STUDY OF THE TYPE-SPECIFIC ANTIBODY RESPONSE TO HERPES SIMPLEX VIRUS TYPE 2 (HSV-2)

BY

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Submitted to the University of Manitoba in Partial Fulfillment of the Requirements for the Degree of

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1-D	one-dimensional
2-D	two-dimensional
aa	amino acid(s)
APS	ammonium persulfate
ATCC	American type culture collection
BIB	baculovirus-expressed gG-based immunoblot
CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate)
CMV	cytomegalovirus
CPE	cytopathic effect
CS	calf Serum
DAB	3,3'-diaminobenzidine tetrahydrochloride
ddH_2O	double distilled water
DTT	dithiothreitol
E	early
EBV	Epstein-Barr virus
FDA	food and drug administration
gB	glycoprotein B
gB-1	glycoprotein B from HSV-1
gB-2	glycoprotein B from HSV-2
gC	glycoprotein C
gC-1	glycoprotein C from HSV-1
gC-2	glycoprotein C from HSV-2
gG	glycoprotein G
gG-1	glycoprotein G from HSV-1
gG-2	glycoprotein G from HSV-2
HCMV	human cytomegalovirus
HHV	human herpesvirus
HPV	human papillomavirus
HSV	herpes simplex virus

HSV1+	patient infected with HSV-1 only
HSV2+	patient infected with HSV-2 only
HSV1+/HSV2+	patient infected with both HSV-1 and HSV-2
ICP35	infected cell polypeptide 35
IE	immediate early
IEF	isoelectrofocalisation
KSHV	Kaposi-sarcoma-associated herpesvirus
L	late
LATs	latency-associated transcripts
MEM	minimum essential medium
MAb	monoclonal antibody
mgG-2	mature gG-2
MOI	multiplicity of infection
MS	mass spectrometry
MW	molecular weight
NC	nitrocellulose
NL	non-linear
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS/T	phosphate-buffered saline with 0.1% Tween20
PFU	plaque forming unit
pI	isoelectric point
PVDF	polyvinylidene fluoride
RSS	rehydration stock solution
RT	room temperature
SDS	sodium dodecyl sulfate
sgG-2	secreted portion of gG-2
TBS	tris buffered saline
TEMED	N,N,N',N'-Tetra-methyl-ethylenediamine
T-TBS	TBS with 0.05% Tween 20
TMR	trans-membrane regions

VP16	virion protein 16
VZV	varicella-zoster virus
WB	western blotting
WBA	western blot assay

ABSTRACT

The diagnosis of Herpes Simplex Virus (HSV) Types 1 and 2 infections relies on serological testing since 70% of genital herpes infections are asymptomatic. The gold standard test, the Western blot, is very sensitive and specific, but is also subjective and time-consuming, which renders it unsuitable for large volume diagnoses. Commercial kits for HSV-type-specific serology are now available, and they are based on the only known type-specific HSV antigen, the glycoprotein G (gG). Unfortunately, there are several limitations to the use of a single type-specific antigen, such as variable titer and timing of seroconversion. For those reasons, gG-based tests may produce a number of discordant results. We therefore implemented two different approaches to discover new HSV-2 type-specific antigens. The first approach, two-dimensional western blotting, allowed the electrophoretic separation of all the HSV-2 proteins. Fifteen potential type-specific proteins were found by comparing the immunoreactivity of 9 serum samples from patients infected with HSV-1 and 7 from patients infected with both HSV-1 and HSV-2. Mass spectrometry analysis to identify those proteins has not been completed yet. The pepscan technique was also explored to map HSV-2 type-specific B cell epitopes on glycoprotein B (gB), which is known to be a strong immunogen but also to be among the most cross-reactive antigens. It was found in this study that gB from HSV-2 is a cross-reactive protein containing no type-specific epitopes. In conclusion, there is a need for the discovery of new HSV type-specific antigens, which could lead to the development of a new ELISA assay that would distinguish unambiguously between HSV-1 and HSV-2 infections.

1. Introduction

1.1 Taxonomy and virology

Reviewed by Roizman and Pellet (2001) and Roizman and Knipe (2001).

Herpes Simplex Viruses (HSV) Types 1 and 2 (HSV-1 and HSV-2) are members of the *Herpesviridae* family. All members of this family have a large, linear double stranded DNA genome and the HSV genome is estimated to be about 150 kilobase pairs. HSV-1 and HSV-2 present a nucleotide sequence similarity of approximately 83%. Their genome has about 90 transcriptional units, of which at least 84 encode proteins. In addition, several of the viral polypeptides undergo post-translational modifications, such as cleavage, phosphorylation, myristylation and O-linked and N-linked glycosylation. At least 30 of the viral proteins are present in the virions and the others are only present during a host cell infection and are thus termed non-structural proteins. HSVs have an icosadeltahedral capsid composed of 162 capsomeres that surrounds the electron-dense core, which contains the viral genome. The tegument, which is a layer of amorphous proteins, is found between the capsid and the envelope. The envelope is a lipid bilayer containing at least 10 viral glycoproteins and several non-glycosylated viral proteins.

Herpesviruses are associated with various animal species. Nine herpesviruses are currently known to have humans as their natural host: HSV-1 (human herpersvirus-1, HHV-1), HSV-2 (HHV-2), Varicella-Zoster Virus (VZV, HHV-3), Epstein-Barr Virus (EBV, HHV-4), Human Cytomegalovirus (HCMV, HHV-5), Herpesviruses 6A, 6B, and 7 (HHV-6A, HHV-6B and HHV-7) and Kaposi-Sarcoma-associated Herpesvirus (KSHV, HHV-8) (Table 1). HSVs are classified within the genus *Simplexvirus* and, along with

VZV, are included in the subfamily *Alphaherpesvirinae*. *Alphaherpesvirinae* are known to replicate quickly *in vitro*, to have a variable host range and to establish latency in sensory ganglia.

Designation	Synonym	Subfamily
Human Herpesvirus-1 (HHV-1)	Herpes Simples Virus-1 (HSV-1)	α
Human Herpesvirus-2 (HHV-2)	Herpes Simples Virus-2 (HSV-2)	α
Human Herpesvirus-3 (HHV-3)	Varicella-Zoster Virus (VZV)	α
Human Herpesvirus-4 (HHV-4)	Epstein-Barr Virus (EBV)	γ
Human Herpesvirus-5 (HHV-5)	Human Cytomegalovirus (HCMV)	γ
Human Herpesvirus-6a (HHV-6a)		β
Human Herpesvirus-6b (HHV-6b)		β
Human Herpesvirus-7 (HHV-7)		β
Human Herpesvirus-8 (HHV-8)	Kaposi-Sarcoma-associated Herpesvirus (KSHV)	γ

Table 1. Taxonomy for the family *Herpesviridae*.

1.2 HSV life cycle

Reviewed by Roizman and Knipe (2001).

Herpesviruses are characterized by two distinct life cyles: the latent and lytic life cycles. The lytic life cycle of HSV is depicted in Figure 1. The HSV virion attaches to the surface of the target cell through glycoprotein C (gC), gB and gD which interact with cellular receptors. The fusion of the viral envelope with the plasma membrane of the host cell is thought to be mediated by gD, gB and the gH-gL heterodimer, releasing the capsid and the tegument proteins in the cytoplasm.

Early in infection, the virus induces a selective shutoff of the cellular protein synthesis, facilitating the transition from cellular to viral protein production. This involves at least one viral protein, the virion host shutoff (vhs) protein, which is the UL41 gene product and is brought into the cell by the infecting virion. Host cell protein

synthesis shutoff is mediated by two main mechanisms: mRNA degradation and inhibition of translation of the remaining mRNA.

The deenveloped capsid is then transported to the nucleus where the DNA is released via the nuclear pores. Transcription, DNA replication and assembly all occur in the nucleus whereas translation takes place in the cytoplasm. Upon entry in the host cell nucleus, the viral genome is transcribed by the host RNA polymerase II and viral factors. Viral gene expression is tightly regulated and three classes of mRNA, α, β and γ, are transcribed in an ordered sequence. The best known transactivator of the α genes is the virion protein 16 (VP16), which is a tegument protein that associates with two cellular proteins upon entry into the host cell to recognize a nucleotide sequence in the promoter region of the viral DNA, triggering transcription by the host cell polymerase.

The α, or immediate early (IE) gene products are transcription factors involved in the regulation of the synthesis of the β or early (E) proteins, which are mainly enzymes and DNA-binding proteins involved in DNA replication and nucleotide metabolism. Viral DNA synthesis occurs by the rolling-circle mechanism and induces the production of the γ or late (L) gene products, which are mainly structural proteins required for viral assembly and egress from the infected host cell at the nuclear membrane.

As for the latent life cycle, herpesviruses are characterized by their ability to enter a latent state in their natural host. In HSV-latently infected neurons, the genome is in a closed circular form and only the latency-associated transcripts (LATs) are expressed. The molecular mechanisms explaining reactivation from latency are not very well understood (Roizman and Pellett, 2001).

Figure 1. The replication cycle of HSV. HSV mRNAs are classified in three groups based on the order of gene expression: α or immediate early, β or early and γ or late genes. The α genes are the first ones to be expressed and they encode transcription activators required for expression of the β and γ genes. The β genes encode proteins that play a role in DNA replication and nucleotide metabolism whereas the γ gene products are involved in viral assembly and egress. Available in Fields Virology, 4th ed., 2001, Fig. 3, p.2410.

1.3 Clinical disease

Reviewed by Whitley, 2001.

1.3.1 Pathogenesis

HSV-1 is mostly associated with orolabial herpes (the common cold sores) whereas HSV-2 is generally associated with genital herpes. Those two viruses are also responsible for a number of other diseases, including neonatal HSV infections, keratoconjunctivitis, skin infections, encephalitis and infections in the immuno-compromised host. The only known reservoir for HSV are humans and infections are transmitted by close personal contact of mucosal surfaces or abraded skin between an infected individual and a non-infected one. As can be seen in Figure 2, herpetic infections

are characterized by an initial infection of epithelial cells and replication of the virus in those cells. This first round of replication can be symptomatic or not, with skin vesicles or mucosal ulcers appearing at the site of infection. Symptoms such as pain, burning and itching appear within 6 hours after the beginning of viral replication followed by vesicle formation within 24 to 48 hours. These herpetic vesicles last for a few days and they result from the lysis of the infected epithelial cells.

The virus then migrates by retrograde transport in sensory nerves to the trigeminal ganglion during a labial infection and in the sacral ganglia in the case of a genital infection. The virus enters a latent state in the ganglia and reactivation can be induced by stimuli such as stress, ultraviolet light and immune suppression, leading to viral replication and migration of the virus back at or near the site of infection by anterograde transport in the sensory neurons. Recurrences occur even in the presence of humoral immunity and are sometimes accompanied by symptoms. The frequency of recurrences differs with each individual and it is believed that the degree of severity of the primary infection correlates with the frequency of the recurrences. Subclinical viral shedding is common for both orolabial and genital infections and is thus one of the major sources of transmission of the virus (Ashley, 1995; Barton et al., 1996).

As with HSV-1 labial infections, HSV-2 viral shedding without symptoms can occur during a primary (first exposure to either HSV-1 or HSV-2 in a seronegative individual), initial (infection with HSV-1 or HSV-2 following an infection with the opposite type) or recurrent infection (recurrence after latency). In fact, at least 75% of genital infections are asymptomatic (Steben and Sacks, 1997). For the individuals who do show symptoms, primary infections are the most severe form of the disease because the

genital lesions last for about 3 weeks and are extremely painful. Initial genital infections, in which a patient infected with one HSV type acquires an infection with the opposite type, are less severe symptomatically and the lesions heal within 2 weeks only. The number of lesions and pain are also lower. Recurrences are the mildest form of the disease and are generally associated with a shorter duration of viral shedding and fewer lesions. Although recurrences can be asymptomatic, all infected individuals will suffer recurrences and may transmit the infection to their sexual partner(s).

Figure 2. Infection cycle of HSV in the human host. HSV is transmitted from human to human through mucosal contact, resulting in a productive infection of the epithelial cells at the primary site of contact. The virus then migrates by retrograde transport to the nucleus of sensory neurons. Sensory neurons are the secondary site of infection and the virus establishes a latent infection in those cells. Upon reactivation, the virus migrates back by anterograde transport to the epithelial cells where it causes a recurrent infection. Available in Fields Virology, 4th ed., 2001, Fig. 11, p.2437.

1.3.2 Epidemiology

It is estimated that up to 90% of the worldwide population is HSV-1 seropositive by the age of 60 and it is also thought that most of those infections are acquired before the age of five (Ashley, 1995). The incubation period varies between 2 and 12 days with

a mean of 4 days. Oral HSV-2 infections, on the other hand, are usually found in patients who have a concomitant HSV-2 genital infection and rarely recur (Lafferty, 2002).

The appearance of HSV-2 genital infections correlates with the age of sexual activity as HSV-2 infections are mainly transmitted by sexual contact. HSV-2 seroprevalence is estimated to be between 7-16% in Europe, 13-40% in the United States, and 30-40% in Africa (Steben and Sacks, 1997). A recent seroepidemiological study performed in two Canadian sexually transmitted disease clinics demonstrated that the prevalences for HSV-1 and HSV-2 were 56% and 19%, respectively (Singh et al., 2005). A prior HSV-1 infection is thought to ameliorate the symptoms during an HSV-2 infection due to the presence of cross-reactive immunity. Nevertheless, a previous HSV-1 infection does not prevent a person from acquiring an HSV-2 infection.

HSV-1 is now known to be responsible for 40% of primary or initial herpetic genital infections (Steben and Sacks, 1997) although an oral infection with HSV-1 may protect individuals from acquiring an HSV-1 genital infection (Lafferty, 2002). Genital infections with HSV-1 are associated with milder symptoms, a decreased number of days of asymptomatic shedding and a lower frequency of recurrences compared to HSV-2 genital infections (Lafferty, 2002).

1.3.3 Treatment

Three antiviral drugs are currently approved for the treatment of genital herpes: acyclovir, and its two derivatives, valacyclovir and famciclovir, the three of them being nucleoside analogs (Tétrault and Boivin, 2000). Topical application can reduce the duration of the symptoms but oral treatment is usually preferred as it is more effective.

Oral drugs can shorten the clinical disease and prevent recurrences (Tétrault and Boivin, 2000) as they are non-toxic and can thus be given as suppressive therapy over several years (Tétrault and Boivin, 2000). Drug resistant mutants have been demonstrated, but their prevalence is still very low. In immunocompetent individuals who followed a suppressive therapy for several years, the prevalence of resistance has been evaluated to be 0.5% compared to 0.3% for the HSV strains isolated before the therapy (Tétrault and Boivin, 2000). The prevalence of resistance of HSV strains isolated from immunocompromised hosts is much higher and has been estimated to be 5% to 10% (Tétrault and Boivin, 2000). In spite of the availability of effective, non-toxic antiviral drugs, herpes simplex infections remain incurable because of the incapacity of the nucleoside analogs to eliminate the latent virus in neuronal cells.

1.3.4 Vaccine development

Several approaches to vaccine development for HSV infections have been investigated, for both prophylactic and therapeutic use: autoinoculation of live HSV, live-attenuated, whole killed, subunit and DNA vaccines (Stanberry, 2004). So far, no vaccine has been demonstrated to be successful in clinical trials but those studies have nevertheless shown that HSV vaccines have a potential utility. Several vaccines are currently planned to enter clinical trials, such as the ImmunoVEX^{HSV-2} vaccine (Bio Vex Ltd), in which genes coding for proteins that block a potent immune response have been deleted. The ImmunoVEX^{HSV-2} vaccine has been shown to be 100% effective in the guinea pig model and clinical trials are planned for 2005/2006 (Thomas et al., 2005). The development of a combined vaccine to prevent both HSV-2 and Human Papillomavirus

(HPV) infections is attractive and currently under investigation (Thomas et al., 2005). Previous HSV vaccine trials have demonstrated that cell mediated immunity might be more important than humoral immunity to protect against HSV infections and recurrences (Stanberry, 2004).

1.4 HSV serological diagnosis

The diagnostic method of choice for genital herpes is to perform a viral culture from the genital lesions followed by viral typing. However, as 75% of the patients are asymptomatic, viral culture is inappropriate for them. Moreover, although those patients do not show symptoms, they still have the potential of transmitting the infection to their sexual partner(s) due to periodic subclinical viral shedding. In fact, the two main factors involved in the transmission of genital herpes are subclinical viral shedding and unrecognized infections (Ashley and Wald, 1999; Barton et al., 1996). One study determined that only 26% of transmitting partners were aware of their infection (Ashley RL and Wald A 1999) and in another one it was shown that in seropositivity discordant couples, the annualized rate of transmission was 10% (Ashley and Wald, 1999). Serological diagnosis is thus required for asymptomatic patients or patients who have an unrecognized genital herpes infection.

