DETECTION AND MOLECULAR CHARACTERIZATION OF HERPES SIMPLEX VIRUS FROM TANZANIA AND NORWAY

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Bergen, September 2006
Mabula Joseph Kasubi

List of original papers

The thesis is based on the following papers referred to in the text by their respective roman numerals.

Paper 1: Kasubi, M.J., Nilsen, A., Marsden, H.S., Bergström, T., Langeland, N., and Haarr, L. A branched, synthetic peptide corresponding to a region of glycoprotein G of HSV-1 reacts sensitively and specifically with HSV-1 antibodies in an ELISA. J. Virol. Methods. 2005; 125:137-143.

Paper 11: Kasubi, M.J., Nilsen, A., Bergström, T., Langeland, N., Marsden, H.S., and Haarr, L. Prevalence of antibodies against herpes simplex viruses type 1 and type 2 in children and young people in an urban region in Tanzania. J. Clin. Microbiol. 2006; 44:2801-2807.

Paper III: Kasubi, M.J., Norberg, P., Nilsen, A., Liljeqvist, JA., Bergström, T., Langeland, N., and Haarr, L. Clinical herpes simplex virus type 2 isolates from Tanzania and Norway cluster in two different genogroups. In manuscript.

Paper IV: Nilsen, A., Kasubi, M.J., Mohn S.C., Mwakagile, D., Langeland, N., and Haarr, L. Herpes simplex virus infection and genital ulcer disease from STD patients in Dar es Salaam, Tanzania. Accepted with some modifications, Acta Dermato-Venereologica.

Abbreviations

HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
VZV	Varicella zoster virus
HCMV	Human cytomegalovirus
HHV-6	Human herpes virus 6
HHV-7	Human herpes virus 7
EBV	Epstein-Barr virus
Nm	Nanometers
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
g	Before a capital letter, glycoprotein B – N (gB - gN)
kbp	kilo base pair
U_L	Long unique sequence of HSV genome
U_S	Short unique sequence of HSV genome
$TR_{S/L}$	Terminal repeat sequences flanking U_S or U_L
Vhs	Virus-host shut-off protein
VP16	Virion protein 16
HveA	Herpes virus entry protein A
TNF	Tumor necrosis factor
Prr1/2	Poliovirus receptor-related proteins-1 or 2
HveB	Herpes virus entry protein B
HveC	Herpes virus entry protein C
MtrI	Morphological transforming region of HSV-1
mtrII / mtrIII	Morphological transforming regions of HSV-2
RRI	Ribonucleotide reductase
HPV	Human papillomavirus
WB	Western blot
ELISA	Enzyme linked-immunosorbent assay
PCR	Polymerase chain reaction
HCV	Hepatitis C virus

HIV	Human immunodeficiency virus
GUD	Genital ulcer disease
STD	Sexually transmitted disease
STI	Sexually transmitted infection

1.0 Herpes simplex virus – General aspects

1.1 Taxonomy

Herpes simplex viruses (HSV) are important human pathogens causing diseases in a variety of different tissues and animal species. There are two antigenic types, HSV-1 and HSV-2, with HSV-1 being most often transmitted non-sexually and HSV-2 most usually sexually transmitted (Umene and Kawana, 2000; 2003). HSV belongs to the family *Herpesviridae.* The family is divided into three subfamilies; α (alpha), β (beta) and γ (gamma) herpesviruses, based on biological properties. At present nine herpesviruses are recognized as natural human pathogens *(*Roizman and Pellet, 2001). Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and Varicella-zoster virus (VZV) belong to the alphaherpesviruses *(alphaherpesvirinae)*, which have a wide host range, a relatively short life cycle and establish latent infections preferentially in sensory ganglia. Human cytomegalovirus (HCMV), and human herpes viruses 6A, 6B and 7 (HHV-6A, HHV-6B and HHV-7) belong to betaherpesviruses *(betaherpesvirinae)*, which have a restricted host range and multiplication of the viruses appears to be slow. These viruses establish latency in lymphoreticular cells. Finally Epstein-Barr virus (EBV) and human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) are B lymphotropic virus belonging to the gammaherpesviruses (*gammaherpesvirinae*), and latency has been detected in lymphoid tissues.

1.2 Structure

Studies have shown that all herpes virions consist of four elements: DNA core, capsid, tegument and glycoprotein-containing envelope (Roizman and Pellet, 2001). A schematic presentation is given in Figure 1. The HSV genome contains approximately 152-kbp (Rajcani et al., 2004). The DNA of HSV-1 and HSV-2 consist of two covalently linked segments called the L (long) and S (short), with unique sequences-U_L (unique long) and U_S (unique short), flanked by large inverted repeat sequences which are designated terminal and internal repeats of the long (TR_L and IR_L) and short (TR_S and IR_S) unique sequences, respectively (Rajcani et al., 2004). Additionally, the unique L and S components can invert relative to one another, yielding four linear isomers, and each of the four is equally virulent (functionally equivalent) in the host cell (Roizman and Knipe, 2001; Whitley and Roizman, 2001; Kimberlin, 2004). The genes of the long and short unique sequences are designated UL1 to UL56 and US1 to US12, respectively (Dolan et al, 1998).

