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Functions of glycoprotein G of herpes simplex virus type 2

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Cover:
Localization of mgG-2 in primary hippocampal neurons from newborn rats infected with HSV-2. The mgG-2 protein is visualized with MAb (red). Neurons are stained with MAP-2 antibody (green) and cell-nuclei are stained with HOECHST (blue).
(photograph by Mona Brantefjord).

Till Sofia, Simon, Amanda och Olivia

Functions of Glycoprotein G of Herpes Simplex Virus Type 2

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Abstract

Background: Herpes simplex virus type 2 (HSV-2) is a common sexually transmitted infection with more than 500 million individuals infected world-wide. A major global health problem is that HSV-2 infection facilitates the spread of HIV. This epidemiological situation warrants the development of a vaccine to reduce the spread of HSV-2. As HSV-2 often is transmitted without symptoms type specific serology is valuable in diagnosis of the infection. The envelope glycoprotein G (gG-2) of HSV-2 is cleaved intracellularly into a membrane bound portion (mgG-2) and a secreted portion (sgG-2). Although the function of mgG-2 is unknown the protein induces a type specific antibody response. This feature is utilized in several serological HSV-2 type specific assays. A general problem is that assays which are reliable in Western world country populations show lower performance for sera from African countries.

Aims: To evaluate sgG-2 as type specific antigen in ELISA for detection of HSV-2 infection in a Swedish cohort and define the performance for both sgG-2 and mgG-2 in ELISA for high HSV-2 prevalence cohorts in Tanzania (paper I and II). To elucidate the function of mgG-2, using HSV-2 mutants, in a genital mouse model (paper III) and to evaluate mgG-2 as a vaccine candidate against genital HSV-2 infection in mice (paper IV).

Methods: Natively produced sgG-2 and mgG-2 proteins were purified by immunosorbent columns from HSV-2 infected cell cultures. For the vaccine studies mgG-2 was purified using *Helix pomatia* lectin affinity chromatography. A frame shift HSV-2 mutant devoid of mgG-2 and an emerald green fluorescent protein labelled sgG-2 and mgG-2 negative HSV-2 were used for functional studies in C57BL/6 mice. Viral load was estimated by virus isolation and by real time PCR. C57BL/6 mice were immunized with mgG-2 and CpG as adjuvant and challenged genitally with wild type HSV-2.

Results: We showed that sgG-2 is a novel antigen that can be used for type specific serological diagnosis of HSV-2 infection and that an ELISA based on mgG-2 can improve the detection of HSV-2 infected individuals from Africa. Both mgG-2 deficient HSV-2 mutants were severely attenuated and all mice survived a genital infection. The viral mutants infected the genital mucosa but did not induce genital disease and were not propagated to the sensory ganglia or to the CNS. Finally, mice immunized with mgG-2 and adjuvant were highly protected against a lethal dose of wild type HSV-2. Protection was associated with an enhanced Th1 response and IFN-γ production in re-stimulated CD4+ T cells.

Conclusion: Native sgG-2 and mgG-2 proteins induce type specific antibody responses and perform well in ELISA in low as well as in high HSV-2 prevalence populations. The mgG-2 protein has an important function in the ability of HSV-2 to infect nervous tissue. As vaccination with mgG-2 protects mice from lethal disease mgG-2 constitutes a promising vaccine candidate against HSV-2 infection. In human trials detection of anti-sgG-2 antibodies may be a valuable tool to discriminate between vaccine induced immunity and natural HSV-2 infections.

Keywords: Herpes simplex virus type 2, glycoprotein G, type specific serology, vaccine, Tanzania.

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II. **Görander Staffan, Mbwana Judica, Lyamuya Eligius, Lagergård Teresa, Liljeqvist Jan-Åke.**
Mature glycoprotein G presents high performance in diagnosing herpes simplex virus type 2 infection in sera of different Tanzanian cohorts. Clin Vaccine Immunol. 2006:13(6):633-9.

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IV. **Görander Staffan, Lindqvist Madeleine, Harandi Ali, Liljeqvist Jan-Åke.**
Vaccination with the mature glycoprotein G of herpes simplex virus type 2 protects against genital and neurological disease. In manuscript.

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Abbreviations

aa	Amino acid/s
ACMC	Antibody-dependent complement-mediated cytolysis
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
CTL	Cytotoxic T-lymphocyte
DCs	Dendritic cells
GAG	Glycosaminoglycan
gG	Glycoprotein G
GM-CSF	Granulocyte macrophage colony-stimulating factor
HPA mgG-2	*Helix pomatia* purified mgG-2
HSV-2 TK−	Thymidine kinase-deficient HSV-2
IFN	Interferon
IFN-αβ	Interferon alfa/beta
IFN-γ	Interferon gamma
IL	Interleukin
i.n.	Intranasal
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
IP-10	Interferon inducible protein-10
IVAG	Intravaginal
LAT	Latency associated transcript
MAbs	Monoclonal antibodies
MCP-1	Monocyte chemoattractant protein-1
mgG-2	Mature portion of gG-2
MIP-1α	Macrophage inflammatory protein-1α
MIG	Monokine induced by interferon-γ
NO	Nitric oxide
nt	Nucleotide
NK cells	Natural killer cells
NKT	Natural killer T cell
NT	Neutralization
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RANTES	Regulated upon activation, normal T cell expressed and secreted
sgG-2	Secreted portion of gG-2
TMR	Transmembraneous region
Th1	T helper type 1 T cell
Th2	T helper type 2 T cell
TLR	Toll-like receptor
wt	Wild type

1. Introduction

The *herpesviridae* belongs to the DNA viruses and constitute a virus family with more than 100 members which are disseminated among animals and humans (Roizman 1996a). The genome is organized as double-stranded DNA and the structure of the virion is illustrated in Fig 1. It is built up by four components: (i) the central electron dense core containing DNA, (ii) an icosahedral capsid organized into 162 capsomeres, (iii) the tegument consisting of an amorphous material and viral proteins and, (iv) the envelope surrounding the viral particle with viral glycoproteins forming protruding spikes. These components have different roles during infection. The envelope enables entry into cells, the capsid is necessary for transportation of DNA into the nucleus of the infected cells, and the tegument proteins are involved in the initiation of the infection by interacting in cellular functions.

Eight herpesviruses have so far been identified in humans: HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7 and HHV-8. Based on molecular phylogeny three subfamilies have been established (McGeoch et al. 1995). These three groups correspond to the current taxonomic classifications based on biological properties and include *alpha-, beta- and gammaherpesviridae.* HSV-1 and HSV-2 belongs to the *alphaherpesviridae* and are classified in this subfamily on the basis of a wide host cell range, efficient and rapid reproductive cycle, and the capacity to establish latency in sensory ganglia (Roizman 1996a).

2. Herpes simplex viruses

2.1. Genome and replication

The HSV genome is organized in two covalently linked segments, the unique long (UL) and the unique short (US). The overall identity of the HSV-1 and HSV-2 genome is 48%. The major difference in length between the two HSV subtypes is located in the US region were the gG-1 gene is 714 nt in length while the gG-2 gene contain 2097 nt. Earlier studies indicate that the HSV genome encodes at least 84 different polypeptides of which 65 map to the UL region and 14 map to the US region (Whitley et al. 1998). The gene, encoding for gG-2, the main issue for this thesis, maps to the US region.

The double-stranded DNA of HSV enters into the nucleus of the cell where replication takes part. A controlled program of viral gene expression is initiated. Although the synthesis of proteins forms a continuum they can be classified into three groups (Roizman 1996b). First, approximately 2 to 4 h after infection, the α-genes are expressed. These immediate early (IE) genes code for proteins which are mostly regulatory in function. They are essential for efficient production of the subsequent proteins. Second, the synthesis of β-genes (early genes) reaches a peak 5 to 7 h post-infection. The proteins encoded by β-genes are involved in viral DNA synthesis and nucleic acid metabolism. The viral DNA polymerase is an example of a β-gene product. Third, the γ-genes (late genes) encode for structural components such as capsid and envelope proteins (Whitley et al. 1998).

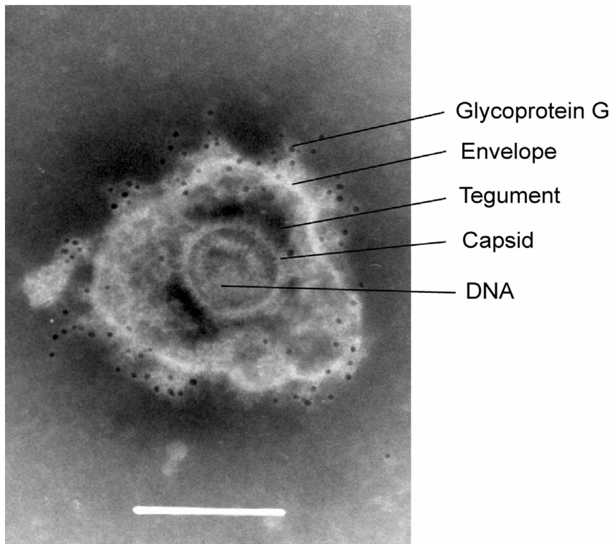

Fig. 1. Electron microscopy picture of an HSV-2 virion. mgG-2 in the envelope is visualized by using a monoclonal antibody (black spots). Bar = 100 nm

2.2. Latency

One of the major features of the HSV infection is the ability of the virus to remain latent in the sensory ganglia. Following an HSV infection viral replication occurs in the oral or genital mucosa and the virus enters sensory nerve endings innervating the mucosal membranes. Viral nucleocapsids are transported to the nucleus of the sensory neurons (ganglia) by the retrograde axonal transportation system via the microtubules. At the nuclear membrane the content of viral DNA is released through the nuclear pores into the nucleus followed by a productive infection or the establishment of latency.

In the latent infection the viral DNA is maintained as a circular episome (Mellerick and Fraser 1987) and the expression of viral genes may be silenced. An 8.5 kb RNA is transcribed from within the long repeat region of the genome and spliced into a family of smaller species designated latency-associated transcript (LAT). The LAT gene promoter shows neuronal specificity and when neurons express LAT they establish latency. Most studies have found no evidence for LAT-directed protein expression. Instead, the LAT function is effected by the transcript itself. Expression of LAT in cultured cells has been shown to reduce lytic (productive) viral gene expression responsible for reactivation and LAT− mutant viruses produce more severe pathology and neuronal death in trigeminal ganglia and cause higher mortality in infected mice as compared with LAT+ virus. In addition, LAT is associated with the prevention of apoptosis of the neuronal cells. This process involves condensation of the chromatin on the lytic gene promotors followed by inactivation (Knipe and Cliffe 2008).

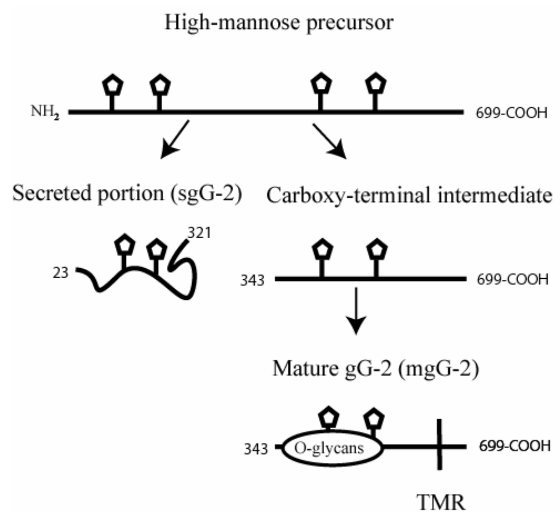

Fig. 2. Processing and cleavage of the gG-2 protein.

3. Glycoprotein G

3.1. Processing and cleavage of gG-2

A protein expressed in HSV-2 infected cells but not in HSV-1 infected was described and mapped to the Us segment of HSV-2 (Marsden et al. 1984). This protein was expressed on the cell-membrane of virus infected cells and was designated as glycoprotein G-2 (gG-2)

(Roizman et al. 1984). The processing of gG-2 is illustrated in Fig. 2. An unglycosylated precursor protein is first produced which is further *N*-glycosylated with high mannose residues followed by cleavage into the high mannose intermediate which was further *O*-glycosylated generating the mature or membrane bound portion of gG-2 (mgG-2) and the product of 40 kDa which is rapidly secreted to the culture medium (sgG-2) (Balachandran and Hutt-Fletcher 1985; Olofsson et al. 1986; Su et al. 1987). The first 22 aminoterminally located aa's of sgG-2 is a signal peptide that is cleaved off from the protein before secretion (Liljeqvist et al. 1999a). Transfection of a mammalian cell line with the gG-2 gene resulted in correct processing and cleavage of the gG-2 precursor protein suggesting that these events are not dependent on other HSV-2 gene products. Based on these findings it was suggested that cleavage of the gG-2 precursor was mediate by a cell-specific protease. Studies based on site-directed mutagenesis have proposed that the precursor protein is cleaved between arginine 321 and alanine 322 and between arginine 342 and leucine 343. These data have not been published but confirmed by R L Courtney (personal communication with Liljeqvist).

