DISCUSSION

CHAPTER FIVE: DISCUSSION

5.1: MOLECULAR DIAGNOSTIC TECHNIQUES

The PCR has been widely used to detect persistent and latent HHV-6 infection in healthy individuals (Lopez *et al.*, 1988; Gopal *et al.*, 1990; Jarrett *et al.*, 1990) and patients with pathological conditions such as AIDS (Buchbinder *et al.*, 1988; Corbellino *et al.*, 1993), HD (Torelli *et al.*, 1991; Levine *et al.*, 1992) and cancer (Bovenzi *et al.*, 1993; Chen *et al.*, 1994b; Wang *et al.*, 1994a; Yadav *et al.*, 1994).

PCR was employed in this study to detect HHV-6 DNA by amplification of the LTP gene sequence in various paraffin-embedded samples. As the PCR is highly sensitive, the utmost care was taken to avoid the occurrence of false positives. In general, false positives are generated from sample to sample contamination or amplicon carryover (Kwok and Higuchi, 1989).

Sample to sample contamination was avoided by aliquotting sample DNA as the last component into its' respective reaction tubes, taking care not to introduce contact with any other surface. The used PCR tip was disposed carefully into detergent quickly without allowing the

potential target DNA that may be present in the tip to escape into the laminar flow hood atmosphere. As an added precaution, positive displacement pipettors and disposable plugged pipette tips were utilized to prevent aerosol formation which could lead to cross-contamination. All items, except the PCR reagents and DNA samples, were UV sterilized in the laminar flow hood prior to PCR.

Amplicon carryover is more serious, requiring stringent procedures to minimize or avoid it. Separate areas were set up for pre-PCR work (DNA extraction of samples, preparation of PCR reagents, sterilization of items essential for PCR) and post-PCR work (analysis of amplified PCR products, restriction enzyme typing, hybridization experiments). Aliquotting of the PCR reaction mix was carried out in a laminar flow hood dedicated solely for PCR reagents.

Other precautions included the changing of gloves frequently, minimum sample handling and quick centrifugation of tubes prior to opening. Negative controls were placed in strategic positions throughout the PCR experiment to detect any form of contamination so that the source of contamination could be easily discerned.

False negatives were minimized by carefully aliquotting reagents and sample DNA into each PCR tube making sure that all reagents were dispensed completely. An internal control, a sequence in the

β-globin gene, was amplified to confirm the integrity of the extracted sample DNA.

Southern hybridization was performed on PCR products with a nonradioactive PCR labelled probe. The plasmid ZVH14 was labelled by the incorporation of dig-dUTP (Boehringer Mannheim). The sensitivity of the probe was equivalent to that of radioactive probes and it permitted speedy detection, with low hazard potential during handling. In addition, the labelled probe could be stored for long periods in contrast to radioactively labelled probes (Lion and Haas, 1990).

In situ hybridization has also been used to detect HHV-6 infection with the added advantage of localizing infected cell types (Fox *et al.*, 1990; Krueger *et al.*, 1990; Levine *et al.*, 1992; Shen *et al.*, 1993; Sumiyoshi *et al.*, 1993; Chen *et al.*, 1994b). It is a sensitive, specific and rapid means for establishing viral presence. In this study, a nonradioactive method for *in situ* hybridization was utilized. The oligonucleotide probes used in the NISH technique were labelled nonradioactively, the 3'-tailing method allowed sensitive detection of viral DNA (Boehringer Mannheim, 1993). The labelled probes could be stored indefinitely without losing their activity but diluted probe solutions lasted for approximately one month.

As a precaution, hybridization buffer containing the probe was sealed in with sample tissue using sealing glue to reduce dehydration

of the tissue. NISH was carried out with a positive control and a 'no probe' control to facilitate the interpretation of positive signals. Due to the lack of a well defined 'gold standard', HHV-6 infected MOLT-3 cells were subjected to NISH and utilized as an external standard of sensitivity and specificity.

