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INNATE AND ADAPTIVE IMMUNITY IN OROLABIAL HERPES SIMPLEX VIRUS INFECTION

by

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To my Family

ABSTRACT

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INNATE AND ADAPTIVE IMMUNITY IN OROLABIAL HERPES SIMPLEX VIRUS INFECTION

Institute of Dentistry, Department of Virology, Postgraduate School of Oral Health Sciences (PeGaSOS), University of Turku, and Department of Virology, Haartman Institute, University of Helsinki, Finland. Annales Universitatis Turkuensis, Medica-Odontologica, Yliopistopaino, Helsinki, 2006.

Oral mucosa is a frequent site of primary herpes simplex virus type 1 (HSV-1) infection, whereas intraoral recurrent disease is very rare. Instead, reactivation from latency predominantly results in asymptomatic HSV shedding to saliva or recurrent labial herpes (RLH) with highly individual frequency. The current study aimed to elucidate the role of human oral innate and acquired immune mechanisms in modulation of HSV infection in orolabial region.

Saliva was found to neutralize HSV-1, and to protect cells from infection independently of salivary antibodies. Neutralization capacity was higher in saliva from asymptomatic HSV-seropositive individuals compared to subjects with history of RLH or seronegative controls. Neutralization was at least partially associated with salivary lactoferrin content. Further, lactoferrin and peroxidase-generated hypothiocyanite were found to either neutralize HSV-1 or interfere with HSV-1 replication, whereas lysozyme displayed no anti-HSV-1 activity. Lactoferrin was also shown to modulate HSV-1 infection by inhibiting keratinocyte proliferation. RLH susceptibility was further found to be associated with Th2 biased cytokine responses against HSV, and a higher level of anti-HSV-IgG with Th2 polarization, indicating lack of efficiency of humoral response in the control of HSV disease. In a three-dimensional cell culture, keratinocytes were found to support both lytic and nonproductive infection, suggesting HSV persistence in epithelial cells, and further emphasizing the importance of peripheral immune control of HSV.

These results suggest that certain innate salivary antimicrobial compounds and Th1 type cellular responses are critically important in protecting the host against HSV disease, implying possible applications in drug, vaccine and gene therapy design.

Key words: herpes simplex virus, saliva, innate immunity, cytokine, HSV persistence, three-dimensional cell culture

TIIVISTELMÄ

Hannamari Välimaa

SYNNYNNÄINEN JA HANKITTU IMMUNITEETTI SUUN ALUEEN HERPES SIMPLEX VIRUS –INFEKTIOSSA

Hammaslääketieteen laitos, Virusoppi, Suun terveystieteiden tutkijakoulu (PeGaSOS), Turun yliopisto ja Virologian osasto, Haartman-instituutti, Helsingin yliopisto. Annales Universitatis Turkuensis, Medica-Odontologica, Yliopistopaino, Helsinki, 2006.

Herpes simplex tyyppi 1 virus (HSV-1) aiheuttaa tavallisimmin suun alueen infektioita. Ensi-infektio saadaan yleensä suun limakalvon kautta, mutta reaktivaatiota seuraava infektio ilmenee vain harvoin suun sisällä. Tavallisimmin reaktivaatiosta on seurauksena joko viruksen oireeton eritys sylkeen tai huuliherpes, joiden ilmenemisessä on suuria yksilöllisiä vaihteluita. Tässä työssä on tutkittu synnynnäisen ja hankitun immuniteetin vaikutusta HSV:n suun alueen ilmentymiin.

Syljen havaittiin sekä neutraloivan HSV:ta, että suojaavan soluja infektiolta. Nämä tapahtumat olivat riippumattomia syljen vasta-aineista. Syljen HSV:ta neutraloiva vaikutus oli suurempi oireettomilla HSV-seropositiivisilla henkilöillä kuin henkilöillä, joilla ajoittain ilmenee huuliherpestä sekä seronegatiivisilla kontrolleilla. Neutralisaation tehokkuus oli ainakin osittain riippuvainen syljen laktoferriinipitoisuudesta. Syljen laktoferriini ja peroksidaasisysteemin tuottama hypotiosyanaatti lisäksi joko neutraloivat HSV:ta tai estivät sen lisääntymistä kohdesoluissa. Lysotsyymillä ei ollut vaikutusta infektion kulkuun. Laktoferriini vaikutti HSV-infektioon lisäksi hidastamalla keratinosyyttien jakautumista. Huuliherpestaipumukseen liittyi taipumus tuottaa Th2-tyypin sytokiinejä sekä korkea määrä Th2-tyypin HSV-IgG-vasta-aineita. Kerrostuneessa epiteelimallissa tehdyssä tutkimuksessa HSV aiheutti sekä lyyttisen että nonproduktiivisen infektion. Tämä viittaa HSV:n mahdolliseen kykyyn aiheuttaa persistoiva infektio myös keratinosyyteissä, jolloin perifeerinen HSV:n immuunikontrolli olisi vielä luultuakin tärkeämpi osa HSV-reaktivaation hallintaa.

Yhteenvetona voidaan todeta, että syljen synnynnäiset puolustustekijät sekä Th1-tyypin immuunivaste suojelevat oireiselta perifeeriseltä HSV-infektiolta. Näitä tuloksia voidaan hyödyntää HSV:n lääkehoito-, rokote- ja geeniterapiasovelluksissa.

Avainsanat: herpes simplex virus, synnynnäinen immuniteetti, sytokiini, HSV persistenssi, kolmiulotteinen soluviljelymalli

TABLE OF CONTENTS

ABBREVIATIONS

APC	antigen-presenting cell
αTIF	alpha trans-inducing factor (VP16)
AU	arbitrary unit
bLF	bovine lactoferrin
bLPO	bovine lactoperoxidase
BrdU	bromodeoxyuridine
BM	basement membrane
BSA	bovine serum albumin
cdc	cell division control
CDK	cyclin-dependent kinase
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DIG	digoxigenin
DNA	deoxyribonucleic acid
DR	direct repeat
ds	double-stranded
ECM	extracellular matrix
eIF	eukaryotic initiation factor
EIU	enzyme immunoassay unit
EV11	echovirus type 11
FcR	Fc-receptor
GAG	glycosaminoglycan
GALT	gut-associated lymphoid tissue
gB-M	glycoprotein B-M
GCF	gingival crevicular fluid
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hLf	human lactoferrin
hMPO	human myeloperoxidase
hpi	hours post infection
HRP	horseradish peroxidase
HS	heparan sulphate
hSPO	human salivary peroxidase
HSV	herpes simplex virus
HVEM	herpesvirus entry mediator
$HOSCN/OSCN^-$	hypothiocyanite
ICP	infected cell protein
IC_{50}	concentration of a substance causing 50% inhibition of a phenomen
IFN	interferon
IL	interleukin
ISH	*In situ* hybridization
IRF	interferon regulatory factor
kbp	kilobasepair = 1000 bp
LAT	latency-associated transcript

Lf	lactoferrin
LN	lymph node
Lz	lysozyme
MDA5	melanoma differentiation associated gene 5
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
mRNA	messenger RNA
MyD88	myeloid differentiation primary-response protein 88
NFκB	nuclear transcription factor-κB
NK cell	natural killer cell
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PFU	plaque-forming unit
PHA	phytohemagglutinin antigen
p.i.	postinfection
PMN	polymorhonuclear neutrophils
pIgR	polymeric immunoglobulin receptor
PRR	pattern recognition receptor
PKR	RNA-dependent protein kinase
Rb	retinoblastoma protein
RIG-I	retinoic acid-inducible gene I
RLH	recurrent labial herpes
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SC	secretory piece
sIgA	secretory IgA
siRNA	small interfering RNA
SCN^-	thiocyanate
SLPI	secretory leukocyte protease inhibitor
snRNP	small nuclear ribonucleoprotein particle
ss	single-stranded
CTL	cytotoxic T lymphocytes
TAP	transporter associated with antigen presentation
TG	trigeminal ganglion
TGF-β	transforming growth factor β
Th	T helper cell
tk	thymidine kinase
TNF	tumor necrosis factor
TLR	toll-like receptor
Treg	regulatory T cell
U_b	unique b sequence within *a* region
U_c	unique c sequence within *a* region
U_L	unique long sequence
U_S	unique short sequence
vhs	virion host shutoff
VP16	virion protein 16 (αTIF)
3D	three-dimensional

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-VI).

I **Syrjänen S, Mikola H, Nykänen M, Hukkanen V.** In vitro establishment of lytic and nonproductive infection by herpes simplex virus type 1 in three-dimensional keratinocyte culture. *J Virol* 1996; 70: 6524-6528.

II **Hukkanen V, Mikola H, Nykänen M, Syrjänen S.** Herpes simplex virus type 1 infection has two separate modes of spread in three-dimensional keratinocyte culture. *J Gen Virol* 1999; 80: 2149-2155.

III **Välimaa* H, Waris M, Hukkanen V, Blankenvoorde MFJ, Nieuw Amerongen AV, Tenovuo J.** Salivary defense factors in herpes simplex virus infection. *J Dent Res* 2002; 81: 416-421.

IV **Mikola H, Waris M, Tenovuo J.** Inhibition of herpes simplex virus type 1, respiratory syncytial virus and echovirus type 11 by peroxidase-generated hypothiocyanite. *Antiviral Res* 1995; 26: 161-171.

V **Välimaa* H, Tenovuo J, Waris M, Hukkanen V.** Lactoferrin and lysozyme, two cationic proteins of the oral innate immunity, differ significantly in their ability to inhibit HSV-1 infection. *Manuscript*.

VI **Välimaa* H, Ilonen J, Seppälä I, Savolainen J, Hukkanen V, Waris M.** Recurrent labial herpes simplex virus infection is associated with Th2 type cellular and humoral immune responses. *Manuscript*.

 *Née Mikola.

INTRODUCTION

Herpes simplex viruses (HSV) are ubiquitous human pathogens, which infect the host via mucosal sites or the skin. The most common manifestations of HSV disease include orolabial infections (typically observed as gingivostomatitis or labial herpes), genital infections, infections of the skin, keratitis and central nervous system infections.

Characteristic for HSV is the ability to establish lifelong latency in the sensory ganglia innervating the initial site of infection. Reactivation of HSV may either lead to clinical disease or asymptomatic shedding of HSV to external secretions, for example saliva. The frequency of reactivation and prevalence of symptomatic disease are highly variable between individuals, and the mechanisms determining the outcome of reactivation are largely unknown.

This thesis aimed to elucidate the role of innate salivary defence mechanisms and adaptive immunity in the modulation of orolabial HSV infection upon establishment of initial infection and the reactivation phenotype. Furthermore, the suitability of a three-dimensional keratinocyte culture, imitating the natural environment of epithelial HSV infection, for *in vitro* HSV studies was investigated.

REVIEW OF THE LITERATURE

1. HERPESVIRIDAE

Family *Herpesviridae* consists of approximately 130 members, which all share similar virion architecture and certain biological properties: They encode enzymes for nucleic acid metabolism, DNA synthesis and protein processing. Synthesis of DNA and capsid assembly takes place in the nucleus, and productive infection leads to destruction of the infected cell. Furthermore, they are all capable of remaining latent within the host. Ten viruses within the family *Herpesviridae* have been isolated from humans this far: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human cytomegalovirus, varicella-zoster virus, Epstein-Barr virus and *Human herpesviruses 6A, 6B, 7* and *8*, and in rare instances, monkey B virus. (Roizman & Pellett, 2001).

2. HERPES SIMPLEX VIRUS TYPE 1

2.1. Structure

HSV-1 virion architecture complies with the general structure of all viruses of the family *Herpesviridae* (Figure 1). The double-stranded (ds) linear DNA genome is packaged in a form of a toroid inside an icosahedral capsid, which is composed of 162 capsomeres arranged in a T=16 symmetry. Between the nucleocapsid and the outmost layer, the envelope, resides a tegument, which contains amorphous material with some proteins necessary for the replication of the virus, including virion host shutoff (vhs) and virion protein 16 (VP16). The envelope is a lipid bilayer, with non-glycosylated and glycosylated proteins projecting as spikes from the virion surface. This far, 11 glycosylated viral proteins have been described, and they are designated as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM. The envelope lipids are derived from the nuclear or cytoplasmic membranes of the host cell. The whole virion diameter is approximately 120-200 nm. (Roizman & Knipe, 2001; Roizman & Pellett, 2001; Whitley, 2001).

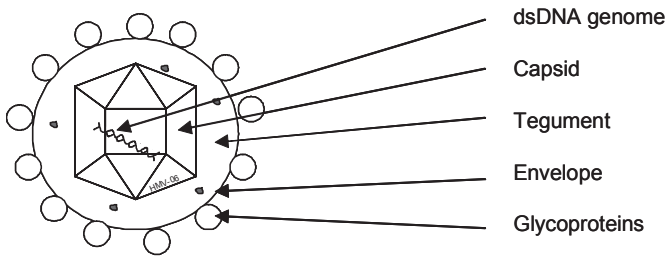

Figure 1. The structure of HSV virion.

2.2. Genome

HSV-1 linear dsDNA genome is approximately 152 kilobasepairs (kbp) in size. The genome contains at least 84 open reading frames (ORFs) that are estimated to encode for about 80 proteins. Approximately half of these ORFs are dispensable for viral growth *in vitro*. The genome consists of two covalently linked long stretches of unique sequences: unique long (U_L) and unique short (U_S) (Figure 2). Each component is flanked by inverted repeat sequences, designated in U_L as *ab* and *b´a´*, and in U_S as *a´c´* and *ca*. The number of *a* sequence repeats is variable at the U_L-U_S junction and at the U_L terminus. The *a* regions in the termini of the linear genome are asymmetrical and are found inverted at the junction of U_L and U_S. The *a* sequence contains two unique regions (U_b, U_c) that are flanked by three direct repeat regions (DR1, DR2 and DR4). Upon circularization of the genome, the asymmetrical *a* sequences form one complete functional DR1. The structure of the *a* sequence is highly conserved but the number of individual repeat elements is variable between different strains of HSV-1. (Roizman & Knipe, 2001; Roizman & Pellett, 2001).

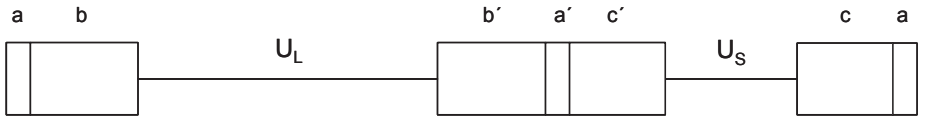

Figure 2. Schematic representation of the arrangement of the HSV-1 genome. The boxes illustrate the inverted repeats and the lines the unique sequences. Modified from Roizman and Knipe (2001).

2.3. Replication

The first step of HSV-1 replication is the attachment of the virus to the cell surface (Figure 3). This initial interaction is mediated by viral envelope glycoprotein gC (Campadelli-Fiume *et al.*, 1990) or gB (Herold *et al.*, 1994). The interaction occurs with glycosaminoglycan (GAG) chains of cell surface proteoglycans, primarily heparan sulphate (WuDunn & Spear, 1989) or other GAGs, including chondroitin sulphate (Gruenheid *et al.*, 1993). In the absence of these GAGs, the efficiency of HSV binding is greatly reduced but cells can still be infected, probably through gB or gD interaction with cell surface receptors (Laquerre *et al.*, 1998; Spear *et al.*, 2000). Subsequent event in the entry process is the interaction of envelope gD with one of its cellular ligands (Ligas & Johnson, 1988) (Table 1). These molecules belong to three different classes, and include herpesvirus entry mediator (HVEM) of the tumour necrosis factor (TNF) receptor family, nectin-1 and nectin-2 of the immunoglobulin (Ig) superfamily, and specific sites in heparin sulphate (3-*O*-S) generated by 3-*O*-sulfotransferases (3-OST-3s), reviewed in Spear *et al.* (2000).

Binding of gD to one of these receptors triggers the fusion of the viral envelope with plasma membrane in a pH-independent manner. Additional viral envelope glycoproteins required for fusion are gB and the gH-gL heterodimer. Additionally, HSV is able to enter some cell types by a low-pH endocytic pathway (Nicola *et al.*, 2003), which is independent of gD receptors, but requires viral glycoproteins gB, gD and gH-gL (Nicola & Straus, 2004).

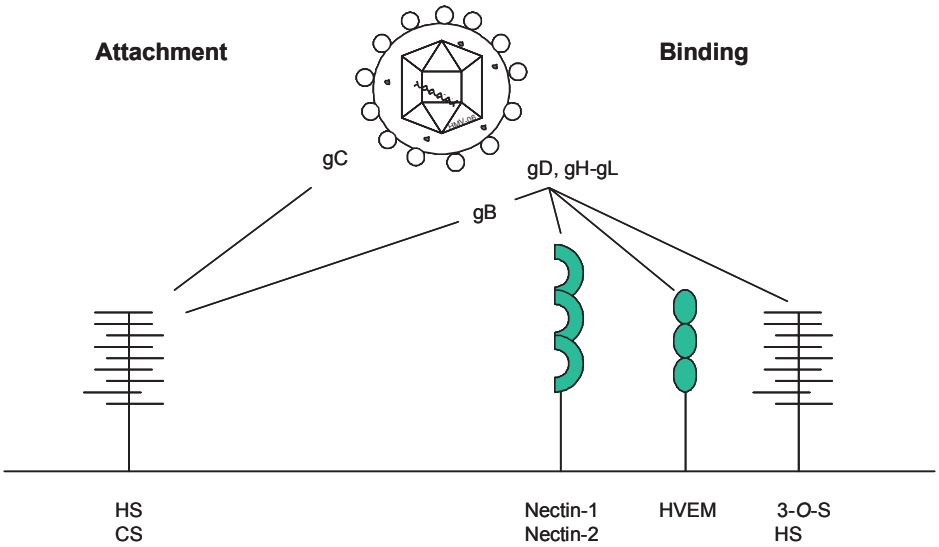

Figure 3. HSV attachment and entry determinants. Initial attachment to cell surface is mediated by envelope glycoproteins gC and gB, which bind to cell surface glycosaminoglycans, such as heparan sulphate (HS) or chondroitin sulphate (CS). Primary attachment facilitates gD binding to one of its receptors, including herpesvirus entry mediator (HVEM) of the tumor necrosis factor family, nectin-1 and -2 of the immunoglobulin superfamily, and the specific sites on HS (3-*O*-S) generated by 3-O-sulfotransferases. This interaction triggers fusion of the viral envelope and plasma membrane together with coordinated action of gB, gD, gH and gL. Fusion of the membranes results in release of tegument proteins and nucleocapsid into the cytoplasm. Low-endocytic pathway is independent of gD, but similarly requires the presence of gB, gH and gL. Modified from Spear et al. (2000).

Table 1. Human proteins that act as gD receptors, or generate gD receptors for HSV-1 entry. Modified from Spear et al (2000).

	ENTRY MEDIATOR		MEDIATES ENTRY OF	
Protein family	Name used in here	Other names	WT	Rid
TNF	HVEM	HveA, ATAR, TR2, TNFRSF-14	yes	no
Ig superfamily	Nectin-1α	HveC, Prr1α	yes	yes
	Nectin-1β	HIgR, Prr1β	yes	yes
	Nectin-2α	HveB, Prr2α, Mphα	no	yes
	Nectin-2δ	Prr2δ, Mphβ	no	yes
Sulfotransferase	3-OST-3_A		yes	no
	3-OST-3_B		yes	no

WT, wild-type; Rid, WT-derived mutant strain of HSV-1 that has a single amino acid substitution in gD at position 25 or 27; HVEM, herpesvirus entry mediator; 3-OST-3, 3-*O*-sulfotransferase; TNF, tumor necrosis factor; Ig, immunoglobulin.

Following fusion, the nucleocapsid and tegument proteins are released into the cytoplasm (Figure 4). Some tegument proteins remain in the cytoplasm (e.g., vhs), whereas others (e.g., VP16) are transported towards the nucleus by microtubules together with the nucleocapsid (Sodeik *et al.*, 1997). Nucleocapsid then docks at the nuclear pore complex, and the viral genome and certain tegument proteins are deposited into the nucleoplasm (Ojala *et al.*, 2000).

HSV genes are expressed sequentially in a temporal cascade (Honess & Roizman, 1974; Honess & Roizman, 1975; Stingley *et al.*, 2000). Cellular RNA polymerase II is responsible for the transcription of all viral genes, but also viral factors contribute to the events at all stages of infection. The viral genes are classified into α (immediate-early), β (early) or γ (late) according to the requirements of expression in viral replication. Transcription of α genes requires no prior viral protein synthesis, and is initiated 0-4 hours postinfection (hpi) coordinately by tegument protein VP16 (α-TIF) and host cell proteins octamer binding transcription factor-1 (Oct-1) and host cell factor-1 (HCF-1) (Narayanan *et al.*, 2005). α gene expression then transactivates β genes, which mainly encode for enzymes that are required for viral DNA synthesis and replication. β gene expression occurs 4-8 hpi. DNA synthesis proceeds from theta replication to replication by a rolling-circle mechanism, which yields concatemeric molecules that are further processed into monomers upon encapsidation. Concatemeric DNA is susceptible to homologous recombination, and crossover events occur between different co-infecting strains of HSV-1 and even HSV-2. The most susceptible genomic regions for homologous recombination are the terminal repeats and the internal inverted repeats. Both α and β proteins are required for γ gene transcription, because DNA replication is a prerequisite for the expression of these genes. γ genes encode for structural proteins, and they can be

further divided into γ_1 (early-late) and γ_2 (true-late) genes. γ_1 gene expression is promoted by the onset of viral DNA synthesis, whereas γ_2 gene expression only takes place after the onset of DNA synthesis. γ gene expression aims to transcribe as many viral structural proteins as possible for the assembly of progeny virus. (Roizman & Knipe, 2001).

The assembly of HSV occurs in the nucleus, reviewed in Mettenleiter (2006). First, capsid proteins assemble autocatalytically into preformed empty capsids with internal scaffolding, which is subsequently lost upon encapsidation of viral DNA. Concatemeric progeny viral DNA is cleaved into unit-length monomers and incorporated into the capsid at modified sites of the inner lamella of the nuclear membrane. Nucleocapsids then bud through the inner lamella acquiring the primary envelope in the process. For the following step in the egress process, two alternate routes have been suggested. In the re-envelopment pathway, the enveloped virus is de-enveloped by fusion to the outer lamella of the nuclear membrane, and the de-enveloped capsid is released to the cytoplasm. The capsid subsequently enters the trans-Golgi network, gains a new envelope while budding through the Golgi membranes, and is finally released through secretory vesicles. In the luminal pathway, the enveloped viral particle is internalized at the outer nuclear membrane either to the vesicles or the lumen of the endoplasmic reticulum. The particle then moves through the trans-Golgi network or Golgi vesicles, where the final maturation of the envelope takes place. The mature viral particles are then either released from the cell to the extracellular space, or to an adjacent cell via adherent junctions. Direct cell-to-cell spread involves viral glycoproteins gB (Laquerre *et al.*, 1998) and gE/gI (Dingwell *et al.*, 1994; Dingwell & Johnson, 1998). The entire productive HSV replication cycle takes approximately 18 h, and leads to the death of the host cell.

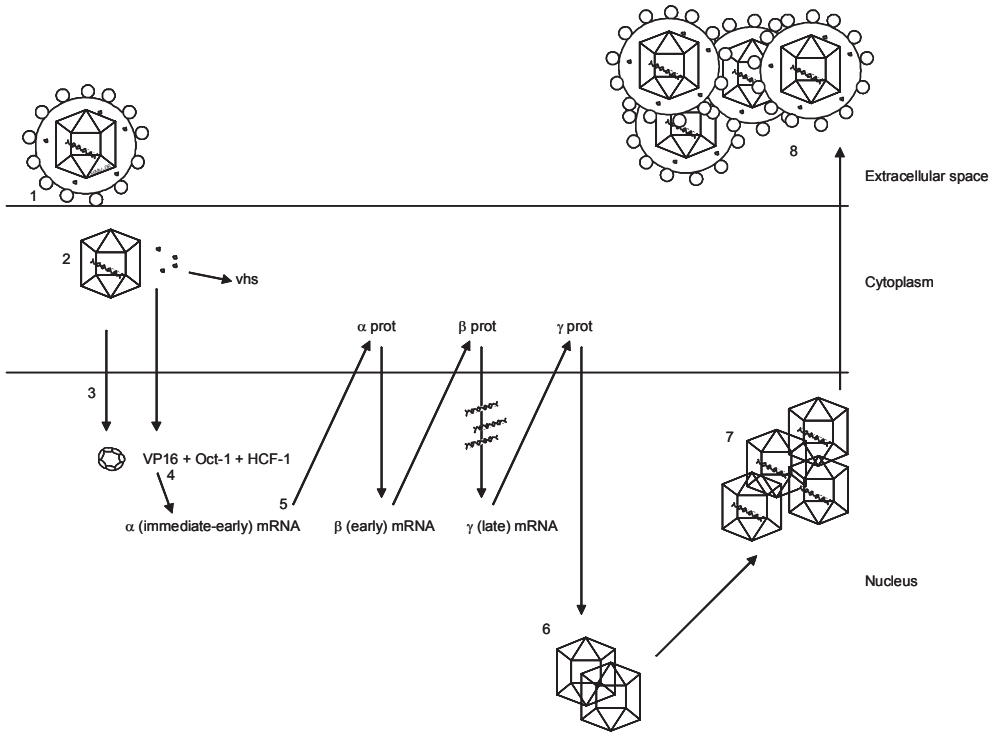

Figure 4. HSV replication cycle. Following fusion of the viral envelope and the plasma membrane (1), tegument proteins and the nucleocapsid are released into the cytoplasm (2). Certain tegument proteins and the viral genome are subsequently transported to the nucleus (3), where viral DNA circularizes. Interaction of VP16 with cellular proteins Oct-1 and HCF-1 stimulates transcription of α (immediate-early) genes by host cell RNA polymerase II (4). Viral genes are then expressed sequentially in a temporal cascade (5). Genes are transcribed in the nucleus, and mRNA translation to protein takes place in the cytoplasm. α (immediate-early) proteins transactivate transcription of β (early) genes. β proteins enable replication of the viral genome and the following γ (late) gene expression. Late proteins are mainly structural proteins. After their synthesis, viral capsids are assembled (6), and viral genomes packaged into the capsids (7). The formed nucleocapsids then bud out of the nucleus, and egress out of the cell either by re-envelopment or luminal pathway, acquiring envelope in the process from nuclear and/or cytoplasmic membranes (8). Vhs, virion host shutoff; VP16, virion protein 16.