3.2. Type-specific properties of gG-2

Already in 1921 Lipschütz suggested that HSV infection etiologically could be divided into herpes febrilis (HSV-1) and herpes genitalis (HSV-2). The antigenic differences were first demonstrated by different biologic activities in complement fixation and neutralization assays (Nahmias and Dowdle 1968; Schneweis 1962). Although antibodies elicited after HSV-1 and HSV-2 infections could be distinguished by using these assays there were difficulties to interpret the results since the subtyping method was based on relative differences. It was however clear that the immune responses against HSV-1 and HSV-2 were both type common (cross-reactive), which means that antibodies against one of the subtypes recognize the other and *vice versa*, and type-specific implicating that antibodies elicited by one subtype do not recognize antigen structures on the counterpart (Bernstein et al. 1985). Attempts were made to find antigen components that could be used for type-discriminating serology. The first candidate antigen was glycoprotein C (gC-1 and gC-2) which were suggested to induce type-specific antibody responses (Arvin et al. 1983; Eberle and Courtney 1981; Vestergaard and Norrild 1978). However, type common determinants were found by using anti-gC MAbs (Zweig et al. 1983). Olofsson et al., in 1981 found that gC-1 could be purified from HSV-1 infected cell lysates by using *Helix pomatia* lectin affinity (HPA) chromatography (Olofsson et al. 1981). By using the same method on lysates from HSV-2 infected cells a protein showing type-specific HSV-2 properties was obtained (Svennerholm et al. 1984). Two years later, this HSV-2 type-specific protein was shown to be mgG-2 (Olofsson et al. 1986).

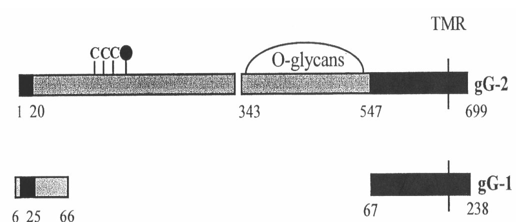

Fig. 3. Schematic presentation of the gG-1 and gG-2 proteins. Regions presenting high similarity are indicated by black colour. The immunodominant region of mgG-2 is located between aa 552 and 574.

HSV-1 and HSV-2 glycoproteins show variable sequence homology. While the type common gB and gD display high similarity (85%) the homology between gG-1 and gG-2 is much lower presenting an overall aa identity of <30%. These observations predicted correctly that gG-2

induced an HSV-2 specific antibody response. Surprisingly, the epitopes for the type-specific antibodies against mgG-2 were not located in the portion of mgG-2 which is lacking in gG-1 but to a region with amino acid similarity with gG-1 (Fig.3). Indeed, within one of the mapped immunogenic regions of gG-1 (Tunback et al. 2000) and the immunodominant region of mgG-2 (Liljeqvist et al. 1998), one linear stretch with high similarity was outlined. This sequence carried nine residues identical between gG-1 and mgG-2 and five type-specific residues. Despite the high similarity, the type-specific reactivity of human anti-gG-1 and anti-mgG-2 antibodies is maintained. By using a pepscan technique and a library of modified gG-1 and mgG-2 peptides, it was demonstrated that the type-specific recognition within this region was maintained in mgG-2 by single type-specific amino acids (proline$_{572}$ or proline$_{573}$) and in gG-1 by dual type-specific residues (leucine$_{92}$ and histidine$_{93}$). By using molecular modelling of the original gG-1 and mgG-2 peptides it was shown that the type-specific residues induced significant structural differences, which may serve to explain the lack of cross-reactivity (Tunback et al. 2005) (Fig. 4).

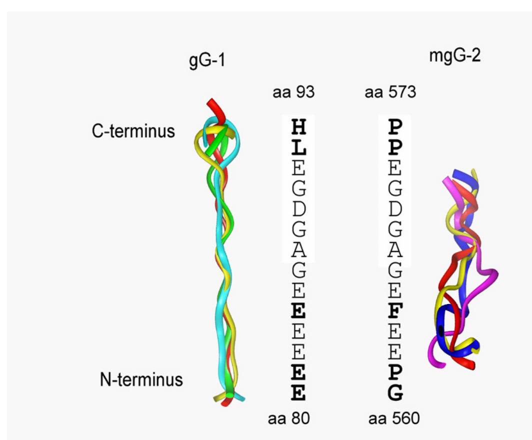

Fig. 4. Four superimposed molecular models of each of the investigated gG-1 and mgG-2 peptides in an aqueous environment. The amino acid sequences are shown vertically and the type-specific residues are displayed in bold.

T cell mediated recognition of HSV glycoproteins has been demonstrated in one study where immunoaffinity purified mgG-2 and recombinantly produced gB-2 and gD-2 were used as antigens. The induced T cell proliferation response against mgG-2 was shown to be type-specific in contrast to gB-2 and gD-2 (Carmack et al. 1996). In collaboration with Kristina Eriksson we have shown that both mgG-2 and sgG-2 elicit type-specific T cell responses (Eriksson et al. 2004). Furthermore, the T cell responses of HSV-1 infected individuals stimulated with HSV-2 particles, were clearly lower (stimulation index of 8) than those of HSV-2 infected individuals (stimulation index of 35) These data suggest that the contribution of HSV-2 cross reactive antigens to T cell proliferation is of minor importance as compared with type-specific components such as mgG-2. In an attempt to localize T cell epitopes a library of synthetic peptides covering the entire gG-2 were used for stimulation. None of the peptides encompassing mgG-2 induced a stimulatory response while for peptides covering sgG-2 a few peptides were positive (Bellner et al. 2005a).

3.3. Glycoprotein G in diagnosis of HSV-2 infection

Virus isolation has for many years been the golden standard method for HSV-1 and HSV-2 diagnosis. This method has now been replaced by the more sensitive PCR technique. A drawback with both methods is that asymptomatic HSV-2 infected patients are seldom diagnosed. Detection of type-specific IgG-antibodies against HSV-2 constitutes therefore an important diagnostic tool. Type-specific serology is necessary for estimation of seroprevalences in defined populations, for monitoring of changes in the seroprevalence, for follow up after HSV-2 vaccine trials and to establish HSV-2 infection during pregnancy and finally for counselling of HSV-2 discordant couples.

Different antigen preparations of mgG-2, including those prepared by recombinant technique and peptides, have been successfully used for HSV-2 specific serological diagnostics (Al-Sulaiman et al. 2009; Ho et al. 1992; Lee et al. 1985; Morrow et al. 2003; Oladepo et al. 2000; Svennerholm et al. 1984). Several commercial assays have been evaluated presenting good performances in populations from developed countries (Ashley et al. 1998; Ashley et al. 2000; Sauerbrei and Wutzler 2007). The most commonly evaluated assays are the HerpeSelect™ Kalon and Biokit. Recently, it has been obvious that an assay performing well in one population not necessarily do so in others (Ashley-Morrow et al. 2004). The most commonly used assay, HerpeSelect™ presents lower specificity for sera from sub-Sahara African countries (Delany-Moretlwe et al. 2009; Gamiel et al. 2008; Gorander et al. 2006b; Laeyendecker et al. 2004). The same problem was described in low risk populations in USA and Vietnam (Mark et al. 2007; Ngo et al. 2008) but not in Poland (Gorander et al. 2008). The reason/s for this unspecific reactivity is unknown but may depend on cross reactivity of HSV-1 Abs, differences in produced mgG-2 antigens interference of IgM-antibodies (see discussion paper II) or by the cut-off level for positive samples. It can be concluded that few if any commercially available assays that have been evaluated so far perform well independent on the population being studied. Further improvements and evaluations of HSV-2 specific serological assays are therefore warranted.

3.4. Functional aspects of the gG-protein of alphaherpesviruses

3.4.1. Secretion and glycosylation

Positional gG homologues have been described within the genomes of most mammalian alphaherpesviruses sequenced to date, excluding varicella-zoster virus (Table 1). Although low degrees of sequence similiarity are shown between gG-2 and other gG homologues, some characteristic features are shared. Three closely spaced cysteines at position 120, 132 and 143 are highly conserved, so also the *N*-glycosylation site at aa position 163 to 165. Several of the investigated gG homologues have been shown to be secreted. In contrast to what has been described for the sgG-2 these proteins contain a transmembraneous region, and it is proposed that these proteins are cleaved off from the cell membrane. For BHV-1 N- and *O*-glycans as well as chondroitine sulfate have been shown to be attached to gG (Keil et al. 1996). In contrast, gG of both EHV-1 (Van de Walle et al. 2009) and EHV-4 (Drummer et al. 1998) contain N-glycans but no *O*-glycans or GAGs. Serine and threonine are residues to which *O*-glycans are attached. The content of theses amino acids for gG of BHV-1 is 10%, for EHV-1 15%, for EHV-4 14%, for simian herpes B virus (SBHV) 15%, and for simian herpesvirus SA8 (SA8) 14%, as compared with 21% for mgG-2 which is heavily *O*-glycosylated. The content of serine and threonine residues of gG-1 is also 21% despite that no *O*-glycans are attached to the protein. We conclude from these observations that it is difficult to predict whether gG is *O*-glycosylated from the content of relevant amino acids.

3.4.2. Functions

Viral chemokine-binding proteins (vCKBP) are a family of proteins which can bind with high affinity blocking the effects of different chemokines. These virokines have no sequence similarity with known chemokines or chemokine receptors. Secreted viral products were first identified for poxviruses but have recently also been described for alphaherpesviruses. Such activity has been described for both membrane bound and secreted gG of EHV-1, EHV-3, PRV,

BHV-1 and BHV-5 which block the binding of a broad range of chemokines to their receptor or blocking the chemokine binding to GAGs. However, no binding was seen of medium from HSV-1 and HSV-2 infected cell cultures or for a truncated form of gG-2 (471 amino acids) produced in baculovirus. These findings suggest that neither sgG-2 nor mgG-2 function as vCKBP (Bryant et al. 2003; Viejo-Borbolla et al. 2010). In a mouse model gG of EHV-1 was shown to inhibit chemotactic functions of the proinflammatory chemokine CCL3. This inhibition was restored in presence of anti-gG-antibodies (Van de Walle et al. 2008). For HSV-2 a 15-mer synthetic peptide corresponding to the amino acids 190-205 of sgG-2 protein was shown to be a chemoattractant for monocytes and neutrophils with production of reactive oxygen species via the formyl peptide receptor. This response inhibited NK cell cytotoxicity and accelerated the apoptotic cell death in NK cell-enriched lymphocyte populations (Bellner et al. 2005b). Although the native sgG-2 protein did not have this effect, the *in vivo* infection may generate the relevant peptide by proteolytic cleavage. In such case, a novel viral escape mechanism is described for sgG-2.

3.4.3. Phenotypic behaviour of gG negative alphaherpesviruses

(i) mgG-2 negative HSV-2 replicates intracellularly in cell culture with approx. 5-10 times lower titer as compared with wt HSV-2. However, the titer of the mgG-2 negative mutant in the supernatant was 100 times lower than wt HSV-2 suggesting that mgG-2 is important for viral egress (Adamiak et al. 2007). mgG-2 is essential for neuronal spread of genital infection in mice (paper III) and protects mice after vaccination (paper IV). Frame shift gG-2 negative clinical HSV-2 isolates can reactivate and induce clinical lesions (Liljeqvist et al. 1999c); (ii) gG-1 negative HSV-1 cannot enter through apical surfaces of polarized epithelial cells (Tran et al. 2000). However, in non polarized cells the growth characteristics are similar to wt HSV-1. In a mouse model, viral titers in sensory ganglia, spinal cord and the brain was approx. 10 times lower for a gG-1 negative HSV-1 as compared with wt HSV-1 (Balan et al. 1994); (iii) An EHV-1 gG negative mutant replicated as well as wt EHV-1 in cell culture and both viral strains presented similar virulence in mice at high doses of infection. In contrast, at a low infective dose the gG negative mutant replicated more efficient in the lungs and induced more severe symptoms than wt EHV-1 (von Einem et al. 2007); (iv) A gG negative PRV mutant showed similar virulence after intranasal infection as wt PRV (Kimman et al. 1992); (v) A BHV-1 gG negative mutant was attenuated in calves after intranasal infection. Infection with this mutant protected from challenge with a wt strain and reactivated after dexamethasone treatment (Kaashoek et al. 1998). BHV-1 gG has been shown to be essential for cell-to-cell spread by maintaining the cell junctions (Nakamichi et al. 2000). However, insertions or deletions within the US4 gene including the promotor and poly-A may also effect the US3 gene as these genes function as a transcriptional unit (Demmin et al. 2001). Thus, manipulations of the US4 gene may disrupt the expression of the US3 gene and observed phenotypic changes may therefore be due to defects in US3 gene products instead of US4.