Similarly, IHC has also been used to recognize active HHV-6 infection in a variety of tissues, including the salivary gland and lung; and in biopsies of renal transplant and AIDS patients (Fox *et al.*, 1990; Krueger *et al.*, 1990; Okuno *et al.*, 1990; Pitalia *et al.*, 1993; Knox and Carrigan, 1994). A relatively simple detection method, IHC relies on the visualization by colour of the antigenic reaction between MAb and viral antigen. As the only obvious problem encountered was dehydration of cells in the tissues, precautions were taken not to allow the tissues to dry between solution changes as this introduced background staining. Sample tissues were placed in a humidified chamber during the experiment to reduce moisture loss. In this study, a positive and negative control was used to determine the success of each IHC experiment.

5.2: DETECTION OF HHV-6 IN ORAL MUCOSAL TISSUES

HHV-6 specific DNA and proteins were found in a high proportion of oral tissues. Nested PCR followed by Southern hybridization revealed the presence of HHV-6 DNA in 40.5% of oral SCC cases, 33.3% of leukoplakic lesions and 14.3% of lichen planus cases. None of the normal tissue amplified viral DNA. Southern hybridization was used in this instance to confirm the specificity of the PCR amplification and also to permit the detection of small amounts of amplified product that may go unnoticed on agarose gels.

Both HHV-6 variant A and B were present in almost equal proportion in the SCC cases. No mixed infection was observed, but this does not reflect the true nature of the viral population in these tissues. Studies have shown that PCR amplification tends to present an imbalance between variants as minority genotypes within a sample cannot be detected (Dewhurst *et al.*, 1992; Aubin *et al.*, 1994).

A study by Yadav *et al.* (1994) on oral SCC showed that HHV-6 DNA was observed in 67% of fresh frozen carcinoma tissue and 71% of formalin-fixed, paraffin-embedded carcinoma tissue by nested PCR and IHC. Though the PCR results do not correspond with Yadav *et al.* (1994), NISH and IHC of the present oral carcinoma tissues revealed

significantly higher percentages of HHV-6 positivity, that is 78.6% and 88.1% respectively.

While the PCR has been touted as a highly sensitive detection method, it is still subject to the starting material used and the amount of target DNA present in a sample. Sampling options for PCR assays are many, including saliva, blood and swabs from body fluids, however the frequency of oral carcinoma patients in the University Hospital and the Seremban General Hospital at any given time was not sufficient to conduct this study. The use of archival formalin-fixed and paraffin-embedded tissue allowed the analysis of a greater sample size and better manipulation of the samples. Not only could PCR studies be carried out, corresponding *in situ* DNA hybridization and viral antigenic assays could be performed on the same samples to help elucidate the role of HHV-6.

A major problem observed in these tissues was excessive formalin-fixation which degrades DNA. Despite this, PCR amplification of short DNA sequences (~100 bp) is still feasible (Shibata *et al.*, 1988; Honma *et al.*, 1993). The primers used in this study (Dewhurst *et al.*, 1993) amplified an approximately 830 bp and 750 bp DNA fragment, which allowed restriction typing of HHV-6 variants. PCR amplification however, may only take place when the target DNA sequence is intact, even though all samples in the study amplified a 268 bp β-globin gene

sequence. In samples that have been subjected to excessive formalin-fixation, the possibility of amplifying an 830 bp target DNA fragment is greatly reduced as the target region may have degraded resulting in false negatives.

Internal primers that amplify a smaller region within the 830 bp sequence could have been selected for nested PCR but this would have compromised the objective of the project, to detect HHV-6 infection and type the variants present. Combining HHV-6 detection with variant identification would give a better understanding of HHV-6 pathogenicity and epidemiology (Agut *et al.*, 1993). A nested protocol was used to increase detection sensitivity. Both primers A and C amplify a highly conserved LTP gene sequence in HHV-6 (Aubin *et al.*, 1991). HS6AE and HS6AF are internal primers just within this region (Dewhurst *et al.*, 1993). Digestion with *Hin*d III permits the detection of variant A and B strains, for which the latter exhibits a restriction site.