2.4. Pathogenesis

HSV-1 is a promiscuous virus that can infect a multiplicity of various cells types. HSV-1 infection is usually acquired early in life, and seropositivity, a generally accepted sign of previous encounter with the virus, is common worldwide. Recently, however, a decline in childhood infections has been observed, particularly in the countries of high hygiene standards (Pebody *et al.*, 2004). In these countries, most people are still seronegative for HSV-1 in teenage.

Epithelial cells are the primary site of replication for HSV-1 (Figure 5). Progeny virus infects the neighbouring cells and enters the underlying sensory neurons. Virus is then transported to the corresponding sensory ganglia by retrograde axonal transport. HSV-1 may further disseminate to and replicate in the brain parenchyma. A particular feature in the life cycle of HSV-1 is its ability to establish a latent infection in sensory ganglia, while maintaining the ability to reactivate. Latency is thought to last for the lifetime of the neuron. The ability to establish latent infections and to reactivate without any need for variation in its antigenic properties, probably largely explains why HSV-1 has been so successful among humans.

The first encounter of a HSV-1 and HSV-2 seronegative host with either HSV-1 or HSV-2 is called a primary infection. Reactivation of HSV is referred to as recrudescence or recurrent infection in the presence of clinical symptoms of HSV infection. Exogenous reinfection by a different strain in the presence of pre-existing antibodies is extremely rare in an immunocompetent host. Instead, initial infection, defined as infection by HSV-1 or HSV-2 in the presence of pre-existing antibodies for HSV-2 or HSV-1, respectively, is common. (Whitley, 2001).

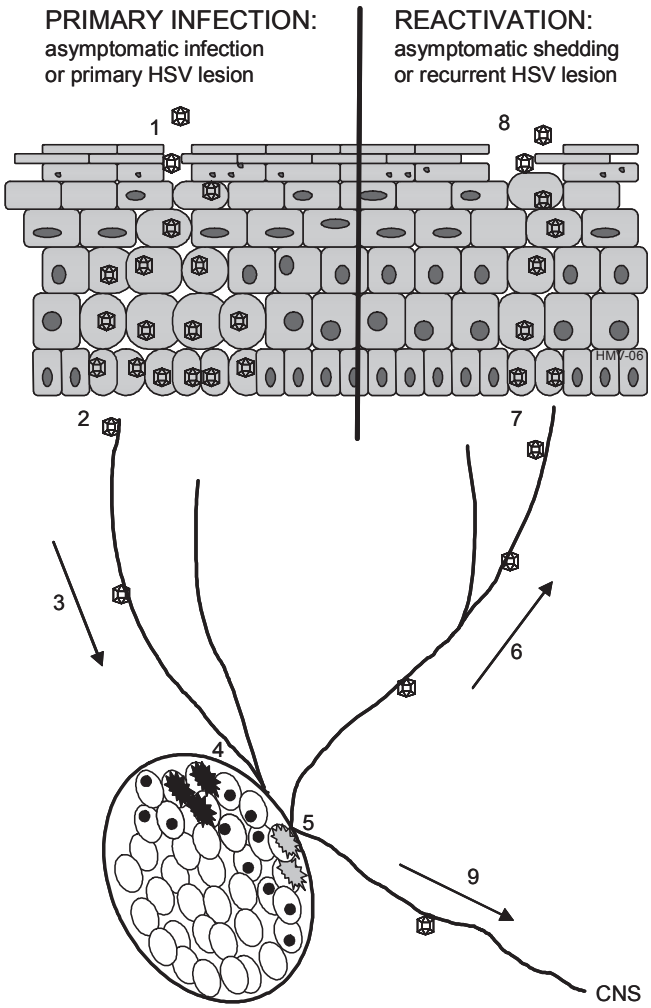

Figure 5. Natural course of HSV infection in the orofacial region. HSV initiates infection in the epithelial cells of the mucosa or the skin (1). Progeny virus from these cells infects neighbouring cells, and enters sensory nerves innervating the area (2). Virus is then transported by retrograde transport within the nerves towards the sensory ganglion (3). In the ganglion, HSV either replicates productively (black explosion), or establishes a latent infection (black circles) in some of the neuronal nuclei (4). HSV can reactivate (5) from latent state (grey explosion) and be transported in anterograde direction along sensory nerves (6) back to the epithelium, where virus begins to replicate productively in the epithelial cells (7). Epithelial viral replication then results in either recrudescent infection or asymptomatic shedding of the virus (8). Occasionally, either upon primary infection of reactivation, infectious HSV may disseminate to the brain parenchyma, where viral replication results in encephalitis (9). CNS, central nervous system.

2.4.1. Effects on the host cell

Histopathologic changes

Replication of HSV in the skin or mucosa induces a similar cytopathology in both primary and recurrent infection (Figure 6), although the extent of pathological changes is typically greater in primary infection. These changes comprise of cellular changes induced by replicating virus and of the associated inflammatory response. Characteristic features of HSV cytopathology are ballooning of the infected cells and formation of multinucleated giant cells (i.e., polykaryocytes), which are formed as a result of disruption of cell membranes. The infected cell nuclei chromatin becomes condensed, and the nuclei are subsequently degraded typically in the parabasal and intermediate layers of the epithelium. Following egress of progeny virus, infected cells eventually lyse, and fluid filled spaces, observed as vesicles, are formed within the epidermis. In addition to cell-free virus, this fluid contains cell debris, inflammatory cells and infected epidermal cells. On mucosal membranes, vesicles are less prominent and rupture sooner because the cornified layer of the mucosal epithelium is thinner compared to the skin. Macroscopically, ruptured mucosal vesicles resemble ulcers with yellowish or greyish floor and red margins.

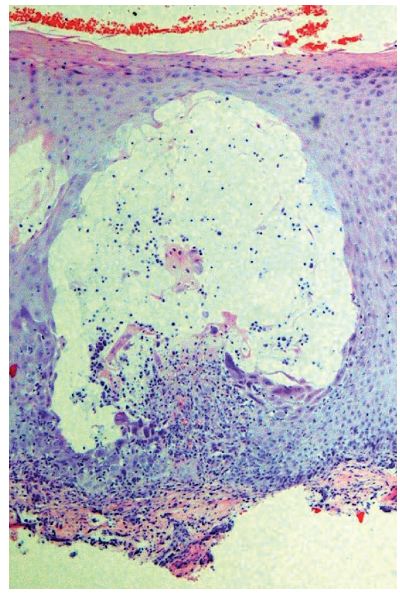

Figure 6. Histopathologic appearance of a HSV lesion in the oral mucosa.

In the early stages of infection, epithelial cells in the roof of the vesicle are spared from infection, whereas cells in the floor of the vesicle are heavily damaged by replicating virus. With the progression of the lesion, the cells of the whole thickness of the epithelium get gradually infected. Typically, the vertical margins of the lesion remain well-defined. In the underlying dermis, an intense inflammatory response is induced. As more inflammatory cells enter the infected area, and the viral replication vanes, the vesicular fluid becomes pustular and eventually a scab is formed. Typically, HSV lesions heal without scarring because vesicles mainly affect the epithelium, not the dermis. (Whitley, 2001).

On the cellular level, cytopathic changes include the initial enlargening and subsequent fragmentation of the nucleolus. Along with these changes, the chromosomes marginate and condense, and the nucleus becomes multilobed. The so-called intranuclear eosinophilic inclusion bodies are formed. These bodies are thought to represent an aggregation of replication proteins, progeny viral DNA and nucleocapsid components. In some cells, the Golgi structures get fragmented and are dispersed as small vesicles around the cytoplasm. Early in infection, microtubule network undergoes changes at the plasma membrane junction, and later on, microtubules rearrange to form bundles around the nucleus. The formation of polykaryocytes appears to be related to changes occurring in the infected cell plasma membranes. In polykaryocytes, microtubules rearrange along the axis of recruitment of new cells. (Roizman & Knipe, 2001).

In the brain, typical histopathological changes include hemorrhagic necrosis and perivascular inflammatory infiltrates. Later, following neuronal death, astrocytosis, gliosis and oligodendrocytic involvement develop. HSV encephalitis is usually focal, but in slowly developing infections brain areas involved are more diffuse. (Whitley, 2001).

Metabolic changes in the host cell

HSV infection has dramatic effects on the host cell metabolism. It shuts off host RNA, DNA and protein synthesis. This effect has been mainly attributed to U_L41 gene product, the vhs protein (Read & Frenkel, 1983). Vhs also accelerates the degradation of both cellular and viral mRNAs (Kwong & Frenkel, 1987). Along with progression of infection, vhs activity declines and the half-life of viral mRNAs becomes longer. Apparently, this is at least in part due to vhs complexing with another tegument protein, VP16, which then blocks vhs from degrading RNA (Lam *et al.*, 1996). From virus point of view this is a good strategy, as along with the shutoff of host cell mRNA transcription, late in infection the principal mRNA found in the cell is of viral origin.

HSV aims to change the transcription to favour transcription of viral instead of cellular genes. Already by 4 hpi, the transcription by all three cellular RNA polymerases has decreased by 50% (Preston & Newton, 1976). HSV inhibits RNA splicing by an α gene U_L54 product, infected cell protein (ICP) 27, by phosphorylating the cellular small nuclear ribonucleoprotein particle (snRNP) U1 (Sandri-Goldin & Hibbard, 1996), which enhances RNA splicing when hypophosphorylated. This has only minimal effect on viral

RNA splicing since three of four mRNAs, which undergo splicing, are expressed as α genes. Later in infection ICP27 function is repressed. This repression allows splicing of U_L15 of the γ kinetic class of genes to take place.

HSV not only shuts off the *de novo* synthesis of cellular proteins, but also contributes selectively to the degradation or stabilization of host proteins involved in the control of the cell cycle. HSV-1 stabilizes cyclins D1 and D3 (Kawaguchi *et al.*, 1997; Van Sant *et al.*, 2001) that is suggestive of an attempt to maintain cells in nonreplicating phase. Consistently, HSV-1 inhibits cyclin D-cyclin-dependent kinase (CDK)4/6- and cyclin E-CDK2-complexes that phosphorylate retinoblastoma protein (Rb), the key regulator of G_1/S transition (Ehmann *et al.*, 2000). Hypophosphorylated Rb prevents members of the E2F transcription factor family to become active and initiate transcription of S-phase cellular genes. Cell division control (cdc) 2 cell cycle kinase, known to phosphorylate viral proteins and control the G_2/M transition, is activated by HSV-1 even though its activating partners, cyclins A and B, are degraded (Advani *et al.*, 2000a). S-phase is not required for HSV-1 replication since HSV-1 does replicate in cells arrested in G_1-phase. HSV-1 has apparently evolved mechanisms to prevent cells in G_1 from entering the G_1/S transition, and mechanisms to prevent cells in S from completing the full round of DNA replication (Ehmann *et al.*, 2000). In fact, evidence exists that S-phase proteins are only transiently active in early phases of infection, and that the expression of these genes is later blocked by posttranslational modification and translocation of the members of the E2F family to inactive compartments, and by the lack of active cdk2 (Advani *et al.*, 2000b). Indeed, HSV-1 is suggested to selectively induce the expression of those S-phase cellular genes that are required for viral replication. For example neurons that are noncycling, and rather support nonproductive than lytic infection, are normally devoid of CDK2 and CDK4 required for G_1/S transition. The stimuli that induce reactivation of HSV-1 in latently infected ganglia, has been shown also to induce expression of CDK2 and CDK4, thereby allowing neurons to support productive infection (Schang *et al.*, 2002).

Another dramatic example of HSV effect on cell metabolism is the function of ICP34.5 protein on protein synthesis. The RNA-dependent protein kinase (PKR) expression is induced by interferon (IFN) and activated by dsRNA. PKR phosphorylates eukaryotic initiation factor (eIF) -2α that normally induces the total shutoff of protein synthesis. HSV ICP34.5 counteracts this PKR function by binding to protein phosphatase 1α and directing it to dephosphorylate eIF-2α, thereby allowing protein synthesis to continue (He *et al.*, 1997). Furthermore, ICP34.5 antagonizes the eIF2α kinase-dependent autophagy through PKR, suggesting that in addition to being activated in response to amino acid starvation for synthesis of essential proteins, autophagy is also an antiviral mechanism (Talloczy *et al.*, 2002).

Apoptosis

HSV-1 encodes for several genes that protect cells from apoptosis, the non-inflammatory programmed cell death. This is important to ensure enough time for viral replication before the death of the cell. However, in dendritic cells (DCs), HSV-1 has been suggested to have a dual effect on apoptosis (Medici *et al.*, 2003). Whereas early phases of infection appear to be anti-apoptotic allowing efficient initial replication of the virus, later phase is characterized by induction of apoptosis. Viral proteins implicated to have anti-apoptotic activity, include α proteins ICP4 (Leopardi & Roizman, 1996) and ICP27 (Aubert & Blaho, 1999), protein kinase U_S3 (Jerome *et al.*, 1999; Leopardi *et al.*, 1997), glycoprotein gD (Zhou *et al.*, 2000), minor glycoprotein gJ (U_s5) (Jerome *et al.*, 1999; Zhou *et al.*, 2000) and ribonucleotide reductase (Langelier *et al.*, 2002). Furthermore, LAT transcript has been shown to have anti-apoptotic functions (Gupta *et al.*, 2006). Viral proteins involved in the initiation of apoptosis are largely unknown, but protein kinase U_L13 and $U_S1.5$, which are expressed with late kinetics, have been shown to possess proapoptotic activity through activation of caspase 3 that is indicative of programmed cell death (Hagglund *et al.*, 2002).

2.4.2. Primary infection

HSV-1 infection is typically transmitted in close contact via exposure of mucosa or scarred skin to infectious virus. The most frequent site of HSV-1 primary infection is the epithelium of the oral region, which gets infected by exposure to oral secretions contaminated by excreted virus or virus from a labial herpes lesion from another person with active disease. Infection of epithelial cells leads to productive HSV-1 replication and release of progeny virus. In the oral region, primary infection is usually subclinical, but can be observed in rare cases as eruption of multiple epithelial vesicles, a condition called HSV gingivostomatitis (see: Orofacial primary infection). In both cases, progeny virus spreads to the adjacent epithelial cells, and enters sensory neurons in the underlying dermis that innervate the infected anatomical area. Within neurons, the nucleocapsid and tegument proteins are transported along the microtubule network in a retrograde direction towards the neuronal cell body (Kristensson *et al.*, 1986). The minus-end-directed motor protein, dynein, as well as its cofactor dynactin, have been implicated in the retrograde transport of HSV-1 capsids (Dohner *et al.*, 2002; Paschal & Vallee, 1987). Upon arrival at nuclear membrane, the nucleocapsids dock at nuclear pore complexes and release viral DNA into the nucleus. In most cases, HSV genome then circularizes and a quiescent form of HSV infection, latency, ensues. In rare occasions, HSV continues to replicate productively in neurons. This may lead to a wider spread of infection within the trigeminal ganglion, or very rarely to spread of the infection to the brain parenchyma resulting in a severe condition called herpes simplex encephalitis. Encephalitis is more likely to occur upon reactivation of HSV.

The ability of HSV to invade from periphery to the neurons of the central nervous system (CNS) and to replicate in neurons, are together referred to as *neurovirulence*. Different strains of HSV may differ in neurovirulence as several viral genes, including genes encoding glycoproteins and genes interfering with host response, are known to contribute to this feature of HSV. These genes also contribute to viral replication in peripheral tissues and in cell culture. Among them, the roles of the genes U_L23 (gene product ICP36 or thymidine kinase, tk) and $\gamma_1 34.5$ (gene product ICP34.5) are the most extensively characterized.

Tk is essential for the replication of HSV in nondividing cells, as it apparently provides resting cells with nucleoside triphosphate precursors for DNA synthesis. Consequently, the neurovirulence of tk-deletion mutants is dramatically decreased. Nonetheless, these mutants can establish latent infection in trigeminal ganglia, but are unable to reactivate indicating that initial replication of HSV in neurons is not required for establishment of latent infection (Coen *et al.*, 1989). Tk-deletion mutants are additionally impaired in the ability to induce innate inflammatory response and disease in HSV-2 encephalitis model in mice (Boivin *et al.*, 2002). Deletion of the so-called neurovirulence gene, $\gamma_1 34.5$, similarly impairs the capability of HSV-1 to replicate in nondividing cells resulting in impaired CNS pathogenicity and decreased ability to reactivate from latency (Chou *et al.*, 1990).

2.4.3. Latency

HSV is able to establish latency in nondividing sensory neuronal cells. The viral genome is rapidly circularized after release into the nucleus to reside there as episomal DNA. The ability to establish latency is dependent on both cellular and viral properties, although none of the viral proteins is known to be indispensable for latency.

Replication of viral DNA is not a prerequisite for establishment of latency. Still, latently infected mouse neurons are shown to constantly harbour HSV DNA genomes in over 20 copy numbers per cell (Simmons *et al.*, 1992; Speck & Simmons, 1991). As terminally differentiated neurons are considered to have no DNA replication functions, this means that either more than one virus enters a neuron during the establishment of latency, or that viral DNA is amplified during latency (Roizman & Sears, 1987). The latter hypothesis is supported by the identification of one host-dependent origin of DNA replication within the HSV genome (Sears & Roizman, 1990). Multiple copy number may also be due to viral DNA replication functions, as latent DNA load is related to the scale of ganglionic viral replication (Thompson & Sawtell, 2000). Furthermore, the amount of peripheral inoculum correlates with viral genome copy number per neuron, and the number of latently infected neurons (Sawtell, 1997). This may be attributed to either bigger number of viruses entering a single neuron, or to greater extent of viral replication taking place either in the periphery or the ganglion.

Upon establishment of latent state, the viral genome becomes silent with only very limited transcriptional activity. The most copious and regularly observed transcripts in

latently infected neurons are the latency-associated transcripts (LATs) (Stevens *et al.*, 1987). Latent state is regarded free of lytic gene expression, and hence replicating virus. Yet, recent data implies that in reality, latency may transcriptionally be a more dynamic form of infection, as limited expression of lytic gene transcripts and proteins has been detected in the absence of production of infectious viral particles. These include the α gene product ICP4, and the β gene products ICP8 and tk (Feldman *et al.*, 2002; Kramer & Coen, 1995; Liu *et al.*, 2000). It is currently unclear, whether the detection of these lytic genes represents early phases of a reactivation, or a still unknown property of latent infection.

Still, LATs are the most abundant genes expressed in latency (Stevens *et al.*, 1987). LATs are expressed under the LAP promoter from the *b* repeat sequence flanking the U_L region of the genome (Stevens *et al.*, 1987). In latently infected neurons, the full-length 8.3 -kb primary transcript is less abundant compared to 2.0 and 1.5 -kb highly stable introns, which are processed from the same transcript, and accumulate in the nucleus. The role of LATs is not well understood, but they appear to be involved in the establishment and maintenance of latency, and the reactivation of HSV from latent state. LATs downregulate the lytic gene expression in sensory neurons (Garber *et al.*, 1997) potentially by expressing a protein product, or by interfering structurally with the viral genome. The 8.3 -kb LAT transcript is antisense to ICP0 mRNA and possibly also ICP4 mRNA. This enables antisense binding between LAT and these α mRNAs, and hence downregulation of lytic viral replication. ICP0 is a promiscuous transactivator. Its optimal activity requires the presence of ICP4, which is essential for efficient expression of all viral transcripts. Inhibition of lytic gene expression may also be dependent on the host cell lacking nuclear transcription factors necessary for α gene transcription (Kristie *et al.*, 1999; Valyi-Nagy *et al.*, 1991). Furthermore, relative to certain lytic viral genes in latently infected mouse sensory ganglia, the LAT promoter has been observed to be hyperacetylated at histone H3 associated with transcriptionally permissive chromatin (Kubat *et al.*, 2004).

The 8.3 –kb LAT encodes for two proteins, ORF P and ORF O cycle (Bohenzky *et al.*, 1993; Yeh & Schaffer, 1993). These proteins have been detected late in productive infection cycle, whereas attempts to detect these proteins in latently infected ganglia have been unsuccessful (Bohenzky *et al.*, 1993; Yeh & Schaffer, 1993). ORF P protein blocks the synthesis of ICP0 and ICP22 (Bruni & Roizman, 1996), whereas ORF O interferes with the binding of ICP4 on its cognate site on HSV-1 DNA (Randall *et al.*, 1997). Furthermore, LATs protect neurons from apoptosis by encoding anti-apoptotic microRNA (Gupta *et al.*, 2006). Even though LATs appear to have a central role in latency, LATs are not indispensable for the establishment of latency (Javier *et al.*, 1988). Deletion of LAT region or ORF P and O may, however, reduce the quantity of latent virus harbored in neurons (Randall *et al.*, 2000; Thompson & Sawtell, 1997), although evidence to the contrary exists as well (Leib *et al.*, 1989a).

2.4.4. Reactivation

The stimulus for reactivation can be either local or systemic. Trauma or stimulation of cells innervated by latently infected neurons and exposure to ultraviolet light, are known to trigger reactivation. Systemic stimuli include emotional and physical stress, immunosuppression, hyperthermia and hormonal changes, such as menstrual cycle. Upon systemic stimulation, virus can reactivate in various ganglia simultaneously. The molecular level signal mechanisms behind reactivation are unknown, but supposedly include viral, cellular and immunological factors. (Roizman & Knipe, 2001).

Reactivation ability of HSV can be compromised by deletion of practically any gene indispensable for replication in cell culture. For example, viruses deleted of LAT (Leib *et al.*, 1989a), $\gamma_1$34.5 (Chou *et al.*, 1990) or the major viral transcriptional activator ICP0 (Leib *et al.*, 1989b) reactivate less efficiently compared to the wild-type virus. Tk- (Coen *et al.*, 1989) and ribonucleotide reductase- (Goldstein & Weller, 1988) deletion mutants are totally unable to reactivate as they cannot replicate in nondividing cells.

HSV gene expression is highly dependent on the status of the cell cycle (Schang *et al.*, 2002) and the availability of cellular factors (e.g., HCF-1 and Oct-1), which evidently differ in nondividing and dividing cells (Preston, 2000). Therefore, also the strategies for initiation of replication within neurons may differ dramatically from those described in dividing cells. In addition to host cell factors, immune responses are apparently critically important for the control of reactivation in the sensory ganglia (see: Immune responses in the CNS).

The frequency of reactivation and recrudescence is highly variable. HSV genomic copy number per neuron and the amount of latent DNA correlates positively with the reactivation frequency (Lekstrom-Himes *et al.*, 1998; Sawtell *et al.*, 1998). The copy number per cell and the number of latently infected neurons is related to the amount of initial viral inoculum (Sawtell, 1997). Furthermore, viral inoculum and replication in the ganglion, and to a lesser extent in the periphery, is related to the number of latently infected sites (Jacobson *et al.*, 1998; Sawtell, 1997; Thompson & Sawtell, 2000). Also the viral strain (Sawtell *et al.*, 1998) and the genetic background of the host (Lopez, 1975) may affect the copy number per neuron and the susceptibility to reactivation. In summary, the amount of infectious inoculum and the ability of HSV to replicate in the ganglion are probably the main virus-associated determinants of susceptibility to reactivation.

The fate of the neuron upon reactivation is debatable. If neurons died upon reactivation, frequent reactivations could be expected to result in the development of an anesthesized mucosal or skin area. Yet, such observation has not been reported, although lack of such finding may also be explained by sprouting of neighbouring nerve fibres. Asymptomatic shedding of HSV occurs very frequently. Therefore, if neurons died upon reactivation, the number of neurons harbouring latent virus would decrease over years resulting in the cessation of shedding and reactivations, unless a more chronic form of mucosal HSV infection is possible. Indeed, HSV DNA has been suggested to persist in extraneuronal tissues, including the human skin (Brice *et al.*, 1994; Miura *et al.*, 1992), after resolution of clinical HSV disease. Furthermore, empirically, genital HSV-2

recurrences have been observed to become less frequent by time, potentially reflecting a decrease in the number of neurons harbouring latent virus, or the improved ability of the immune system to resist reactivation due to gradual education by repeated exposure to HSV. Thus far, the studies regarding the fate of the neuron upon reactivation have been inconclusive. (Roizman & Knipe, 2001).

2.5. Immune responses

HSV infection evokes a multitude of innate and adaptive immune responses in the host. However, even before exposure to these mechanisms, HSV has to combat the physico-chemical barrier formed by the epithelium of the skin or the mucosa and by the body fluids covering mucosal surfaces that constitute the very first line of defence. Healthy epithelium prevents HSV from reaching the most susceptible epithelial cell layer, the metabolically active basal cells, and therefore HSV is most likely to infect the host via mucosal surfaces or abraded skin. Various body fluids, including saliva, tears and vaginal secreta contain antiviral soluble molecules (e.g., defensins and natural antibodies), which can reduce the amount of HSV inoculum reaching the epithelium even before the activation of innate immune responses.