Table 1. Characteristics of positional gG homologues in mammalian alphaherpesviruses.

Virus	Size aa	Cell-to-cell spread	Secretion	Clusters of *O*-glycans	Viral chemokine binding protein	Symptoms
HSV-2	699					genital lesions
-mgG-2	~357	No	No (TMR)	Yes	No	
-sgG-2	~320	?	Yes (no TMR)	No	No	
HSV-1	238	No	No (TMR)	No	No	oral/genital lesions
EHV-1	411	No	Virion-68kDa+12kDa (C-term), 60 kDa secreted (N-term), (TMR)	No	Yes	abortion, respiratory disease, encephalitis
EHV-3	448	?	? (TMR)	?	Yes	gential lesions
EHV-4	405	?	Virion-68kDa+12kDa (C-term), 60 kDa secreted (N-term), (TMR)	No	No	abortion, respiratory disease, encephalitis
PRV	498	No	Only secreted, 180 kDa + 75kDa + 50 kDa, (TMR)	Yes	Yes	meningoencephalitis, pneumonia
BHV-1	444	Yes? (see text)	Only secreted, 65 kDa+90-200 kDa, (TMR)	Yes, + GAGs	Yes	respiratory disease, abortion, encephalitis, vulvovaginitis
BHV-5	430	?	Only secreted, 65 kDa+90-200 kDa, (TMR)	Yes, + GAGs	Yes	resp.disease, abortion, encephalitis
SHBV	674	?	? (TMR)	Yes, PTTTP motifs	?	oral/genital lesions
SA8	605	?	? (TMR)	Yes	?	genital lesions

EHV: equine herpesvirus, PRV: pseudorabies virus, BHV: bovine herpesvirus, SHBV: simian herpes B virus, SA8: simian herpesvirus SA8.
TMR = transmembraneous region. GAGs = glycosaminoglycans.

4. Epidemiology and clinical manifestations of genital HSV-2

HSV-2 is one of the most commonly sexually transmitted infections (STI). The seroprevalence show great variations dependent on the population studied with the highest numbers of infected in some African countries (Malkin 2004; Smith and Robinson 2002). Changes in prevalence have been studied and for Sweden and USA there are data supporting an increasing prevalence (Fleming et al. 1997; Forsgren et al. 1994). However other epidemiologic data also from Sweden collected during the 1990s indicated a decline in seroprevalence among younger women (Persson et al. 1995). These results have been confirmed in a recent study among pregnant women (Berntsson et al. 2009). However, globally there is a considerable burden of disease and in a review based on data from 12 regions it was estimated that in 2003 536 million people aged between 15 to 49 years were infected with HSV-2 and that 23.6 million were infected during 2003 (Looker et al. 2008).

From a clinical standpoint genital HSV-2 infection can be divided into primary and recurrent infection. Primary infection is when a patient with no previous HSV-2 infection is infected with HSV-2 while recurrent HSV-2 infection occurs in a patient already infected with HSV-2. After resolving a primary HSV-2 infection genital HSV-2 reactivate with an annually mean frequency of 4 to be compared with 1.4 and 0.25 for oral and genital HSV-1 infection, respectively (Lafferty et al. 1987). Men have about 20% more recurrences than women and almost all of the patients with a symptomatic primary infection have recurrent infections (Benedetti et al. 1994).

However, the majority of HSV-2 infections (60%) are transmitted without causing any symptoms (asymptomatic infection) (Mertz et al. 1992) and the number of new HSV-2 infections among HSV-2 seronegative sexually active individuals was shown to be 5.1 per 100 person-years, implicating that most of patients are not aware of being infected with HSV-2 (Langenberg et al. 1999). By using daily genital sampling from HSV-2 seropositive individuals HSV-2 shedding can be detected by PCR on up to 20% of the days. The majority of these episodes were asymptomatic and half lasted for less than 12h (Mark et al. 2008).

Besides causing painful lesions genital HSV-2 can cause psychosexual problems (Mindel 1996) and facilitate the spread of HIV. HSV-2 infected patients have been estimated to have three times higher risk to acquire HIV (Freeman et al. 2006). They also present higher HIV viral load (Serwadda et al. 2003) and HSV-2 reactivation enhances HIV transmission by increasing HIV target cells in the genital tract (Rebbapragada et al. 2007). Additionally, antiviral treatment against HSV reduces genital and plasma HIV-1 RNA (Nagot et al. 2007).
Other severe complications like meningitis or myelitis are occasionally seen (Skoldenberg et al. 1975). More rarely, encephalitis is associated with HSV-2 (Gorander et al. 2006a). HSV-2 can also cause severe perinatal infection a feared complication which occurs approximately in one of 3500 births (USA) most often associated with maternal primary HSV-2 infection (Brown et al. 2003).

As the majority of HSV-2 infected subjects are unaware of their infection prevention against spread of HSV-2 is troublesome. In these situations serological assays are the only method to achieve a diagnosis. Since the diagnosis of HSV-2 infection can add psychological stress to the patients it is important that the assays used have high specificity. In addition, for correct comparison of prevalences and for vaccine follow up in different parts of the world, it

is important that the selected assays perform equally in different populations.

Finally, and important for prevention of HSV-2 spread, it has been shown that the use of condoms and treatment with antiviral drugs can significantly reduce transmission of HSV-2 (Corey et al. 2004; Martin et al. 2009). However, a vaccine that protects individuals from being infected or prevent from spread between individuals would be the best way to decrease HSV-2 related morbidity among humans.

5. Immune responses after HSV infection

Although the different defence lines of the host in the natural HSV infection most likely are closely integrated we separate the protective arms of the immune response into the natural or innate and the adaptive defence systems. The latter being further subdivided into the T cell mediated and the humoral or B cell mediated systems. A brief description of these immune systems is presented below.

5.1. Innate immunity

The innate immune responses represent the first line of defence against invading microorganism. It is rapidly induced and function without prior exposure to the foreign antigen. Three different phases can be distinguished: (I) action by inherently secreted proteins; (II) early induced cytokine response; (III) response mediated by recruited cellular effectors (Duerst and Morrison 2003).

5.1.1. Secreted proteins

Proteins that build up the complement system are present not only in the circulating blood but also in the lumen of vagina. They can induce direct lysis of the virus as well as enhancing the neutralization of viral particles or infected cells. The complement cascade starts before viral attachment and consists of reactions finally leading to formation of a membrane attack complex directed against lipid layers of cells or enveloped virions. The complement system can be divided into three different parts; (i) the classical pathway; (ii) the lectin pathway and (iii) the alternative pathway (Pangburn et al. 2008). The classical pathway is activated by antibodies and starts with binding of C1q to the Fc-portion of an antigen-antibody complex. The lectin pathway uses mannan-binding lectin (MBL) which binds to viral glycoproteins and initiates the complement cascade. This pathway has been suggested to be important in control of recurrent human HSV-2 infection as patients with clinical lesions show MBL-deficiency more often then asymptomatic individuals (Gadjeva et al. 2004). Finally, in the alternative pathway the system is activated by foreign polysaccharides and lipopolysaccharides (Pangburn et al. 2008).

5.1.2. Interferon, chemokines and defensins

HSV-2 entry into cells followed by replication triggers an early response by production of secreted proteins such as IFN-αβ (Duerst and Morrison 2003), chemokines (Thapa and Carr 2008) and defensins (John et al. 2005; Soboll et al. 2006). The different molecules and their major function in innate immunity are summarized in Table 2. The purpose of this early response is to limit virus replication and spread by for example up-regulation of adhesion molecules on endothelial cells and activation of macrophages.

Type I interferons (IFN-αβ) play an important role since they are produced within hours after infection. They are produced by various cells including epithelial cells, macrophages and dendritic cells (Eloranta and Alm 1999; Malmgaard 2004; Rasmussen et al. 2007). Rapid IFN-

αβ production is initiated by recognition of HSV-2 through pattern recognition receptors (PRRs) belonging to the TLR family (TLR9) but can also be induced independently of TLRs stimulation (Rasmussen et al. 2007). Type I IFNs set the cells into an "antiviral state" (Duerst and Morrison 2007; Pestka et al. 2004) which prevent from viral spread in the infected host (Pestka et al. 2004). It has been shown that differences in susceptibility to HSV-2 seen for different mouse strains correlates with the amount of IFN-αβ produced (Ellermann-Eriksen et al. 1986; Svensson et al. 2007).

The chemokines are small peptides that are secreted in response to infection acting by recruiting effector cells to the site of infection (chemotaxis) (Table 2) (Thapa and Carr 2008). Chemokine production is induced by recognition through the PRRs as described for IFN-αβ and besides mediating chemotaxis chemokines are involved in T cell differentiation and angiogenesis. Recent studies, using genetically modified mice lacking expression of chemokine ligands or receptors, have revealed important roles for the CXCL9 and CXCL10 ligands (Thapa et al. 2008) and their receptor CXCR3 (Thapa and Carr 2009) in the first line of defence against genital HSV-2 infection and disease.

Defensins are small proteins that are produced in granulocytes and macrophages. The expression of defensins in uterine and vaginal tissues from mice has been demonstrated by using reverse transcriptase PCR (Soboll et al. 2006). *In vitro* (Sinha et al. 2003) and *in vivo* (Hazrati et al. 2006; John et al. 2005) experiments have shown that defensins may be important in the defence against HSV infection. In the study by John et al., it was suggested that defensins in cervicovaginal secretions from humans contributed to innate resistance against lethal IVAG HSV-2 challenge in mice (John et al. 2005).

Table 2. Molecules involved in innate immunity with focus on early induced responses to HSV-2 infection.

Component	Production and functions	References
Type 1 interferons (IFN-αβ)	Expressed by most cells and set the cells in an "antiviral state". Acts by stimulating immune cells (T cells, NK cells and macrophages) and increase the expression of MHC-I, activate pro-apoptotic genes and inhibit anti-apoptotic mechanisms.	(Ellermann-Eriksen 2005; Pestka et al. 2004; Svensson et al. 2007)
Type 2 interferon (IFN-γ)	Produced by NK cells, macrophages, B cells, and professional antigen-presenting cells. IFN-γ has antiviral activity but acts mostly by activation of macrophages antigen-presenting cells as well as NK cells and inhibits CD4+ Th2 cells, resulting in a Th1-like cell-mediated response.	(Ellermann-Eriksen 2005)
TNF-α	Produced by macrophages leading to an increased cytokine production and phagocytosis.	(Malmgaard et al. 2004)
T-bet	Transcription factor controls the IFN-γ production early during the differentiation of naïve CD4+ T cells into Th1 cells. T-bet is important in both the innate and acquired immune protection against genital HSV-2 infection.	(Svensson et al. 2005)
MyD88	Adaptor molecule for TLR. MyD88 is important for innate early control of genital HSV-2 infection in the C57BL/6 mouse but not required for induction of acquired immunity following vaginal immunization with HSV-2 TK−.	(Tengvall and Harandi 2008)
CXCL1	Keratinocyte-derived chemokine expressed locally within the genitalia. Involved in the initial immune response through the recruitment of neutrophils.	(Thapa and Carr 2008)
CXCL9 (MIG) and CXCL10 (IP-10)	Produced by several cell types in a tissue specific manner, for example epithelial cells, DCs and astrocytes. Binds to the CXCR3 receptor expressed on activated T cells, NK cells, monocytes, DCs, and B cells. Mice deficient in CXCL9 or CXCL10 are more sensitive to HSV-2 infection and show reduced NK cell and virus-specific CD8+ T cell mobilization and reduced CTL activity in the brain. Deficiency in CXCL9 expression may be compensated by the expression of CXCL10.	(Liu et al. 2000; Thapa et al. 2008)
CCL2 (MCP-1)	Produced by microglia and a chemoattractant for monocytes, macrophages and T cells that express the receptor CCR2. Associated with inflammatory CNS disorders, lung infections, and viral infections including HSV-2. CCL2 is induced rapidly in the infected brain of mice. The association of CCL2 and CCR2 in neuropathogenesis has also been demonstrated in other disease models including experimental autoimmune encephalomyelitis (EAE).	(Izikson et al. 2000; Thapa and Carr 2008)
CCL3 (MIP-1α)	Produced by macrophages, DCs, neutrophils, astrocytes, and fibroblasts. During HSV-2 infection, CCL3 is elevated in the infected tissue of mice. CCL3 drives Th1 development through IFN-γ production.	(Shao et al. 2005; Thapa and Carr 2008)
CCL5 (RANTES)	Produced by T cells and signals through four different receptors CCR1, CCR3, CCR4 and CCR5 which are expressed by T cells, NK cells, monocytes, and memory T cells. CCL5 support the recruitment of memory CD4+ T cells as well as Th1 differentiation. Following genital HSV-2 infection, CCL5 expression significantly increases in vaginal tissue as well as in the spinal cord and brain stem. The application of anti-CCL5 antibody to mouse hepatitis virus-infected mice suggests CCL5 expression promotes inflammation and has an overall detrimental impact on CNS infection.	(Thapa and Carr 2008)
CXCR3 (receptor)	Expressed on activated T cells, NK cells and macrophages. Signals through CXCL9, CXCL10. Mice lacking expression of CXCR3 showed higher mortality, higher viral titer in the vaginal tissue and the spinal cord, reduced CTL effector function through impaired expression of T-bet, perforin, and granzyme B by CD8+ T cells. CXCR3 expression might be required for appropriate recruitment of DCs in the draining lymph nodes.	(Thapa and Carr 2009)
CCR5 (receptor)	Expressed on T cells, NK cells, macrophages and DCs. Interacts with CCL3 and CCL5. Appears to be a necessary receptor within the nervous system to control genital HSV-2 infection in mice primarily through NK cell trafficking.	(Thapa and Carr 2008)
Defensins	Synthesised by neutrophiles and macrophages present in the mouse vagina. Blocks binding and entry but also post-entry events.	(Hazrati et al. 2006; John et al. 2005; Soboll et al. 2006)