Amplification of HHV-6B DNA with the internal primers (Dewhurst *et al.*, 1993) led to an approximately 750 bp fragment, which when subjected to *Hin*d III digestion, cleaved into 2 fragments of approximately 530bp and 220bp. This was readily discerned on a 2% agarose gel. Restriction enzyme digestion of a smaller sequence would have resulted in two fragments of similar sizes which would be difficult to

identify on 2% agarose gels. Thus, primers HS6AE and HS6AF were indispensable to the study.

Low copy number of viral DNA in sample tissues might have been responsible for the reduced detection of HHV-6 by PCR (Kondo *et al.*, 1991; Honma *et al.*, 1993). Similarly the distribution of HHV-6 in a tissue sample also influences PCR amplification. In the event that the virus is present in a small interior portion of cells within a tissue , loss of the virus-infected tissue during sampling may have resulted in false negatives (An and Fleming, 1991). Nevertheless in this context, analysis of HHV-6 prevalence in formalin-fixed, paraffin-embedded tissue by PCR, when combined with NISH and IHC provides a more complete understanding of the virus.

NISH performed on corresponding formalin-fixed paraffin-embedded oral tissues revealed much higher rates of HHV-6 infection. The ability to detect HHV-6 DNA and determine the infected cell type may help assess the role of HHV-6 in these tissues. The localization of HHV-6 DNA was carried out using 3'-tail labelled oligonucleotide probes that distinguished variant A from B. Of the 78.6% of oral SCC cases positive for HHV-6, almost half exhibited DNA of both A and B genotypes. This was not observed in restriction typing of PCR products. The increase in detection sensitivity could be attributed to the oligonucleotide probes and

labelling method used. The 3'-tail labelling resulted in high probe sensitivity due to incorporation of at least 5 digoxigenin molecules per 50 nucleotides. The probes were 18 bp in size and tails varied between 10-100 nucleotides. The probe size was also advantageous for tissue permeabilization, allowing easy access to DNA within a cell. Even fragmented viral DNA could be detected thus downplaying the effects of formalin-fixation on these tissues.

Our study shows that while both HHV-6A and B were observed in oral carcinoma, HHV-6B was more common. HHV-6 variant B appears to be the causative agent for ES; it is found in healthy children and adults (Dewhurst *et al.*, 1992; Dewhurst *et al.*, 1993; Yamamoto *et al.*, 1994), transplant recipients (Yalcin *et al.*, 1994), immunosuppressed patients (Drobyski *et al.*, 1994) and Sjogren's Syndrome (Ranger-Rogez *et al.*, 1995). Although the HHV-6A prototype, GS, was isolated from lymphoproliferative disorders and AIDS, a clear association with any disease process is yet to be seen (Salahuddin *et al.*, 1986). However, it is not known if the predominance of variant B strains in oral carcinoma is due to any growth advantage over variant A strains.

The paraffin-embedded oral tissue were also analyzed by IHC. This technique allows the detection of viral antigens at the time of fixation during active viral infection. Latent HHV-6 infection cannot be

detected (Lusso and Gallo, 1995). In contrast, PCR assays do not discriminate latent from active viral infection. It is interesting to note that in the oral SCC tissue, expression of at least one viral protein was observed in 88.1% of cases.

Both the MAbs used in the present study were reactive to viral antigens conserved among various HHV-6 strains, reacting with U1102 (A type) and Z29 (B type) type strains. HHV-6 p41 is an early-late protein homologous to HCMV ICP36, a processivity factor that stimulates viral replication. The ability of p41 to bind single-stranded DNA suggests a similar function for HHV-6 replication. Therefore, p41 DNA-binding protein essential for viral propagation, was expressed in half of the HHV-6 cases positive for IHC. The expression of gp116/64/54, the gB homologue for HHV-6 was also detected concurrently except in 4 cases. The MAb 6A5G3 reacts with gp116 and its cleavage products, gp64 and gp54 (Balachandran *et al.*, 1989). This major envelope glycoprotein complex was expressed in most of the oral SCC cases and is important for viral entry. Thus the expression of both antigens indicated the status of HHV-6 in these tissues was probably that of active replication.

