome contains 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..[13] (B) Reiteration-specific sequences, as indicated by the superscript numbers, were derived from the following HSV-1 strains: 1, MP17; 2, USA-8; 3, F.

these regions varies. Re-I, -III, -IV and -VII 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 between HSV-1 strains.[27,28] Re- I and III are located within the "a" sequence of the repeat regions flanking the unique short sequence. ReIV is present twice within the HSV-1 genome, located within introns of both the genes US1 and US12, whereas ReVII is located within the protein-coding region of US10 and US11.[3,11,13]

This report describes the development of a PCR method to discriminate between HSV-1 strains, based on the variability of reiterated sequences within the HSV-1 genome. This approach was successfully used to discriminate between 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 swabs were obtained from 37 patients with herpetic keratitis at the Rotterdam Eye Hospital (The Netherlands) for diagnostic purposes. From 5 patients sequential samples (n=2) were obtained (mean time interval 19 months, range 9-38 months), in four cases from the same eye, and in one case from different eyes. Virus was grown on human embryonic lung fibroblasts and harvested when approximately 75% of the monolayer displayed a cytopathic effect. All culture samples were confirmed HSV-1 positive by PCR (data not shown). To determine the stability of the hypervariable regions, 24 subclones were generated from HSV-1 strain 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 (Janssen Chemika, The Netherlands), mixed, and incubated for 10 min at room temperature. The celite-bound DNA was washed twice with wash buffer, twice with 70% (v/v) ethanol, once with acetone and subsequently dried. DNA was extracted by resuspending the pellet in 150 µl water at 56°C for 10 min. A volume of 5 µl of the resulting DNA suspension was used per PCR reaction.

PCR amplification.

Primers were designed to amplify distinct regions in the HSV-1 genome, containing Re-I, III, IV or VII. PCR amplification was performed using several combinations of primers (Table 1). The PCR reactions were performed in 50 µl volumes. The reaction mixture contained 1.25 U cloned *Pfu* DNA polymerase (Stratagene Europe, Amsterdam, The Netherlands), corresponding buffer supplemented with 5% (v/v) dimethyl sulfoxide (DMSO), 1 µM of each of the primers and 200 µM of each dNTP, containing an equimolar amount of dGTP and 7-deaza-2'-dGTP (Boehringer Mannheim, Mannheim, Germany). A 5 µl sample of DNA suspension was added and the reaction mixtures were overlaid with 50 µl 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; see 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 PCR reactions were performed in a Perkin Elmer 480 thermocycler (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Table 1. Primers used for amplification and detection of HSV-1 reiterations.

Genome region	Primer[a]	Anneal temp(°C)	Sequence (5' 3')	Position in genome[b]
"*a*" sequence	RelF	72	GCCGCCACCGCTTTAAAGGGCCGC	125976-125999 / 152234-152257
	RelR		GTGCTCTGTTGGTTTCACCTGTGGCAGC	126368-126395 / 151838-151865
"*a*" sequence	RelIIF	72	TCTCTACCTCAGTGCCGCCAATCTCAGGTC	126742-126771 / 151462-151491
	RelIIR		CGAAGACGCAATAAACGGCAACAACCTG	127171-127198 / 151035-151062
US1	ReIVUS1F	64	TCCGACGACAGAAACCCACC	132333-132352
	ReIVUS1R		GTCCCGGAGGACCACAGTGG	132615-132634
US12	ReIVUS12F	58	TTTTTGCACGGGTAAGCAC	145853-145871
	ReIVUS12R		TGGTGTCCAGGAAGGTGTCC	145535-145554
US10/11	ReVIIUS1011F	56	AGCGTATGCTCCATGTTGTG	144697-144716
	ReVIIUS1011R		CGAGAACCTAGGGAACCCA	144928-144946
Rel	Rel probe	37	CCGCTCCTCCCC	
RelII	RelII probe	37	CCGCCCCTCGCCCCCTC	
RelV	RelV probe	37	CCCCCTCCTCCACCCCCGCGTC	
ReVII	ReVII probe	37	CCCCGGTCTCCCCGGGAG	

[a] F, forward; R, reverse.
[b] Positions correspond to the genomic HSV-1 sequence HE1CG (NID nr. G1944536).

Detection of amplified products.

Amplicons were size-fractionated in 2% agarose gels and visualized by ethidium bromide staining. Specificity of the amplicons was confirmed by Southern blotting.[20] Briefly, the electrophoresed samples were transferred onto Hybond N+ (Amarsham, Pharmacia Biotech). Hybridization was performed overnight at 37°C using [γ-^{32}P] ATP-labeled Re-specific oligonucleotides (Table 1). Posthybridization washes were performed twice with 2x SSC, 0,1% SDS 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 of individual samples, the DNA fragments were electrophoresed on denaturing (8 M urea) 6% acrylamide gels.[20] The length of the amplicons was 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.

RESULTS

Amplification of hypervariable genomic HSV-1 regions containing reiterations I, III, IV and VII.

On basis of documented variability and stability,[27,28] hypervariable regions containing ReI, -III, -IV and -VII were selected as candidate templates for PCR-mediated discrimination between unrelated HSV-1 strains.Amplification of these regions was not possible or insufficient using standard PCR reaction conditions (data not shown). Alternative conditions, selected to decrease the formation of secondary structures due to the high G/C content of these sequences, improved amplification of the target sequences and allowed direct visualization of the amplicons with ethidium bromide. Specificity of the amplicons was 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 stability of Re-I, -III, -IV and -VII, PCR amplification of these regions was performed on 24 separate subclones of the HSV-1 strain F and compared with the amplicons of the parental strain (Fig. 2). For the regions containing Re-IV and -VII, amplicons from all 24 subclones were identical in size to those of their parental strain, indicating the stability of Re-IV and -VII during the two limiting dilution rounds. For the regions containing Re-I and -III, not all separate subclones showed the same amplicon length as their parental strain, differences being greatest for ReIII (Fig. 2). Consequently, Re-IV and -VII were further used to discriminate between 37 unrelated HSV-1 isolates obtained from keratitis patients. The results of the analyses performed on all 37 clinical corneal HSV-1 isolates are summarized in Table 2. As an example, differences in amplicon lengths between unrelated clinical isolates from ten patients are shown in Fig. 3A. The variability in the US10/11 region (ReVII) was low, showing only three different alleles. Regions US1 and US12 (ReIV) showed a wider variety with 14 and 15 different alleles detected among the 37 samples analyzed respectively (Table 2).

Combining the results of the three amplified regions showed that 34 of the 37

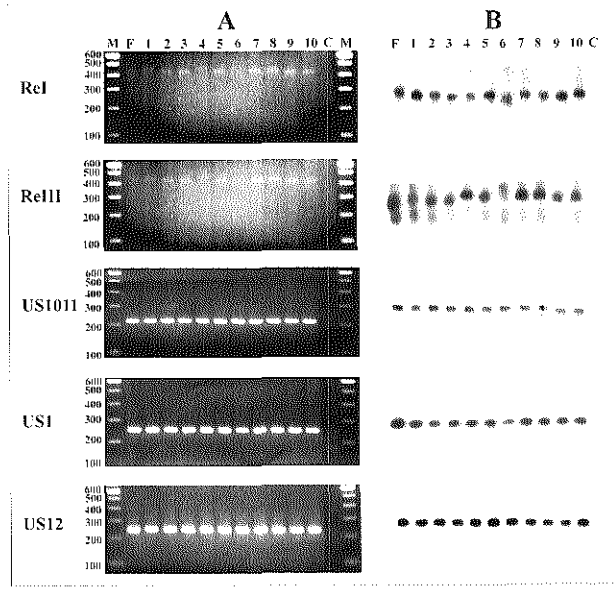


Figure 2.
Amplification of hypervariable regions within the HSV-1 genome. (A) PCR amplification on regions containing Re-I, -III, -IV and -VII was performed on DNA from various HSV-1 (strain F) subclones. Amplicons were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Ten representative samples from 24 subclones analyzed are shown. Lane F, HSV-1 parental strain F; 1-10, HSV-1 (strain F) subclones; lane C, water control; lanes M, 100 bp molecular size marker. Numbers on the left are in bp. (B) Autoradiogram of DNA in gel from panel A after Southern blot transfer and hybridization with a Re-specific probe.

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

Isolate and patient nr.	Estimated amplicon length (bp)		
	Region US10/11 (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 HSV-1 isolates			
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

[a] Three pairs of patients (patients 8 and 9, patients 10 and 11, and patients 12 and 13) were infected with clinically unrelated strains with identical DNA patterns.

isolates (92%) displayed a unique combination of amplicons. For some clinical samples, no PCR product could be detected with ethidium bromide staining, or multiple fragments appeared. This was probably due to poor quality of 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 variance in length. However, the amplicons from the sequential samples from each individual were identical.

DISCUSSION

In the present paper, we present a PCR based approach that allows the rapid and accurate discrimination between unrelated HSV-1 strains. The method generally used to discriminate between HSV-1 strains is RFLP analysis.[7,15,17-19,24,29-31] This method requires virus culture, is time-consuming and highly labor-intensive. Furthermore, culture requires viable virus, which is not always obtainable from certain types of clinical samples (e.g. cerebrospinal and intra-ocular fluids). More recently, a system using PCR amplification and subsequent RFLP analysis has been developed to facilitate discrimination between HSV-1 strains, eliminating the necessity of virus culture. This method, however, is not significantly less time-consuming and labor-intensive than conventional strain differentiation.[31]

Conventional RFLP analysis, using restriction endonucleases recognizing 6 base pairs (6-bp REs), is insufficient to differentiate HSV-1 strains of a predominant genotype. Using 4-bp REs and RFLP analyses of reiteration sequences greatly improved the differentiation rate.[28] As in our study, the RFLP analysis of reiterated sequences was based on varying numbers of repeats. Usage 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

between strains of other herpes viruses like Epstein-Barr virus and Human Cytomegalovirus.[23,36]

To be applicable in a PCR based assay to discriminate between different HSV-1 strains, these regions should show a considerable degree of variability, and remain stable during a relatively short time of replication. We tested the suitability of several HSV-1 hypervariable regions to discriminate between 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 reaction 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 replacing 100% dGTP, and 5% DMSO resulted in the most consistent amplification of the large alleles. Specificity of the amplicons was confirmed by hybridization with a Re-specific probes after Southern blotting.

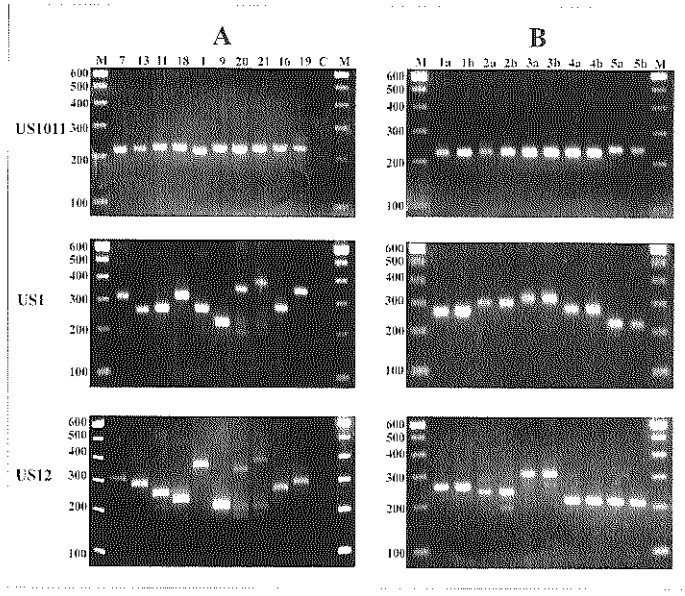


Figure 3.
Variability of Re-containing regions US1, US12 and US10/11 between unrelated and sequential corneal HSV-1 isolates. PCR amplification was performed on HSV-1 DNA from corneal virus isolates. Amplicons were analyzed as described for Fig. 2. Results from 10 representative samples out of 37 analyzed are shown on the left panel. The right panel shows amplicons from sequential samples from 5 individuals. Lane C, water control, lanes M, 100 bp molecular size marker. Numbers on the left are in bp.

Analysis of subclones of HSV-1 strain F showed that stability of the sequences Re-I and III was too low to be used for discrimination between HSV-1 strains. In contrast, ReIV and ReVII were shown to be stable during this procedure. Thus, regions US1 (ReIV), US12 (ReIV) and US10/11 (ReVII) were chosen for the discrimination between unrelated corneal HSV-1 isolates.

In agreement with previous studies, the variability in the US10/11 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 US10/11 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.

From five individuals with recurrent herpetic corneal infections, sequential corneal isolates were analyzed. For each individual, sequential samples showed identical DNA patterns while differing between patients. These results implicate 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 varying numbers of repeats. More detailed analysis, like sequencing 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 usually no viable virus can be detected.[4] Re-specific PCR analyses were performed on DNA isolated from affected corneal buttons and rims obtained from HSK patients during therapeutic keratoplasty. The PCR approach proved to be sensitive enough to amplify 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 between 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 to address questions regarding reactivation and the modes of transmission of HSV-1. For example, it could be used in assessing the risk of HSV-1 transmission through cornea transplantation and other manifestations of HSV-1 recurrent infections.

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Chapter 3

Herpes Simplex Virus type 1 (HSV-1) Induced Retinitis Following Herpes Simplex Encephalitis: Indications for Brain-to-Eye Transmission of HSV-1

Jeroen Maertzdorf, Allegonda Van der Lelij, G. Seerp Baarsma, Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

Annals of Neurology ***2000***;*48:936*

ABSTRACT

Herpes simplex encephalitis (HSE) is a severe neurological disease with high mortality and morbidity. Reactivated herpes simplex virus (HSV) can cause relapses and might even spread to the retina where it can induce a potentially blinding eye disease, known as acute retinal necrosis (ARN). In the present study, the HSV-1 strains in the brain and eye of two patients with ARN following an episode of HSE were genotyped. The HSV-1 strains in both the brain and eye were identical in each patient, but differed interindividually. The data suggest brain-to-eye transmission of HSV-1 in these patients.

INTRODUCTION

Herpes simplex encephalitis (HSE), caused by an infection of the brain by herpes simplex virus (HSV) is a severe disease with high mortality and morbidity[1]. Reactivation and neuronal translocation of HSV can result in relapses of HSE or new infections at anatomically different sites like the eye. Clinical data suggest that HSE may be a risk factor for the development of acute retinal necrosis (ARN), a rapidly progressing and potentially blinding eye disease induced by HSV[2-4].

Two patients with HSE who later in life developed ARN were included in this study. The HSV-1 strains involved in both disease manifestations of each patient were genotyped using a newly developed PCR method[5] and subsequent nucleotide sequence analyses. The data implicate that in both patients HSE and ARN were caused by a single HSV-1 strain, suggesting trans-neuronal spread of the virus from brain to eye.

PATIENTS AND METHODS

Patients

Patient #1 was a 68 year-old male who had been admitted to hospital in a somnolent state. A viral encephalitis was suspected and computed tomographic scans showed a hypodense in the right temporal region. A CSF sample showed leukocyte counts of $73x10^6$/l. Diagnosis of HSE was confirmed by detection of HSV-1 DNA, determined by PCR using virus-specific primers as described[6], and HSV-specific antibodies in the cerebrospinal fluid (CSF). Intravenous treatment with 10 mg/kg acyclovir three times daily for 2 weeks resulted in slow recovery. However, nine months after discharge from the hospital he developed a unilateral acute decrease of visual acuity (VA). The diagnosis ARN was made on clinical grounds and confirmed by detection of HSV-1 DNA and local HSV-specific antibody production in the aqueous humor (AH) as described previously[6]. Again the patient was treated with acyclovir and maintenance therapy with valcyclovir resulted in a slight improvement with a remaining visual acuity of 0.5.

Patient #2 was a 64 year-old female hospitalized because of progressive headache with vomiting and aphasia. Scans showed a hypodense and space occupying process in the left temporal region. A CSF sample showed a leukocyte count of $44x10^6$/l and the diagnosis of HSE was confirmed by detection of HSV-1 DNA[6] and HSV-specific antibodies in the cerebrospinal fluid (CSF). A slow recovery was achieved after intravenous treatment with 10 mg/kg acyclovir three times daily for 2 weeks. Only ten days after being discharged from the hospital this patient developed a unilaterally decreased VA and ARN was diagnosed two weeks later. An AH sample contained HSV-1 DNA as determined by PCR whereas no local HSV-specific antibody production could be detected[6]. Again, this patient was given antiviral treatment with acyclovir. However, despite maintenance therapy, the remaining visual acuity was only finger counting at 3 meter.

HSV-1 strain differentiation

Isolation of DNA from the CSF and AH samples from both patients, taken for diagnostic purposes, was performed as described previously[6]. The HSV-1 strains in these samples were genotyped with a recently developed PCR-based DNA fingerprint assay which allows the rapid and accurate discrimination of up to 92% of unrelated HSV-1 strains[5]. The

assay is based on the amplification of hypervariable regions within the HSV-1 genes US1 and US12. These regions contain strain-to-strain differences in the number of DNA repeats, termed reiteration IV (Re IV)[7], resulting in variable amplicon lengths between HSV-1 strains. Size and specificity of the PCR products were determined on an agarose gel and Southern blotting with ReIV-specific probes. Nucleotide sequence analyses of gel-purified HSV-1 US12 gene amplicons was performed with both PCR primers on a Perkin Elmer automated sequencer using a commercially available kit according to the manufacturer's instructions (DYEnamic™ ET Terminator; Amersham Pharmacia, Cleveland OH).

RESULTS

The CSF- and AH-derived HSV-1 strains from both patients were genotyped using a recently developed PCR assay[5]. Whereas different between the patients, the HSV-1 US1 and US12 amplicons amplified from both CSF- and AH-derived DNA samples from each patient were of similar size (Fig.1).

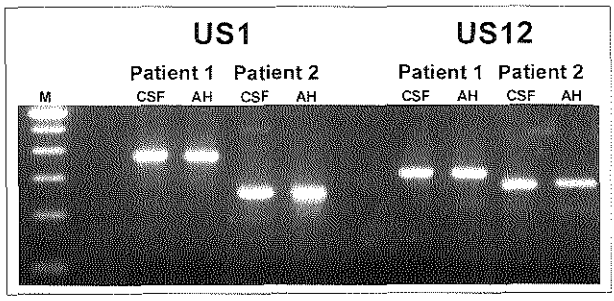

Figure 1: *PCR-mediated genotypic analyses of HSV-1 strains located in cerebrospinal fluid (CSF) and aqueous humor (AH) samples of two ARN patients with a history of HSE. Amplification and detection of hypervariable regions in the HSV-1 genes US1 and US12 was performed as described previously[5]. Left lane: 100-bp molecular size*

The nucleotide sequences of the US12 amplicons were determined and aligned with the corresponding sequence of HSV-1 strain 17 (HS1US; GenBank accession number 291490) (Fig. 2). The DNA sequence analyses revealed identical nucleotide sequences in CSF and AH samples from each patient. Comparison between the patients revealed, next to a difference in the number of ReIV elements (2- and 3-times for patient # 1 and #2, respectively), four separate and unique point mutations (Fig. 2). These data suggest that in each patient the same HSV-1 strain was involved in the pathogenesis of both HSE and ARN. Interestingly, next to the 22-bp long repeating elements (ReIV), a new 45-bp long repeating element was identified in the US12 sequences. This 45-bp element (designated here as ReVIII) was repeated 2- and 3-times in the HSV-1 strains obtained from patients #1 and #2, respectively. In the US12 gene sequence of HSV-1 strain 17 the number of ReIV and VIII repeats are 5 and 1, respectively.