5.1.3 Cellular components

Immune cells involved in innate immunity are activated and recruited to the infected site. These are neutrophiles followed by monocytes and finally NK cells and γδT cells. Neutrophils have the ability to degrade HSV particles by phagocytosis (Van Strijp et al. 1990) and are recruited to the site of infection by IL-8, TNF-α, IFN-γ and GM-CSF secreted by epithelial cells and macrophages. Recruitment is also mediated by complement cleavage products (C3a and C5a). Data from animal experiments show that neutrophils accumulate in the mucosa shortly after infection and constitute an important component in the innate immune response against HSV-2 (Milligan 1999). Depletion of neutrophils results in higher vaginal HSV-2 titers during the first days after challenge but only with a slightly delayed resolution of infection supporting the importance of these cells in the early phase of infection (Milligan 1999; Milligan et al. 2001). Taken together, these findings support that neutrophils contribute to the control of HSV-2 infection.

Beside a role as antigen presentation cells macrophages/monocytes (MO) have the capacity to ingest and destroy HSV virions by phagocytosis and play an important role in the production of cytokines e.g., (IFN-αβ and TNF) (Ellermann-Eriksen 2005) (Fig. 5A). In addition, activated macrophages produce ROS in a first wave of response to IFN-αβ. However, production of ROS has not been shown to be of major importance in viral infection (Ellermann-Eriksen 2005). NO is produced in the second wave induced by IFN-γ (Fig. 5B) (Ellermann-Eriksen 2005). Mice treated with an iNOS inhibitor showed increased genital disease after IVAG challenge with wt HSV-2 and an accelerated mortality (Benencia et al. 2003). In addition, *in vitro* experiments have shown that IFN-γ can inhibit HSV-1 replication by NO activity (Komatsu et al. 1996). These finding suggest that NO production is important in the antiviral responses to HSV-2 infection.

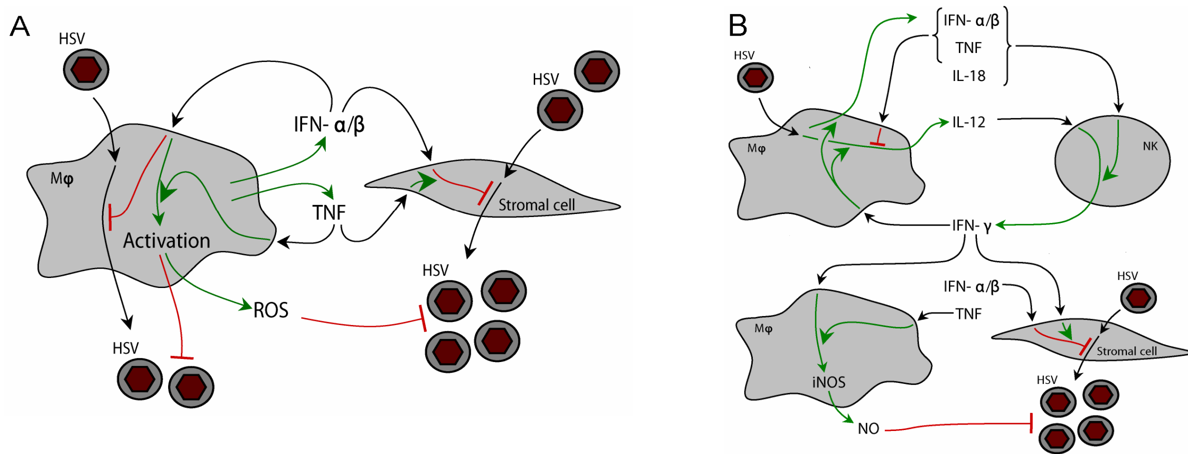

Fig. 5. The first (A) and second (B) wave of cytokine responses in macrophages after an HSV infection (Modified from (Ellermann-Eriksen 2005)).

NK cells represent an important part of the innate immune response to HSV-2. They are recruited to sites of viral infection 2-4 days after infection. NK cells are activated by Th1 produced cytokines, IL-12, -15 and -18. Activated NK cells produce IFN-γ (Fig. 5B) and have the capacity to kill virus infected cells by perforin/granzyme-mediated cytolysis. Most studies on NK cell cytotoxicity have been performed on non-virus infected cells showing that the cytotoxic effect is dependent on down regulated MHC I expression (Storkus et al. 1987). In accordance to these findings down-regulation of MHC-1 expression by the ICP47 protein (see immune evasion strategies) have been suggested to explain the increased NK cell mediated cytotoxicity against HSV infected cells. This suggestion were based on experiments performed 48 h after infection. However, other experiments have shown that recognition and killing of HSV-1 infected cells occurs early during infection (18 h) and is dependent on IE gene expression (Fitzgerald-Bocarsly et al. 1991). Recently it was shown that expression of ICP0 was sufficient for early NK cell mediated cytotoxicity and that this effect was not dependent on down regulation of MHC-1 molecules (Chisholm et al. 2007).

In vivo experiments in animals have shown that NK cells are important in protecting the host from HSV-2 infection and disease. Antibody-mediated depletion of NK cell activating cytokines (IL-12 and IL18) results in higher viral titers and earlier death after vaginal challenge with HSV-2 (Harandi et al. 2001). Mice genetically deficient in IL-15 or NK cells also show higher viral titers and increased mortality after vaginal challenge with HSV-2 (Ashkar and Rosenthal 2003). Finally, the impact of NK cells in control of HSV-2 related infection and disease in both the vagina and CNS was shown using C57BL/6 mice deficient in CCR5 (Thapa et al. 2007).

5.1.4. Links to the adaptive immunity

DC forms a link between innate and adaptive immunity. Skin-derived DCs include epidermis-derived Langerhans cells, classical dermal DCs and a langerin-positive CD103+ dermal subset. After an infection, monocyte-derived DCs are also recruited to site of infection. The DCs are involved in antigen presentation to the T cells and they are producers of IFN-αβ, cytokines and chemokines. They migrate from the site of infection to regional lymph nodes. During this migration they mature and present viral peptides, up-regulate expression of co-stimulatory molecules and produce cytokines. In the lymph node DCs present the antigen by the MHC I and MHC II pathway to CD4+ and CD8+ T cells. Binding occurs between MHC complex and the T cell receptor as well as between co-stimulatory molecules. The Langerhans cells have been suggested to be the most important DC presenting antigen to CD8+ T cells controlling immunity to skin derived antigens. However, in a flank model in mice infected with HSV-1 it was recently established that langerin-positive CD103+ dermal DCs were the principal cells to induce immunity (Bedoui et al. 2009). The role for the Langerhans cells is therefore unclear but they may be involved in presentation of antigens to CD4+ T cells.

Another newly discovered important function of T regulatory (T reg) cells (suppressive T cells) has been described for the vaginal infection of HSV-2 in mice. These cells are activated both at the local site of infection and in the genital lymph nodes. When T reg cells were blocked significantly higher viral titers were detected in vagina and in the spinal cord with a higher mortality rate. This blockage was associated with retention of NK cells, DCs and T cells in the genial lymph nodes and a delay in the arrival to the vagina with

low levels of IFN-α produced by DCs and IFN-γ produced by NK cells and CD4+ T cells (Lund et al. 2008).

γδT cells are present only in small numbers in peripheral lymphoid tissues but are abundant in the epithelial layer of reproductive organs such as the uterus and vagina. In a study using the IVAG infection model in mice the absolute numbers of γδT cells increased in the vagina to a peak on day 3 in C57BL/6 mice. They have also high ability to produce IFN-γ which help protect against HSV-2 infection by promoting a systemic CD4+ Th1 response to HSV-2 (Nishimura et al. 2004).

5.2. T cell mediated immune responses

T cells are divided into two subsets based on the presence of two different surface co-receptor molecules the CD4 and CD8. The CD4+ T cells, also known as helper T cells, are further subdivided into Th1 and Th2 cells and the cytokines they produce are named Th1-type (e.g. IFN-γ, IL-2 and IL-12) which promote inflammatory immune responses. However, IL-10 is also a Th1 cytokine which has an anti-inflammatory action by suppressing macrophage functions. Th2 type cytokines (e.g. IL-4, -5 and IL-13) promote B cell activation and antibody production. Finally, the CD8+ T cells also known as cytotoxic lymphocytes (CTL) recognize and kill cells presenting foreign antigen together with MHC I. In the IVAG infection of mice with HSV-2 several studies have demonstrated that protective mechanisms against primary infection with HSV-2 are mainly mediated by MHC II-restricted CD4+ Th1 cells secreting IFN-γ (Milligan and Bernstein 1997; Parr and Parr 2003a).

Studies on mice have shown that activated HSV specific and IFN-γ producing CD8+ T cells infiltrate and persist in latently infected trigeminal ganglia (Khanna et al. 2003; Khanna et al. 2004). In a vaccine study based on a single MHC-I restricted CD8+ CTL epitope protection was obtained against challenge with a lethal dose of HSV-2 (Blaney et al. 1998). Another possible protective mechanism involving a CD8+ T cell function is that uninfected bystander DCs can phagocytize HSV infected apoptotic DCs (Jones et al. 2003) and by cross presentation stimulate virus specific CD8+ T cell clones (Bosnjak et al. 2005). Systemic HSV-2 specific CD8+ T cells producing IFN in the spleen have been shown to correlate to protection in vaccinated mice. As the number of CD8+ T cells are too low in the genital tract and in DRG other models are used. For example, trigeminal ganglia have been investigated after vaccination followed by ocular challenge showing that the number of IFN-γ producing CD8+ T cells correlate to protection (Hoshino et al. 2005). Furthermore, in a recent study using *ex vivo* cultivated, latently HSV-2 infected, trigeminal ganglia the CD8+ T cell number were shown to correlate with viral reactivation measured as cell free virus released from ganglion (Hoshino et al. 2007). Memory T cells are important to protect a host from a re-infection with the same microorganism. The relationship between peripheral and re-circulating memory cells was recently elucidated. Gebhardt et al. showed, using a mouse model and HSV-1, that a memory CD8+ T cell subset present after an acute infection remained to the epithelial and latently infected sensory ganglia protecting from re-infection (Gebhardt et al. 2009). These T cells are phenotypically and functionally different from their circulating counterparts and reside in the tissue without antigen stimulation. Taken together, these observations strongly suggest that CD8+ T-lymphocytes play an important role in the control and resolution of HSV infection.