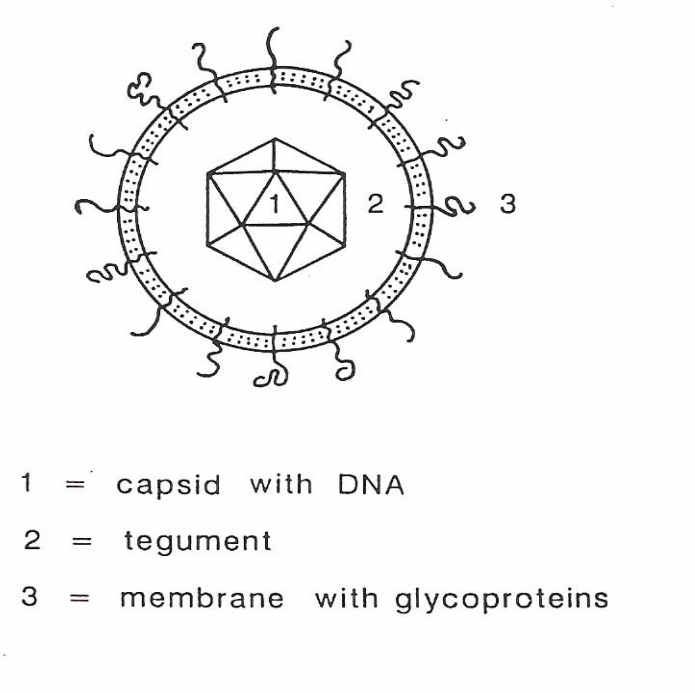

Fig.1. Schematic diagram of the HSV-1 virion (from Haarr and Skulstad, 1994)

The capsid is a structurally well-defined icosahedron, an important function of which is to contain and protect the viral genome. It is 125 nm in diameter and approximately 15 nm thick, and it is organized into 162 capsomers, of which 150 are hexavelent capsomers (hexons), and 12 pentavalent capsomers (pentons) (Trus et al., 2001; Mettenleiter, 2002). All capsomers are connected in groups of three by trigonal nodules or triplexes on the capsid surfaces (Newcomb et al., 2003; 2005; Singer et al., 2005). Three types of capsids are reported (Warner et al., 2000; Desai, 2000). The C capsid are mature forms and contain viral DNA. The B capsids do not contain DNA, but do contain the scaffold proteins and are believed to be an early stage of viral assembly. The A capsids are empty. These are thought to be capsids that failed in the packaging process. In cell free system a fourth form of capsids has been identified, termed procapsid and this may be a precursor of these three capsids (Newcomb et al., 2000; Chi and Wilson, 2000; Sheaffer et al., 2000).

The tegument is the least understood component of the virion in relation to its structure and function, its role in virus entry, and mechanisms of its assembly and incorporation into virions. However, cryoelectron micoroscope analyses showed that at least the innermost part of the tegument that is located adjacent to the capsid may also exhibit icosahedral symmetry (Zhou et al., 1999). The tegument contains at least 19 different HSV proteins (Mettenleiter,

2002; Miranda-Saksena et al., 2002). While the precise functions of most of the individual tegument proteins are not yet clear, it seems likely that they perform dual roles in the virus replication cycle, providing activities both at the onset of infection, as the capsid-tegument enters the cell, and during virion assembly, as virus matures and exits the cell. UL 31 and UL 34 tegument proteins form a complex which plays an important role in envelopment of nucleocapsids (Reynolds et al., 2001). UL 41 has been implicated in virally induced host-cell shut off by degradation of host mRNAs soon after infection (Whitley and Roizman, 2001; Mettenleiter, 2004). The UL48 protein is the α-transinducing factor, αTIF, responsible for the trans-activation of immediate early genes (Mettenleiter, 2004). It is also reported that UL48 plays a direct role in virion assembly and egress (Mossman et al., 2000). The UL 46 and UL 47 proteins modulate the activity of the αTIF protein (Donnelly and Elliott, 2001, 2001; Kopp et al., 2002). The UL6, UL15, UL17, UL25, UL28, UL32, and UL33 proteins are required for cleavage and packaging of viral DNA (White et al., 2003).

On the outer side of the tegument is the envelope, which is a lipid bilayer derived from the host cell. HSV specifies at least 12 glycoproteins designated gB, gC, gD, gE, gG, gH, gI, gK, gL, gM and gJ and gN (Whitley and Roizman, 2001; Foster et al., 2001). These glycoproteins function in several important roles, including pH-independent virus entry via fusion of the virion envelope with cellular membranes (Milne et al., 2005), egress of infectious virion particles (Foster et al., 2001, 2003; Neubauer and Osterrieder, 2004; Melancon et al., 2005), cell-to-cell spread (Rauch et al., 2000; Johnson et al., 2001), virus induced cell fusion (Bender et al., 2005) and immune evasion (Trybala E., 2000; Saldanha et al., 2000; Rux et al., 2002). Antigenic specificity is provided by gG, with the resulting antibody response allowing for the distinction between HSV-1 (gG-1) and HSV-2 (gG-2) (Eriksson et al., 2004).

1.3 Regulation of gene expression

During productive infections, transcription of the viral genome occurs in a cascade-like fashion resulting in immediate-early, early, and late viral mRNAs. The α (immediate-early) genes, require no prior viral protein synthesis for their expression and are responsible for the expression of the other genes in a regulated way. β (early) genes, whose expression is totally independent of viral DNA, encode proteins and enzymes which are directly involved in DNA synthesis and nucleotide metabolism. The beta (β) genes induce the activation of the last group of genes, the gamma (γ) or late genes, coding for many of the structural proteins in the

HSV virion, including capsid proteins, which are translated in the cytoplasm and then imported into the nucleus where capsid assembly occurs (Mettenleiter, 2004).

