CHAPTER 5

DISCUSSION

5.1 Polymerase chain reaction and Southern blot hybridization

HPV research have been severely hampered in the past by inadequate detection methods. Standard viral detection methods are deterred by the lack of in vitro cultivation system for HPV (Gravitt and Manos, 1992). As a consequence, investigators have depended predominantly on molecular biological techniques for the detection, identification and analysis of HPV infections.

PCR has been successfully applied to detect and identify HPV in routine pathological specimens ranging from exfoliated cells of cervical scrapes (van den Brule et al., 1990), fresh or frozen cervical biopsies (Li et al., 1988; Manos et al., 1989) and archival cervical smears (Rakoczy et al., 1990) to formalin-fixed, paraffin-embedded archival cervical specimens (Shibata et al., 1988; Cornelissen et al., 1989).

Fresh frozen cervical biopsies, non-cervical specimens and cervical scrapes from women without cytological abnormality were successfully analysed in this study by PCR for the presence of the highly oncogenic genital HPV types 16 and 18. Crudely prepared DNA lysates are generally sufficient for amplification with PCR (Wright and Manos, 1990; Kawasaki, 1990). However most of the specimens obtained for this study contained blood on visual examination. Blood has been known to inhibit PCR amplification (Gravitt and Manos, 1992). Thus in addition to non-ionic detergent or protease digestion, sample preparation included DNA purification with phenol-chloroform extraction. All extracted DNA were quantified spectrophotometrically. The resulting A260/A280 value ranging from 1.8-2.0 indicated

a relatively pure DNA sample suitable for use as template in PCR amplification. Samples with the ratio of below 1.6 were subjected to further purification with phenol-chloroform.

All DNA extracted from clinical specimens collected for this study were evaluated for integrity of DNA in PCR amplification for β-globin genes. Amplification of the 268 bp product from the β-globin sequences indicates that the cellular DNA are adequate and do not contain inhibitors for HPV analysis by PCR (Bauer et al., 1991). Almost all of the DNA extracted from cervical cancer biopsies, cervical scrapes and non-cervical specimens demonstrated the characteristic β-globin gene amplification, indicating the suitability of these samples for HPV analysis by PCR. Thus samples positive for β-globin but negative for HPV DNA were indeed true negatives.

In this study, rigorous precautions were taken to prevent false-positives in PCR. DNA extraction, preparation of reaction mixtures, PCR amplification and analysis of amplified products were spatially separated. Preparation of reaction mixture was carried out in a sterile laminar flowhood (Gelaire, Australia). Positive displacement pipettes were used. The set of pipettes used for analysis of the amplified products was different from that used for reaction mixing. To monitor potential contamination of the reagents, negative controls (sterile deionized water which did not contain any input DNA) were strategically included in each PCR run. Any batch of results with contaminated negative control were dismissed and discarded. Amplifications were repeated until results were satisfactory.

Detecting and identifying HPV in a genital sample can be very challenging due to the heterogenous pool of HPV that might be present (Bernard et al., 1994). HPV type-specific primers have been utilized for separate and specific amplification of HPV 6, 11, 16, 18 and 33 nucleotide sequences (Shibata et al., 1988; Melchers et al.,

1989; Young et al., 1989). However the number of many different primers required to amplify DNA specifically from every HPV type would render PCR impractical for use in investigations of clinical specimens and in large scale epidemiological studies (Fujinaga et al., 1991).

This study employed a generalized PCR-based method using a pair of L1 consensus (degenerate) primers to detect a broad spectrum of genital HPV genotypes. Numerous studies using similar approach have been previously described by others (Snijders et al., 1990; Manos et al., 1989; Gregoire et al., 1989). The MY09 and MY11 are designed to generally amplify an approximately 450 bp fragment within the 3' terminal third of the L1 gene from at least 25 distinct genital HPVs, including HPV types 16 and 18 (Manos et al., 1989; Gravitt and Manos, 1992; Hildesheim et al., 1994).

The identity of the resulting amplification product on gel electrophoresis was confirmed by Southern blot hybridization with two digoxigenin-labelled probes specific for HPV 16 and 18. Two cervical carcinoma and 2 normal cervical smear samples (Table 4.1) which produced a clear amplified product but did not hybridize with either probe may harbour HPV DNA other than that of HPV 16 and 18.