Serology for the diagnosis of HSV infections is a challenge for several reasons: the high degree of genetic similarity between HSV-1 and HSV-2, the high prevalence of HSV-1 orolabial infections in the population, the overlapping epidemiology of HSV-1 and HSV-2 and the complexity of the antibody immune response against the numerous herpes simplex antigens.

As mentioned earlier, HSV-1 and HSV-2 are closely related antigenically, and this results in a cross-reactivity of the antibody response against the two virus types. Furthermore, given that around 90% of individuals are HSV-1 positive (HSV1+), antibodies raised against HSV-1 can cross-react with the HSV-2 antigens used in a serological assay, resulting in a false positive diagnosis for an HSV-2 genital infection because of the sequence similarity of most HSV antigens.

Moreover, there is an overlap in the epidemiology of the two viruses, with some oro-labial infections caused by HSV-2 and some genital infections caused by HSV-1 (Ashley and Wald, 1999;Steben and Sacks, 1997). This complicates even more the serological diagnosis of those infections. Conversely, it has been observed that the majority of patients who have an oral HSV-2 infection also have a genital infection (Lafferty, 2002) and that HSV-1 genital infections are usually subclinical (Lafferty, 2002).

It has also been shown that the antibody immune response to HSV is very complex, with most patients having IgG antibodies to at least 15 to 20 viral antigens, the majority being type-common antigens (Lopez C et al., 1993). As can be seen in Table 2, the time of response to different antigens is also variable and they are usually classified in three categories, early, convalescent and late. The time of response to a given antigen is categorized as early if 50% or more of the patients are seropositive for this antigen within two weeks, convalescent if they seroconvert within two months and late if more than 50% of the patients seroconvert after more than two months for a given antigen (Lopez C et al., 1993).

Serological tests for the diagnosis of genital herpes should thus be based on well-defined type-specific antigens, which would allow the distinction between an HSV-1 and an HSV-2 infection, or a dual infection with both types.

Table 2. HSV-2 major immunogens in patients with a primary HSV-2 genital infection. Western blot profiles for IgG against various HSV proteins in patients with a primary HSV-2 genital infection. The time of response is considered early if more than 50% of patients seroconvert within two weeks, convalescent if more than 50% of patients seroconvert within two months and late if more than 50% of patients seroconvert after more than 2 months. ICP35: infected cell polypeptide 35. Available in The Human Herpesviruses, 1993.

1.4.1 The western blot assay

The gold standard for the serological diagnosis of herpes simplex infections is generally considered to be the western blot assay (WBA), such as the one designed by Rhoda Ashley et al. at the University of Washington, Seattle for which there have been extensive validation studies (Ashley et al. 1988; Ashley, 1993;Ashley and Militoni, 1987;Ashley and Wald, 1999). In the WBA, sera are reacted against electrophoretically separated proteins from both HSV-1 and HSV-2 cell extracts. In patients infected with HSV-1, pre-absorption with HSV-1 antigens is required to determine if the subject also has an HSV-2 infection because of the HSV-1 anamnestic response to type-common

antigens. Pre-absorption with the HSV-1 antigens prior to the WB procedure removes the cross-reactive anti-HSV-1 antibodies, thus unmasking the anti-HSV-2 antibodies if present. In the case of a dual infection, the intensity of the bands should decrease dramatically in the HSV-1 blot only (all of the HSV-1 antibodies are bound to the HSV-1 antigens from the pre-absorption step) whereas in the case of an infection with HSV-1 only, there should be no reactivity anymore with both HSV-1 and HSV-2 antigens in the blots (Ashley, 2001). An example of an HSV1+/HSV2+ western blot before and after pre-absorption with HSV-1 antigens is shown in Figures 3A and 3B, respectively.

Unfortunately, the WBA is unsuitable for high-throughput testing diagnoses or seroepidemiological studies because it is time-consuming and can be subjective in some cases. To overcome those disadvantages, several tests have been developed that are based on type-specific antigens.

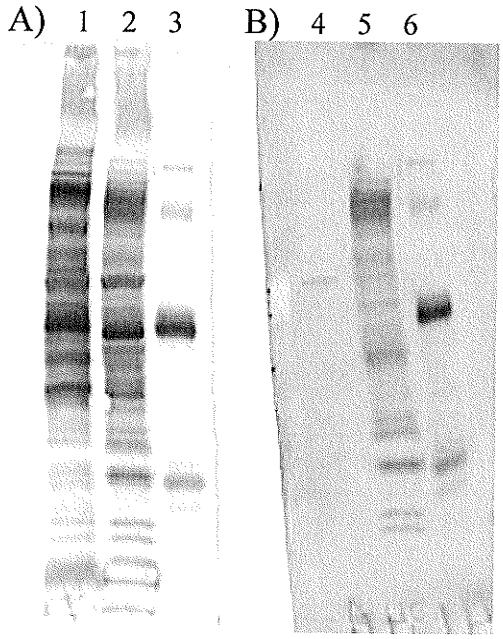


Figure 3. Western blot assay for the serological diagnosis of HSV infections. This figure represents a typical antibody response of a human patient who is infected with both HSV-1 and HSV-2. Lanes 1 and 4 contain HSV-1-infected cell lysate antigens, lanes 2 and 5 contain HSV-2-infected cell lysate antigens and lanes 3 and 6 contain non-infected cell lysate antigens with human IgG as a control. In panel A, it can be noticed that the patient has antibodies that react strongly with both HSV-1 and HSV-2 antigens, but not with the control antigens. However, at this point it is difficult to determine whether the reactivity to both virus types is due to a dual infection or cross-reactivity of the antibodies. Panel B represents a WB for the same serum sample after pre-absorption with HSV-1 antibodies. This time all cross-reactive antibodies have been removed and because the antibodies are still reactive against HSV-2 antigens, it can be concluded that the patient is infected with both HSV-1 and HSV-2.

1.4.2 Type-specific serology

Currently, the only known type-specific antigen is glycoprotein G (gG-1 for HSV-1 and gG-2 for HSV-2). Those two glycoproteins have evolved differently, with gG-1 containing 238 aa and gG-2 having 699 aa (Liljeqvist et al., 1998). In addition, gG-2 is processed differently, with the amino-terminal segment being secreted (sgG-2) and the heavily O-glycosylated carboxy-terminal part being anchored in the viral envelope to

form the mature gG-2 (mgG-2) (Liljeqvist et al., 1998). gG-1 lacks the amino-proximal region of gG-2 that is secreted (Figure 4) (Liljeqvist et al., 1998).

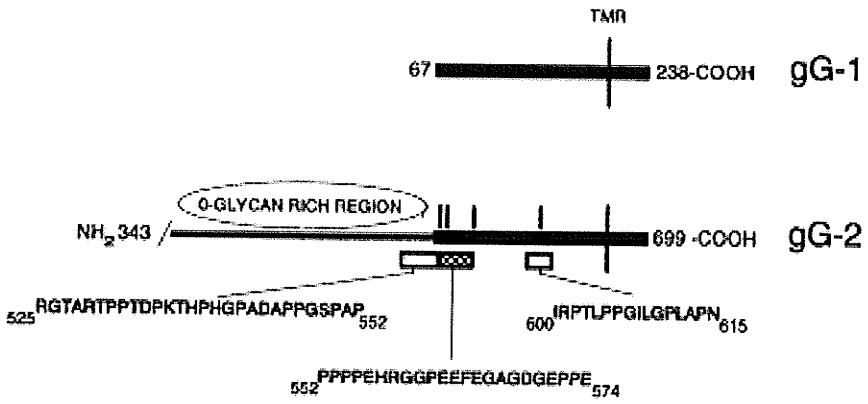


Figure 4. Schematic representation of gG-1 and gG-2. Filled boxes represent the homologous regions between gG-1 and gG-2. Three type-specific immunoreactive regions have been identified on gG-2. The open boxes represent gG-2 epitopes that are immunoreactive with the majority of purified human anti-gG-2 antibody samples. The hatched box represents the immunodominant region of gG-2 that reacts with all HSV-2 sera. This gG-2 immunodominant epitope is located in a region that is homologous to gG-1. TMR: trans-membrane regions. Source: J Virol. 79 :1215-1224, 1998. Reproduced with permission from the publisher.

The first report on the use of gG for HSV type-specific serology was published by Lee et al. in 1985. The authors used affinity-purified gG-2 from an infected cell lysate to perform an immunodot enzyme assay. It was demonstrated that the specificity of the assay was 100% and the sensitivity was 99% for recurrent genital cases and 93% at 11 to 20 days after the onset for primary cases (Lee et al., 1985). Later on, tests based on recombinant gG were developed to overcome the problem of antigen availability when using a purified antigen (Sanchez-Martinez et al., 1991).

HSV type-specific tests that are based on gG are now available commercially. The first gG-based test to be approved by the Food and Drug Administration (FDA) was the ELISA from the Gull Laboratories/Meridian Diagnostics in 1999. However, this test was withdrawn from the market in early 2001. The HerpeSelect-1 and HerpeSelect-2

ELISA kits, from Focus Technologies (formerly MRL Diagnostics), have also been approved by the FDA in February 2000. Those two kits use baculovirus recombinant gG-1 or gG-2 in a 96-well plate format. Focus Technologies also offers this test in the immunoblot strip format. In the HerpeSelect Immunoblot, gG-1, gG-2 and a mixture of type-common antigens are applied in bands on the nitrocellulose strip. The HerpeSelect kits are the only gG-based HSV serological tests currently approved by the FDA in addition to biokit HSV-2 (biokit, Lexington, MA) and Captia EIA (Trinity Biotech).

Prince et al. compared the Focus gG-1 and gG-2-based ELISA kits with the WB gold standard and demonstrated that the sensitivity of the kits was 100% for both types and the specificity was 98.3% and 95.7% for HSV-1 and HSV-2, respectively (Prince et al., 2000). Six of 155 samples were found to give false positive results with the gG-2 ELISA. Since those were in the low-positive range, it was suggested that any sera with low-positive index values should be assayed by WB to confirm that the sample is truly positive or not. The gG-2 HerpeSelect test was also evaluated based on clinical and virological evidence of infection by Ashley-Morrow et al. (Ashley-Morrow et al., 2003). It was found that its sensitivity was 100% for recurrent HSV-2 but only 86% for first HSV-2 episodes while its specificity was 93%. Another study, published in 2004 by Ashley-Morrow et al., evaluated the performance of the gG-1 and gG-2 HerpeSelect ELISA kits among women in various geographical areas worldwide. The concordance between the HSV-1 HerpeSelect ELISA and the WB assay was 97%, with a sensitivity of 99% and specificity of 78%. For the HSV-2 HerpeSelect ELISA, the concordance was 92%, with a sensitivity of 97% and specificity of 89%. Most of the discordant results were positive in the HerpeSelect tests, but negative in the WBA. The authors suggested

that those cases might have occurred because of the fact that HerpeSelect detects seroconversion earlier than the WBA (Ashley-Morrow et al., 2003). Sample mix-up and geographical origin of the specimens were also suggested to explain the discordant results (Ashley-Morrow et al., 2004). It was nevertheless concluded that the HerpeSelect assays are sufficiently accurate for use in seroprevalence studies. Several other studies also evaluated the performance of the HerpeSelect kits (Ribes et al., 2001; Ribes et al., 2002; Turner et al., 2002). However, because they used other type-specific tests rather than the WBA as the reference standard, they will not be discussed here.

The HerpeSelect ELISA assays have also been evaluated in the context of pediatric sera (mean age of 5.7 years with a range of 1 to 13 years). It was determined that these tests are reasonably accurate for use in children (for HSV-1, a sensitivity of 80% and a specificity of 97.4% were determined whereas for HSV-2 they were of 87.5% and 100%, respectively) (Leach et al., 2002). The use of the HerpeSelect assays is however currently recommended for sexually active adults and pregnant women only.

Taken together, those evaluations of the performance of gG-based tests demonstrated that those assays have a good specificity and sensitivity but they also documented discrepant and false positive results.

1.4.3 Limitations to the use of a single antigen for HSV type-specific serology

There are several limitations to the use of a single type-specific viral antigen in the serological diagnosis of herpes simplex infections:

1) Recombinant antigens from bacterial or baculovirus expression vectors may lack immunodominant epitopes.

Some gG-based assays use native affinity-purified gG-1 and gG-2 antigens (e.g. Gull/Meridian ELISA) whereas others produce them as recombinant proteins in non-mammalian expression vector systems, such as the bacterial and baculovirus systems (e.g. HerpeSelect ELISA). Recombinant antigens produced from those vectors can lack epitopes recognized by human antibodies due to the difference in the glycosylation mechanisms of mammalian and non-mammalian cells (Ashley and Wald, 1999; Martins et al., 2001).

2) The type-specificity of an antigen might be altered due to variability in its gene sequence.

Several studies have investigated the stability of the gG gene among various clinical isolates and the presence of anti-mgG-2 antibodies in patients infected with different virus strains. For example, Rekabdar et al. demonstrated by DNA sequencing that 2 of 11 HSV-1 clinical isolates had a point mutation in an immunodominant epitope and that the purified anti-gG-1 antibodies from the patients carrying this mutation were less reactive in a gG-1 based ELISA assay compared to the control patients (Rekabdar et al., 1999). On the other hand, the same group demonstrated three years later that although two major gG-1 genetic variants were identified based on DNA sequencing and phylogenetic comparison of 108 clinical isolates, no difference in the antibody reactivity was observed when sera from patients carrying either genetic variant were compared using a gG-1 based ELISA assay (Rekabdar et al., 2002). Regarding gG-2, Liljeqvist and colleagues reported 5 clinical isolates with no or incomplete expression of the protein (Liljeqvist et al., 1999). Moreover, 3 of the 5 patients carrying those strains showed no antibodies against mgG-2 and it was suggested that the two other patients must have been

infected with multiple strains. The lack of gG-2 expression was found to be caused by single frameshift mutations resulting in a premature termination codon as shown by sequencing. The authors tested more clinical isolates in a later study and demonstrated that those carrying a mutated gG gene resulting in no expression of the protein occur only rarely. Moreover, it was shown that most mutations that do not prevent gG expression occur outside of the known dominant epitope regions, supporting the use of gG in HSV type-specific serology (Liljeqvist et al., 2000).

A recent study has examined this time the molecular basis for type-specificity in gG-1 and gG-2 using synthetic peptides covering the dominant epitope regions, including peptides carrying residue substitutions (Tunback et al., 2005). Those peptides were subsequently tested for reactivity with human sera. This experiment demonstrated that two residues only are required for binding to gG-1 whereas all mgG-2 type-specific amino acids (aa) were required. This implies that one or a few point mutations only are sufficient to give a false-positive or false-negative result when gG alone is used for HSV serological diagnosis.

Altogether, those results suggest that both gG-1 and gG-2 are genetically stable, but that a small proportion of HSV clinical isolates carry a mutated gG gene. Rarely, patients infected with those virus strains will lack antibodies against gG either because gG is not expressed or because it carries mutations in the immunodominant region(s). As a result, those patients are diagnosed as HSV negative when tested with a gG-based assay solely.

3) Titer and timing of seroconversion to a single antigen is variable in different individuals.

As mentioned earlier, although a trend can be observed for the timing of seroconversion to different HSV antigens, not all individuals will seroconvert to a given antigen at the same time (Ashley and Wald, 1999; Lopez et al., 1993). For example, the time of response to gG-2 in patients with a primary HSV-2 genital infection is characterized as convalescent, with 69% of patients seroconverting within one to two months (Table 2) (Lopez et al., 1993). gB and gI are the only antigens to which most patients were found to seroconvert early after infection, with more than 50% of the individuals developing IgG within two weeks (Table 2) (Lopez et al., 1993).

4) Reversion from a seropositive to seronegative status over time.

Using a baculovirus-expressed gG-based immunoblot (BIB) assay, Schmid et al. reported that 6.6% of the HSV-1 positive and 14.9% of the HSV-2 positive subjects from a cohort of Thai military recruits reverted from a seropositive to a seronegative status (Schmid et al., 1999). Seroreversion was explained by errors in specimen handling for some cases only. A portion of the samples was also retested with 3 other gG-based assays to confirm the results obtained with the BIB. It was concluded that until the cause of those seroreversion cases is understood, gG-based tests should be employed with caution.

Some tests are also available that are based on crude infected-cell lysates or whole virus. Although those tests can detect an HSV infection, they cannot distinguish between an HSV-1 and an HSV-2 infection or a dual infection with both types. Those tests are sometimes said to be type-specific by measuring the relative intensity of the antibody response but they have been shown to be much less accurate than the gG-based assays and should not be used for the diagnosis of genital herpes (Martins et al., 2001).