1.4 Entry of HSV into host cells

The entry of HSV requires binding of virus to receptors on the cell surface and fusion of the virion envelope with the cell plasma membrane (Spear et al., 2000; Spear and Longnecker, 2003). The initial attachment is mediated through viral glycoprotein C (gC) and/or gB to cell surface heparan sulfate proteoglycans (Langeland et al., 1990; Sakisaka et al., 2001; Cheshenko and Herold, 2002). The fusion of the viral envelope with the plasma membrane requires gB, gD, gH, and gL (Muggeridge, 2000; Pertel et al., 2001; Avitabile et al., 2003). Three cell surface receptors for HSV have been identified: herpes virus entry mediator A (HveA or HVEM) is a member of the tumor necrosis factor receptor (TNFR) family. The other two receptors are HveB and HveC (Lopez et al., 2000; Connolly et al., 2001; Terry-Allison et al., 2001; Krummenacher et al., 2004; Jogger et al., 2004). It is also reported that HveC allows viral entry by directly interacting with gD, as shown for HveA (Connolly et al., 2001; Milne et al., 2001; Whitbeck et al., 2001). Recently both HveB and HveC were found to be components of a novel cell-cell adhesion system, and to belong to the Ig superfamily. They were therefore renamed as nectin-1 and nectin-2, respectively (Lopes et al 2000; Satoh-Horikawa et al., 2000; Takahashi et al, 2001).

1.5 Life cycle of HSV

HSV replicates by three rounds of transcription that yields: immediate-early (α) proteins that mainly regulate viral replication; early (β) proteins that synthesis and package DNA; and late (γ) proteins, most of which are virion proteins.

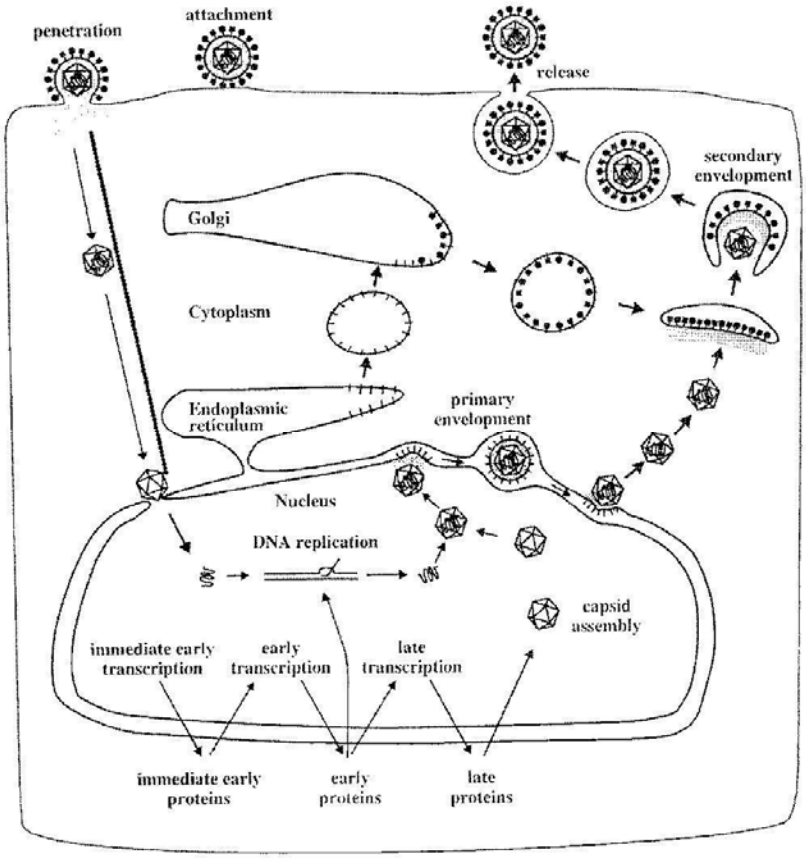

Fig. 3. Diagrammatic representation of the herpesvirus replication cycle (Metteinletter, 2004).

1.6 HSV infections

HSV is a natural pathogens for humans, with particular affinity for the nervous tissue. The virus spread from person to person by infected secretions, classically oral secretions for HSV-1 and genital secretions for HSV-2. There are three types of herpetic infections: lytic infection, latent infection and transforming infection.

In a lytic infection, virus multiply inside the nucleus of infected cell. This is followed by production of infectious virions before lysis of infected cells, partly due to suppression of host protein synthesis by a structural protein named virus host shutoff (vhs) protein, encoded by the UL41 gene (Whitley and Roizman, 2001; Mettenleiter, 2004).

In latent infection, viral DNA is maintained in a non-replicative state and persists in the nucleus as an episome for the entire life of the individual (Roizman and Knipe, 2001). Virus

may reactivate following a variety of local or systemic stimuli to cause recurrent disease (Sawtell and Thompson, 2004). During latency, the viral lytic genes are extremely repressed and only a single transcription unit encoding the latency-associated transcripts (LATs), remain active (Steven et al., 1987; Thompson and Sawtell, 2000; 2001; Peng et al., 2005). The most abundant LAT is a 2.0-kb RNA (Rødahl and Haarr, 1997; Inman et al., 2001), which is also detected during productive infections (Ahmed et al., 2002). The other LATs are 1.4- and 1.5-kb long which can only be detected during latency (Kang et al., 2003). The molecular mechanisms controlling latency and reactivation remain poorly understood and is a focus of active investigation.