2.5.1. Immune responses in the periphery

Innate immunity

In the absence of pre-existing immunity, innate immune responses have a critical role in protecting the body in the early phases of infection, because adaptive T-cell responses take as long as 3-5 days and antibody responses more than a week to develop.

Innate immune recognition of a virus evokes antiviral immune responses, reviewed in Malmgaard (2004). HSV infection of the keratinocytes leads to secretion of cytokines and chemokines, which then activate and attract macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DC) to the site of infection. Macrophages and neutrophils phagocytose HSV-infected cells, and release mediators of inflammation, toxic substances (e.g., nitric oxide and hydrogen peroxide) and chemoattractants. Together these molecules mediate the immediate protective response, recruit immune cells to the infected tissue, and steer the development of adaptive immune responses.

The initial host cell recognition of a virus is mediated through interaction of pathogen-associated molecular patterns (PAMPs) with host cell pattern recognition receptors (PRRs), reviewed in Takeda *et al.* (2003). This interaction leads to intracellular signalling-cascade and activation of nuclear transcription factor-κB (NFκB) leading to production of proinflammatory cytokines, chemokines and type I interferons (α/β), and to subsequent activation of the adaptive immunity. These recognition mechanisms, as well as

responses, may vary between host and pathogen species. PRRs are differentially expressed according to the cell type, and they also differ in requirements for activation, such as the replication state of the virus.

Toll-like receptors (TLR) are a recently identified group of PRRs found to activate adaptive immunity in humans, reviewed in Takeda *et al.* (2003). PRRs known to be involved in HSV-induced innate responses include TLR2, TLR7 and TLR9.

TLR9 has been shown to interact directly with HSV PAMPs. TLR9 recognizes unmethylated dsDNA CpG motifs, such as genomic HSV DNA, in endosomal cellular compartments (Wagner, 2004). This interaction results in cytokine secretion including type I interferons (IFN) and the Th1 polarizing cytokine IL-12 (Krug *et al.*, 2004; Lund *et al.*, 2003). TLR9 is expressed in humans primarily on professional antigen-presenting cells (APCs) including plasmacytoid dendritic cells (pDCs) and B cells (Wagner, 2004). pDCs have been identified as the most potent IFN-α/β producing cells in viral infection. Remarkably, these cells can express IFN-α/β immediately upon stimulation due to the constitutive expression of interferon regulatory factor-7 (IRF-7), whereas in other cell types, IRF-7 induction phase is required (Malmgaard, 2004). Although potent, pDCs probably only produce a minor proportion of the total IFN produced in response to viral infection (Hochrein *et al.*, 2004). Furthermore, HSV-1 induced IFN-α responses have been further shown to occur via both TLR9-independent and –dependent pathways depending on the cell type and cell tissue source (Hochrein *et al.*, 2004; Malmgaard *et al.*, 2004). Consistently, a novel cytoplasmic DNA recognition pathway, also activated by HSV DNA, was recently shown to activate innate responses through IRF-3 resulting in type I IFN production independently of TLRs (Ishii *et al.*, 2006; Okabe *et al.*, 2005; Stetson & Medzhitov, 2006). Since TLR9 is only expressed in highly specialized subpopulation of PBMCs, it is vitally important that keratinocytes and fibroblasts, the primary targets of HSV-infection, possess efficient TLR9-independent mechanisms for the immediate production of type I IFNs.

TLR2 recognizes a variety of microbial structures (Takeda *et al.*, 2003). Although TLR2 has been implicated also in HSV infection, HSV ligand for TLR2 is currently unidentified. TLR2-HSV ligand interaction apparently does not require viral replication, since UV-inactivated HSV has been shown to activate NFκB through TLR2 (Kurt-Jones *et al.*, 2005). HSV-induced TLR2-mediated responses have been shown to induce production of inflammatory cytokines (Kurt-Jones *et al.*, 2004). TLR2 has been also suggested to be responsible for the sepsis-like outcome of neonatal HSV infection characterized by high levels of serum cytokines and multiorgan failure (Kurt-Jones *et al.*, 2005). Data on TLR2 effect on HSV-1 CNS pathology is conflicting, since in mice, TLR2 has been suggested to either enhance (Kurt-Jones *et al.*, 2004) or have no effect (Mansur *et al.*, 2005) on the outcome. The discrepancy between these two studies may at least partly be influenced by the differences in the route of infection, the viral strain and the infectious dose.

TLR7 and TLR8 belong to the TLR9 subfamily, and are expressed by pDCs, myeloid DCs (mDC) and macrophages (Takeda *et al.*, 2003). Single-stranded RNA (ssRNA) viruses and several synthetic compounds structurally related to nucleic acids,

activate TLR7 and TLR8 which results in production of inflammatory cytokines (Takeda *et al.*, 2003; Wagner, 2004). HSV induces TLR7 expression in corneal epithelial cells (Li *et al.*, 2006) suggesting an active role for epithelial cells through TLR7 in immune activation against HSV and possible occurrence of ssRNA intermediates during HSV replication.

Viral dsRNA stimulates IFN type I responses by various mechanisms, and promotes maturation of DCs (Takeda *et al.*, 2003). Transcription of DNA viruses may result in accumulation of dsRNA as a result of transcription of complementary viral mRNA transcripts (Jacquemont & Roizman, 1975) or formation of ds structures by single viral mRNA (Jacobs & Langland, 1996). dsRNA is a ligand for TLR3 (Takeda *et al.*, 2003). Additionally, dsRNA is recognized by PKR (see: Metabolic changes in the host cell) and the cytoplasmic RNA helicases, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) (Yoneyama *et al.*, 2005).

Finally, HSV-1 gD has been shown to induce IFN-α in peripheral blood mononuclear cells (PBMC) (Ankel *et al.*, 1998) possibly through interaction with cell surface mannose receptor (Milone & Fitzgerald-Bocarsly, 1998).

Among all cytokines and other mediators of inflammation produced in the first wave of immune responses, type I IFNs appear to have a pivotal role in defence against viruses (Malmgaard, 2004). These IFNs induce an antiviral state within the cell, warn neighbouring cells to the presence of infection, induce maturation of DCs and antigen presentation via major histocompatibility complex (MHC) class I, enhance cytotoxic T cell responses, and stimulate production of chemokines, which recruit inflammatory cells to the site of infection.

The importance of type I IFNs in confining resistance to HSV-1 infection was originally shown *in vivo* in mouse studies. The observed resistant phenotype of C57BL/6 mouse strain and the susceptibility to infection of BALB/c strain (Lopez, 1975) was shown to be attributable to a more rapid type I IFN response in C57BL/6 mice (Zawatzky *et al.*, 1982). Type I IFNs delay the spread of infection from the inoculation site to peripheral nervous system (Halford *et al.*, 2004), and administration of antibodies against type I IFNs enhances viral replication in mice (Halford *et al.*, 1997; Su *et al.*, 1990). Interestingly, this effect seems transient, as HSV-1 enters, replicates, spreads and establishes latent infections with equal efficiency in these two genetically different mouse strains (Halford *et al.*, 2004).

Type I IFN expression requires the concerted action of certain latent transcription factors, including NFκB, c-Jun/ATF-2, IRF-3 and IRF-7 (Malmgaard, 2004). The crucial role of IRF-7 for type I IFN responses in HSV-1 infection was recently shown by dramatic decrease in IFN response by pDCs negative for IRF-7, but not for IRF-3, which is constitutively produced in most cell types (Honda *et al.*, 2005). Further, the survival of IRF-7 -negative mice infected with HSV-1 was dramatically impaired compared to mice lacking either IRF-3 or myeloid differentiation primary-response protein 88 (MyD88; a cytoplasmic adaptor molecule upstream of IRF-7 in TLR9 signalling pathway). This observation correlated with a decrease in serum IFN level in IRF-7 -negative mice, indicating an essential role for IRF-7 and TLR9-independent IFN activation pathway in

anti-HSV responses against intravenously inoculated HSV. In another study, lack of MyD88 in mice of the same genetic background resulted in remarkably poorer survival when HSV-1 was inoculated intranasally (Mansur *et al.*, 2005). The differential outcome of HSV infection in MyD88-negative mice in these studies suggests that the route of infection, the cells that HSV-1 initially encounters and the pathogenic properties of the virus strain may all have a pivotal impact on the following IFN responses.

Innate resistance to HSV-1 infection is further profoundly related to NK cell function (Malmgaard, 2004). APCs and cytokines produced in the first wave of innate immune responses activate NK and NKT cells, which are the main IFN-γ producing cells in the innate phase of immune response. In addition to secreting IFN-γ, NK cells cause direct lysis of infected cells. Also NK cells express TLRs and contribute to the maturation of DCs (Moretta, 2005). Depletion of NK cells from C57BL/6 mice results in dramatically increased mortality (Ghiasi *et al.*, 2000). Further, mice lacking NK and/or NKT cells or interleukin-15, a cytokine essential for function of these cells, are substantially more susceptible to HSV-2 infection (Ashkar & Rosenthal, 2003).

Adaptive immunity

DCs, macrophages and B cells function as APCs, and have thereby a crucial role in the activation of adaptive immunity. Of these cells, DCs are the most efficient APCs. Maturation of DCs themselves can be initiated by various cytokines, notably IFN-α/β. Although DC maturation can be initiated by cytokines secreted by other cells, evidence exists that in order to activate a fully differentiated T helper type 1 (Th1) cell response, a direct pathogen-DC contact is required (Sporri & Reis e Sousa, 2005). Further, in genital HSV-2 infection, induction of a robust Th1 type immunity is only generated if DCs receive stimuli from stromal cells that have sensed viral infection through their own TLRs (Sato & Iwasaki, 2004). Recognition of HSV-2 through TLR on DC alone is not sufficient. In mucosa or skin, Langerhans´ cells (LC) or submucosal DCs take up antigen, get activated and subsequently migrate to draining lymph nodes (LN) to present peptide antigens to naïve CD8+ and CD4+ T cells. At the site of infection, some of the DCs become infected by HSV themselves and enter apoptosis. Apparently, these DCs are taken up by invading submucosal DCs, which then present antigens to naïve T cells through cross-presentation in the LN (Bosnjak *et al.*, 2005).

Differentiation of naïve Th cells into Th cell subclasses is driven by cytokines produced by APCs, reviewed in Tato & O'Shea (2006). Th1 cell differentiation is stimulated by IL-12, and Th2 by IL-4. Th1 cells characteristically produce IFN-γ, and their proper function is crucial for the defence against intracellular pathogens. IL-4 is the cytokine produced by Th2 cells, and associated with potent induction of antibody responses and allergy. Recently two additional Th lineages were described. Transforming growth factor β1 (TGF-β1) and IL-6 induce the differentiation of Th17 cells, which produce IL-17. These cells are proinflammatory and are thought to have a major role in autoimmune processes. IL-23, which has been suggested to have a role in the maintenance

or expansion of Th17 cells, as well as in the stimulation of IFN-γ production, has been shown to be upregulated in the mRNA level in trigeminal ganglia of HSV-1 infected mice in the acute phase of infection (Broberg *et al.*, 2002). An inverse role has been attributed to regulatory T cells (Treg), which produce TGF-β1, and function to suppress inflammatory reactions. In HSV infection, Tregs suppress CD8+ T cell function (Suvas *et al.*, 2003).

Activated T cells are attracted to the site of infection by the released mediators of inflammation. Various cytokines, including IFN-α/β/γ, IL-1, IL-6, IL-10, IL-12 and tumour necrosis factor α (TNF-α), and chemokines (e.g., CCL5/RANTES, the major immune cell-attracting chemokine) are detectable in HSV lesions (Cunningham & Mikloska, 2001). IFN-γ is a type II IFN that can be detected in high levels within HSV lesions and the vesicle fluid (Cunningham *et al.*, 1985; Torseth & Merigan, 1986). Prior to the influx of activated T cells, IFN-γ is mainly produced by NK cells. IFN-γ partially restores MHC class I associated antigen presentation in infected epidermal cells by increasing the production of cellular TAP, reviewed in Cunningham & Mikloska (2001). It also stimulates MHC class I and II expression on infected cells, and activates macrophage function. The level of IFN-γ produced by PBMC has been further shown to correlate positively with the length of interval between consecutive HSV recurrences (Cunningham & Merigan, 1983).

CD4+ Th cells enter HSV lesions before CD8+ T cells (Cunningham *et al.*, 1985). Cytokines secreted by DCs, macrophages and Th cells modulate CD8+ T cell and B cell responses. Influx of cytotoxic CD8+ T cells correlates with the clearance of HSV infection (Cunningham *et al.*, 1985; Koelle *et al.*, 1998). The proper function of these cells is therefore regarded essential in the control of spread and termination of infection.

Antibodies appear to be relatively inefficient against HSV infection, because even high levels of pre-existing antibodies do not provide protection against HSV recurrences (Spruance *et al.*, 1995). Furthermore, serum antibody levels remain relatively constant both quantitatively and qualitatively despite orofacial recurrences (Zweerink & Stanton, 1981). Antibodies have, however, been shown to limit spread of HSV from the dorsal root ganglion to the periphery *in vitro* (Mikloska *et al.*, 1999) and from the periphery to the CNS in mice infected via corneal route (Halford *et al.*, 1997).

2.5.2. Immune responses in the CNS

Following primary infection HSV-1 replication begins in the trigeminal ganglion (TG) 2-3 days after corneal infection of mice. At this point, the only components of the immune system present both in the eye and the TG, are the components of the innate immunity. Depletion of macrophages, nitric oxide, TNF-α or IFN-γ during the first five days after corneal infection, has been shown to greatly increase viral replication and the number of infected neurons in the TG (Kodukula *et al.*, 1999). Absence of γδ T cells similarly increases the viral titres and the spread of infection in the TG, but does not affect the duration of replication (Kodukula *et al.*, 1999; Sciammas *et al.*, 1997). In contrast,

depletion of αβ T cells does not enhance viral titres within the TG, but results in prolonged low titre viral replication, transmission to the brain and lethal encephalitis (Sciammas *et al.*, 1997). All of these innate defence factors critically contribute to the latent viral load in the TG, and thereby to the reactivation frequency of HSV-1.

The inflammatory reaction in the TG continues for prolonged time periods after viral markers for replicating HSV are no longer detectable (Halford *et al.*, 1996; Liu *et al.*, 1996). The long-term residence of T lymphocytes and T cell-derived cytokines and immune cell-attracting chemokines in latently infected ganglia imply a role for T cells in the maintenance of latency and reactivation. Significant levels of IL-2, IL-10 and IFN-γ mRNA (Halford *et al.*, 1996), and TNF-α and IL-6 protein (Shimeld *et al.*, 1997) have been detected for extended periods of time in latently infected mouse ganglia, and IFN-γ, IL-6 and TNF-α mRNA in human ganglia (Theil *et al.*, 2003). The continued presence of cells of the immune system in latently infected ganglia (Liu *et al.*, 1996; Shimeld *et al.*, 1995) may partially be explained by the high level expression of chemokine CCL5/RANTES, (Theil *et al.*, 2003). Furthermore, it was recently shown, that CD8+ T cells are found selectively around latently infected neurons in the human TG (Theil *et al.*, 2003).

In the context of reactivation, information regarding TG cytokine expression is very limited. UV-irradiation of mouse corneas has been reported to increase the expression of TNF-α and IL-6 indicating a change in TG cytokine balance (Shimeld *et al.*, 1999). In the same experiment, IL-4 expression in the TG was detectable from day three onwards with increasing number of positive cells, whereas IFN-γ was only occasionally detected.

CD8+ T cells (Liu *et al.*, 2000) and the lymphocyte derived cytokine, IFN-γ (Liu *et al.*, 2001), can block HSV-1 reactivation in explant cultures of latently infected mouse ganglia even in the presence of γ gene expression (Decman *et al.*, 2005). The control of reactivation by CD8+ T cells is at least partly mediated by IFN-γ and happens apparently without destruction of neurons (Liu *et al.*, 2000; Liu *et al.*, 2001). In the TG, the majority of CD8+ T cells are specific for a single immunodominant epitope on gB ($gB_{498-505}$), express the recent activation marker CD69, and show T cell receptor polarization near the T cell-neuron junction (Khanna *et al.*, 2003) that may be driven by the low level expression of HSV-1 lytic genes. MHC class I expression is inducible in neurons (Neumann *et al.*, 1995) and HSV-1 has been shown to induce this expression in mouse sensory neurons in acute infection (Pereira *et al.*, 1994; Pereira & Simmons, 1999). Increasing evidence suggests that CD8+ T cells and IFN-γ have a crucial role in the control of HSV-1 reactivation.

2.6. Immune evasion mechanisms

HSV-1 has developed multiple mechanisms to evade host immune responses to persist successfully within the host and to further spread to susceptible individuals. For persistence, the above described latent form of infection efficiently hides HSV-1 from the

destructive immunological responses and can be viewed as one mechanism of immune evasion. Other immune evasion strategies of HSV-1 include interference with DC function, inhibition of MHC class I and II peptide presentation, inhibition of type I IFN response and inhibition of complement- and antibody-mediated defence.

DCs act as major antigen presenting cells and are pivotal for the initiation of specific immune responses. Infection of DCs by HSV-1 interferes with DC activation, downregulates the expression of the key cell surface co-stimulatory molecules, and impairs the cytokine production critical for the activation of T cells (Kruse *et al.*, 2000; Salio *et al.*, 1999). Importantly, HSV infected DCs secrete less IL-12 (Salio *et al.*, 1999), which is a critical cytokine for the polarization of naïve T cells into Th1 cells which characteristically produce high amounts of IFN-γ. HSV-1 also downregulates the expression of chemokine receptors CCR7 and CXCR4, which leads to impaired migration of DCs to the peripheral lymph nodes and reduced presentation of antigens to naïve T cells (Prechtel *et al.*, 2005).

Antigen presentation to both CD4+ and CD8+ T cells is affected by HSV-1, since HSV-1 infection impairs the presentation exerted by both MHC class I and II molecules. This is very detrimental to the function of DCs as they are presenting antigens to both CD4+ and CD8+ T cells. MHC class I effect is mediated by HSV-1 α protein, ICP47 (Hill *et al.*, 1995). ICP47 inhibits antigen processing by forming a complex with transporter associated with antigen presentation (TAP) thereby blocking presentation of antigen peptides by MCH class I on the cell surface. Furthermore, cells infected with HSV-2 vhs-deletion virus are recognized by CD8+ cytotoxic T lymphocytes (CTL) less efficiently (Tigges *et al.*, 1996). IFN-γ has been shown to partially restore MHC class I presentation in fibroblasts and keratinocytes, further indicating a central role for this cytokine in defence against HSV (Cunningham & Noble, 1989; Koelle *et al.*, 2001; Tigges *et al.*, 1996). HSV-1 interrupts antigen processing by MHC class II by downregulating the expression of the invariant chain, and by gB forming complexes with HLA-DM and HLA-DR polypeptides resulting in inhibition of HLA-DR-gB complex transportation to the cell surface (Neumann *et al.*, 2003). MHC class II expression may be further downregulated by induction of TGF-β1 expression in HSV-1 infected mononuclear cells (Mendez-Samperio *et al.*, 2000). ICP22 has been shown to greatly reduce the antigen presentation efficiency of human B lymphoblastoid cells to CD4+ T cells (Barcy & Corey, 2001). CD4+ T cells have been shown to be critical for the priming of CTLs both at the initial expansion phase of HSV-specific CTL clone, and upon generation of memory CTLs and immunity to HSV (Smith *et al.*, 2003).

Antibodies seem to provide relatively inefficient protection against HSV infection. This may be attributed at least in part to Fc-receptor (FcR) activity of HSV. Heterodimer of envelope glycoproteins gE-gI form a high-affinity FcR for the Fc domain of both monomeric and aggregated IgG, whereas gE alone has lower affinity and only binds IgG complexes (Dubin *et al.*, 1990). Upon binding of anti-HSV-1-IgG to its specific target by Fab fragments, Fc domain is bound to HSV-1 FcR (Dubin *et al.*, 1991). This interaction prevents the effector cells from recognizing the antiviral antibody and complement system

C1q binding to Fc, whereby infected cells are protected from antibody-dependent cellular cytotoxicity and complement-mediated lysis, respectively.

Type I interferons, IFN-α and IFN-β, interfere with viral replication at multiple steps, but HSV has developed several mechanisms to overcome these effects. HSV ICP34.5 is able to activate a phostaphatase, which dephosphorylates eIF-2α. This leads to reverse action of PKR, the interferon-inducible dsRNA-activated protein kinase R, resulting in continuing of the translational activity and arrest of autophagy (see: Metabolic changes in the host cell). Recently it was shown that for complete IFN-mediated translational arrest, in addition to ICP34.5, also U_s11 is required (Mulvey *et al.*, 2004). Furthermore, HSV vhs blocks the IFN-inducible phosphorylation and activation of Stat1, Stat2 and Janus kinases (Chee & Roizman, 2004; Yokota *et al.*, 2001), and ICP0 inhibits the induction of multiple IFN-stimulated genes (Eidson *et al.*, 2002) through degradation of promyelocytic leukemia protein (Chee *et al.*, 2003), an organizer of nuclear domain 10 structures. All in all, HSV has a dual effect on the expression of IFN-stimulated genes and IFN-α/β: Whereas HSV virions induce the expression, viral protein synthesis has an inhibitory effect (Mossman *et al.*, 2001; Nicholl *et al.*, 2000). Antagonism of IFN response is indicative of an immune evasion mechanism in the very early stages of infection.

HSV-1 has developed means to evade both the classical and the alternative pathways of the complement system. Envelope glycoprotein, gC, binds a central component of the complement system, C3, and its activation products C3b, iC3b and C3c, and also accelerates the decay of the alternative complement pathway C3 convertase (Fries *et al.*, 1986; Kostavasili *et al.*, 1997). Since gC is expressed both on cell-free HSV-1 virions and on the plasma membrane of an infected cell, it protects both the virion from complement-mediated neutralization and the infected cell from destruction. Furthermore, HSV-1 gC interference with C5 and properdin binding to C3b inhibits the complement activation cascade (Fries *et al.*, 1986; Kostavasili *et al.*, 1997).

As a novel immune evasion mechanism, the secreted part of HSV-2 gG was recently reported to activate phagocytes to release reactive oxygen species, which were shown to inhibit NK cell cytotoxicity and to accelerate apoptosis of NK cell enriched lymphocyte populations (Bellner *et al.*, 2005).

HSV-1 is capable of infecting activated T lymphocytes, which may then be killed by HSV-specific CTLs (Raftery *et al.*, 1999). This fratricide may serve as an additional immune evasion mechanism since in this model, infected T cells serve as decoy targets and kill each other instead of keratinocytes, which are mainly responsible of HSV-1 replication in the infected skin or mucosa.

3. ORAL ENVIRONMENT IN HSV INFECTION

3.1. Structure of oral mucosa

Oral mucosa forms the first line of defence against orally invading pathogens and toxic or irritant agents, and provides protection against mechanical stress and excessive dehydration (Figure 7). Oral epithelium is continuously bathed by saliva which further reinforces the protective function of the mucosa (see: Defence mechanisms in the oral cavity).

3.1.1. Epithelium

Oral epithelium is typed as stratified squamous epithelium, reviewed in Squier & Kremer (2001). It is further regionally specialized in order to meet the varying requirements for mechanical stress in different parts of the mucosa. *Lining mucosa* is the predominant type of oral mucosa covering up to 60% of the total oral mucosal area. This type of mucosa is nonkeratinized, very elastic and movable. It is loosely attached to underlying structures by connective tissue. This arrangement allows the movements required for speech, mastication and bolus formation before swallowing. *Masticatory mucosa* covers the hard palate and gingiva, which are areas having to endure great masticatory forces. Masticatory mucosa is keratinized and directly attached to the underlying periosteum without intervening submucosa. This firm and rather inelastic anatomical structure is called a mucoperiosteum. The third type of mucosa is a mixture of both keratinized and nonkeratinized epithelium. Such *specialized epithelium* covers the dorsum of the tongue, and is firmly attached to the muscles of the tongue. Extraorally, oral mucosa is delineated to the skin at the vermilion border of the lip, the site where extra-oral red or pink lip tissue joins the facial skin.

Stratified oral epithelium consists of multiple cell layers in which cells undergo differentiation while moving from the deepest basal layer towards the surface, reviewed in Squier & Kremer (2001). The only cells that divide within the epithelium are the basal cells. These cells provide epithelium with new cells while the differentiated cells are desquamated from the surface. Tissue homeostasis prevails when these two events are in balance. The basal progenitor cells fall into two functional groups. The smaller population of these cells have a low rate of mitosis, and represent the epithelial stem cells that provide epithelium with the major population of progenitor cells, the transient amplifying cells (Bickenbach, 1981; Hume & Potten, 1979; Hume & Potten, 1983). Transient amplifying cells undergo a limited number of mitosis and enter subsequently the differentiation process. The proliferation and maturation activity is influenced by the cytokines and growth factors available (Feliciani *et al.*, 1996), and the switch from proliferation to differentiation by multiple substances, including ions, retinoic acid and vitamin D (Dotto, 1999). Turnover time, the time it takes for a basal cell to transit through

the epithelium and desquamate, is longer in keratinized than in nonkeratinized epithelium. Consequently, turnover time varies in different sites of oral mucosa being for example approximately 14 days in the buccal mucosa and 24 days in the hard palate (Thomson *et al.*, 1999).