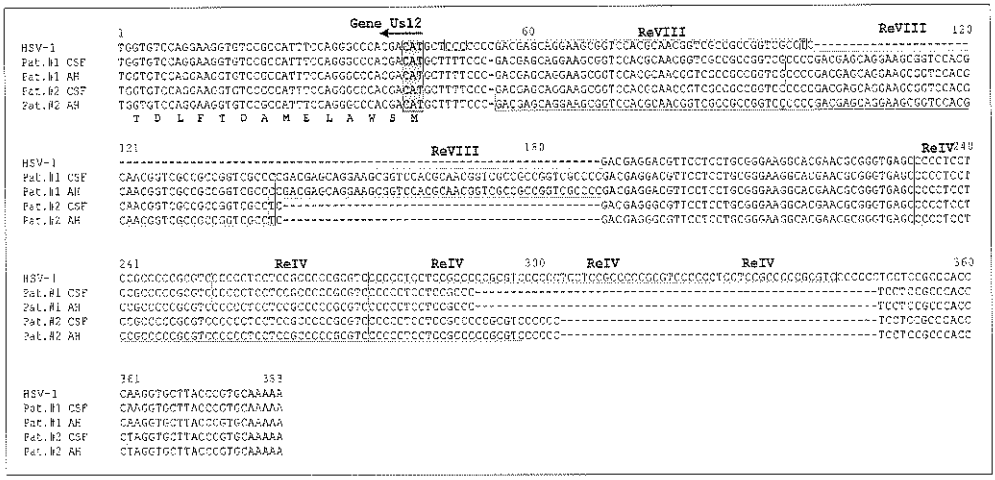

Figure 2: *DNA nucleotide sequences of the HSV-1 US12 gene amplicons obtained from the AH and CSF samples of two ARN patients with a history of HSE.Comparison of the sequences between the patients and the homologous sequence of HSV-1 strain 17 revealed a high degree of variety within the US12 region. The HSV-1 strain 17 US12 sequence was obtained from the GenBank database (HS1US; accession GI 291490: nucleotides 6683-6391). Reiterations (Re) IV and VIII are boxed and unique point mutations are shaded. The start codon and the encoded US12 amino acid sequence are indicated. The DNA sequences obtained have been submitted to the Genbank database (accession AF 290017 and AF290018 for patient # 1 and # 2 respectively).*

DISCUSSION

Several studies have reported on the development of HSV-induced ARN following an episode of HSE[2-4]. It has been hypothesized that the induction of ARN in these patients was due to reactivation of latent HSV within the brain and subsequent infection of the retina. Studies on the experimental ARN mouse model have provided evidence for this assumption. Herein, intra-ocular inoculation of mice with HSV-1 resulted in infection of the brain and subsequent ARN in the contralateral eye. The virus was shown to reach the retina of the contralateral eye by transaxonal spread via the optic nerve[8].

Here, two ARN patients with a previous episode of HSE were studied to determine whether a similar mode of brain-to-eye transmission of HSV-1 had occurred. Detailed genotypic analyses of the HSV-1 strains located in the brain and eye samples from these patients strongly suggest that the viruses found in both anatomic sites of each patient were identical, but differed interindividually. To our knowledge, this is the first study to provide molecular evidence that a single HSV-1 strain can cause HSE and subsequently ARN in a single individual. Analogous to the ARN mouse model, this suggests that the virus may have spread from the brain to the eye, probably through the optic nerve.

The potential of HSV-1 to establish latency in the brain[9] and reactivate from neural cells poses a lifetime threat of recurrent infections. Our findings should alert neurologists to the possibility that HSE may be followed by ARN, since only prompt and specialized medical care may prevent the loss of sight in such patients. Patients recovering from HSV brain infections should be closely monitored for viral eye infections, probably for the rest of their lives.

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Chapter 4

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

Lies Remeijer, Jeroen Maertzdorf, Johannes Buitenwerf,
Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

Investigative Ophthalmology & Visual Science ***2002;43:358***

ABSTRACT

Purpose: Herpetic keratitis is a common sequel of a corneal infection with herpes simplex virus type 1 (HSV-1). Recrudescent herpetic keratitis may result in irreversible damage of 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 recrudescent herpetic keratitis (RHK).

Methods: From 30 RHK patients 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 of the patients scored retrospectively were: ophthalmologic history, clinical picture during recurrences, number and time points of penetrating keratoplasty (PKP) and steroid/acyclovir treatment.

Results: Whereas the sequential corneal HSV-1 isolates of 19 (63%) out 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 penetrating keratoplasty (PKP) was significantly different between the patient groups. While no patients of group 1 had been transplanted between sampling, 4 out of 11 patients of group 2 underwent PKP in the inter-recurrence period of 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 following 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 form 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 transplanted for reasons unrelated to HSV infection, suggesting the possibility of HSV-1 transmission through cornea transplantation[9]. These issues underline the clinical importance to know 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 (U_L) and a unique short (U_S) 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), which 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 variation appears as variation in length of cleaved fragments derived from Re-containing genomic HSV-1 regions[11]. Among the 8 Re 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 facilitated the differentiation of up to 92% of unrelated HSV-1 strains[12,15]. The aim 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 inoculated on 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

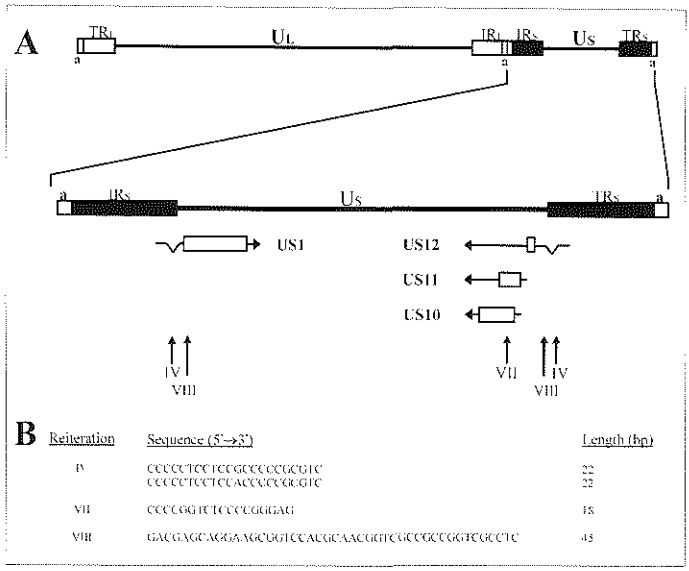

Figure 1

Map of the herpes simplex virus (HSV-1) genome including the location and sequences of the reiterated sequences (Re) 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 as V-shaped indents. Protein coding regions are shown as open boxes. Vertical arrows indicate locations of reiterations, and Roman numerals indicate their locations as defined previously[10,12]. (B) Reiteration-specific sequences have been described previously[10,12]. Reiteration IV exists as two forms that differ from each other in a single residue[10].

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: anatomical 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 PKP and PKP 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/US11 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 Celite solution (Jansen Chemika, Beers, Belgium), as described previously[15]. The PCR primers and conditions for amplifying and detection by Southern blotting of the hypervariable regions of the HSV-1 genes US1, US10/US11 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). To confirm similarities or differences in amplicon length, all samples were finally electrophoresed in order of increasing length.

Statistical analyses

The statistical evaluation of the results was performed using the Fisher's 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 RHK patients included in this study consisted of 13 female and 17 male patients (mean age 58.1 years, range 17 – 78 years). 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 months). Patient 22 had a bilateral herpetic keratitis (Table 1).

Table 1. Patient characteristics and genetic characterization of sequential corneal HSV-1 isolates from patients with recrudescent herpetic keratitis.

			Estimated amplicon length (bp)[c]									Months between HSV-1 isolates[d]		Eye
			US10/11 region			US1 region			US12 region					
Patient	Sex[a]	Age[b]	a	b	c	a	b	c	a	b	c	a – b	b – c	Difference[e]
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	-
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	-

[a] F, female and M, male.

[b] Age in years.

[c] 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 underlined. Based on the genotypic analyses, patients 1 – 19 were designated as group 1 (genotype sequential isolates identical) and patients 20 – 30 as group 2 (genotype sequential isolates different).

[d] Patient 22 had a bilateral herpetic keratitis. In case of patient 5, the first and second isolate were obtained from the left eye and the third isolate was sampled from the right eye.

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

To differentiate whether RHK is due to reactivation of latent HSV-1 or super-infection with another HSV-1 strain, the sequential corneal HSV-1 isolates of the RHK patients 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 1-5 and 20-24 are shown in Fig. 2. The sequential corneal HSV-1 isolates of 19 (63%) out of the 30 patients and 11 (37%) out of 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 RHK patients analyzed was superinfected with a different HSV-1 strain. In 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 3 amplified genomic regions showed that the majority of the distinguishable HSV-1 isolates displayed unique combinations of amplicons (Table 1). In case of patient 22, the data indicate 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 sampling episode the strain identified during the second recurrence was isolated (Table 1).

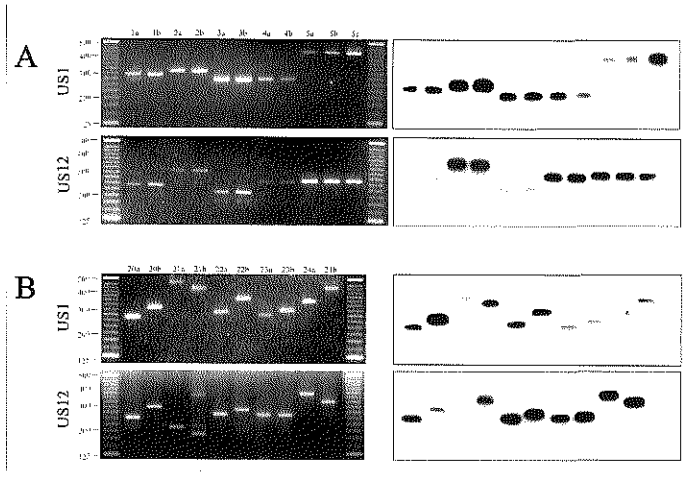

Figure 2
Amplicons of the hypervariable regions US1 and US12 amplified from sequential corneal HSV-1 isolates from patients with recrudescent herpetic keratitis. ***Left panel****, Amplicons were electrophorezed on a 2.5% agarose gels and were visualized by ethidium bromide staining. Representative sequential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

Comparison of clinical characteristics of RHK patients in patient group 1 and 2

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

The clinical characteristics of the patients in group 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), previous ocular history and clinical picture at time of recurrences were not statistically different between both groups (Table 1 and 2).

Comparison of therapeutic regimen of RHK patients in patient group 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).

While 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 patient in group 1 received a corneal transplant between the sampled recurrences, 4 out of the 11 patients of group 2 underwent a PKP during the inter-recurrence period of 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

Herpes simplex viruses 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 herpetic keratitis risk the development of HSK, a leading cause of corneal blindness worldwide[2,3]. The objective of the present study was to address the two types of etiologies, 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 of 30 RHK patients demonstrated that 63% of the patients (patients 1-19; designated as group 1) had evidence for reactivation of the same HSV-1 strain. From 5 patients of group 1, the isolates were obtained from separate eyes. HSV-1 infection of the contralateral cornea has most likely occurred via the external route (cross-infection). Interestingly, 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. HSV, like 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 8 HSV-1-specific Re described varies extensively[11]. Genotypic analyses of HSV-1 single-plaque clones compared to 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]. This, together with the mean inter-recurrence period of patient group 1 (30.4 months), and the proofreading activity of *Pfu* DNA polymerase, implicate that the intra-individual HSV-1 genotype differences are most likely not due to a genetic alteration of the initial strain or errors in amplifying these highly GC-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 studies have used RFLP analyses using 6-bp recognizing restriction enzymes (RE)[4-6]. The lower efficacy of 6-bp RE, compared to 4-bp RE, to differentiate HSV-1 strains may account for the different frequencies of HSV superinfection described[11].

Table 2.
Clinical characteristics and cornea transplantations performed on patients with recrudescent herpetic keratitis

Patient[a]	History of HSV-mediated eye disease[b]	Diagnosis at time point of sampling[b] Anatomical site			Penetrating keratoplasty	
		Time point a	Time point b	Time point c	No. PKP[c]	PKP between samples[d]
1	None	blepharitis	blepharitis		0	No
2	None	IEK	IEK		0	No
3	Rec ISK	IEK	IEK		0	No
4	Rec ISK	IEK	IEK		0	No
5	Bil ISK	IEK	IEK	IEK	0	No
6	Rec ISK and uveitis	IEK	NSK	NSK	3	No
7	Rec ISK	IEK and ISK	IEK and ISK		0	No
8	NSK in PKP	IEK and ISK	IEK and ISK		3	No
9	Rec ISK	IEK and ISK	IEK		0	No
10	bil/rec ISK	IEK in PKP	IEK in PKP		1	No
11	bil/rec ISK and uveitis	IEK in PKP	IEK in PKP		2	No
12	New HSV in PKP	IEK in PKP	IEK in PKP		1	No
13	NSK in PKP	IEK in PKP	NSK in PKP		2	No
14	Rec ISK	NSK in PKP	IEK in PKP		1	No
15	New HSV in PKP	New HSV in PKP	IEK in PKP		2	No
16	NSK	IEK and uveitis	IEK		0	No
17	Rec ISK in PKP	IEK in PKP	IEK in PKP		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
20	ISK and uveitis	Blepharitis	IEK		0	No
21	Endothelitis	IEK	IEK		0	No
22	Rec IEK and ISK in PKP	IEK	IEK		2	No
23	New HSV 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	IEK and uveitis	IEK and uveitis		1	No
29	NSK in PKP	ISK in PKP	IEK in PKP		3	Yes
30	New HSV in PKP	NSK IN PKP	ISK in PKP	NSK in PKP	6	Yes

a The sequential corneal HSV-1 isolates of patients 1 - 19 (designated as group 1) and 20 – 30 (designated as group 2) had identical or different genotypes, respectively.

b 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. The prefixes "Rec", "Bil" and "New" indicate recurrent, bilateral and newly acquired, respectively.

c Total numbers of preceding penetrating keratoplasty (PKP) performed on each patient are given.

d 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's exact test; $p = 0.012$). The PKP of patient 30 was performed between time point a and b.

In general, corneal HSV-1 infections result in the development of herpetic epithelial keratitis in about two-third of the patients[2]. In the present study, however, the patient cohort consisted predominantly of patients with severe entities of herpetic keratitis (Table 2). Selection for individuals with a higher susceptibility for corneal HSV-1 infection may have occurred. Alternatively, the patients of 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. While no patients of group 1 had been transplanted between sampling, 4 out of 11 patients of group 2 underwent PKP during the inter-recurrence period of 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.* have demonstrated HSV-1 transmission through PKP in an experimental rabbit model[18]. HSV-1 DNA was detected in recipient corneal rims and the innervating TG of naive rabbits that received corneal allografts from latently infected rabbits. Moreover, infectious HSV-1 was recovered from the tear film of the transplanted rabbits[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, prior to PKP. In animal model studies, corneal trauma (like 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 superinfections in RHK patients. Although we could not determine the source or mode of corneal HSV-1 superinfection in patient group 2, the data suggest that PKP might 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 for both strains, providing conclusive evidence for graft-to-host transmission of HSV-1 through a corneal allograft[23].

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Herpes Simplex Virus Type 1 Transmission Through Corneal Transplantation

Lies Remeijer, Jeroen Maertzdorf, Peter Doornenbal,
Georges M G M Verjans, Albert D M E Osterhaus

Lancet ***2001***;*357:442*

ABSTRACT

Genetic characterisation 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 represents a leading cause of blindness worldwide[1]. The current therapy of choice for this condition is penetrating keratoplasty (PKP). Infectious agents reported to be transmitted by PKP are the Creutzfeldt-Jakob agent, hepatitis type B and C viruses, cytomegalovirus and rabies virus[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 non-herpetic 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. Post-operative treatment consisted of prednisolone 0.5% eye drops 8-times daily. Immediately after surgery, an epithelial defect developed which showed only slow recovery despite additional treatment with bandage lenses and frequent lubrication with autologous serum. Three months post PKP, a large epithelial defect re-occurred from which HSV-1 could be isolated (data not shown). After 3 months, the eye lost visual function due to uncontrollable herpetic keratitis with graft failure in combination with choroidal effusion after glaucoma surgery. Diagnostic HSV-1 PCR and serologic tests were performed retrospectively on the donor-derived corneoscleral rim and serum of the patient, respectively. HSV-1 DNA was detected in the rim, and no HSV-specific IgM and IgG antibodies were found in his serum before surgery (data not shown). Collectively, the data suggested transmission of HSV-1 from the allograft to the HSV naive 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 recently developed PCR-based DNA fingerprint assay[5]. 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 US1 and US12[5]. The PCR products of the D- and R-derived HSV-1 strains were of the same size (data not shown). The respective US1 and US12 PCR products were sequenced. The DNA sequences were identical for both strains, with multiple unique mutations as compared to the corresponding sequences of the HSV-1 F strain (Fig. 1).

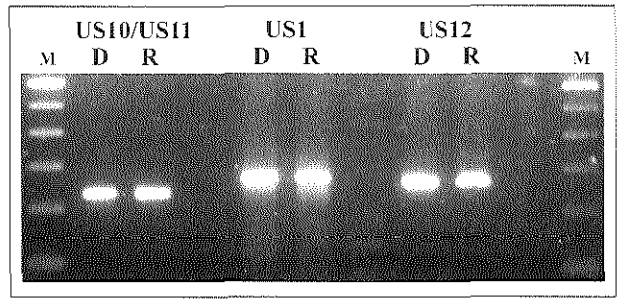

Figure 1
DNA nucleotide sequences of the HSV-1 US1 and US12 gene amplicons obtained from the donor-derived corneoscleral rim and post-PKP corneal HSV-1 isolate of the recipient.
Comparison of the HSV-1 US1 (A) and US12 (B) DNA sequences of the donor (rim) and patient (cornea) derived samples and the homologous sequence of HSV-1 strain 17 revealed a high degree of variety within both genomic regions. The HSV-1 strain 17 sequences were obtained from the GenBank database (HS1US; accession GI 291490). Reiterations (Re) IV and VIII are boxed and unique point mutations are shaded. The start codon and the encoded US12 amino acid sequence are indicated.

Donor-to-host transmission of infectious agents via corneal transplantation poses a real risk to the recipient. Serological screening of cornea donors for a number of viruses, as well as bacterial and fungal culturing prior to transplantation, is currently standard practice in

most eye banks to limit transmission of infections by PKP. 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 infectious virus loads or poor reactivation of putative latent virus in the allograft. Since extra-neuronal HSV-1 latency in the cornea remains enigmatic, 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 previous history of HSV infection has also been described, implicating the possibility of HSV-1 transmission by PKP[3,4]. Here, we present a HSV naive patient who lost his vision in his only functional eye due to graft-to-host transmission of HSV-1, leading to graft failure in combination with choroidal effusion after glaucoma surgery. To our knowledge, this is the first study 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. Especially transmission of HSV-1 to a seronegative recipient may be dangerous, as the virus may cause - among other complications – potentially blinding eye infections in patients under immunosuppressive treatment. To prevent HSV-1 transmission to HSV-1 naive donors, serologic matching of donor and recipient is an option. If for a HSV seronegative patient PKP is urgently requested and no seronegative donor is available, at least during immunosuppressive regimen, administration of prophylactic doses of acyclovir would be recommended. Our observations stress the need for further studies on the localisation and latency of HSV-1 in corneoscleral tissue.

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Chapter 5

Characterization of Herpes Simplex Virus Specific intra-Corneal T Cells at the Single-Cell Level in Human Herpetic Stromal Keratitis

Jeroen Maertzdorf, Georges M.G.M. Verjans, Lies Remeijer, Alexander van der Kooi, Albert D.M.E. Osterhaus

Journal of Infectious Diseases (in press)

ABSTRACT

Purpose. Herpetic stromal keratitis (HSK) is a T cell-mediated inflammatory disease triggered by herpes simplex virus (HSV) infection of the cornea. Although this inflammatory process has been well characterized in experimental mouse models, little is known about the specificity and subsets of intracorneal T cells in human HSK. Here we describe a more elaborate characterization of HSV-specific T cells recovered from human HSK-diseased corneas at the single-cell level.

Methods. T cell lines (TCL) were generated from inflammatory cells recovered from corneas of 12 HSK patients. Characterization of individual T cells in the TCL was performed by stimulating TCL with mock –infected or HSV type 1 and 2 -infected autologous BLCL and subsequent flow cytometric analyses. Virus-specificity, phenotype and clonality of the T cells were identified by simultaneous detection of intracellular IFN-γ, CD4 and CD8, and T cell receptor (TCR) Vβ gene expression, respectively.