The possibility that herpes simplex virus has transforming potential has been a focus of interest. Numerous studies have shown that, both HSV-1 and HSV-2 are able to transform the morphological phenotype of rodent cells. Transformation by HSV-1 does not require the entire viral genome, but is attributed to a region located between map units 0.31 and 0.42 designated as morphological transforming region of HSV-1 (*mtr-I*) (Das et al., 1994). Failure to detect viral DNA in transformed cells led to the hit-and-run hypothesis of HSV-1 transformation (McDougall, 2001). Within the HSV-2 genome there are two unique morphological transforming regions designated as *mtr II* and *mtr III* located between map units 0.585 and 0.63 and 0.42 and 0.58 (Jones, 1995). *MtrII* and *mtrIII* encompass for the large subunit of viral ribonucleotide reductase (RR1) which has transforming potential (Aurelius., et al 1998; 1999; Smith et al., 2000). It has been proposed that HSV-2 participates as a cofactor in the development of invasive cervical carcinoma (Smith et al., 2002). No human tumours has so far been shown to be directly caused by HSV (Lehtinen et al., 2002).

1.7 Human disease

HSV-1 and HSV-2 are common human pathogens that can cause primary and recurrent infections of mucous membranes. Primary HSV infections are usually symptomatic but may be sub clinical. Recurrent infections are generally less severe than the primary infection. The most commonly seen clinical manifestations include oro-facial and genital lesions. Ocular infections may include any part of the eye including the retina, conjuctiva, cornea and eyelids (Ganatra et al., 2000; Liesegang, 2001; Umene et al., 2003). Meningitis is usually benign, but the HSV encephalitis has been associated with high mortality (Tyler et al., 2004). Neonates are particularly at risk for serious HSV infections; early treatment appears to be an important

determinant of the outcome (Kimberlin et al., 2001; 2001). In addition, immunocompromised patients are at risk of developing more severe HSV infections. Cutaneous HSV infections are uncommon in healthy persons but may be seen in a number of skin disorders such as eczema herpeticum and atopic dermatitis (Wollenberg et al., 2003; Yoshida and Umene, 2003). Herpetic whitlow is infection of fingers among dentists and other health care workers (Szinnai et al., 2001) whereas infection on bodies of wrestlers is called herpes gladiatorum (Dworkin and Shoemaker, 1999). Erythema multiforme is frequently associated with HSV infection (Sun et al., 2003; Aurelian et al., 2005; Ono et al., 2005).

1.8 Epidemiology of HSV infection

The prevalence of HSV-2 infection among health adult populations is higher in the USA than in Europe. Furthermore, HSV-2 seroprevalence varies widely among European countries (Malkin, 2004). In some, but not all countries, HSV-2 seroprevalence appears to be increasing (Malkin, 2004). The most recent data available in the United States demonstrate a 30% increase of HSV-2 seroprevalence over the past two decades (Malkin, 2004; Bünzli et al., 2004). The higher HSV-2 seroprevalence has been reported among patients attending sexually transmitted disease (STD) clinics (Gottlieb et al., 2002; Weiss, 2004). The situation is not well characterized on African content but available data suggest that HSV-2 seroprevalence is higher than in the United State (Smith and Robinson, 2000; Weiss, 2004).

HSV is now a major health concern, confirmed by the epidemic of genital HSV and enhanced acquisition of human immunodeficiency virus (HIV) infections in association with HSV infections (Mbopi-Keou et al., 2002; Celum, 2004). HSV-2 is a common cause of genital ulceration (Wawer et al., 1999). In an HIV-negative individual, genital ulcer might increase susceptibility to HIV infection by disrupting the mucosal barrier and by inflammatory changes, which increase recruitment of HIV target cells to the ulcer (Rottingen et al., 2001).

1.9 Treatment

There is currently no curative therapy of HSV infection. In fact, no drugs are available acting on the virus during latency in the dorsal root ganglia. The yet most successful drugs such as acyclovir, valacyclovir, famciclovir are all nucleoside analogues inhibiting replication. These drugs have similar modes of action. They first undergoes monophosphorylation by virally encoded thymidine kinase (TK) and concentrates in infected cells. Host enzymes, cellular

kinases further phosphorylate the drug to the active triphosphate form which inhibits viral DNA polymerase and consequently viral replication (Chosidow et al., 2001; Tyring et al., 2002; Coen and Schaffer, 2003). Foscarnet is a pyrophosphate analogue which directly inhibits viral DNA polymerase without prior activation by thymidine kinase. In contrast to nucleoside analogues, foscarnet is a non-competitive inhibitor of the DNA polymerase (Coen and Schaffer, 2003).

Various vaccine against HSV infection have been tested without success, focusing on primary prevention and on immunotherapy for those already infected. Continue search for an effective vaccine is still on going (Stanberry, 2004; Jones and Cunningham, 2004; Aurelian, 2004; Hoshino et al., 2005).

2.0 Diagnosis of HSV infection

Efficient laboratory testing is an essential component for management and development of strategies to prevent transmission of HSV infection. Current laboratory methods used to diagnose HSV infections include: virus detection, antigen detection, DNA detection and serological tests. Virus detection methods through culturing and DNA detection, particularly using polymerase chain reaction (PCR), are applicable during active infection in patients presenting with lesion. Antigen detection method can be nearly as sensitive as culture methods (Ashley and Wald, 1999), and the most sensitive strategy is to perform both tests. Serological tests allows identification of silent carriers of HSV infection and provide useful information in symptomatic patients when virological tests such as culture, antigen detection and PCR are not helpful (Woolley et al., 2000; Ashley, 2001). The application of HSV type-specific serological tests has been difficult due to strong serological cross-reactivity caused by the extensive antigenic similarities between the two viruses (Schmid et al., 1999; Tunbäck et al., 2000). The identification of type-specific glycoproteins G-1 (gG-1) and gG-2 in the mid-1980s seemed to resolve this difficult (Marsden, et al., 1984; Roizman, et al., 1984), because it is antigenically distinct for the two viruses. Since the demonstration of two antigenic types, numerous test formats have been developed to detect type specific antibodies (Ashley, 2001).