1.5 Two-dimensional western blotting

Two-dimensional (2-D) gel electrophoresis is a powerful technique that is employed to study complex protein mixtures, including cell extracts. This technique separates the proteins based on two properties: their charge and their molecular weight (MW). The power of resolution of 2-D gel electrophoresis is thus at least one order of magnitude higher than that of an ordinary one-dimensional (1-D) gel electrophoresis, allowing hundreds of proteins to be visualized on a single gel. The first dimension, isoelectrofocusing (IEF), is based on the notion that all proteins are amphoretic, meaning that they have either a positive, negative or neutral charge, depending on the pH in the environment. During IEF, a protein mixture is placed on a pH gradient gel and when a current is applied, the proteins move in the gel until their charge becomes zero, which is called the isoelectric point (pI). For example, if a protein has a net positive charge, it will move toward the cathode, until it reaches its pI. The second orthogonal dimension, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separates the proteins further, this time based on their MW. The proteins are transferred from the pH gradient gel (which is in the format of a strip that can be applied on the top of the SDS-PAGE gel) to the SDS-PAGE gel and when a current is applied, the proteins migrate in the SDS-PAGE gel according to their MW.

Several HSV polypeptides have been described to be immunogenic, including both structural and non-structural proteins (Ashley and Wald, 1999; Eberle and Mou, 1983; Kühn et al., 1987; Lopez et al., 1993). As can be seen in Figure 3, when performing WB from a 1D gel with an HSV-infected cell lysate, not all of the HSV proteins and of their post-translationally modified forms can be resolved. We therefore

decided to perform 2D gel electrophoresis prior to the standard WB procedure to resolve all the immunogenic HSV proteins. We hypothesized that by comparing human serum samples from patients infected with either HSV-1 or both HSV-1 and HSV-2 (dual infection), we could identify all the immunoreactive HSV antigens, and of most interest, to distinguish between the type-specific and type-common antigens.

1.6 Antibody epitope mapping

Immunoreactive epitopes on a protein can be mapped by several methods, including chemical or proteolytic cleavage, use of systems for the expression of recombinant proteins, phage display libraries or overlapping synthetic peptides (pepscanning) (Harlow and Lane, 1999). A literature search allowed us to identify several studies that employed some of those techniques to identify type-common and type-specific epitopes on several herpes simplex glycoproteins (Ackermann et al., 1998; Eing et al., 2002; Grabowka et al., 1999; Levi et al., 1996; Liljeqvist et al., 1998; Liljeqvist et al., 2002; Marsden et al., 1998; Tunbäck et al., 2000; Tunback et al., 2005). For example, Grabowska et al. used both phage display library mapping and pepscanning, including soluble and membrane-bound peptides, to identify the epitopes on gG-2 that are recognized by monoclonal and human polyclonal antibodies (Grabowka et al., 1999). Both approaches allowed the mapping of the same immunodominant epitopes. Nevertheless, the authors concluded that membrane-bound peptides had an increased sensitivity compared to soluble peptides, probably because of the use of a chemiluminescence detection system or because of the difference in the antigen presentation of the two techniques. Following a review of those studies, we decided to

use the pepscan technique because it appeared to be efficient and also because it was relatively simple, inexpensive and less time-consuming than the other techniques available. Moreover, we did not have a library of gene fragments prepared in an expression vector, and as our primary goal was epitope mapping, the phage display library was not suitable for us as it requires extensive cloning and sequencing. The major drawback of the pepscan method is that it is biased towards the detection of linear epitopes, but given that human anti-HSV antibodies are known to react with denatured antigens in the immunoblot assay, we did not consider this a limitation.

In the pepscan technique, a set of synthetic overlapping peptides issued from the linear protein sequence of a given protein is created and arrayed on a membrane support (Frank, 1992). This array of peptides can then be assayed for reactivity with the test antibody, in our case a serum sample, and the bound antibodies are detected using an enzyme-labeled secondary antibody. The membranes can be stripped of antibody and detection reagent for reuse (Frank, 1992).

The presence of type-specific and type-common regions on HSV antigens cannot be entirely predicted from the degree of homology in their genetic sequences and consequently Bergström and Trybala proposed that mapping of the human B-cell epitopes might be the solution to the problem (Bergström and Trybala, 1996). This is supported by the fact that the immunodominant epitope recognized in a type-specific fashion for gG-2 resides within the homologous regions of the protein and not in the divergent regions (gG-1 and gG-2 are 50% similar) (Levi et al., 1996; Liljeqvist et al., 1998). In our case, antibody epitope mapping may allow the identification of the type-

specific and/or type-common region(s) on a single protein by comparing the reactivity of serum samples from patients with either an HSV-1 or a dual infection with both types.

1.7 Objectives and hypothesis

In order to increase the sensitivity and specificity of the currently available type-specific serological assays for the diagnosis of herpes simplex infections, the discovery of new type-specific antigens is required. Because HSVs produce nearly a hundred proteins, one-dimensional WB does not permit the separation of all the viral proteins. On the other hand, 2-D gel electrophoresis allows the resolution of hundreds of proteins on a single gel. We hypothesized that this technique could be used in conjunction with Western blotting to identify the type-specific and type-common viral proteins recognized by humans during a genital herpes infection by comparing patients who are infected with HSV-1 with patients who have a dual infection with both HSV types. However, because the majority of the viral proteins contain type-common epitopes, the antibody epitope mapping technique was also employed to identify possible type-specific epitopes. We hypothesized that epitope mapping could be used to identify type-specific epitopes within a viral protein that would appear as a type-common protein in a 2-D western blot analysis.

2. Materials and Methods

Figure 5 presents an overview of the 2-D western blotting experimental approach.

2.1 Two-dimensional western blotting

2.1.1 Cell culture, viruses and virus infection

Vero African green monkey kidney cells (American Type Culture Collection, ATCC CCL-81) were grown in filter sterilized Eagle's minimum essential medium (MEM) (pH7.2) (Invitrogen, Burlington, ON) with 5% heat-inactivated calf serum (CS) (Invitrogen), 1% 200mM L-glutamine (Invitrogen) and 2% 7.5% (w/v) sodium bicarbonate solution (Invitrogen). The cells were grown in 175-cm^2 flasks and kept in a 37°C humidified 5% CO_2 incubator.

For the last passage before infection and during infection, the CS concentration was decreased to 2% and antibiotics (10,000 units/ml penicillin G and 10,000 µg/ml streptomycin, Invitrogen) were added at a concentration of 1%. Confluent Vero cells in T-150 flasks were infected at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell with HSV-2 strain G (ATCC VR-734) using supplemented MEM as diluent. After an incubation of 1 hr at 37°C, 5% CO_2 to allow adsorption of the virions on the cells, 35ml of supplemented MEM was added, and the cultures were incubated in a 37°C humidified 5% CO_2 incubator until 95% cytopathic effect (CPE) was noted (typically 18 hours post-infection for HSV-2).

2.1. 2 Virus stock preparation

HSV-2-infected cells were collected at a 95% CPE and centrifuged at 4°C for 5 min at 500 xg. The supernatant was discarded and the cells were resuspended in 1 ml

phosphate buffered saline (PBS) per T-150 flask. The virus was released from cells by freeze-thawing 3 times in acetone/dry ice and 37°C water bath. The tube was then centrifuged at 4°C for 10 min at 2000 xg, the supernatant was collected and aliquots were stored at -80°C until use.

2.1.3 Plaque assay

Vero cells were grown as for cell culture (see above) in 8 Petri dishes. Confluent Vero cells were infected with serially diluted HSV-2. The serial dilutions were prepared by adding 0.2 ml of the virus stock into 1.8 ml MEM prepared for infection (see above) and then transferring 0.2 ml into the next tube containing 1.8 ml MEM, up to a 10^{-7} dilution. Vortexing was performed before each transfer. 0.2 ml of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions was added to the Petri dishes in duplicate and the plates were rocked in a circular motion to cover them entirely. During adsorption (one hour at 37°C, 5% CO_2), the agar overlay was prepared by mixing autoclaved 2X MEM (4.5g MEM in 250ml ddH_2O) with autoclaved 1.5% agar in a 1:1 ratio. The 2X MEM was supplemented with 8% CS, 4% L-glutamine, 8% of 7.5% sodium bicarbonate solution and 4% antibiotics (10,000 units/ml penicillin G and 10,000 µg/ml streptomycin, Invitrogen) just before use. After adsorption, the agar overlay was poured on the Petri dishes and allowed to solidify for 5 min, after which the cultures were incubated in a 37°C humidified 5% CO_2 incubator for 42 hours. After 42 hours, the agar overlay was removed and the cells were fixed with formalin and stained with crystal violet. The following formula was used to determine the PFU count for the dilution for which plates contained between 20 and 200 PFUs:

$$PFU/mL = \frac{\text{mean PFU count}}{0.2ml} \text{ X dilution factor}$$

2.1.4 Antigen preparation

HSV-2-infected cells from T-150 flasks were collected at a 95% CPE and centrifuged at 4°C for 5 min at 500 xg. The pellet was resuspended in chilled PBS and centrifuged at 4°C for 10 min at 500 xg. The pellet was resuspended in rehydration stock solution (RSS) (250µl per flask) containing 8M urea and 2% (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate) (CHAPS) (usb Corporation, Cleveland, OH) in ddH_2O supplemented with proteinase inhibitor cocktails (1:50) (Sigma-Aldrich, Saint Louis, MO). To help protein solubilization, sonication (Vibra Cell, Sonics and Materials, Danbury, CT) was performed 5 times for 20 s at a 60% output power with a 1 min cooling period on ice in-between each sonication. Aliquots were frozen at -80°C until use.

For non-infected Vero cells antigen preparation, the cells were washed once with chilled PBS, and they were scraped in 5ml chilled PBS per flask. The cells were then centrifuged at 4°C for 5 min at 500 xg. The pellet was resuspended in RSS as above supplemented with proteinase inhibitor cocktails (1:100). Sonication was also performed to help resuspension as above. Aliquots were frozen at -80°C until use.

Protein precipitation was performed using the 2-D Clean-Up Kit (Amersham Biosciences, Buckinghamshire, UK) to selectively separate proteins in the sample from interfering substances. 0.5% IPG buffer (Amersham Biosciences) with a pH range matched to the pH range of the IPG strip to be used for IEF was added to the sample. The sample was incubated for 30 min at room temperature (RT) and it was then centrifuged at

4°C for 5 min at 13,000 xg. The supernatant was then transferred to a new tube. The procedure B for the 2-D Clean-Up Kit was then followed with centrifugation at 13,000 xg. The resuspension solution contained 0.5% IPG buffer in RSS. The volume of resuspension solution used was the same as the volume of the original sample. Sonication was performed at 20% output power as above.

Following protein precipitation, protein concentration was determined using the PlusOne 2-D Quant Kit (Amersham Biosciences) according to the manufacturer's directions, using 2.5µl or 5µl of the sample in duplicate and centrifugation at 12,000 xg.

2.1.5 Patients and sera

Two groups of human serum samples were used in this study: archival sera from patients with an HSV-1 infection (HSV1+) and from patients with both HSV-1 and HSV-2 infections (HSV1+/HSV2+), as determined by traditional western blotting. Serum samples from patients infected with HSV-2 only were not used in this study as this was a preliminary study and that those samples are rare.

2.1.6 Two-dimensional gel electrophoresis

2.1.6.1 First dimension: isoelectrofocalisation

For the first dimension, IEF, 18 cm Immobiline DryStrip gels with a pH range of 3 to 10 non-linear (NL), 3 to 7 or 6 to 11 were used (Amersham Biosciences). The IPG strips were loaded with a known quantity of proteins in rehydration solution containing 1.2% DeStreak Reagent (hydroxyethyl disulfide) (Amersham Biosciences), 0.5% or 1% IPG buffer (see below), and 0.002% bromophenol blue. The conditions were different

depending on the use of the gels: for 2-D WB, 500µg of proteins were loaded on the strip with an IPG buffer concentration of 0.5% whereas 750 µg were loaded with a 1% IPG buffer concentration for silver staining.

IEF was performed according to the manufacturer's directions using the Ettan IPGphor II Isoelectric Focusing System (Amersham Biosciences). The running conditions for the non-linear pH 3-10 Immobiline DryStrip gels when 2-D WB was to be performed were the following: step and hold at 0.5 kVh, step and hold at 1 kVh and lastly step and hold at 30.5 kVh for a total of 32 kVh at 20°C after 10 to 15 hours of rehydration. For gels intended for silver staining, the protocol was slightly different: step and hold at 0.5 kVh, gradient at 2 kVh, gradient at 13.5 kVh and finally step and hold at 17.5 kVh for a total of 33.5 kVh at 20°C after 10 to 15 hours of rehydration. When the IPG strips were not to be used directly for the second dimension, they were frozen at -80°C.

2.1.6.2 Second dimension: SDS-PAGE

The second dimension, SDS-PAGE, was performed using the PROTEAN II xi Cell System (Bio-Rad Laboratories). Gels were composed of 10% acrylamide cross-linked with N,N'-methylenebisacrylamide, 0.375M 1.5M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS) and 0.05% TEMED (N,N,N',N'-Tetra-methyl-ethylenediamine, Bio-Rad Laboratories). When MW markers were used, 20µl of MagicMark™ XP Western Protein Standard (Invitrogen) for WB or BenchMark Pre-stained Protein Ladder (Invitrogen) for silver staining was added in a well on the side of the gel.

Prior to electrophoresis, equilibration of the IPG strips was required. The equilibration solution contained 6M urea, 2% SDS, 50mM 1.5M Tris-HCl pH 8.8, 30%w/v87%v/v glycerol and 0.002% bromophenol blue and aliquots were stored at –20°C until use. The IPG strips were placed in individual tubes with 10ml of the equilibration solution to which 0.1g dithiothreitol (DTT) (Amersham Biosciences) was added just prior to use. The IPG strips were equilibrated twice for 15 min on a rocking platform. According to the manufacturer's instructions (Amersham Biosciences), the IPG strip was positioned on its edge on the top of the gel and the IPG strip was overlaid with freshly prepared 0.5% agarose. Electrophoresis was performed at a constant current of 24 mA per gel (VWR 3000P power supply, VWR International, West Chester, PA) for approximately 5 hrs 30 min.

2.1.7 Western blotting

For WB, electrophoretic transfer of proteins from the gels to polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore, Billerica, MA) or nitrocellulose (NC) membranes (Bio-Rad Laboratories) was carried out. The gel and the membrane were sandwiched together with the paper facing the anode. Proteins were transferred electrophoretically at 0.125A per cassette overnight in 25mM Tris-HCl/192mM glycine/20% methanol. Blots were always used immediately. Following transfer, the membranes were blocked with 0.1% Tween20 in PBS (PBS/T) for a minimum of 5 hrs at RT. The membranes were then incubated with 10 ml of 1:50 dilution for chromogenic detection and 1:500 dilution for chemiluminescence of human serum in PBS/T overnight. Antibody incubations were performed in sealed plastic bags to minimize the volume of

serum used. After the membranes were washed twice for 10 min in PBS/T, the membranes were incubated with 10 ml of 1:500 dilution for chromogenic detection and 1/2500 dilution for chemiluminescence of peroxidase-conjugated anti-human IgG (Fc specific) (Sigma-Aldrich, Saint Louis, MO) for 45 min at RT. Two 15 min washes in PBS/T were performed, and bound antibodies were visualized using either a chromogenic substrate, or chemiluminescence. For the chromogenic detection, the color reagent solution contained 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) solution (1.25% DAB in 0.05M Tris-HCl, pH 7.6) and 0.024% nickel chloride in PBS. Just prior to use 0.003% hydrogen peroxide was added to the color reagent solution. The developing reaction was stopped by adding ddH_2O to the color reagent solution. Chemiluminescence was performed according to the manufacturer's directions (ECL™ Western Blotting Detection Reagents Kit, Amersham Biosciences), with film exposures of 10 sec and 30 sec (Hyperfilm, Amersham Biosciences). When MW markers were used, the procedure was the same as for the sera (see above).

For monoclonal antibodies (MAb) experiments, different dilutions of the primary antibody were used and the dilution for each MAb is mentioned in the results section. The following MAbs have been used: MAb 1107 and 1106 (mouse anti-gG-1 and gG-2, respectively) (Rumbaugh-Goodwin Institute, Plantation, FL) and AP-1 (mouse anti-gG-2) (supplied by Tony Minson, University of Cambridge, UK). As for the secondary antibody, a peroxidase-conjugated goat anti-mouse antibody was used at a 1:10,000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA).

For the analysis of the 2-D western blots probed with human sera, the ImageMaster 2D Platinum, version 5.00 (Amersham Biosciences) was employed. The

western blot images or films were scanned with ImageScanner II via LabScan 5 (Amersham Biosciences).

2.1.8 Identification of the viral proteins

2.1.8.1 Gel staining

Silver staining was performed according to the manufacturer's directions (PlusOne Silver Staining, Amersham Biosciences) with the following modifications: prior to fixation, the gels were washed twice with ddH_2O for 30 min at RT and fixation was performed overnight. When spots were to be excised from the gel, glutardialdehyde and formaldehyde were not added to the sensitization and silver solutions, respectively.

Colloidal Coomassie staining was also performed according to the manufacturer's directions (GelCode Blue Stain Reagent, Pierce, Rockford, IL).

