CHAPTER 4 : DISCUSSION

4.1 Overview

HHV6 has a widespread distribution in the world and being a herpes virus, can bring about either lytic or latent infection. Methods are still being established to definitively diagnose the reactivation of this virus. Diagnosis of HHV6 infections today is largely based on serology where minimal cross-reactivity and easy standardisation methods are important matters to be considered.

HHV6 infections are by and large self-limiting, and treatment is usually symptomatic. In brain infections and multiple sclerosis patients, HHV6 infections have been treated with antiviral drugs such as acyclovir. Though not associated with any fatal outcome of a disease, there is now concern of the possible role of HHV6 infections in syndromes such as AIDS, mononucleosis and multiple sclerosis. These factors have revitalised studies of HHV6 especially on its antigens and immunology. Most HHV6 prevalence studies use serological assays, such as immunofluorescence assay (Morris *et al.*, 1988) and anti-complement immunofluorescence assay (ACIF) (Okuno *et al.*, 1989; Robert *et al.*, 1990). ACIF was found to provide a lower background signal and stronger positive signal than IFA (Lopez *et al.*, 1988). In spite of their similar sensitivities, ACIF and IFA probably do not explore the same antibody or the same antigen. Such assays make use of HHV6 antigens prepared by infecting cells such as peripheral blood lymphocytes, cord blood lymphocytes or cell lines like HSB2 with HHV6. The infected cells are then harvested for use. Specific immunogenic proteins of HHV6 would be more

sensitive and specific antigens that can be used in diagnostic tests. The purpose of this study was to identify antigenic immunodominant sites of HHV6, with emphasis on the carboxyl terminal protein, p101.

Antigenic site or Epitope

An epitope or an antigenic site is defined as the region which interacts specifically with antibodies. In an antigen-antibody reaction, the antibody-binding site often accommodate a portion of the antigen. The part of the antigen that is the target of the antibody-binding is an antigenic determinant. One molecule may have more than one antigenic determinant (Berzofsky and Berkower, 1993). The types of antigenic sites have been categorized as sequential, continuous and discontinuous (Figure 1.5). Sequential determinants are short linear amino acids, as they occur in the protein antigens. Such determinants are rare in globular protein but can be found in fibrous proteins (Crumpton *et al.*, 1974). A continuous site is where the amino acid is spatially adjacent in a continuous peptide bond linkage. Antigenic sites consisting of amino acid residues that are widely separated but brought together on the surface by the folds (secondary or tertiary folding) are known as discontinuous antigenic sites, because they are assembled from different parts of the sequence and exist only in the surface topography of the native molecule (Benjamin *et al.*, 1984; Berzofsky *et al.*, 1985).

Antigenicity, Immunogenicity and Immunodominance

It is important to distinguish between antigenicity and immunogenicity of peptides. Antigenicity of a peptide refers to the ability of a protein or a peptide region to

bind specifically to an antibody whereas immunogenicity refers to the ability of an antigenic site to elicit antibody production. Antigenicity is an intrinsic characteristic of a peptide while immunogenicity is dependant on the quality of the T- helper cell function, immunization protocol, the nature of B and T-cell interactions and other extrinsic factors (Milich *et al.*, 1989). Certain sequences or even individual residues on the surface of proteins have been identified as immunodominant sites i.e. sites to which most of the immune response is directed (Benjamin *et al.*, 1984). Several mechanisms have been suggested to explain immunodominance. One is that special structural properties intrinsic to certain regions make these regions immunodominant. A basic view is that immunodominance is dependant on the regulatory mechanism of the host, including tolerance to structures resembling self, immune response genes and specificity of the T-cell help (Benjamin *et al.*, 1984).

With much research done in the antigen-antibody binding field, several conclusions have emerged, although many remain unanswered (Benjamin *et al.*, 1984). Antigenic determinants of proteins consist of amino acid residues in a 3-D array. In any binding, the amino acid has to make contact with complimentary residues in the antibody-binding site. Such residues are usually exposed on surface of protein (Berzofsky and Berkower, 1993). In order to mimic a protein epitope with a small synthetic peptide, it is appropriate to choose a sequence that is hydrophilic and surface-orientated. The N-termini and C-termini of proteins are generally surface orientated since they contain charged groups, i.e., NH_3^+ and COO^-. These termini are often chosen as candidates for synthesis as they possess hydrophilic and surface orientated properties.

4.2 Immunofluorescence Assay (IFA) on HHV6 Infected Human Cord Blood Mononuclear Cells (HCBMC)

Despite multiple reports on HHV6 seroepidemiology, there is a lack of standardisation in the detection and quantification of this virus. Immunofluorescence is the standard HHV6 serological method.