3.0 Rationale for the present study

The present study focused on the following four points:

3.1 Establishment of a new peptide ELISA for detection of HSV-1 antibodies

Problems with the present diagnostic methods:

Considerable homology exists between HSV-1 and HSV-2 genomes. As a result, most polypetides specified by one viral type are antigenically related to polypeptides of the other viral type (Schmid et al., 1999; Kimberlin, 2004). One of the consequences is that antibodies directed towards antigens from one type of the virus will often cross-react with antigens from the other serotype. In addition, tests based on recombinant gG from bacteria or baculovirus expression vectors (Parkes, et al., 1991), may not detect all the antibodies elicited in humans by exposure to gG, because recombinant gG may lack epitopes that depends upon glycosylation mechanisms available in mammalian cells. Therefore, few tests are available to date for specific detection of HSV antibodies. The most specific and reliable diagnostic test for HSV-1 or HSV-2 specific tests are Western blotting (WB) and Polymerase chain reaction (PCR) (Schmid et al., 1999; Madhavan et al., 1999). However, both tests are laborious and /or expensive and require expensive equipment and well trained personnel, and are therefore not suitable for routine use in clinical laboratory settings.

New possibilities:

Two new diagnostic approaches have recently been available. Firstly, methods have been developed using either the complete glycoprotein gG (gG) or specific epitopes in gG for detection of HSV-2 antibodies (Marsden, et al., 1998; Ashley, 2001). The question was if epitopes of this glycoprotein in HSV-1 could also be used for detection of HSV-1 specific antibodies. Secondly, branched peptides corresponding to the amino acid sequences of certain epitopes have been tested and found to be useful for specific detection of antibodies against hepatitis C virus (HCV) and HSV-2 (Bhattacherjee, et al., 1995; Marsden, et al., 1998). Here we have explored new diagnostic approaches in an attempt to establish a new diagnostic method for specific detection of HSV-1 antibodies using branched synthetic oligopeptides corresponding to an immunodominant region on gG-1of HSV-1 as antigens.

3.2 Detection of HSV specific antibodies in sera from children and young persons

Most adult people are infected with HSV-1 which is the usual cause of oro-labial herpes, but to and increasing extent infections of the genital area. Genital herpes is usually caused by HSV-2, and most primary HSV-2 infections do occur later in life. Reports from the African continent indicate that the percentage of HSV-2 infected adults has increasing in recent years (Weiss, 2004). However, the situation is not well characterized in children and young persons in developing countries, although several studies from smaller geographical areas and from selected population groups suggest that there is a strong association between HSV-2 infections and HIV (Nilsen et al., 2005). Here we have analysed sera from Tanzanian children and young persons aged 1 – 20 years old in order to determine the acquisition patterns of HSV infections.

3.3 Genetic diversity of clinical HSV-2 isolates from Tanzania and Norway

As already mentioned, HSV-2 is the commonest cause of genital ulcer disease. Classically, primary infection usually occur later in life. Variations in gG-2 might alter the antibody response in the HSV-2 type-specific serological assays based on gG-2 as antigen. This could be expressed as a low sensitivity in serotesting of patients harbouring strains carrying one of the other gG-2 gene variants if the antigen used as a diagnostic tool is based on the sequence of the other genetic variant. So far, the variability of HSV-2 strains has not been extensively studied, although (Liljeqvist et al., 2000) found a gG-2 epitope, as determined by using a monoclonal antibody, to be highly conserved among 2,400 HSV-2 isolates, these isolate were all from northern Europe, and no studies have evaluated the relatedness of HSV-2 isolated in Africa. Recently, we focused on DNA sequencing analysis of the glycoproteins G (gG-2), and in addition on genes encoding gI-2 and gE-2 to add the number of sequences available for phylogenetic comparison.

3.4 Aetiology of genital ulcers among patients attending STD clinic in Tanzania

Although it is generally recognized that sexually transmitted infections (STIs), particularly those causing genital ulceration, facilitate HIV transmission (Mbopi-Keou et al., 2002; Celum, 2004), and HIV infection may simultaneously prolong or augment the infectiousness of individual with (STIs), yet little information exist on the causative agents of genital ulceration among STD patients in Tanzania. Four infectious agents have so far been identified

as common causes of GUD: herpes simplex virus type 2 (HSV-2), HSV-1, *Treponema pallidum*, which is known to cause syphilis, and *Haemophilus ducreyi*, which causes chancroid. Currently, the diagnosis of GUD in many African countries is based primarily on the clinical presentation of the ulcer. Since clinical diagnosis of GUD is difficult due to the diversity of clinical manifestations of genital herpes, and occurrence of multiple and mixed infections, we have analysed material from genital ulcers to identify the causative agent(s) which could help in designing appropriate control measures of STDs including HIV infection.

4.0 Aims of the study

- To evaluate the performances of three synthetic branched oligopeptides for specific detection of HSV-1 antibodies (Paper 1).

- To use the newly established method, as well as other ones, to determine acquisition patterns of HSV-1 and HSV-2 infection among Tanzanian children and young persons aged 1 to 20 years old (Paper 2).

- To investigate genetic variability of clinical HSV-2 isolates from Dar es Salaam, Tanzania and Bergen, Norway and to compare the strains from each region (Paper 3).

- To analyse material from genital ulcers to determine which proportion is caused by HSV, Treponema pallidum and Haemophilus ducreyi (Paper 4).