Results. Intra-corneal TCL from 10 of the 12 patients contained HSV-specific T cells. Both $CD4^+$ and $CD8^+$ HSV -reactive T cells were identified in these TCL. The majority of the TCL showed a restricted Vβ usage. T cells expressing a single TCR Vβ domain dominated the HSV-1 reactivity in TCL of some of the patients analyzed.

Conclusions. Corneas of HSK patients were shown to be infiltrated by both $CD4^+$ and $CD8^+$ HSV-specific T cells, which might play a role in the immunopathogenesis of humans HSK. The data suggest that the intracorneal HSV-reactive T cell response is dominated by a restricted number of T cell clones.

INTRODUCTION

HSV-1 corneal infections are a leading cause of non-traumatic blindness in developed countries.[1] Replication of the virus within the cornea can lead to an immunopathologic disorder, known as HSK, which results in the development of corneal lesions and blindness. Current knowledge on the immunopathogenic processes in the development of HSK has greatly benefited from studies in experimental mouse models, which mimic the human situation. Investigators in the field generally agree that HSK in the mouse model is induced by activated $CD4^+$ T cells of the Th1 phenotype.[2-4] These cells are believed to orchestrate the major influx of neutrophils into the corneal stroma characteristic for HSK. These cornea infiltrating neutrophils are considered to be the main cell type directly involved in corneal destruction.[3-5] Other cell types that play an important role in the immunopathogenesis of HSK are Langerhans cells (LCs)[6] and macrophages.[7,8]

Studies in murine models have shown that both virus and host factors play a role in the susceptibility for, and the severity of HSK development. Although the crucial involvement of $CD4^+$ Th1 cells has been clearly established in the HSK mouse model, there is still debate concerning the antigen-specificity of the T cells involved. The group of Cantor *et al.* has proposed that HSK is an autoimmune disease triggered by a cross-reactivity between a corneal autoantigen and an HSV-1 UL6 peptide.[9,10] In sharp contrast are the findings by the group of Rouse *et al.* indicating an inflammatory process in HSK in the absence of viral antigen recognition, referred to as bystander T cell activation.[11-14]

Functional studies on cornea infiltrating T cells in human HSK are limited. Studies by our group[15,16] have shown the presence of HSV-1 –specific T cells and surprisingly high percentages of $CD8^+$ T cells in the majority of corneas form HSK patients. This was confirmed by the isolation of HSV-1 –specific $CD4^+$ and CD8+ T cell clones (TCC) by Koelle and co-workers.[17] Most of the human HSV-specific TCC isolated by both groups appeared to be $CD4^+$ T cells of the Th1 or Th0 phenotype.[15,17] Neither group could detect any reactivity against the UL6 peptide or a corneal autoantigen.[16,17]

A major drawback of these studies on cornea infiltrating T cells in human HSK is the relatively low number of TCC analyzed, which does not provide an accurate insight into the phenotype and clonality of T cells involved in the inflammatory process in human HSK. We therefore aimed to make a more broadened analysis of the TCL generated from human HSK diseased corneas. In the present study we analyzed the phenotype and clonality of intracorneal HSV-specific T cells, using four colour flow cytometry. Staining of intracellular IFN-γ was used as a marker for T cell activation and antigen-specificity. Staining on CD4 and CD8 indicated the subtype of T cell involved while staining with mAbs against different TCR Vβ domains was used as an indication for the clonality of the HSV-1 specific T cells present within the TCL.

MATERIALS AND METHODS

Clinical samples and processing

Heparinized peripheral blood and corneal buttons were obtained from 13 patients with HSK at the time of therapeutic corneal transplantation. Patient characteristics and disease classifications are outlined in Table 1. Corneal buttons were obtained immediately after surgery and divided in two. One half was used for DNA extraction and standard diagnostic detection of HSV DNA by PCR as described previously.[16] The other corneal half was used for recovery of intra-corneal inflammatory cells. Generation of TCL from these specimens by mitogenic stimulation of recovered inflammatory cells, isolation of PBMC and generation of BLCL by transformation with Epstein-Barr virus were essentially performed as described previously.[15] Virus stocks with cell lysate antigen preparations of the HSV-1 MacIntyre strain (American Type Culture Collection [ATCC] VR-539) and the HSV-2 MS strain (ATCC VR-540) were generated and titrated on Vero cells. Mock infections were performed with preparations from similarly treated Vero cells only. The present study was performed in agreement with the Declaration of Helsinki, and informed consent was obtained from all patients.

Table 1. HSK patients' characteristics

Patient	Sex [a]	Age [b]	Diagnosis [c]	Disease status	HSV-1 PCR	Time since HSK first diagnosed [d]	Time since last recurrence [e]	Topical pre-operative treatment	
								Steroids	Acyclovir
1	F	25	ISK	Quiescent	-	10	17	Yes	Yes
2	M	68	NSK	Active	+	17	0	Yes	Yes
3	F	78	NSK	Active	+	25	0	Yes	Yes
4	F	72	ISK	Quiescent	-	9	12	Yes	Yes
5	M	71	NSK	Quiescent	-	11	36	Yes	Yes
6	M	72	ISK	Quiescent	-	59	28	Yes	No
7	F	28	ISK	Quiescent	-	5	22	No	Yes
8	M	56	NSK	Active	+	13	0	Yes	Yes
9	M	43	ISK	Quiescent	+	6	16	Yes	No
10	F	64	ISK	Quiescent	-	8	36	Yes	No
11	F	50	ISK	Active	+	0	0	No	No
12	F	52	ISK	Quiescent	-	40	14	No	No
13	F	40	ISK	Quiescent	-	13	17	Yes	No

[a] M; Male. F;Female

[b] Age in years

[c] ISK: immune stromal keratitis, NSK: necrotizing stromal keratitis. PNU: perforated neurotrofic ulcer

[d] Time in years

[e] Time in months

Stimulation of intra-corneal TCL

BLCLs were infected with cell lysate antigen preparations of HSV-1, HSV-2 or uninfected cell control (mock) for 1 hour at 37°C in pure virus stock preparation and left overnight in an incubator (37°C; 5% CO_2) in RPMI-1640 medium supplemented with 10% FCS. After UV-irradiation of the BLCLs, $2x10^5$ virus- or mock –infected BLCLs were cocultured with $3x10^5$ T cells from the cornea-derived TCL in 96-well tissue culture plates in

RPMI-1640 supplemented with 10% heat-inactivated human pooled serum. After 1 hr., monensin (2 μg/ml; BD Pharmingen, San Diego, CA) was added to block the transport of newly synthesized proteins to the cell surface. Accumulation of intracellular IFN-γ was allowed for 6 hrs. or overnight at 37°C before staining with fluorescent label conjugated mAbs and subsequent flow cytometric analyses. As a positive control for stimulation, TCL were incubated with phorbol myristate acetate (PMA; 50 ng/ml) and calcium-ionophore (500 ng/ml) (both obtained from Sigma, St Louis, MI).

Staining and FACS analyses

Fluorescent staining of TCL was performed with the following monoclonal antibodies (mAbs): IFN-γ- allophycoctanin (APC) (BD Pharmingen), CD3- phycoerytherin (PE) (Dako, Glostrup, Denmark); CD4-fluorescein isothiocyanate (FITC) (Dako); CD8-peridinin chlorophyll A protein (PerCP) (BD Biosciences, San Jose, CA). Determination of the TCR Vβ repertoire was performed by staining with TCR Vβ -specific unconjugated mAbs, followed by incubation with a PE-labeled goat-anti-mouse IgG preparation (Dako). A total of 21 mAbs against different TCR Vβ domains were pooled into six separate mixes to limit the number of samples to be analyzed. Content of the different mixes with equivalent amounts of mAbs against TCR Vβ domains are as follows (indicated as [TCR (clone)]: Mix 1 [Vβ3 (CH92) and 5.1 (IMMU1570)], Mix-2 [Vβ8.1/8.2 (56C5.2), 12.2 (VER2.23.1) and 17 (E17.5F3.15.13)], Mix-3 [Vβ2 (MPB2D5), 16 (TAMAYA1.2) and 23 (AHUT7)], Mix-4 [Vβ6.1 (CRI304.3), 7.1 (ZOE), 9.1 (FIN9), 11.1/11.2 (C21) and 14 (CAS1.1.3)], Mix-5 [Vβ13.6 (JU74.3), 18 (BA62.6), 20 (ELL1.4), 21.3 (IG125) and 22 (IMMU546)] and Mix-6 [Vβ35.2/5.3 (4H11), 6.7 (OT145) and 13.1/13.3 (BAM13)]. These TCR Vβ mAb mixes were designed to stain ~10% of $CD3^-$ cells from PBMC, covering 60-65% of all PBMC $CD3^+$ cells.[18]

Stimulated cells were washed with PBS with 3% FCS and incubated in the dark on ice for 30 min with the different TCR Vβ mAb mixes as indicated above. After blocking of unbound goat antibodies with PBS containing 2% normal mouse serum, cells were similarly stained for the different surface markers. Cell preparations were subsequently fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) and stained for intracellular IFN-γ according to the manufacturer's instructions. Following another wash step, cells were resuspended in PBS with 1% FCS and approximately 2.10^4- 4.10^4 viable cells were analyzed on a FACScalibur flow cytometer (Beckton Dickinson).

RESULTS

Phenotypic Characterization of Intracorneal TCL of HSK Patients

Diagnostic analyses were performed on corneas from 12 patients with immune- or necrotizing stromal keratitis (Table 1), immediately after therapeutic cornea transplantation. HSK classification was defined on the basis of clinical criteria.[19] Age of the patients was 55 ± 17 (years; mean ± stdev) and all except one (patient 11) had a history of recurrent episodes of HSK (Table 1). Standard diagnostic PCR analysis revealed HSV-1 DNA in 7 corneas, irrespective of pre-operative treatment with acyclovir (Table 1). HSV-1 DNA was detected

in corneas of all four patients with active disease (patients 2, 3, 8 and 11) and in three with a quiescent disease status (patients 4, 9 and 10).

To analyze the antigen-specificity and subsets of T cells involved in human HSK, TCL were generated by recovery of intra-corneal T cells, which were expanded twice by mitogenic stimulation. Cells within the intra-corneal TCL from all patients were predominantly $CD3^+$ and contained both $CD4^+$ and $CD8^+$ T cells. The ratio of $CD4^+$ and $CD8^+$ T cells varied considerably between patients (Table 2). The clonality of the individual TCL was determined by analyzing their TCR Vβ domain usage within both $CD4^+$ and $CD8^+$ T cell populations. A panel of 21 TCR Vβ domain-specific mAb were divided into 6 TCR Vβ mAb mixes, each covering ~10% of peripheral blood $CD3^+$ T cells.[18]

Table 2. Phenotype and TCR Vβ expression of intra-corneal TCL from HSK patients.

Patient	% Cells with phenotype [a]		% Cells expressing TCR V domains [b]															
			Mix 1		Mix 2		Mix 3		Mix 4		Mix 5		Mix 6		Total TCR$^+$		Individual mAbs [c]	
	CD3/ CD4	CD3/ CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
1	35	43	3	6	3	7	1	3	3	3	<1	**11**	**72**	**18**	83	48	**73** (Vβ13.1/13.3)	**14** (Vβ13.1/13.3)
2	22	74	2	<1	3	<1	4	<1	1	<1	<1	<1	4	<1	15	<1	-	-
3	40	54	<1	<1	**53**	**86**	<1	<1	<1	<1	3	<1	3	5	59	91	**53** (Vβ17)	**87** (Vβ12.2)
4*	64	19	<1	<1	7	**22**	5	**43**	<1	<1	<1	<1	8	<1	20	66	-	ND
5	85	5	<1	<1	<1	<1	3	<1	<1	<1	<1	<1	<1	<1	3	<1	-	-
6	76	26	1	<1	**80**	3	**1**	<1	<1	<1	<1	2	<1	1	83	7	**83** (Vβ8.1/8.2)	-
7	85	11	<1	3	3	6	9	3	2	<1	10	1	2	5	26	18	-	-
8	41	57	<1	2	1	**23**	6	6	<1	<1	**28**	**57**	**32**	**24**	67	106	**29** (Vβ13.6) **26** (Vβ13.1/.3)	**19** (Vβ8.1/8.2) **27** (Vβ13.6) **21** (Vβ20) **17** (Vβ13.1/.3)
9	37	60	<1	4	**40**	**63**	5	2	1	4	<1	6	1	5	48	84	**42** (Vβ17)	**62** (Vβ17)
10*	62	36	<1	<1	**83**	2	<1	<1	<1	<1	<1	<1	<1	<1	83	2	**75** (Vβ17)	-
11	86	11	**44**	<1	**12**	**11**	8	**58**	<1	6	1	<1	4	1	69	76	**42** (Vβ5.1)	**53** (Vβ2)
12*	49	50	<1	<1	8	**84**	<1	2	<1	<1	<1	<1	5	3	**14**	89	-	ND
13*	95	2	<1	3	<1	5	<1	2	<1	<1	<1	<1	**11**	**19**	11	29	-	ND

[a] Gated on live cells
[b] Gated on live cells and CD4 or CD8
[c] Dominant TCR Vβ domain identified.
* Expression of TCR Vβ was analyzed in a single experiment
ND, not done.

Screening with the available panel of TCR Vβ mAbs, indicated in most cases a skewing towards a particular TCR Vβ mix (Table 2). Whereas in TCL from 6 patients (1, 3, 6, 9, 10 and 12) TCR Vβ usage was confined to a single dominant TCR Vβ mix, TCL from patients 4, 7, 8 and 11 appeared to contain a more or less oligoclonal distribution of TCR Vβ domains. For patients 2 and 5, no significant staining for the different TCR Vβ domains could be detected. The most predominant TCR Vβ mix staining positive in most TCL was mix 2, either in the $CD4^+$ or $CD8^+$ subset alone, or in both T cell populations (Table 2). To a lesser extent TCR Vβ mixes 3 and 6 also appeared to stain a significant portion of T cells present within several TCL. Only TCR Vβ mix 4 did not show any significant staining in any of the TCL analyzed. Subsequently, TCL showing positive staining in dominant TCR Vβ mixes were analyzed for their usage of individual TCR Vβ domains within the relevant TCR Vβ mixes (Table 2). Unfortunately, this analysis could not be performed on TCL from patients 4 and 12 due to limited availability of cells. This analyses indicated that positive

staining in the matching TCR Vβ mixes was in all but one cases attributable to a single TCR Vβ domain (Table 2). Only staining of TCR Vβ mix 5 in the CD8 fraction of TCL from patient 8 was caused by usage of two different TCR Vβ domains (TCR Vβ 13.6 and TCR Vβ 20), furthermore underlining the oligoclonality of this TCL. The most frequently observed TCR Vβ domains used were TCR Vβ17 (in patients 3, 9 and 10), TCR Vβ8.1/8.2 (in patients 6 and 8) and TCR Vβ13.1/13.3 (in patients 1 and 8). Positive staining of both $CD4^+$ and $CD8^+$ subsets in a particular TCR Vβ mix (in patients 1, 3 ,8 and 9) was in most cases attributable to the same individual TCR Vβ domain, with the exception of TCL from patient 3, which expressed different TCR Vβ domains in its $CD4^+$ and $CD8^+$ populations.

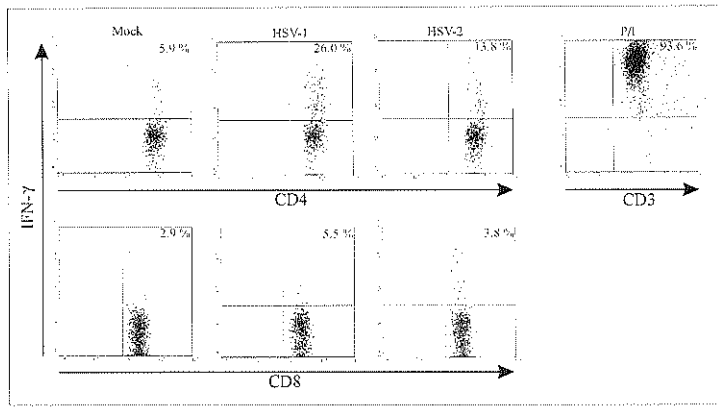

Figure 1.
Detection of HSV-specificity in the $CD4^+$ and $CD8^+$ T subsets in intra-corneal TCL from HSK patient 9. TCL were cocultivated with mock-, HSV-1 or HSV-2 –infected autologous BLCL. Staining of intra-cellular IFN-γ was used as an activation marker for antigen-specific T cells and TCL was analyzed by flowcytometry. Results shown were gated on live cell population. P/I indicates stimulation of TCL with PMA and calcium-ionophore as a positive control for IFN-γ production.

Phenotypic characterization of HSV –specific intracorneal T cells.

HSV antigen specificity of the intra-corneal TCL was analyzed by co-cultivation with mock-, HSV-1- or HSV-2 –infected autologous BLCL as antigen presenting cells (APCs). Subsequent detection of intracellular IFN-γ was used as a measure for T cell activation to identify antigen-specific cells. Figure 1 shows the results of a representative flow cytometric analysis on the intracorneal TCL from patient 9. TCL from 10 of the 12 patients showed reactivity against HSV-1 antigens, while 6 of the 9 TCL analyzed for HSV-2 -specificity showed specific T cells for HSV-2 (Table 3). All TCL analyzed for both HSV-1 and HSV-2, except one (patient 6), were reactive against both HSV serotypes.

HSV antigen-reactivity could be attributed to the $CD4^+$ subset (patients 2, 8 and 12), the $CD8^+$ subset (patients 5, 6 and 10) or both (patients 3, 4, 7 and 9). While the majority of cells in all other TCL were capable of producing IFN-γ, TCL from patient 2 only showed a marginal number of IFN-γ producing T cells upon stimulation with P/I in multiple experiments (not shown), despite the observation that cells looked healthy under normal light-microscopic examination and by tryphan blue staining.

Clonal analyses of HSV-1 –specific intracorneal T cells.

A fourth fluorescent parameter, TCR Vβ domain expression, was included to determine the clonality of the cornea-derived HSV-1 –specific T cells. Figure 2 shows the results of a representative analysis of the TCL from patient 3. Analysis of 5 of the intra-

Table 3. Phenotype of HSV-specific intra-corneal T cells from HSK patients

Patient	% IFN-$^+$ T cells [a]			
	HSV-1 [b]		HSV-2 [b]	
	CD4+	CD8+	CD4+	CD8+
1	0.1 ± 0.1	0.6 ± 0.7	ND	ND
2	**4.5 0.7**	0.1 ± 0.1	**3.3 2.0**	0.2 ± 0.2
3	**4.3 0.9**	**44.0 7.5**	ND	ND
4	**4.1 1.8**	**16.6 5.1**	0.4	**13.6**
5	0.3 ± 0.5	**7.1 1.6**	0.4 ± 0.3	**6.7 2.1**
6*	0.2	**4.6**	0.0	0.7
7	**7.5 2.2**	**6.0 1.1**	**2.2 0.8**	**2.3**
8	**3.1 1.8**	0.7 ± 0.5	ND	ND
9	**17.7 3.5**	**2.3 0.5**	**8.5 0.8**	**1.3 0.5**
10*	0.2	**20.3**	ND	ND
11	0.3 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
12*	**5.3**	0.4	**8.2**	0.5
13*	0.1	0.0	0.1	0.0

ND; not done

[a] Gated on live cells and CD4 or CD8 / corrected for mock-infection

corneal TCL from HSK patients (Table 4) showed that usage of a single TCR Vβ domain dominated the T cell response to HSV-1 in 2 of these individuals (patient 3; $CD8^+$/Vβ12.2 and patient 9; $CD4^+$/Vβ17). Despite the high prevalence of TCR Vβ17 in the $CD4^-$ fraction of the TCL from patient 3 (see Table 3), the antigen-reactivity in this fraction could not be assigned to a T cell subset using a particular TCR Vβ domain. The same goes for HSV-1 –specificity within the $CD8^+$ fraction of TCL from patient 9. For the other 3 individuals, no predominant HSV-1 -specific TCR Vβ domain was detected (Table 4). From all TCL with proven HSV-reactivity, patients 2 and 5 are not included because no dominant TCR Vβ usage could be detected in their TCL (see Table 2). Similarly, patients 6 and 10 were excluded. Although a profound skewing of TCR Vβ usage was seen in the $CD4^+$ fraction of these TCL (see Table 2) , HSV-reactivity was only observed in the $CD8^+$ T cell subset (see Table 3). Only for patient 8, the clonality of HSV-1 –specific T cells could not be determined due to unavailability of sufficient numbers of cells.