4.2a Detection of Specific Antibodies to HHV6 in HCBMC

4.2a (i) Serum IgG

In our work, IFA was used to confirm the presence of IgG-HHV6. All 98 infant sera tested for IgG antibody were positive. This is not in agreement with Yadav *et al.* (1990) who found a positive rate of 34% in Malaysian Kadazans. The results obtained, however, are in agreement with those reported by Chua *et al.* (1996) who found a positive rate of 83.7% in healthy Malaysians and Ablashi *et al.* (1988) who sampled on healthy donors from the US, Canada and Europe. Our data differ from data from Japan, where the positive rate was found to range from 70 to 83% for all age groups (Okuno *et al.*,1989). Besides the probable differences in technical procedures, alternative explanations for discrepancies in the prevalence could be due to geographical or ethnic differences. The possibility of high prevalence clusters and the influence of age could also significantly modify the results of epidemiological studies (Briggs *et al.*,1988; Yanagi *et al.*,1990).

The results we obtained for the presence of IgG-HHV6 antibodies in the 98 infant sera is in line with the persistence of maternal antibodies. As we obtained 100% positivity for IgG-HHV6 in the 98 sera, we conclude that maternal antibodies to HHV6

are consistently found in infants. As reported by Ward *et al.*(1993), after birth, almost all children have IgG to HHV6, presumed passively acquired maternal antibodies thus reflecting the prevalence of antibodies in the adult population as a whole. These antibody levels progressively decline over the few weeks after birth so that by the age of 6 months, many children are seronegative and hence, susceptible to HHV6 infection. Briggs *et al.* (1988) and Knowles and Gardner (1988) further confirm this. The pattern of antibody response of infants suggested that as maternal antibodies disappear, they are rapidly replaced by antibodies produced in response to antigen challenge (Pietroboni *et al.*,1988)

4.2a (ii) Salivary IgA

In our study, all 7 saliva samples were negative for IgA by IFA on human cord blood mononuclear cells infected with HHV6, which is contrary to a study by Levy *et al.* (1990), on residents of diverse areas of the world. In Levy *et al.* (1990), HHV6 infected adult peripheral blood mononuclear cells (PBMC) or MT-4 cells were used as antigens to measure the seropositivity of anti-HHV6 antibodies. In his study, Levy found that HHV6 can be found in cell-free saliva at very high frequency (>85%) and substantial titer in healthy and infected patients.

4.2a (iii) Breast milk IgA

Kusuhara *et al.* (1997) eliminated breast milk as a mode of HHV6 transmission. In our study, we tested 20 breast milk against the HHV6 infected cord blood mononuclear cells and we obtained 9 positive and 11 negative results. The finding of IgA

HHV6 in breast milk suggests that breast milk may provide another means of passive immunity besides transplacental IgG.

4.2b Age and Prevalence of Serum IgA and IgG to HHV6

We compared the seroprevalence of IgA and IgG antibodies in persons aged between 0 months and 20 years. 240 serum samples were used in this study. We found that IgA profiles do not follow the observed pattern for IgG, which is in contradiction to reports by other researches (Pietroboni *et al.*, 1988; Ward *et al.*, 1993). In their studies it was found that IgG antibody prevalence was highest in sera in the first four years of life, and consistently declined subsequently. From the data obtained, we found no correlation between the prevalence of IgA and IgG antibodies with age. However, a test of line of best fit was carried out and resulted in correlation between the IgG and IgA prevalence in subjects between 0 - 20 years old.

From Chua's study, HHV6B prevalence was lowest at the first two age groups (0-1 years, 1-2 years). Both the age groups scored seroprevalence of 77.3%. in our study, HHV6B IgG prevalence was 80% at age 0-1years and 55% at 1-2 years. Almost all have maternally derived IgG against HHV6B and protected from HHV6B infection. This was in agreement with reports from Yoshikawa *et al.*(1989), whereby infants aged 0-2 months old had maternal antibodies.

In the present study, the IgG level declined at age 1-2 years probably due to the waning of maternal antibody in the body. Starting from age 2-3, the prevalence of IgG increases until it reaches the adult level.

The early seroconversion in life is in agreement with Levy *et al.* (1990), Huang *et al.* (1992) and Ward *et al.* (1993) and suggests the possibility of HHV6B infections through saliva and oral secretion.

IgA binding to all the selected peptides were much lower than with IgG. Our study showed that IgA increased in the first 4 years of life, from 20% to 55%. At the ages of 4-5, the IgA was found to drop drastically to 20% and at age 5-6 increased to 55%. It may be possible that the IgA levels increased after an episode of reactivation. Following the increase, the antibody level drops again and a sudden increase is observed at age 9-10, after which the level consistently declines.

Morris *et al.* (1988) observed that IgA antibodies for herpes simplex, cytomegalovirus and varicella-zoster were only found when specific IgG was also detected in the serum. Thus, there may be a possibility that the presence of IgG contributes to the presence of IgA in the sera, although there has been no report on such an investigation.