2.1.8.2 Mass spectrometry

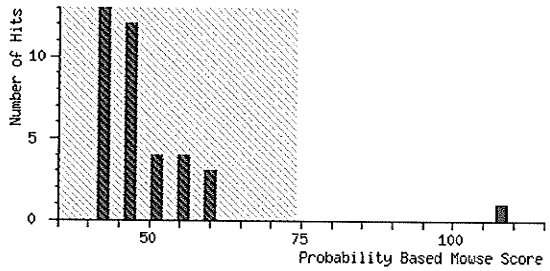
Stained spots were excised from a 2-D gel using a cut pipette tip in a biological safety cabinet to minimize keratin contamination of the samples. Siliconized tubes that were rinsed twice with 100% methanol and air dried in a chemical hood to remove residual unbound silicone from the tubes were used to prevent binding of the proteins to the surface of the tube. The spots were transferred to the siliconized tubes containing 100 µl sterile ddH_2O and the samples were either stored at -20°C or sent directly to the Institute for Biomolecular Design at the University of Alberta, Edmonton, Canada. Sample preparation for mass spectrometry (MS) was performed at the University of Alberta. Briefly, the protein samples were de-glycosylated and an automated in-gel tryptic digestion was performed on a Mass Prep Station (Micromass, UK). The gel pieces

were de-stained, reduced (DTT), alkylated (iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides extracted from the gel and analyzed via LC/MS/MS. LC/MS/MS was performed on a CapLC HPLC (Waters, USA) coupled with a Q-ToF-2 mass spectrometer (Micromass, UK). Tryptic peptides were separated using a linear water/acetonitrile gradient (0.2% formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip) (New Objectives, MA, USA), with an in-line PepMap column (C18, 300 micron ID x 5 mm), (LC Packings, CA, USA) used as a loading/desalting column. Protein identification from the generated MS/MS data was done searching the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK). The search was against all species and the search parameters included carbamidomethylation of cysteine, possible oxidation of methionine (partial) and one missed cleavage per peptide.

The MS/MS instrument first measured the MW of the peptides issued from the enzymatic digest of the protein sample to obtain a peptide mass fingerprinting spectrum. Peptides that had the highest intensity peaks were then selected for sequencing and a fragment ion spectrum was acquired for those peptides (MS/MS). The experimental peptide mass values were compared with the calculated ones from a specified database. By using an appropriate scoring algorithm, the closest match or matches could be identified, which often include proteins that exhibit the closest similarity to the protein of interest, such as equivalent proteins from related species. The Mascot public server (http://www.matrixscience.com/) was used to analyze the MS results. As described by Matrix Science, «Mascot incorporates a probability based scoring called the Mowse

scoring algorithm. The total score is the absolute probability that the observed match is a random event and is expressed as $-10*LOG_{10}(P)$, where P is the absolute probability. For example, a score of 200 indicates that there is a probability of 10^{-20} that the match is a random event. Mascot also considers that an event is significant if it is expected to occur randomly with a frequency of less than 5% and it thus reports that all scores greater than an X score value (this value differs for each experiment) will be significant ($p<0.05$)».

Here is a typical histogram of the score distribution:



In this case, individual ion scores > 74 indicate identity or extensive similarity ($p<0.05$). Proteins that share the score of 108 have the same set of peptide matches and probably share the same sequence, such as homologous proteins from different species.

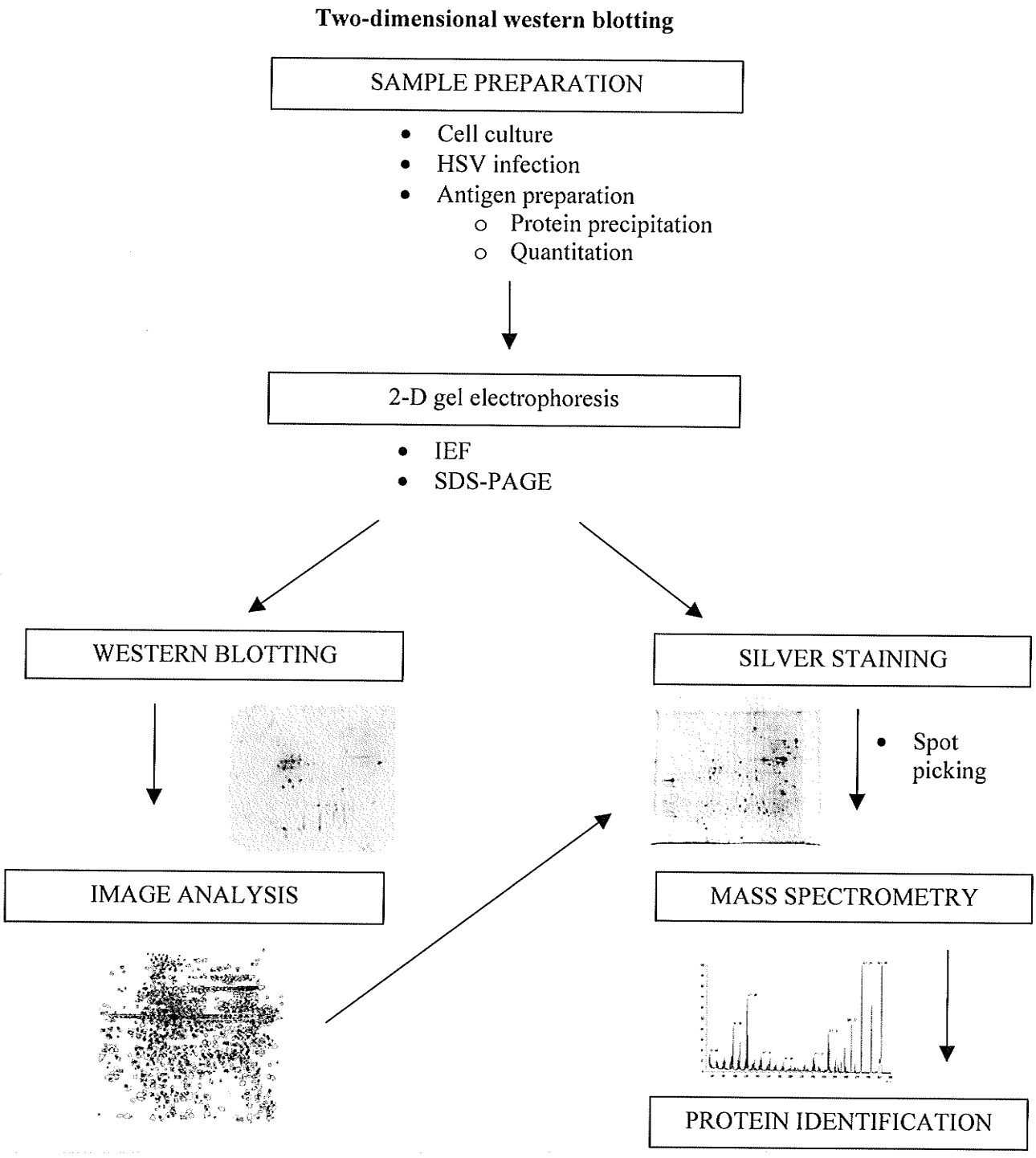


Figure 5. Experimental plan for the two-dimensional western blot approach. Sample preparation for 2-D gel electrophoresis includes cell culture and infection, protein extraction from the cell lysate, protein precipitation to remove interfering substances. 2-D electrophoresis is followed either by western blotting and analysis or silver staining to excise the proteins that were found to be immunogenic in the western blots. Those proteins are subsequently identified by mass spectrometry analysis.

2.2 Antibody epitope mapping

2.2.1 Pepscanning

Three HSV-2 proteins were chosen to be tested with the pepscan epitope mapping technique: gB, gC and ICP35. Serum samples were screened for binding using a panel of overlapping peptides (15mers with 12 aa overlaps) (JPT Peptide Technologies, Berlin, Germany). This produced 298 peptides for gB which is 914 aa long, 156 peptides for gC which is 480 aa long and 106 peptides for ICP35 which is 329 aa long. The peptide sequences were from HSV-2 strain G for gB and from strain HG52 for gC and ICP35. The cysteine residues were replaced by serines to avoid oxidation during the incubation steps. N-acetylation was performed as well because peptides are then more stable to degradation and also because the uncharged N-acetyl group better represents the region in the native antigen than a charged amino group. New membranes were stored at -20°C until use. They were then rinsed with a small volume of methanol for 5 min to avoid the precipitation of hydrophobic peptides during the following Tris buffered saline (TBS) washing procedure. The membranes were subsequently washed three times with an appropriate volume of TBS (50 mM TRIS, 137mM NaCl, 2.7 mM KCl, pH 8.0) for 10 min. Blocking was performed in 1% blocking buffer (50mL of Blocking Stock Solution, BM Chemiluminescence Blotting Substrate (POD), Roche Diagnostics, Penzberg, Germany in 450 mL TBS) for at least 2 hours at RT or overnight at 4°C. The membranes were incubated with 100mL of 1:5,000 dilution of human serum in 0.5% Blocking Buffer for 3 hours at RT. The membrane was washed once with T-TBS (0.05% Tween-20 in TBS) for 10min to reduce the background. The membrane was then incubated with 100mL of 1:10,000 of peroxidase-conjugated anti-human IgG (Fc specific) (Sigma-

Aldrich) in 0.5% Blocking Buffer for 2 hours at RT. The membrane was washed three times with TBS for 5 min. The bound antibodies were visualized by chemiluminescence according to the manufacturer's directions (BM Chemiluminescence Blotting Substrate (POD), Roche Diagnostics), with film exposure of 10 sec. Incubated membranes that were to be used again after only a few days were washed three times with T-TBS for 10min and kept in a small volume of T-TBS in a closed container at 4°C. Incubated membranes that were stored for a longer period of time were regenerated, washed with methanol twice, air dried and kept at -20°C in a sealed bag.

Before testing a new serum sample on a membrane, regeneration of the membrane was performed using the regeneration protocol I suggested by the manufacturer (JPT Peptide Technologies). The membrane was washed three times with ddH_2O for 10 min followed by four 30 min washes with Regeneration Buffer I (62.5mM TRIS, 2% SDS, 100mM 2-mercaptoethanol, pH6.7) at 50°C without shaking. The membrane was then washed three times with PBS (9.2mM $Na_2HPO_4x12H_2O$, 1.6mM $NaH_2PO_4xH_2O$, 150mM NaCl, pH7.2) for 20 min, three times with T-TBS for 20 min and three times with TBS for 10 min at RT. Success of the regeneration protocol was verified by rinsing the membrane in the substrate solution and then exposing the membrane to a film for 1min. If spots were still detected, the regeneration was repeated. If spots were not detected, then no secondary antibody remained on the membrane. The membrane was then re-incubated with the secondary antibody and the substrate solution as above and exposed for 1min to ensure that the primary antibody had been completely removed. If spots were still detected, the regeneration protocol was repeated.

2.2.2 Data analysis

Following scanning of the images using ImageScanner II via LabScan 5 (Amersham Biosciences), the raw intensity of each spot was measured with Quantity One, version 4.2.3 (Bio-Rad Laboratories). The relative intensity for each spot was determined by dividing the raw intensity of a given spot by the sum of all the raw spot intensities on that particular membrane.

$$\text{Relative intensity} = \frac{\text{raw intensity of a given spot}}{\Sigma \text{ raw intensities of all spots on that membrane}}$$

Figure 6. The pepscan technique. This technology uses synthetic overlapping peptides coupled to a cellulose membrane. Reactivity of antibodies to the peptides can be measured by incubating the antibodies with the membrane followed by incubation with a secondary antibody conjugated to a peroxidase for example. Detection can be done by chemiluminescence and regeneration of the membrane is possible to test further serum samples. Available at: http://www.jpt.com/content/pep/peptide_arrays/pepspots.htm

3. Results

In order to identify new HSV-2 type-specific antigens that could be used in combination to gG-2 for the serological diagnosis of genital herpes, we used two different approaches. The first one, 2-D western blotting, was used to distinguish between type-specific and type-common HSV-2 proteins. The second approach, antibody epitope mapping, was employed to map type-specific B cell epitopes on proteins that were known to be type-common as a whole.

3.1 Discovery of HSV-2 type-specific proteins by two-dimensional western blotting

In order to identify new HSV-2 type-specific antigens, we implemented a 2-D Western blotting technique, which would allow the electrophoretic separation of all the HSV-2 proteins on a single gel. Serum samples from patients infected with either HSV-1 or both HSV types were then tested by WB to determine which antigens were type-common (i.e. both groups of patients react to those antigens) and type-specific (i.e. only the double-positive cohort reacts to those viral proteins).

Nine serum samples from patients infected with HSV-1 were compared with 7 samples from patients infected concurrently with HSV-1 and HSV-2. 2-D WBs from one HSV1+ and one HSV1+/HSV2+ serum samples can be seen in Figures 8A and 8B, respectively. The 16 blots were analyzed using the ImageMaster 2D Platinum software, version 5.00 (Amersham Biosciences). Two reference blots were thus created, one for each patient group. Each reference blot is a synthetic blot representing any spot present in one or more of the blots in a given group (Figure 7). Moreover, each 2-D blot in a group was linked to its reference blot and statistics could thus be obtained as to how many

patients in a group reacted to a given HSV-2 protein. The two reference blots were also paired together to identify which antigens were immunoreactive with one group of patient and not the other.

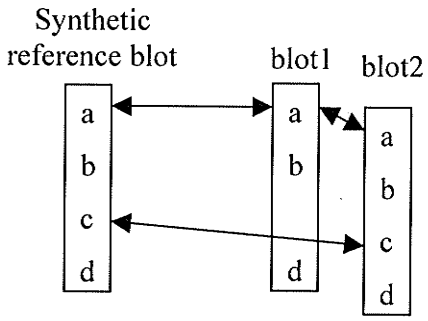
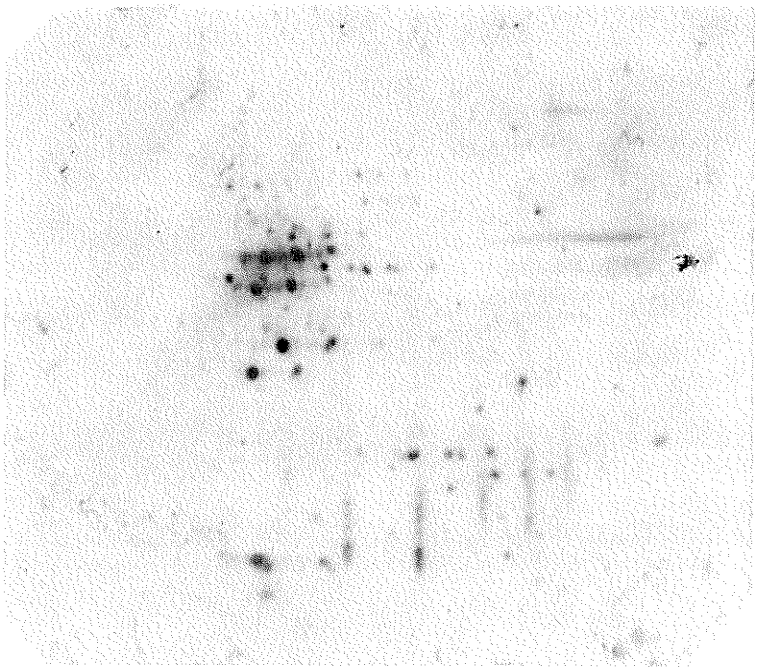


Figure 7. A synthetic reference gel represents a superimposition of all the blots in a group.

A)



B)

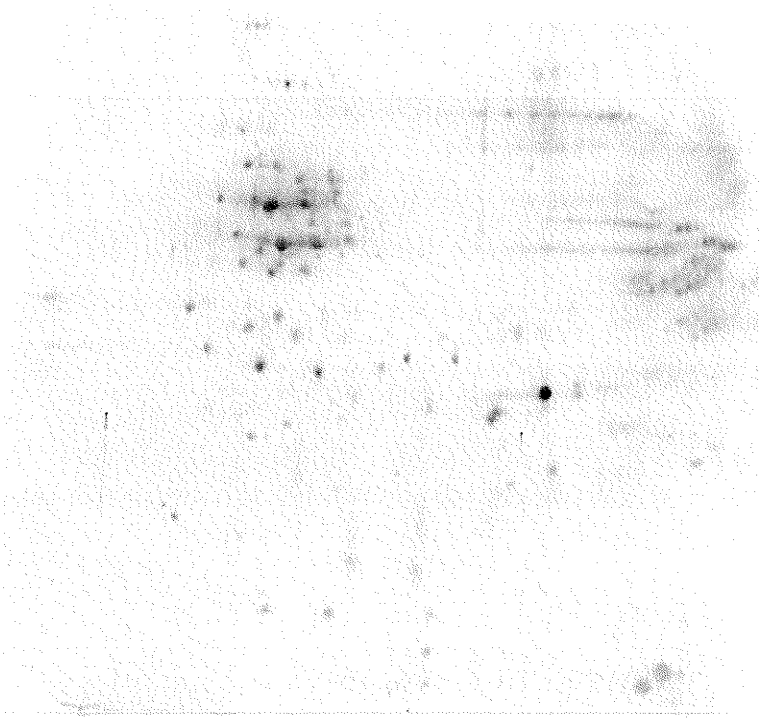


Figure 8. Visualization of immunogenic HSV-2 antigens by two-Dimensional western blotting. Five hundred µg of proteins from extracts of HSV-2-infected Vero cells were loaded on 18cm IEF strips with a pH range 3-10NL. The primary antibody for WB was a human serum sample (1:500) and the secondary antibody was an anti-human IgG conjugated to a peroxidase (1:2500). Detection of the antigens was performed by chemiluminescence. A) 2-D WB for a patient infected with HSV-1 only (serum no. 8683). B) 2-D WB for a patient infected with both HSV-1 and HSV-2 (serum no. 12017).