5.0 Summary of results

Paper I

This paper describes an evaluation of an enzyme-immunoassay for detection of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG-1)-specific antibodies in human sera. The assay is based on a 16mer peptide (presented as branched tetramer) representing an immunodominant sequential region of glycoprotein G of HSV-1. The selection process involved a core peptide to march an immunodominat region of gG-1. One N-terminally and one C-terminally elongated peptide were also tested for suitability. Sera grouped into four classes according to their results with a commercial HSV immunoblot using recombinantly expressed gG-1 and gG-2 were tested with the peptide assay. Furthermore, the peptide assay results were compared with results obtained by an immunoassay using recombinant gG-1. The results show that no differences were found between the peptide corresponded in sequence to the immunodominant region and the assay with recombinant antigen. This findings suggests that the peptide corresponded in sequence to the immunodominant region was as specific and sensitive in an ELISA as were the commercial methods.

Paper II

In this work we took advantage of the findings described in paper I and used the newly established HSV-1 peptide-ELISA as well as other ones to investigate the prevalence of specific HSV antibodies. To approach this problem, sera were collected from Tanzanian children and young people aged 1 to 20 years old and thereafter divided into five different age groups using intervals of 4 years with at least 100 individuals in each age group. Sera were analyzed by using the WB-UB method which has shown to perform well in comparison with a commercial Western blot assay as well as peptide-ELISA methods for specific detection of HSV antibodies. Due to strong correlation between HIV and HSV-2 (Celum, 2004), all sera were also analyzed in the laboratory of Bionor for the detection of HIV-1 and HIV-2 antibodies. Our results show that HSV-1 infections occur early in life in Tanzania, as 74% of the individuals aged 2 to 4years old had specific antibodies, and more than 80% of children in the age group of 5 to 8 years had specific antibodies. The prevalence of HSV-2 antibodies was surprisingly high, as 15% of the children were infected at an age of 8 years, with an increase to 40% for the age group of 17 to 20 years old, suggesting that non-sexual transmission of HSV-2 is common than previously thought. No statistical significant association between HSV-2 and HIV seropositivity.

Paper III

Genetic diversity among herpes simplex virus type 2 (HSV-2) is of potential importance for diagnostics and vaccine development. However, little is known. In the present work, we have sequenced gene segment coding for gG, gI and gE as well as the non-coding region between g I and gE from 11 Tanzanian isolates and 10 Norwegian isolates. The sequences were compared to the reference HSV-2 strain HG52. Overall, sequence variation was low, with an overall similarity between the two most distant isolates of 99.6%. Phylogenetic analysis revealed that HSV-2 strains are classified into two genogroups, designated Africa (A) and European (E) supported by high bootstrap values. All strains classified in A genogroup were isolated in Tanzania, while the E genogroup contained both Tanzanian and Norwegian strains for gI/gE as well as for gG trees. This findings suggests that three clinical HSV-2 isolates collected from patients in Tanzania presenting a genetic pattern consistent with homologous recombination between the A and E genotypes. Mutations within the epitope regions I and II identified by monoclonal antibodies were detected in 2 Norwegian isolates 5 and 7[*]. Further investigation of possible influence of alteration in gG in diagnosis was beyond the scope of the present study, but it forms the bases for future studies.

Paper IV

The most common etiologic agents of genital ulcer disease (GUD) are herpes simplex virus type 1 (HSV-1), HSV-2, *Treponema pallidum,* and *Haemophilus ducreyi*. Genital ulceration is recognized as a risk factor for the heterosexual transmission of HIV-1 infection (Rottingen et al., 2001). Although a significant association with chancroid and syphilis has been reported earlier, there are studies showing that HSV-2 is increasingly associated with HIV-1 infection (Mbopi-Keo et al., 2002; Celum, 2004). In an outpatient clinic for sexually transmitted diseases in Dar es Salaam, Tanzania, specimen from 301 patients with GUD were collected. Of 301 patients recruited into the study, herpes simplex virus type 2 infection was detected in 64%, herpes simplex virus type 1 in 5.9%, *Haemophilus ducreyi* in 3.9% and *Treponema pallidum* in 3.3%. No pathogens were detected in 30% of patients. 6.6% of patients had mixed infections. The results show that HSV-2 is the predominant cause of genital ulcers among Tanzanian STD patients, and HSV-1 is as least as common as the agents causing syphilis and chancroid.

[*] There was originally a printing error here, stating that 5/5 genotype A strains presents with amino acid changes within the suggested immunodominant region. This was not consistent with the results in paper III.

6.0 General discussion

Peptide ELISA for detection of HSV-1 antibodies

The ability to discriminate between HSV-1 and HSV-2 infections by using reliable HSV type-specific serological method is essential for several purposes, including seroepidemiological studies, to evaluate the efficacy during trials of HSV prophylactic agents such as potential vaccines. An important part of these studies is determination of the frequencies of type-specific seroconversions. Seroconversion may also be an important parameter in attempts to estimate the optimal dosage of antiviral treatment. Although previous evidence suggested that the majority of genital herpes infections were HSV-2-related, recent reports suggested an increase in HSV-1 genital infection, sometimes outnumbering cases caused by HSV-2, especially in women (Stanberry et al., 1999; Taylor et al., 1999). Development of sensitive, specific and reliable diagnostic method is of utmost importance, since HSV-1 and HSV-2 genital infections are less clearly clinically separate than once was the case.