In a study done by Rumbo *et al.,* (1998) on antibodies specific to food antigens, he obtained higher levels of IgG in serum compared to saliva and breast milk. However, levels of IgA obtained were higher in milk compared to saliva and serum. He concluded that the different degrees of specificity among IgA in serum , saliva and breast milk that was observed suggested that the local IgA producing populations are functionally different in the different tissues of an organism. This could be a reason to explain difference between our IgA and IgG data.

4.3 Multipin Peptide Synthesis of the HHV6 p101 Carboxyl Terminal Protein

The development of multipin peptide synthesis on polyethylene pins (Geysen, 1987) has enabled epitope mapping of immunogens, which may be relevant to vaccine design and also to the development of better diagnostic assays to be carried out.

The p101 nucleocapsid protein synthesized in this study was discovered in 1990 (Yamamoto *et al.*) and its immunogenicity was confirmed by Pellet *et al.* (1993). It was discovered that human sera which lacks HHV6 specific antibody and which is seropositive for any other human herpesvirus failed to react with this protein, indicating that it is a specific serological marker for HHV6 infections. (Yamamoto *et al.*, 1990). As this protein is found specific for variant B (Yamamoto *et al.*, 1990; Pellet *et al.*, 1993), by mapping the protein and identifying its antigenic regions/peptides, a more accurate detection is possible and the occurrence of misdiagnosis may be avoided.

In this work, the 858 amino acids of the p101 protein was subjected to hydropathy analysis and from the plot obtained, 334 amino acids from the carboxyl terminal was selected for synthesis. This selection was because the carboxyl terminal gave the highest hydropathy readings and as mentioned by Walter *et al.* (1986), the carboxyl terminus of a protein is more often than not, the most immunogenic region.

In evaluating the long-term stability of the synthesized peptides to repeated ELISA testing, Geysen *et al.* (1987) found the usefulness of the set of peptides to be limited to 30-60 tests. Bearing this in mind, the pins were subjected to no more than 60 cycles before screening was stopped.

4.3a Antigenic regions of HHV6 carboxyl terminal recognized by serum IgG

On testing the synthesized peptides of p101 against 25 sera from infants, peptides 6-7, 16-19, 22-23, 26-28, 30-31 and 44-45 were found to show higher antibody binding in the pin-ELISA (modified ELISA) (Figure 3.10 and 3.11). Since nonspecific reactions are the biggest problems with pin-ELISA, we have carried out exhaustive testing to eliminate the possible sources of nonspecific binding. The conjugate test (Figure 3.6) carried out showed very low nonspecific reactivities between conjugates and the pins, indicating the peptide synthesis to have been satisfactorily achieved.

Although both IFA-positive and IFA-negative sera reacted in the pin-ELISA, the profile between IFA-positive and IFA-negative were distinctly different (Figure 3.12). The IFA-positive sera had more dominant epitopes, and the ELISA readings for the positive samples ranged from 0.7 to 2.3 while the negative readings ranged from 0.3 to 1.1. Interestingly, there was no overlap of reactive epitopes between the positive and negative sera. The cut off value for serum ELISA was taken as 1.4, which was obtained using the mean + SD formula. The peaks for IFA-positive sera were peptides 6-7, 16-19, 22-23, 26-28, 30-31 and 44-45, all of these peptides were not significant reactive peptides against the IFA-negative sera.

Factors Influencing on Peptide Antigenicity:

There are several postulations as to why some epitopes are more reactive than others and what factors contribute to the antigenicity of peptides.

Overlapping of two peptides

Along the polypeptide chain, the most and the least reactive residues are separated by residues with average reactivity (Getzoff *et al.*, 1988). On the surface of a molecule, the most reactive residues cluster into patches, likewise the least reactive and the two sets of patches are separated by areas with average reactivity residues (Getzoff *et al.*, 1988). This phenomenon may account for the reactions observed in regions like peptides 16-19, whereby peptide 19 shows the highest reactivity. Peptide 16 and 17 showed lower reactivities but peptide 18 showed the lowest reactivity compared to the other 4. The region spanning peptide 44-45 is of special interest as it showed consistently high sensitivity and reactivity by recognizing 24 of the 25 IFA-positive sera. Peptide 45 though less reactive than peptide 44 also showed reactivity. The overlap between peptide 44 and 45 consists of 7 amino acids : DEETVPG. It is possible that the reactive site is actually the overlap of the 2 peptides.

PEPTIDE 44 : IRQDGETDEETVPG

PEPTIDE 45: DEETVPGPGVAESL

OVERLAP: DEETVPG

Peptides 6-7 also showed high frequency (68%) as well as high reactivity. The overlap between these two peptides is DSGDATE. However, of the three reactive peptides discussed here, peptide 44-45 with the reactivity of 96% has the strongest possibility of being immunogenic and immunodominant.