Every patient was found to have antibodies that reacted to over 150 proteins present on the 2-D gels. Knowing that HSV-2 produces over 84 proteins and that many of them undergo post-translational modifications, this high number of immunoreactive proteins was not unanticipated. For example, some proteins are phosphoproteins, which results in a change in the pI of the protein and can be seen as a horizontal chain of spots that have the same MW but different pI (Figure 8B, upper right quadrant). Others are glycosylated, resulting in a change in the MW of the proteins, which is seen on a 2-D gel as a vertical chain of spots with the same pI but different MW (Figure 8A, lower half). Given that the antigen source for the 2-D WBs was an infected cell lysate, it is expected to observe those different post-translationnaly modified proteins.

For the HSV1+ group, the reference blot contained 336 spots that were immunoreactive with one or more of the nine serum samples in this group (Figure 9A, in red). Of those, 217 were paired to spots on the HSV1+/HSV2+ reference blot (Figure 10, HSV1+ spots in red, HSV1+/HSV2+ spots in purple). From the 119 non-paired spots, 46 were present on one or two blots only and 76 were present on more than 2/9 blots. This high number of non-paired spots on the HSV1+ blots was surprising as the other group is also infected with HSV-1 and should thus react to the same antigens as the HSV1+ group. Several reasons might explain this discrepancy: 1) only a small number of patients in each group was screened and increasing the sampling number might show that the HSV1+/HSV2+ patients also have antibodies against those antigens 2) the history of the patients in unknown (primary, initial, recurrent or asymptomatic infections) 3) several of those antigens were found in more crowded regions where it is thus difficult to match the spots from the two reference blots.

As for the HSV1+/HSV2+ group, the reference blot included 297 spots, which reacted with at least one of the seven serum samples in this group (Figure 9B, in purple). Of those, 217 spots were paired to the HSV1+ reference blot (Figure 10, HSV1+ spots in red, HSV1+/HSV2+ spots in purple). Out of the remaining 89 antigens that were not paired, 51 were present on 1/7 blots only and 38 were found on two or more blots. Of those 38 HSV-2 proteins, 15 were suggested to be potential HSV-2 type-specific antigens as the spot intensity was sufficiently high and there was absolutely no spot on the HSV1+ reference blot that could have been paired to these antigens (Figure 10,in green and Table 3). In particular, spot ID 95 was immunogenic for 5/7 serum samples and spots ID 99 and 205 reacted with 6/7 samples.

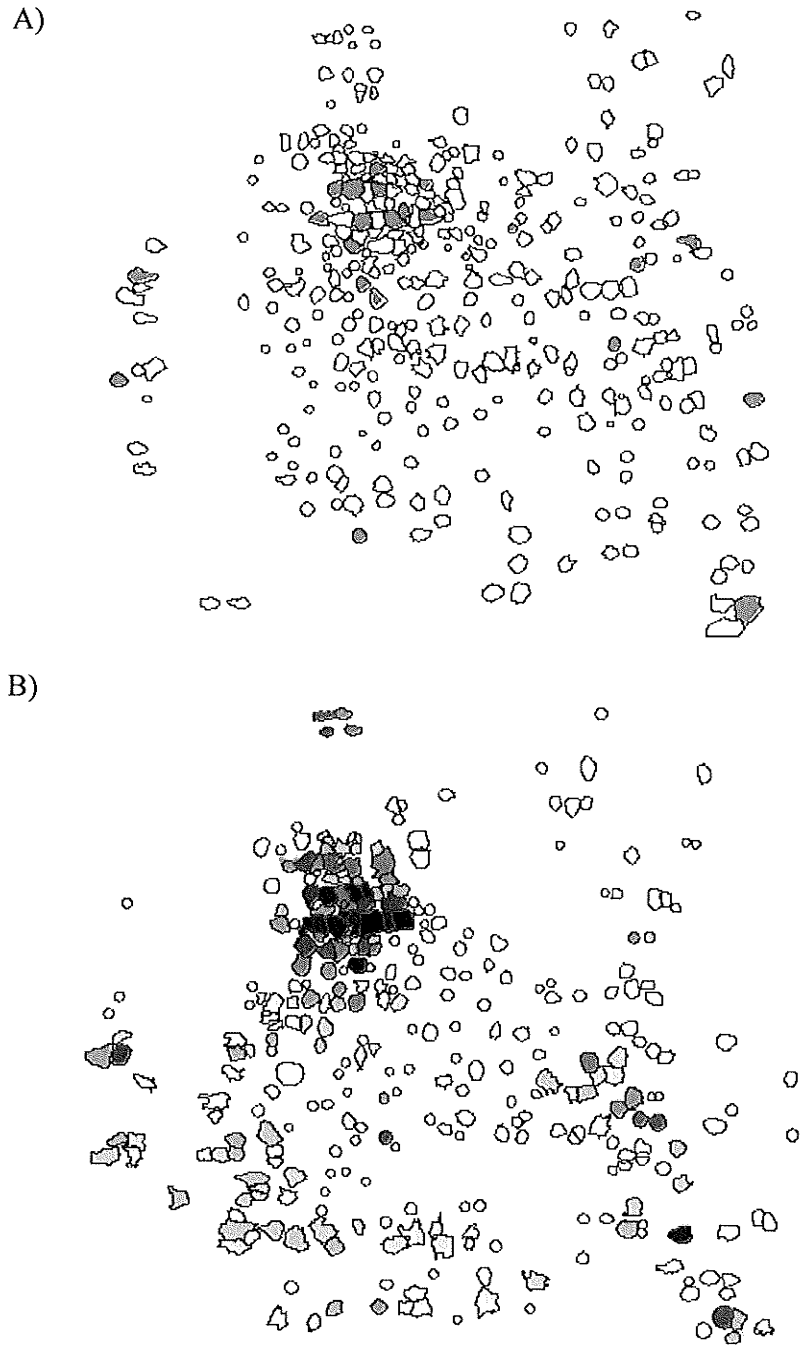


Figure 9. HSV1+ and HSV1+/HSV2+ reference blots. A) The HSV1+ reference blot represents a superimposition of the nine HSV1+ blots (in red). B) The HSV1+/HSV2+ reference blot represents a superimposition of the seven double-positive blots (in purple). Those synthetic gels have been created using the ImageMaster 2D Platinum software, version 5.00 (Amersham Biosciences). To create a reference blot, one blot in a given group had to be chosen as the starting template and those spots thus have the intensity of the spots in the original blot.

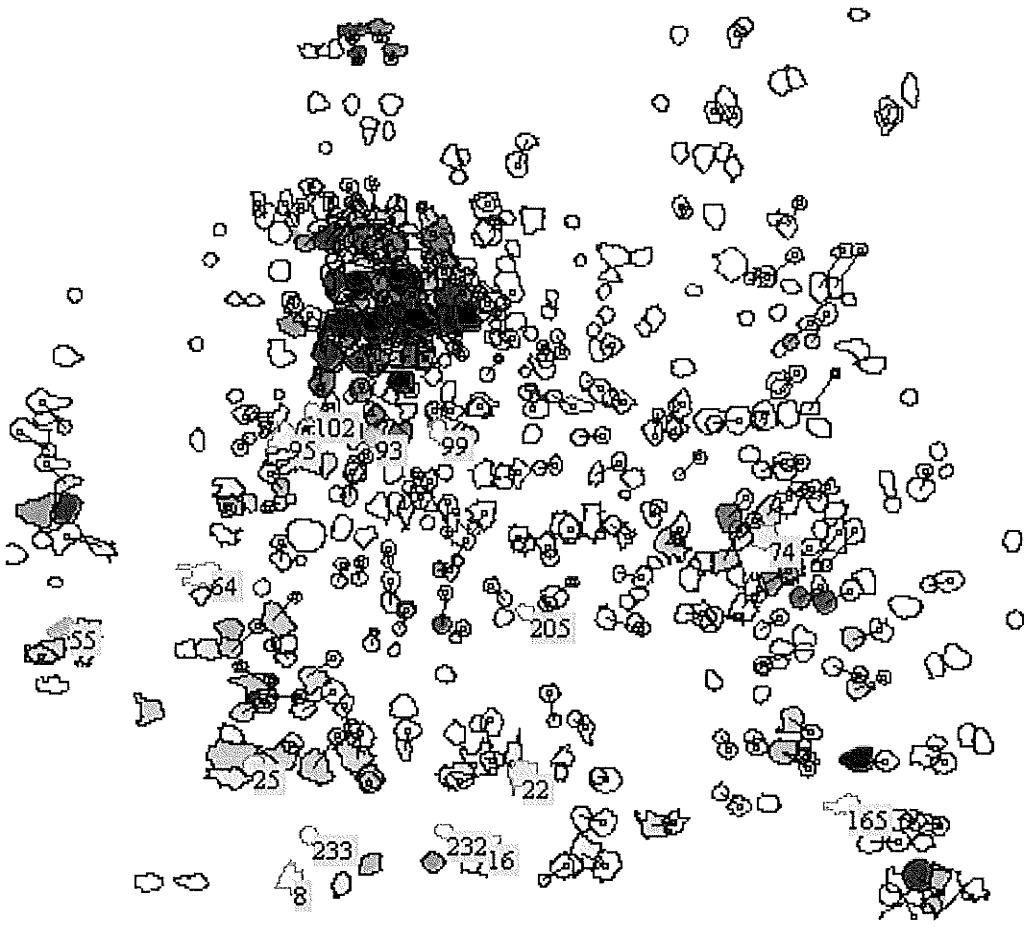


Figure 10. Superimposition of the paired HSV-1 and double-positive reference gels. The two reference gels were superimposed and the spots were paired together using the ImageMaster 2D Platinum software, version 5.00 (Amersham Biosciences). The spots from the HSV1+ reference gel appear in red whereas the ones from the double-positive reference gel appear in purple. The 15 potential HSV-2 type-specific antigens (non-paired spots from the double-positive reference gel) appear in green and the spot IDs are indicated for those proteins only.

	Double-positive serum samples							
	Serum no.							Ratio of positive
Spot ID	12013	12031	12017	12008	12458	8703	8690	samples
8		X					X	2/7
16			X	X			X	3/7
22	X			X			X	3/7
25					X		X	2/7
55		X					X	2/7
64					X		X	2/7
74	X		X				X	3/7
93		X	X	X			X	4/7
95		X		X	X	X	X	5/7
99	X		X	X	X	X	X	6/7
102	X	X					X	3/7
165		X	X	X				3/7
205	X	X	X	X	X	X		6/7
232	X		X					2/7
233	X		X	X	X			4/7

Table 3. Potential HSV-2 type-specific proteins. Comparison of nine 2-D blots from HSV1+ patients and seven 2-D blots from HSV1+/HSV2+ patients led to the identification of at least 15 potential HSV-2 type-specific proteins that reacted with double positive sera. None of those proteins reacted with HSV1+ sera.

Control western blots using a non-infected cell lysate as the antigen source were also performed to ensure that the reactive spots on the gels were truly HSV-2 and not cellular proteins. Three serum samples from patients infected with both HSV-1 and HSV-2 were tested (from patient no. 12052, 8690 and 50072). As can be seen in Figure 11, variable results were obtained depending on the serum sample tested. Serum samples from patients no. 12052 and 8690 (Figure 11, B and D) reacted to only a few proteins from a non-infected cell extract whereas the one from patient no. 50072 (Figure 11, F) reacted to many proteins all over the blot. However, the reactivity pattern was completely different from the one observed for an HSV-2-infected cell extract (Figure 11, E and F). Whether this serum sample represents an exception or not remains to be answered by testing more serum samples.

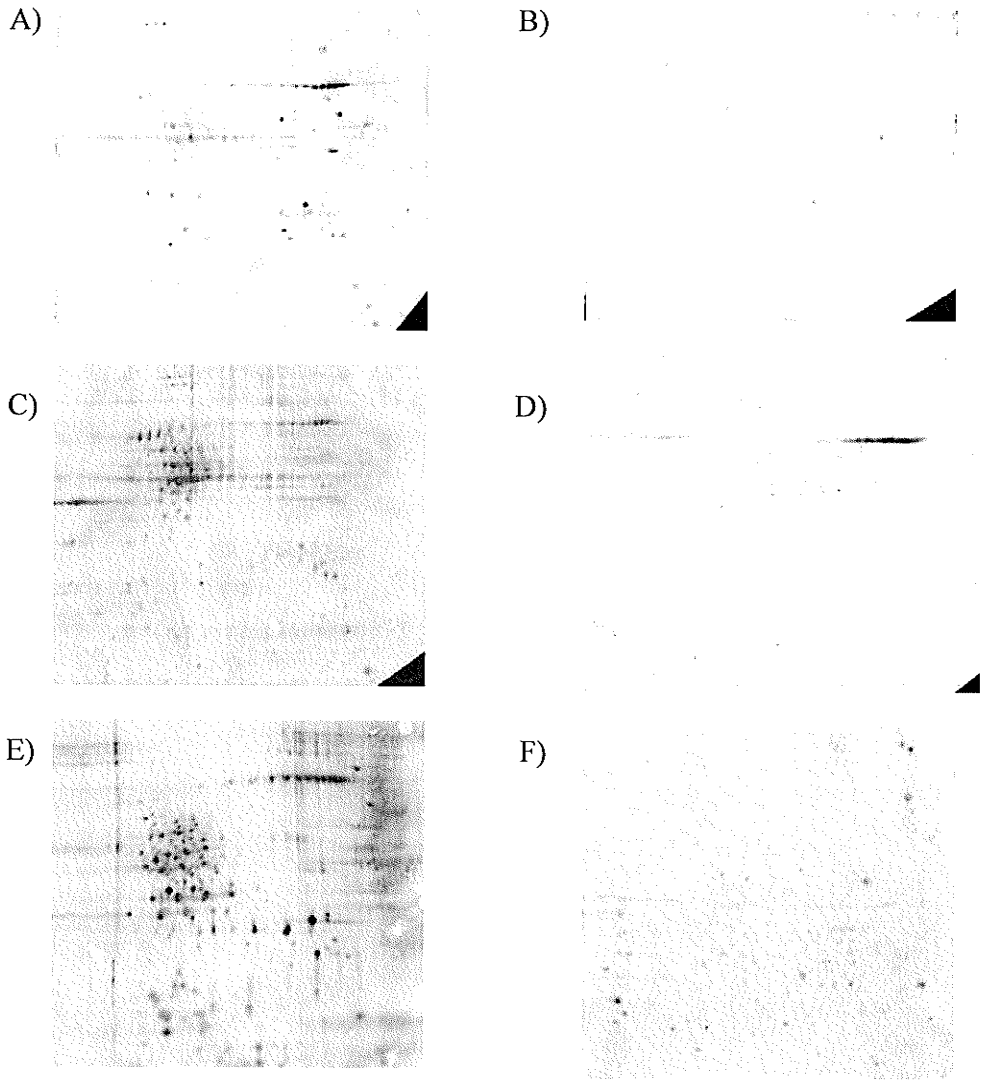


Figure 11. Control 2-D WBs using non-infected cell extracts as the antigen source. Five hundred μg of proteins from extracts of HSV-2-infected (A, C, E) Vero cells or non-infected cells (B, D, F) were loaded on 18cm IEF strips with a pH range 3-10NL. The primary antibody for WB was a human serum sample (1:500) and the secondary antibody was an anti-human IgG conjugated to a peroxidase (1:2500) Detection of the antigens was performed by chromogenic detection for A, B, C and D and by chemiluminescence for E and F. A and B) 2-D WB for patient no. 12052. C and D) 2-D WB for patient no. 8690. E and F) 2-D WB for patient no. 50072.

3.2 Identification of the viral proteins

Following the discovery of potential HSV-2 type-specific antigens on the 2-D WBs, the next step was to identify them by picking the spots on a stained 2-D gel and submitting the spot to mass spectrometry analysis. Moreover, as gG-2 is already known to be a type-specific antigen, we wanted to demonstrate its presence on the 2-D WBs using monoclonal antibodies specific to this protein.