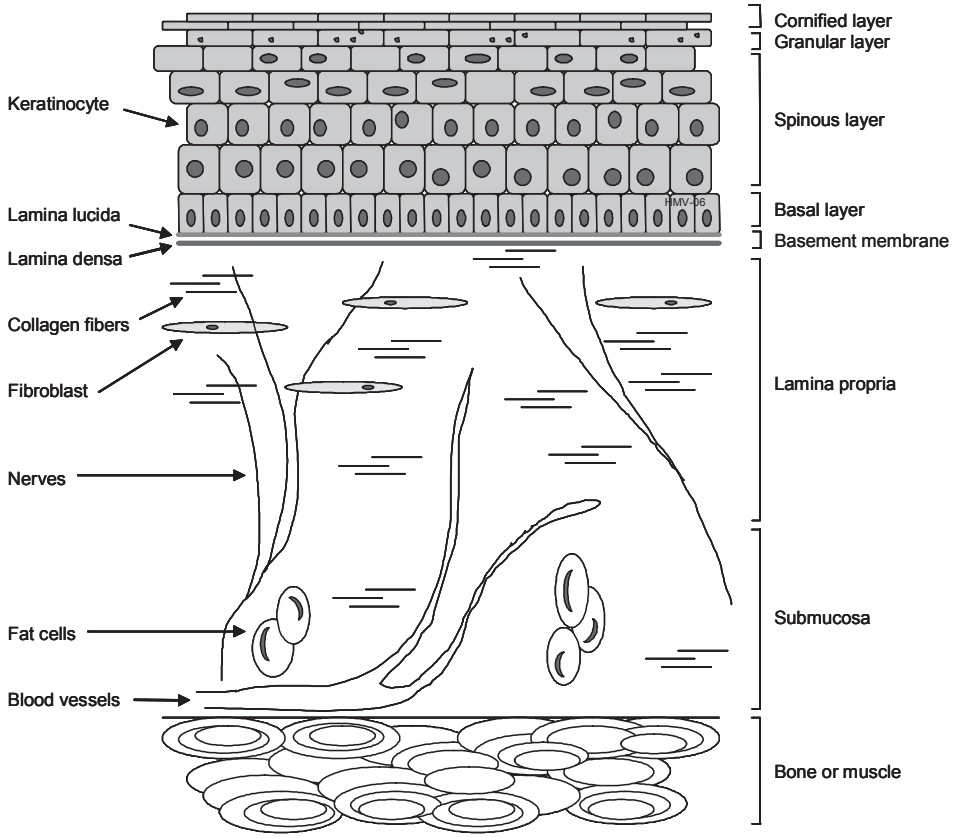

Figure 7. The structure of oral mucosa.

Basal cells are attached to the *basement membrane* (BM) by hemidesmosomes, reviewed in Scully *et al.* (2005). BM is a thin layer of specialized extracellular matrix composed of two structurally and functionally distinct layers: Lamina lucida provides attachment for epithelial cells via hemidesmosomes, and the underlying lamina densa mediates attachment to connective tissue by collagenous anchoring fibrils. When basal cells start to differentiate, they loose this attachment and begin to migrate, reviewed in

Squier & Kremer (2001). Migrating cells are intercellularly connected by desmosomes, which serve as anchorage sites for intermediate filaments of keratinocytes, the keratin filaments. Differentiation involves changes in the intracellular proteins, such as the cytokeratins (Upasani *et al.*, 2004).

Basal cells of the *basal layer* are cuboidal in shape, but as cells start to differentiate, they first become bigger and then flatten gradually. In the above *spinous layer*, cells start to accumulate lipids, and retain certain precursor proteins for the thickening of the cell membrane in the upper layers. Accumulation of keratohyalin granules is typical for the cells in the above *granular layer*. These cells also synthesize proteins, including profilagrin, loricrin, involucrin and cornifin that are known to be important for the final stages of epithelial differentiation. In the outer granular layer and in the topmost *cornified layer*, plasma membranes get reinforced on the cytoplasmic surface by cross-linked protein layer. The lipids are extruded from the cells at this layer interface. This interface also marks the boundary between metabolically active inner epithelium and the dead outmost layer, the cornified layer. Cells in the cornified layer have lost the nucleus, are packed with proteins (e.g., cytokeratin filaments) and surrounded by extracellular lipids. In the nonkeratinized epithelium, the most superficial cells also flatten but still retain the nucleus and other cellular organelles, and do not accumulate protein filaments to the same extent as the terminally differentiated cells.

In addition to keratinocytes, a variety of other cells have been identified in the oral epithelium. These include the pigment-producing melanocytes, Merkel cells associated with sensory functions, Langerhans´ cells which act as professional APCs, and a varying collection of inflammatory cells, most frequently lymphocytes. These nonkeratinocytes constitute about 10% of the total cell number of the oral epithelium, reviewed by (Squier & Kremer, 2001). Evidently, keratinocytes influence the function of the epithelial nonkeratinocytes and the cells of the underlying connective tissue by secreting various cytokines (Feliciani *et al.*, 1996).

3.1.2. Connective tissue

Connective tissue or mesenchyme is separated from the epithelium by the BM. Connective tissue provides epithelial cells with nutrients and gives mechanical support. Importantly, mesenchymal-epithelial interactions also affect epithelial growth and differentiation (Smola *et al.*, 1993).

The outmost layer of the connective tissue, the *lamina propria*, consists of sparsely distributed cells, mainly fibroblasts, and of an extensive extracellular matrix (ECM) composed of different macromolecules including collagen, elastic fibres and various glycoproteins. Collagen fibres, the most abundant component of the ECM, provide lamina propria with structural support and elastic fibres with resilience. Glycoproteins bind water and participate to the diffusion of nutrients and various signalling molecules within the tissue. The thickness and the collagen content of lamina propria varys greatly between different oral regions (Squier & Kremer, 2001). Whereas it is very thin but densely packed with collagen fibres in the areas of masticatory mucosa, it is thicker but contains

fewer collagen bundles in the areas of lining mucosa. Lamina propria also harbours nodules of lymphoid tissue, which can be extensively infiltrated by lymphocytes or plasma cells. In some people, sebaceous glands can be seen submerged in the lamina propria in the upper lip and buccal mucosa.

Lamina propria is difficult to delineate from underlying tissues in the oral region because of the lacking muscularis mucosae (Squier & Kremer, 2001). Where present, *submucosa* consists of loose fatty or glandular connective tissue containing blood vessels and nerves serving the mucosa. In addition, minor salivary glands, which are connected to oral cavity via short ducts, reside in submucosa. From below, submucosa is demarcated by either muscle or bone.

3.2. Orolabial HSV infections

The most common way to get infected with HSV in the oral region is either by mucocutaneous contact to a HSV lesion or by exposure to infectious secreta. Infectious virus may originate from a primary or recurrent lesion or be shed asymptomatically to saliva.

3.2.1. Epidemiology

The epidemiology of HSV infections is changing. Earlier, the primary HSV infection was typically an orofacial HSV-1 infection, and the seroprevalence reached 75-85% already in the five-year-olds. With the commencement of sexual life, people then gradually became infected with HSV-2, and seroprevalence in adults has commonly been approximately 20% in Western countries. Recent studies indicate that particularly in the countries of high hygiene standards, primary HSV infection is at present gained considerably later: In Northern European countries, most teenagers are still HSV-seronegative (Pebody *et al.*, 2004). For example, only about 20-30% of Finns of 20 years of age are HSV-seropositive. Subsequently, primary HSV infection is increasingly more likely to be a genital rather than an orofacial infection. Also the frequency of oral infections by HSV-2 is increasing. Whereas earlier HSV-1 was almost the exclusive HSV type isolated from the oral region (Docherty *et al.*, 1985), recently up to 15-20% of oral HSV infections have been reported to be due to HSV-2 (Wolff *et al.*, 2002). The frequency of genital HSV-1 infections has increased reciprocally. These changes are hypothesized to be at least in part attributable to changes in sexual habits. The identified risk factors for oral HSV-2 infection are the male sex, human immunodeficiency virus (HIV) infection and homosexuality (Wald *et al.*, 2004).

3.2.2. Orofacial primary infection

In the majority of the cases, primary infection in the oral region is subclinical, but still inevitably leads to the establishment of latent infection in the trigeminal ganglion. Less than 10% of those affected have clinical symptoms in association with primary infection, and only in about 1% symptoms of classical HSV *gingivostomatitis* develop. This condition is characterized by eruption of multiple vesicles that subsequently develop into ulcers. Lesions are very painful and extensively distributed on areas of both lining and masticatory mucosa. Also lips and perioral skin may be affected. Development of lesions is accompanied by malaise, fever and lymphadenopathy. Particularly in small children, the severe oral pain may lead to refusal of eating and drinking, which creates a risk for dehydration. Symptoms associated with gingivostomatitis typically last for 10-14 days. (Whitley, 2001).

In young adults, primary infection may lead to pharyngitis and mononucleosis-like disease. Of all oral HSV infections, pharyngitis-like disease appears to be most commonly associated with HSV-2 primary infection (Corey *et al.*, 1983). Pharyngitis is clinically observed as mild erythema, or in severe cases as ulcerations or exudative lesions of the pharynx. HSV pharyngitis has also been reported to lead to airway obstruction (Tustin & Kaiser, 1979).

3.2.3. Reactivation

HSV can reactivate in the orofacial region either with (*recrudescence*) or without (*recurrence*) symptoms. Recurrent disease is estimated to occur in about 20-40% of seropositive individuals (Wheeler, 1988). Upon reactivation, latent HSV begins replication in the sensory ganglion, and progeny virus is transported along the axon in the anterograde direction towards the periphery. Released virus then infects the epithelium at the innervation area of the sensory neuron. Although the exact mechanisms of HSV reactivation are largely unknown, it is empirically acknowledged that orolabial recrudescence can be triggered by factor like stress, exposure to sunlight, drying of lips, trauma, hormonal changes, and immunosuppression. UV-irradiation given experimentally to lips led to the eruption of herpetic lip lesion within 1 week in 43% of subjects with history of recurrent disease following sun exposure (Kriesel *et al.*, 1994).

The most typical symptom ensuing reactivation is *recurrent labial herpes* (RLH) or “cold sore”. It begins with prodrome symptoms, which include itching, tingling or burning sensation in the epithelial area corresponding to the innervation area of the neuron in which reactivation has occurred. Sometimes reactivation is terminated at this stage (abortive lesion). If the development of a lesion continues, an erythematous area can soon be observed. Erythema is followed by sequela of papule, vesicle, ulcer and crusting stages, and finally healing. The episode takes 8-10 days, and throughout the vesicle stage, infectious virus is present in the lesion. Similar to primary infection, lesions are a risk for

autoinoculation of the eye and also to herpetic whitlow, painful infection of the periungal region of the finger. (Whitley, 2001).

HSV type and the anatomical site appear to be critical determinants for oropharyngeal and also genital reactivation frequency (Lafferty *et al.*, 1987): Following primary infection, oropharyngeal HSV-1 reactivation occurs less frequently than genital HSV-2 reactivation. Furthermore, HSV-1 reactivates at a significantly higher rate (0.12 times per month) in the oropharyngeal region compared to HSV-2 (0.001 times per month) and visa versa in the genital region. Substituting HSV-2 with HSV-1 LAT results in HSV-2 reactivation rate comparable to that of HSV-1 in rabbit trigeminal ganglion (Yoshikawa *et al.*, 1996) suggesting that efficient function of LAT is anatomically restricted.

The most common site of recrudescence is the vermilion border of the lip. Depending on the nerve where reactivation has taken place, recrudescent lesions can develop also on any other extra-oral skin area, but rarely intraorally. Intraoral recrudescences are most commonly observed in the immunocompromised hosts or after a trauma to orofacial tissues from procedures, such as dental operations or surgery of the trigeminal nerve. The appearance of these trauma triggered lesions peak at 2-3 days after manipulation (Raborn & Grace, 1999). Intra-oral lesions erupt almost solely in the areas of masticatory mucosa, unless the host is severely immunocompromised. In the immunocompetent host, oropharyngeal recrudescences are self-limited and considered very local, although recent data suggests that labial herpes does result in most instances also to HSV viremia (Youssef *et al.*, 2002).

HSV reactivation has also been suggested to attribute to inflammatory disease of the periodontium. HSV antigens have indeed been detected in stratified gingival epithelium in these conditions, in particular in patients with aggressive periodontitis (Ehrlich *et al.*, 1983; Prato *et al.*, 2002). Pathogenetic mechanisms have been suggested to include HSV-induced increase in the inflammatory process or direct destruction of these tissues by replicating virus. Even cooperation between certain known parodontopathogens and herpesviruses has been suggested (Saygun *et al.*, 2004). The exact contribution of HSV to these diseases remains debatable, because HSV antigens have been detected also in up to 40% of samples of healthy gingiva from subjects with unknown HSV-serostatus, with infectious HSV isolated from only one sample (Amit *et al.*, 1992). Furthermore, the data on causality between herpesviruses and periodontal disease is still lacking.

Despite the apparent frequency of HSV recrudescences, the asymptomatic recurrences are even far more common. 2-5% of the immunocompetent population sheds HSV asymptomatically to saliva (Whitley, 2001) and frequently with high copy numbers (Kaufman *et al.*, 2005; Miller *et al.*, 2005). Naturally, shedding is far more common, if only HSV seropositive subjects are included, although some seronegative subjects have also been suggested to shed HSV (Kaufman *et al.*, 2005). Conflicting data exists on the impact of history of recurrent disease on shedding frequency (da Silva *et al.*, 2005; Knaup *et al.*, 2000). HSV shedding in association with false prodromes has been reported to be as high as 60% (Spruance, 1984), whereas other studies suggest that shedding occurs independently of even labial herpes recrudescences (da Silva *et al.*, 2005; Kriesel *et al.*, 1994).

Some of the factors empirically known to trigger labial herpes have also shown to increase shedding of HSV to saliva. By viral isolation, the observed shedding frequency was as high as 20% in oral surgery patients, and 38% in the immunocompromised patients compared to 4.5% in healthy subjects (Kameyama *et al.*, 1988). Further, the duration of shedding is prolonged in these patients, and in some immunocompromised patients shedding may even be considered to be almost continuous (Kameyama *et al.*, 1988). Increased shedding in association with oral surgery procedures or trauma has been confirmed by other studies (Kameyama *et al.*, 1989; Spruance, 1984; Tateishi *et al.*, 1994). Even a routine dental procedure enhances greatly both asymptomatic shedding and clinical reactivation of HSV (Miller *et al.*, 2004). Shedding frequency in association with common cold has been reported to be 21% (Spruance, 1984). Furthermore, shedding appears to be related to the age of an individual, as those younger than 10 or older than 60 years of age, have been shown to shed the most frequently (Tateishi *et al.*, 1994). Genetic factors involving immune functions may also contribute to the susceptibility to reactivation in the orofacial region (Seppanen *et al.*, 2001).

The special characteristics of oral shedding of HSV-2 have not been elucidated much yet. Consistent with infrequent clinical recrudescence of HSV-2 in the orolabial region (Lafferty *et al.*, 1987), oral HSV-2 shedding has been reported to be far more infrequent than HSV-1 shedding (Wald *et al.*, 2004). Similar to HSV-1 infection, immunosuppression has been shown to be a risk factor also for HSV-2 shedding (Wald *et al.*, 2004). Oral shedding occurs frequently in association with the first episode of genital HSV-2 or during genital recurrence of HSV-2 (Wald *et al.*, 2004).

3.2.4. Source of HSV in saliva

It is generally accepted that HSV contaminates saliva as a result of reinfection of oral epithelium after reactivation of the virus in the ganglion. As an alternative or additional possibility, it has been suggested that HSV could remain dormant also in oral tissues, including oral epithelium and salivary glands, and reactivate therefrom. In support of this view, HSV antigens have been detected in healthy gingival and periodontal tissues suggesting extraneuronal residence for nonreplicating HSV (Amit *et al.*, 1992; Zakay-Rones *et al.*, 1973; Zakay-Rones *et al.*, 1986). However, antigen detection techniques may give false positive results, because tissues may harbour APCs carrying HSV antigen (Ono *et al.*, 2005). In only one study, HSV has been isolated from healthy gingiva (Amit *et al.*, 1992). Alternatively, salivary glands and ocular tissue have been proposed as sites for dormant HSV, intermittent HSV replication and the origins of salivary HSV (Kaufman *et al.*, 1967).

3.3. Defence mechanisms in the oral cavity

Oral cavity is the major portal of entry for numerous infectious agents. Oral mucosa forms the mechanical barrier against invading micro-organisms. Cells of the epithelium and the lamina propria also actively participate in the immunological defence of the oral cavity. Serum-derived components of oral defence predominantly enter the oral cavity mixed with *gingival crevicular fluid* (GCF), which accumulates around the cervical areas of the teeth. Moreover, oral mucosa is continuously bathed by saliva, which lubricates the mucosal surfaces facilitating speech and bolus formation, and provides physical and chemical protection against toxic and microbial agents.

Saliva is a mixture of secreta from minor and major salivary glands, reviewed in Pedersen *et al.* (2002). These glands can be further classified according to the type of gland secretory end-piece, the *acini*, that can be either serous, mucous or mixed. Major salivary glands are paired organs. The parotid gland acini are uniformly serous. Sublingual and submandibular glands are mixed, although mucous acini predominate in the sublingual and serous acini in the submandibular glands. Minor salivary glands, hundreds in numbers, are scattered around the oral submucosa, and secrete mainly mucous saliva, although the palatal glands are uniformly mucous and the lingual glands serous. These minor glands apparently account for a certain degree of baseline salivary secretion without responding significantly to stimulation.

Acini secrete fluid composed of water and electrolytes, including bicarbonate that mainly accounts for the buffer effect in saliva (Ericsson, 1959). Acini are also the major site of salivary protein and glycoprotein synthesis, which explains the differences in organic salivary components between different types of glands.

Saliva is released to *ductal system* that is comprised of various ductal cell types, reviewed in Laine (1991). Listed from the acinar end to the oral cavity, these may include intercalated, granular, striated and excretory ducts depending on the gland. While in passage through the complex ductal system, the primary fluid is extensively modified by the ductal cells, which actively change the electrolyte and ion content of saliva without reabsorbing any water to produce a final hypotonic fluid that is excreted to the oral cavity. Unstimulated saliva is mainly modified by excretory ducts and stimulated saliva by striated ducts, which partly explains the marked differences in the contents of these two types of saliva. Granular ducts contribute to the modification the salivary protein composition. Secretion rate affects the concentration of both inorganic and organic saliva constituents (Ferguson, 1981). Therefore, secretion rate has a pivotal role in determining saliva composition, although many other factors, such as hormones, additionally contribute to the final contents of saliva (Laine, 1991).

All in all, whole saliva bathing oral mucosa is a complex blend of water, electrolytes and salivary gland -derived organic compounds that mix in the oral cavity with exfoliated epithelial cells, leukocytes, cytokines and growth factors, gingival fluid, food debris and a rich mixture of various microbes.

3.3.1. Innate defence mechanisms

Innate defence mechanisms need no earlier contact to the antigen to be fully active, reviewed in Tenovuo (1998). They target antigens in a nonspecific manner, and are functional almost at full capacity already in early childhood. Innate cellular mechanisms include NK cells and the major phagocytic cells, the polymorhonuclear neutrophils (PMN) and the macrophages. PMNs are recruited to the site of inflammation and phagocytose targets opsonised by complement or antibodies. Macrophages, derived from monocytes, are long-lived in tissues and specialized in phagocytosing intracellular organisms. NK cells kill their target cells by releasing perforin. The numerous soluble innate defence factors in oral tissues and cavity are derived either from saliva, gingival crevicular fluid or the cells within the supporting tissues (Table 2).

Table 2. The major soluble salivary constituents, their origins and the main known targets of antimicrobial activity. Modified from Van Nieuw Amerongen *et al.* (2004).

Salivary protein	Tissue of origin	Antimicrobial activity
Immunoglobulins	B cells; all salivary glands, GCF	Bacterial, fungal
Lactoferrin	all salivary glands, PMN	Bacterial, viral
Lysozyme	all salivary glands, cells of the myeloid lineage	Bacterial
Salivary peroxidase	parotid, submandibular gland	Bacterial, viral*
Myeloperoxidase	PMN cells	Bacterial, viral*
Cystatins	major salivary glands, GCF	Bacterial, viral
Amylase	parotid, sublingual gland	Non known
Mucins	minor and major salivary glands	Bacterial, fungal, viral
Histatins	parotid, submandibular glands	Fungal, bacterial
Proline-rich glycoprotein	parotid gland	Bacterial, viral
Agglutinin	all major salivary glands	Bacterial
Defensins	all salivary glands, epithelial cells, PMN	Bacterial, viral
SLPI	minor salivary glands, mucosal epithelial cells	Viral

GCF, gingival crevicular fluid; SLPI, secretory leukocyte protease inhibitor

* The main antimicrobial activity of the oxidation products from reactions catalyzed by the enzyme.

Defence mechanisms of saliva

The most salient soluble salivary defence mechanisms with regard to this thesis are introduced below.

Peroxidase systems

Peroxidase activity is found in various exocrine secretions of the body. Saliva contains two different peroxidases, salivary peroxidase (hSPO) and myeloperoxidase (hMPO), reviewed in Ihalin *et al.* (2006). hSPO is secreted by two of the major salivary glands, the parotid gland and the submandibular gland, whereas hMPO is released from the PMNs. In whole saliva, these two peroxidases are mixed although hMPO is thought to represent the major proportion of the total activity in saliva (Thomas *et al.*, 1994). Enzymatically active peroxidases are also retained in the oral biofilm, which covers the teeth and the mucosal surfaces (Pruitt & Adamson, 1977; Tenovuo *et al.*, 1977).

hSPO and hMPO catalyze the oxidation of halides iodide (I^-), bromide (Br^-) and thiocyanate (SCN^-) to hypohalides in the presence of hydrogen peroxide (H_2O_2). In addition, hMPO also oxidizes chloride (Cl^-). Salivary H_2O_2 originates from the parotid gland, PMNs and oral microbes, predominantly the facultative anaerobes. H_2O_2 concentration varies greatly due to its extremely reactive nature, but is estimated to be 8-13 µM (Pruitt *et al.*, 1986). In saliva, the major substrate for the reaction is SCN^- for both of the peroxidases. SCN^- concentration varies considerably because it is influenced heavily by factors like diet and smoking habits. In non-smokers, the concentration is in the range of 0.5-2 mM, but can reach 6 mM in heavy smokers (Tenovuo & Makinen, 1976). At pH 6-8, the major oxidation product of SCN^- is hypothiocyanite ($OSCN^-$) (Aune & Thomas, 1977; Pollock & Goff, 1992), which is in equilibrium in the aqueous solution with HOSCN (pK_a value 5.3) (Thomas, 1981). SCN^- reaction with H_2O_2 in the oral cavity can be summarized as follows:

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O$$
$$OSCN^- + H^+ \rightarrow HOSCN$$

The consumption of H_2O_2 protects tissues from toxicity of oxygen species, and peroxidase systems exert a broad range of antimicrobial properties, including antibacterial, antiviral and antifungal activity, reviewed in Ihalin *et al.* (2006). The antimicrobial effect of oxidized halides is greater in the protonated form (Thomas, 1981), presumably due to the greater capability of non-ionized molecules to permeate hydrophobic membranes. Furthermore, acidic pH is known to enhance the antibacterial effects of HOSCN/$OSCN^-$ (Thomas *et al.*, 1983).

hMPO system inactivates and modifies all major proteins of influenza virus (Yamamoto *et al.*, 1991). Pretreatment of poliovirus (Belding *et al.*, 1970), HSV (Courtois *et al.*, 1990) or HIV (Pourtois *et al.*, 1990) with bovine lactoperoxidase (bLPO) system

has been shown to inactivate these viruses. hMPO and bLPO system -mediated inactivation of HIV virions is time and concentration dependent (Chochola *et al.*, 1994; Yamaguchi *et al.*, 1993). The virucidal effect of monocyte-derived hMPO system on HIV is decreased following differentiation of these cells to macrophages due to decrease in intracellular level of hMPO (Chase & Klebanoff, 1992).

Lactoferrin

Lactoferrin (Lf) is a 78 kDa monomeric glycoprotein capable of binding two iron atoms, reviewed in Weinberg (2001). Lf is present in many exogenous secretions in humans, including saliva, milk and tears. It is also the major constituent of the secondary specific granules of the PMNs. Lf is a multifunctional protein with antimicrobial, immunomodulatory and tumour growth suppressing properties.

The antibacterial effect is mainly attributed to efficient deprivation of iron from bacteria. Since acidic pH enhances the ability of Lf to retain iron, this deprivation is particularly efficient at sites of inflammation. Inhibition of viral replication presumably depends on different mechanisms. The main inhibitory mechanism reported this far is mediated by inhibition of viral attachment to host cell receptors by either Lf binding to the virion or the receptor. Such inhibition has been observed for HSV-1, HSV-2, human cytomegalovirus, HIV, hepatitis C virus, human papillomavirus, hantavirus, and poliovirus (Drobni *et al.*, 2004; Harmsen *et al.*, 1995; Ikeda *et al.*, 1998; Marchetti *et al.*, 1996; Marchetti *et al.*, 1999; Murphy *et al.*, 2000). The inhibition of HSV-1 attachment has been suggested to be mediated by Lf binding to heparan sulphate (Andersen *et al.*, 2004). Lf has been shown to block DC capture of HIV (Groot *et al.*, 2005). Furthermore, the inhibition of HSV (Marchetti *et al.*, 1998) and HIV (Puddu *et al.*, 1998) has been shown to correlate with the degree of Lf iron-saturation, whereas iron alone has no inhibitory effect. The effect of iron-saturation is thus likely to result from conformational changes occurring upon iron binding to Lf rather than being the effect of iron as such.