C and N terminal residues

For a peptide to show high reactivity, its C and N terminal residues sometimes play an important role in enhancing the binding of an antigenic determinant (Benjamini *et al.*, 1972). In our work, an example may be peptides 26-28. The reactivity of peptide 27 is higher compared to 26 and 28. An explanation may be that the residues at the carboxyl terminal of peptide 26 and the residues at the amino terminal of peptide 28 may have played a role in enhancing the reactivity of peptide 27. Likewise, we can say the same about peptide 30 and 31, whereby the carboxyl terminal of peptide 30 may have contributed to the higher reactivity of peptide 30 (FKNFKLQNDLDSVQ), which in turn caused peptide 31 (NDLDSVQSPFRLPN) to show higher reactivity with sera.

Cascade interaction of polyclonal antibodies

Another explanation for the continuous high reactivity observed in several peptides is that interaction of polyclonal antibodies can be envisioned as a cascade, which at times functions to uncouple side-chain interactions and perhaps, destabilize the original protein conformation. Thus, the binding of an antibody to one epitope would help induce co-operative conformational changes in adjoining epitopes, making the binding of antibodies to the second site more favourable, while disrupting the stability or activity of the protein antigen (Paterson *et al.*, 1985; Parham *et al.*, 1984). An example may be seen in peptides 16-19 whereby the highest reactivity is shown by peptide 19. Peptide 16, 17 and 18 showed high reactivity but the highest was observed for peptide 19. Thus, the binding of one epitope helps induce changes in the adjoining epitopes, making the

binding to the next site more favourable. For peptides 30-31, peptide 31 shows more reactivity.

For IgG testing of sera, peptide 44 showed a frequency of 96%, peptide 16-19, 84% and peptide 6-7 gave 68%. Besides showing high frequencies, they also showed high reactivity suggesting those are immunodominant determinants. However, intrinsic factors such as hydrophilicity, surface accessibility and secondary structure would have to be looked into before designating them as potential peptides for more effective testing of HHV6 infection.

4.3b Antigenic Regions of p101 Protein recognized by Breast Milk IgA

Similar to sera, breast milk samples were IFA-positive or negative to HHV6 in the immunofluorescence assay. However, when peptides were tested against IFA-positive and IFA-negative breast milk samples, a similar profile was obtained. This suggests that the reactive IgA population detected in the IFA was not reactive in the pin-ELISA. Thus, we could not differentiate between the IFA-positive and IFA-negative breast milk using pin-ELISA on the peptides.

A cut-off value of 0.8 was calculated using the mean + standard deviation (SD) formula. Peptide number 13 was of special interest as it showed consistently high reactivity with all the IFA-positive breast milk samples tested. It was not significantly reactive with the IFA-negative breast milk, suggesting that it could contain an HHV6-specific epitope.

Peptides 2-3 and 44 were picked up by both IFA-positive and IFA-negative samples and thus was designated not HHV6-specific.

Takahashi reported the acquisition of HHV6 infection by 9 months of age in breast-fed infants (Takahashi *et al.*, 1988). Kusuhara demonstrated that there was no difference in seroprevalence between the bottle-fed and breast fed infants, suggesting that milk-borne transmission is not a significant factor in early HHV6 acquisition (Kusuhara *et al.*, 1997). A study by Dunne (1993) demonstrated that none of the 120 breast milk samples tested using PCR yielded the 830bp amplicon indicative of HHV6 DNA. The primers used were the A and C primers (Aubin *et al.*, 1991). Our results suggest that besides transplacental IgG contributing towards passive immunity, IgA may also be a means of passive immunity. Table 4.1 shows a summary of the detection of HHV6 in breast milk.

4.3c Saliva IgA Reactivity to HHV6 p101 Peptides

The saliva samples we worked on were collected from healthy subjects at the Institute of Postgraduate Studies and Research, University of Malaya. All 7 salivary samples tested by IFA for IgA showed negative results. Three of the 7 IFA-negative samples were positive in pin-ELISA.

Table 4.2 shows a summary of HHV6 that has been detected in saliva. This may be in agreement with Di Luca *et al.* (1995b) who forwarded the hypothesis that it is possible that HHV6 antigen could be present in saliva but could not be detected by IFA.

Mukai *et al.* (1994) showed that primary infection of HHV6 in young children can be from mothers secreting HHV6 in their saliva. Di Luca *et al.* (1995b) showed that HHV6 is not shed in large quantities in the saliva, indicating that another route of

transmission may exist and that the likelihood of salivary transmission would depend on the rate of reactivation and the load of infectious particles.