3.2.1 Monoclonal antibodies against gG-2

Because gG-2 is the only known type-specific antigen and that it has been very well described in the literature, it was important to demonstrate its presence in the 2-D gels from a lysate of HSV-2-infected Vero cells but also to determine whether the serum samples we tested were reactive to gG-2. The processing of gG-2 is fairly complex as the precursor protein (115 kDa) is cleaved to yield the amino-proximal portion, sgG-2 (40 kDa) and the carboxy-terminal portion (Liljeqvist et al., 2002). The latter is a high-mannose intermediate (76 kDa) which is processed by O-glycosylation to yield the mature gG-2 (120 kDa) (Liljeqvist et al., 2002).

We had three monoclonal antibodies available: 1106 (anti-gG-1) (Rumbaugh-Goodwin Institute), 1107 (anti-gG-2) (Rumbaugh-Goodwin Institute) and AP-1 (anti-gG2) (supplied by Tony Minson, University of Cambridge, UK). Unfortunately, the three of them reacted with numerous proteins on a 2-D WB. MAb 1106 was also tested with a non-infected cell extract as the antigen source with the same results.

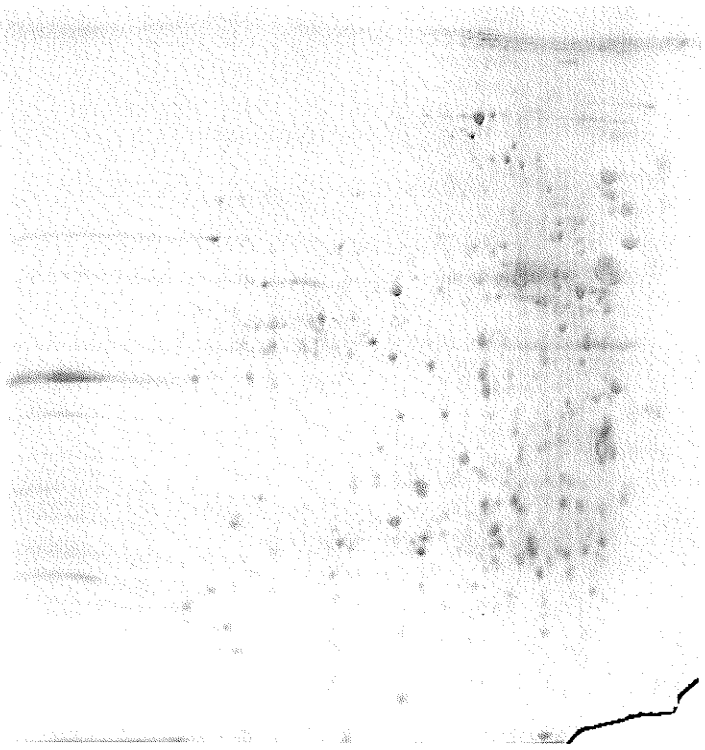
To ensure that the problem was coming from the MAbs and not from the goat anti-mouse secondary antibody, a membrane containing an HSV-2 cell extract was

incubated with the secondary antibody alone (1:2500). Only a few spots were visible (data not shown), ruling out this hypothesis.

3.2.2 Silver staining and mass spectrometry analysis

Please refer to section 2.1.8.2 for an explanation of the MS analysis that was performed. The selection of the spots for MS analysis was based on the matching of the western blots with a stained gel or matching of two stained gels from an infected or non-infected cell extract.

A)



B)

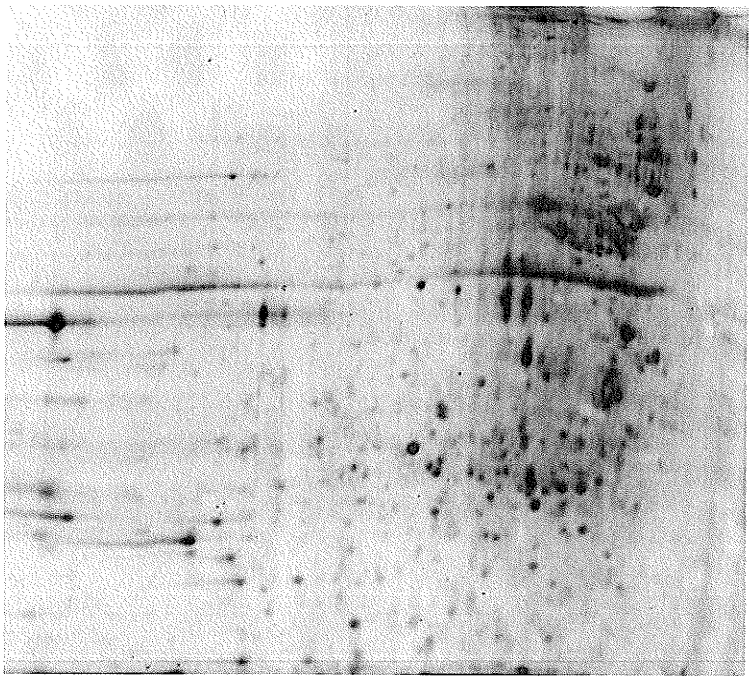


Figure 12. Silver stained two-dimensional gels using HSV-2-infected and non-infected cell lysates as the antigen source. A) 750 µg of proteins from an HSV-2-infected Vero cell extract were loaded on 18cm IEF strips with a pH range 3-10NL. B) A) 750 µg of proteins from a non-infected Vero cell extract were loaded on 18cm IEF strips with a pH range 3-10NL.

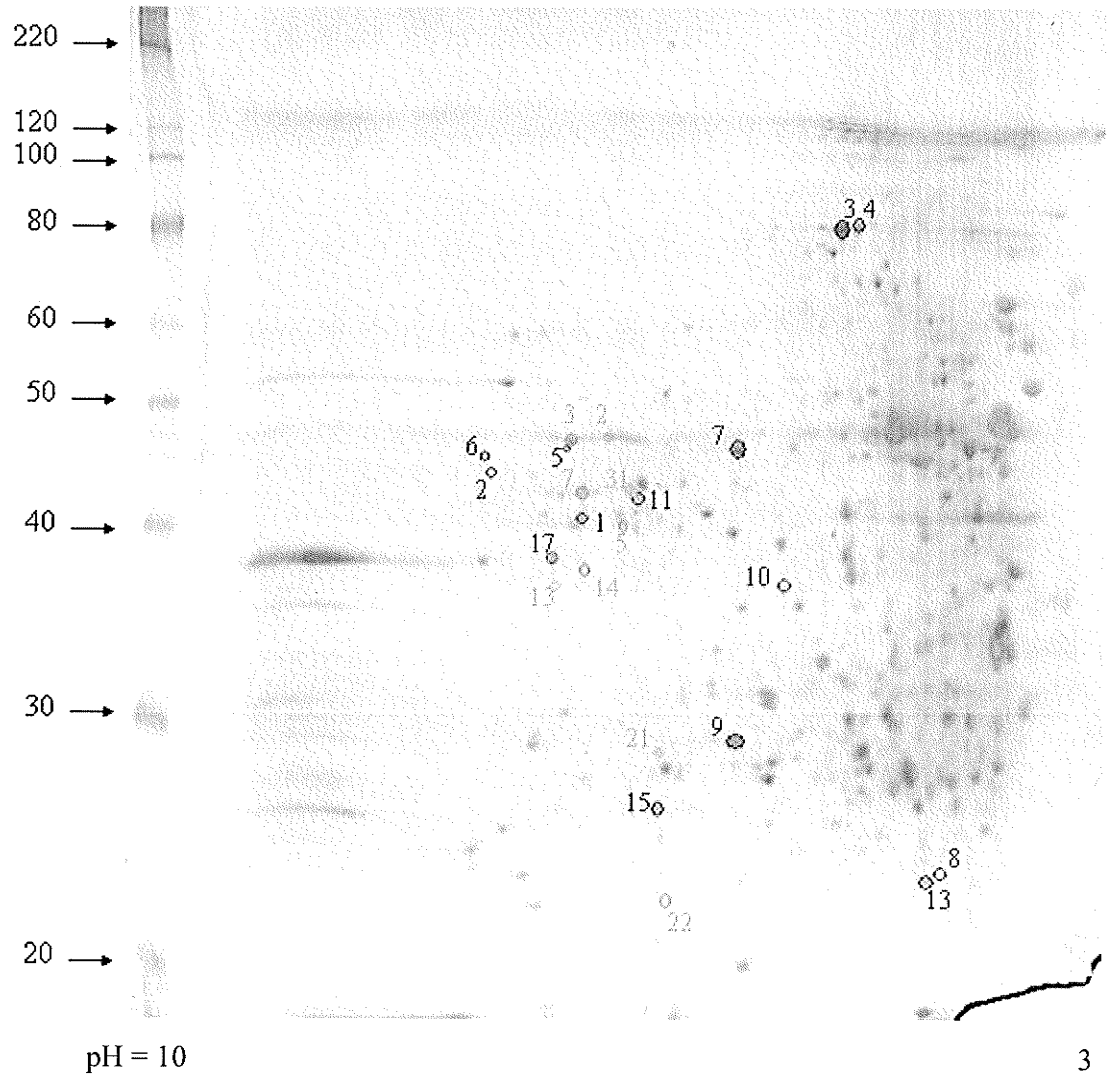


Figure 13. HSV-2 and cellular proteins identified by mass spectrometry. The identity of the protein species contained in 22 spots has been determined by mass spectrometry analysis. Spots outlined in red contain cellular proteins whereas spots outlined in green contain HSV-2 proteins. See text for details.

Four proteins were identified as HSV-2: U_L50 (deoxyuridine triphosphatase, dUTPase), U_L51, U_L26/ $U_L26.5$ and U_L12 (alkaline exonuclease).

Spot ID5 had a mascot score of 585 for U_L50 (MW: 38.943 kDa, pI: 6.50). The second highest score for this sample was 148 for alkaline exonuclease (predicted MW: 66.739 kDa, apparent MW: 85 kDa, pI: 8.80) (Martinez et al., 1996). According to the spot location on the gel, it is expected that the identified protein should have a MW around 40kDa. Although the MW of alkaline exonuclease did not fall in that range, unlike U_L50, the 4 peptides that matched alkaline exonuclease were not the same as the ones that matched U_L50, suggesting that a proteolytically processed form of alkaline exonuclease was also present in the sample. There is also an alternative transcript of the $U_L12.5$ gene, called $U_L12.5$, that produces proteins of 60 kDa and 54 kDa (Martinez et al., 1996). However, those cannot explain the presence of the protein at 40 kDa.

Spot ID22 was identified as U_L51 (MW: 25.809 kDa, pI: 5.88), with a mascot score of 314. The spot location was around 22 kDa, which correlates with the expected MW of U_L51. Three other proteins had a significant score: trypsin precursor (score of 96), keratin (score of 80) and the phosphorylase family protein from *Coxiella burnetii* RSA 493 (score of 51). The latter one matched only one peptide that also matched U_L51 and can therefore be considered as an artifact. The presence of trypsin is expected in MS since it is used as the digest enzyme during sample preparation for MS. The presence of keratin is also not uncommon due to human contamination during handling of the samples.

Several spots (ID 2, 3, 7, 13, 21 and 31) were identified as U_L26 or $U_L26.5$ gene products, which is not surprising due to the complex processing of those two proteins (Figure 14).

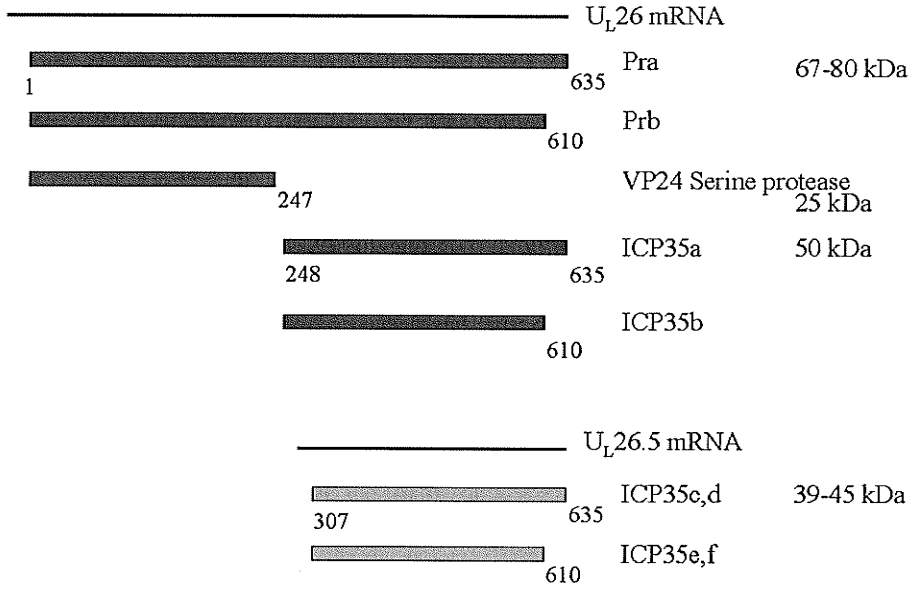


Figure 14. Post-translational processing of U_L26 and $U_L26.5$ proteins from HSV-2. The U_L26 gene consists of two open reading frames, U_L26 and $U_L26.5$. The U_L26 gene generates one full-length mRNA which encodes a 635 aa long precursor protein, known as Pra with an apparent MW of 67 to 80 kDa (Braun et al., 1984; Liu and Roizman, 1993; Liu and Roizman, 1991). Pra cuts itself at position 610/611 to yield Prb. Pra and Prb appear as a double band on a 1-D gel (Liu and Roizman, 1991). Pra also cuts itself at position 247/248, yielding an amino-terminal portion, Prn (VP24), which retains the protease activity (aa 1 to 247) and has a MW of 25 kDa. The carboxy-terminal portion, VP21, is a scaffolding protein with a MW of 50 kDa. VP21 is known to have two isoforms, the ICP35a (aa 248 to 635) and ICP35b, which arises by carboxyl-terminal cleavage of ICP35a (aa 248 to 610). The smaller ORF, designated $U_L26.5$, overlaps with the U_L26 ORF, initiating within the U_L26 ORF and is 3' coterminal with it. $U_L26.5$ produces 4 additional ICP35 isoforms denoted ICP35c,d,e and f. ICPc,d (pre-VP22a) (aa 307 to 635) are post-translationally processed by the $U_L26.5$ protease to yield ICP35 e,f (VP22a) (aa 307 to 610). The MWs of those isoforms vary between 39 and 45 kDa (Braun et al., 1984). Moreover, several of the members of the ICP35 family have been demonstrated to be phosphorylated (Braun et al., 1984).

Spots ID 2, 3, 7, 13, 14 and 31 all contained peptides that matched the carboxy-region of U_L26. Spots ID2 and 3 had scores of 183 and 167, respectively, and were both located at approximately 47 kDa on the 2-D gel. They could thus represent any of the ICP35 isoforms, most likely ICP35a,b. Spots ID7 and 31 had a MW around 42 kDa and could thus represent ICP35c,d whereas spot ID13, which was localized at approximately 37kDa, could represent ICP35e,f.

On the other hand, only peptides from the amino-proximal region of U_L26 matched spot ID21, suggesting that only Pra, Prb or Prn could have been present in the sample. Given that the MW of Prn is known to be 25 kDa and that the location of the spot on the 2-D gel is around 28 kDa, it can be suggested that Prn was the protein present in the sample. Confirmation that Prn was the protein present in the sample remains to be done by repeating the experiment a second time.

Spot ID14 was identified as the U_L12 gene product, alkaline exonuclease (MW: 66.729 kDa, pI: 8.8) with a score of 52. One peptide matched that protein. Another HSV-2 protein also had a significant score for this spot. U_L26 had a score of 49 with two peptide matches. Because another sample had been run previously that was identified as U_L26 (spot ID21), it was unsure whether its presence was real or was a carry over contaminant from sample 21. However, the peptide matches for spot ID21 covered aa 47 to 62 and aa 94 to 106 (Figure 14, sequences in orange), whereas those for spot ID14 covered aa 430 to 442 and aa 471 to 497 (Figure 14, sequences in red and turquoise, respectively). Given that the location of the spot ID14 was around 38 kDa, it is impossible that it contained the full-length U_L26 protein (Figure 13). It can thus be concluded that the presence of U_L26 was real and that this might represent one of the $U_L26.5$ protein isoforms (ICP35c,d,e and f). Spot ID13 also had alkaline exonuclease as the second highest ranked hit (score of 59). However, because only one peptide matched that protein and that spots ID13 and 14 are near each other on the gel, there is also a possibility that this was a contamination during spot picking.