Table 4. TCR Vβ usage of HSV-1 –specific intra-corneal T cells of HSK patients.

Patient	% IFN-$^+$ T cells expressing TCR V gene segments [a]													
	Mix 1		Mix 2		Mix 3		Mix 4		Mix 5		Mix 6		Individual mAbs [b]	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
3	0	0	<1	**44.0**	0	<1	0	0	1	0	0	<1	-	**45.4** (Vb 12.2)
4	0	0	0	<1	<1	**9.3**	0	0	0	0	0	0	-	ND
7	<1	0	<1	<1	1.1	<1	<1	0	<1	0	<1	0	-	-
9	0	<1	**18.6**	<1	2.7	0	<1	<1	<1	<1	<1	<1	**19.5** (Vb 17)	-
12	0	<1	<1	<1	0	0	0	0	0	0	<1	0	-	-

[a] Cells specific for HSV-1; Gated on live cells and CD4 or CD8

[b] Dominant TCR Vβ domain identified.

ND; not done

DISCUSSION

HSK is an immunopathologic disease elicited by HSV infection of the corneal stroma. Both viral antigens and factors of the host immune system play crucial roles in the development of the inflammatory processes in HSK. Viral replication within the cornea is essential for the development of HSK, since UV-inactivated and replication defective HSV mutants fail to induce HSK in mice.[20] On the other hand, a functional T cell repertoire of the host's immune system is required, as T-cell deficient nude mice do not develop HSK upon corneal HSV infection.[21] Although the crucial involvement of $CD4^+$ Th1 cells has been clearly established in experimental mouse models of HSK,[2-4] little is known about the specificity and subsets of T cells involved in the human situation. In order to make a more elaborate evaluation of intra-corneal T cells from HSK patients at the single-cell level, we used an alternative approach without the need of generating TCCs. The methodology developed involves four-color flowcytometric analyses of intra-corneal TCL and enables simultaneous analysis of HSV-1 specificity (IFN-γ production), subtype (CD4 and CD8) and clonality (TCR Vβ domain) of individual cells. Staining of intracellular IFN-γ was used as a T cell activation marker to detect HSV-specificity as previous studies have shown that both $CD4^+$ and $CD8^+$ HSV-specific T cells from corneas of HSK patients secrete IFN-γ upon antigenic stimulation.[15,17] A similar experimental method has been used successfully before, as described in a recent report on the clonotypic structure of the human $CD4^+$ memory T cell response to cytomegalovirus in PBMC.[22] Analysis of virus-specificity and phenotype at the single-cell level mainly has the advantage over T cell cloning in that vast numbers of cells can be screened in short time.

A total of 12 HSK patients from whom intra-corneal TCL had been generated were included in this study. HSV-1 DNA could be detected in diseased corneas of 7 patients and intracorneal T cells of both $CD4^-$ and $CD8^+$ subsets were observed in variable ratios. The fact that a small portion of cells is neither $CD3^+/CD4^-$ nor $CD3^+/CD8^+$ (see Table 2) was due to contamination by BLCL in the live gate set during flowcytometric analyses. In agreement with previous studies,[15-17] HSV-reactive T cells were observed in corneas of most HSK patients included in this study, either in the $CD4^+$ or $CD8^+$ population alone, or in both T cell subsets. Surprisingly, the TCL of several of the patients we analyzed contained relatively high numbers of $CD8^+$ HSV-specific T cells. These data are in contrast with results from studies performed in experimental mouse models of HSK, which have indicated that in murine HSK, cornea infiltrating T cells are predominantly of the $CD4^+$ subset.[2-4] Only one study on intra-corneal T cells in human HSK has previously indicated the presence of HSV-1 –specific $CD8^-$ T cells in the diseased cornea,[17] albeit in very low numbers. Recent findings suggest that the antigen-specific frequency in the original samples used in that study might have been considerably higher, partly because CTL effectors undergo activation-induced cell death in limiting dilution cultures,[23] as employed in the study mentioned. This might in part explain the discrepancy between the low percentages of HSV –specific $CD8^+$ TCC found by Koelle and co-workers[17] and the relative high frequencies of HSV-specific $CD8^-$ T cells found in the present study. Alternatively, the mitogenic stimulation of T cells applied in order to obtain sufficient numbers of cells for our analyses invokes the risk of selective growth of certain T cell subtypes and clones. Similarly, the more extended growth potential of $CD8^-$ over $CD4^+$ T cells following a non-specific stimulus[24] might have caused the high frequencies of $CD8^-$ cells in some of our TCL. On the other hand, clonal expansion of $CD4^+$ T cells was also frequently observed. One explanation for this might be related to the overall

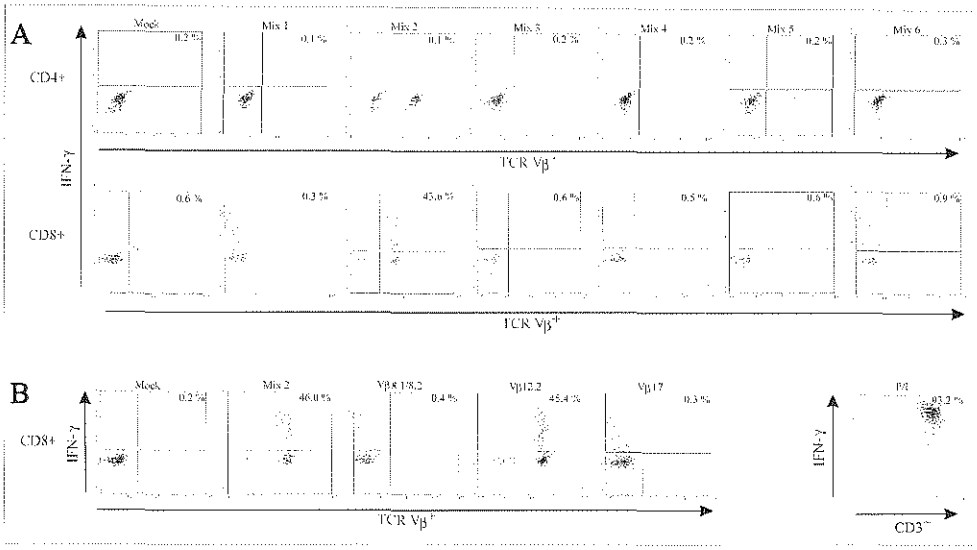

Figure 2.
Analyses of clonality of HSV-1 –specific T cells in intra-corneal TCL from patient 3. Cells were similarly treated as in Fig. 1 and were stained for: (A) different TCR Vβ mAb mixes or (B) individual TCR Vβ mAbs, to identify the HSV-1 -specific subset of T cells in this TCL. Cells were gated on live cells and on either CD4 or CD8. Mock stimulated cells were incubated with 2% normal mouse serum instead of a TCR Vβ mix to correct for background staining of mAbs.

age of the patients and the fact that HSK generally develops upon recurrent corneal HSV infections over a prolonged period of time. That is, clonal expansion of particularly $CD4^+$ T cells requires chronic antigenic stimulation, as is the case in elderly people infected with herpes viruses, which are carried for longer periods of time and can regularly cause recurrent infections.[23]

The most striking observation in our study was the skewing towards the usage of a limited number of TCR Vβ domains by T cells present within intra-corneal TCL of the majority of patients. In line with a previous study,[15] HSV reactivity also appeared to be almost completely accountable to T cells with a single TCR Vβ domain in some of the patients analyzed. As we did not perform sequencing analysis of the TCR Vβ gene segments of each single cell, although likely, we cannot be certain that usage of the same TCR Vβ domain in a particular TCL reflects a pure monoclonal T cell population. This skewing of a particular TCR Vβ domain might have, at least in part, been due to the stimulation protocol used to grow intra-corneal T cells. On the other hand, elderly people are known to frequently exhibit usage of a single or few TCR Vβ domains in their antigen-specific T cell repertoire.[23] A similar clonotypic dominance of virus specific T cells has also been observed by others in fresh PBMC obtained from cytomegalovirus-infected individuals.[22]

A second limitation of this study is the fact that the available panel of mAbs against the different TCR Vβ domains does not cover the whole spectrum of Vβ gene segments expressed by human T cells. This imperfection probably accounts for the majority of T cells from which the TCR Vβ domain could not be identified. In intra-corneal TCL from some patients, the majority of T cells was not recognized by any of the six TCR Vβ mixes. Still, a

clear antigenic response against HSV could be measured in TCL from these patients (see Table 2), implicating that a more or less dominant HSV-specific T cell response, with a TCR Vβ domain for which a mAb was not available to us, existed in these TCL.

Although the antigen specificity of cornea infiltrating T cells in the mouse HSK model remains unclear, HSV-specific T cells have been observed in murine corneas.[6] Few data however are available on the clonality of these T cells. Studies by Heiligenhaus *et al.* though have indicated that in murine HSK, similarly to what we observed in humans in this study, the cornea is infiltrated with a dominant subtype of T cells.[25,26] These investigators found mainly activated $CD4^+$ T cells expressing the TCR Vβ8 domain in inflamed corneas of susceptible mice. They argued that this dominant and clonal T cell subtype was involved in mediating HSK in mice. Although in the murine HSK model HSV antigens are no longer detectable at the time corneal disease manifests itself, HSV antigens have been detected in corneas of human HSK patients during the active phase of the disease, predominantly in patients with ulcerative necrotizing HSK.[15,27] Especially the high frequency and clonality of HSV-specific T cells in some TCL in this study argues for an enrichment of HSV-specific T cells in HSK diseased human corneas by antigen-specific mechanisms. It remains however speculative whether these HSV-specific T cells are the actual trigger for the development of HSK in humans or whether alternative mechanisms are at work.

In summary, the present study demonstrates that four-color flowcytometry can be used to analyze intra-corneal T cell fractions from HSK patients at the single cell level, without the need of subcloning of T cells. HSV-specific T cells of both $CD4^+$ and $CD8^+$ subsets were observed in intra-corneal TCL from most HSK patients. Our data demonstrate the infiltration and local enrichment of HSV-specific T cells and indicate a profound skewing of TCR Vβ usage by HSV-1 -specific intra-corneal T cells in these HSK patients. We hypothesize that HSV-specific T cells may have an important function in the development and/or perpetuation of the inflammatory processes in human HKS.

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Chapter 6

IL-17 Expression in Human Herpetic Stromal Keratitis: Modulatory Effects on Chemokine Production by Corneal Fibroblasts

Jeroen Maertzdorf, Albert D.M.E. Osterhaus, Georges M.G.M. Verjans

Journal of Immunology (in press)

ABSTRACT

Herpetic stromal keratitis (HSK) is an immunopathologic disease triggered by infection of the cornea with herpes simplex virus. Key events in HSK involve the interaction between cornea infiltrating inflammatory cells and resident cells. This interaction in which macrophages, producing IL-1 and TNF-α, and IFN-γ producing Th1 cells play a crucial role, results in the local secretion of immune-modulatory factors and a major influx of neutrophils causing corneal lesions and blindness. The Th1-derived cytokine IL-17 has been shown to play an important role in several inflammatory diseases characterized by a massive infiltration of neutrophils into inflamed tissue. Here we show that IL-17 is expressed in corneas from patients with HSK, and that the IL-17 receptor is constitutively expressed by human corneal fibroblasts (HCF). IL-17 exhibited a strong synergistic effect with TNF-α on the induction of IL-6 and IL-8 secretion by cultured HCF. Secreted IL-8 in these cultures had a strong chemotactic effect on neutrophils. IL-17 also enhanced TNF-α- and IFN-γ-induced secretion of macrophage inflammatory protein (MIP-1)α and MIP-3α, while inhibiting the induced secretion of RANTES. Furthermore, considerable levels of IFN-γ-inducible protein (IP)-10 and matrix metalloproteinase (MMP)-1 were measured in stimulated HCF cultures, while the constitutive secretion of monocyte chemotactic protein (MCP)-1 remained unaffected. The data presented suggest that IL-17 may play an important role in the induction of the immunopathologic processes in human HSK by modulating the secretion of pro-inflammatory and neutrophil chemotactic factors by corneal resident fibroblasts.

INTRODUCTION

Herpes simples virus (HSV) infection of the cornea can lead to the development of a chronic inflammatory disease of the cornea called herpetic stromal keratitis (HSK), a leading cause of non-traumatic blindness in developed countries.[1] HSK is considered to result from an immunopathologic process in the cornea involving both innate and adaptive immune responses to the replicating virus.[2] Solid support for this viewpoint comes from studies in experimental mouse models. In immunocompetent mice the disease is associated with a bi-phasic cellular infiltrate in the corneal stroma. In the pre-clinical phase, when HSV-1 replicates in the corneal epithelium, neutrophils invade the underlying corneal stroma.[3] This transient response, triggered by replicating virus,[4,5] is thought to control HSV replication and limit viral spread into peripheral tissues.[6,7] During the second phase, i.e. the clinical phase, a second wave of inflammatory cells, predominantly consisting of neutrophils, infiltrate the corneal stroma.[8] An essential factor for the development of HSK is the involvement of IL-2- and INF-γ- secreting $CD4^+$ T cells, orchestrating the extravasation and activation of these neutrophils. These neutrophils are considered to be directly involved in corneal destruction.[9-11] Additionally, Langerhans cells (LC) and macrophages, secreting the pro-inflammatory cytokines IL-1 and TNF-α, are essential mediators.[12-15]

Extensive studies in murine models of HSK have provided insight into the roles multiple chemokines play in the development of the disease.[5,8,16,17] These studies demonstrated the temporal expression of chemokines like IL-8, RANTES, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein (MCP)-1 and IFN-γ-inducible protein (IP)-10 in affected murine corneas. These chemokines are likely to contribute to the recruitment and activation of lymphocytes, dendritic cells and neutrophils, initiating the clinical phase of HSK. Although the cellular source of the chemokines involved in HSK remains ill-defined, they may be produced by infiltrating inflammatory cells and/or resident corneal cells. Several studies have implicated that the interaction with inflammatory cells mainly involves fibroblasts within the corneal stromal layer. The stromal cell layer of the cornea is made up of fibroblasts providing mechanical strength to the cornea by supporting a framework of extracellular matrix. Large numbers of IFN-γ and TNF-α producing cells have been observed in the stromal layer of HSV-infected murine corneas but not in the epithelial or endothelial layers.[18,19] This corresponds to the location of neutrophils that mainly infiltrate the stromal fibroblast cell layer of the cornea. Similarly, the induction of IL-8, which exhibits neutrophil chemotactic properties, has been shown to be associated with HSV replication in corneal fibroblasts but not in epithelial cells.[20]

Evidence is accumulating that activated tissue resident cells, including fibroblasts, are involved in modulating local immune responses by expressing adhesion molecules and secreting regulatory factors like cytokines and chemokines. Macrophage-derived cytokines like IL-1 and TNF-α, and Th1 cytokines like IFN-γ, have been shown to activate mesenchymal cells.[21-26] Interestingly, another recently described Th1 cytokine IL-17, can exert strong synergistic and/or antagonistic effects with the aforementioned pro-inflammatory cytokines. These effects include the modulation of MHC and adhesion molecules, and the induction of a variety of cytokines and chemokines by mesenchymal cells.[21-24,27-30] This modulatory effect of IL-17 could therefore have an indirect but profound effect on the recruitment and activation of different inflammatory cells into the corneal stroma during human HSK. To address this issue, we analyzed the induction of the cytokine IL-6, various chemokines and matrix metalloproteinase (MMP)-1 by human corneal

fibroblasts (HCF) upon stimulation with IL-1β, TNF-α and IFN-γ. The combinative effect of IL-17 with these cytokines on HCF activation was emphasized.

Our date show that IL-17 is expressed in corneas of HSK patients and has a regulatory effect on the induction of immune-modulatory factors by cytokine stimulated HCF. These results indicate that the interaction of IL-17 with HCF may play a significant role in the initiation and perpetuation of the inflammatory processes in human HSK.

MATERIALS AND METHODS

Cytokines and mAbs

Human recombinant IL-1β, IL-17, TNF-α and IFN-γ were obtained from Peprotech (London, UK). For blocking experiments, neutralizing mAbs directed to human IL-17 (R&D Systems, Abingdon, UK), IL-8 and isotype matched control mAbs were used (Peprotech).

Human intra-corneal T cell lines and HCF cultures

The generation of HSV-specific T cell lines (TCL) used in this study, obtained from affected corneas of two HSK patients, has been described previously.[31] To determine IL-17 mRNA expression, TCL were either left unstimulated or stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml). Primary HCF cultures were generated from three individual donor corneas that had been rejected for transplantation use due to low endothelial cell counts, and from one transplanted HSK diseased cornea. The central part of each cornea was finely minced and digested with collagenase essentially as described elsewhere.[31] Adherent cells were cultured in 6 wells plates in medium consisting of a 1:1 ratio of DMEM and Ham F12 nutrient mixture (Gibco BRL, Breda, the Netherlands) supplemented with 10% FCS and antibiotics. Growing HCF cultures, with a fibroblast-like morphology, were grown in bulk in 162 cm^2 flasks and cryopreserved. All cells stained positive for vimentin by immunofluorescence staining, whereas almost no reactivity was found for both the acidic and basic subfamilies of cytokeratin (data not shown), indicating that HCF cultures were not contaminated with corneal epithelial or endothelial cells. Third- or fourth-passage corneal fibroblasts were used in all experiments. For cytokine stimulation experiments, HCF from donor corneas were grown in medium of DMEM and Ham F12 with 10% FCS and antibiotics. At confluence, medium was replaced with a serum-free medium consisting of DMEM supplemented with 2 mM L-glutamin, 20 μg/ml cholesterol and 0.3 % BSA (all obtained from Sigma, St Louis). HCF were left for 5 days on serum-free medium prior to stimulation with cytokines. Serum-free medium was used to maintain a more native biosynthetic phenotype and appearance (32) and to reduce background levels of cytokine and chemokine production (data not shown). Stimulatory cytokines were added to the HCF cultures and supernatants were collected after 48 h. All tested stimulatory conditions were repeated in at least eight individual experiments unless stated otherwise. For blocking experiments, the stimulating cocktail of cytokines was pre-incubated with a neutralizing anti-IL-17 mAb (10 μg/ml) at room temperature 30 min prior to addition to the cell culture. Inhibition of cytokine and chemokine expression was induced by pre-treatment of HCF cultures with 10 ng/ml of the anti-inflammatory drug dexamethasone (Sigma) for 2 h.

RNA isolation and RT-PCR analyses

Total cellular RNA was extracted from human HSK corneas, cultured HCF or intra-corneal TCL with TriZOL reagent (Gibco BRL) according to the manufacturer's protocol. For RT-PCR analyses, total RNA was converted into cDNA using oligo(dT) and reverse transcriptase. The following synthetic oligonucleotides were used for PCR amplification: for IL-17 amplification, primers 5'-ATCTCCACCGCAATGAGGAC-3' and 5'-GTGGACAATCGGGGTGACAC-3' (232-bp amplicon); for IL-17 receptor, primers 5'-CTAAACTGCACGGTCAAGAAT-3' and 5'-CTGAGCTCATGCATGGCGTGG-3' (456-bp amplicon). As an internal control for the amount of cDNA, the GAPDH gene was amplified with primers 5-GGTGAAGGTCGGAGTCAACG-3' and 5'-CAAAGTTGTCATGGATGACC-3' (496-bp amplicon). Amplification was performed in standard PCR buffer with 1 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Groningen, The Netherlands), 200μM dNTPs and 25 pmol of each primer in a total volume of 50 μl. DNA amplification was started with a 5 min incubation at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final extension was at 72°C for 7 min. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Southern blotting and labeling with a specific [^{32}P]-labeled oligonucleotide probe was performed to confirm the specificity of the amplicons.