Yadav *et al.* (1994) observed the expression of gp116/64/54 in 7/7 (100%) paraffin-embedded oral SCC tissue localized in the transformed cells while PCR of the same cases detected HHV-6 DNA in

71% of the tissues suggesting that IHC is more sensitive in the detection of HHV-6-specific antigens than PCR for the amplification of HHV-6 DNA. This is a similar scenario for the present study. NISH, however seems to be on par with IHC thus allowing comparisons between the two methods. The importance of these techniques lie in the indication of the type of cells infected by the virus than merely acknowledging its' presence in a tumour.

While HHV-6 DNA was observed in 78.6% of oral SCC tissues by NISH, HHV-6-specific proteins were expressed in 88.1% of these cases. The difference in percentage indicates the detection of HHV-6 antigens in the absence of HHV-6 DNA. Since IHC detects active viral infection, the inability to support HHV-6 antigen expression with viral DNA is suggestive of low viral copy number.

The distribution of HHV-6 DNA and antigens was mostly in the transformed cells of the oral SCC. The staining of the outer layer of cells surrounding keratin pearls was of much interest. Invasive carcinomas such as well differentiated SCC contain neoplastic cells that function by forming keratin which appear as circular masses or 'pearls' (Lucas, 1976). The selective intense immunohistochemical staining of some keratin pearls for both gp116/64/54 and p41/38 was surprising as it suggested that the keratin producing cells were either infected by HHV-6 or the MAbs were

non-specifically binding to the keratin. Adsorption studies to show that the MAbs used were specific for HHV-6 proteins confirmed the lack of non-specific binding. Also, the same MAbs did not react with the keratin pearls in cervical carcinoma. Upon NISH, it was noted that the transformed outer layer and cells surrounding the keratin pearls contained HHV-6 DNA sequences. Although the significance of this observation is not clear, viral replication in the cells outside the keratin pearls produce viral proteins which may be extruded into the keratin due to a natural affinity to the keratin serving to alter its' function and disrupt the structural integrity of the tissue.

Active HHV-6 replication was mostly observed in the transformed cells, occasionally viral proteins and DNA were noted in the normal columnar epithelial cells, lining glandular ducts. The presence of HHV-6 in the glandular cells is probably reflective of the viral persistence in salivary glands (Fox *et al.*, 1990; Krueger *et al.*, 1990). The results suggest that HHV-6 may have ascended through the ducts of the salivary gland where it persists, to infect the glandular cells found in the oral tissue. These cells may have a role in the propagation and distribution of the virus. However, it is not known if the virus spreads from the glandular cells to the transformed cells or vice versa.

Interestingly, the squamous epithelial cells of half the histologically normal oral tissue also showed the presence of viral DNA and proteins. The observation of active infection in these normal tissues suggests that the virus may infect a cell without causing any noticeable changes. Therefore, the inclusion of premalignant oral lesions was essential to determine the role of the virus in relation with SCC and normal tissues. Commonly observed premalignant lesions are leukoplakia and lichen planus. Most (92.3%) of the premalignant tissues were NISH positive for HHV-6 DNA sequences in the sites of leukoplakic and lichen planus lesions.

A mucocutaneous disease of the mouth, lichen planus forms oral lesions in which the epithelium is atrophic. One to ten percent of oral lichen planus cases lead to carcinoma (Lucas, 1976). Leukoplakia is referred to as a white plaque on the oral mucosa which cannot be attributed to other diagnostic entities. Prominent dysplastic lesions observed in leukoplakia are considered as surface markers associated with malignancy in which different proportions of leukoplakia have become carcinomatous in various populations. The average rate for malignant transformation is six percent (Lucas, 1976; Eveson, 1983; Pillai *et al.*, 1994).