5.3. B cell responses

During viral infection antibodies function by recognize of specific viral antigenic determinants (epitopes). Antibodies can interact with free virions or by virus-infected cells. Antiviral effects to free virions include NT-activity and Fc-mediated complement-mediated lysis and phagocytosis. The "coating model" implies that several Abs with high affinity to the antigens block attachment and entry of the virus which prevent infection. In the infected cell antibodies can exert antiviral effect via a wide range of mechanism (see Fig. 6). Fc-mediated effector mechanisms include cellysis by ADCC or ACMC. Abs can exert inhibition of the infection from the outside of the cell through blockage of release and spread of virus from the infected cell. Abs can also inhibit virus replication inside the cell by the binding to viral antigens expressed at the cell membrane. This mechanism, probably by blocking signalling pathways, has been described for clearance of alphaviruses (a togavirus) (Levine et al. 1991) and for measles virus (Fujinami and Oldstone 1979) in neurons. IgA, IgM and IgG are actively transported across mucosal epithelium. During this transport the Abs might bind to intracellular viral antigens neutralizing viruses. An interesting observation is that non-neutralizing Abs can bind to non-structural viral antigens expressed on the virus-infected cell but not on the virions. This mechanism has been described for tick borne encephalitis virus (TBEV) and dengue virus. Indeed, protection against TBEV has been correlated to such an antibody response (Burton 2002). Three different functions of antibodies directed to HSV will be discussed more detailed: (i) Neutralization; (ii) ADCC and Fc receptors; and (iii) ACMC.

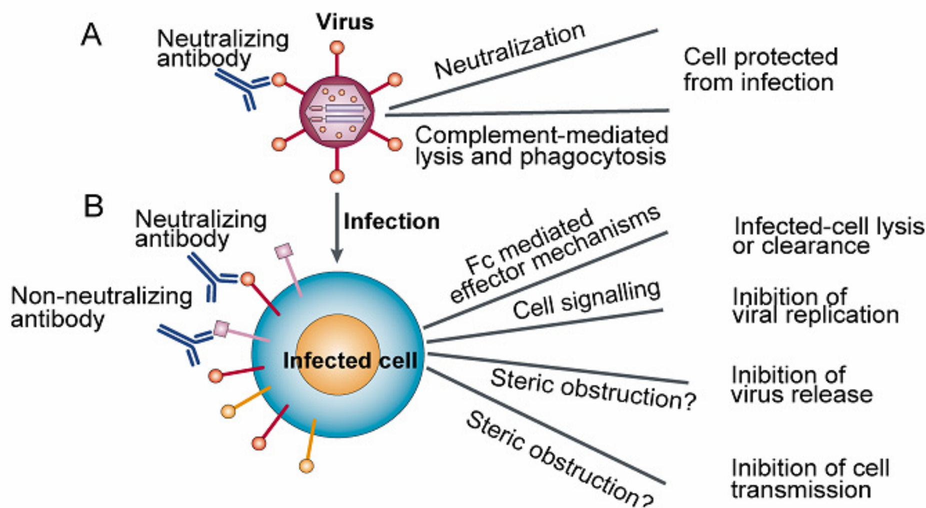

Fig. 6. Antiviral effects of antibody mediated immunological mechanisms directed against the virus particle (A) and the virus infected cell (B) (modified from (Burton 2002)).

5.3.1. Neutralization

Anti-HSV MAbs directed to gB, gD and gH (Fuller et al. 1989; Para et al. 1985) display neutralizing activity with or without complement. Most of the MAbs directed to gG, gC and gE exert no such activity (Balachandran et al. 1982; Para et al. 1985). Thus, MAbs targeting proteins which are coded by genes which are indispensable for infection are mostly neutralizing while MAbs directed to envelope proteins which are coded by dispensable genes are not (gG, gC, gI and gE). We investigated 2,400 clinical HSV-1 and 2,400 HSV-2 isolates, using a type-specific anti-gC-1 MAb, a type-specific anti-mgG-2 MAb and a type-common gE MAb. All HSV-1 isolates were reactive with the anti-gC-1 MAb and only 5 of the HSV-2 isolates were mgG-2 negative (Liljeqvist et al. 1999b; Liljeqvist et al. 1999c). The gE MAb was unreactive with 3 of 4,800 isolates. It is obvious that these proteins have important functions in the life-cycle of the HSV-infection, and that non-neutralizing MAbs defined in commonly used cultured cells may have NT-activity if relevant model systems are used. For example, Adamiak et al., showed recently that anti-gC-1 human antibodies and a type-specific anti-gC-1 MAb, targeting the heparan sulfate-binding domain of gC-1, are neutralizing in keratocytes and cells with restricted expression of glycosaminoglycans (GAGs) but not in GMK-AH1 cells expressing larger amounts of GAGs (Adamiak et al. 2009). Two anti-mgG-2 MAbs and a rabbit hyperimmune serum raised against affinity-purified mgG-2 have been shown to be devoid of neutralizing activity (Balachandran et al. 1982; Balachandran et al. 1987; Para et al. 1985). Similarly, four anti-mgG-2 MAbs produced at our laboratory (Liljeqvist et al., 1998) and purified human anti-mgG-2 Abs exerted no NT-activity without or with complement in GMK-AH1 cells (unpublished observation).

5.3.2. ADCC and Fc-receptors

ADCC is the process by which NK cells, monocytes/macrophages and granulocytes kill virus infected cells coated with antibodies directed against antigenic structures on the cell surface. ADCC represents therefore a mechanism by which antibodies can mediate an antigen-specific response together with effector-cells that lack antigen-specific capacity by themselves. Receptors recognizing the Fc-portion of IgA, IgE and IgG-antibodies are designated Fcα Fcε and Fcγ, respectively. We will focus on Fcγ-receptor interactions. In humans different effector cells interact with IgG-antibodies through different Fcγ receptors. For NK cells the low affinity Fcγ RIII (CD16) is the most important in mediating ADCC while for monocytes the high affinity Fcγ RI (CD64) and medium affinity Fcγ RII (CD32) are the most important. Macrophages use CD16, CD32 and CD64 and neutrophils CD32 as Fc-receptor. When stimulated with IFN-γ neutrophils also express CD64 and CD16 (Woof and Burton 2004).

Few studies on ADCC have been performed in mice, probably due to difficulties in preparation of effector cells and weak responses. However, in a mouse model using passively transferred anti-HSV-2 MAbs (gB, gC, gD, gE and mgG-2) followed by a lethal footpad inoculation a good correlation was observed between protection and ADCC. The mgG-2 MAb induced low ADCC-activity (Balachandran et al. 1982). None of our own produced anti-mgG-2 MAbs presented ADCC-activity (unpublished observation). Recently, Chu et al., showed that IgG-antibodies isolated from the serum of HSV-immune mice induced protection in normal mice against HSV-2 disease (higher survival rate) when administered prior to genital HSV-2 inoculation. Protection was significantly diminished in Fcγ-receptor deficient mice supporting that

Fcγ-dependent mechanisms such as ADCC were important (Chu et al. 2008). In humans there are several studies which suggest that ADCC is an important mechanism in the immunologic defence against HSV infection. In 1982 it was reported that human leukocytes and antibodies given i.p. protected mice against challenge with lethal dose of HSV-1 (Kohl and Loo 1982). Maternal or neonatal anti-HSV ADCC antibody levels were associated with an absence of disseminated HSV infection (Kohl et al. 1989). In 1992 a case of severe, recurrent mucocutaneous HSV-2 infection associated with ADCC dysfunction was reported (Wise et al. 1992). Moreover, in a prospective study of patients with frequently recurrent genital HSV-2 infection low levels of ADCC mediating IgG1 and IgG3 antibodies were associated with recurrences (Seppanen et al. 2006). Finally, it was stated that the relatively poor efficacy observed in clinical HSV-2 vaccine trials, using gD and/or gB as immunization antigens, may partially have been the result of failure to elicit high levels of Abs capable of mediating ADCC (Kohl et al. 2000). Taken together, these data support, ADCC as an important immunologic mechanism in the control of the HSV infection.

5.3.3. ACMC

The interest in studying ACMC and protection against HSV-2 infection has been low. To our knowledge only one study has been published. Balachandran et al., (1982) described that protection by passively transferred MAbs correlated well with ADCC-activity but not with ACMC or NT-activity. The mgG-2 MAb was not protective in contrast to the other MAbs which induced protection in varying degrees. We have confirmed these results in that four additional anti-mgG-2 MAbs (Liljeqvist et al. 1998) did not induce protection when passively transferred i.p. followed by IVAG-challenge. Furthermore, neither the anti-mgG-2 MAbs nor purified human anti-mgG-2 Abs presented ACMC (unpublished observations). In contrast, passively transferred MAbs directed against gC of HSV-1 followed by a lethal challenge of HSV-1 i.p. conferred protection which correlated to the ability of the MAbs to induce ACMC (Bystricka et al. 1997).

6. Immune responses in sensory ganglia and CNS

6.1 The innate system

Several molecules involved in the innate defence system have been described to be important to control the HSV infection in the brain. For example, the lack of type I IFN receptor (Shen and Iwasaki 2006), TNF-α and IL-1β (Sergerie et al. 2007), using knock-out mice, results in higher viral load in the brain with development of CNS symptoms much more rapidly than for wt mice. Mice lacking TLR2 show reduced mortality to a lethal HSV-1 challenge despite that similar viral load was detected in the brain as compared with wt mice suggesting that the TLR2-mediated cytokine response to HSV-1 is detrimental to the host (Kurt-Jones et al. 2004). In contrast, it was recently suggested that an intact TLR3 is important for resistance against HSV-1 encephalitis in human (Zhang et al. 2007). Myeloid differentiation factor 88 (MyD88) is an adaptor protein that mediates TLR-activation and is essential for the production of inflammatory cytokines. In the vaginal infection model MyD88-negative mice were shown to be less resistant to HSV-2 infection than wt mice (Tengvall and Harandi 2008) and MyD88 negative mice are highly susceptible to HSV-1 infection in the brain (Mansur et al. 2005). A recent study suggested that a synergistic action of TLR2 and TLR9 are responsible for activation of the MyD88-dependent anti-HSV activity during an *in vivo* HSV-2 infection in the brain but not

in vagina or the spinal cord (Sorensen et al. 2008).

6.2. Immune cells

Depletion of NK cells following genital HSV-2 infection results in an elevation of virus recovered in the vaginal tissue and brain stem but not in spinal cords of wt mice. Furthermore, CCR5-/- IVAG infected mice presented significantly more virus and expressed higher levels of TNF-α, CXCL1, CCL2, CCL3, and CCL5 in the vagina, spinal cord, and/or brain stem than did wt mice. This response was associated with a reduction in NK cell expansion, infiltration, and activity into the brain stem whereas residing CD4+ T cells or CD8+ T cells were similar as for wt mice (Thapa et al. 2007; Thapa et al. 2008). Sensory ganglia lack a blood-nerve barrier and the neurons are enclosed by a high number of satellite glial cells (SGC). Human trigeminal SGC were recently shown to resemble CNS microglia both phenotypically and functionally implicating their origin from myeloid progenitor cells and not from the neuroectoderm. SGC are therefore tissue resident APCs expressing MHC II. As neurons do not express MHC II infiltrating CD4+ T cells most likely interact with SGC. Virus-specific CD8+ T cells are directly juxtaposed to latently infected neurons producing cytokines such as IFN-γ to control reactivation of HSV from latency either by direct contact with the neuron or via the SGC. Trigeminal ganglia-infiltrating CD8+ T cells express the T cell inhibitory molecules CD94/NKG2A and PD-1 which may explain that latently infected neurons are not damaged (van Velzen et al. 2009).

6.3. Antibodies

To examine the role of Abs in protection FcγR knock-out mice and passively transferred hyperimmune serum collected from gD-2 immunized mice followed by IVAG HSV-2 challenge or a highly neutralizing gD-specific MAb were used. Mice received the Abs or MAb as a single i.p. injection 2 days prior to IVAG HSV-2 challenge. The authors concluded that animals pre-treated with polyclonal hyperimmune serum or MAb were infected in the genital tract but were significantly protected against disease and infection of the sensory ganglia. For mice which received polyclonal hyperimmune serum this protection was largely dependent of host FcγR expression while protection seen after MAb treatment was independent of the FcγR expression (Chu et al. 2008). In addition to granulocytes, monocytes and NK cells, FcγR1 has been detected on primary sensory neurons. Cross-linking of FcγR1 bound Ab was shown to mediate intracellular signalling and release of neurotransmitters (Andoh and Kuraishi 2004). A possible protective role for neuron-expressed FcγR1 during HSV infection has however not been investigated. Finally, an anti-gD Mab and sera to HSV-1 gB and gD but not by rabbit anti-gE or anti-gG sera neutralized HSV-1 released from the axon of cultured human foetal dorsal root ganglia inhibiting the infection of the epidermis (Mikloska et al. 1999).

7. Immune evasion strategies

During time of co-evolution HSV has evolved several mechanisms to escape from the immune defences described above. The most distinctive feature of HSV is the establishment of latency and limited gene expression in sensory ganglia which enables the virus to avoid recognition by the immune system.