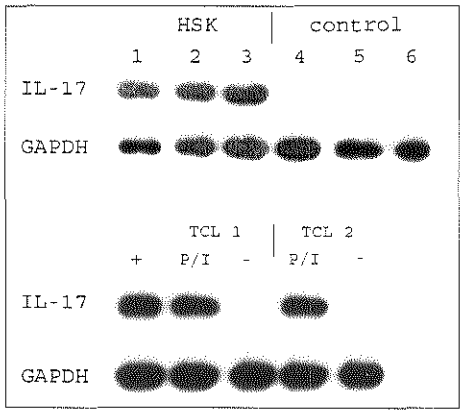

Figure 1.
Expression of IL-17 mRNA expression in human HSK corneas and intra-ocular T cell lines analyzed by RT-PCR and southern blotting. ***(A)*** *RNA was isolated from corneas from three patients transplanted during active HSK (1-3) and from three healthy donor control corneas (4-6).* ***(B)*** *HSV-1-specific intra-corneal T cell lines from two other HSK patients were cultured and RNA from a total of 10^6 mitogen-stimulated (P/I) or nonstimulated (-) cells was extracted for analysis. "+" indicates positive control on RNA from mitogen-stimulated PBMCs.*

ELISA

Culture supernatants from corneal fibroblasts stimulated for 48 h, referred to as conditioned medium, were harvested and cleared of cellular debris by centrifugation. Secretion levels of IL-6, IL-8, RANTES, MIP-3α, IP-10 and metalloproteinase-1 (MMP-1) was measured with commercially available ELISA kits (R&D Systems). MIP-1α content was measured in an ELISA using a coating mAb, recombinant human MIP-1α as a standard and a HRP-conjugated detection mAb (R&D Systems). The amount of MCP-1 in culture supernatants was determined using a commercially available ELISA kit obtained from BioSource (Nivelles, Belgium).

Neutrophil chemotaxis

Polymorph mononuclear cells (PMN) were isolated from fresh peripheral blood of healthy, adult volunteers using Polymorph Prep (Nycomed, Oslo, Norway) and residual erythrocytes were lyzed. To analyze the chemotactic activity of conditioned medium of HCF, PMN were brought to a final concentration of $2x10^6$ cells/ml in serum free medium. The chemotaxis assay was performed in a 24-well Transwell system

(Costar, Badhoevedorp, Netherlands). The bottom wells of the chamber were filled with 100 µl of either recombinant human IL-8 (10 ng/ml) as a positive control for neutrophil chemotaxis, the control solution or conditioned medium from stimulated HCF cultures, 10 times diluted in serum free medium. The upper wells, holding a polycarbonate filter with a pore size of 3 µm were placed on top and filled with 150 µl of neutrophil suspension. The Transwell system was incubated in humidified air with 5% CO_2 at 37°C for 45 min, and the number of cells that had migrated through the filter into the bottom well were counted. Inhibition of IL-8-mediated neutrophil recruitment involved a pre-incubation of the conditioned medium with 10 µg/ml anti-IL-8 mAb 30 min prior to the experiment at room temperature. As a control, conditioned medium was incubated under similar conditions with an irrelevant isotype matched control mAb.

RESULTS

IL-17 expression in corneas and intra-corneal T cell lines obtained from HSK patients

Whereas the normal healthy cornea is essentially devoid of T cells, HSK is considered an immunopathologic disease orchestrated by corneal infiltrating $CD4^+$ Th1 cells secreting IL-2 and IFN-γ.[9-11] To assess the possible role of the Th1 cytokine IL-17 in human HSK, the expression of IL-17 mRNA was determined in corneas from 3 patients with fulminant HSK who underwent corneal transplantation to restore sight. IL-17 transcripts could be detected in all HSK corneas, while no IL-17 was detected in control corneas (Fig. 1A). Mitogenic stimulation of 2 intra-corneal TCL generated from corneas of two other HSK patients induced IL-17 mRNA expression. This demonstrates that corneal infiltrating T cells, at least in part, are able to express IL-17 mRNA upon activation (Fig. 1B).

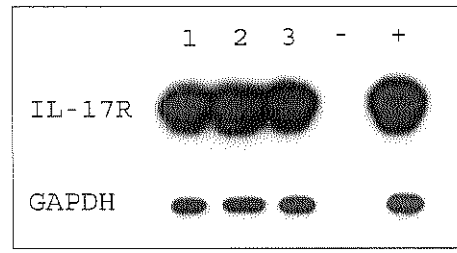

Figure 2.
Corneal stromal fibroblasts constitutively express IL-17 receptor mRNA. Corneal fibroblasts were cultured from two individual healthy donor corneas (1 and 2) and one transplanted non-HSK cornea (3). RNA was isolated from 10^6 cells from each culture and assayed for IL-17R mRNA expression by RT-PCR and southern blotting. "-" indicates the water control, "+" indicates positive control.

HCF constitutively express the IL-17 receptor

The keratogenic properties of Th1 cytokines in HSK may in part be due to their modulatory effect on corneal resident cells. Corneal infiltrating T cells are mainly observed in the corneal stroma, suggesting an interaction between T cells and corneal fibroblasts by means of direct cell-cell contact or soluble factors like IL-17. To evaluate whether corneal fibroblasts express the IL-17 receptor, RT-PCR was performed on RNA extracted from three primary HCF cultures. Two primary HCF cultures had been generated from two healthy donor corneas and one from a transplanted HSK diseased cornea. RT-PCR analysis showed that HCF constitutively express the IL-17 receptor (Fig. 2).

Synergistic effect of IL-17 on TNF-α -induced IL-6 and IL-8 secretion by HCF

Studies in HSK mouse models have shown a strong induction of IL-6 and IL-8 after HSV-1 infection of the cornea.[5,20] By mimicking the inflammatory situation in the corneal stroma during HSK development *in vitro*, we determined the modulatory effect of IL-17 (100 ng/ml) on IL-1β (100 ng/ml), TNF-α (50 ng/ml) and IFN-γ (100 U/ml) to induce secretion of IL-6 and IL-8 by HCF. Optimal concentrations of stimulatory cytokines had been determined in preliminary experiments (data not shown).

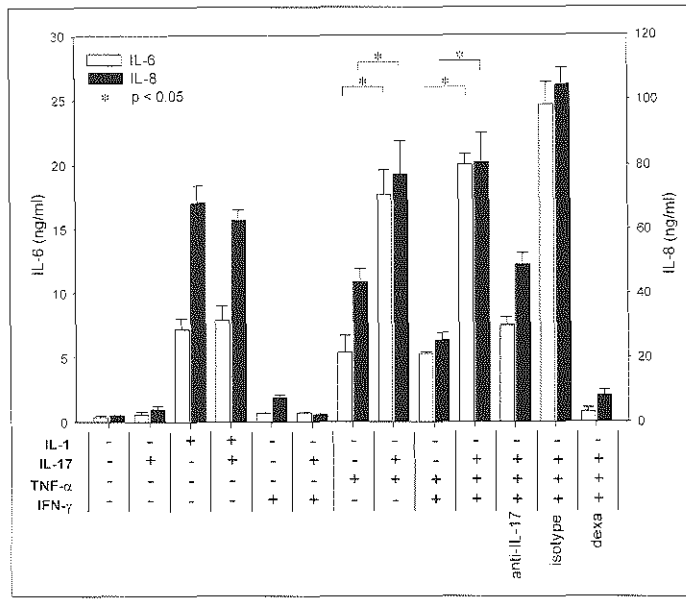

Figure 3.
IL-6 and IL-8 secretion by stimulated HCF. Cultured HCF were stimulated for 48 hr with the cytokines indicated and IL-6 and IL-8 levels in conditioned medium were determined by ELISA. Cytokine mixtures were pre-incubated, half an hour prior to addition to the cell cultures, with an anti-IL-17 mAb or an isotype matched control mAb (isotype) to block IL-17 activity. Dexa indicates a 2 hour pre-incubation of the cells with dexamethasone (10 ng/ml). Data shown are representative for a total of 8 individual experiments.

HCF showed a low level of background secretion of both IL-6 and IL-8, while stimulation with IL-17 or IFN-γ alone or in combination did not show any considerable increase in either IL-6 or IL-8 levels (Fig. 3). Stimulation of HCF with IL-1β or TNF-α however did have a clear stimulatory effect on the secretion of both factors. Although IL-17 itself had no effect, incubation of HCF with combinations of stimulating cytokines showed that it had a strong synergistic effect on the TNF-α-induced secretion of both IL-6 and IL-8. This synergistic effect was almost completely neutralized by pre-incubating the stimulating cytokine cocktail with an anti-IL-17 mAb, but not with an isotype matched control mAb (Fig. 3). Furthermore, a 2 hr pre-treatment of HCF with dexamethasone, an immunosuppressive drug commonly used in treating human HSK, almost completely abolished the induced secretion of IL-6 and IL-8 (Fig. 3).

Modulatory effect of IL-17 on HCF secretion of chemokines and matrix metalloproteinase-1

In addition to IL-6 and IL-8, several other chemokines are expressed in affected corneas, and are assumed to play a critical role in the development of HSK.[5,8] As HCF are a probable cellular source for these chemokines, we also analyzed the effects of stimulation

with IL-1β, TNF-α, IL-17 and IFN-γ on chemokine production (including MCP-1, RANTES, MIP-1α, MIP-3α, IP-10) and MMP-1 by HCF. The observation by Tumpey *et al.*[33] that MIP-1α knockout mice fail to develop HSK suggests that this chemokine plays a key factor in the development of the disease. MIP-1α secretion by HCF, although in low amounts (up to 30 pg/ml), was solely observed upon co-stimulation of IL-17 with TNF-α and/or IFN-γ (Fig. 4). Whereas MIP-1α is mainly chemotactic for neutrophils, MIP-3α is a powerful chemoattractant for LC precursors (34). Both IL-1β and TNF-α induced the secretion of MIP-3α by HCF, whereas neither IL-17 nor IFN-γ stimulated MIP-3α secretion (Fig. 4). IL-17 showed a clear synergistic effect with TNF-α on MIP-3α secretion by HCF, but not with IL-1β or INF-γ.

Intra-corneal expression of RANTES and IP-10 is restricted to the pre-clinical phase of HSK. Both these chemokines are known to induce T cell migration.[35] Secretion of RANTES could only be induced by TNF-α, whereas IP-10 production was augmented by TNF-α and IFN-γ (Fig. 4). IL-17 showed a remarkable inhibition on the secretion of RANTES by HCF upon stimulation with TNF-α and/or IFN-γ, while IP-10 production was only slightly inhibited by IL-17 (Fig. 4).

Although the role of MCP-1 is not clear at this point, it has been reported to play a role in $CD4^+$ T cell recruitment, and appears to be rapidly upregulated and persistently expressed in HSV-1 infected corneas.[5] In our experiments, MCP-1 was found to be constitutively secreted by HCF and no evident changes were observed by incubation with

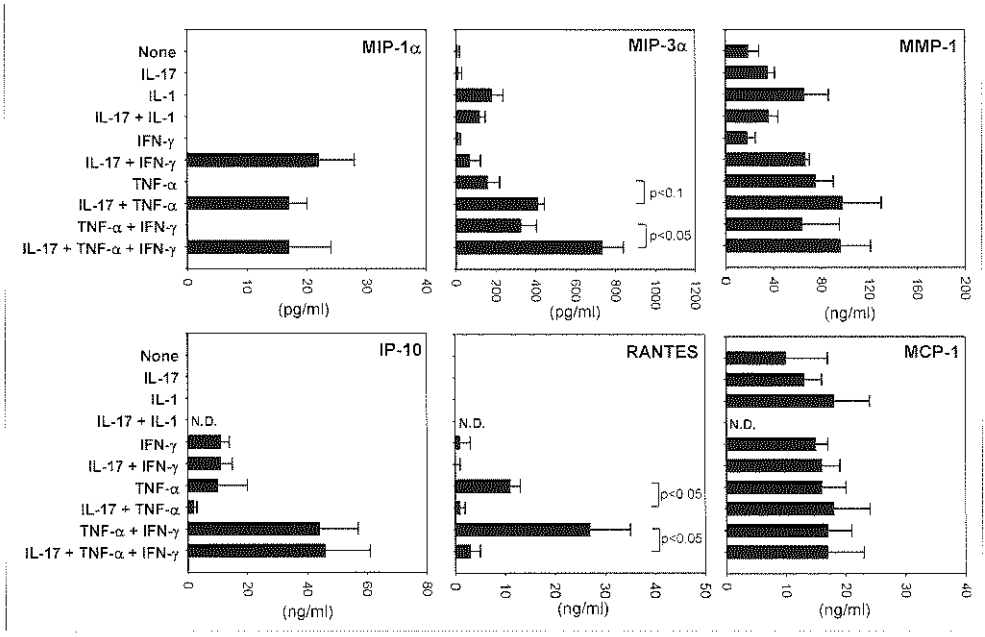

Figure 4.
Chemokine and MMP-1 secretion by stimulated HCF. Cultured HCF were stimulated for 48 hr with the indicated cytokines and chemokine and MMP-1 levels in conditioned medium were determined by standard ELISA analyses. Data shown are representative for a total of 4 individual experiments. N.D. = not done.

stimulatory cytokines (Fig. 4). Although MMP-1 is not a chemokine, it was included in this study because MMP-1 is known to degrade fibrilar collagens,[36] an essential component of the corneal stroma. HCF showed a constitutive secretion of MMP-1, which was not affected by IFN-γ. Compared to the mild induction by IL-17, both IL-1β and TNF-α induced a strong enhancement of MMP-1 secretion. Interestingly, IL-17 had a clear synergistic effect with IFN-γ on MMP-1 secretion, while showing a slight inhibitory effect in combination with IL-1β or TNF-α (Fig. 4).

HCF induce neutrophil recruitment through IL-8 release

Chemokines expressed within HSV-1 infected cornea are believed to induce the corneal infiltration of neutriphils involved in the development of corneal lesions.[3,5,8,16,17] To test the PMN chemotactic properties of chemokines secreted by HCF, conditioned medium from control and cytokine -stimulated HCF cultures were tested in a neutrophil chemotaxis assay. Conditioned medium from HCF stimulated with the combination of IL-17, TNF-α and IFN-γ was used because it contained the highest levels of IL-8 and MIP-1α (see Fig. 3 and 4), both potent PMN chemoattractants. Recombinant IL-8 (10 ng/ml) was used as a positive control, while fresh serum free medium served as background control. Compared to the medium control, conditioned medium from stimulated HCF induced a strong migration of PMNs. This effect could be blocked by pre-treatment of the conditioned medium with a neutralizing anti-IL-8 mAb 30 min prior to the experiment (Fig. 5). Conditioned medium from non-stimulated fibroblasts caused a mild migration of PMNs, probably due to a low level of background production of IL-8 in these cultures (see Fig. 3).

DISCUSSION

Herpetic stromal keratitis is an HSV-induced corneal disease characterized by complex interactions between infiltrating inflammatory cells and corneal resident . These cellular interactions are assumed to result in the induction and perpetuation of a chronic inflammatory process resulting in corneal damage.[2,3,7,8] Stimulation of mesenchymal cells with pro-inflammatory cytokines of macrophage/monocyte (IL-1β and TNF-α) or Th1 cell (IFN-γ) origin, has previously been shown to activate and modulate the secretion of chemokines,[21-26] suggesting a role of resident cells like fibroblasts in the recruitment and activation of inflammatory cells to sites of inflammation. Recent observations have furthermore shown that activation of resident cells can be heavily modulated by the Th1-derived cytokine IL-17.[21-24,27-30] Accordingly, the potential interactive role of IL-17 and corneal fibroblasts in the induction and perpetuation of inflammatory processes in human HSK was hypothesized.

The expression and pathogenic properties of IL-1β, TNF-α and IFN-γ in HSK have been extensively studied in the experimental HSK mouse model. These pro-inflammatory cytokines probably do not have a direct immunopathologic effect by recruiting and stimulating inflammatory cells, but rather exert their effects through interaction with corneal resident cells in an autocrine or paracrine fashion. Interleukin 17 is secreted by activated $CD4^+$ T cells, mainly Th0 and Th1,[27] and exhibits pleiotropic biological activities on various human tissue resident cells including fibroblasts. IL-17 shares mainly properties with IL-1β and TNF-α in that these three cytokines activate the transcription factor NFκB in a variety of

cell types and all stimulate secretion of pro-inflammatory factors in mesenchymal cells.[27] The present study shows IL-17 expression in affected corneas of HSK patients, while being undetectable in control corneas. Mitogenic stimulation of intra-corneal TCL obtained from corneas of HSK patients induced IL-17 expression. The HCF constitutively expressed the IL-17R, consistent with its broad tissue distribution.[37]

IL-17 indirectly stimulates granulopoiesis both *in vitro* and *in vivo*,[37,38] and has also previously been marked in playing an important role in the pathogenesis of other immune-mediated diseases associated with a massive infiltration of neutrophils into inflamed tissues.[39-44] The functional relevance of IL-17 signaling in host defense has recently been demonstrated in an experimental model of *Klebsiella pneumoniae* lung infection in IL-17R knockout mice.[45] IL-17R signaling appeared to be critical for the local induction of G-CSF and MIP-2 and subsequent PMN recruitment into the alveolar space. The murine C-X-C chemokines MIP-2 and KC, homologues of human IL-8, have also been associated with the development of experimental murine HSK.[5] Neutralization of MIP-2 decreased corneal PMN infiltration and significantly reduced corneal pathology, demonstrating that MIP-2 is the major chemokine that attracts PMN into the HSV-1 infected cornea in mice.[17] Interestingly, in our experiments, conditioned medium from HCF stimulated with the combination of TNF-α, IFN-γ and IL-17 induced chemotaxis of PMN *in vitro* (Fig 5). Despite the presence of multiple other chemokines in these conditioned media, this chemotactic activity on PMNs was mainly induced by IL-8, since pre-incubation of conditioned medium with a neutralizing IL-8 mAb almost completely abrogated PMN migration.

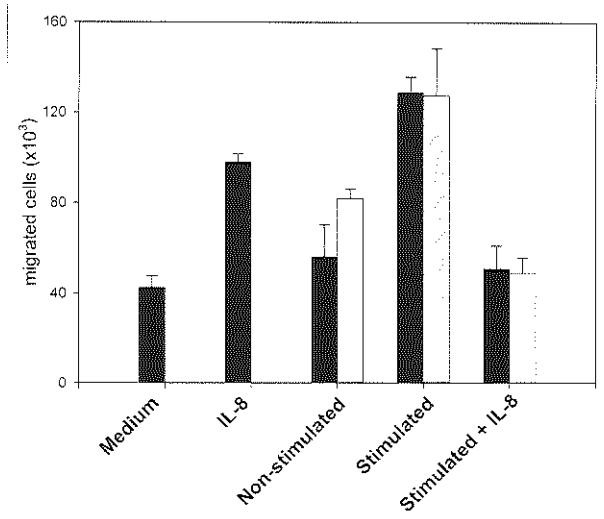

Figure 5.
Conditioned medium from stimulated HCF cultures induces neutrophil migration. Serum free medium served as the background control and rhIL-8 (10 ng/ml) was used as a positive control for neutrophil migration. Non-stimulated indicates conditioned medium from cells incubated with serum free medium alone; stimulated conditioned medium was obtained from cells stimulated with IL-17, TNF-α and IFN-γ simultaneously. Blocking neutrophil migration was achieved by a 30 min pre-incubation of the conditioned medium with an anti-IL-8 mAb (10 μg/ml). Black and grey bars represent conditioned media from two different stimulation experiments and are representative for 4 individual experiments.