Based on NISH and IHC, HHV-6 infection was detected in a large proportion of oral SCC and premalignant tissues and in about half of the normal tissue studied. Infection with HHV-6 in the normal tissues does

not predispose a tendency towards tumourigenicity. Rather the presence of the virus in these tissues is probably indicative of reactivation or reinfection. However the high prevalence of HHV-6 in the premalignant lesions suggests a role for the virus since NISH shows that the virus-producing cells are concentrated in the dysplastic lesions.

HHV-6 may play a cofactorial role in tumour progression. HHV-6 DNA sequences with the ability to neoplastically transform nontumourigenic mouse fibroblasts (NIH3T3) and human epidermal keratinocytes (RHEK-1) *in vitro* have strengthened the possibility of a viral aetiology in oral carcinogenesis (Razzaque, 1990; Razzaque and Puri, 1992; Razzaque *et* al., 1993). Oral carcinogenesis is a multistep process involving multiple cofactors, including oncogenes.

Proto-oncogene activation by biological, chemical or physical carcinogens results in oncogenes that are associated with different stages of carcinogenesis. Proto-oncogenes are important in the growth regulation of normal tissues. Oncogene activation in the form of mutation caused by viruses, chemicals or ionizing radiation leads to the formation of abnormal products or altered gene expression, thus losing all normal growth constraints (Scully, 1992). Mutations of H-*ras* and p53 have been found in oral carcinomas, especially in smokers. Also, the elevated expression of *bcl*-1, c-*erbB*-1, *int*-2 and c-*myc* at various stages may correlate with

malignancy (Berenson *et al.*, 1989; Field *et al.*, 1991; Saranath *et al.*, 1991).

The p53, a tumour suppressor oncogene on the chromosomal band 17p13 is of great interest as recent evidence indicate that it is also the site of HHV-6 integration (Luppi *et al.*, 1994; Torelli *et al.*, 1995). Integration of HHV-6 was noted at the short arm of chromosome 17 (17p13) in HD, NHD and multiple sclerosis (Torelli *et al.*, 1995). However, the integration status of HHV-6 in the oral tissues is unknown. Elevated expression of p53 has been observed in SCC of the upper aero-digestive tract which may have been caused by carcinogens found in tobacco or betel nut quid (Field *et al.*, 1991). In oral carcinoma, p53 over-expression is moderate to highly prevalent suggesting a role for HHV-6 in the dysregulation of p53 by its' integration at chromosome 17p13 (Langdon and Partridge, 1992; Warnakulasuriya and Johnson, 1992; Torelli *et al.*, 1995).

Our study included cancer biopsies of patients from the three major ethnic races, and all tissues were obtained for a defined period of time. To maintain an unbiased study, the medical reports of the biopsied patients were kept double blind prior to HHV-6 studies and all tissues in a consequential series were used. It was found that most of the oral carcinoma patients were Indians with a history of betel nut/tobacco quid

chewing, a common practice among the older generation. The risk of oral cancer development is almost eight times higher in chewers, and the development of the tumour appears to be a direct result of carcinogens in the betel nut/tobacco quid (Jussawalla and Deshpande, 1971) which together with other cofactors, possibly viral, induce carcinogenesis (Yadav *et al.*, 1994).

In line with multifactorial carcinogenesis, HHV-6 may also act in concert with HPV and HSV-2, two viruses that have long been associated with oral carcinoma. Not only are HPV and HSV strongly associated with oral carcinoma, both viruses contain DNA sequences with oncogenic capabilities in the presence of cofactors (Iwasaka *et al.*, 1988; Maden *et al.*, 1992). Indeed, HHV-6 contains transactivators that upregulate the expression of HPV-18 E6 and E7 genes (Chen *et al.*, 1994a). The E6 gene product can bind to p53 protein and inactivate its normal function leading to uncontrolled cell proliferation (Scully, 1992). HHV-6 may, via synergistic mechanism, contribute to oral carcinogenesis in HPV-infected cells.