Proteolytic digestion of human or bovine Lf by pepsin yields a 10-47 amino acid long N-terminal fraction called lactoferricin (Weinberg, 2001). Physiologically such cleavage may occur while Lf is in transit through gastrointestinal tract, or alternatively by neutrophilic phagolysosome derived pepsin-like proteases. This peptide is highly cationic, and has been shown to display diverse iron-independent antimicrobial activity and immunomodulatory functions.

Lysozyme

Salivary lysozyme (Lz) is derived from cells of the myeloid lineage and salivary gland epithelial cells. It is a 14.3 kD protein with high cationic net charge and muramidase activity, reviewed in Hannig *et al.* (2005). Lz hydrolyzes the β(1,4)-glycosidic bond between *N*-acetyl muramic acid and *N*-acetylgucosamine in the bacterial cell wall

peptidoglycan layer. Degradation of this integral cell wall results in electrolyte and osmotic changes and eventually cell death. Consequently, Gram-negative bacteria are less susceptible to the enzymatic effects of Lz. Furthermore, Lz inhibits anionic glucosyltransferase activity, and has a dual role on the bacterial adherence, since it can either enhance or inhibit bacterial adherence to the oral biofilm. Lz also displays enzyme independent antimicrobial activity by its polycationic properties, as shown by Lz activation of bacterial autolysins (Laible & Germaine, 1985). Lz retains its activity also in oral biofilms (Orstavik & Kraus, 1974), and is active in a wide pH range (Davies *et al.*, 1969).

Although, antibacterial effects of Lz are well characterized, very little is known about the possible antiviral efficiency. Results on the anti-HSV activity of native Lz are contradictory (Cisani *et al.*, 1989; Oevermann *et al.*, 2003), but evidence exists on Lz preventing HIV replication possibly through its hydrolytic activity (Lee-Huang *et al.*, 1999).

Cystatins

Cystatins are proteins that possess cysteine peptidase inhibitor activity, reviewed in Amerongen & Veerman (2002). Cystatins bind to cysteine peptidase tightly, but reversibly. This binding leads to formation of enzymatically inactive complex that contributes to the control of proteolytic enzyme activity presumably both in host tissues and micro-organisms. Cystatin family is large, and ubiquitously present in many tissues and body fluids. Salivary cystatins are mainly derived from the major salivary glands, and to lesser extent from the gingival crevicular fluid. At least nine cystatin isoforms have been identified in saliva. Of these, the anionic cystatin S is the most abundant, and is present in three or four isoforms. The other well characterized cystatins are the neutral cystatin SN, moderately anionic cystatin SA, cationic cystatin C and the most recently identified cystatin D. Cystatins S, SA and SN are often together referred to as S–like cystatins and with cystatin D, SD-like cystatins. Unlike other cystatins, cystatin A is present also in the gingival crevicular fluid in addition to saliva (Blankenvoorde *et al.*, 1997).

Cystatin C is present only in trace amounts in saliva under healthy conditions. Under inflammatory conditions, like periodontitis, elevated level of cystatin C has been detected in saliva (Henskens *et al.*, 1996a). Consistently, cystatin C levels were shown to normalize following treatment (Henskens *et al.*, 1996b). Unlike cystatin C, which appears to be associated with inflammatory conditions, the level of cystatin S, the major salivary cystatin seems to be higher in periodontitis-free patients (Henskens *et al.*, 1996a), suggesting a protective function for cystatin S against periodontal disease.

Cystatin C inhibits HSV-1 replication at molar concentrations lower than that required for acyclovir (Bjorck *et al.*, 1990). Also cystatin S interferes with HSV-1 replication, although less efficiently than cystatin C (Gu *et al.*, 1995). Moreover, cystatin C (Collins & Grubb, 1991) and D (Collins & Grubb, 1998) inhibit coronavirus replication.

The possible mechanism for the inhibition of viral replication could be interference with capsid maturation for which host or viral cysteine peptidases are required.

Amylase

α-Amylase is the most abundant salivary enzyme, reviewed in Hannig *et al.* (2005). Most of it is secreted by the parotid gland and the minor proportion by the sublingual gland. In whole saliva, 5% of the total protein content consists of amylase, and it is the major constituent of oral biofilms. Amylase catalyses the hydrolysis of α(1,4)-glucosidic linkages in dietary starch.

Amylase is not known to possess any antimicrobial activity. Meanwhile, it does bind to various oral bacteria facilitating adherence to oral biofilms, and provides bacteria with glucose by breaking down starch.

Mucins

Mucins constitute the major protein fraction of mucous secreta covering epithelial surfaces, making up to 20-30% of all proteins in unstimulated saliva, reviewed in (Offner & Troxler (2000). Mucins give saliva its viscoelastic and lubricating properties, and provide epithelial cells protection from invading micro-organisms and noxious substances. Mucins are an integral part of oral biofilms.

Mucins are large mono- or multimeric glycoproteins with a polypeptide backbone and covalently linked carbohydrate side chains. The 11 known mucins are divided into three groups: the large gel-forming mucins, the large membrane-associated mucins and the small soluble mucins. To date, four mucins have been identified in oral tissues. The most abundant salivary mucin, MUC5B (former MG1), is a high-molecular-weight large gel-forming mucin (M_r 10-30 MDa). It is an elongated multimeric protein with abundant carbohydrate side chains which yield a high degree of hydrophobicity. MUC5B is secreted by the salivary gland mucous acinar cells (Veerman *et al.*, 2003). MUC7 is a member of the small soluble mucins (M_r ~130 kDa), and was earlier referred to as low-molecular-weight mucin or MG2. It is a monomeric protein with short carbohydrate side chains. It is secreted by demilune cells of the (sero)mucous salivary glands and by serous acinar cells of the submandibular gland (Veerman *et al.*, 2003). The most recently discovered oral mucins, MUC1 and MUC4, belong to the family of large membrane-associated mucins, but are also secreted to some degree. RNAs of these mucins have been detected in all major salivary glands and labial glands (Offner & Troxler, 2000), suggesting that these mucins may be expressed by both serous and mucous acini. Moreover, MUC1 and MUC4 transcripts are expressed by buccal epithelial cells (Offner & Troxler, 2000).

Mucins have also been implicated in antimicrobial defence. Despite diverse carbohydrate chains potentially providing binding sites for bacterial adhesins, MUC5B

appears to bind to relatively few oral bacterial species, whereas MUC7 displays a more diverse bacterium binding capability, reviewed in Van Nieuw Amerongen *et al.* (2004). Furthermore, mucins have been shown to inhibit HIV replication, aggregate HIV virions (Bergey *et al.*, 1994) and neutralize HIV (Bolscher *et al.*, 2002).

Defence mechanisms of the epithelium

Epithelial cells have an active role in the first-line defence against microbes. Keratinocytes express MHC class II molecules and secrete proinflammatory cytokines and chemokines during inflammation which leads to attraction of immune cells to the site of infection. Via these molecules they also activate adaptive immune responses. Further, resident epithelial Langerhans´ cells are professional APCs and serve as a connective link between innate and adaptive immune responses.

Oral epithelium has been shown to express potent antimicrobial peptides and proteins. These antimicrobial peptides comprise two main families, the defensins and the cathelicidines, reviewed in MasCasullo *et al.* (2005). Defensins are further divided into α(1-6)- and β(HBD1-4)-defensins, which all contain a three intramolecular disulfide motif.

α-defensins 1-4 (HNP1-4) are expressed by leukocytes and 5-6 (HD5-6) by certain epithelia. In oral mucosa, only leukocyte-associated α-defensins have been detected, reviewed in Dale & Fredericks (2005). Instead, oral keratinocytes constitutively express β-defensin HBD1, whereas HBD2 and HBD3 expression is induced by bacterial or proinflammatory stimuli. Probably due to continuous presence of prominent commensal oral bacterial flora, normal stratified gingival epithelium expresses ubiquitously both HBD1 and HBD2 throughout the differentiating epithelium. Only basal cells and the poorly differentiated junctional epithelium are negative for these β-defensin peptides. All α-defensins possess antiviral, including, anti-HSV activity, and HSV-2 gB has been identified as a HNP1-3 binding site, reviewed in MasCasullo *et al.* (2005). Similarly, β-defensins are effective against certain viruses, but studies have failed to demonstrate any direct effect against HSV by HBD1-2. Still, β-defensins, as well as α-defensins, may modulate the course of HSV infection through their multiple effects on immune cells of both innate and adaptive immunity, reviewed in Oppenheim *et al.* (2003).

The only known human cathelicidin is LL-37. It is expressed in neutrophils in oral tissues. LL-37 modulates inflammatory reaction by attracting neutrophils, monocytes and T cells. It has further been shown to neutralize HSV-1 (Gordon *et al.*, 2005). LL-37, as well as defensins, are also secreted by salivary glands and/or ducts.

Secretory leukocyte protease inhibitor (SLPI) is a 107 kDa antimicrobial protein recently shown to be constitutively expressed by oral keratinocytes (Jana *et al.*, 2005). In oral tissues, also macrophages and neutrophils secrete SLPI. Among other known antimicrobial activity, SLPI has been shown to inhibit HSV-2 and HIV infection, reviewed in MasCasullo *et al.* (2005). Interestingly, HSV-2 has been suggested to

downregulate SLPI expression in cells isolated from genital cervical area (MasCasullo *et al.*, 2005) implying to yet another immune evasion mechanism by HSV.

3.3.2. Adaptive defence mechanisms

Common to all adaptive defence mechanisms is the requirement for preceding antigen exposure, and antigen specificity in the following immune responses. In the oral region both humoral and cell associated responses are functional, but entail some features that are specific for mucosal immunity.

Immunoglobulins

Saliva contains secretory, locally produced and serum-derived antibodies. Secretory salivary antibody production can be induced either in the gut-associated lymphoid tissue (GALT) or locally in the lymphoid tissue found in close proximity to the salivary glands. From GALT, the antigen-sensitized B cells home to the lymphoid tissue in the mucosa or to the glandular epithelium of the alimentary tract, including salivary glands, reviewed in Challacombe & Shirlaw (2005). After homing, B cells undergo clonal expansion and mature into immunoglobulin producing plasma cells under T cell influence. Local induction of secretory antibodies takes place in the lymphoid tissue located adjacent to acinar and ductal cells of the salivary gland. Antigens move in retrograde direction into the salivary duct and are subsequently endocytosed through epithelial cells to the local lymphoid tissue.

The predominant plasma cell type in salivary glands is the IgA isotype producing plasma cell, and IgA is the predominant antibody isotype in saliva. Pure secretion of the major salivary glands may also contain IgM and little IgG (Brandtzaeg *et al.*, 1970). About 20% of salivary IgA is synthesized in the minor, and 80% in the major salivary glands. IgA is synthetized as a dimeric molecule joined by a J-chain. This complex binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral cell membrane of the salivary gland epithelial cell, and the whole complex is transported across the cell in an endocytic vacuole. The vacuole fuses with the apical cell membrane, and pIgR gets proteolytically cleaved. A part of pIgR, the secretory piece (SC), and the dimeric IgA are released together as secretory IgA (sIgA). SC makes sIgA more resistant against proteolytic cleavage. IgM may be similarly transported to saliva by pIgR as secretory IgM.

The most thoroughly described biological function of sIgA is the inhibition of bacterial adherence, reviewed in Challacombe & Shirlaw (2005). sIgA also neutralizes bacterial toxins, and exhibits immune exclusion by binding to antigens thereby facilitating their removal. sIgA has been shown to neutralize certain viruses by inhibiting their binding or penetration into the host cells. Furthermore, it has been proposed that IgA may neutralize virus also intracellularly in cells expressing pIgR (Mazanec *et al.*, 1992).

Certain leukocyte subsets express FcαR, and IgA has been suggested to function as an immunomodulatory molecule for these cells. sIgA does not activate the classical complement pathway, but some indication exists on the alternative pathway activation. Since saliva contains only trace amounts of complement, this functional deficiency of sIgA is probably not significant in the oral environment.

Another important source of immunoglobulins in saliva is the gingival crevicular fluid (GCF), reviewed in Challacombe & Shirlaw (2005). Although GCF contributes by only 0.5% to the total whole saliva volume, the immunoglobulins in this fluid have been estimated to constitute about 5% of the total salivary IgA and about 50% of the total salivary IgG. In addition to locally produced substances, GCF contains serum components that have transudated through endothelial capillaries. Inflammation of these tissues mounts transudation as a result of increased endothelial permeability. Antibodies in GCF are derived from the serum or are produced locally as indicated by the observed local plasma cell infiltrates in periodontal disease, reviewed in Kinane & Lappin (2002). Serum contains antibodies directed against various oral pathogens. Not much is known about the role of these immunoglobulins in oral defence, but serum-derived IgG have been suggested to provide protection against certain oral microbes, including cariogenic bacteria (Aaltonen *et al.*, 1985).

IgM is normally only synthesized in the acute phase of infection. However, in some IgA-deficient individuals, salivary IgM and IgG levels are continuously elevated presumably as a compensatory mechanism (Norhagen *et al.*, 1989).

Cell-mediated defence mechanisms

In the oral environment, cell-mediated immune responses are mainly restricted to the surrounding soft tissues, although few leukocytes can be detected also in saliva. Moreover, in saliva, leukocytes are short-lived due to the hypotonicity of saliva. In addition to classical immune cells, oral keratinocytes and fibroblasts contribute to the immunological processes by expressing and secreting various cytokines and compounds of the innate immunity, reviewed in Feliciani *et al.* (1996). Non-immune cells act also as APCs. In the oral mucosa, αβ T cells predominate, and only very few T cells represent the γδ T cell pool (Vroom *et al.*, 1991). After antigen has been presented to T cells, they become activated, proliferate and execute either helper functions (CD4+ T cells) or cytotoxic activity (CD8+ T cells) against the given pathogen (see: Immune responses).

AIMS OF THE STUDY

People differ greatly in susceptibility to orolabial primary and recurrent HSV-1 infection, but the factors explaining these individual differences are largely unidentified. This study was designed to elucidate the immunological mechanisms involved in defence against mucocutaneous HSV-1 primary infection and reactivation in the orolabial region, and the epithelial pathogenesis of HSV-1 infection. Hypothesis of this study was that individual differences in oral innate defence factors and adaptive responses against HSV-1 explain at least partly the varying susceptibility to HSV-1 transmission and disease. Furthermore, to be able to study the interplay of HSV-1 and oral immune mechanisms *in vitro* in conditions imitating the natural environment of epithelial HSV-1 infection, it was hypothesized that a three-dimensional organotypic keratinocyte culture mimicking stratified oral epithelium can be used for HSV-1 infection studies.

The specific aims were:

1) To establish an *in vitro* model imitating the natural environment for studies of epithelial HSV-1 infection.
2) To study the capacity of human saliva to inhibit HSV-1 infection and to identify salivary factors mediating the inhibitory effect.
3) To study susceptibility of HSV-1 to certain innate salivary defence factors with known wide-spectrum antimicrobial activity.
4) To study the effect of peroxidase-generated hypothiocyanite on HSV-1 in comparison with viruses with differing primary target organ.
5) To study the role of innate salivary defence factors, and humoral and cellular immunity in the control of recurrent HSV disease in the orolabial region.

MATERIALS AND METHODS

Elaborate description of the methodology has been given only for those areas of the thesis that lack sufficient detail in the original publications.

1. STUDY SUBJECTS (III, VI)

Altogether 177 individuals were initially tested for serum anti-HSV-IgG antibodies. Of these, 88 subjects additionally donated a saliva sample (median age, 20 yrs) and 45 subjects heparinized venous blood (median age, 21 yrs). The vast majority of the participants were medical or dental students at the Faculty of Medicine at Turku University. The individuals that donated samples of saliva or heparinized blood were also asked to fill in a questionnaire regarding the clinical history HSV reactivations, factors predisposing to reactivations, oral inflammatory disease and mucosal lesions, chronic illnesses, medication, and smoking habits. Subjects participated in the study with informed consent, and the study protocols were approved by the Ethics Committee of the Medical Faculty of Turku University.

2. CLINICAL SAMPLES (III, VI)

2.1. Saliva (III)

Saliva samples were collected by stimulation with paraffin chewing, and salivary flow rate was recorded. Immediately after sample collection, hypothiocyanite concentration and salivary buffer capacity were measured. Aliquots of saliva were directly frozen at -20°C for the analysis of lysozyme and lactoferrin. The rest of the sample was centrifuged at 20.000 x g for 10 min at 4°C, aliquoted and stored at -20°C and -70°C for further analysis. Participants were asked not to eat, drink or smoke for 1 h preceding sample collection.

2.2. Serum (III, VI)

Venous blood samples were drawn and allowed to coagulate at room temperature (RT). The samples were subsequently centrifuged at 460 x g for 10 min at RT in order to separate serum for antibody analysis. Sera were stored at -20°C until analysis.

2.3. Peripheral blood mononuclear cells (VI)

PBMC were freshly separated from heparinized venous blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient-centrifugation. Cells were suspended in RPMI

medium (Gibco BRL, NY, USA), washed twice, and the number of cells was determined by Trypan Blue exclusion. Fresh cells were plated for T cell proliferation and cytokine stimulation assays, and used for the analysis of lymphocyte subpopulations.

3. CELLS (I-V)

All fibroblasts used in the studies were primary fibroblasts isolated either from healthy gingiva (human gingival fibroblast; HGF; (Kahari *et al.*, 1991)), foreskin (human foreskin fibroblast; HFF) or skin (human skin fibroblast; HSF) using standard procedures. Fibroblasts were passaged in Dulbecco´s modified Eagle´s medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS), Hepes, gentamycin and fungizone. For cell monolayer infection experiments, the amount of FCS was reduced to 1%. Fibroblasts were used at passage 5-15 for the experiments.

Vero cells are isolated from a normal kidney of an African Green Monkey (ATCC, Bethesda, MD, USA). The cells were passaged in Medium 199 (Gibco BRL), which was supplemented similar to DMEM for fibroblast cultures.

HEp-2 (ATCC) is an epithelial cell line established via HeLa cell contamination. HeLa cells originate from cervical adenocarcinoma, and are known to contain human papilloma virus 18 sequences. DMEM (Gibco BRL) based media, with the same supplements as for fibroblasts cultures, were used for HEp-2 cell cultures.

HaCaT keratinocytes are isolated from normal skin excised with a 1.5 cm marginal to melanoma tissue (Boukamp *et al.*, 1988). These cells have undergone spontaneous transformation *in vitro*. They are immortal but nontumorigenic, and have maintained epidermal differentiation capacity. HaCaT keratinocytes have a mutation in both alleles of the p53 gene: a heterozygous C>T mutation in codon 179 of exon 5, and a consecutive heterozygous CC>TT mutation in codons 281-282 of exon 8 (Lehman *et al.*, 1993). The cells were maintained in DMEM supplemented with 10% FCS, 1% nonessential amino acids, 2 mM L-glutamine, 50 μg/ml streptomycin and 100 U/ml penicillin.

4. VIRUSES (I-V)

F and KOS strains of HSV-1 are both wild-type viruses. Strain F was originally isolated from a facial (Ejercito *et al.*, 1968) and strain KOS from a labial herpetic lesion (Smith, 1964). Rid1 and gC$^-$39 are spontaneous mutants of the parental KOS wild-type virus. Rid mutants have a single amino acid substitution in gD at position 25 or 27 (Dean *et al.*, 1994) that disables this virus to use HVEM as an entry mediator (Montgomery *et al.*, 1996; Yoon *et al.*, 2003). gC$^-$39 is a gC-null mutant virus (Holland *et al.*, 1984). Viruses Rid1, gC$^-$39 and the corresponding wild-type viruses were generously donated for these studies by Prof. Patricia Spear, Northwestern University, and by Dr. William Goins, University of Pittsburgh. All HSV-1 strains were propagated in HEp-2 cells or HFF, and titrated in either HGF, HFF, Vero or HaCaT cells using rapid HSV-1 culture (see below).

Respiratory syncytial virus (RSV; strain Randall) originates from the laboratory of Dr. Taylor-Robinson (Harvard Hospital, Salisbury, UK). The virus was plaque-purified and propagated in HGFs for the studies. Stock virus was titrated in HGF by rapid viral culture (see below).

Echovirus 11 (EV11; strain Gregory) was purchased from ATCC. The plaque-purified virus is sensitive to neutralization with specific antiserum (Auvinen & Hyypia, 1990). EV11 was propagated in HGFs for the studies and the titer of viral stock was determined by rapid viral culture in HGFs (see below).

5. RAPID VIRAL CULTURE (I-V)

For rapid viral culture, a previously published method with slight modifications was used (Waris *et al.*, 1990; Ziegler *et al.*, 1988). Briefly, susceptible cells were seeded in 24- or 12-well plates and grown until confluence. The cells were inoculated by centrifugation at 740 x g for 45 min at RT. Stock virus, virus suspensions, culture supernatant or homogenates of three-dimensional keratinocyte cultures were used for inoculation. Cultures were incubated for 16-20 h (HSV-1, EV11) or 46-48 h (RSV) and fixed with methanol or acetone, respectively. Monoclonal horse-radish peroxidase (HRP)-conjugated antibodies (Waris *et al.*, 1990; Ziegler *et al.*, 1988) were then added on the fixed cell monolayers. The used HRP-conjugated HSV-1 monoclonal antibody is specific for gC. Therefore, another unlabelled monoclonal antibody was used sequentially with an appropriate HRP-labelled secondary antibody to detect gC deletion virus in the studies. For the detection of EV11, an unlabelled rabbit polyclonal antibody to EV11 was first added onto the cells followed by incubation with HRP-labelled swine anti-rabbit antibodies. Finally, to visualize the infected cells, a substrate solution containing 3-amino-9-ethylcarbazole diluted in dimethylformamide, acetate buffer and H_2O_2 was added. The viral plaques were counted by light microscopy.

6. CELL CULTURE ASSAYS (I-IV)

6.1. Effect of hypothiocyanite on HSV-1, RSV and EV11 (IV)

Oxidation of thiocyanite by H_2O_2 yields hypothiocyanite ($HOSCN/OSCN^-$) in a peroxidase-catalyzed reaction. $HOSCN/OSCN^-$ solution was generated by drop-wise addition of various amounts of H_2O_2 to VMG buffer (pH 7.1 or 6.0) supplemented with potassium thiocyanate (KSCN) and bovine lactoperoxidase (bLPO). An equal volume of virus suspension (HSV-1, RSV or EV11) was immediately combined with this solution, and incubated for 30 min at 37°C. HGF grown in 24-well plates were then inoculated by centrifugation with ten-fold dilutions of virus-$HOSCN/OSCN^-$ suspension. Controls included in the study were: 1) VMG buffer alone, 2) culture supplemented with the highest concentration of H_2O_2 used in the assay, 3) bLPO and KSCN only and 4) whole

bLPO system but no virus. Infected cells were detected in the cultures by immunoperoxidase staining. The amount of the generated HOSCN/$OSCN^-$ was verified by measuring spectrophotometrically the reaction with the coloured anionic monomer of 5, 5´-dithio-bis(2-nitrobentzoic acid) ($(Nbs)_2$) (Aune & Thomas, 1977; Pruitt *et al.*, 1986).

6.2. Effect of lysozyme and lactoferrin on HSV-1 infection (V)

The effect of hen egg-white derived lysozyme (Lz) and human milk derived non-iron-saturated and iron-saturated lactoferrin (Lf) (all purchased from Sigma Chemicals Co., St. Louis, MO, USA) on HSV-1 infection was studied in a series of cell culture assays. Assays with Vero cell monolayers included 1) preincubation of HSV-1 with Lz/Lf prior to inoculation of the culture 2) addition of Lz/Lf to the culture after absorption of the virus to the cell surface and 3) Lz/Lf treatment of cultures for 24 h before inoculation with HSV-1 with or without removal of these supplements before inoculation. Infectious virus was detected by immunoperoxidase staining. Three-dimensional keratinocyte cultures were supplemented with Lz or Lf from the establishment of collagen-fibroblast gel onwards (see below). The cultures were infected with HSV-1 either 24 h before or 30 min after lifting of the cultures to the air-liquid interface. Lifted cultures were maintained for 1 week. Some uninfected control cultures were supplemented with bromodeoxyuridine (BrdU; Dako A/S, Glostrup, Denmark) one day before termination of the cultures. Vero cell cultures were infected with HSV-1 strains KOS, Rid1 and gC^-39, whereas three-dimensional keratinocyte cultures were infected with strain F only.

6.3. Salivary cell-protective assay (III)

Vero cells grown in 24-well plates were first overlaid with centrifuged saliva mixed with 2 x culture medium in a 1:2 dilution. After overnight incubation, cells were washed and inoculated by centrifugation with 100 PFU of HSV-1 (F). Infected cells were visualized by immunoperoxidase staining 20 h post infection (p.i.). A culture without saliva treatment was included as a control.

6.4. Salivary HSV-1 neutralization assays (III)

Centrifuged saliva was mixed with 200 PFU of HSV-1 (F) and incubated for 30 min at 37°C. Subsequently, Vero cells on 24-well plates were inoculated by centrifugation with this virus suspension in a 1:10 dilution, and infected cells were detected by immunoperoxidase staining the following day. Saliva was replaced by PBS for the control virus suspensions.