The data presented here demonstrate that cytokine stimulated HCF secrete chemokines associated with HSK. Chemokine secretion was differentially regulated by Th1 cell- and macrophage/monocyte-derived cytokines. Compared to IFN-γ and IL-17, IL-1β and TNF-α were more effective in stimulating HCF to secrete IL-6, IL-8, MIP-3α and MMP-1. Whereas simultaneous treatment with TNF-α and IFN-γ induced significant secretion of RANTES and IP-10 by HCF, no detectable amounts of MIP-1α were observed upon

stimulation with these cytokines. Neither Th1 cell- or macrophage/monocyte-derived cytokine however seemed to significantly alter the constitutive expression of MCP-1

The induced secretion of IL-6 and IL-8 by HCF stimulated with either IL-1β or TNF-α was consistent with observations previously described.[46,47] Similarly, induction of IL-6 secretion upon stimulation with IL-1β or TNF-α has also recently been observed in myofibroblast cells (48). In addition, such cells do not secrete any MIP-1α upon stimulation with IL-1β or TNF-α,[26] comparable with our results. In comparison to another report on chemokine production by HCF[49] however, we observed some contradiction in the induction of RANTES and MCP-1 secretion by treatment of HCF with IL-1 or TNF-α. In this report stimulation of HCF with IL-1α or TNF-α resulted in increased levels of RANTES and MCP-1 production. In contrast, stimulation of HCF in our experiments with IL-1β did not induce secretion of detectable amounts of RANTES, nor did IL-1β or TNF-α significantly alter MCP-1 secretion. Altough the different effects of IL-1α and IL-1β on HCF are understandable, the differential effect of TNF-α on MCP-1 production might be related to the usage of serum in cell cultures which might contain costimulatory factors, absent in our experiments. In a recent report about rheumatoid arthritis in man, RANTES and MCP-1 have been shown to induce IL-6 and IL-8.[50] These chemokines thus appear not to solely play a role in inflammatory cell migration, but also to be involved in the activation of synoviocytes. Consequently, the secretion of the chemokines detected in conditioned medium of cytokine-stimulated HCF may only in part be directly induced by the cytokines added.

As in human HSK corneas, IL-17 expression has been observed in other inflamed tissues like the lungs in asthma[51] and the synovium in arthritis.[52] IL-17 exerts a modulatory effect on IL-1-, TNF-α- and IFN-γ -induced chemokine secretion by human mesenchymal cells.[21-24,27-30] Furthermore, IL-17 has been described to stimulate the secretion of IL-1β and TNF-α by macrophages.[53] In our experiments, IL-17 had a synergistic effect on TNF-α -induced HCF secretion of IL-6, IL-8 and MIP-3α, which was similar to observations by others.[23,25,27,51,54] Additionally, a synergistic effect was observed for IL-17 with TNF-α and IFN-γ on the induction of MIP-1α, while similar to findings in keratinocytes[21] IL-17 antagonized TNF-α- and/or IFN-γ-induced secretion of RANTES. In contrast however with some of these reports,[21,27] no synergistic effect of IL-17 with IL-1β or IFN-γ was observed, which might be a cell type specific phenomenon.

As its name suggests, IP-10 is known to be induced by IFN-γ, as was also observed in our experiments. Strikingly, IP-10 secretion by HCF was strongly increased upon co-stimulation with both IFN-γ and TNF-α, which is in accordance to a previous report.[55] Whereas a previous report[56] showed no significant stimulatory effect of IL-17 on IP-10 induction in keratinocytes, our results showed a slight inhibitory effect of IL-17 on IP-10 secretion by HCF. Secretion of IL-6 and IL-8, induced by HCF stimulation with a combination of IL-17, TNF-α and IFN-γ - resulting in the highest secretion levels (Fig. 2) - was inhibited by pretreatment with an anti-IL-17 mAb to levels similar to those of TNF-α plus IFN-γ, demonstrating the specific modulatory effect of IL-17. Glucocortocoids, like dexamethasone, are widely used in the treatment of corneal inflammatory diseases. The data presented here show that secretion of IL-6 and IL-8 by cytokine stimulated HCF can be completely blocked by pretreatment with dexamethasone. This is compatible with the idea that the beneficial effects of glucocortocoids in clinical practice are due to inhibition of exaggerated cytokine production. This effect of glucocortocoids is mediated by their antagonistic action on transcription factors, like NF-*k*B, required for cytokine transcription.[57]

The collection of chemokines found to be secreted by HCF upon cytokine stimulation exhibit a broad range of leukocyte recruiting and activating potentials. These activities include the migration and activation of T and NK cells (e.g. RANTES, MCP-1, MIP-3α and IP-10), Langerhans cells (e.g. MIP-3α), macrophages/monocytes (e.g. MCP-1) and PMN (e.g. IL-8 and MIP-1α). Studies on expression profiles of different chemokines in the HSK mouse models have shown that most pro-inflammatory cytokines and chemokines are expressed throughout both the pre-clinical and clinical phase of HSK.[8] Exception to this are the chemokine MIP-1α, which was only detected during the clinical phase, and the chemokines IP-10 and RANTES, that can only be detected early after HSV-1 infection of the cornea, but not during the clinical phase of the disease.[8] Remarkably, in the present study IL-17 exerted a strong stimulatory signal on the induction of IL-8, MIP-1α and MIP-3α, whereas it inhibited the secretion of RANTES and to a lesser extent IP-10. This IL-17 specific inhibitory effect on RANTES and IP-10 secretion might reflect a down-regulatory signal by activated T cells to limit the recruitment of additional T lymphocytes, and corresponds with the transient detection of these chemokines in the cornea early after infection. On the other hand, increased secretion of IL-8 and MIP-1α would create an inflammatory environment in the corneal stroma with a high potential for PMN extravasation and activation, where they directly or indirectly cause tissue damage. Although MMP-1 is not a chemokine, its secretion in the corneal stroma might explain some clinical aspects of HSK. As described previously, IL-1 and TNF-α augmented secretion of MMP-1 in human fibroblasts.[58] MMP-1 degrades structural type I and type II collagen and its production in the corneal stroma during HSK might thus contribute to the development of corneal lesions. Accordingly, IL-17 has previously been implicated in synovium matrix destruction in rheumatoid arthritis by similar modes of action.[59]

In conclusion, our study shows the ability of cytokine stimulated HCF to secrete inflammatory mediators which are potentially involved in the immunopathogenesis of HSK. The Th1 cytokine IL-17, expressed in human HSK corneas, modulated these responses in a synergistic or antagonistic fashion. We speculate that during the development of HSK, in addition to corneal infiltrating cells, corneal fibroblasts are an important cellular source of pro-inflammatory cytokines and chemokines. Among these IL-8, which primarily mediates the extravasation and activation of pathogenic PMN into the cornea. Furthermore, our results suggest that IL-17 might be an important player in the immune activation of HCF. Future studies in murine models might elucidate any potential role of IL-17 in the inflammatory processes leading to HSK.

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Chapter 7

General Discussion

INTRA-TYPICAL DIFFERENCES AND DISCRIMINATION OF HSV-1 STRAINS

Herpes simplex virus is a ubiquitous pathogen in humans. State of the art molecular biological techniques has allowed the genetic characterisation of the virus and to study the epidemiology of HSV infections in detail. HSV is assumed to be derived from a common ancestor virus, and heterogeneity among viral strains of each serotype is probably the result of genomic variation arisen throughout evolution. From a clinical point of view, the use of molecular biology is important in determining whether independently isolated HSV-1 strains originate from the same source. Genetic differentiation and characterisation of HSV-1 strains can be performed in two ways. The first type of variation, i.e. distinct type variation, is based on the presence or absence of particular restriction endonuclease (RE) sites in the viral genome. Such assays, referred to as restriction fragment length polymorphism (RFLP), have been widely applied in studying the spread of HSV-1 in human populations.[1-3] Studies on HSV-1 DNA dynamics suggest a host-linked evolution of herpes viruses, and a close association of a certain HSV-1 genotype with a particular historically related human population[4] seems thus clear. Spread of the virus within human populations can be illustrated by the demonstration that based on distinct type variation, different HSV-1 strains can be found in geographically separate countries or anthropologically different races.[2]

The second type of variation, i.e. common type variation, is characterised by variability in copy numbers of tandem repeats within the viral genome, known as reiterated sequences (Re). Such sequences within the HSV-1 genome can be used as genetic markers for a particular HSV-1 strain (ref. 5 / this thesis). The accuracy of RFLP analysis depends on the type of restriction endonucleases (REs) used. The usage of 6-base pair recognising REs will result in a lower discrimination rate than with 4-base pairs recognising REs, as was also demonstrated for epidemiologically related HSV-1 strains in a study by Umene *et al.*[6] Restriction sites, especially those of 6-base pairs recognising REs are more or less conserved within a certain HSV-1 genotype and therefore not quite suitable for strain differentiation between infected individuals within a certain population. On the other hand, the differences in number of reiterated sequences as used in our method (described in chapter 2), probably are much more variable within HSV-1 strains in a certain human population. These sequences were shown to remain stable after a limited number of replication cycles (chapter 2) and thus probably within a certain individual but not within a whole population.

In conclusion, RFLP is the method of choice in large-scale epidemiological studies comparing HSV isolates in distinct human populations. On the other hand, our newly developed PCR method is more suitable for studies focusing on person-to-person transmission of HSV-1 strains and spread of the virus within a single individual.

The conventional methods of RFLP analyses and the analyses of polymorphism on the basis of changes in numbers of Re as used in the past, have several major disadvantages. First of all, these kinds of analyses are laborious, time-consuming and expensive. Second, these methods depend on the isolation of the virus to be able to obtain sufficient amounts of

viral DNA. Several important questions concerning the spread and transmission of HSV-1 still remain that can not be answered by traditional methods. Such methods of strain discrimination like RFLP can only be used when virus can be isolated during active replication. Characterisation of the strains involved is thus not possible when HSV-1 is latent in its human host or infectious virus has been cleared from the tissue of interest.
For this reason, we set out to develop a new method to discriminate between HSV-1 strains based on PCR amplification of Re. This approach obviates the disadvantages mentioned above in traditional methods of strain differentiation. The development and evaluation of this new approach as described in chapter 2 has an accuracy of discriminating up to 92% of HSV-1 strains from unrelated sources. The fact that this approach can also be used on samples from which virus could not be isolated is demonstrated by its successful use in the studies described in chapter 3 and 4.

Our method is based on PCR amplification of ReIV and ReVII within the HSV-1 genome. In accordance with studies by Umene *et al.*,[5] ReIV and ReVII appeared to be variable enough to discriminate between different HSV-1 strains. At the same time, these sequences also remained stable after a limited period of viral replication, indicating their usefulness to function as genetic markers. This observation was further strengthened by the finding that both ReIV and ReVII remained stable over prolonged periods of time in patients with recurrent herpetic keratitis (see also chapter 4). In contrast, ReI and ReIII were discarded as genetic markers as they were found to be too unstable after even several rounds of viral replication to function as proper genetic markers. The variability of ReIV appeared to be much more pronounced than that seen in ReVII. This is not surprising considering that ReIV is located within the introns of HSV-1 genes US1 and US12, while ReVII is part of the coding sequence of gene US10/11. By using ReIV and ReVII as genetic markers, we genotyped 37 unrelated clinical HSV-1 isolates from corneal swabs of herpetic keratitis patients. Combining the analysis of ReIV and ReVII for each clinical sample showed that 34 out of these 37 isolates displayed a unique combination of PCR fragments. It should be noted that HSV-1 strain differentiation based on PCR amplification alone does not suffice for conclusive evidence of virus transmission. A differentiation rate of 92% in our method gives a good indication for whether a single or different HSV-1 strains are involved. However, PCR amplification should be complemented with detailed nucleotide sequencing analysis to obtain conclusive evidence for virus transmission.
Such nucleotide sequencing analysis also revealed the presence of a newly identified Re, referred to as ReVIII (see chapter 3). ReVIII is located between ReIV and the startcodon of genes US1 and US12. Besides their role in genomic recombination processes, Re may also have a modulatory role on the transcription and/or translation of proteins encoded by flanking genes. Considering the importance of the proteins encoded by genes US1 and US12 (ICP22 and ICP47 resp.) in immune evasion of the virus, ReVIII might thus have a profound impact on the function of these proteins and persistence of the virus in its host.

One might ask what the clinical or scientific significance is to know whether HSV-1 infections are caused by endogenous virus reactivated from latency or reinfection with a new exogenous virus (i.e. superinfection). First of all, by differentiating HSV-1 strains on the basis of a viral DNA fingerprint is probably the only decisive method of indicating direct person-to-person transmission of the virus. It also enables more detailed study on viral recombination processes and its consequences. For example, recombination of two different HVS-1 strains infecting the same anatomical site might give rise to new and more virulent strains.[7] Similarly, recombination between intertypic viruses has been observed, which can

result in the rise of viruses with new pathogenic properties.[8] The acquirement of HSV strains resistant against antiviral therapy is another aspect to be considered in HSV superinfection. Any potential clinical consequences for ocular HSV superinfections have been illustrated by studies in rabbits showing that different HSV-1 strains can cause different types of ocular lesions.[9]

HSV-1 TRANSLOCATION FROM BRAIN TO EYE

Both HSV serotypes can infect humans at various anatomical sites resulting in distinct disease entities. Translocation of HSV-1 within an individual is generally considered to occur by cell-to-cell spread within the same tissue or through auto-inoculation from a lesion to an anatomically different site. Alternatively, HSV may spread within a single individual by transneural transport and cause distinct infections. Such spread of a single HSV-1 strain within the human host was demonstrated using our newly developed PCR method for differentiation of HSV-1 strains. In chapter 3 we describe brain-to-eye transmission of HSV-1 in two patients who suffered from acute retinal necrosis (ARN), which is induced by HSV-1, following a previous episode of herpes simplex encephalitis (HSE). The pathway by which the infecting virus reaches the eye is usually unclear in patients with ARN. In this study, we concluded that in each patient the virus had most likely translocated from the brain to the eye, possibly through the optic nerve. This conclusion was partially based on findings in experimental mouse models. Investigations by two different groups have shown that in mice HSV-1 can spread through the central nervous system and travel up the optic nerve to the eye.[10-12]

Another explanation for our findings could be that ARN in these patients resulted from reactivation of the same HSV-1 strain that had been in a latent state on a different anatomical location within the patient. Seemingly aberrant from this theory however is that, despite the fact that ARN is a very rare disease, a high percentage of patients with HSV-1 induced ARN have a history of HSE.[13] A correlation between HSV -induced ARN and a previous episode of HSE has also been indicated in multiple case reports.[13-18] Also considering the fact that in most of these cases ARN disease developed only short after HSE suggests that both disease manifestations are at least closely linked. Although we can not formaly exclude the hypothesis of reactivation from a different anatomical site, the usually short time-interval between HSE and ARN and the demonstration of viral translocation in the mouse model suggests that brain-to-eye transmission through the optic nerve remains a probable explanation. One should not exclude however that the HSV-1 infections in HSE and ARN might have been caused by a virus that originated from the eye. In concurrence with findings in the experimental mouse model, the infecting virus might have originated from one of the eyes, spread to the brain and subsequently travelled back up the optical nerve to the retina of the contra-lateral eye.

Regardless of which route the infecting virus might have followed, the clear link between ARN and a previous episode of HSE should provide a warning for clinicians. Both physicians and ophthalmologists should be aware that persons with a history of HSE are at risk for developing ARN. On the other hand, neurologists treating patients with HSE should point out the increased risk of future ocular complications due to HSV. Awareness of this risk may improve early recognition and prompt specialised medical care to prevent loss of sight in such patients.

CORNEAL SUPERINFECTIONS

In contrast to HSV infections at different sites of the body, recurrent HSV infections at the same anatomical location are more common and pose a serious health problem in clinical practise. In such cases of recurrent HSV disease, infections may be caused by reactivation of endogenous virus or reinfection with an exogenous virus, referred to as HSV superinfection. Superinfections by different viral strains of both HSV serotypes have been demonstrated in humans before.[19-21] In contrast to the low frequency of superinfection described in these studies, our data, as presented in chapter 4, indicate that HSV-1 superinfections of the cornea occur in at least one third of individuals with herpetic corneal disease.

Part of this discrepancy may be explained by the method used to discriminate between different virus strains. We used a PCR method based variation in Re as described in chapter 2, in contrast to other studies that have used conventional RFLP analyses. As stated in the previous section, RFLP patterns are quite stable within a population and mutations outside RE recognition sites are missed in this procedure. Thus, the actual rate of superinfection in humans is probably higher than estimates based on conventional distinct type variation by RFLP analyses. A second explanation for this high frequency of superinfection in our patient cohort would be selection of individuals with a higher susceptibility for corneal HSV-1 reinfection. This might be related to the predominantly severe entities of herpetic keratitis in this group of patients. Alternatively, the location of the HSV-1 infections from which isolates were obtained, i.e. the cornea, may be more vulnerable to HSV superinfections than other anatomical sites.

Apart from the surprisingly high percentage of superinfections observed in our study, the data also suggest that individuals with corneal herpetic disease have a higher risk of acquiring a superinfection after cornea transplantation (PKPL). Four out of the 11 patients with different HSV-1 strains in subsequent episodes of corneal infection had undergone PKPL in between these episodes. In contrast, in the group of 19 patients with recurrent infections with the same viral strain, none had undergone PKPL in between episodes of corneal infection. As indicated previously, superinfections with a new HSV strain increases the risk for the patient of acquiring strains resistant to antiviral drugs or more virulent HSV strains with a different disease pattern. From a healthcare point of view, such superinfections may pose new or additional complications in diagnosis and treatment of corneal HSV disease.

Remains the question what the origin and route of infection of this new virus (HSV-1 strain) was. The most obvious explanation would be an infection with exogenous virus. In general, exogenous corneal infections occur on the corneal surface in the epithelial layer. Especially the severe disease entities in our patient cohort might render the cornea more susceptible for new infections. This would suggest however, that both groups of patients, either undergoing PKPL or not, are equally at risk of acquiring superinfections. The data however suggest that recurrent infections in the patient group without PKPL between viral isolations are all attributable to reactivation of latent HSV-1 strains. So, apparently, PKPL leads to an increased susceptibility of HSV-1 superinfection of the cornea.
One explanation might be that the natural defence of the cornea against HSV infection is distorted after surgical intervention in PKPL or due to corticosteroid treatment. One of the

components of this natural defence of the cornea is the secretion of HSV neutralising antibodies of the IgA isotype in the tear fluid. There are however no indications that IgA levels in tear fluid and corneal defence are decreased after PKPL. Still, exogenous virus probably is in most cases the cause of corneal superinfection in these patients.
Alternatively, these patients may have been latently infected with multiple HSV-1 strains simultaneously. Multiple HSV-1 strains may reside in a latent form either within distinct parts of the innervating sensory ganglion (trigeminal ganglion) or even extraneural in the cornea or eyelids. Studies in rabbits and rats have indicated that corneal trauma like corneal transplantation is a powerful inducer of viral reactivation.[22, 23] Subsequent corneal HSV-1 infection after PKPL might then be caused by any of the latent HSV-1 strains present in the host. An alternative route of corneal infection could involve the transmission of a new HSV-1 strain through the corneal graft itself. Studies in animal models have shown that donor-to-acceptor transmission of HSV-1 through the corneal graft is probable.[24] Clinical observations in humans also have shown that some patients may develop corneal HSV-1 infections after PKPL performed for reasons unrelated to HSV.[25-27]

The data presented in chapter 4 show that donor-to-acceptor transmission through a corneal graft with subsequent replication of HSV-1 also occurs in humans. Although PKPL is nowadays a routine and frequent procedure, the risk of acquiring an HSV-1 infection through PKPL is probably low. In contrast to this is the relative high percentage of HSV-positive donor grafts. In eye banks, up to 38% of donor corneas have been reported to harbour HSV-1 DNA, although in very few cases viral replication could be demonstrated upon culturing.[28-30] Moreover, most of these HSV-1 positive donor corneas are rejected for transplantation due to endothelial abnormalities. Nevertheless, despite critical screening in eye banks, HSV-1 can be latently present in corneal tissue and corneal grafts containing HSV-1 DNA are occasionally transplanted.[31, 32] Thus the risk remains that HSV is transmitted through PKPL and can reactivate in the recipient.
Our data clearly show that transmission of HSV-1 through corneal grafting does occur. More importantly, they indicate that HSV-1 transmission through PKPL can be followed by active viral replication, inducing a new infection and corneal disease. In the patient described, corneal disease resulted in complete loss of vision. These findings stress the potential hazardous consequences of HSV-1 transmission through a corneal graft. It is not clear whether in this case the HSV-1 superinfection resulted from actual latent virus or from a low-level replication in the cornea.