All these observations coupled with the data obtained in the present study lend support to the hypothesis of multifactorial carcinogenesis for oral carcinoma in which HHV-6 may very likely be a cofactor.

5.3: DETECTION OF HHV-6 IN SALIVARY GLAND TISSUES

The salivary gland has been the subject of much excitement since the isolation of HHV-6 from saliva. The frequent detection of the virus in healthy adult populations, with seroprevalence ranging between 80-100% and the early age of seroconversion all suggest that HHV-6 is transmitted via saliva (Levy *et al.*, 1990; Harnett *et al.*, 1990; Jarrett *et al.*, 1990). The potential site of viral latency and persistence has been determined as the salivary glands following *in situ* DNA and protein studies (Fox *et al.*, 1990; Krueger *et al.*, 1990).

Salivary gland carcinoma tissue and benign tumours (pleomorphic adenoma, adenolymphoma) were studied to determine the frequency of HHV-6 infection. Due to the scarcity of biopsies of normal salivary gland tissue, only five cases were available. Surprisingly only one carcinoma tissue was HHV-6 positive by PCR and Southern hybridization. HHV-6 was detected in two normal salivary gland tissue only after hybridization.

Again NISH proved to be a better detection technique for these tissues. Similar to the oral tissue, most of the carcinoma cases (85.7%) and benign tumours (83.3%) were HHV-6 positive. The virus was localized in the diseased region of both the malignant and benign tumours.

In the normal salivary gland tissue, only 60% of the cases revealed HHV-6 DNA sequences. The virus was detected in the acinic cells and also in the columnar epithelial cells surrounding ducts. This distribution is in accordance with the other two studies on HHV-6 in salivary glands (Fox *et al.*, 1990; Krueger *et al.*, 1990) with Fox and colleagues showing a high percentage (76.9%) of HHV-6 infection in their histologically normal samples.

Corresponding immunohistochemical experiments revealed very interesting data; all the salivary gland tissues expressed HHV-6 gp116/64/54 regardless of whether the tissue was cancerous, benign or normal. Active viral infection was observed in the tumour cells and the ductal cells of carcinoma tissue and benign tumour tissue. In the normal tissue, antigen expression was observed in the ductal cells of most samples, and not in the serous and mucous acinic cells. As HHV-6 DNA was detected in the acinic cells, the lack of HHV-6 protein expression within these cells might be indicative of latent infection.

Thus as suggested by Fox and coworkers (1990), the salivary gland may well be the site of HHV-6 persistence. The virus may establish latency in acinic cells following primary infection and remain dormant until it is reactivated by various factors. Reactivation results in active viral replication leading to asymptomatic viral shedding. In fact, intermittent

HHV-6 viral shedding in otherwise healthy adults has been reported and this supports the idea of saliva being the source of transmission (Levy *et al.*, 1990). Hence active viral replication in the columnar epithelial cells surrounding ducts may be an ingenious method by which the virus may disperse within the host to other tissues, that is via the glandular ducts. Tissues from the surrounding regions of the oral cavity were studied in this context to help define the extent of HHV-6 infection.

5.4: DETECTION OF HHV-6 IN LARYNGEAL AND NASOPHARYNGEAL CARCINOMA TISSUES

In this section, laryngeal and nasopharyngeal tissue were analyzed to detect HHV-6 infection. The unavailability of normal tissue from these regions hindered comparisons between normal and diseased tissues. Thus, this remains a provisional study of the tissues.

NISH and IHC revealed the presence of HHV-6 DNA and antigens in all the laryngeal carcinoma cases studied. Active viral replication was observed in the transformed regions of these tissues with HHV-6B being the common variant. A PCR study by Kositanont *et al.* (1993) in NPC biopsies revealed only 14.7% of HHV-6 DNA as compared to 40% by our study.