6.5. T cell proliferation assay (VI)

PBMC were suspended in RPMI (Gibco BRL) supplemented with human AB serum (Finnish Red Cross, Helsinki, Finland), Hepes, glutamine and gentamycin. Cells were

plated on a microtiter plate at a density of 7 x 10^5 cells/well. To test the cultures for the presence of HSV-specific T cells, inactivated HSV-1 antigen (1 μg/ml) was then added to the culture medium. Cultures supplemented with either control antigen (1 μg/ml), phytohemagglutinin antigen (PHA; 100 μg/ml) or medium alone served as controls in the assay. The cultures were maintained for 4 days, and ^{3}H-thymidine (Amersham Biosciences, Buckinghamshire, UK) was added 18 h before harvest. Cells were harvested with a Tomtec 93 Mach III Manual harvester (Tomtec, Orange, CT, USA), and incorporated radioactivity was measured with a Micro-Beta counter (Wallac, Turku, Finland). HSV-1 and control antigens were prepared from HSV-1 or mock infected cultures as previously described (Ilonen, 1979). Briefly, HSV-1 (strain VR; ATCC) or mock infected Vero cells were lysed, and cell debris was removed by centrifugation. After ultracentrifugation of the supernatant, the antigen pellet was recovered and treated with β-propiolactone to inactivate any infective viral particles.

6.6. Cytokine mRNA assay (VI)

PBMCs were suspended in RPMI medium supplemented as described above for the T cell proliferation assay. Cells were plated on a 24-well plate at a density of 2 x 10^6/well. Cultures were supplemented with either inactivated HSV-1 antigen (2.5 μg/ml), control antigen (2.5 μg/ml), PHA (100 μg/ml) or medium alone. HSV-1 and control antigens were prepared according to previously described method (Ilonen, 1979). Cells were stimulated for three days, pelleted and suspended in Ultraspec™ (Biotecx Laboratories, Houston, TX, USA). Samples were stored at –70°C until extraction of mRNA for cytokine expression analysis.

6.7. Three-dimensional (3D) keratinocyte culture (I-II, V)

3D keratinocyte cultures were prepared according to previously published technique by Assalineau and Prunieras (1984) with slight modifications (Figure 8). First, a collagen solution was prepared by mixing Vitrogen 100 collagen (Celtrix Pharmaceuticals, Inc., Santa Clara, CA, USA) with 10 x DMEM. The pH was adjusted to neutral with 0.1 M NaOH. Primary HSF were immediately suspended in the collagen solution at a density of 400.000 cells/ml, and 0.7 ml of this suspension was laid into wells of a 24-well plate. The collagen was allowed to gel for 1 h at 37°C, before addition of Green´s medium. Some of the cultures were supplemented with either Lz or Lf. After one week, 200.000 HaCaT keratinocytes/well were added on top of the fibroblast-collagen gel. Keratinocytes were grown submerged in the medium for three days to reach confluence. Then, the cultures were placed on a metal grid on a semipermeable membrane to the air-liquid interface to enable the stratification of the epithelium. At this point, cultures were fed from below. The cultures were infected with 0.1-10^5 PFU of HSV-1 (F) at various time-points either 52-78 h before or 0.25-6 h after lifting of the cultures. The cultures were harvested one week after lifting. Each culture was partially snap-frozen for viral nucleic acid analysis,

and partially fixed with formalin for immunohistochemistry and *in situ* hybridization studies. Samples from cultures and culture medium were also collected for viral culture.

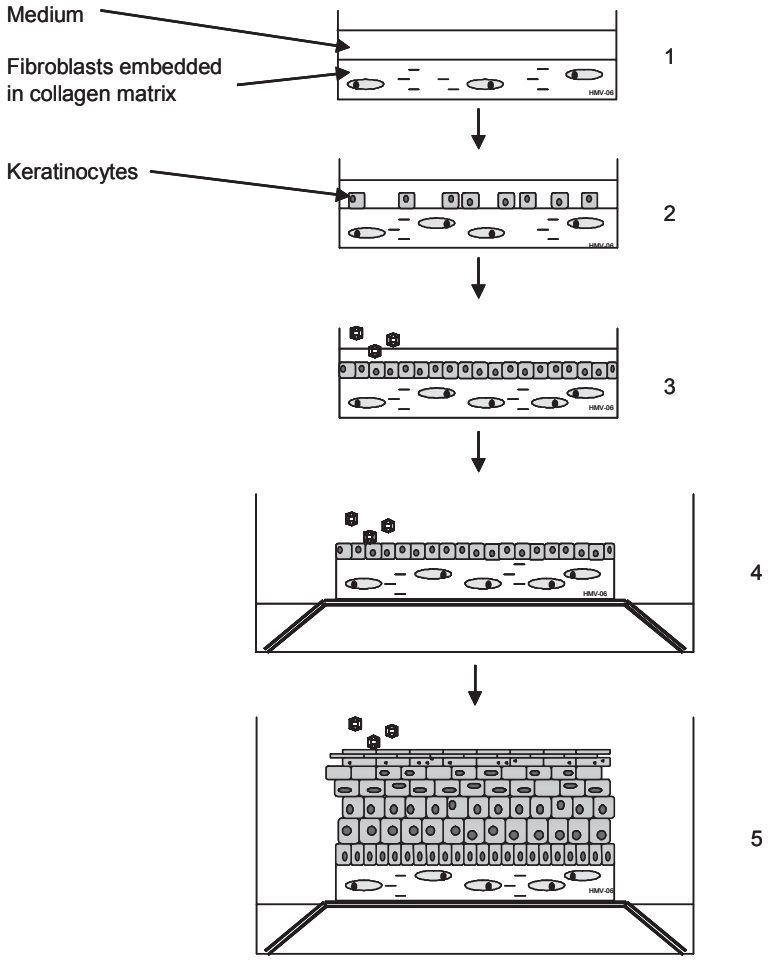

Figure 8. HSV infection of the 3D keratinocyte culture. Fibroblasts are first grown alone embedded in collagen (1). After 5-7 days, keratinocytes are added on top of the collagen bed (2). Upon reaching confluence (3), cultures are lifted to the air-liquid interface on a stainless-steel grid on a semipermeable membrane, and fed from below thereafter (4). When in contact with the air, keratinocytes begin to stratify (4). Cultures were harvested 1 week after lifting. HSV-1 was inoculated to the cultures when keratinocytes had reached confluence either 24h prior to lifting into air-liquid interface (3), or at various time-points after lifting (4-5).

7. FLOW CYTOMETRIC ANALYSIS (VI)

PBMCs were double-stained with fluorescently labelled antibodies for the analysis of activated T cells (HLA-DR/CD3+), helper CD4+ T cells (CD29+/CD4+), cytotoxic CD8+ cells (CD11b+/CD8+), and naïve CD4+ cells (CD45RA+/CD4+). CD11b and CD45RA antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ, USA) and other antibodies from Beckman Coulter (Fullerton, CA, USA). For each sample, 5 x 10^5 cells were incubated with the appropriate antibody pair for 30 min at 4°C. Cells were washed twice and fixed with 1% formaldehyde before analysis by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA). The subpopulation of cells of interest was gated, and results were analyzed by CellQuest 3.3 software (Becton Dickinson, San Jose, CA, USA).

8. IMMUNOGLOBULIN ASSAYS (III, VI)

8.1. Serum antibodies (III, VI)

For measurement of anti-HSV-IgG antibodies, microtiter plates were coated with HSV-1 (strain VR) envelope antigen, which reacts with both anti-HSV-1 and -2 antibodies (Martin *et al.*, 1972). Before addition of sera, wells were blocked by incubation with 0.5% bovine serum albumin (BSA) in PBS for 1 h at RT. Sera were added at a dilution of 1:1000 and 1:100 for the measurement of total anti-HSV-IgG and anti-HSV-IgG subclass antibodies, respectively. Plates were then incubated for 2 h at 37°C and washed thereafter. For the determination of IgG-subclass antibodies, anti-human-IgG subclass-specific mouse monoclonal antibody (Sarnesto, 1983; Seppala *et al.*, 1984) was then added to the wells and incubated for 1 h at 37°C. Plates were washed and HRP-conjugated rabbit anti-human IgG (total anti-HSV-IgG assay: Dako A/S) or anti-mouse (anti-HSV-IgG subclass assay: Dako A/S) antibodies were added and incubated for 1 h at 37°C. Ortho-phenylene-diamine (Kem-En-Tec A/S, Copenhagen, Denmark) supplemented with H_2O_2 was used as the substrate, and the enzymatic reaction was stopped after 30 min by addition of 1 M HCl. The results were read on a standard plate reader and displayed as arbitrary units (AU) or enzyme immunoassay units (EIU) using a standard curve as a reference. In this standard curve, a pool of sera with high anti-HSV titre (designated as 100 U) and a pool of negative sera (0 U) form standards containing 0, 20, 40, 70 and 100 U (Koskinen *et al.*, 1987).

8.2. Salivary antibodies (III)

A capture antibody assay was used to determine the amount of different antibody isotypes in saliva (Lehtonen *et al.*, 1984). Briefly, microtitre wells were coated with isotype-specific antibodies. Diluted saliva was added, and HRP-conjugated isotype-specific antibodies were used to detect the bound salivary antibody (Dako A/S). Antibody

concentrations were determined by using human serum (NOR-Partigen; Behringwerke AG, Marburg, Germany) derived standard curve as a reference.

Anti-HSV-IgG test for serum samples was slightly modified for testing salivary anti-HSV antibodies. In this assay, wells were post-coated overnight at 4°C with 1% BSA. Saliva was diluted in 2-fold serial dilutions from 1:2 to 1:256. HRP-conjugated rabbit anti-human IgA, IgM and IgG antibodies (Dako A/S) were used to detect the bound HSV-specific antibody. The result was read as end-point titre which was defined as the dilution in which the absorbance of the sample cut the curve formed by the mean +3SD of salivary samples of HSV-seronegative subjects.

9. CHEMICAL ANALYSES OF SALIVA (III)

9.1. Buffer capacity

Buffer capacity was determined immediately after saliva collection using a Dentobuff Kit (Orion Diagnostica, Espoo, Finland), which shows the buffering capacity of saliva by colour change on an indicator strip.

9.2. Hypothiocyanite (HOSCN/$OSCN^-$)

The amount of HOSCN/$OSCN^-$ was determined spectrophotometrically by a reaction with the coloured anionic monomer of 5,5´-dithio-bis$(Nbs)_2$ (Aune & Thomas, 1977; Pruitt *et al.*, 1986). This analysis was done immediately following sample collection.

9.3. Thiocyanate (SCN^-)

SCN^- ion content of saliva was determined from centrifuged saliva by allowing ferric nitrate to react with salivary SCN^- (Betts & Dainton 1953). The amount of the reaction product, $FeSCN^{2+}$, was then measured spectrophotometrically using a KSCN standard curve as a reference.

9.4. Salivary peroxidase (hSPO) and myeloperoxidase (hMPO)

Samples of centrifuged saliva were tested for hSPO activity by following spectrophotometrically the oxidation rate of 5-thio-2-nitrobenzoic acid (Nbs) to $(Nbs)_2$ by $OSCN^-$ ions that are generated by oxidation of SCN^- by H_2O_2 in a hSPO catalyzed reaction (Wever *et al.*, 1982). SCN^- was replaced by Cl^- in the assay for the measurement of hMPO activity, since Cl^- is oxidized by hMPO but not hSPO (Mansson-Rahemtulla *et al.*, 1986).

9.5. Lysozyme (Lz)

Lysozyme concentration was measured from noncentrifuged saliva by Micrococcus lysodeikticus agar diffusion plates (Lysozyme Kit, Kallestad Laboratories, Chaska, MN, USA) in which lyophilized human urine lysozyme is used as a reference.

9.6. Lactoferrin (Lf)

Lactoferrin was quantified from non-centrifuged samples by a noncompetitive avidin-biotin enzyme immunoassay (Vilja *et al.*, 1985). Human colostral lactoferrin (Sigma Chemicals Co.) was purified by affinity chromatography and used as a standard.

9.7. Mucin/MUC7

Centrifuged samples were used for the determination of MUC7 (MG2) activity by indirect enzyme immunoassay (Bolscher *et al.*, 1999). The antiserum used in this assay was raised against a synthetic peptide of the C-terminus of MUC7. It recognizes MUC7 independently of its carbohydrate moiety. Purified MUC7 was used as a standard.

9.8. Cystatin

Cystatin activity was determined from centrifuged samples by measuring the inhibition of hydrolysis of α-N-benzoyl-DL-arginine-β-naphtylamine substrate by papain standard (Henskens *et al.*, 1993). Chicken egg white cystatin was used as a standard. Cystatin S concentration was measured with a capture type enzyme-linked immunosorbent assay (Henskens *et al.*, 1996b).

9.9. Amylase

α-Amylase activity was measured from centrifuged saliva with a kinetic determination kit (Behring, Marberg, Germany) according to manufacturers instructions with minor modifications (Henskens *et al.*, 1996b). Lin-Trol reagent (Sigma Chemical Co.) was used as a standard in the assay.

9.10. Protein

A colorimetric method employing Folin phenol reagent was used for the measurement of protein content of centrifuged saliva (Lowry *et al.*, 1951).

10. SPECIMEN PREPARATION FROM 3D CULTURES (I-II, V)

Samples of 3D keratinocyte cultures were fixed with buffered 10% formalin for 24 h and embedded in paraffin. The samples were subsequently sectioned onto organosilanated slides for hematoxylin and eosin staining, immunohistochemistry and *in situ* hybridization.

11. IMMUNOHISTOCHEMISTRY (IHC) (II, V)

Sections of 3D keratinocyte cultures were stained with an automated slide-processing instrument (Dako TechMate 500). HSV-1 was detected in cultures with a polyclonal rabbit anti-HSV-1 antibody (BioGenex Laboratories, San Ramon, CA, USA) and with HSV-1 anti-ICP0 and anti-tk antibodies (generous gift from Prof. Bernard Roizman, University of Chicago, USA). HSV-positive labial herpes biopsy specimen was used as a positive control. Primary antibodies were omitted for the staining of the negative controls.

12. IN SITU HYBRIDIZATION (ISH) (I-II)

Samples of 3D keratinocyte cultures were hybridized with a commercial biotinylated probe (Enzo Diagnostics, N.Y: USA) for HSV DNA according to a previously published protocol (Syrjanen *et al.*, 1988). HSV-1-positive biopsy specimen served as a positive control. Incubation with the specific probe was omitted in negative control slides.

Sliced samples of 3D keratinocyte cultures were hybridized with ssRNA digoxigenin(DIG)-labelled probes for HSV-1 LAT, α0 and VP16 mRNA as previously described (Hukkanen *et al.*, 1990). The LAT RNA probe contained the sequences from the 0.5 kb *HpaI-SalI* subfragment of the *BamHI* fragment B of HSV-1 DNA. LAT probe was digested with *EcoRI*, and transcribed with SP6. The same plasmid was linearised with *SalI* and transcribed with T7 to yield the probe for α0 RNA. VP16 RNA probe was transcribed from pRB3717 by T7 polymerase (McKnight *et al.*, 1987). A ganglion from a mouse infected with HSV-1 was used as a positive control and a ganglion from an uninfected mouse as a negative control. In addition, RNase and DNase pretreated control slides were included.

13. PCR ASSAYS (I-II, VI)

13.1. Cytokine mRNA amplification and detection of amplified products by time-resolved fluorometry (VI)

Total RNA was extracted according to manufacturer´s instructions from cells suspended in Ultraspec™ (Biotecx Laboratories, Inc., TX, USA). After RT-PCR of mRNA, the

levels of IFN-γ, IL-4, -12 and -13 and the constitutively expressed β-actin were determined using PCR and subsequent time-resolved fluorometry employing lanthanide labelled probes as previously described (Halminen *et al.*, 1999; Nieminen *et al.*, 2002). Briefly, mRNA was reverse-transcribed and the resulting cytokine and β-actin cDNAs were simultaneously amplified in a triplex-PCR reaction (IFN-γ, IL-4 and β-actin or IL-12, IL-13 and β-actin). One primer of each primer pair was biotinylated enabling capture of PCR products on the streptavidin coated microtitre plates. The products were detected with lanthanide-labelled probes in a solution hybridization reaction. The level of bound probe was measured using time-resolved fluorometry. Cytokine specific counts were normalized to β-actin to represent the relative amount of cytokine mRNA transcribed per cell. PHA stimulated PBMCs served as a positive control.

13.2. PCR for HSV-1 DNA (I, II)

DNA was extracted from frozen samples of three-dimensional keratinocyte cultures by ethanol precipitation (Miller *et al.*, 1988). For the amplification of HSV-1 DNA, a modification of a protocol originally used for the detection of HSV in cerebrospinal fluid was used (Aurelius *et al.*, 1991). The primers (5´-ATC ACG GTA GCC CGG CCGTGT GACA and 5´-CAT ACC GGA ACG CAC CAC ACAA) and the internal hybridization probe (5´-TAC GAG GAGGAG GGG TAT AAC AAA GTC TGT) were derived from HSV-1 glycoprotein D gene (Aurelius *et al.*, 1991). The PCR started by incubation at 95°C for 5 min. The subsequent steps in the PCR were 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, for 41 cycles with extension of the incubation at 72°C of the last cycle by four minutes. The PCR products were ran on an agarose gel and stained with ethidium bromide to enable visualization of a 221-bp PCR product. The specificity of the product was then confirmed by Southern blotting with a DIG-labelled oligonucleotide probe (Hukkanen *et al.*, 2000). One PFU of HSV-1 (strain F) served as a positive and distilled water as a negative control.

14. NORTHERN BLOT (I)

RNA was extracted from snap-frozen 3D keratinocyte cultures by TRIzol (Gibco BRL). Samples of total RNA and DNase-treated total RNA were run on an agarose gel. RNA was transferred to a nylon filter and the filter was hybridized with a [^{32}P]CTP-labelled ssRNA probe for HSV-1 LAT RNA (Hukkanen et al. 1990). Latently infected ganglia from BALB/C mice were used as a positive and uninfected cultures and ganglia as negative controls.

15. STATISTICAL ANALYSES

In studies III and VI, the nonparametric Kruskal-Wallis and Mann-Whitney U-tests were used for the comparison analysis, and Spearman rank correlation test for the correlation analysis of different study groups. These analyses were performed with StatView 4.02 software (Abacus Concepts, Inc., Berkeley, CA, USA). Data from study V were analysed by Student´s two-tailed paired t-test by Excel 2002 (Microsoft Corp., Redmond, WA, USA). Two-sided p-values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

1. SUITABILITY OF 3D CULTURES FOR HSV INFECTION STUDIES (I-II, V)

1.1. Keratinocytes support nonproductive and lytic HSV-1 infection in organotypic 3D keratinocyte culture model

HSV infection studies have been traditionally carried out either on cell culture monolayers or in animal models. Monolayer cultures do not, however, represent the natural environment of HSV epithelial infection, which normally affects stratified epithelium. Animal models on the other hand are very laborious, and for ethical reasons, should only be performed when absolutely required for the understanding of a complex biological phenomenon, such as latency and reactivation. Organotypic 3D keratinocyte cultures mimic the structure of a stratified epithelium (Asselineau & Prunieras, 1984). A series of studies was performed to examine the suitability of this culture system, imitating more closely the natural environment of epithelial HSV infection, for HSV-infection studies.

Organotypic keratinocyte cultures were established with skin-derived primary fibroblasts embedded in collagen, and with spontaneously immortalized HaCaT keratinocytes. HaCaT cells were seeded at a density that reached confluence at 60 h after seeding. Upon becoming confluent, cells became proliferation-arrested. Morphologically cells appeared flattened at this stage. This phenotype was maintained up to 120 h after seeding.

Lifting the cultures to the air-liquid interface initiates the differentiation of the keratinocytes (Asselineau & Prunieras, 1984). HaCaT cells formed a stratified 8-12 cell layers thick epithelium within 7 days of culture. The formed epithelium was not terminally differentiated, as only slight parakeratosis was observed. However, the initiation of terminal differentiation was observed as flattening of cells in the most superficial layer (study I, Figure 1a). BrdU positive cells were present in all cell layers indicating hyperproliferation (V). In a normal epithelium, only basal cells proliferate. Consistently, HaCaT keratinocytes have been earlier shown to express cytokeratins 17 and 19, indicative of epithelial immaturity, throughout the whole thickness of the epithelium in a 3D HaCaT culture with skin-derived fibroblasts and with a collagen matrix equivalent to the one used in the current study (Merne & Syrjanen, 2003). In this previous study, only scattered cell clusters were observed to express cytokeratin 10, a marker of initiation of differentiation (Merne & Syrjanen, 2003), further conforming the poor differentiation status achieved with 3D HaCaT cultures.

1.2. Outcome of HSV infection is dependent on the dose and time of viral inoculation

3D cultures were infected with $0.1\text{-}10^5$ PFU of HSV-1 (F) at various time points either before, or after initiation of stratification induced by lifting the cultures to the air-liquid interface. The titre of the stock virus was determined on Vero cells. HaCaT cells were

found to be about 10 times more sensitive to HSV-1 compared to Vero cells (II, Table 2). Therefore, inoculum used was in reality in the range of 1-10^6 PFU/ culture.

Infecting cultures 48-120 h after seeding of the keratinocytes, while cultures were still submerged in the medium, resulted always in a lytic HSV infection of the keratinocytes (Figure 9). This was verified by positive viral isolation from the cell culture supernatant and the culture homogenate. The highest viral titres were recorded from confluent cultures infected 72 h post seeding. Morphologically cultures displayed typical epithelial HSV infection induced changes, including ballooning of the cells, multinucleated cells and intraepithelial vesicles through the whole thickness of the epithelium (I, Figure 1c-d). Lytic infection was further verified by positive staining of keratinocytes by IHC for HSV-1 antigen, and by ISH for HSV-1 DNA (I, Figure 2a-b), and LAT, α0 (I, Figure 4c-d) or αTIF RNA (II) of the areas with HSV-associated morphological changes. Fibroblasts were negative for HSV-1 antigen by IHC (II), and for HSV-1 DNA by ISH (I, Figure 2a-b). LAT and α0 RNA expression of fibroblasts could not be evaluated due to high background signal associated with the collagen matrix (I, Figures 4c-d). PCR for HSV-1 DNA was positive from these culture samples (I, Figure 3).

Interestingly, cultures infected after initiation of differentiation displayed different phenotypes both morphologically and in the molecular level depending on the time point of viral inoculation (Figure 9). Cultures were infected with 5 PFU of HSV-1 15 min-6 h after lifting (15 min, 30 min, 1h, 2h, 4h, 6h). All time points, excluding 30 min, resulted in a lytic HSV infection determined as described above. However, those cultures that were subconfluent at the time of lifting due to hLf effect yielded lytic HSV infection also if infected at 30 min after lifting (V). All other cultures infected at 30 min after lifting exhibited morphology similar to those of the uninfected cultures (I, Figure 1a-b). No infectious virus could be detected in the culture supernatants or culture homogenates from these cultures, and keratinocytes and fibroblasts were negative for HSV-1 antigen by IHC (II, Figure 1a-b), and for HSV-1 DNA by ISH (I, Figure 2c). Similarly, the transcripts associated with lytic HSV infection (α0 and αTIF RNA) showed no signal (I, Figure 5b). Surprisingly, however, keratinocytes were positive for HSV LAT RNA by ISH (I, Figure 5a). LAT RNA expression was further confirmed by Northern blot (I, Figure 6), and HSV-1 DNA positivity of the cultures by PCR (I, Figure 3). Remarkably, LAT RNA appeared to be predominantly expressed in the cytoplasm. In the Northern blot analysis, only the 2.0 kb species of LAT was detected. Instead, in latently infected neurons, LAT RNA is typically localized to the nucleus, and expressed as the full-length 8.3 –kb primary transcript and the two spliced 2.0 and 1.5 -kb introns. The unexpected HSV phenotype of cultures infected 30 min after initiation of differentiation, as well as the effect of hLf-mediated subconfluence to the culture phenotype, suggest that the outcome of infection may be dramatically influenced by the status of the host cell.

Cultures infected 1–6 h or later after lifting had a distinctive model of spread and viral gene expression. In the superficial layers, lytic infection seemed to spread from foci extending laterally along the superficial layer (II, Figure 3a). In the margins of the cultures, infection yielded a vertically sharply demarcated area through the whole thickness of the epithelium, and extended further laterally to the epithelium growing over

the periphery of the 3D culture (II, Figures 2a, d). The areas in between these marginal and central focal zones remained free of any morphological changes associated with an epithelial HSV infection, as well as of any specific markers of lytic HSV infection. In these areas, keratinocytes were, however, positive for HSV LAT RNA by ISH (II, Figure 2b), and thus represented the same HSV infection phenotype as the cultures infected 30 min after lifting, that is, a nonproductive infection (II, Figure 2b). This observed mixed infection further suggests that the status of the host cell contributes significantly to the outcome of infection.

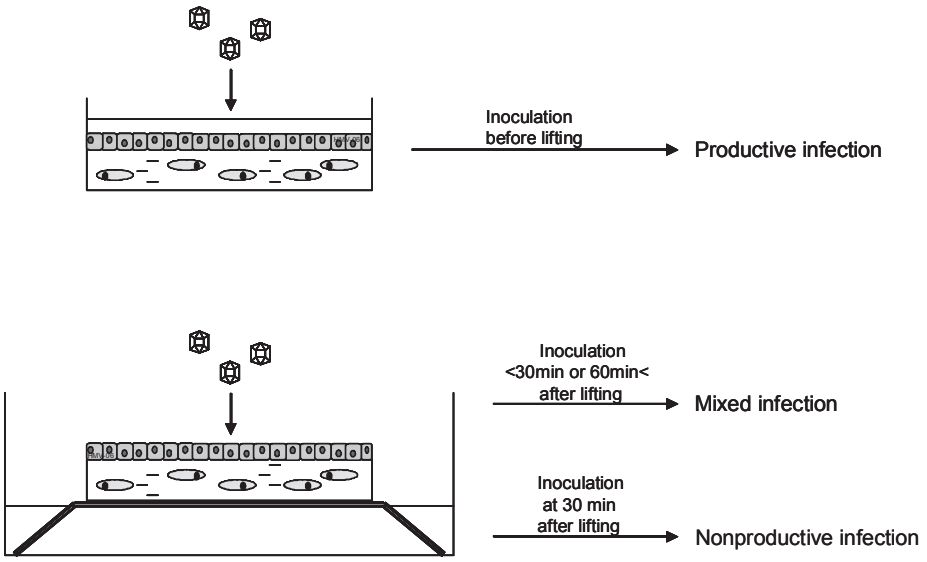

Figure 9. The outcome of HSV-1 infection in 3D keratinocyte culture. Inoculating cultures before lifting resulted in productive HSV infection of the keratinocytes. When infected after lifting, the outcome was dependent on the time-point of HSV inoculation: Inoculation at 30 min resulted in nonproductive HSV infection, whereas inoculation before or after 30 min yielded in most cases a mixed infection with features of both productive and nonproductive infections.