The patient concerned was sero-negative for both serotypes of HSV at the time of corneal surgery and consequently probably more vulnerable for infection. It remains questionable however, whether pre-existing antibodies against HSV-1 in sero-positive individuals can protect against superinfection. Protection against exogenous infection on the corneal surface is a role for virus neutralising IgA in tear fluid. Such antibodies however do not help against virus directly introduced into the cornea through an infected corneal graft. There is no doubt that pre-existing immunity against HSV-1 surely aids in limiting the spread of the virus after inoculation through the corneal graft. On the other hand, the lack of blood vessels in the healthy cornea hampers the contribution of virus neutralising antibodies in limiting viral replication in the cornea. Moreover, virus neutralising antibodies can not prevent direct cell-to-cell spread of the virus within the tissue. Furthermore, it should be noted that the cornea, and especially its epithelial layer, is one of the tissues most densely innervated with neuron endings. This dense innervation gives the virus all opportunities to infect neurons and establish a latent infection in the trigeminal ganglion. Vaccination studies in mice and rabbits have actually indicated that pre-existing immunity and anti-HSV-1

antibodies are correlated with decreased severity, recurrence of corneal HSV-1 infection and establishment of latency.[33-37] Complete protection against infection and establishment of latency by an exogenous virus is though highly unlikely. Individuals susceptible for recurrent corneal HSV-1 infections will therefore never be fully protected against the risk of acquiring HSV-1 through a corneal graft.

ANALYSIS OF INTRA-CORNEAL T CELLS IN HUMAN HSK

Studies in the experimental mouse models of HSK have indisputably demonstrated the essential role of T cells in the development of HSK. This critical involvement of T cells has been apparent ever since the observation that HSK did not develop in T-cell-deficient, athymic (nude) mice.[38] Nevertheless, it has taken over a decade before more clarity emerged on which T cell subset is of primary importance in the pathogenesis of HSK. Studies by the groups of B.T. Rouse and R.L. Hendricks have been the main contributors to the agreement that $CD4^+$ Th1 cells are the major inducers of pathogenicity.[39-42] The cytokine IL-12, which is closely linked to induction of Th1 rather than Th2 cells, appeared to be upregulated very early in HSV-infected corneas.[43] The same investigators also showed that CTL function in HSK diseased corneas is mainly executed by $CD4^+$ T cells. In contrast, others found that Th2 cells are equally well capable of inducing HSK.[44, 45] They showed that $CD4^+$ Th2 clones from CAL-20 mice rendered HSK-resistant C.B-17 mice susceptible after transfer into these animals.

During the resolution phase of the disease, the intra-corneal T cell response is more Th2-like.[46] Treatment of HSK diseased corneas with the Th2 cytokine IL-10 minimises ocular inflammation, indicating the central role of this cytokine to initiate the resolution phase of HSK.[47] More recent reports also suggest that HSK is not initiated by a classic Th1 response, but rather caused by a mixed Th1 and Th2 infiltrate in recurrent HSK.[33, 48]
The antigen specificity of the T cells that initiate HSK in these experimental mouse models is still a matter of debate. Triggering T cells may be solely directed to viral antigens or may be crossreactive to both the HSV-1 UL-6 protein and a corneal auto-antigen. A more detailed outline on this issue is discussed in the final section of this chapter.

Due to the scarce availability of HSK diseased corneal tissue from humans and the imperfect methods to efficiently isolate and expand T cells from these biopsies, functional studies on cornea infiltrating T cells in human HSK are limited. More so, cornea transplantation in HSK patients is not carried out at fixed time points after onset of disease, making interpretation of the data precarious.
Verjans and co-workers observed that intra-corneal T cell lines (TCL) from the majority of HSK patients contained HSV-1 reactive T cells.[49, 50] These TCLs consisted of both $CD4^+$ and $CD8^+$ T cells. HSV-1 specific T cell clones (TCC), generated from intra-corneal TCL of 2 HSK patients, were all $CD4^+$ Th0 cells. Detailed analyses of the T cell receptor (TCR) α and β hypervariable regions of these TCC suggested that the intra-corneal T cell response was oligoclonal.[49] Similar experiments performed by Koelle *et al.* indicated that amongst a majority of HVS-1 -specific $CD4^+$ T cells, human HSK corneas are also infiltrated by $CD8^+$ T cells reactive to HSV-1.[51] TCC in these experiments turned out to be of the Th1 phenotype. Studies from both groups failed to provide evidence for potential cornea autoreactive intra-corneal T cells in human HSK.[50, 51]

The above mentioned studies however have several limitations that should be noted. First of all, the studies focusing on analyses of TCC contained limited numbers of patients.[49-51] Second, The limited number of TCC analysed may not be expected to provide an accurate insight into what kind of T cell responses dominate the inflammatory processes in human HSK. In the other study on TCL by Verjans *et al.*,[50] although a larger group of patients was studied, no discrimination could be made on what cell types were specific for HSV-1 antigens.

In chapter 6 we aimed to make a more elaborate analysis of the HSV-reactive T cells in TCLs generated from the cornea of human HSK patients. The methodology developed involves four-colour flowcytometric analyses of TCL incubated with autologous mock- and HSV-infected BLCL. HSV-reactivity was monitored by intracellular IFN-γ production, combined with phenotypic (CD3, CD4 and CD8) and clonal (TCR Vβ gene expression) analysis of T cells. This approach enabled simultaneous analysis of HSV-1 specificity, subtype and TCR usage of individual cells within the intra-corneal TCL. Analysis of virus-specificity and phenotype on a single-cell level has advantages over T cell cloning in that vast numbers of cells can be screened in short time.

Out of a total of 12 intra-corneal TCLs from HSK patients analysed, 10 showed reactivity against HSV antigens as determined by intra-cellular detection of IFN-γ production. Most of these TCLs showed reactivity against both serotypes of HSV, although the frequency of T cells specific for HSV-2 appeared to be lower than for HSV-1. This suggests that if the development of HSK in these patients was a viral antigen specific event, corneal infection by HSV-1 was most likely the antigenic trigger. Cross-reactivity with HSV-2 is in that case most likely the result of epitope similarity between both HSV serotypes. HSV reactivity in these TCLs was attribtued to T cells of both $CD4^+$ and $CD8^+$ subsets. Although few HSV-1 specific $CD8^+$ TCC had also been observed by Koelle *et al.*,[51] TCLs from some patients in our study appeared to contain solely $CD8^-$ HSV-specific T cells. These data oppose results from studies performed in experimental mouse models of HSK,[52-54] which have indicated that in murine HSK, cornea infiltrating T cells are predominantly of the $CD4^+$ subset.

Our data furthermore indicate a remarkable skewing of the usage of a limited number of TCR Vβ gene segments by the T cells in these TCLs. In some patients, the HSV-specificity in their TCLs appears to be dominated by a single subset of T cells expressing one particular TCR Vβ gene segment. Because we did not perform sequencing analysis of the TCR Vβ regions of each single cell, we cannot be certain however that usage of a single TCR Vβ gene segment reflects a pure monoclonal T cell response.
In general, the high frequency and clonality of HSV-specific T cells in some TCLs suggests that clonal expansion and T cell activation within the cornea might have been an antigen-specific phenomenon.

Some critical remarks about these findings should however be made. First of all, in order to obtain sufficient numbers of cells, the initial T cells obtained from the HSK diseased corneas had to be stimulated twice in a non-specific fashion. Such non-specific stimulation of TCL invokes the risk of selective growth of certain T cell subtypes and clones. Similarly, the more extended growth potential of $CD8^+$ over $CD4^+$ T cells following a non-specific stimulus,[55] might have caused the surprising high frequencies of $CD8^-$ cells in some of our TCLs. The relative high frequencies of HSV-specific T cells of both $CD4^+$ and $CD8^-$ subsets detected after expansion of the TCL nevertheless argues for an enrichment of HSV-specific

T cells in HSK diseased human corneas. As on the skewing of TCR Vβ usage in the TCLs examined, one explanation for this might be related to the age of the patients in our study and the observation that HSK generally develops upon recurrent corneal HSV infections over a prolonged period of time. Earlier studies have indicated that, as in mice, aged humans frequently exhibit expansion of one or several TCR Vβ families which is most prominent among $CD8^+$ cells.[56-58] On the other hand, clonal expansion of $CD4^+$ T cells in particular requires chronic antigenic stimulation, as is the case in elderly people infected with herpes viruses which are carried for longer periods of time.[59] A second limitation in this study is the fact that the available mAbs against the different TCR Vβ regions did not cover the whole spectrum of Vβ gene segments expressed by human T cells. This imperfection may well have accounted for the percentage of T cells from which the TCR Vβ usage could not be identified.

ROLE OF IL-17 IN THE IMMUNOPATHOGENESIS OF HSK

HSK is an immunopathologic disorder, triggered by HSV infection of the cornea that leads to the development of corneal inflammation and eventually corneal blindness. Replication of the virus within the cornea is essential since UV-inactivated and replication defective mutants fail to induce HSK in mice.[60, 61] Viral replication within the cornea triggers the influx of inflammatory cells into the corneal stroma, initiating an immunopathologic process in the cornea characteristic for HSK.
The development and maintenance of a chronic inflammatory response requires a distorted balance between leukocyte recruitment, retention and proliferation versus cell death and efflux. The pro-inflammatory microenvironment in the cornea, which develops after HSV-infection, induces the recruitment and retention of inflammatory cells in the stromal layer. These inflammatory cells, most notably T cells of the Th1 phenotype, induce the activation of resident fibroblasts and their secretion of multiple chemokines that enhance further recruitment and retention of neutrophils and T cells within the corneal stroma. Thus, the inflammatory process in HSK appears to persist as a direct result of sustained recruitment, retention and survival of mainly Th1 cells mediated, at least in part, by stromal fibroblast-derived factors.

Previous studies have indicated the crucial interaction of corneal resident cells with T cells and macrophages in the development of HSK in the mouse model.[40, 42, 54, 62, 63] Several studies have implicated that the interaction of inflammatory cells mainly involves fibroblasts within the corneal stromal layer. Large numbers of IFN-γ and TNF-α producing cells have been observed in the stromal layer of HSV-infected murine corneas but not in the epithelial or endothelial layers.[48, 64] This corresponds to the location of neutrophils that predominantly infiltrate the stromal fibroblast cell layer of the cornea.[65] Similarly, the induction of IL-8, which exhibits neutrophil chemotactic properties, has been shown to be associated with HSV replication in human corneal fibroblasts but not in epithelial cells.[66] Considering this, corneal fibroblasts are a potential source of the chemokines responsible for the recruitment of inflammatory cells, i.e. T cells, macrophages, Langerhans cells and neutrophils, within the cornea during HSK.

We have tried to extend these views to the human situation by analysing the cytokine and chemokine secretion profiles of cultured human corneal fibroblasts stimulated

with Th1- and monocyte/macrophage-specific cytokines (Chapter 5). Our results indicate that the secretion of pro-inflammatory cytokines and chemokines by human corneal fibroblasts reflects the elaborate network of factors found in HSK mouse models. Furthermore, we found that IL-17, which is produced by activated T cells, has a strong modulatory effect on the secretion of most of these factors. These findings are in agreement with other observations indicating a role for IL-17 in other immunopathologic diseases characterised by a major influx of neutrophils.[67-71]
The IL-17 receptor (IL-17R) is expressed in a wide variety of tissues,[72] whereas IL-17 itself is strictly confined to activated T cells, mainly of the Th1 or Th0 phenotype.[73-75] Considering this distribution of the IL-17R and IL-17 secretion, this cytokine probably plays a distinct role in local inflammatory processes. In this respect, IL-17 probably does not play a direct potent pro-inflammatory role but might rather act as a "fine-tuning" cytokine in inflammatory microenvironments.
We speculate that during the development of HSK, in addition to cornea infiltrating cells, corneal fibroblasts are an important cellular source of pro-inflammatory cytokines and chemokines. Furthermore, our results suggest that IL-17 might be an important player in the immune activation of human corneal fibroblasts. Future studies in murine models of HSK might elucidate a potential role of IL-17 in the inflammatory processes leading to HSK.

It is clear that HSK represents a very complex immunopathological process. The genotype of the host and the virus can quantitatively and qualitatively influence the inflammatory processes that occur in the HSV-1 infected cornea. Factors within the microenvironment of the infected cornea are crucial to the development and outcome of disease. Factors such as cytokine and chemokine production, expression of adhesion molecules and antigen presentation all appear to contribute significantly to HSK susceptibility. Approaches designed to modify cytokine and chemokine expression and APC function within the microenvironment of the HSV-1 infected cornea may have therapeutic value in treating HSK. Individuals with HSK can benefit from topical treatment with corticosteroids,[76] as is currently standard clinical practice. The use of corticosteroids to treat HSK however has the disadvantage of thinning of the epithelium layer and an increased risk for opportunistic infections.

GENERAL CONSIDERATIONS ON HSK IN MICE AND HUMANS

HSV is a virus typically adjusted to the human host. The evolutionary co-existence of HSV and its human host has resulted in adaptation of both the human immune system to challenge HSV infections and the virus to persist in its natural host. As a consequence, the infectious process of HSV and the fine-tuning of the host's immune response might be quite distinct from the situation in experimental mouse models.
In most cases, the pathogenesis of experimental corneal HSV-1 infection in the mouse is not the same as that seen in humans (reviewed in ref. 77). HSV-1 infection is usually lethal in the mouse, and secondary or recurrent infections in surviving mice cannot always be induced consistently. Qualitative and quantitative responses might also be quite different between mice and humans. ADCC for example has a far more potent effect on antibodies and cells in humans. Furthermore, it is not unrealistic to suppose that relevant antigenic targets in mice are quite distinct from the targets of human CD4 and CD8 T cells. The basic genetic

difference between humans and mice might well account for such differences in epitope recognition.
Finally, these animal studies were performed using inbred mouse strains with distinct genotypes, which does not resemble the genetically heterogeneous human population.

Why certain strains of mice develop HSK after corneal HSV infection while others appear resistant remains yet to be defined. Thomas *et al.* did however indicate that resistant strains of mice do possess potentially pathogenic $CD4^+$ T cells.[61] Transfer of $CD4^+$ cells from resistant C.B-17 mice can induce HSK in SCID mice that are themselves incapable of inducing inflammatory responses in the cornea.
Another puzzling observation in experimental murine HSK which needs clarification is that the severity of HSK and the type of T cells involved, also vary between mouse strains and the HSV-1 strains used.[39-42, 44, 45] The initiation and severity of HSK and the type of T cells involved also vary between mouse strains and with the HSV-1 strains used. While Rouse and colleagues used BALB/C mice, infected with HSV-1 KOS and RE strains, others found that Th2 cells are equally well capable of inducing HSK.[44, 45] They showed that CD4+ Th2 clones from CAL-20 mice rendered HSK-resistant C.B-17 mice susceptible after transfer into these animals.

Although little is known about the characteristics of HSK development in humans, they seem to resemble those found in the mouse model. Several differences however have been observed and there are some important uncertainties to keep in mind. For example, in the HSK mouse model, replicating virus and/or viral antigens are absent in the cornea after about five to seven days post infection at the time of onset of lesion progression.[42, 78] In human HSK diseased corneas, viral antigens in contrast have been demonstrated weeks after onset of disease.[50, 79]
Second, there are differences in the mouse models used to study corneal HSV-1 infection. Most research groups, like those from Rouse *et al.*,[39-41,43] Hendricks *et al.*,[42] and Heiligenhaus *et al.*[44-46] have used a model of primary corneal HSV-1 infection, while others have focused on recurrent infection.[33, 48, 80] HSK in humans generally develops upon reactivation of latent virus, and the characteristics as observed in the mouse model on recurrent infection appear to more closely resemble the human situation.[81]

Despite the fact that there is general agreement about the triggering agent that causes HSK, there is still controversy about the immunopathologic processes involved in HSK development. Though most investigators in the field agree that HSK is triggered by HSV infection, there are partially conflicting theories concerning the antigen specificity of the T cells initiating and perpetuating the inflammatory processes within the cornea.
The most general theory implies the basic immunological principle that the inflammatory process is initiated and enduringly mediated by viral antigen-specific T cells. Arguing against lasting inflammation in this theory is that virus replication and consequently production of viral antigen is no longer detectable in the murine cornea around the time T cells infiltrate the cornea. On the other hand, low amounts of viral antigen might still be present in the stroma, sufficient to activate infiltrating virus-specific T cells but undetectable by immunohistology.

Another theory, put forward by the group of Cantor and co-workers, proposes that HSK is rather an autoimmune disease. They found that HSK-inducing $CD4^+$ T cell clones in mice were reactive against both a corneal tissue autoantigen and an epitope within the HSV-

1 UL6 peptide.[82] The authors proposed that the inflammatory process is induced by the release of a sequestered keratogenic peptide in the cornea sharing molecular mimicry with an epitope of the HSV-1 UL6 protein. They also stated that resistance for HSK in certain mouse strains is associated with allotypic variation in immunoglobulin genes. In resistant mice bearing the $IgG2a^b$ allotype, T cells specific for corneal tissue autoantigens were probably cross-tolerised by $IgG2a^b$ –derived peptides.[83] Susceptibility for or resistance to HSK is thus based on what the authors define as "three party molecular mimicry" between an HSV-1 UL6 peptide, a corneal autoantigen and a peptide in a particular IgG2a allotype. Although this tantalising theory as an HSK-inducing mechanism is supported by solid evidence, a potential similar mechanism for the induction of HSK in humans remains to be explored. So far, no reactivity against the HSV-1UL6 protein could be detected in intra-corneal T cells from human HSK patients.[49, 50]
A third theory, as proposed by the group of Rouse and co-workers, emphasises an inflammatory process in HSK in the absence of antigen recognition by virus-specific T cells, referred to as "bystander activation" of non-specific T cells.[84-87] They used transgenic mice back-crossed with SCID mice, bearing a TCR specific for a single ovalbumin peptide. Although these mice failed to recognise HSV-1 antigens, they still showed a major influx of T cells in the cornea and development of HSK lesions upon corneal HSV-1 infection. The investigators propose a mechanism of inflammation by cytokine/chemokine –induced bystander activation of $CD4^+$ Th1 cells in a virus-induced inflammatory environment.

As supporters of each of the above theories continue to dispute each other's ideas, conclusive evidence on which of the above theories most accurately approximates the *in vivo* situation remains to be elucidated. Recently, the latter research groups seem to come to some agreement that though molecular mimicry might play a role in the induction of disease in mice, the inflammatory process may well be proceeded and perpetuated via a bystander mechanism.[88, 89]

As for the human situation, there is no clear indication what processes lead to T cell activation. Although HSV-1 specific T cells in human HSK have been described (refs. 49-51 / this thesis), the role of cross-reactivity with a potential corneal autoantigen and T cell activation through a bystander effect can not be ruled out. Notably, not all intra-corneal TCL from HSK patients tested showed HSV-specific T cell reactivity (ref. 50 / this thesis). Irrespective of the antigenic stimulus for activation of cornea infiltrating T cells in the onset to human HSK, the perpetuation of the intra-corneal inflammatory process is probably not so much the result of ongoing antigen presentation but rather of the lack of inhibitory signals in a pro-inflammatory microenvironment.

In humans, HSK is frequently associated with bacterial superinfection. This is most likely due to breakdown of the corneal epithelial integrity and disturbed lachrymal immunity. As a matter of fact, bacterial infiltrates have been observed during pathological examination in some of the corneas of HSK patients we studied in chapter 5. Various bacteria are known to produce and secrete superantigens. Superantigens are proteins with a high activating potential on T cells in an antigen-independent fashion. Unlike conventional antigens, superantigens bind to MHC class II molecules and particular TCR Vβ gene segments, resulting in proliferation and activation of T cells expressing these TCR Vβ gene segments.[90] As a consequence, superantigens stimulate a substantial repertoire of the host's T-cell repertoire. This results in a massive release of pro-inflammatory cytokines which play a pivotal role in the pathogenesis of diseases provoked by superantigen-producing

microorganisms. The frequent association of human HSK with corneal bacterial superinfection might thus suggest a potential role of superantigen in the activation of T cells in the HSV-infected cornea. Bacterial superinfections of the cornea might thus, rather than trigger, perpetuate or increase the severity of the inflammatory process and deteriorate the clinical outcome of HSK.