MASAEMRERLEAPLPDRAVPIYVAGFLALYDSGDSGELALDPDTVRAALPPENP
LPINVDHRARCEVGRVLAVVNDPRGPFFVGLIACVQLERVLETAASAAIFERRG
PALSREERLLYLITNYLPSVSLSTKRRGDEVPPDPTLFAHVALCAIGRRLGTIVTYD
TSLDAAIAPFRHLDPATREGVRREAAEAELALGRTWAPGVEALTHTLLSTAVNN
MMLRDRWSLVAERRRQAGIAGHTYLQASEKFKIWGAESAPAPERGYKTGAPGA
MDTSPAASVPAPQVAVRARQVASSSSSSFPAPADMNPVSASGAPAPPPPGDGSYL
WIPASHYNQLVTGQSAPRHPPLTACGLPAAGTVAYGHPGAGPSPHYPPPPAHPYP
GMLFAGPSPLEAQIAALVGAIAADR**QAGGLPAAAGDHGIR**GSAKRR**RHEVEQP**
EYDCGRDEPDRDFPYYPGEARPEPRPVDSRRAARQASGPHETITALVGAVTSQ
QELAHMRARTHAPYGPYPPVGPYHHPHADTETPAQPPRYPAEAVYLPPPHIAPP
GPPLSGAVPPPSYPPVAVTPGPAPPLHQPSPAHAHPPPPPPGPTPPPAASLPQPEAP
GAEAGALVNASSAAHVNVDTARAADLFVSQMMGSR

Figure 15. Amino acid sequence of the U_L26 protein. Peptides covering aa 47 to 62 and aa 94 to 106 only matched spot ID21 (in orange). The peptide encompassing aa 409 to 423 matched spots ID 2, 3, 7 and 31 (in blue). The peptide covering aa 430 to 442 matched spots 2, 3, 7, 13, 14 and 31 (in red). The peptide encompassing aa 471 to 497 (in turquoise) matched spot ID 14 and the one covering aa 624 to 636 matched spots ID 2 and 3 (in green).

Several proteins have been identified as cellular proteins (Table 4). Although almost all of them were identified as belonging to the *Homo sapiens* species during the sequence database search, we consider them to be simian proteins because we used Vero (African monkey kidney cells) for viral infection and also because the simian proteins have not all been sequenced yet.

Spot ID	Protein identified	Mascot score	MW of the protein (kDa)	Spot location (kDa)	Next highest match	Mascot score	MW of the protein (kDa)
1	Pyruvate kinase, M2 isoenzyme	425	58,4	41	Pyruvate kinase (*Elaphe* sp.)	170	
	Pyruvate kinase	92	58,4	41	Unnamed protein product (*Rattus norvegicus*)	79	58,2
9	Pyruvate kinase	515	58,4	28	Putative pyruvate kinase (*Elaphe* sp.)		
2	Phosphoserine aminotransferase	87	35	44	Mitochondrial outer membrane protein TOM40 (*Homo sapiens*)	85	38,2
3	Heat shock 70KDa protein 9B, precursor	421	73,9	80	Heat shock 70KDa protein 9B precursor variant	398	
4	MTHSP75	137	74,019	80			
7	MTHSP75	485	74,019	47	Heat shock protein 9B (*Danio rerio*)	251	
5	Alpha-enolase	219	47,421	47	FUSE binding protein 2 (*Homo sapiens*)*	73	68,7
6	Migration-inducing gene 10 protein	128	44,9	46	Phosphoglycerate kinase (*Lepidosiren paradoxa*)	52	
8	Trypsin precursor	74			Chaperonin (HSP60)	52	61,1
10	Nucleolin (protein C23)	173	76,3	37	Trypsin precursor	103	
17	Aldose reductase	68	34,6	38			
11	Pyruvate kinase	233	58,4	42	M2 pyruvate kinase (*Rattus norvegicus*)	222	
13	Chaperonin (HSP60)	281	61,1	23	Heat-shock 60kD protein 1 (*Danio rerio*)	200	
15	Glutathione S-transferase P (*Macaca mulatta*)	203	23,7	25	Glutathione S-transferase (*Homo sapiens*)	161	
5	UL50	585	38,9	40	Alkaline exonuclease	148	66,7
	UL50	153	38,9	40			
2	UL26 protease	183	67	47	Keratin		
3	UL26 protease	167	67	47	Alpha-enolase	135	47
		61	67	47			
7	UL26 protease	92	67	42	UL26.5	92	33,9
13	UL26 protease or UL26.5	125	67	37	Alkaline exonuclease	59	66,7
14	Unnamed protein product (*Mus musculus*)	78	27	38	Alkaline exonuclease	52	66,7
21	UL26 protease	111	67	28	Triophosphate isomerase (*Macaca mulatta*)	99	27
31	UL26 protease	100	67	42			
22	UL51	314	25,8	22	Trypsin precursor	96	

* This protein was ranked 5th but appeared to be present in the spot. See text for more details.

Table 4. HSV-2 and host cell proteins identified by mass spectrometry analysis. Spot ID numbers in red were identified as cellular proteins whereas the ones in green were identified as HSV-2 proteins. Only the two proteins that were ranked the highest for each protein sample are represented here.

3.3 Antibody epitope mapping

One important finding of the 2-D western blotting work was that the majority of the HSV proteins appeared to be type-common antigens. However, when looking at the 1-D western blots after pre-absorption, it was noticed that for a patient infected with both HSV-1 and HSV-2, the number of bands in the HSV-2 lane was the same as before the pre-absorption (pre-absorption removes the cross-reactive anti-HSV-1 antibodies by incubating the serum sample to be tested with an HSV-1 cell lysate prior to the WB procedure) (Figure 3). Instead, it was the intensity of the bands that decreased, suggesting that the cross-reactive anti-HSV-1 antibodies and the anti-HSV-2 antibodies were binding the same proteins. This raised the question as to whether the cross-reactive anti-HSV-1 antibodies and the HSV-2 antibodies were binding the same region(s) on a given protein. To answer this question, the pepscan technique, which uses a series of overlapping synthetic peptides coupled to a solid membrane support, was employed to map the B cell epitopes on three HSV-2 proteins, gB, gC and ICP35.

In order to identify the immunoreactive epitopes on gB-2, 15-mer peptides, overlapping by 12 aa and covering the entire gB-2 sequence were synthesized and bound to a cellulose membrane. Two identical membranes were used and they were regenerated after each serum sample tested. Given that each serum sample might react to different epitopes on the protein, the gB-2 peptides were tested for seroreactivity with a panel of 10 well-characterized human sera, 5 from patients infected with HSV-1 and 5 from patients infected with both HSV-1 and HSV-2. As can be seen on Figure 16, which represents one HSV-1-positive (Figure 16A) and one double-positive (Figure 16B) serum samples, not all serum samples react to the same peptides.

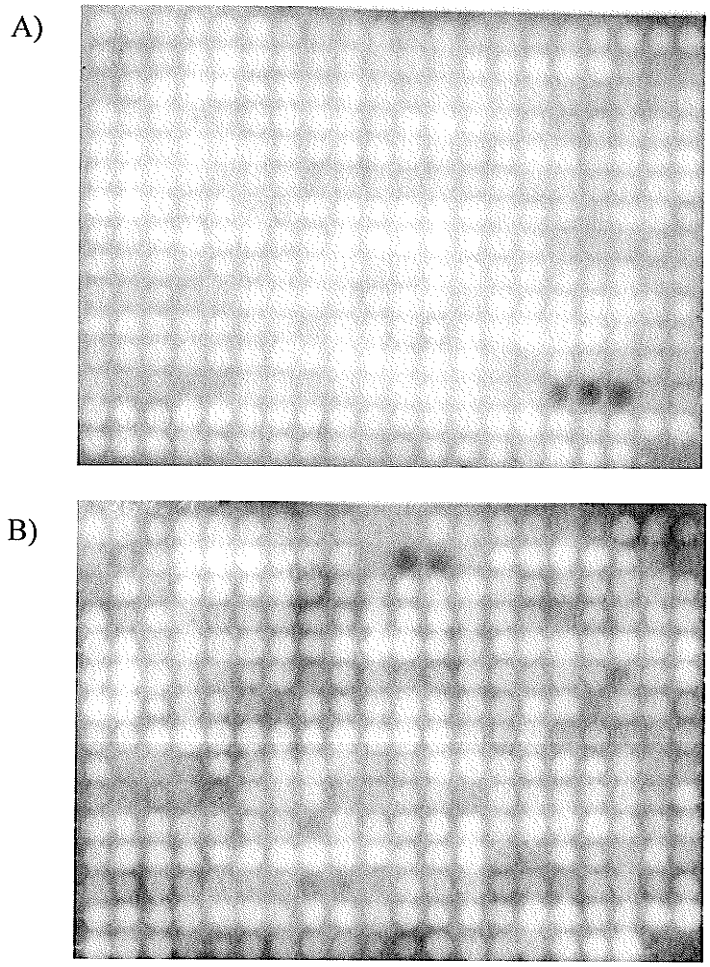


Figure 16. Reactivity of two serum samples with overlapping peptides covering gB-2 and coupled to a cellulose membrane. The antibodies for each patient were incubated with the membrane (1:5,000) followed by incubation with a secondary antibody conjugated to a peroxidase (1:10,000). Reactivity was detected by chemiluminescence (dark spots). A) Serum sample 8707, from a patient infected with HSV-1 only, reacted strongly with the peptides covering aa 766-792. B) Serum sample 8690, from a patient infected with both HSV-1 and –2, reacted with peptides encompassing aa 91 to 108.

The overall reactivity of both groups of sera (i.e. HSV-1-positive and double-positive) for each peptide is represented in Figure 17, in which each peak represents the average of the spot intensities obtained with 9 HSV1+ sera (in red) and 7 HSV1+/HSV2+ sera (in blue). In particular, peptides 38, 39, 40 and 41, covering aa 114 to 136 and peptides 289, 290 and 291, covering aa 864 to 884, were found to be immunodominant with both groups of patients, suggesting that those regions contain type-common epitopes Figure 17 and Table 5. This is not surprising as those two regions are 100% identical in their amino acid sequence (Figure 18). No dominant peptide was identified as being type-specific.

gC and ICP35 (U_L26.5 gene product) from HSV-2 were also screened for the presence of linear epitopes that would react with sera from patients infected with either HSV-1 or both HSV-1 and HSV-2. Those two proteins were chosen because although antibodies against those antigens appear only late during an infection (Table 2) (Ashley RL and Wald A 1999) (Lopez C et al. 1993), the reactivity is known to be very strong and the aa sequences are more divergent from their HSV-1 homologs (Figures 19 and 20) compared to gB-2. However, regeneration of the membranes was not possible for both proteins, even after a second regeneration process and no conclusions can thus be drawn from the only two serum samples tested.

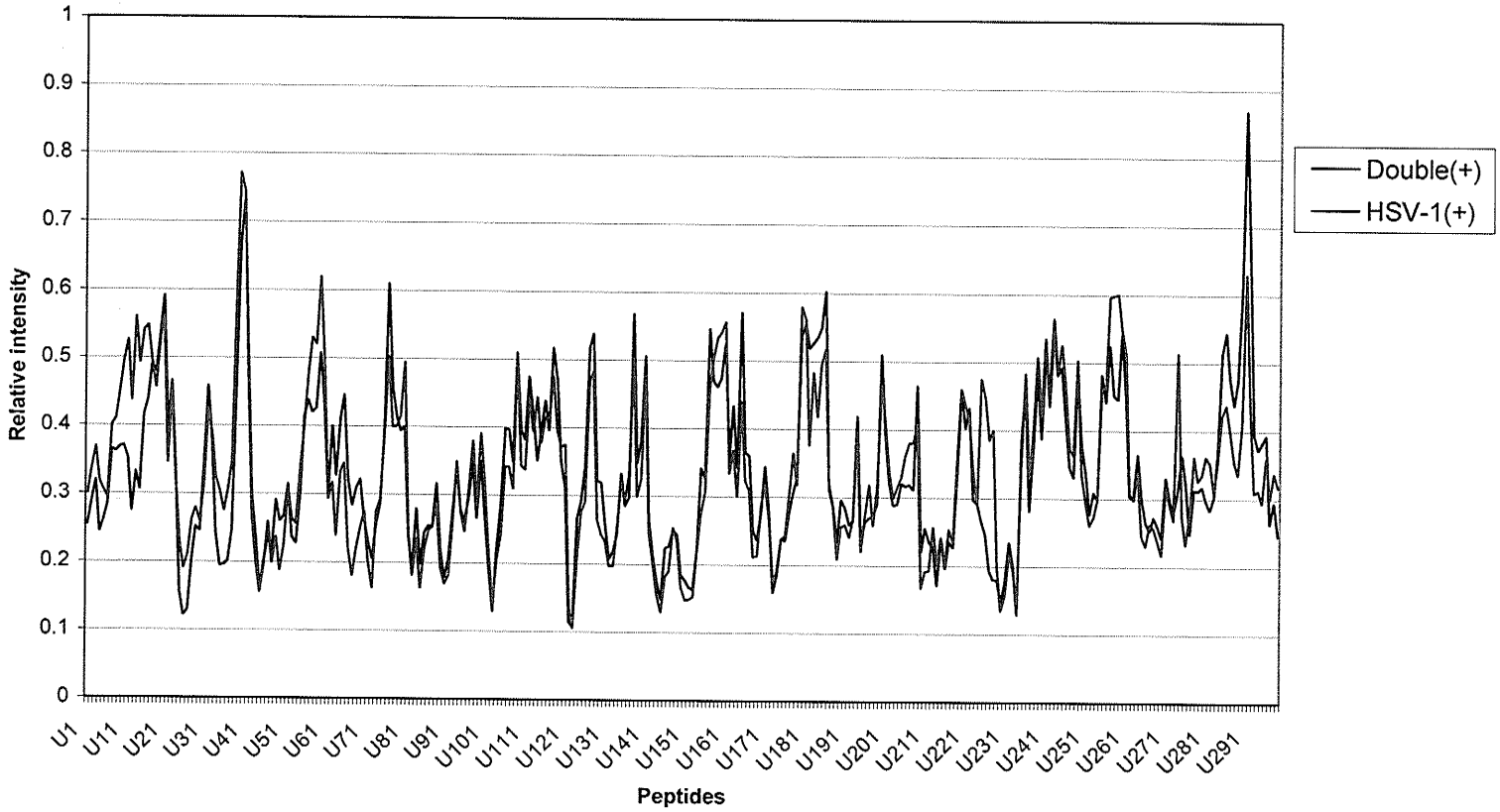


Figure 17. Percent intensity of each gB-2 peptide for the two patient groups. The reactivity of human sera with a series of 298 overlapping 15mer peptides derived from the gB-2 sequence is illustrated. Each serum group, HSV1+ (in red) and HSV1+/HSV2+ (in blue), includes five serum samples. For each peptide, the percent intensity of the reactivity for each group is represented. The percent intensity of each peptide was determined by dividing the raw intensity of each spot by the sum of the raw intensities for all spots on the membrane.

		HSV1+/HSV2+ sera					HSV1+/HSV2- sera				
Peptides	Peptide sequences	8690	12458	12013	12017	12008	8707	8683	8713	8696	8612
38	$_{112}$PPPTGATVVQFEQPR$_{126}$	-	++	-	+	++	++	+	+++	+	+
39	$_{115}$TGATVVQFEQPRRSP$_{129}$	++	+++	-	+++	+++	+++	++	+++	++	++
40	$_{118}$TVVQFEQPRRSPTRP$_{133}$	+++	+++	+	++	+++	+++	++	+++	++	++
41	$_{121}$QFEQPRRSPTRPEGQ$_{136}$	+	++	+	-	+++	-	+	++	+	-
289	$_{865}$KKGTSALLSSKVTNM$_{879}$	+	-	+++	-	-	+	+	-	-	+
290	$_{868}$TSALLSSKVTNMVLR$_{882}$	+++	+	+++	-	-	+++	+++	+	+	+++
291	$_{871}$LLSSKVTNMVLRKRN$_{885}$	+++	+	+++	-	-	-	+	-	++	+

Table 5. gB-2 type-common immunodominant peptides identified by pepscanning. The reactivity of 5 HSV-1-positive only and 5 double-positive human sera for two regions on gB-2. Binding of sera to each peptide is scored as negative (-), weak (+), strong (++) or very strong (+++). The peptide sequences are represented using the standard amino acid code from the N to the C terminus and the position of the first and last aa of each peptide is indicated by subscript numbers. Two regions were identified as immunodominant for both groups of patients and were thus classified as type-common.