7.1. Virus induced apoptosis and anti-apoptosis

The infected host can limit viral replication by triggering the apoptosis program. HSV has evolved to inhibit apoptosis which is a prerequisite for continuous replicating in

the cell but will also result in escape from the apoptosis induced by cytotoxic lymphocytes (Aubert and Jerome 2003; Jerome et al. 1998). One example of anti-apoptotic proteins are the products of HSV-1 genes US3 and US5 (Jerome et al. 1999). In a recent study US3, US5 as well as US12 were shown to inhibit different T-lymphocyte cytotoxic effects such as cytolysis and apoptosis (Aubert et al. 2006).

In contrast, HSV can infect antigen presenting cells such as DCs and cause apoptosis. HSV infection of DCs also results in down-regulation of co-stimulatory molecules, adhesion molecule (CD54), cytokine production and MHC-I molecules. Inhibition of MHC-I is mediated by the viral protein ICP47 which acts by binding to a cellular protein and thereby hindering expression of viral peptide in conjugation with MHC class I molecules (Früh et al. 1995; Hill et al. 1995; Tomazin et al. 1996; Tomazin et al. 1998). This leads to impaired antigen presentation and interferes with recognition of DCs by CD8+ T cells (Novak and Peng 2005; Salio et al. 1999). However, the ICP47 effect can be reversed by IFN-γ secreted by CD4+ T cells which also are present in the HSV lesion (Bosnjak et al. 2005).

7.2. Evasion from interferon

HSV have evolved mechanisms to evade the effects of type I interferons. The virus host shutoff protein (vhs) produced by the UL41 has endoribonuclease activity and down-regulates protein synthesis by mRNA degradation and inhibits the IFN-αβ response early after HSV-2 but not after HSV-1 infection (Murphy et al. 2003). In addition, HSV-1 ICP0 and ICP34.5 proteins contribute to viral immune evasion by blocking important steps in type I interferon production (Eidson et al. 2002; Leib 2002; Lin et al. 2004; Pasieka et al. 2006).

7.3. Evasion from complement

HSV has also a strategy to avoid the effects of the complement system. Both gC-1 and gC-2 bind to the complement C3b component, thereby conferring protection against complement-mediated neutralization of viral infectivity (Friedman et al. 1996; McNearney et al. 1987). In addition, the gE protein and the gE/gI complex function as receptor for the Fc-domain of anti-HSV antibodies and therefore inhibit Fc-mediated immune functions, both *in vitro* and *in vivo* (Dubin et al. 1990; Dubin et al. 1991; Nagashunmugam et al. 1998).

7.4. Other immune evasion mechanisms

It was recently discovered that HSV-1 and HSV-2 down-regulates secretory leukocyte protease inhibitor (SLPI), an anti-inflammatory mediator of mucosal immunity. This inhibition occurred within 6h suggesting that the down-modulation was not dependent on viral replication and vhs activity. Instead, the effect was caused by immediate-early gene-products such as ICP4 or ICP0 (Fakioglu et al. 2008). A recent study has shown that HSV-1 can induce suppression of cytokine signalling-1 (SOCS-1) in keratinocytes and thereby block the effect of IFN-γ on these cells. It was concluded that SOCS-1 plays an important role in the inhibition of the antiviral effect of IFN-γ in keratinocytes infected with HSV-1 (Frey et al. 2009).

8. The primary genital infection in non immunized mice

There is to date no human HSV-2 vaccine available although several vaccine candidates have been evaluated. This may be due to that the critical viral components essential for protective immune responses not have been defined. Studies on genital HSV-2 infection, mostly using mice, rats or guinea-pigs, are therefore important animal models to further elucidate these

issues. A proposed model of the genital HSV-2 infection in mouse (A-D) is illustrated in Fig. 7 and described in detail;

A. The initial step in the HSV-2 infection is transmission of infectious HSV-2 particles to the genital epithelial cells via a cellular receptor. In cultured cells several receptors have been defined binding to the envelope gD, for example, (i) herpes virus entry mediator (HVEM), a member of the tumour necrosis factor family; (ii) nectin 1 and 2, members of cell adhesion molecules; and (iii) specific sites in cellular heparan sulfates generated by 3-*O*-sulfotransferases. Recently, it was shown that infection of the vaginal epithelium in mice was dependent on either HVEM or nectin-1. When both receptors were absent no infection was detectable (Taylor et al. 2007). After the first round of replication virions are spread by cell-to-cell contact and released from the basolateral surface of the epithelial cells to free nerve endings located in the epidermis. This transport is dependent on gE (shown for HSV-1 gE) (McGraw and Friedman 2009). Using DRG from human embryos Erik Lycke et al., at our laboratory, showed that the HSV-2 particle enters the axon via cell fusion of the envelope with the cell membrane releasing the nucleocapsid and associated inner tegument proteins into the cytoplasm of the neuron (Lycke et al. 1988). It is currently unknown whether the fusion process involves gD and the same cell membrane receptor/s as described for other cultured cells.

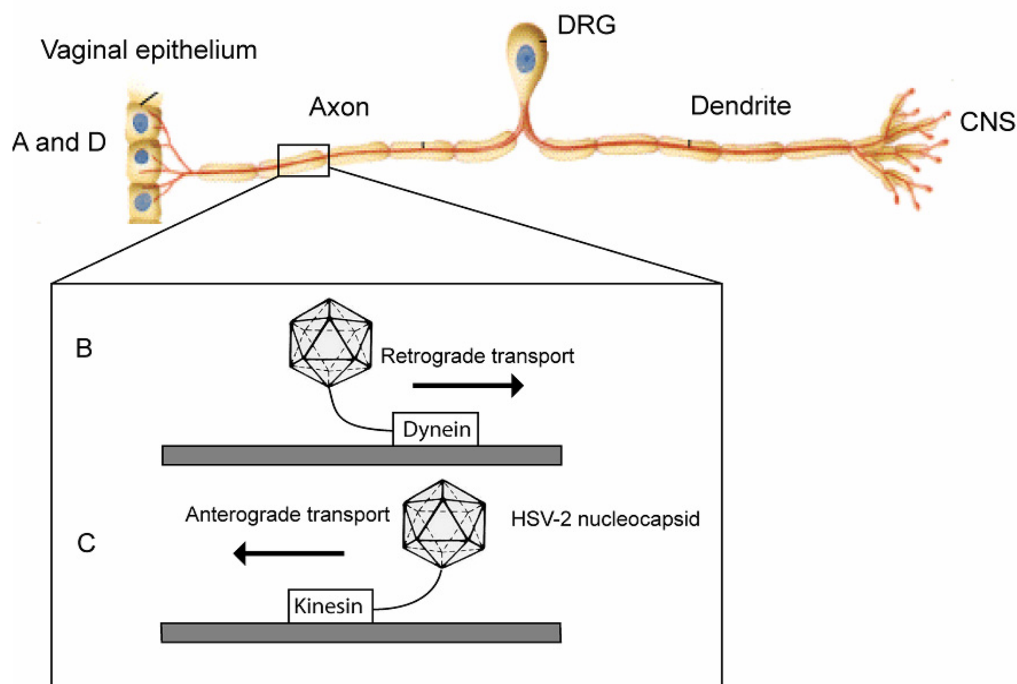

Fig. 7. A proposed model of vaginal HSV-2 infection in mouse. HSV-2 replicates in the epithelial layer (A) and enters the neuron followed by retrograde transport by dynein motor protein (B). Reaching the DRG a second round of replication occurs followed by anterograde transportation by the kinesin motor protein back to the genital mucosa (C) where a third round of replication is initiated (D).

B. The capsids are actively transported in the retrograde direction to the cell body via a transport system driven by dynein motor proteins using the microtubulus. The cytoplasmatic dynein motor protein complex consists of a microtubulus domain, an ATP-binding chain and a chain which binds the cargo. Based on binding studies different viral components have been suggested to bind to the dynein motor complex (e.g., pUL34, pUL9, pUL46 and pUL35). However, some of these proteins are not contained in the nucleocapsid-tegument protein complex**.** Capsids lacking the proposed protein have still retained transport activity (for review see) (Diefenbach et al. 2008). We conclude that the molecular basis for retrograde transport needs further studies.

C. Reaching the nucleus the HSV-2 DNA establishes latency or begins to replicate a second round. It is still a controversy whether the nucleocapsid, tegument and envelope proteins are transported separately in alphaherpesviruses followed by assembly at the axon termini or alternatively, transported as mature enveloped particles. In PrV, a virus infecting pigs, the complete mature virion seems to be transported, while in HSV-1 the different components seems to be transported separately. For HSV-1, nucleocapsid, tegument proteins as well as envelope proteins are detected at the axon termini. Recently, gE-1 and gI-1 were shown to be essential for transport of HSV-1 using a compartmentalized primary neuron culture system (McGraw et al. 2009). The anterograde transport system is similar as for retrograde transport except that the cellular motor protein kinesin binds to the cargo. The anterograde as well as retrograde transport systems are rapid. Capsid proteins of HSV-1 have been estimated to reach the axon termini from the nucleus within 24h after production (Miranda-Saksena et al. 2000). In the mouse, the HSV-2 particles are transported back to the periphery via the axon and to the spinal cord via the dendrite extension, a feature which explains why the animal succumbs. In humans, the HSV-1 and HSV-2 particles are transported only to the periphery but rarely further to the spinal cord and brain giving clinical manifestations. The basis for this block of transport to CNS is unclear. After the transport to axon termini the HSV-2 particle is released to the extracellular compartment via the same mechanism as described for non-neuronal cells i.e., by exocytosis.

D. After entry into the epithelial cells a third round of replication is initiated. Several studies have shown that the clinical lesions recognized after an HSV infection are dependent on this neuronal round-trip. Using infection with HSV-1 in a mouse flank model, it was recognized that the clinical lesions were dependent on anterograde spread of viruses and that the lesions were detected distant from the inoculation site (Simmons and Nash 1984). Similarly, in the HSV-2 genital infection model using guinea-pigs, it was established that after primary infection lesions were dependent on neuronal spread of virus to the periphery (Bernstein and Stanberry 1986; Stanberry et al. 1982).

Finally, treatment of guinea-pigs with capsaicin, a sensory neuron and axonal

anterograde transport blocker, before IVAG infection with HSV-2, ameliorated cutaneous lesions but not the replication in vagina or ganglionic infection (Stanberry 1990).

9. The mouse model for protective vaccine studies

9.1. Background

Although the genital tract mucosa in the mouse differs from other mucosal sites in that it lacks mucosal lymphoid nodules the mouse vagina is part of a common mucosal immune system. Thus, immunity induced at one mucosal site can be directed to sites distant from the initial antigen exposure occurred. As a consequence, vaginal immunity can be obtained by immunization either via the i.n. or the IVAG route. Langerhans cells (LC) are antigen-presenting cells in the mucosal epithelial layer. In a primary HSV-2 infection the LC take up and process HSV-2 antigen, migrate to the local lymph presenting antigen via MHC to naïve T-cells (Parr et al. 1994). The B cells encounter the HSV-2 antigen in the lymph nodes either as free intact antigen or antigen presented by dendritic cells. Antigens are taken up by B cells by receptor-mediated endocytosis and presented to CD4+ helper T cells by the MHC class II pathway. The CD4+ helper cells secrete cytokines which stimulate B cells to clonal expansion and differentiation to antibody secreting plasma cells and memory B cells.

9.2. Intravaginal (IVAG) infection

The model for IVAG challenge used in this work was originally described by McDermott et al., in 1984 (McDermott et al. 1984). Parr et al. (Parr et al. 1994) showed that the age dependent resistance to IVAG HSV-2 challenge was circumvented by treatment with progesterone (DepoProvera). After this treatment, HSV-2 replicates in the genital mucosa and the virus is rapidly spread in the perineal region followed by infection of multiple neuronal sites including the autonomic ganglia, DRG and spinal cord leading to severe neurological illness and death (Parr and Parr 2003b).

9.3. Route of immunization

Systemic immunization protects against neurological disease and death (Parr and Parr 1998) whereas mucosal immunization via the i.n. route induces local and long lasting immunity in the vaginal tract (Gallichan and Rosenthal 1998; Gallichan et al. 2001; Rosenthal and Gallichan 1997). Vaccination with attenuated TK^{-} virus using the IVAG route induced stronger vaginal immunity in mice as compared with mice vaccinated i.n. (Parr and Parr 1999). As the IVAG route alone has been shown to be less effective for subunit vaccines (Kwant and Rosenthal 2004) we used a combination of the parenteral and i.n. route to obtain optimal protective immunity.