Infection of a full thickness epithelium at 7 days after lifting resulted predominantly in the infection of the entire epithelium. The amount of inoculum correlated with the degree of histomorphologic changes. With an inoculum as high as 10^4-10^5 PFU, even occasional fibroblasts became infected. Since the samples in this setting were only harvested at one time point in which the entire epithelium had already become infected, these experiments do not reveal the initiation site of infection. It has been suggested that virus would need to penetrate the epithelium and infect the proliferating

basal cells in order to initiate lytic infection *in vivo*. Morphology of biopsy samples from HSV blisters similarly suggest initiation of infection at the basal or the parabasal layer, and subsequent vertical spread as indicated by sharply demarcated vertical walls of these lesions. This idea is further supported by another 3D keratinocyte culture model, in which infecting full thickness epithelium resulted in the initiation of HSV infection in the basal and suprabasal cells and subsequent limited lateral spread of the infection (Visalli *et al.*, 1997). The proposed pivotal role of basal cells in the initiation of HSV infection would not be possible, however, to study in the 3D HaCaT keratinocyte model used in this study, because proliferating cells are present throughout the whole thickness of the epithelium. The hyperproliferative and immature phenotype of this epithelium may affect the outcome of the infection, because differentiation modulates the keratinocyte expression of intracellular proteins (Squier & Kremer, 2001), and certain molecules involved in the defence functions against microbes (Dale & Fredericks, 2005). However, the highly lytic infection in the margins of the cultures does suggest that active horizontal proliferation of cells enhances HSV replication.

Epithelial polarization has been shown to modulate dramatically the susceptibility of cells to HSV infection. Polarization and formation of tight junctions decrease substantially the susceptibility of epithelial Madin-Darby canine kidney and human Caucasian colon adenocarcinoma cell lines to apical HSV infection (Hayashi, 1995; Marozin *et al.*, 2004; Topp *et al.*, 1997). However, chemical disruption of tight junctions abolishes the resistant phenotype for apical inoculation (Hayashi, 1995; Marozin *et al.*, 2004; Topp *et al.*, 1997). Similarly, mechanical disruption of tight junctions results in enhanced viral replication (Hayashi, 1995). Disruption of adherence junctions results in redistribution of nectin-1 and in enhanced viral entry (Yoon & Spear, 2002). Moreover, polarization of epithelial cells affects the distribution of HSV receptors on the cell surface (Sears *et al.*, 1991), and the release of progeny virus from the cells (Hayashi, 1995). Together these findings suggest that epithelial injury *in vivo* may result in more efficient entry of HSV and initiation of infection.

In summary, increasing body of evidence is necessitating the use of methods with differentiating and polarized cells for the studies of HSV epithelial pathogenesis. In stratified epithelium, HSV spread appears to follow the growth or differentiation direction of epithelial cells. The epithelial layer specific proliferation activity of keratinocytes in stratified epithelia as such presumably affects replication of HSV, since HSV is known to interact with the host cell proteins involved in the control of cell cycle (see: Metabolic changes in the host cell). Further, polarization of cells has been shown to modulate the distribution of viral entry mediators on the cell surface and consequently, the susceptibility of cells to HSV infection. Moreover, as earlier shown, differentiation may alter the expression pattern of certain innate defence mechanisms of the keratinocytes, such as the defensins, reviewed by Dale & Fredericks (2005). Similarly, differentiation may further modulate the expression and function of other constituents of innate immunity, for example the Toll-like receptors. Therefore, the differential outcome of HSV-1 infection in the 3D cell culture model in this study may be associated with factors like cell cycle status, polarization and differentiation, and the expression pattern of innate defence factors.

1.3. HSV may persist in extraneuronal sites

The results of the established 3D culture model suggest that in the absence of lytic viral replication, keratinocytes may be able to support persistent or latent HSV infection. Previously, extraneuronal persistence has been implicated by the presence of HSV DNA in various human tissues from *post mortem* samples from individuals that had been asymptomatic for HSV before death (Chen & Hudnall, 2006; Crouse *et al.*, 1990), from healed skin areas with previous recurrent HSV infection or erythema multiforme (Brice *et al.*, 1994; Miura *et al.*, 1992) and by the presence of HSV antigen in gingival tissue (Amit *et al.*, 1992; Zakay-Rones *et al.*, 1973; Zakay-Rones *et al.*, 1986). Particular care should be taken when interpreting findings based on non-*in situ* PCR techniques only, due to possibility of positive findings being a result of the presence of HSV antigen within the tissue simply because of its association with circulating cells of lymphoid origin (Ono *et al.*, 2005). Epithelial persistence has been also suggested in association with UV-induced labial lesions appearing within just a few hours after irradiation (Spruance *et al.*, 1991).

In a mouse model, HSV DNA has been shown to persist for several weeks in the epithelial cells the eye by *in situ* PCR (Maggs *et al.*, 1998; Mitchell *et al.*, 1994). Importantly, HSV was also shown to reactivate from such persistent state of infection in a rabbit eye model (Cantin *et al.*, 1992). HSV DNA persisted in mouse footpads for more than two weeks after disappearance of infectious virus and viral antigen after primary infection (Simmons *et al.*, 1997). Interestingly, with increasing time from the viral inoculation, HSV DNA positive cells were found to localize gradually to more superficial layers of the epithelium, most likely reflecting migration of the epithelial cells, and the nonreplicative state of the viral genome. LATs were not detected at any time point after inoculation. Furthermore, HSV has been reported to reactivate from mouse skin explants after months of recovery from primary infection (Clements & Subak-Sharpe, 1988; Hill *et al.*, 1980).

The suggested concept of extraneuronal persistence of HSV is controversial. Persistence of HSV DNA may simply indicate a transient status of the cells migrating from basal to more superficial layers within the epithelium for the time of epithelial turnover thereby being an indicator of a recent acute infection of a particular epithelial site. However, even longer times of persistence have been suggested. Even if HSV was able to persist in extraneuronal tissues, it is unclear weather HSV would be able to reactivate from such state of infection. If HSV was able to persist in oral mucosa and reactivate therefrom, the concepts of HSV latency and the frequently detectable salivary HSV shedding and HSV transmission by saliva would have to be totally reformulated.

1.4. 3D keratinocyte culture provides a novel model for in vitro epithelial HSV infection studies

Results from this series of studies inevitably show that 3D keratinocyte cultures can be used for studies of lytic HSV-1 infection. Furthermore, the observed nonproductive HSV infection in this culture model may provide an *in vitro* model for studies of epithelial persistence and reactivation. The model can be more conveniently used for studies of lytic

HSV infection compared to nonproductive infection, since the establishment of nonproductive infection is only confined to a very limited time-frame.

These results strongly suggest that the status of the host cell is a major determinant for the outcome of infection, and further necessitate the use of differentiating and stratified cell culture models for the studies of epithelial HSV-1 pathogenesis. This culture system can be extensively used for studies of effects of cellular factors, such as innate defence mechanisms, and viral genes on epithelial HSV infection in a model closely mimicking the natural site of primary infection, as achieving epithelial polarization and differentiation evidently has a dramatic impact on the outcome of HSV infection. A more mature and better differentiated epithelium can be achieved for these purposes by using primary keratinocytes (Visalli *et al.*, 1997). Furthermore, to better mimic the conditions in the oral environment, cells should originate from oral tissues. HaCaT cells were used for this study because this cell line is relatively well characterized and known to have preserved well its stratification capacity (Lehman *et al.*, 1993; Schoop *et al.*, 1999). Further, in this model, the effects of cellular determinants related to reactivation susceptibility in anatomically distinct sites (intraoral vs. labial), as implicated by empirical observations, can be investigated by use of cells isolated from different anatomical regions. The critical effect of the anatomical site for susceptibility to reactivation has been previously shown by the differential reactivation frequency for HSV-1 and HSV-2 in oral and genital regions (Lafferty *et al.*, 1987).

The use of 3D cultures and the interpretation of results also entail several limitations. Altogether, this culture technique is very sensitive regarding handling. 3D cultures are comprised of multiple variable components, including cells, matrix and growth conditions. Variation in these components has been shown to dramatically affect the phenotype of the stratified epithelium (Merne & Syrjanen, 2003). Therefore, the results from different 3D culture studies may be difficult to compare. Further, the keratinized epidermis formed in 3D cultures has been shown to be about 10 times more permeable than epidermis *ex vivo* (Regnier *et al.*, 1990), which probably has an effect, for example, on viral access to the epithelium. The major drawback for microbiological studies in this system is the lack of cells of the immune system. Keeping the above limitations in mind, 3D keratinocyte culture model provides a valuable instrument for *in vitro* studies of epithelial HSV-1 pathogenesis.

2. EFFECT OF SALIVA ON HSV INFECTION IN VITRO AND IN VIVO (III-V)

HSV is known to be frequently shed asymptomatically to saliva in seropositive subjects (Whitley, 2001). This frequency varies from about 5% of immunocompetent individuals to almost continuous shedding in some immunocompromised individuals. This asymptomatic shedding is thought to account partly for the transmission of HSV. However, HSV appears to be inactivated in saliva to some extent, although the mechanisms mediating this inhibition are largely unknown.

2.1. Innate salivary defence factors may contribute to the outcome of HSV reactivation phenotype

This series of studies aimed to elucidate the role of saliva and several innate antimicrobial salivary compounds in the regulation of establishment of initial HSV-1 infection and reactivation from latency in HSV-seropositive subjects with or without history of RLH and HSV-seronegative subjects.

Depending on HSV-serostatus and history of RLH, whole saliva was found to neutralize 56-67% of infectious HSV-1 *in vitro* within 30 min at 37°C (III, Figure 2b). Neutralization capacity was significantly higher ($p=0.05$) in saliva from asymptomatic HSV seropositive subjects compared to subjects with history of RLH, suggesting that composition of saliva may contribute to the HSV reactivation phenotype. In this group, neutralization capacity correlated positively ($p=0.026$) with salivary Lf concentration, and also a tendency for correlation with peroxidase-generated hypothiocyanite was observed ($p=0.094$). Importantly, salivary anti-HSV-1 antibody levels did not correlate with salivary HSV-1 neutralization capacity, indicating that constituents other than anti-HSV antibodies are predominantly responsible for HSV neutralization in saliva.

Previously, saliva has been shown to neutralize several viruses *in vitro*, including HSV (Gyselink *et al.*, 1978) and HIV (Fultz, 1986). Submandibular/-lingual saliva has been shown to possess greater HSV (Bergey *et al.*, 1993b) and HIV (Bergey *et al.*, 1993a) neutralizing capacity than saliva from the parotid gland. These results suggest that certain components of saliva that are mainly secreted by submandibular/-lingual glands (e.g., mucins and cystatins) may contribute significantly to the viral neutralization. Furthermore, anti-HSV-1-IgG antibodies have been suggested to have a major role in the neutralization of HSV in saliva (Gyselink *et al.*, 1978). However, in this study, neutralization capacity was independent of mucin, cystatin and anti-HSV-IgG concentration of saliva.

Preincubation of cells with saliva resulted in 50-60% reduction in HSV infectivity depending on HSV-serostatus and history of RLH (III, Figure 2a). Protective effect did not differ significantly between the groups. The protective effect was independent of the level of studied innate defence factors and salivary immunoglobulins. Previously, both unstimulated whole saliva (Heineman & Greenberg, 1980) and submandibular/-lingual saliva with even greater efficiency than parotid saliva (Bergey *et al.*, 1993b) have been shown to protect cells from HSV-1. Contrary to results obtained in the current study, Heineman and Greenberg (1980) found the cell protective effect to be higher in saliva of

seropositive subjects without history of RLH. The discrepancy of these findings may result from the use of differentially collected saliva with regard to stimulation, since stimulation of saliva secretion is known to contribute significantly to the composition of saliva (Ferguson, 1981). However, they similarly found the cell protective effect to be independent of anti-HSV salivary immunoglobulin levels (Heineman & Greenberg, 1980).

The absolute concentrations and the concentrations adjusted to salivary secretion rate of the studied salivary innate defence factors were comparable in all groups (III, Table 1). Whole saliva is a rich blend of various proteins and enzymes with potential anti-HSV-1 activity (see: Table 2 in Innate defence mechanisms). Therefore, it is unlikely that any single constituent would have a critical role in determining the outcome of oral HSV-1 infection. Indeed, innate salivary defence factors have been shown to act in both synergistic and additive manner, reviewed by (Tenovuo 1998). Salivary antimicrobial proteins and enzymes also become enriched in oral biofilms (Hannig *et al.*, 2005). Binding to these biofilms have been further observed to alter the antimicrobial activity of certain enzymes (Hannig *et al.*, 2005). Therefore salivary levels of these compounds do not reflect well the effective concentrations in the oral mucosa, which is the critical microenvironment in the prevention of HSV infection. However, our study does suggest that innate salivary defence factors may contribute to the control of orolabial HSV-infection. The role of innate defence factors may be greater at the time of initial infection when acquired mechanisms are still absent. In this situation, innate factors may contribute either by totally preventing the initial infection or by decreasing the initial infective dose that has been shown to be one critical factor in determining the reactivation susceptibility from the ganglion (Sawtell 1997). Importantly, along with natural antibodies, salivary innate defence factors are completely active already in small children, and have been shown to act fully and even partially compensate for immune deficiencies in immunocompromised patients, reviewed by (Tenovuo 1998).

2.2. Peroxidase-generated hypothiocyanite displays variable inhibitory activity against different types of viruses

Salivary peroxidase system constitutes a complex innate salivary defence system with broad antimicrobial properties. Salivary peroxidases predominantly oxidase SCN^-. The major oxidation product from this reaction and the antimicrobial effector molecule of this system is hypothiocyanite ($HOSCN/OSCN^-$). Moreover, consumption of H_2O_2 by this system protects mucosal cells from toxicity of reactive oxygen species.

The sensitivity of different types of viruses to $HOSCN/OSCN^-$ was found to differ dramatically. HSV-1 and RSV, which are both enveloped viruses, were highly susceptible to the inhibition by $HOSCN/OSCN^-$ in a dose-dependent manner (IV, Figures 1-2). In contrast, EV11, a non-enveloped enteropathogen, was highly resistant to this effect in physiological concentrations (IV, Figure 3). In the oral region, HSV-1 is in contact with saliva upon transmission, as well as EV11, which is typically transmitted through feco-oral route. EV11 is further known to endure well any physico-chemical stress, as it retains its infectivity in passage through the gastrointestinal tract. RSV, by contrast, infects the

host via nasal mucosa thereby omitting the contact with saliva before establishing an infection.

Normal salivary concentration of HOSCN/$OSCN^-$ varies in the range of 10-300μM (Tenovuo *et al.*, 1982; Tenovuo *et al.*, 1986). The concentration needed for inactivation of HSV-1 was minimal, as IC_{50} was 8.5 μM at pH 6.0, and 20 μM at pH 7.1. The inhibition of RSV was strongly pH-dependent. Whereas IC_{50} was 8 μM at pH 6.0, RSV was highly resistant to HOSCN/$OSCN^-$ at pH 7.1. Even the highest concentrations used (175 μM) did not yield an IC_{50} value. HOSCN/$OSCN^-$ had no effect on EV11 at pH 7.1. Surprisingly, however, at pH 6.0, the inhibition of EV11 was inversely proportionate to HOSCN/$OSCN^-$ concentration. The individual components required for the generation of HOSCN/$OSCN^-$ had no antiviral activity in the concentrations used. Furthermore, HOSCN/$OSCN^-$ concentrations up to 300 μM have been shown to cause no damage to human cells *in vitro* (Tenovuo & Larjava, 1984) further suggesting that the inhibitory effect was indeed mediated by HOSCN/$OSCN^-$.

Salivary pH varies in the range of 5-8. Decrease in pH from neutral to 6.0 enhanced the inhibitory effect of HOSCN/$OSCN^-$. This was more dramatically observed with RSV, as HSV-1 was readily inactivated already at pH 7.1 at very low concentrations of HOSCN/$OSCN^-$. RSV was dramatically inactivated by low pH under pH 6, therefore lower pH conditions for HOSCN/$OSCN^-$ -mediated inhibition could not be tested. HSV-1 and EV11 were more resistant to varying pH conditions in the pH range of 6.0-7.1. Slightly acidic pH actually appeared to preserve EV11 infectivity better than pH 7.1. Together these experiments show that even a subtle change in pH has a dramatic impact on antiviral action of HOSCN/$OSCN^-$. Furthermore, changes in the pH conditions may greatly affect the infectivity of a virus.

In accordance with our results, HOSCN/$OSCN^-$ has been previously shown to inactivate HSV-1 at a 100 μM concentration at pH 7.4 (Courtois *et al.*, 1990), but clearly even a significantly lower concentration is sufficient to completely inactivate HSV. The greater susceptibility of the both studied enveloped viruses, HSV-1 and RSV, may be associated with the sensitivity of the envelope lipid bilayer to highly oxidizing HOSCN/$OSCN^-$. The pK_a value for HOSCN/$OSCN^-$ is 5.3, indicating that in lower pH, the equilibrium of the reaction favours formation of uncharged HOSCN (Thomas, 1981). The uncharged HOSCN can penetrate biological membranes more readily than the charged $OSCN^-$. Slightly acidic conditions have similarly been shown to potentiate antibacterial effect of HOSCN/$OSCN^-$ (Lumikari *et al.*, 1991). The more efficient inhibition of HSV-1 and RSV at lower pH similarly suggests that antiviral activity of HOSCN/$OSCN^-$ can be enhanced by lowering pH. Furthermore, the changes in the pH microenvironment in the oral cavity may contribute to the survival of viruses. The nucleic acid composition of the viral genome did not seem to have any role in the inhibition, since RSV and EV11 containing a single stranded RNA genome differed greatly in susceptibility to inhibition by HOSCN/$OSCN^-$.

HOSCN/$OSCN^-$ concentrations measured from saliva in the clinical study varied between 0-92.8 μM (III, Table 1). The concentrations did not differ significantly between HSV-seropositive subjects with or without history of RLH or HSV-seronegative subjects.

Moreover, the discovered HSV-1 neutralizing or cell protective activity found in saliva was independent of HOSCN/OSCN$^-$ concentration, although a tendency for a positive correlation with neutralization capacity (r_s=0.43, p=0.094) was observed in the group of HSV-seropositive subjects without history of RLH.

2.3. Cationic salivary proteins, lactoferrin and lysozyme, differ dramatically in the ability to inhibit HSV-1 infection

Lactoferrin (Lf) and lysozyme (Lz) are abundant constituents of saliva. They are both known to possess wide spectrum antibacterial and antifungal activity, whereas less is known about their antiviral properties. Both of these proteins have a highly cationic charge which makes negatively charged cell surface molecules, such as GAGs, the major attachment receptor for HSV, attractive attachment sites for them. Indeed, both human (hLf) and bovine (bLf) have been shown to possess anti-HSV activity (Andersen *et al.*, 2004; Hasegawa *et al.*, 1994; Marchetti *et al.*, 1996; Marchetti *et al.*, 2004), but only little is known about LZ effect on HSV infection (Cisani *et al.*, 1989; Oevermann *et al.*, 2003).

Human lactoferrin (hLf) was found to interfere with progression of HSV-1 infection at multiple steps of replication cycle in a dose-dependent manner, whereas Lz did not display significant anti-HSV-1 activity in any of the assays (V). Therefore, the cationic nature of a protein is most likely not the key determinant of anti-HSV-1 activity of salivary proteins.

IC_{50} values for hLf applied at different stages of wt HSV-1 infection were in the range of 50-500< μg/ml. IC_{50} values were found to be lower when HSV-1 virion was incubated with hLf compared to pretreatment of cells with hLf (V, Figures 2-3). Earlier studies have strongly suggested that the main inhibitory activity of hLf is mainly mediated through competitive binding of Lf to cell surface GAGs, heparan (HS) and chondroitin sulphate (CS) (Andersen *et al.*, 2004; Marchetti *et al.*, 2004). However, bLf has been shown to neutralize HSV-1 in even lower concentration (Andersen *et al.*, 2004), and HSV-1 has been shown to bind to bLf (Marchetti *et al.*, 1996). Together these results suggest that both viral neutralization and interference with viral adsorption may contribute significantly to inhibition of HSV-1 infection. Consistently, HSV-1 infection was inhibited most efficiently in hLf pretreated cultures with hLf present at the time of viral inoculation and continuously thereafter throughout the entire replication cycle (V, Figure 1).

HSV-1 neutralization is at least in part mediated by hLf binding to gC and gD, since both the gC-negative strain, gC$^-$39, and gD-mutant strain, Rid1, were found to be more resistant to neutralization compared to wt HSV-1 (V, Figure 2). This is in accordance with previously reported resistant phenotype of gC$^-$39 to Lf-mediated inhibition (Marchetti *et al.*, 2004). Preincubation of gC$^-$39 with non-iron-saturated hLf significantly enhanced infectivity in low concentrations, suggesting that hLf binding to virion component other than gC, may mediate formation of contacts between virion bound hLf and cell surface hLf receptors facilitating interaction between HSV-1 virion and the target cell.

Wt and gC$^-$39 strains were equally sensitive to inhibition by pretreatment of cells with hLf (V, Figure 3), indicating that hLf-mediated inhibition of attachment is not only gC-restricted, but probably gB, the other major attachment receptor (Herold *et al.*, 1994), also contributes to this event. Surprisingly, Rid1 was more resistant to inhibition compared to wt HSV-1 and gC$^-$39. This observation suggests that also post-attachment events in the replication cycle are sensitive to hLf-mediated inhibition.

Supplementing cultures after viral attachment resulted in differential virus strain and Lf-iron-saturation dependent inhibition of infection (V, Figure 4), further suggesting that in addition to attachment, hLf has anti-HSV-1 activity also in the later stages of viral replication. In this assay, gC$^-$39 was found to be resistant to hLf effect after attachment, which is consistent with the major role of gC in viral attachment but not entry. Rid1 infectivity decreased about 60% at a concentration of 500 μg/ml of iron-saturated Lf, whereas non-saturated Lf had less inhibitory activity. Lf effect on Rid1 at post-attachment steps of viral replication is in accordance with the major role of gD in viral entry. Wt HSV-1 was even more sensitive to inhibition at this step with an IC_{50} of 100-250 μg/ml of iron-saturated Lf. At a concentration of 500 μg/ml infectivity was inhibited by 80%. Inhibition of wt HSV-1 was similarly dependent on the degree of Lf iron-saturation.

Earlier, however, bLf has been shown not to affect differentially on the entry of wt and Rid1 strains as observed by a β-galactosidase assay (Andersen *et al.*, 2004). Therefore, the gD-associated inhibition in our assay may rather yield from hLf interference with one of the other viral glycoproteins involved in fusion/entry, or post-entry events than interference with gD upon entry. Indeed, hLf has been shown to be internalized in the cells by HS-dependent nucleolin transport (Legrand *et al.*, 2004). Further, some of the previously reported lack of Lf-mediated inhibition of HSV-1 in cells deficient of proteoglycans (Andersen *et al.*, 2004) may result from reduced endocytosis of Lf, since nucleolin-associated transport has been shown to require the presence of HS on

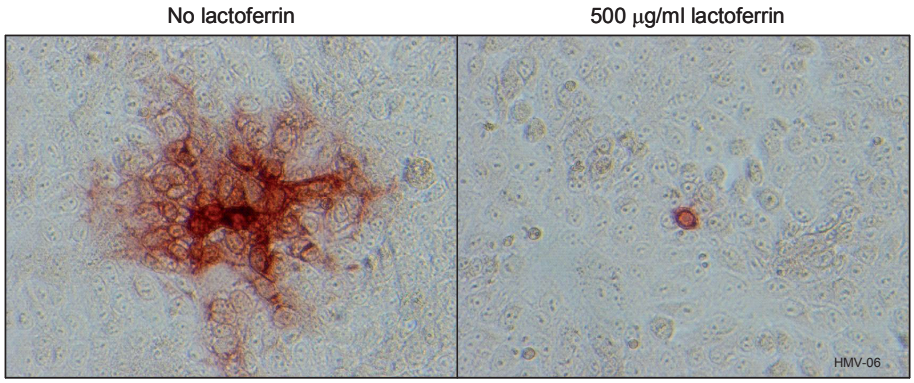

Figure 10. Lactoferrin inhibits efficiently cell-to-cell spread of HSV-1 in Vero cells. HSV-1 infected cells are stained with peroxidase-conjugated anti-HSV-antibody 20 h post infection.

the cell surface (Legrand *et al.*, 2004). Nucleolin is shuttling between cell surface and the nucleus, and contributes thereby presumably also to the dynamics of Lf localization and function in cells. In addition to GAGs, other cell surface molecules have been implicated in Lf binding to cell surface (Ji & Mahley, 1994; Suzuki *et al.*, 2001). Of these, the lipoprotein receptor-related protein can bind to Lf also in the absence of HS (Ji & Mahley, 1994). Therefore, Lf binding and internalization may to some extent be cell type specific depending on the expression pattern of a specific Lf receptor. A nuclear localization signal in the N-terminal region of Lf has been identified as well (Penco *et al.*, 2001). Subsequently, hLf interference with post-attachment and -entry events may involve interference with either HSV-1 replication events or progeny glycoproteins within the cell, or with the release of progeny virus at the cell surface.