A controversial issue is the presence of HHV6 and HHV7 in the saliva. The majority of published studies attempted virus isolation from saliva, but with contrasting results. Some reported the exclusive presence of HHV6 (Levy *et al.*,1990; Harnett *et al.*., 1990) whereas others isolated only HHV7 (Wyatt and Frenkel, 1992; Hikada *et al.*, 1993). Kido (1990) reported low prevalence of HHV6 in salivary specimens. However, these results conflict with other studies which detected HHV6 in the majority of salivary samples analysed (Gopal *et al.*,1990; Jarrett *et al.*, 1990; Levy *et al.*,1990; Cone *et al.*, 1993). A possible explanation could be the amount of saliva analysed. If the virus load is low, the analysis of small amounts of saliva would give negative negative results, underestimating the prevalence of HHV6 (Di Luca *et al.*,1995b). There is also a discrepancy on the amount of HHV6 DNA sequences present in salivary fluid. Jarrett (1990) estimated that fewer than 100 target molecules were present in 1 ml of saliva, and Cone (1993) estimated an average of more than 50,000 HHV6 DNA molecules per ml. Our study showed the presence of HHV6 antibodies in saliva as we obtained positive IFA results.

Di Luca also suggested that HHV6 and HHV7 are present in salivary glands and that saliva of healthy adults harbour HHV7 but not HHV6, suggesting that HHV6 establishes a latent or chronic infection with low levels of replication or occasional reactivation. HHV7, by contrast, was reported to undergo an active replication in salivary glands with continuous shedding of cell-free infectious virus (Di Luca *et al.*,1995b).

Huang *et al.* (1992) tested sera for IgG antibody against HHV6 by IFA. 80% of mothers were positive for IgG HHV6 at delivery. Babies were found to lose maternally derived antibodies by 6 months. This agrees with our finding whereby as age increases, the prevalence of antibody decreases (Table 3.3b). At age 0-1, the antibody level was 80% and between ages 1-2, it dropped to 55%. Huang postulated that the high chance of infection from 6-12 months may be related to the frequent oral exploration of the environment by children of this group that allows contact with saliva.

Table 4.1 :Detection of HHV6 and HHV7 Antigens and Antibodies in Breast Milk

Detection method	Reference	HHV6	HHV7
PCR	Dunne *et al.*, 1993	_	ND
Indirect immunofluorescence assay	Kusuhara *et al.*, 1997	_	ND
PCR	Fujisaki *et al.*, 1998	ND	+

Table 4.2 :Detection of HHV6 in Saliva

Detection method	Reference	HHV6
Antibody assays Immunofluorescence assay (IFA)	Pietroboni *et al.*, 1988	+
Immunofluorescence assay (IFA)	Levy *et al.*, 1990	+
Antigen assays In situ hybridisation and immunohistochemistry	Fox *et al.*, 1990	+
Virus cultivation	Harnett *et al.*, 1990	+
Polymerase chain reaction (PCR)	Jarrett *et al.*, 1990	+
Restriction enzyme polymorphism	Wyatt and Frenkel, 1992	–
PCR	Cone *et al.*, 1993	+
PCR	Di Luca *et al.*, 1995	+
Nested (PCR)	Aberle *et al.*, 1996	+
Cell culture studies	Levy, 1997	+

Wyatt and Frenkel failed to isolate saliva-borne HHV6. Levy, who propagated HHV6 in PBMC, found that HHV7 can activate HHV6 in PBMC (Levy *et al.*, 1990). Wyatt and Frenkel also found that HHV6 replicates more efficiently than HHV7 and limited propagation of mixtures of both viruses results in selection of HHV6 (Wyatt and Frenkel, 1992). Table 4.2 shows a summary of HHV6 that has been detected in saliva.

4.4 Comparison of HHV6 Epitopes Defined by Serum, Breast Milk and Saliva Antibodies

It was observed that peptides 23, 25, 37 and 41 reacted with breast milk but not with saliva. Reactivities of different samples, in this study sera , saliva and breast milk, may be directed to different HHV6 antigenic binding sites. It is known that the recognition site is also dependant on the type of antibody. Antibodies differ from one another in their molecule size, electrical charge and antigenic properties.

Of the 7 salivary IFA IgA-negative samples, 3 were subjected to pin-ELISA and found to bind to peptides 2, 9, 21 , 30 and 44. Peptide 44, which binds to 2 of the 3 saliva samples tested gave the highest reactivity. As the number of samples we used was very low, the percentage error is correspondingly large. A larger sample number would be required to confirm that these peptides are indeed immunogenic. But, due to the inaccuracy of the duplicacy of the pin-ELISA readings, we could not do more saliva samples.