An initial study of the binding of antibodies to three different HSV-1 peptides (paper I) were performed in order to select the peptide which was most specific and sensitive in the ELISA format. Our results indicate that the peptide corresponding in sequence to the immunodominant region in gG-1 (peptide 2048.8) was suitable for the specific detection of serum antibodies to HSV-1. However, when analyzing sera from young children (paper II), this ELISA was less sensitive and specific than was peptide 55 ELISA for specific detection of HSV-2 antibodies. The reasons are not clear. One explanation could be that the peptide used did not mimic the epitope as well as peptide 55 mimics the HSV-2 epitope. Furthermore, it is not known why addition of three flanking amino acids to either end of the peptide reduced both the specificity and the sensitivity. It would have been interesting to investigate whether reducing the number of amino acids in the peptide might help to improve the performance of the assay. However, it was beyond the scope of this study to investigate the effects of such modifications. African sera are known to produce a high degree of false positive results regardless of the method used for analysis of antibodies (Hogrefe et al., 2002). Could it be due to amino acid differences in gG, since many assays in which gG is used as antigen are based on isolates from Europe and America. However, in our study (paper I) comparable results were obtained for Scandinavian and African sera when analyzing them with peptide HSV-1 ELISA, commercial ELISA kit and a commercial immunoblot kit for Scandinavian and African sera.

Detection of HSV specific antibodies

Herpes simplex virus type 1 (HSV-1) is transmitted by close contact through mucosas in the oro-pharyngeal region. In contrast, the vast majority of infections with herpes simplex virus type (HSV-2) are transmitted sexually. The seroprevalence of HSV-2 antibodies in adults has been studied in several places in the world and shown to vary by country and group of population. Reports from the African continent indicate that the percentage of HSV-2 infected adults has increased in recent years. This is alarming, when considering the association between infection with HSV-2 and HIV as mentioned earlier.

Little is known about HSV-2 infections in Africa in childhood and adolescence. Although most HSV-1 infections are acquired in childhood, very few studies from Africa have focused on the pattern and time of acquisition, as we did in paper II. In addition, we wanted to investigate the performances of the two newly developed ELISA methods for specific detection of HSV-1 and HSV-2 antibodies. To study the acquisition pattern of HSV infections, we analyzed more than 500 sera from children and young people aged 1 to 20 years using methods that discriminate between HSV-1 and HSV-2 antibodies. The methods were an in-house Western blot designated WB-UB which was evaluated against a commercial Western blot, and two ELISA methods in which the antigenic oligopeptides were specific for either HSV-1 or HSV-2 (Kasubi et al., 2005; Marsden et al., 1998). Due to a strong correlation between infections of HSV-2 and HIV, all sera were also screened for HIV antibodies.

The work described in paper II show that prevalence of HSV-1 antibodies was more than 70% in the age group 1-4 years old, increasing to about 90% in the age group 17-20 years, indicating that HSV-1 infections occurs early in life in Tanzania compared to similar studies conducted in Sweden and Germany (Tunbäck et al., 2003; Wutzler et al., 2000). About 20% in age group 1-4 years had HSV-2 antibodies. The detected antibodies in this age group could include maternal antibodies, since the figures decreased to 14% in age group 5-8 years. Thereafter the HSV-2 prevalence increased continuously to reach 40% in the age group 17-20 years. The high percentage of HSV-2 infection in children and young people was unexpected, suggesting that non-sexual means of HSV-2 spread might play a major role of HSV-2 transmission in young children. The present work did not investigate how non-sexual means of HSV-2 spread might occur. One possibility is through contaminated fingers. Recently,

haematogenous vertical transmission of HSV-1 has been described in mice (Burgos et al., 2006). However, it is not clear whether similar mode of transmission would be possible in human. The idea of non-sexual transmission of HSV-2 has also been suggested earlier by other investigators (Löwhagen et al., 2002).

Statistically, no significant association between HSV-2 and HIV was observed (paper II), although other investigators have demonstrated such association in adult cohorts. This difference could possibly be explained by the probability that relatively more children had congenital HIV infections than had sexually active adult.

Ashley, (2001), as well as manufacturers of commercial kits, have reported difficulties in interpretation of results when analyzing sera from persons younger than 14 years old for the presence of HSV specific antibodies. Similar findings have been reported in the present work, except that the problems seem to be more limited to sera from young children (paper II). The reason is unknown. However, one explanation might be that the patterns of immunoglobulins are different in sera from children and from adults. Specifically, textbooks in immunology indicate that the level of IgG in younger age group could be relatively low, and then increases with age. Consequently, the ratio between antibodies directed to a specific pathogen, and the total amount of immunoglobulins, could be different from the ratio later in life. In addition, the amount of IgM could possibly play a role, because it is the most efficient immunoglobulin in agglutination. At the age of one year, the infant produces 75% of what is the adult IgM level, thereafter the IgM level increases with age.

Genetic diversity of gG-2, gE-2 and gI-2

The identification of extensive sequence diversity and the presence of multiple HSV-2 subtypes co circulating within a defined population may be of importance for the design and application of effective vaccines. In addition, divergent isolates may escape detection by currently available screening assays. Due to a strong associations between HSV-2 and HIV, identification and characterization of divergent HSV-2 isolates is important with respect to evaluation and possible modification of diagnostic assays in the future, and for development of effective vaccines. However, our knowledge in this area is still very limited. The results in paper III shows that the HSV-2 isolates from Tanzania and Norway had genetic similarity of approximately 99.6%. Similar findings of conserved genetics have been reported by other

investigators for HSV-1 and VZV (Norberg et al., 2004; Loparev et al., 2004). The conserved genetics of clinical HSV-2 isolates from the two regions probably reflects the low mutation rate in herpes viruses compared to RNA viruses such as HCV (Kato et al., 1994).