Indeed, hLf addition after the attachment step furthermore resulted in dramatic changes in the ability of HSV-1 to spread from cell-to-cell. This effect was highly dependent on the viral strain, but independent of Lf degree of iron-saturation. Rid1 plaque-size remained unchanged, whereas at 500 μg/ml of hLf wt HSV-1 and gC$^-$39 plaque size was dramatically reduced (Figure 10). Cell-to-cell spread is known to involve primarily gB and gE/gI, but not gC directly (Dingwell *et al.*, 1994; Dingwell & Johnson, 1998; Laquerre *et al.*, 1998). The susceptibility of gC-negative HSV-1 strain to inhibition of cell-to-cell spread may result from the removal of the gC masking effect, which may allow hLf interference with gB binding to heparan sulphate at cell junctions (Laquerre *et al.*, 1998). A comparable inhibition of cell-to-cell spread of wt HSV-1 but not Rid1 suggests that gD may also have a role in direct cell-to-cell spread. Cell-to-cell spread is a major determinant in viral pathogenesis, and therefore this observation further establishes the significant role of Lf as an innate anti-HSV compound.

Taken together, in accordance with hLf inhibition of HSV-1 infection at multiple steps of viral replication cycle (Figure 11), HSV-1 infection was inhibited most efficiently in hLf pretreated cultures with hLf present at the time of viral inoculation and continuously thereafter throughout the entire replication cycle.

In addition to monolayer cultures, hLf effect on HSV-1 infection was studied in 3D keratinocyte culture in order to study hLf effect on HSV-1 infection in a culture model mimicking more closely the natural epithelium (V). As expected, cultures infected prior to lifting produced HSV-1 progeny determined by viral isolation from culture homogenates. Surprisingly, however, viral yields were the highest from cultures supplemented with hLf. Furthermore, cultures supplemented with hLf were also positive by viral isolation when infected 30 min after lifting, whereas control cultures and cultures with Lz supplement remained negative. Based on the earlier HSV infection experiments in this 3D model (I, II), cultures infected 30 min after lifting were expected to remain negative for HSV by viral isolation (see: Suitability of 3D cultures for HSV infection studies). The highest amount of progeny virus was constantly detected in cultures with iron-saturated hLf. Thus, instead of inhibiting HSV-1 replication, the presence of hLf appeared to enhance viral replication.

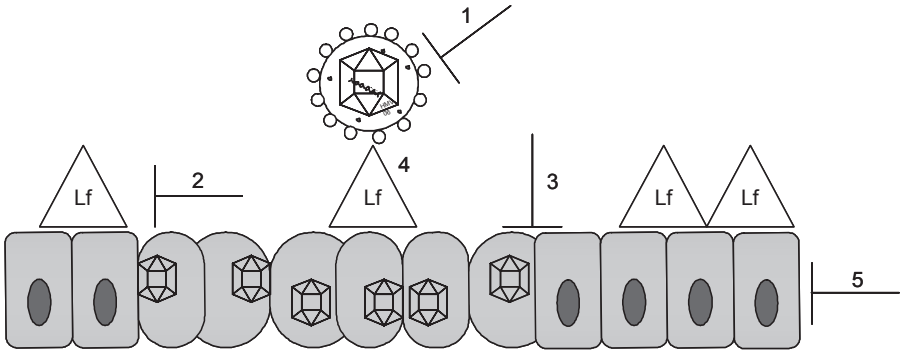

Figure 11. Lactoferrin (Lf) modulates HSV-1 infection at multiple steps of replication. Lf neutralizes free HSV-1 virions preventing their subsequent attachment to cell surface receptors (1). Lf can additionally mask HSV-1 cell surface attachment sites (2), and inhibit cell-to-cell spread of progeny virus (3). In the absence of the primary attachment-mediating viral glycoprotein, gC, Lf may act as a mediator and facilitate HSV-1 interaction with cell surface receptors and thereby promote the initiation of infection (4). Lf may further modulate HSV infection through its effects on the proliferation activity of the cells (5).

This effect may be attributed to the different status of the hLf supplemented HaCaT monolayer at the time of viral inoculation compared to the control cultures, since development of confluent monolayers was delayed in cultures supplemented with hLf in an hLf iron-saturation dependent way (Figure 11). Consequently, hLf supplemented HaCaT cultures were subconfluent at the time of viral inoculation. The pattern of BrdU positivity in stratified 3D culture was, however, not different between the control, and the hLf or Lz supplemented cultures, mostly likely reflecting the quantitative insensitivity of the method. Lf has previously been shown to inhibit cell proliferation (Damiens *et al.*, 1999). Histomorphologically, the control, and the hLf or Lz supplemented cultures were not distinct from each other. This result further suggests that differentiation and proliferation status of the host cell may critically contribute to the outcome of HSV-1 infection. It also further indicates that access to proliferating and horizontally growing basal cells (e.g., through epithelial scars) may enhance the initiation of HSV-1 infection.

In vivo studies similarly implied a role for Lf in the inhibition of oral HSV infection (III). The measured salivary Lf concentrations were in the range of 3.5-76.0 μg/ml (III, Table 1). No significant differences were observed between HSV seronegative and seropositive subjects with or without history of RLH. However, as stated above, salivary HSV-1 neutralization capacity did correlate positively ($p=0.026$) with Lf content of saliva in seropositive subjects without history of RLH, suggesting that salivary Lf may influence the reactivation phenotype of HSV-1 in the oral region.

Based on this series of studies, Lf appears to have a dual role in oral HSV-1 infection. It possesses inarguably anti-HSV activity at the initial stages of infection but also apparently at multiple later steps in the viral replication cycle. Iron-saturation is unlikely to have a major role in anti-HSV-1 effects exerted by Lf, although the saturation percentage appears to contribute to this activity at certain steps of viral replication, as indicated by the present and some earlier studies (Hasegawa *et al.*, 1994; Marchetti *et al.*, 1996; Marchetti *et al.*, 1998). Iron supplementation as such apparently has no significant direct effect on HSV-1 viral replication (Marchetti *et al.*, 1998). Furthermore, degree of iron-saturation-associated minor differences in anti-HSV-1 activity have been rather attributed to conformational changes due to iron-binding than to presence or absence of iron as such (Hasegawa *et al.*, 1994; Marchetti *et al.*, 1996; Marchetti *et al.*, 1998). Therefore, the mechanism of inhibition is very distinct from the well established Lf-effect on bacteria, which is predominantly based on iron-depletion. Furthermore, the effect is not primarily related to the cationic nature of hLf, since the other studied cationic protein, Lz, had no significant effect on HSV-1 replication, although cationic proteins have affinity for negatively charged cell surface proteins, such as GAGs.

In an earlier study, Lz has been shown to inhibit HSV syncytia-formation (a step involving cell-to-cell spread of HSV-1) with a higher concentration of Lz than was used in the current study, by a mechanism related to the enzymatic activity and cationic nature of Lz (Cisani *et al.*, 1989). In the *in vivo* study (III), Lz content of saliva did not correlate with HSV-1 neutralization capacity or cell protective effect, and the level of salivary Lz was comparable in all groups regardless of serological status for HSV or history of recurrent labial herpes (III, Table 1).

The concentration of whole salivary Lf does not always reach that required for efficient inhibition of HSV-1 infection observed *in vitro*. However, in certain oral microenvironments, like the gingival crevices and the biofilm covering the mucosa, Lf concentration can be significantly higher (Friedman *et al.*, 1983). These microenvironments are the critical sites regarding oral HSV infection, and therefore Lf presumably may modulate substantially the course of oral HSV infection.

3. ADAPTIVE IMMUNITY IN OROLABIAL HSV INFECTION (III, VI)

Th cells are currently divided into four functionally distinct subclasses (see: Adaptive immunity). Of these subclasses, Th1 and Th2 cells are the most thoroughly studied in association with various infectious diseases. Th1 cells are stimulated by IL-12. These cells characteristically secrete IFN-γ and TNF-β, and mediate proinflammatory or cell-mediated immune responses. Th2 cell function is stimulated by IL-4. Th2 cells secrete IL-4, -5. -9 and –13, and promote humoral immune responses. Th1 and Th2 type cytokines are mutually antagonizing. Th1 and Th2 type cytokines have been further indicated in the immunoglobulin isotype synthesis. In humans IgG1 is downregulated and IgG2 induced by IFN-γ (Kawano *et al.*, 1994), whereas IgE and IgG4 are induced by IL-4 (Lundgren *et*

al., 1989), indicating IgG1, IgG4 and IgE association with Th2 and IgG2 association with Th1 type immune responses.

3.1. Subjects with Th2 biased cytokine responses to HSV are susceptible to recurrent labial herpes

PBMCs from subjects with history of RLH episodes responded to inactivated HSV antigen by producing significantly ($p<0.02$) higher amount of IL-4 mRNA compared to asymptomatic subjects (VI, Figure 1a). Furthermore, IFN-γ/IL-4 ratio was significantly ($p<0.04$) lower in subjects with RLH (VI, Figure 1b), indicating a bias towards Th2 type response. These observations are consistent with previously reported finding of subjects with RHL producing less IFN-γ protein (McKenna *et al.*, 2001; Spruance *et al.*, 1995). Together these results suggest Th2 predominance over Th1 in Th cell responses against HSV in subjects with RLH, and a critical role for Th2 bias in determining the peripheral outcome of reactivation.

The overall T cell proliferation response was comparable in subjects with RHL and historically asymptomatic subjects (VI, Figure 2). Interestingly, an additional group of subjects with comparable T cell proliferation response in the absence, or with an extremely low level of anti-HSV-IgG-antibodies, was identified. This is consistent with previously described seronegative immune subjects for hepatitis C virus (Koziel *et al.*, 1997), HSV-2 (Posavad *et al.*, 2003) and Epstein-Barr virus (Savoldo *et al.*, 2002). Together, these findings suggest, that seroconversion may not be a consistent indicator of previous exposure to a viral pathogen.

3.2. Anti-HSV antibodies are relatively inefficient in protecting the host from recrudescences in the oral region

The overall HSV-seroprevalence among medical students, who participated in the study, was 39.5% (III). It has to be taken into account, that the eagerness to participate may have been influenced by a subject´s phenotype for recurrent HSV infection. However, the observed seroprevalence is consistent with recently reported seroprevalence numbers in this age group in Finland (Pebody *et al.*, 2004). Due to reduced childhood HSV transmission, the likelihood of having genital infection as the primary HSV infection has increased.

The level of anti-HSV-IgG antibodies in serum ($p=0.0005$) and saliva ($p=0.042$) was significantly higher in HSV-seropositive subjects with a history of RLH compared to asymptomatic seropositive subjects (III, Table 2). This is consistent with the observed increased level of Th2 cytokine, IL-4, in subjects with RLH. The level of anti-HSV-IgG did not correlate with the frequency of RLH. A role of antibodies in defence against HSV disease in not well understood. Previous studies are in accordance with our results, and similarly suggest that pre-existing antibodies do not provide efficient protection against recurrent disease (Spruance *et al.*, 1995). Furthermore, anti-HSV-antibody level and composition of antibody pool remains relatively constant despite repeated attacks of RLH (Zweerink & Stanton, 1981). A role for antibodies in interfering with spread of HSV

between latency sites and the periphery has been implied by animal studies (Halford *et al.*, 1997; Mikloska *et al.*, 1999).

IgG subclasses were differentially distributed in asymptomatic subjects and subjects with RLH (VI, Figure 3b). The levels of anti-HSV-IgG3 ($p<0.02$) and –IgG4 ($p<0.01$) were significantly higher, and anti-HSV-IgG2/IgG4 ratio smaller in subjects with history of RLH compared to asymptomatic subjects, further suggesting Th2 polarization of immune response in subjects with RLH. Consistently, IgE has been shown to associate with RLH (McKenna *et al.*, 2001). These results also indicate that the higher level of total anti-HSV-IgG in subjects with RLH is associated with the higher amount of anti-HSV-IgG3 and -IgG-4. IgG3 and IgG4 are normally the minor constituents of the total IgG pool in serum. Previous studies have similarly suggested an association between anti-HSV-IgG4 and frequent HSV reactivations (Ljungman *et al.*, 1988; Sundqvist *et al.*, 1984).

3.3. Salivary anti-HSV antibodies are mainly of IgG isotype

As expected, IgA class antibodies were the most predominant antibody isotype present in saliva (III, Table 1). However, most of the anti-HSV-antibodies were of IgG isotype (III, Figure 1, Table 2). The level of salivary anti-HSV-IgA was so low that not all HSV-seropositive subjects were positive for salivary anti-HSV-IgA by the test used. Instead, all subjects positive for serum anti-HSV-IgG were also positive for salivary anti-HSV-antibodies. This is in accordance with previously suggested major role of anti-HSV-IgG instead of anti-HSV-IgA in immunoglobulin-mediated salivary neutralization of HSV (Douglas & Couch, 1970; Gyselink *et al.*, 1978). The redundant role of sIgA in oral health have been shown in subjects with selective IgA deficiency, who do not appear to have any enhanced risk for oral infections, reviewed by (Tenovuo, 1998).

The level of salivary anti-HSV-IgG antibodies did not correlate with the serum antibody level. This is surprising, since IgG antibodies have been suggested to predominantly enter oral cavity passively as serum transudate via gingival crevices (Brandtzaeg *et al.*, 1970; Challacombe *et al.*, 1978). Accordingly, level of salivary antibodies should correlate with serum antibodies. The level of salivary IgG has also previously been shown to be independent of serum levels (Gronblad & Lindholm, 1987). Furthermore, significant proportionate variation exists in IgG subclass distribution between saliva and serum (Berneman *et al.*, 1998), and edentulous patients have significant amounts of IgG in saliva (Gronblad & Lindholm, 1987; Kraus & Sirisinha, 1962). Some of the salivary IgG is derived from the major salivary glands, but pure secretion from these glands contains only little IgG (Brandtzaeg *et al.*, 1970). In oral mucosa, local IgG production has been earlier observed in patients with periodontitis, reviewed in (Kinane & Lappin, 2002). Accordingly, possibility exists, that during HSV reactivation episodes, significant amount of salivary HSV-IgG is synthesized locally in mucosal plasma cells and released to saliva.

4. HYPOTHETICAL MODEL FOR PERIPHERAL CONTROL OF HSV DISEASE IN THE ORAL REGION

Based on the above and earlier studies, a following model for peripheral control of primary infection and reactivation in the oral environment is proposed (Figure 12). In primary infection, the constitutively secreted innate salivary proteins, mainly derived from the salivary glands and the keratinocytes, and the natural antibodies contribute to the neutralization of HSV-1 prior to contact with the mucosal epithelial cells. Some of the innate proteins (e.g., lactoferrin) can subsequently inhibit the attachment of HSV-1 to target cells by competitive binding to its cell surface receptors. Beyond this point, innate salivary defence factors together with innate defence mechanisms of the keratinocytes can further interfere with replication of HSV-1 and spread to neighbouring cells. Keratinocytes and Langerhans´ cells, the resident APCs of the epithelium, activate other DCs, and attract neutrophils and NK cells to the site of infection. Together these cells further attract and activate adaptive immune responses via antigen presentation and secretion of various cytokines and chemokines. Once the acquired immunity is activated, the lesions are cleared by CD8+ T cells.

The function of innate defence factors is critical in limiting the initial viral inoculum and mucosal replication, since the amount of virus entering the ganglion is known to correlate positively with the extent of ganglionic replication, the number of established latent sites and the subsequent susceptibility to reactivation.

The suppression of reactivated latent ganglionic infection is predominantly controlled by the action of CD8+ T cells and IFN-γ. Factors, like stress or glucocorticoids, which are able to transiently compromise CD8+ T cell function, may allow lytic HSV replication following reactivation to continue thereby allowing HSV transportation to the periphery along the neurons. In the periphery, similar to the primary infection, the professional cells of the innate defence system limit epithelial infection. Innate immunity is further supported by the function of innate defence mechanisms of the keratinocytes and intraorally by salivary compounds. In the presence of pre-existing immunity, adaptive immune functions are recruited quickly as well. HSV-specific CD4+ Th cells contribute either by producing Th1 type cytokines providing efficient help for CD8+ T cells known to be critically important for the clearance of HSV lesions, or by producing Th2 type cytokines inducing suppression of CD8+ T cell response and enhancement of humoral responses, which results in inefficient clearance of infection and manifestation of recurrent disease. Therefore it is suggested, that whereas reactivation at the ganglion level is primarily controlled by the resident ganglionic CD8+ T cells, in the periphery, the balance between Th1 and Th2 cell functions in addition to function of innate defence mechanisms, may be critically important in determining weather reactivation results in asymptomatic or symtomatic infection. These differentiating T cell and innate immunity functions may similarly contribute to the prolongation of subclinical epithelial infection, as suggested by limited data indicating HSV epithelial persistence. Furthermore, the replication and differentiation status and the anatomically defined type of the keratinocyte may have dramatic effects on the outcome of infection.

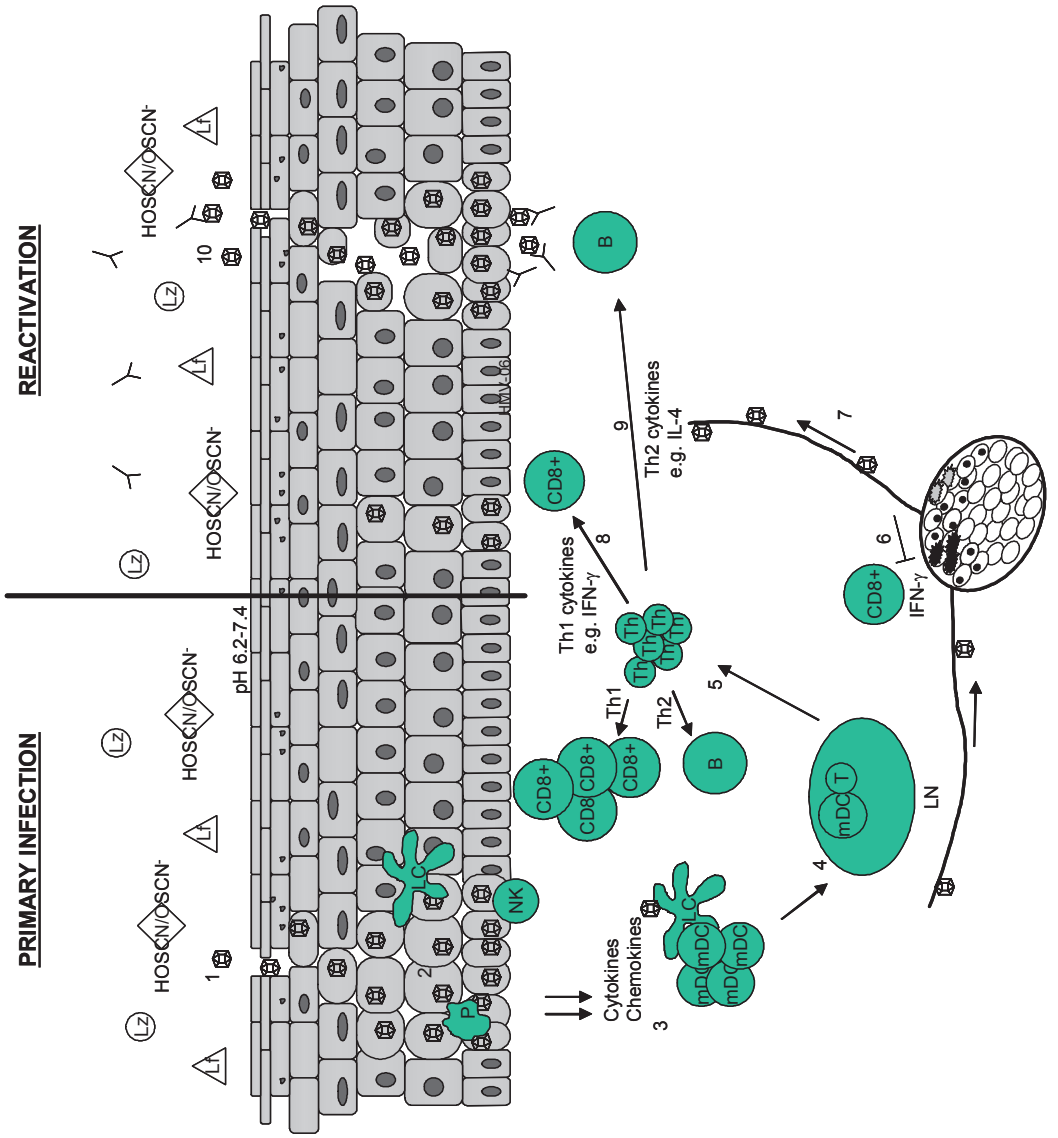
REACTIVATION
PRIMARY INFECTION
HOSCN/OSCN-
Lz
Lf
pH 6.2-7.4
HMW-Mucin
1
2
3
4
5
6
7
8
9
10
LC
NK
P
CD8+
Th1
Th2
Th1 cytokines
e.g. IFN-γ
Th2 cytokines
e.g. IL-4
IFN-γ
Cytokines
Chemokines
B
mDC
T
LN

Figure 12. Hypothetical model for peripheral control of HSV-1 in the oral region. Oral mucosa is bathed by saliva. Saliva contains various innate defence factors, which contribute to the initial inactivation of cell free HSV-1 and hinder attachment to cell surface (1). Furthermore, occasional acidic salivary pH conditions inactivate HSV-1. Infection of keratinocytes evokes production of a multitude of cytokines and chemoattractants and activates innate immune functions in keratinocytes (2). Resident Langerhans cells (LC) and invading phagocytes (P), including monocytes and neutrophils, phagocytose infected keratinocytes, and NK cells kill HSV-infected cells. Some LCs also get infected by HSV-1 themselves. Dendritic cells of myeloid origin (mDC) are further attracted to the site of infection (3). DCs phagocytose infected cells, including LCs, and migrate to draining lymph nodes (LN) to present antigens to naïve CD4+ and CD8+ T cells (4). Activated T and B cells migrate to the site of infection (5). CD4+ T helper (Th) cells reach the site first, and secrete cytokines to further amplify and direct immune responses. Th cells are followed by an influx of CD8+ T cells, and finally B cells. Lesion is cleared from infection by CD8+ T cells.

Reactivated infection in trigeminal ganglion is primarily controlled by CD8+ T cells and cytokines it secretes, largely IFN-γ (6). Transient debasement of immune control leads to appearance of infectious HSV-1 and transport to the epithelium (7). In the presence of pre-existing immunity, memory T and B cells get activated, and home to the site of recurrent infection. Th cell pool is again the first to be activated, and orchestrates the function of CD8+ T cells and B cells. Predominantly Th1 type cytokine production results in efficient CD8+ T cell response and termination of infection (i.e., abortive lesion) without development of symptoms or asymptomatic secretion of virus (8). Th2 type biased cytokine response supports B cell stimulation and results in inefficient limitation of HSV-1 infection, and recrudescent HSV disease (9). Released progeny virus is further inactivated by innate and adaptive salivary immune mechanisms (10).

SUMMARY AND CONCLUSIONS

Oral cavity is the major portal of entry for HSV-1. Within oral cavity, HSV comes in contact with saliva and the biofilm lining mucosal surfaces, the typical site for initial HSV replication.

Saliva was found to exert anti-HSV-1 activity independent of anti-HSV antibodies. The constitutively secreted salivary substances, lactoferrin and peroxidase-generated hypothiocyanite, were found to either neutralize HSV-1 or interfere with HSV-1 replication at multiple steps, whereas lysozyme displayed no anti-HSV activity. Furthermore, lactoferrin was found to modulate HSV-1 replication by affecting proliferation of keratinocytes. The more efficient salivary HSV-1 neutralization capacity was found to be associated with historically asymptomatic HSV infection, and the neutralization capacity to be independent of anti-HSV antibodies and at least partially dependent on the salivary lactoferrin content, suggesting that innate defence factors may also contribute to the reactivation phenotype of HSV-1 in the oral region. Therefore, although asymptomatic oral HSV shedding is very common, the presence of saliva may at least partly explain the infrequency of intraoral clinical disease upon reactivation, as well as that of primary infection. The recurrent disease susceptible phenotype was further found to be associated with Th2 type biased cytokine and antibody responses, indicating the lack of efficiency of humoral response in the control of HSV disease. Three-dimensional organotypic culture model was found to be valuable for studies on HSV-1 infection of keratinocytes. In this model, keratinocytes were found to support both lytic and nonproductive infection, suggestive of HSV persistence in epithelial cells. This observation further emphasizes the significance of peripheral immune responses in the control of HSV.

Identification and functional characterization of salivary innate proteins against HSV-1 is important for understanding anti-HSV functions in external secretions in general, since the same antimicrobial proteins are ubiquitously present in various mucosal secretions. Effective neutralization of microbes in the oral cavity and other mucosal surfaces provides protection for the whole body before initiation of an infection. Information on innate anti-HSV substances can be further utilized for the development of topical treatment for HSV disease. Such applications would be most needed for subjects suffering from reduced salivary secretion. Results regarding the relative efficiency of B cell and different type of Th cell responses may have indications for future vaccine and HSV-based gene therapy applications.

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