Figure 4.1 shows the regions that are recognized by serum IgG, salivary IgA and

VSLIKDLRDKDGFRKQKKLDLLGSWTKEKNDKAIVHSREVT
HSREVT
RDKDGFRKQKKLDLLGSWTKE
KQKKLDLLGSWTKE

GDSGDATETVTARDSPVLRKTKHANDIFAGLNKKYARDVSR
GDSGDATETVTARDS
PVLRKTKHANDIFAGLNKKYARDVSR
PVLRKTKHANDIFA

GGKGNSRDLYSGGNAEKKETSGKFNVDKEMTQNEQEPLPNL
FNVDKEMTQNEQEPLPNL
GGKGNSRDLYSGGNAE

MEAARNAGEEQYVQAGLGQRVNKILAEFTNLISLGEKGIQDI
MEAARNAGEEQYVQAGL LAEFTNLISLGEKGIQDI
ISLGEKGIQDI

LHNQSGTELKLPTENKLGRESEEANVERILEVSDPQNLFKNFK
LHN LPTENKLGRESEEANVERILEVSDPQNLFKNFK
LHN LPTENKLGRESEEA VSDPQNLFKNFK
LPTENKLGRESEEA

LQNDLDSVQSPFRLPNADLSRDLDSVSFKDALDVKLPGNGER
LQNDLDSVQSPFRLPN
LQNDLDSVQSPFRLPNADLSRDL
GNGER

EIDLALQKVKAGERETSDFKVGQDETLIPTQLMKVETPEEKD
DLALQKVKAGERET QLMKVETPEEKD
EIDLALQKVKAGERET QLMKVETPEEKD

DVIEKMVLRIRQDGETDEETVPGPGVAESLGIAAKDKSVIAS
IRQDGETDEETVPGPGVAESL
DV IRQDGETDEETVPGPGVAESLGIAAKDK
DVIEKMVLRIRQDGETDEETVPG

Figure 4.1 : Comparison of reactive regions recognized by serum IgG (coloured in red), breast milk IgA (coloured in blue) and saliva IgA (coloured in green).

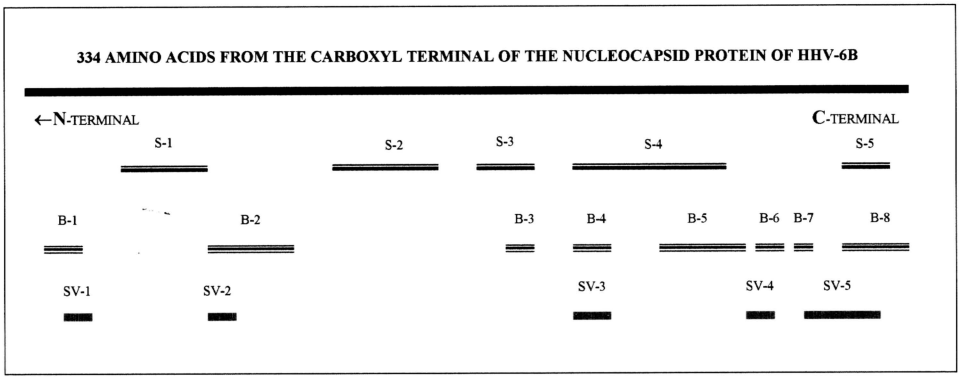

Figure 4.2 : The profile of HHV-6B sequence showing the epitopes as detected by various sources of sera (S-1→S-5), breast milk (B-1→B-8) and saliva (SV-1→SV-5).

breast milk IgA. Breast milk IgA and salivary IgA recognize similar epitopes, but, some peptides recognized by serum IgG are different from those recognized by salivary or breast milk IgA.

4.4a Potential Use of HHV6 Peptide ELISA for HHV6 Infection Investigation

In an analysis by Sloots *et al.* (1996), results obtained by IFA and ELISA were in close agreement, suggesting that ELISA provides a suitable test method for the determination of HHV6 IgG antibodies in routine clinical labs. Of the 502 samples they tested, 79% were positive, 14% negative and 7% gave different results obtained by IFA and ELISA. Further absorption of sera prior to assay revealed that the majority of these results were false positive or false negative in IFA (true positive or negative in ELISA). According to Sloots *et al.* (1996), the poorer performance of the IFA may be a reflection of the subjective nature of interpretation of this test.

The virus strain used for infection was the Hashimoto strain (from Dr. Chua K.B.) of HHV6 isolated from an exanthem subitum patient. The method of titration of HHV6 is as described by Asada *et al.* (1989). The viral preparation method is specific for HHV6 and thus does not allow any cross-reactivity (Kondo *et al.* 1991). Through the virus preparation method, it is clear that the IFA test is specific, but not the pin-ELISA method. Cross-reactivity in pin-ELISA is unlikely as it does not use propagated whole viruses.