Although a small number of HSV-2 isolates were studied, and despite their genetic similarity, two phylogenetically distinct groups were clearly detected and designated European (E) and African (A). The laboratory strain HG52 was included as reference. The sequences in the two groups were geographically separated. However, exception to this were the Tz_2032, Tz_1855 and Tz_2737 sequences which clustered in group A in one phylogenetic tree and in group E in the other tree. We concluded (paper III) that these three isolates might represent recombination events between African and European genotypes indicating that Dar es Salaam city is populated with a mixture of strains. The process of recombination is important for generating diversity and genome stability, as it is essential for repair of some types of DNA lesions (Yao and Elias, 2001). It would have been interesting to study larger numbers of isolates both from Norway and from Tanzania.

Aetiology of genital ulcer diseases

Several seroprevalence studies of HSV-2 and syphilis have been conducted in Tanzania among individuals at high risk of STI (Langeland et al., 1998; Mwansasu et al., 2002; Nilsen et al., 2005). However, a smaller number of studies have been conducted to investigate the specific aetiology of GUD among patients attending STD clinics.

Laboratory diagnosis of GUD is not done routinely in Tanzania due to lack of the necessary laboratories or facilities and experienced personnel. Hence, in many areas of Tanzania the diagnosis of GUD is based on clinical appraisal. Reports on the most common causative agents in given areas are limited. The use of PCR has improved the diagnosis of GUD. Previously, the detection of some of the causative agents required Gram staining and/or culture which are less accurate and sensitive procedures (Dangor et al 1990). The advantage of PCR include the ability to transport samples off-site for testing as well as its increased sensitivity relative to culture, presumably due to the difficulties in growing fastidious organism (Morse et la., 1997; Orle et al., 1996). Most of the genital ulcers were caused by HSV-2 in our studied population (paper IV). This findings is in agreement with a previous study done in Dar es Salaam, Tanzania, where HSV-2 DNA was detected in 64% of patients

with GUD (Mwansasu et al., 2002) and much higher than that reported in a recent study in Mbeya region, Tanzania, among female bar and hotel workers where a prevalence of 13.5% in 52 women with GUD was reported (Reidner et al., 2003). These findings in Tanzania support the conclusion that HSV-2 is the leading cause of GUD worldwide (Celum et al., 2004) and that HSV-2 infection is common in our studied population. One possible reason for this is that efficient syndromic treatment has been targeted to bacterial but not viral genital infections. Improved microbiological diagnostic methods for identification of single pathogens, particularly HSV, might also have played a role in the apparent increase in prevalence of these pathogens. Although the numbers for the other pathogens are small, HSV-1 was apparently at least as important as *H. ducreyi* and *T. pallidum*.

No pathogen was detected in 30% of the ulcer specimens (paper IV). These ulcers may have been caused by other unrecognized ulcerative diseases such as lymphogranuloma venerum or granuloma inguinale, which were not examined for, or by sexual trauma. Alternatively lack of serous fluid in the ulcers, inadequate specimen collection, and improper storage may have occurred. Furthermore, the duration of the ulcers might influence the probability of detecting the causative agent. Similar findings have been documented by other investigators, who have used multiplex PCR. In a study done in the Netherlands, 37% of ulcer specimens were negative (Bruisten et al 2001), 32% in a study done in Atananarivo, Madagascar (Behets et al., 1999a), 20% in a study in Jackson, Mississippi (Mertz et al., 1998b).

7.0 Conclusions

Data from this thesis shows that:

- A branched, synthetic oligopeptide has been used successfully as antigen for specific detection of HSV-1 antibodies. The peptide contained exclusively type-specific-epitopes identified in gG-1, and displayed no cross-reactivity when 16 HSV-1 negative/HSV-2 positive sera were analysed in our initial study (paper I). Thus, this peptide has the potential to be another antigen for the detection of type-specific human anti-gG-1 antibodies.

- HSV-1 infections occurs early in life in Tanzania. The high percentage of HSV-2 infection especially in young children is a cause of great concern and suggest that non-sexual means of HSV-2 transmission is more common than expected. Although the peptide-ELISA methods for detection of HSV-1 or HSV-2 antibodies performed well, their sensitivities and specificities were reduced when analysing sera from children.

- HSV-2 isolates from Tanzania and Norway seemed to be divided in two genetic variants designated A (Africa) and E (European), as shown by phylogenetic trees based on the sequences of the genes encoding gG-2, gI-2 and gE-2. By comparing the trees obtained from the different genes, evidence of recombination events between the two genetic variants was found.

- HSV-2 is becoming the most important cause of GUD in Tanzania as in other African countries. These results suggest the need for integrating HSV-2 treatment in the syndromic management of GUD in Tanzania. Treatment of STI including HSV-2 could also play a role in preventing HIV infection, and it should be used together with other approaches, such as promotion of condom use and behaviour change intervention. Currently, some programs for HIV treatment in Africa include antiviral compounds against HSV, but the results are not yet known.

8.0 Future perspectives

- Since addition of amino acids to either C-terminal or N-terminal ends of the peptide reduced both the sensitivity and specificity of the assay, it would be interesting to investigate the functional implication of the antibody binding to peptides containing reduced numbers of amino acids.

- Although our study suggested the possibility of non-sexual transmission of HSV-2, we could not explain the mechanisms of such transmission. Further studies into the possible non-sexual routes of HSV-2 transmission are warranted.

- Given the limited number of clinical HSV-2 isolates collected from each country, that the Tanzania isolates were all from Dar es Salaam and only smaller portions of the genome were sequenced, this findings should be interpreted with some caution. To obtain more firm conclusions, it would have been useful to collect clinical HSV-2 isolates from the rural areas of Tanzania to be sure that the HSV-2 isolates represented the original Tanzanian HSV-2 strains, and to sequence larger portions of the genome.

9.0 References

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