Similarly, the detection of active HHV-6 infection in most of the nasopharyngeal carcinoma tissue, 80% by NISH and 60% by IHC, is interesting and may be of importance as the infection was found in the tumour lesions. Nasopharyngeal carcinoma is an excellent example of multistep carcinogenesis in which EBV is strongly associated (Wolf *et al.*, 1984).

While it may be prudent not to speculate the role of the virus in these tissues, the presence of HHV-6 in carcinomas surrounding

the oral mucosa is interesting. Further studies involving a greater sample size and appropriate controls are necessary to define the relationship between HHV-6 and these carcinomas.

5.5: DETECTION OF HHV-6 IN CERVICAL MUCOSAL TISSUES

Cervical carcinoma is strongly associated with HPV, whereby a viral aetiology has been suggested for carcinogenesis. Infection with HPV is essential; over 90% of cervical carcinomas are infected with high risk HPVs but this alone is not sufficient for cancer development (zur Hausen, 1989). Various cofactors have been linked, including smoking and HSV-2 (Brinton *et al.*, 1986; Hildesheim *et al.*, 1994). Recently, a study by Chen *et al.* (1994a) showed that HHV-6 could infect cervical epithelial cell lines and transactivate the expression of HPV E6 and E7 oncogenes.

In our study, HHV-6 DNA and antigens were demonstrable in tumour cells and normal columnar epithelial cells of cervical tissues. PCR and Southern hybridization revealed HHV-6 infection in 33.3% of the cervical carcinoma tissue and 12.5% of the normal tissue studied. 90% of the infection was typed as variant B. Wang *et al.* (1994a) found 2/8 SCC cases positive for HHV-6 by PCR in which one was variant A and the other, B.

HHV-6 DNA sequences were localized in 33.3% and HHV-6-encoded proteins were demonstrated in 46.7% of cervical carcinoma cases by NISH and IHC respectively. Though both PCR and NISH detected HHV-6 DNA in an equal number of samples, NISH revealed viral DNA in

more keratinizing SCC cases than PCR and occasionally the sensitive PCR technique detected HHV-6 DNA that may have been present in minute amounts in other carcinoma tissue. Also, IHC was the more sensitive detection method as viral-encoded proteins were observed in a greater number of carcinoma cases. Once again, low viral copy number may account for the failure to detect viral DNA in the tissues that were positive for protein expression.

The classification of HHV-6 types by NISH disclosed that variant B was the more prevalent variety in cervical carcinoma tissue as was noted in oral carcinoma tissue. While HHV-6 infection was observed in fewer cases in both keratinizing and nonkeratinizing SCC; most of the adenocarcinoma and carcinoma *in situ* cases harboured HHV-6 DNA and proteins. Whether the preferential infection of a subset of cervical carcinomas is coincidental is not known.

The expression of HHV-6 gp116/64/54 late antigen was detected in more cases compared to p41/38 early-late antigen suggesting that the two virus-encoded proteins necessary for complete virus production are not expressed together in cells. This may be related to the status of the virus and/or transformed cell. The lack of reactivity of MAb 9A5D12 to p41/38 in the cervical carcinoma tissue may also be due to the loss or modification of the reactive epitope as a result of formalin-fixation. Yet, it

is more likely that the absence of this antigen is due to non-expression of the viral gene.

Recent evidence show that 10% of women shed HHV-6 DNA in vaginal fluids but the cellular source of the virus was not identified (Leach *et al.*, 1994). In our study, HHV-6 DNA was detected in 12.5% of normal cervical tissue by PCR; NISH localized the endocervical columnar epithelial cells lining ducts and some cells in the submucosa as the source of virus. Similarly, virus-encoded antigens were observed in the endocervical columnar epithelial and submucosal cells of 62.5% normal cervix uteri. It appears that the virus persists in low copy numbers in the antigen-expressing cells and are only detected if viral DNA exceeds the limit of sensitivity for a positive NISH signal. It appears that two-thirds of normal women harbour HHV-6 in the cervix while virus shedding occurs in about 10%. Further work is warranted to identify the factors responsible for the viral shedding and to determine whether this could be a route of transmission.