According to Dahl *et al.* (1990), high ELISA IgG titers were usually found in sera with high IFA titers but no significant correlation was observed with the degree of antibody titer. The ELISA antigen used by Dahl *et al.* (1990) consisted of whole infected

cells. In our study, we used peptides. IFA detects antibodies to native proteins whereas the peptides would detect antibodies to linear short sequences. It is therefore not surprising that some discrepancies between IFA and pin-ELISA were observed.

Through pin-ELISA, which was done for serum, saliva and breast milk, specific peptides were selected as reactive for each body fluid. One peptide that showed consistently high reactivity with all three body fluids was peptide 44. The question that arises here is whether detection of peptide 44 (IRQDGETDEETVPG) in pin-ELISA is sufficient for a patient to be considered positive for HHV6 infection. Our studies indicate that peptide 44 is a reliable marker for HHV6 infection investigation. When the 25 sera samples were tested against the peptides, highest reactivity was obtained for peptide 44. Of the 25, 24 sera samples (96%) reacted with peptide 44. Peptide 44 may be useful for a more rapid and accurate detection of HHV6 infection. However, for breast milk IgA, peptide 44 was recognized by both IFA-positive and negative samples and thus could not be designated as a marker for infection. For IgA, peptide 13 seemed to be a more likely candidate as an HHV6 infection marker.

Identification of the epitope boundary of peptide 44 will determine the exact location and number of amino acids that contribute to this antigenic site. This can be done by synthesizing a set of peptides of different lengths spanning the length of the peptide.

4.4b IgA-HHV6 in Breast Milk

The epitope regions that were found reactive for IgG in a cord blood sample were similar to the region bound by IgG in infant sera. This is in agreement with the fact that IgG is transferred through maternal antibodies from mother to infant (Ward *et al.*, 1993).

Our finding is in agreement with the findings of Huang *et al.* (1991) who found that babies lose maternal antibodies within 6 months of birth. Takahashi *et al.* (1988) found that infants acquire exantham subitum within 9 months of birth, probably as maternal antibody wanes.

However, when cord blood was tested for IgA antibodies, negative results were obtained, demonstrating that IgA does not get transferred in maternal antibodies.

4.5 Association of Hydropathy, Surface Accessibility, Secondary Structure And Pepscan Analysis with HHV6 p101 Peptides

The first step in defining the chemistry of antibody binding to protein antigens is to identify the intrinsic features of a protein that distinguish the immunologically most frequently reactive and least frequently reactive sites. Among commonly known methods of prediction, are that described by Hopp and Woods (1981,83) and that by Kyte and Doolittle (1982). However, it was apparent that no single parameters set was able to predict all antigen sites (Parker *et al.*, 1986). As mentioned earlier, other proposed parameters are hydrophilicity, hydrophobicity, secondary structures, amino acid specificity and surface accessibility. Each of the factors may contribute in enhancing or decreasing the reactivity of the site and these properties are closely associated with one another (Getzoff *et al.*, 1988).

Hydrophilicity

In order to limit the number of peptides to be tested in this study, the peptides present in the hydrophilic region were selected to be synthesized. Since antigenic epitopes usually occur on the surface of folded globular proteins, they are usually made

up of hydrophilic amino acids. Thus, it was of interest to delineate the hydrophilic regions/amino acids. This was carried out using Hopp & Wood (1981) hydrophilicity plot (Figure 3.25). This approach was based on the supposition that most epitopes will be found on the protein surface and that relative hydrophilic stretches of the protein are more likely to be on the surface. Globular proteins generally fold, so many of the hydrophobic residues will be buried in the protein core, while many of the hydrophilic residues are located on the protein surface in order to maximize good energy interactions with the aqueous environment.

Immunogenic epitopes, on the other hand, need not be present on the surface, as it was found with Epstein-Barr virus (Abu Samah, Thesis, University of Malaya, 1997) and with the foot and mouth disease virus (Meleon and Barteling, 1986). Our results show that the significant peptides, 6-7 , 16-19 and 44-45 for IgG and peptides 13 for IgA reactivity fell in the hydrophilic regions. In the case of serum, reactive peptides 30-31 and 22-23 were hydrophobic residues since they did not fall within the hydrophilic region.

It has been suggested that hydrophobic areas enhance the binding through a hydrophobic interaction with antibodies (Singer 1965). This implies that in addition to having an area complementary to the antigenic determinant, the antibody combining site contains hydrophobic areas complementary to the hydrophobic areas of the antigenic determinant (Benjamini *et al.*, 1972). Proteins cannot exist in aqueous solutions as stable monomers with too many hydrophobic residues on the surface. When a hydrophobic residue in a protein antigenic determinant interacts with a corresponding hydrophobic residue in the antibody-combining site, the water molecules in contact with each other are excluded and this results in stabilization of the interaction (Sela, 1969). This may be the

reason for reactivity in hydrophobic regions such as peptides 22-23 and 30-31. Thus, hydrophilicity alone cannot be used to determine antigenicity as there are evidence of hydrophobic residues involved in antigenic sites.