9.4. Adjuvant

Adjuvant stimulate the immune system in different ways (Simms et al. 2002). Unmethylated CpG dinucleotide (CpG ODN) patterns are frequently present in bacterial DNA (Klinman et al. 1996) and serve as a potent adjuvant by activation of the TLR 9 receptor present intracellularly in the endosomes and at the cell membrane (Malmgaard et al. 2004). We used CpG ODN 1826 which contains two CpG motifs (TCC ATG ACG TTC CTG ACG TT) (Bhat et al. 2009). The adjuvant effect of CpG ODN is characterized by induction of innate and acquired immunity with a Th1 like cellular immune response (Klinman et al. 2009; Tengvall et al. 2006).

9.5. Earlier vaccine studies in mice

One important issue to be addressed is if immunization can limit or even completely protect the host from being infected (sterile immunity in the mucosa).

9.5.1. Live attenuated HSV-2

Using IVAG immunization with TK^{-} HSV-2 followed by challenge with a lethal dose of wild type HSV-2 complete protective immunity against disease was obtained with very low levels of HSV-2 detectable in the vaginal epithelium (Parr and Parr 2003a; Svensson et al. 2005). However, other studies have shown that although TK^{-} HSV-2 induced complete protection against disease, a substantial amount of HSV-2 was detected in vagina i.e., the immunization did not protect against infection (Milligan and Bernstein 1995; Milligan et al. 1998).

9.5.2. Replication-defective mutants of HSV-2

Morrison et al., using a replication-defective mutant of HSV-2 (ICP8 negative), showed that mice were completely protected from disease and very low titers (<10 PFU) were detected in vaginal samples (Morrison et al. 1998). Da Costa et al., used three different deletion mutants for immunization which all protected completely against disease but not against viral infection in vagina (Da Costa et al. 1999).

9.5.3. Subunit vaccines

The most well documented subunit vaccine is based on gD. Initially, natively produced gD-2 was tested together with adjuvant in the mouse model using i.p. immunization and footpad or i.p challenge with HSV-2. The survival rate was 80% (Dix and Mills 1985) or 100% (Long et al. 1984). Recent studies using gD and different adjuvant as immunogens followed by IVAG challenge reported complete protection from disease but not from infection of the genital mucosa (Del Campo et al. 2009; Lindqvist et al. 2009; Tengvall et al. 2008).

9.6. Protection from latency

Another vital issue in vaccine development against genital HSV-2 is if vaccinated mice are protected from latent infection in the DRG and CNS. The lack of establishment of latency is favourable for a vaccine against HSV-2. Two methods are frequently used for detection of HSV-2, HSV-2 DNA detection by PCR or real time PCR and virus isolation which mostly have been performed on explant cultivated DRG from mice surviving challenge with wt HSV-2. We have found two studies which analyzed latent HSV-2 DNA in DRG after immunization of mice followed by IVAG challenge with wt HSV-2. One study used a DNA vaccine based on gB-2 and gD-2 genes (Lee et al. 2002) while the second study used ICP8 replication-defective HSV-2 for immunization (Morrison et al. 2001). Qualitative or semi-quantitative PCR were used for detection of HSV-2 DNA. Although the vaccines induced reduction in clinical disease they failed to block latent infection in DRG. Using different replication-defective HSV-2 mutants, Da Costa et. al., showed that vaccination with these mutants reduced latent HSV-DNA in trigeminal ganglia significantly measured by a semi-quantitative PCR method (<100 HSV-2 DNA genome copies/ganglion), (Da Costa et al. 1999). Several studies have used the genital model in guinea-pig. Wachsman et al., used a growth-compromised ICP10 deleted HSV-2 for immunization and challenged IVAG with wt HSV-2. No latent virus was detected in DRG by using explant co-cultivation. However by using PCR technique the average HSV-2 DNA copy number per ganglia, 60 days after challenge, was $3x10^{3}$ for immunized mice as compared with $2x10^{5}$ for control animals (Wachsman et al. 2001). Similar

reductions of HSV-DNA (approx. 2 $\log_{10}$) in DRG were shown for guinea-pigs immunized with gD-2 and adjuvant as compared with controls (Bourne et al. 2005; Hoshino et al. 2005).

9.7. Conclusions based on earlier experiments in mice

The primary goal for a protective vaccine is that the immune responses elicited after immunization abort the HSV-2 infection at the mucosal surface. However, sterile immunity in vagina after IVAG challenge is difficult to obtain irrespective which immunization strategy is chosen. The second goal is that the vaccine should prevent transport and/or establishment of latent HSV-2 DNA in DRG. This goal has also been difficult to obtain with used vaccination strategies.

10. Aims

Paper I

- To evaluate immunosorbent purified sgG-2 as a novel type-specific ELISA-antigen for serological HSV-2 diagnosis.

Paper II

- To evaluate immunosorbent purified sgG-2 and mgG-2 as type-specific ELISA-antigens using sera from Tanzania.
- To compare the performance of sgG-2 and mgG-2 ELISA with a commercial assay in Tanzanian populations.

Paper III

- To elucidate the function of mgG-2 by using mgG-2 negative viruses in a genital mouse model.

Paper IV

- To evaluate mgG-2 as vaccine candidate against HSV-2 infection and disease in a mouse model.

11. Discussion and future prospects

11.1. Paper I

The aim was to evaluate sgG-2 as ELISA antigen for detection of type-specific IgG-antibodies against HSV-2. The performance was compared with the HerpeSelect 2 ELISA (FOCUS2). An earlier study by Liljeqvist et al., suggested that sgG-2 could constitute a novel antigen for type discriminating serology (Liljeqvist et al. 2002). Moreover, as sgG-2 is secreted rapidly after infection it was suggested that antibodies against sgG-2 might be detect earlier than those against mgG-2. This would constitute an improvement as antibodies against the mgG-2 protein in some cases are detectable relatively late (1-6 months) after a primary HSV-2 infection (Ashley et al. 1988; Lopez et al. 1993). We showed that sgG-2 is immunogenic and presented high sensitivity and specificity (≥98%) in an ELISA format of sera collected from individuals with recurrent HSV-2 disease. For sera from individuals with a primary HSV-2 infection the antibody response against sgG-2 was detected somewhat later as compared with Abs directed to *Helix pomatia* lectin purified mgG-2. We conclude that sgG-2 is a novel antigen for type discriminating HSV-2 serology. A future problem using mgG-2 as immunization antigen in vaccine trials in humans is to distinguish HSV-2 infection from vaccine induced immunity. As sgG-2 ELISA detects type-specific Abs it should be possible to diagnose HSV-2 infection among vaccinated subjects. However, before use in vaccine studies the assay must be evaluated among asymptomatic HSV-2 infected individuals and for primary HSV-2 infections in already HSV-1 infected patients.

An observation which is not discussed in the paper is that only 3 of 21 individuals with primary HSV-2 lesions, confirmed by positive isolation, and with no anti-mgG-2 Abs were HSV-1 positive. We can identify two possible explanations. First, prior HSV-1 infection protects against HSV-2 infection. This issue is still a controversy. While some authors have shown a decreased transmission rate of HSV-2 among individuals infected with HSV-1 as compared with HSV-1 negative (Mertz et al 1992, Stanberry et al, 2002) others have not demonstrated such an association (Langenberg et al. 1999, Corey et al., 1999). Second, Langenberg et al., (1999) showed that prior HSV-1 infection increased the likelihood to acquire asymptomatic HSV-2 infection by a factor of 2.6 suggesting that individuals with a symptomatic HSV-2 infection are more often HSV-1 negative.

11.2. Paper II

We evaluated ELISAs based on immunosorbent purified sgG-2 and mgG-2 in two different Tanzanian cohorts and compared the results with a commercially available HSV-2 specific assay based on recombinantly produced gG-2 (FOCUS2). Sera drawn from blood donors (BD) and patients with genital ulcer disease (GUD) were analyzed. The mgG-2 ELISA showed higher performance than the sgG-2 ELISA and significantly higher specificity for sera from blood donors as compared with FOCUS2. Surprisingly, for sera collected from GUD-patients the mgG-2 ELISA and FOCUS2 performed equally well. A general problem with serological assays based on mgG-2 is that many assays perform well in western world countries but not in African populations where the specificity usually is significantly lower. We will discuss different aspects of this problem.

i) *Cross-reactive IgM-antibodies.* In paper I we showed that the FOCUS2 assay presented lower specificity among sera which contained IgM-Abs against other microorganisms. A

possible explanation to the lower specificity for the FOCUS2 assay in paper II might be due to higher levels of IgM-Abs in sub-Saharan African countries with the risk for unspecific binding. Lugada et al., showed that the IgM-levels (g/l) in Uganda and Tanzania were significantly higher as compared with individuals from Norway and Canada and that HIV-positive patients have higher amounts of IgM-Abs irrespective of citizenship (Lugada et al. 2004). However, as the unspecific reactivity of the FOCUS2 assay was more pronounced in sera collected from HIV-negative individuals (blood donors) the presence of cross-reactive IgM-Abs seems less plausible. On the other hand, a recent study showed that HIV-positive sera reduced the specificity of FOCUS2 to 30% among a South African population (Delany-Moretlwe et al. 2009).

ii) *Genetic divergence of HSV-1 isolates.* All commercial and other assays are based on mgG-2 sequences deduced from HSV-2 isolated from individuals living in the western world. A few critical amino acids have been defined to maintain the type-specific antibody responses to gG-1 and mgG-2 (Tunback et al. 2005). A relevant question is whether clinical HSV-1 isolates from African populations have mutations within the critical residues (histidine and leucine). This seems not to be case. We and others have shown that the gG-1 gene is conserved in European as well as in African isolates with not a single mutation of the critical amino acids (Bowden et al. 2004; Rekabdar et al. 2002). It is therefore unlikely that genetic variability of the gG-1 can explain the low specificity described for some mgG-2 based assays from sera collected in African populations.

iii) *Cross-reactivity due to the production system of mgG-2.* Cross reactivity in an mgG-2 based assay can occur from HSV-1 antibodies but also from antibodies directed against other antigen structures that are similar but not identical to mgG-2 (molecular mimicry). The production method of the antigen may influence the performance of an assay. In our study we used natively produced immunosorbent purified mgG-2. A similar antigen was evaluated in an earlier study using sera from USA showing a sensitivity and specificity of 99% and 100% (Lee et al. 1985). In another study nine HSV-2 specific serological assays were evaluated with 330 serum samples from sub-Saharan Africa. Four of the five assays presenting the highest specificity were based on purified natively produced mgG-2 while in the same study the FOCUS2, based on baculovirus expressed sgG-2 and mgG-2, were among the less specific (van Dyck et al. 2004). Based on these data we suggest that assays based on purified native antigens are better than those using baculovirus expressed recombinant antigens.

A more native-like recombinant protein can be produced in mammalian cell lines, such as CHO-cells. These cells may generate proteins that are properly folded and glycosylated (Demain and Vaishnav 2009). To our knowledge CHO-cells expressed gG-2 has so far not been evaluated for large serum panels from Europe and Africa. We suggest that the mgG-2 and/or sgG-2 antigens produced in CHO-cells have the potential to be the best ELISA antigen for detection of HSV-2 specific antibodies in European and African sera. We plan to produce the gG-2 proteins in CHO-cells and evaluate these antigens in an ELISA format using sera from Sweden and Africa.

iv) *The cut-off level for positive results.* One way to improve specificity is to increase the cut-off level which will eliminate low reactive false positive samples. However, this will in most cases result in lower sensitivity since true positive sera showing weak antibody-reactivity will be judged as false negative. The influence on the performance using different cut-off values can be illustrated as shown in Fig. 8 by (A) plotting the OD-values for each sample or by (B) a receiver operator curve (ROC). We showed, based on a ROC plot, that the same cut-off values as used for sera from Sweden could be used with retained sensitivity and specificity for sera from Tanzania. For FOCUS2, on the other hand, the specificity could be increased by raising the cut-off level for sera from Africa. However this adjustment resulted in lower sensitivity (Laeyendecker et al. 2004).