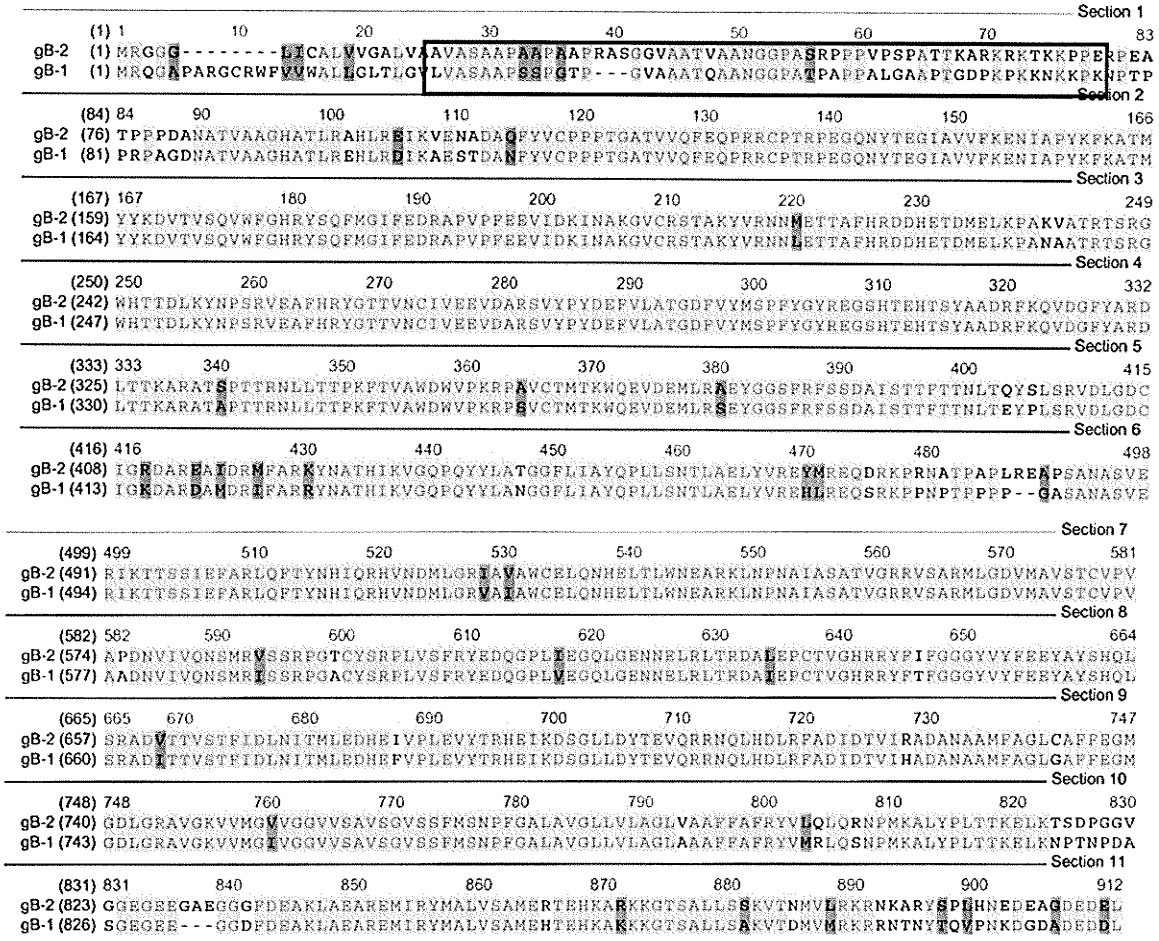


Figure 18. Sequence alignment of gB-1 and gB-2. The sequences were aligned using VectorNTI using gB-1 from strain F and gB-2 from strain G. The blue box highlights amino acids 18 to 75 from gB-2, which were shown to contain type-specific epitope(s) by Goade et al., 1996. Amino acids highlighted in yellow are identical amino acids whereas the ones highlighted in green are conservative substitutions.

4. Discussion

The diagnosis of HSV-1 and HSV-2 infections relies on serological testing since 70% of genital herpes infections are asymptomatic. The gold standard test, the Western blot assay, is very sensitive and specific, but is also subjective and time-consuming, which renders it unsuitable for large volume diagnoses. Commercial kits for HSV-type-specific serology are now available, and they are based on the only known type-specific HSV antigen, gG. Unfortunately, although specific, the antibody response against gG is not the strongest, and it occurs relatively late after HSV infections (Ashley and Wald, 1999; Lopez et al., 1993). For this reason, gG-based tests may produce a number of discordant results. We have therefore implemented two different experimental approaches, 2-D western blotting and antibody epitope mapping, to identify HSV-2 type-specific antigens to distinguish unambiguously between an infection with HSV-1, HSV-2 or both types concurrently.

4.1 Two-dimensional western blotting

The first approach, 2-D western blotting, was used to electrophoretically separate all HSV-2 proteins on a single gel and to then test the reactivity of different human serum samples to those HSV-2 proteins. This is the only report to date on the use of the 2-D WB technique to study the type-specific antibody response to HSVs. The 2-D WB technique had to be optimized several times during the study. For example, we switched from using a chromogenic substrate to chemiluminescence for the detection of the bound antibodies because this greatly increased the sensitivity of detection. We also had some issues regarding entry of the proteins into the Immobiline DryStrip gels during the first dimension and we resolved this problem by increasing the concentration of the IPG buffer

from 0.5 to 1% in the rehydration solution prior to loading of the protein sample to the strips. Nevertheless, it was found that over 300 proteins from the western blots were immunoreactive with human serum samples. Moreover, 15 of those proteins were identified as potential HSV-2 type-specific antigens as at least 2 serum samples from patients infected with both HSV-1 and HSV-2 reacted to those proteins. None of the serum samples from patients infected with HSV-1 only reacted to those proteins. The 2-D western blots were also matched to stained gels in order to perform spot picking from the stained gels for MS analysis. However, some difficulties arose from this.

4.2 Two-dimensional gel analysis of HSV-2 polypeptides

The major problem with the identification of the viral proteins from a silver stained gel is that this gel includes both the viral and the cellular proteins. A region where only one spot appears on the WB might contain several more proteins on a stained gel. Moreover, the intensity of a spot on a WB does not necessarily correlates with the intensity on a stained gel. It thus becomes more difficult to pick the right spot for MS identification. Hence, we tried to compare silver stained gels containing infected and non-infected cell lysate antigens to identify the viral proteins. However, some of those were cellular proteins, such as the heat shock proteins, that were upregulated during viral infection.

To overcome this limitation, I decided to attempt to pick the proteins from an immunostained NC membrane rather than a stained gel for MS analysis. The western blot-mass spectrometry technique has been described for the first time in 2002 by Klarskov and colleagues (Dufresne-Martin et al., 2005) (Klarskov and Naylor, 2002). Regrettably, this technique is still at the early stages of development and although we sent

some samples to Klarskov's group (University of Sherbrooke, Sherbrooke, Canada), no protein was identified.

Because of the complexity of the antigen samples, which resulted in crowding of the proteins in some regions of the gels and western blots (Figures 8 and 12), we have attempted to use narrower pH range strips for the first dimension (pH 4-7 and pH 6-11) rather than using strips with a pH of 3-10NL. However, the results were not as good as expected since half of the protein sample was lost because the pH range of the strip was not adequate for those proteins. To overcome this limitation, we also tried to fractionate the samples further using a Multi-Compartment Electrolyser (MCE) (Proteome Systems, Sydney, Australia) prior to the use of the narrower pH range strips. The MCE system allows a liquid-based separation of the proteins based on their pI and it is then possible to either perform 2-D gel electrophoresis with narrow-pH range strips or to even apply this fractionated sample on a 1-D gel. On the other hand, further separation of the proteins into fractions might only add to the complexity of the sample rather than simplifying it since the lower abundance proteins are revealed. Nevertheless, we tried the MCE procedure but as the sample is diluted greatly during the procedure, we decided to concentrate the samples following MCE, however unsuccessfully. Using a much higher amount of the infected-cell extract proteins for fractionation might overcome this limitation but also renders this approach more time-consuming as the cell extract samples need to be cleaned and quantified prior to the MCE procedure.

Because of the difficulty of excising the right spot when using an infected cell extract as the antigen for gel electrophoresis, an alternative method to identify HSV-2 type-specific proteins was also explored. The immunoprecipitation technique was used to capture HSV proteins using human serum bound to a protein A column. Unfortunately,

the extremely low concentration of anti-HSV antibodies in human serum did not allow the capture of sufficient amounts of the HSV proteins to permit their visualization by either silver staining or western blotting.

Only a few published studies have described so far the pattern of HSV polypeptides on a 2-D gel (Haarr and Marsden, 1981; Palfreyman et al., 1983; Sathananthan et al., 1996). Still, Haarr and Marsden identified ^{35}S-labeled HSV proteins from an infected-cell lysate based on mobility in an IEF gel and apparent MW on an SDS-PAGE gel (Haarr and Marsden, 1981) whereas we employed mass spectrometry to identify the viral proteins. Our data though support the results presented by Haarr and Marsden who stated that 230 viral polypeptides could be identified by 2-D gel analysis (Haarr and Marsden, 1981). Still, our results cannot be compared to those as we used the 2-D technique developed by Görg and colleagues (Görg et al., 1988) while Haarr et al. employed the original technique developed by O'Farrell (O'Farrell, 1975)and Klose (Klose, 1975) in 1975, in which the first-dimension was performed in carrier ampholyte-containing polyacrylamide gels cast in narrow tubes. In addition, the authors used non-equilibrium pH gradient electrophoresis (NEPHGE) to analyze extremely basic proteins. In the NEPHGE approach, the protein sample is applied at the acid end and all the proteins being positively charged, they migrate towards the basic side of the gel. Those variations in the technique employed thus results in a completely different protein pattern on the 2-D gel.

4.3 Antibody epitope mapping

The second approach we adopted was to look at the type-specificity of epitopes contained in a single protein rather than in the protein as a whole. Our hypothesis was that

a single protein might contain both type-common and type-specific epitopes and would thus appear as a type-common protein on a 2D-Western blot. To test this hypothesis, we used overlapping synthetic peptides and we tested the reactivity of human sera from patients infected with either HSV-1 only or both HSV-1 and HSV-2, as for the 2D-WB. Pepscanning was chosen to map the B cell epitopes on gB-2 as it has been successfully employed to map the type-specific epitopes on gG-1 and gG-2 (Grabowka et al., 1999; Levi et al., 1996; Liljeqvist et al., 1998; Liljeqvist et al., 2002; Tunbäck et al., 2000).

gB-2 and its HSV-1 homologue, gB-1, present an 86% linear amino acid sequence similarity. Nevertheless, the region at the N-terminus of the protein is quite divergent (Figure 18). Additionally, gB-2 is known to induce an antibody response early during the course of the disease in human patients (Table 2) (Ashley and Wald, 1999; Lopez et al., 1993) and would thus be an ideal candidate for type-specific serology if it were identified as a type-specific protein.

One study, performed by Goade et al. in 1996, analyzed the B cell epitopes found on gB-2 using three overlapping constructs expressing either the amino-, middle or carboxy- portion of gB-2 in an *E. coli* system. All of the tested serum samples from HSV2+ patients (18/18) reacted strongly with the amino-proximal portion of gB-2 (aa 18 to 228) whereas none (23/23) of the HSV1+ patients reacted. To further delineate the immunoreactive epitopes, additional plasmids were created that expressed smaller peptides issued from the amino-proximal segment. The region covering aa 18 to 75 was identified as being preferentially recognized by HSV-2 rather than HSV-1 antibodies (58/58 (100%) HSV2+ sera vs. 3/33 (9.1%) HSV1+ sera). On the other hand, our results demonstrated that although this region appears to be type-specific, only 1/5 HSV1+/HSV2+ serum samples strongly reacted to the peptide sequences encompassing

aa 18 to 75 and 0/5 HSV1+ serum samples reacted. The solely reactive serum sample recognized two peptides, covering aa 61 to 76 and 64 to 79, respectively. The region delimited by aa 18 to 75 might not have been identified as recognized predominantly in an HSV-2 type-specific manner in our study either because of the small number of samples used or because of the difference in the study design employed by Goade et al. and ours.

Goade et al. also identified the carboxy-terminal region covering aa 819 to 904 as containing a dominant type-common epitope or epitopes. This corroborates with our results, which show that the gB-2 segment covering aa 864 to 884 contains one or more type-common epitope(s). However, the second dominant type-common region (aa 114 to 136) that we identified was not tested by Goade et al. with the nested sets of serially deleted gB-2 recombinant constructs and our results can thus not be confirmed.

It should be mentioned that although no immunodominant type-specific epitope was mapped on gB-2 using the pepscan technique, there is still a possibility that gB-2 contains type-specific epitopes, but that a different technique is required to identify them.

As for gC and ICP35, antibodies against gC-2 and ICP35 from HSV-2 appear to be induced rather late during the course of an infection, with 91% of patients being seropositive for both proteins after 6 to 12 months only (Table 8) (Lopez et al., 1993). Nevertheless, both gC and ICP35 present a greater dissimilarity with their HSV-1 counterparts than gB. gC-2 and ICP35 have also been suggested as potential type-specific antigens (Arvin et al., 1983; Bergström and Trybala, 1996; Dolter et al., 1992).

Unfortunately, the regeneration of the membranes for both gC and ICP35 was unsuccessful, preventing us from testing more than two serum samples. To overcome this problem, pooling of the serum samples in each group for an initial screening of the

immunoreactive peptides could have been useful. The identified peptides could then have been synthesized and coupled to a membrane to test more serum samples individually, as was done by Tunbäck et al., 2000. However, given the cost of the membranes, we decided not to repeat the experiment.

In conclusion, the use of overlapping synthetic peptides coupled to a solid support, although appealing, was found to have several drawbacks. One major limitation of the pepscan technique is that it detects linear epitopes, not discontinuous ones. It is also quiet expensive, especially when one wants to study several proteins and that multiple samples are to be tested, such as human serum samples. This method might be more convenient for monoclonal antibodies. Another limitation is that extensive glycosylation of some HSV proteins, such as gC and gG, may influence the immunogenicity of the protein, by masking or unmasking epitopes for example. Moreover, some samples also give high background. Purifying the antibodies with the protein of interest prior to incubation with the membrane can reduce the background, although this adds an extra step and cost to the procedure.

4.4 Summary

There is a need for the discovery of new HSV-2 type-specific antigens in addition to gG. The identification of new antigens could lead to the development of a new serological assay that would unambiguously distinguish between HSV-1 and HSV-2 infections.

The first approach we implemented to achieve this goal was 2-D western blotting and this is the first report of the use of this method to identify HSV type-specific antigens. By comparing the reactivity of 9 serum samples from patients infected with HSV-1 only

with 7 serum samples from patients infected with both HSV types, more than 300 polypeptides have been found to be immunogenic. Moreover, 15 potential HSV-2 type-specific proteins have been identified. However, mass spectrometry analysis to characterize those proteins has not been completed yet due to difficulties in matching the blots with the stained gel from which spots can be picked for MS analysis. The second approach was to perform antibody epitope mapping on a protein, gB-2, that was known to be a type-common antigen but also to be an early and strong immunogen. This was done in hope to identify some type-specific epitopes within gB-2 that appears to be type-common by 2-D WB. Unfortunately, it was found that glycoprotein B from HSV-2 is a cross-reactive protein containing no type-specific epitopes. Nevertheless, one immunodominant type-common epitope was identified.

4.5 Future directions for HSV type-specific serology and 2-D gel electrophoresis

My project was only a preliminary study of the type-specific antibody response to HSV-2. Regarding the 2-D WB approach, it would be interesting to also test serum samples from patients infected with HSV-2 only. Moreover, those 2-D WBs could also be useful to determine if patients with a primary, initial or recurrent infection have different patterns of immunoreactive viral proteins. This project could also be repeated for HSV-1 and other herpesviruses.

Once the potential antigens are identified, it will be possible to produce them recombinantly to perform further studies with human sera to confirm our preliminary results. If those studies reveal new type-specific antigens, this could lead to the development of a new type-specific serological assay that would distinguish unambiguously between HSV-1 and HSV-2. The same would apply if type-specific

epitopes are found using pepscanning, although in this case peptides would be used rather than proteins. Furthermore, the identification of immunodominant type-common epitopes, such as the one identified on gB-2, could lead to their use for the diagnosis of HSV for example.

Nonetheless, even if novel HSV type-specific antigens are found, it will be important to study the possibility of cross-reactivity with other human herpesviruses, which are also common in the population. Some studies have already presented data demonstrating cross-reactivity of the human antibody response to proteins from HSV and other herpesviruses (Balachandran N et al., 1987; Kuhn et al., 1990; Lopez et al., 1993).

Furthermore, the 2-D technique for HSVs could be applied to the study of virus-host interactions by comparing viral and cellular protein expression patterns at various time points during a cell culture infection. Microarrays for HSV gene expression are also done in our laboratory and protein expression profiles from 2-D gel electrophoresis could be a way of confirming the results obtained from the gene expression experiments. Only one study has been published so far that looked at cellular protein expression profiles during an HSV-1 infection (Greco et al., 2000). No study has been published yet on changes in protein expression during an HSV-2 infection and none on viral protein expression patterns for both HSV-1 and HSV-2.

In conclusion, the development of the 2-D WB technique is not only useful for the study of the antibody response to HSV but the 2-D gel electrophoresis procedure could also be applied to study virus-host interactions, as there are still many questions that remain to be answered regarding the effect of many HSV proteins on the host cell.

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