A number of hydropathy scales have been proposed for the predictions of antigenic determinants. The predictive methods rely on the correlation between structural properties of proteins and experimentally determined antigen site in proteins of known structure. Prediction also depends on accessibility and flexibility of parameters to produce a composite surface profile (Parker 1986).

Hopp and Woods (1981) assigned each amino acid in the sequence of a protein sequence a hydrophilicity value. The hydrophilicity values are then repetitively averaged down the length of the polypeptide chain, generating a series of local hydrophilicity value. First, an average of 6 hydrophilicity value was used, which resulted in 100% correct prediction (Hopp and Woods, 1981). However, in 1983, Hopp and Woods suggested that it may be more prudent to synthesize more than the six residues with the highest hydrophilicity average as other investigators found that addition of amino acids flanking the antigen sequence often enhance the antigenic reactivity of a given sequence, probably by imparting a more native conformation to the sequence (Crumpton *et al.*,1974).

Secondly, it is a possibility that the predicted proteins may be immediately to one side of the natural antigenic determinant, so several additional residues are required to ensure a good overlap of the synthetic peptide on the antigenic site (Hopp and Woods, 1981).

Surface accessibility

It has been established that the binding of antibodies to molecules is likely to occur on sites at the exposed part of the protein. Surface accessibility is one of the requirements for an antigenic determinant to be bonded by an antibody specific for the native conformation, without any requirement for unfolding of the structure (Berzofsky *et al.*, 1985). For an antigenic site to be contained completely within a single continuous segment of protein sequence, the site is likely to have to protrude from the surface, as otherwise residues from other parts of the sequence would fall within the area of the contacting antibody (Barlow, 1986).

From our work, peptide 6-7, 13 and 44-45 all fell within the accessible area. As they were both hydrophilic as well as accessible, it was not surprising that they showed the highest reactivities with antibodies. An interesting observation was that, peptides 16-19 did not fall within the accessible region, although it was hydrophilic.

Secondary structure predictions

Hydropathy profile usually reveals too many hydrophilic regions. In such a case, secondary structures would provide additional help in locating the most likely region to be antigenic. It is appropriate to consider secondary structure prediction methods simultaneously with hydrophilicity methods because protein secondary structure (helices, strands/sheet and bends/coil) depend on the hydrophobic nature of local protein chain segments and are therefore inextricably related to the hydrophilicity methods. Short helices have been indicated as highly exposed segments and are therefore likely to contain antigenic sites (Benjamini *et al.*, 1972). Turns (coils) in a secondary structure also

frequently exist as protrusions from the main body and may preferentially be recognized by antibodies.

The additional two parameters, mobility and coil structure were used to support the findings using hydrophilicity, accessibility and secondary structure. From the present study, analysis of both peptide 13 and 44, which are both potential antigenic sites, fell within the mobile segment (Table 3.15, 3.16). Notably, these two peptides are also hydrophilic, surface accessible and having secondary structures of helix-coil-helix and coil-helix-coil structure respectively. As for the coil parameter, both peptides 13 (residue 84-98) and 44 (302-329), correspond to coil structures at residues 80-97 and 308-321 respectively. The coil structure parameter was used to support the fact that the potential antigenic regions were indeed made up of coils (besides helices). In our study, the significant peptides are peptides 6-7, 13 and 44-45. All were made up of coils and helices only (Tables 3.13 and 3.14 and Appendix 2).

However, there is no proof that secondary structure considerations are useful in limiting the number of possible choices given by hydropathy profiles. It has been documented that identical pentapeptides gave completely different secondary structures in different proteins (Walter 1986). It has been pointed out that secondary structure can only be predicted with 56% accuracy.

Hydrophilicity studies of the p101 nucleocapsid protein predicted 6 amino acid regions to be hydrophilic. Of these 6 regions, 3 considered significant being peptides 6-7, 16-19 and 44-45 for serum IgG. As has been explained earlier we consider peptides 6 and 44 to be immunodominant and reactivities of peptides 7 and 45 are probably due to the overlapping amino acids. Peptide 44 showed higher reactivity and higher frequency

(96%) than peptide 6 and 45, and the more likely candidate as a diagnostic reagent. Predicted secondary structure of the 6 hydrophilic regions and the most reactive region from our study were combinations of sheets, coils and helices. The most reactive peptide from our study is peptide 44, with a coil-helix-coil structure.

In the case of IgA reactivity with breast milk, only one region, peptide 13 was identified as antigenic. Peptide 13, which has a coil-helix-coil, structure. This peptide showed a very high frequency and is a likely candidate as a diagnostic reagent.