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Chapter 8

Summary

Herpes simplex virus (HSV) infections of the human eye can cause a variety of disease manifestations. Generally, the infecting pathogen in ocular HSV infections is of the first subtype (HSV-1). A common site of HSV infection of the eye is the cornea. HSV infections of the superficial epithelial layer of the cornea are usually transient and without permanent ocular damage. However, when the infection involves the stromal layer of the cornea, a chronic inflammatory disease called herpetic stromal keratitis (HSK), can develop. This type of ocular disease is a leading cause of non-traumatic blindness in developed countries.

Although HSK is a relatively rare disease, patients with recurrent and severe episodes of epithelial HSV infections have an increased risk of stromal involvement. Recurrent corneal HSV infections can be caused either by reactivation of a latent virus, or by a re-infection with a new viral strain. Such infections are referred to as endogenous or exogenous viral infections, respectively. In the light of medical care for patients with corneal HSV infections, it can be of importance for an ophthalmologist or physician to know which type of infection (either endogenous or exogenous) is involved in recurrent corneal disease. To date, recurrent corneal HSV-1 infections are commonly considered to be caused by an endogenous, latent viral strain.

In **chapter 2**, we describe the development of a PCR-based molecular technique, which allows the distinction between both types of HSV-1 infection. This technique provides a genetic "fingerprint" of each HSV-1 strain and enables to track whether recurrent HSV-1 infections are caused by the same or a different virus. Evaluation of this technique showed that it provides a discrimination rate of 92% of unrelated clinical HSV-1 isolates. This technique provides a quick and convenient method for determining the origin of HSV-1 infections. Combined with amino acid sequence analysis of the targeted genomic region of HVS-1, an even much more accurate and higher rate of discrimination can be achieved. Moreover, this PCR-based technique obsoletes the need for isolation of the infectious agent, as is required for conventional methods.

The method was successfully employed in a study, described in **chapter 3**. This study involved two patients with another type of ocular HSV disease, known as acute retinal necrosis (ARN), and a previous episode of herpes simplex encephalitis (HSE). Our PCR-based "fingerprinting" technique enabled to provide a direct relation between the HSV-1 infections in the eye and the brain. The data suggested transmission of the virus from the brain to the eye, a route of HSV-1 translocation within a host, which has been clearly demonstrated in an experimental mouse model. Our data, together with multiple similar clinical cases described in literature should warn neurologists and ophthalmologists that individuals with HSE have an increased risk of developing ARN.

In **chapter 4**, we investigated the frequency of endogenous and exogenous infections in patients with recurrent corneal HSV-1 infections. Besides showing a strikingly high frequency of exogenous re-infections in recurrent corneal HSV-1 infections, our data implicated that corneal transplantation increases the risk of acquiring an exogenous HSV-1 strain. This finding suggested the possibility of donor-to-host transmission of HSV-1 through corneal grafting. Our suspicion was validated, as shown in the second report described in

chapter 4. This report involves a case study of an individual who suffered from cornea epithelial defects, caused by an HSV-1 infection, shortly after receiving a corneal transplantation. This corneal HSV-1 infection subsequently led to the development of HSK, leaving this patient permanently blind. Employment of our PCR-based technique as described in chapter 1 and subsequent DNA sequencing revealed that the donor graft contained the same HVS-1 strain as the one causing cornea epithelial defects in the recipient shortly after transplantation.

The clinical presentation of HSK is considered to result from an immunopathologic process in the cornea involving both innate and adaptive immune responses to the replicating virus. Solid support for this viewpoint comes from studies in experimental mouse models. In immunocompetent mice the disease is associated with a bi-phasic cellular infiltrate in the corneal stroma after corneal HSV infection. While the first - smaller and transient - infiltrate is not associated with any clinical symptoms, a massive cellular infiltrate of mainly neutrophils invades the corneal stroma several days later, initiating the clinical phase of HSK. These neutrophils are considered to be directly involved in the disruption of the stromal tissue integrity. Such tissue damage can lead to corneal scarring and corneal blindness.

An essential factor for the development of HSK is the involvement of cornea infiltrating $CD4^+$ T cells with a Th1 phenotype. These cells orchestrate the extravasation and activation of infiltrating neutrophils during the clinical phase of the disease.
The antigen specificity of these infiltrating T cells, which drive the immunopathological process in the cornea, however remains a mystery. There are several (partially) conflicting theories on the antigen specificity of these T cells involved in HSK as seen in experimental mouse models. And although multiple reports have addressed this issue, the controversy still remains. In contrast, only very few data are available on the antigen-specificity of cornea infiltrating T cells in HSK in humans. In **chapter 5** we describe a more detailed study on the specificity, subtype and clonality of T cells invading the cornea in patients with HSK. We found that cornea-derived T cell lines (TCL) from the majority of HSK patients contained high numbers of HSV-specific T cells. HSV-reactivity was HSV type common, and the majority of the TCL showed a restricted usage of T cell receptor Vβ domains (TCR Vβ). Our data demonstrate that both $CD4^+$ and $CD8^+$ T cells are potentially involved in the intra-corneal HSV-specific T cell response in corneas of HSK patients, and suggest restricted TCR Vβ usage of cornea residing HSV-specific T cells.

Key events in the development of HSK involve the interaction between cornea infiltrating inflammatory cells and resident corneal tissue cells. This interaction, in which macrophages and Th1 cells play a crucial role, results in the local secretion of immune-modulatory factors. Secretion of these factors results in the recruitment and retention of additional inflammatory cells in the corneal stroma and perpetuation of the immunopathological processes.

In **chapter 6** we show that macrophage and/or Th1 cell –derived cytokines induce the secretion of these pro-inflammatory cytokines and chemokines by resident corneal fibroblasts. We found that the induction and secretion of some of these immune-modulatory factors was strongly modulated by another Th1 cell-derived cytokine, IL-17. We also found IL-17 mRNA expression in corneas of HSK patients, while no IL-17 mRNA was observed in control corneas. The IL-17 receptor was constitutively expressed by corneal fibroblasts. The

data presented indicate that IL-17 has strong modulatory effects on the production of pro-inflammatory cytokines and chemokines by resident corneal fibroblasts and that IL-8 is the main chemotactic factor for neutrophils secreted by these cells.
Our results suggest that IL-17 may play an important role in the induction of the immunopathologic processes in human HSK by modulating the secretion of pro-inflammatory and neutrophil chemotactic factors by resident cornea fibroblasts.

Samenvatting

Een infectie van het menselijk oog door een Herpes Simplex Virus (HSV) kan een verscheidenheid aan ziektebeelden veroorzaken. In de meeste gevallen is subtype 1 van HSV (HSV-1) de veroorzaker van herpetische oog-infecties en regelmatig betreft het hierbij een infectie van het hoornvlies. Een HSV infectie van de oppervlakkige epitheel cel laag van het hoornvlies is meestal van korte duur en blijft zonder permanente schade aan het oog. Wanneer echter de dieper gelegen stromale cel laag van het hoornvlies geïnfecteerd raakt, kan zich een chronische ontstekingsreactie van het hoornvlies voor doen. Dit ziektebeeld wordt omschreven als herpetische stromale keratitis (HSK) en is een van de meest voorkomende oorzaken van non-traumatische blindheid in de Westerse wereld.

Hoewel HSK een relatief zeldzame ziekte is, lopen met name patienten met veelvuldig terugkerende en ernstige vormen van epitheliale HSV infecties een verhoogd risico een stromale infectie te ontwikkelen. Terugkerende HSV infecties van het hoornvlies kunnen veroorzaakt worden door reactivatie van een latent virus, of door een infectie met een nieuwe virus stam. Zulke infecties worden respectievelijk aangeduid als endogeen en exogeen. In het kader van de medische behandeling van patienten met een terugkerende HSV infectie van het hoornvlies, kan het voor een oogarts van belang zijn te weten met welk type infectie men van doen heeft. In het algemeen wordt bij terugkerende HSV infecties van het hoornvlies aangenomen dat het een endogeen, gereactiveerd virus betreft.

Hoofdstuk 2 beschrijft de ontwikkeling van een moleculaire techniek, gebaseerd op PCR amplificatie van delen van het HSV-1 genoom, waarmee een onderscheid kan worden gemaakt tussen beide vormen van infectie. Deze techniek levert een zgn. "fingerprint" op van individuele virus stammen en kan uitwijzen of terugkerende HSV-1 infecties veroorzaakt worden door hetzelfde virus of door een andere virus stam. Evaluatie toonde aan dat met deze nieuwe techniek een onderscheidend vermogen van 92% voor niet gerelateerde HSV-1 isolaten kan worden behaald. Daarmee levert deze techniek een snelle en eenvoudige methode om de oorsprong van HSV-1 infecties te achterhalen. Om een nog nauwkeuriger beeld te verkrijgen, kan in combinatie met DNA sequentie analyse een nog groter onderscheidend vermogen worden bereikt. Bovenal vervalt bij deze techniek de noodzaak om het virus in kwestie te isoleren, hetgeen voor conventionele technieken wel vereist is.

Deze methode werd met succes toegepast in de studie beschreven in **hoofdstuk 3**. Deze studie omhelsde de analyse van twee patienten met een ander type HSV-geïnduceerde oog ziekte, aangeduid als acute retina necrose (ARN). Beide individuen hadden een voorgeschiedenis van herpes simplex encefalitis (HSE), een HSV infectie van de hersenen. Toepassing van onze nieuwe techniek leverde een directe relatie op tussen de HSV infecties in het oog en de hersenen: de resultaten wijzen op transmissie van het virus tussen de hersenen en het oog. Een vergelijkbare transmissieroute is duidelijk aangetoond in muizen waarbij het virus vanuit de hersenen via de oogzenuw het netvlies kan bereiken. In de literatuur zijn bovendien een aanzienlijk aantal gevallen beschreven van individuen met ARN, vooraf gegaan door een episode van HSE. Met dit in het achterhoofd zouden onze resultaten een waarschuwing moeten afgeven naar neurologen en oog artsen dat patienten met HSE een verhoogd risico lopen voor het ontwikkelen van ARN.

Hoofdstuk 4 handelt over een onderzoek naar de frequentie van endogene en exogene HSV-1 infecties in patienten met terugkerende infecties van het hoornvlies. Onze data laten een verrassend hoog aantal exogene her-infecties zien bij deze terugkerende HSV-1 infecties van het hoornvlies. Bovendien suggereren deze resultaten dat de kans om een infectie met een nieuwe (exogene) HSV-1 stam op te lopen wordt vergroot wanneer de patient een hoornvlies transplantatie ondergaat. Deze bevinding suggereerde dat er in sommige gevallen mogelijk sprake was van transmissie van HSV-1 van de donor naar de patient via het transplantaat. Deze hypothese werd bevestigd in een ander onderzoek welk eveneens in hoofdstuk 4 beschreven staat. Dit onderzoek betreft een individu dat kort na een hoornvlies transplantatie last kreeg van ernstige defecten aan het hoornvlies epitheel, veroorzaakt door een HSV-1 infectie. Deze infectie leidde vervolgens tot de ontwikkeling van HSK, waardoor deze persoon permanent blind werd. Toepassing van de PCR-techniek, als beschreven in hoofdstuk 1, toonde aan dat de virus stam die HSK veroorzaakte in de patiënt die de hoornvlies transplantatie onderging dezelfde was als de stam die aanwezig was in het donor-materiaal.

Het klinische beeld van HSK wordt veroorzaakt door een immuunpathologisch proces in het hoornvlies, waarbij zowel primaire als adaptieve immuun reacties tegen het virus een rol spelen. Het overgrote deel van de kennis omtrent deze immuunpathologische reacties is afkomstig uit studies gebruikmakend van experimentele muis modellen. In immuun competente muizen wordt de ziekte gekarakteriseerd door een twee-ledige infiltratie van immuun cellen in het hoornvlies na infectie door HSV. Tijdens de eerste fase, direct na HSV infectie van het hoornvlies, is een milde en tijdelijke infiltratie van cellen te zien, hetgeen geen klinisch ziektebeeld veroorzaakt. Enkele dagen later echter, tijdens de tweede fase, treedt er een veel massalere infiltratie van immuun cellen in het hoornvlies op. Dit infiltraat bestaat voornamelijk uit neutrofielen en luidt de klinische fase van HSK in. Men gaat er van uit dat deze neutrofielen direkt betrokken zijn bij de verstoring van de integriteit van het stromale weefsel van het hoornvlies. Dergelijke schade aan het weefsel kan leiden tot de vorming van littekenweefsel, met als gevolg een vertroebeling van het hoornvlies en blindheid.

Essentieel voor de ontwikkeling van het ziektebeeld van HSK is de infiltratie van het hoornvlies door $CD4^+$ T cellen met een Th1 fenotype. Deze cellen zorgen voor de infiltratie en activatie van neutrofielen tijdens de klinische fase van de ziekte. Alhoewel er algemene consensus is over de rol van deze cellen in de ontwikkeling van het immuunpathologische proces in het hoornvlies, blijft de antigeen specificiteit van deze T cellen onderwerp van discussie. Zo zijn er verschillende theorieen over de aard en oorsprong van de antigenen waartegen de T cellen in de HSK muizen modellen gericht zijn. En ondanks het grote aantal studies waarin getracht is een eenduidig antwoord op dit vraagstuk te vinden blijft de controverse bestaan. In tegenstelling tot het grote aantal studies in muizen modellen van HSK zijn er zeer weinig data voorhanden over de antigeen specificiteit van T cellen bij de ontwikkeling van HSK in mensen. **Hoofdstuk 5** beschrijft een meer gedetailleerde studie aangaande de antigeen specificiteit, klonaliteit en het subtype van T cellen die werden geisoleerd uit humaan hoornvlies met HSK. Onze resultaten laten zien dat T cel lijnen (TCL) gegenereerd uit deze humane hoornvliezen een relatief hoog percentage HSV-specifieke T cellen bevatten. Deze TCL bevatten zowel $CD4^+$ als $CD8^+$ HSV-specifieke T cellen. De meerderheid van deze HSV-specifieke T cellen vertoonde bovendien een beperkte expressie in het repertoire van T cel receptor Vβ (TCR Vβ) genen. Deze studie

geeft aan dat zowel $CD4^+$ als $CD8^+$ T cellen een mogelijke rol spelen in de HSV-specifieke T cel respons in het hoornvlies van HSK patiënten. Verder lijken deze responsen een min of meer klonaal karakter te hebben.

In de ontwikkeling van het ziektebeeld van HSK speelt de interactie tussen infiltrerende cellen van het immuun systeem en weefsel van het hoornvlies zelf een cruciale rol. Deze interactie, waarbij Th1 cellen en macrofagen een centrale rol vervullen, resulteert in lokale secretie van immuun modulerende factoren. Deze factoren zijn op hun beurt verantwoordelijk voor de verdere infiltratie van immuun cellen in het stroma van het hoornvlies en daarmee voor de voortzetting en versterking van het immuunpathologische proces.

De studie beschreven in **hoofdstuk 6** toont aan dat bepaalde factoren, welke door macrofagen en Th1 cellen uitgescheiden worden, de secretie van zulke cytokinen en chemokinen induceren. Onze resultaten tonen aan dat de inductie en secretie van enkele van deze immuun-modulerende factoren sterk beinvloed worden door een ander cytokine, IL-17, dat uitsluitend wordt geproduceerd door geactiveerde T cellen. Expressie van IL-17 mRNA werd aangetoond in hoornvliezen van HSK patienten, terwijl controle hoornvliezen geen expressie van dit mRNA lieten zien. De receptor voor IL-17 werd bovendien constitutief geexpresseerd door fibroblasten afkomstig uit het stroma van verschillende humane hoornvliezen. De data beschreven in hoofdstuk 6 geven aan dat IL-17 een sterk modulerend effect heeft op de lokale productie van cytokinen en chemokinen door fibroblasten in het hoornvlies. Van deze factoren blijkt verder met name IL-8 een voorname rol te spelen in de rekrutering van neutrofielen.
Onze bevindingen omtrent de modulatie van de secretie van ontsteking inducerende en neutrofiel rekruterende factoren door fibroblasten in het hoornvlies geven aan dat IL-17 mogelijk een belangrijke rol speelt in de inductie en continuering van de immuunpathologische processen bij HSK in de mens.

Dankwoord

Zo, nu alles op papier staat rest me nog dit dankwoord. Hoewel mijn naam voor op dit boekje staat, hebben vele anderen eveneens een aanzienlijke en soms onmisbare bijdrage geleverd aan de totstandkoming van dit proefschrift. Bij deze wil ik graag de volgende direct en indirect betrokkenen bedanken voor hun bijdrage en steun.

Beste Ab, zo zie je maar, zelfs zo'n rare Limburger kan de eindstreep halen. Veel dank voor alle mogelijkheden en de vrijheid die je mij geboden hebt dit promotie-onderzoek uit te kunnen voeren.

Zonder enige twijfel gaat de meeste dank uit naar Georges voor zijn onmisbare bijdrage aan mijn promotie-onderzoek. Aangezien ik jouw eerste aio was, was dit ook voor jou iets nieuws. Alle lof voor je inzet en enthousiasme als begeleider, Georges! En even zozeer dank voor het plezier en goede gezelschap op onze uitstapjes naar de diverse congressen. Sorry dat ik je met mijn slechte karma voor vliegreizen de nodige stress-volle momenten heb bezorgd.

Een eervolle vermelding gaat tevens uit naar Lies, met wie ik een aantal jaar bijzonder prettig heb mogen samenwerken en diverse artiekelen samen heb gepubliceerd. Lies, dank voor je hulp met alle hoornvliesjes, discussies en klinische inzichten. Dit proefschrift was niet mogelijk geweest zonder jouw medische expertise en je enthousiaste inbreng bij het onderzoek.

Ook veel dank aan al mijn collega's van de afdeling virologie voor alle hulp, discussies, de gezellige borrels en bovenal een bijzonder aangename werksfeer. Daarbij wil ik wel nog even de mensen van ons lunch-groepje extra bedanken voor alle gezelligheid tijdens het nuttigen van mijn saucijzebroodje.

Al mijn vrienden en vriendinnen voor ruim voldoende plezier in en buiten de Zaak, op feestjes en festivals, overheerlijke barbecues en ontspannende vakanties in de bergen. En ondanks alle geroddel en geouwehoer draaide het regelmatig toch ook uit op nachtelijke discussies over T cellen, virussen of wetenschap in het algemeen. Geloof het of niet, maar ook dat alles heeft een wezenlijke bijdrage geleverd aan dit proefschrift.

En waar zou ik zijn zonder mijn Broer en grote Zus! Die bijzondere en hechte band onder ons drieën zal altijd blijven.

Maar de meeste dank zal toch altijd blijven uitgaan naar mijn lieve ouders, die mij altijd volop gesteund hebben en zonder wie ik nooit zover was gekomen.
Ans en Wiel, deze is voor jullie!

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 3 april 1971 te Utrecht. Het diploma Gymnasium B werd behaald in 1990 aan het Stedelijk Lyceum te Maastricht. In datzelfde jaar begon hij aan de Universiteit Utrecht met de studie Biologie. De specialisatiefase omvatte twee stages: Bij de vakgroep Moleculaire Microbiologie, Universiteit Utrecht (Prof. dr. W.P.M. Hoekstra), en bij het Immunologisch laboratorium van de afdeling Kindergeneeskunde, Leids Universitair Medisch Centrum (onder leiding van Dr. M.J.D. van Tol). Tevens werd een additionele stage uitgevoerd bij de afdeling Immunologie van het Hospital Clinic te Barcelona, Spanje (onder leiding van Dr. F. Lozano). Het doctoraal examen werd behaald in augustus 1996. Vervolgens heeft hij gedurende een periode van een half jaar werkervaring opgedaan bij de afdeling Immunotoxicologie, Universiteit Utrecht (onder leiding van Dr. R. Pieters). In januari 1998 is hij als assistent in opleiding (AIO) in dienst getreden bij de afdeling Virologie van de Erasmus Universiteit Rotterdam onder leiding van Prof.Dr. A.D.M.E. Osterhaus en Dr. G.M.G.M. Verjans. In deze functie werd het onderzoekt verricht waarover dit proefschrift rapporteert. Nadien heeft hij gedurende de periode van een half jaar (juni-november 2002) onderzoek verricht aan de afdeling Medische Virologie van de Universiteit van Washington te Seattle, Verenigde Staten (onder leiding van D.M. Koelle).

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