HHV-6 contains at least two separate sequences which can neoplastically transform epithelial cell lines, (Razzaque, 1990; Razzaque *et al.*, 1993). However, the virus was detected in less than half of the cervical carcinomas and also in normal epithelial cells suggesting the possibility of incidental transmission from the normal to transformed cells.

pZVH14, a HHV-6 clone with the ability to transactivate the HIV-1 promoter can also transactivate HPV-18 promoters in HeLa cells (Chen *et al.*, 1994a; Wang *et al.*, 1994b). In Malaysia, 90% of cervical tumours contain HPV DNA in which the majority are HPV-16 or HPV-18 genotypes (Yadav *et al.*, 1995). Both these HPV types are known oncogenic agents of the anogenital region (zur Hausen, 1989). However, HPV infection alone does not cause cancer. HHV-6 may be involved in multistep carcinogenesis of certain cervical carcinomas by upregulating the expression of HPV-18 E6 and E7 transforming genes (DiPaolo *et al.*, 1994). However additional studies are required to determine the function of HHV-6 in HPV-infected carcinomas.

5.6: STUDY OF HHV-6 IN BREAST CARCINOMA TISSUE

Breast carcinoma tissue was analyzed for HHV-6 detection by the techniques mentioned earlier. Neither HHV-6 DNA nor antigens were detected in any of the tissues studied. Indeed, viral transmission via breast milk was ruled out (Dunne and Jevon, 1993). The lack of viral infection suggests that HHV-6 does not play a role in breast carcinogenesis. Absolute confirmation however, requires the analysis of more samples as only ten cases were studied in this exercise.

5.7: CONCLUSION

The detection of HHV-6 was carried out by PCR, NISH and IHC on formalin-fixed, paraffin-embedded tissue. All the techniques employed were nonradioactive and yielded reproducible results. PCR revealed fewer HHV-6 positive cases probably due to low viral copy number or the degradation of target DNA as a result of excessive formalin-fixation. NISH and IHC were effective methods of viral detection. It was observed by NISH that both HHV-6 A and B were found with variant B, the causative agent for ES (Yamanishi *et al.*, 1988), being more common in oral and cervical carcinoma. HHV-6B is found in healthy populations and immunosuppressed patients (Pellett *et al.*, 1992). The detection of mixed HHV-6 infection in these carcinoma cases shows that the infections of either variant is not mutually exclusive.

Both MAb 6A5G3 and 9A5D12 react with all HHV-6 strains tested. HHV-6 p41/38 is an early protein involved in stimulating viral replication while gp116/64/54 is a late protein necessary for viral entry, the detection of both viral antigens appear to be valid markers for active or reactivated infection (Chang and Balachandran, 1991; Iyengar *et al.*, 1991; Thompson *et al.*, 1994a). Also, the use of two MAbs allowed better

detection of the virus as not all cells expressed both antigens together. This reduced the chances of false negatives.

Based on studies of the salivary glands, HHV-6 probably establishes latency in the salivary glands following primary infection. Upon reactivation, the virus may be transmitted in the saliva and also infect other regions of the body through blood CD4 T lymphocytes and other cells. HHV-6 was highly prevalent in various carcinomas with the exception of breast carcinoma. The role of HHV-6 in these carcinomas may be related with the multistep development of the cancer.

HHV-6 possesses various strategies for the conversion of normal cell to the malignant phenotype. The strategies may include transactivation, transforming genes and misrational activation of proto-oncogenes as discussed above. Carcinogenesis is probably the result of a multistep process with a multifactorial aetiology. While carcinogens may play an active role in causing molecular aberrations in cellular genes, other synergistic factors may facilitate the transformation processes. HHV-6 probably acting as a cofactor may provide the final step in the change of the initiated cell to the cancer cell. These observations raise the possibility of a direct role for the virus in the transformation of a subset of epithelial carcinomas. Further studies are warranted to analyze the molecular mechanisms in HHV-6 associated tumours.