11.3. Paper III

A relevant question is if the significant biological effects described in the manuscript using the two different gG-2 negative mutants can be assigned to the lack of sgG-2, mgG-2 or both. Most experiments were performed with the mutant 333+AC9gG which harbours a frame shift mutation in the gene segment coding for mgG-2. As described, the sgG-2 protein was secreted into the media of 333+AC9gG infected cell cultures detected by ELISA. In further experiments, using western blot from cell lysate and from medium of 333+AC9gG infected cell cultures, sgG-2 was detected from both compartments although at somewhat lower levels as compared with wt 333 (unpublished observation). In the *in vivo* model sgG-2 was poorly immunogenic in mice which survived from a sublethal dose of wt 333. Poor immunogenicity of sgG-2 in the genital mouse model probably explained why mice infected with high doses of 333+AC9gG did not develop anti-sgG-2 Abs. The gG-2 negative HSV-2 strain “EGFP-gG-2 neg HSV-2” lacks the expression of both sgG-2 and mgG-2 and presented similar results when used in the genital model of infection in mice i.e., no genital inflammation or lesions, no HSV-2 in the nervous system and complete survival. Although not proven we suggest that the 333+AC9gG mutant expresses sgG-2 not only in cell cultures but also in the *in vivo* infection and that the behaviour of the mutant described in the manuscript is due to the lack of the mgG-2 protein. The problem might be definitely solved by the construction of separate HSV-2 mutants lacking the expression of sgG-2 or mgG-2. The design of such mutants is complicated by the fact that the cleavage site/s between the two gG-2 proteins has only been proposed in an abstract and not been confirmed in published studies.

The lack of HSV-2 in DRG and CNS suggests that the mgG-2 negative HSV-2 particles are not released from the epithelial cells or are not able to enter into the free nerve endings. Defects in anterograde transport in the neurons are also less likely as in such case HSV-2 should be retained in the cell body and detected in DRG. Furthermore, clinical HSV-2 isolates devoid of mgG-2 can induce clinical lesions implying that HSV-2 can be transported from the cell body to the periphery without mgG-2 (Liljeqvist et al. 1999a).

Work is now in progress to investigate whether mgG-2 is important for sorting, transport and/or egress of HSV-2 virions from basal surfaces in polarized epithelial cells using both 333+AC9gG and EGFP-gG-2 neg HSV-2 mutants. In a recent study the spread of the HSV-1 infection from polarized epithelial cells to the neurites was shown to be dependent on gE (McGraw and Friedman 2009). We use polarized Madin-Darby canine kidney

(MDCK) epithelial cells and ovary epithelial cells (OvCAR) cultivated on inserts containing a membrane with 3µm pore-size which is used within a 12-well plate. The polarization is followed by the trans-epithelial resistance measurements using an Ohm-meter. The cells can be infected from both the apical and the basal surfaces and the yield can also be measured from both sides of the cell membrane. In preliminary results we found that infectious wt HSV-2 (strain B4327UR) are released from the basal surface of the cell membrane when the cells are infected apically. Another possible function of mgG-2 is that the protein is ligand for a cellular receptor on the sensory free nerve endings. This issue can be investigated by culture of primary sensory neurons from ganglia in a dual chamber culture system.

11.4. Paper IV

Several epidemiological studies collecting consecutive HSV-2 isolates from the same individual have shown that re-infection with a new HSV-2 strain of an already HSV-2 infected individual is rare. This means that a protective immune response is elicited after a primary HSV-2 infection which precludes new HSV-2 strains to re-infect the host. This information is fundamental because it means that it should be possible to develop an HSV-2 vaccine by mimicking the natural protective immune response. The outcome of a human vaccine against HSV-2 infection can be described as follows;

i) The most favourable outcome is the induction of sterile immunity i.e., no infection, no disease, no latency and no reactivation. This goal has been difficult to obtain both in animal studies as mentioned and in human vaccine trials. The biological explanation might be that sensory free nerve endings are located in epidermis of the genital mucosa and therefore infected early in the infection process.

ii) Lower rate of disease, latency and reactivation. This scenario is a realistic goal for a vaccine.

iii) No protection against infection, disease, latency and reactivation.

iv) Lower rate of disease but establishment of latency and reactivation to the same extent as a natural infection. This situation may be the worst scenario as such a vaccine may contribute to increase the spread of HSV-2 in the population.

Considering the promising findings described in paper IV the final goal is to use mgG-2 as antigen in human trials. As the outcome of a vaccine in a mouse model poorly predicts the outcome in humans the effect of protection using mgG-2 as immunogen in humans can only be tested in humans. The immunization schedule in the mouse model was optimized to induce both systemic (s.c) and mucosal immunity (i.n). As the mucosal component of immunity seems to be low future studies should focus on the induction of a strong systemic immunity by subcutaneous or intramuscular immunizations. Dose titration of the antigen, toxicological studies and evaluation of different adjuvant which can be used in humans such as alum need also to be evaluated in animal models before human trials can be initiated.

In the vaccine study in mice it was surprising that no NT-activity was detected for hyperimmune sera collected from mice immunized with mgG-2 alone and with mgG-2-CpG. We have extended these observations in that four anti-mgG-2 MAbs and purified human anti-mgG-2 Abs were tested for NT-activity in a GMK-AH1 cell culture system. Similarly as described above no NT-activity was detected (unpublished). In the human vaccine trial using both gB-2 and gD-2 (Chiron) strong neutralizing antibody titers were detected in vaccinated individuals but the levels did not correlate to protection. However, it was speculated that the low protection rate was

due to low ADCC activity. The majority of mice immunized with mgG-2-CpG presented macrophage mediated ADCC. Although we were unable to correlate ADCC activity to survival, data stimulated us to investigate ADCC of human purified mgG-2 antibodies. As shown in Fig. 9 these Abs presented ADCC activity mediated by purified NK cells (A), monocytes (B) as well as PBMC (C).

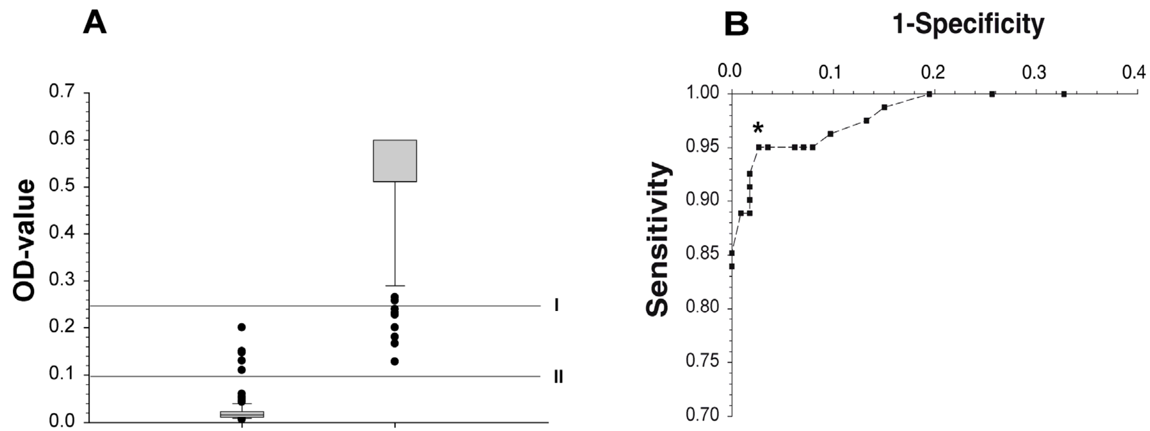

Fig. 8. The variation in sensitivity and specificity using different cut-off values in ELISA. Results are illustrated for hypothetical negative and positive panels of sera by (A) a box-plot and (B) ROC plot. The upper horizontal line (I) in the box-plot figure marks a high cut-off level of 0.25 resulting in 94% sensitivity (six of 100 false negative) and 100% specificity while the lower cut-off level (II) of 0.1 results in 100% sensitivity and 96% specificity (four of 100 false positive). The ROC plot shows the relation between sensitivity and 1-specificity at incremental cut-off steps. The optimal cut-off value is indicated by an asterisk (*).

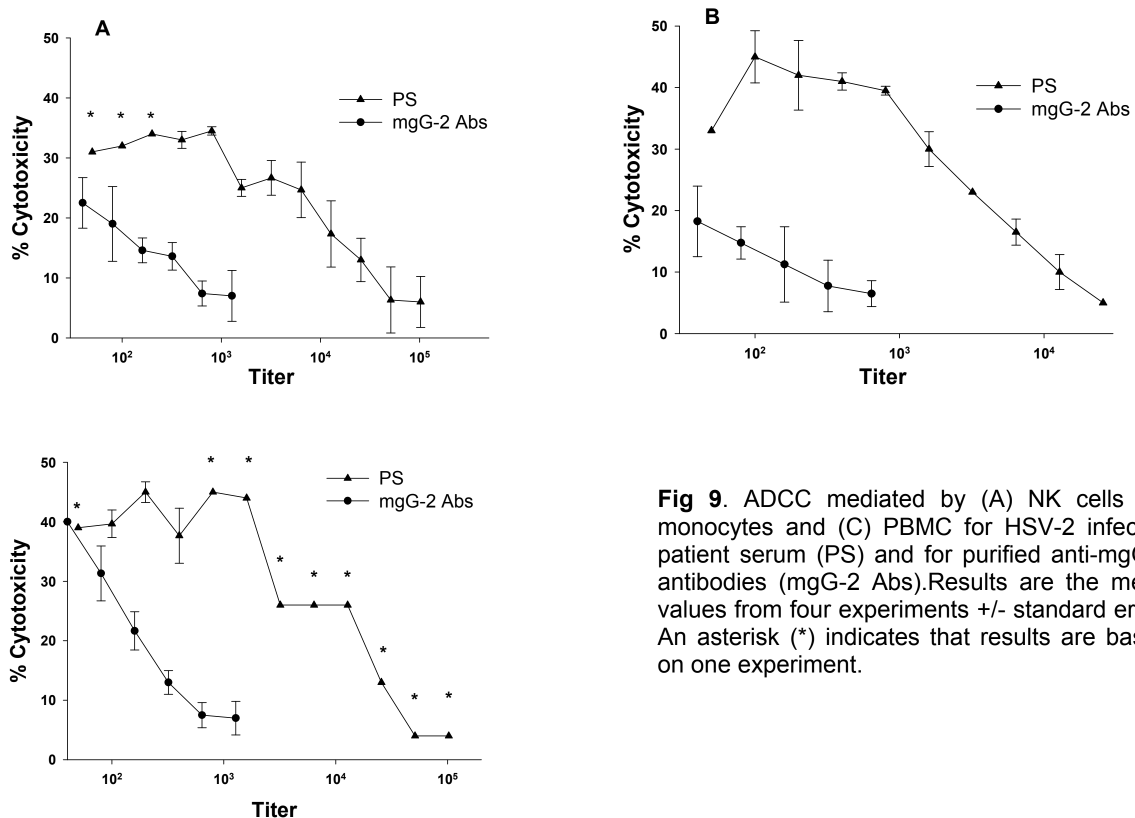

Fig 9. ADCC mediated by (A) NK cells (B) monocytes and (C) PBMC for HSV-2 infected patient serum (PS) and for purified anti-mgG-2 antibodies (mgG-2 Abs).Results are the mean values from four experiments +/- standard error. An asterisk (*) indicates that results are based on one experiment.

12. Concluding remarks

HSV-1 and HSV-2 are closely related viruses that enter target cells using similar surface receptors followed by establishment of latency in sensory nerve ganglia. From this state they reactivate to cause mucocutaneous lesions and various CNS manifestations. HSV-1 and HSV-2 mainly present type-common antigenic structures, i.e. antibodies directed against one HSV subtype also recognize the counterpart. However, as HSV-2 infects a previously HSV-1 infected individual the type-common immune responses are not sufficient to induce cross-type immunity.

The work of this thesis has focused on the antigenic differences between HSV-1 and HSV-2 and the possible functional consequences. Both viruses show type-specific antigenic properties through their respective gG proteins; these encoding genes show the most profound nucleotide type-difference across the whole HSV genome. The work presented here addressed questions regarding the specificity of the antibody response to sgG-2 and mgG-2, the role of mgG-2 for protective immunity and the function of this protein for HSV-2 infection *in vitro* and *in vivo*.

In the thesis we have shown that the sgG-2 shows type-specific properties and can be utilized as a novel antigen for HSV-2 specific serology (Paper I). In addition, we showed that highly purified mgG-2 used as ELISA antigen can improve serological diagnosis in those samples where other assays perform less well, for example for individuals from sub-Saharan Africa (Paper II). We have extended our understanding of the function of the mgG-2 protein by showing that this protein is of importance for the propagation of HSV-2 from the mucosa to the sensory ganglia and to the CNS (Paper III). Finally, we showed that the immune response induced by vaccination with mgG-2 significantly protected mice from neurological disease and death (Paper IV). Thus, of the 11 HSV-2 envelope glycoproteins, the mgG-2 protein carries unique immunological and functional roles of importance for HSV-2 infection, and for protective immunity against this virus. Based on these findings, we propose that the mgG-2 protein constitutes a promising vaccine candidate against HSV-2 infection in humans.

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