It was noted that on two occasions (both for DNA from invasive carcinoma), the PCR product was not visible under ultraviolet light, following ethidium bromide staining of the gel but the DNA was detectable with Southern hybridization with specific homologous probes. The absence of the clear PCR amplicon in both of these samples could be due to very low copy numbers of HPV in the original tumour samples. This also meant that amplification of HPV DNA in these samples had occurred at a level below the level of detection by ethidium bromide staining. Hybridization increases the sensitivity of detecting small amounts of amplified DNA

which are too faint to be visible on a gel (Demmler et al., 1988). Morrison et al. (1992) discovered that hybridization increased the sensitivity of HPV 16 detection by a factor of 100.

The specificity of the primers and probes utilized in this study was validated. Amplified product of CaSki (containing 400-500 integrated copies of HPV 16 per cell) and SiHa (containing 1-2 integrated copies of HPV 16 per cell) and HeLa (containing 10-50 copies of HPV 18 10-50 copies of HPV 18 per cell) hybridized intensely with their respective probes. No band was detected when CaSki and SiHa were crosshybridized with the probe specific for HPV 18 or when HeLa was crosshybridized with the probe specific for HPV 16. The absence of the band indicated that nonspecific hybridization did not take place between HPV 16 and 18 sequences. The probes did not detect HPV sequences unrepresented by them and were indeed type-specific.

Strict measures were taken to prevent the occurance of any cross-reactivity between different HPV types that might be present in the samples. An appropriate choice of post-hybridization wash conditions permits selective destabilization and dissociation of imperfectly matched nucleic acid duplexes. High temperatures balanced with low salt concentration result in lower hybrid stability (Wetmur and Davidson, 1986; Lancaster and Norrild, 1989).

In this study, a PCR-labelling method was employed for the production of digoxigenin-labelled HPV 16 and18 probes. The traditional methods for probe-labelling which are tedious, laborious and time-consuming involve bacteria growth, plasmid vector extraction, purification of the insert and finally labelling with an appropriate molecule. The PCR-labelling method is based on a one-step procedure which involved the incorporation of the nonradioactive compound (dNTP labelled with

digoxigenin) into DNA synthesized by PCR (Griffais et al., 1990; Lion and Haas, 1990). Utilizing a small amount of starting material (50 ng of cloned plasmid containing the insert of HPV 16 or 18), this method allowed for simultaneous amplification and labelling of DNA, thus facilitating the generation of large amounts of labelled HPV 16 or 18 DNA within 3 hours. The simple procedure only required a reaction mixture containing cloned plasmid in its circular form, specific primers, DIG-dUTP, buffer,taq polymerase and a thermal cycler. It provided an efficient and direct labelling of the 450 bp DNA. Since the size of the DNA fragment is specified by the primers, no fragment of intermediate sizes were produced.

The PCR-labelled DNA probes were used successfully in this study for the detection of HPV sequences on positively-charged nylon membrane without further purification. PCR-labelling is superior to other labelling methods in that it ensures that all molecules are successfully labelled, so during hybridization, no unlabelled DNA are present to compete with the labelled ones. This results in higher sensitivity (Morris et al., 1990).

5.2 In situ hybridization

Using digoxigenin-labelled probes in in situ hybridization, 74 formalin-fixed, paraffin-embedded sections from various cervical lesions ranging from CIN 1, CIN 2, Cin 3/CIS, SCC and ADC were successfully examined for the presence of HPV 16 and 18 sequences.

As with Southern blot hybridization, the sensitivity of in situ hybridization was largely determined by the type of labels used for the probes (de Villiers, 1992a). This study utilized the same probes used in Southern blot hybridization described earlier. Digoxigenin-labelled probes specific for HPV 16 and 18 were optimized to 10 ng/ul for

use in this study and were utilized separately. Higher amounts of probe concentration resulted in nonspecific background, which masked the detection of positive hybridization signals within infected nuclei. Lower amounts, however, gave weak, insufficient signals.

The digoxigenin-labelled HPV DNA probes used here were physically large and long. Accessibility to target nucleic acid was aided by the enzymatic digestion of cell membranes by proteinase K. A concentration of 100 ug/mlof proteinase K for 20 minutes at 37°C was optimal for this study. Lower amounts required longer times for the enzymatic reaction to be completed. The addition of 50% (w/v) dextran sulphate in the prehybridization solution increased the hybridization rate.

The specificity of the digoxigenin-labelled probes used in in situ hybridization were assured. As with Southern blot hybridization, positive and negative contros were included in every batch. HPV-positive cell lines were grown or fixed onto sterile, activated slides. CaSki and HeLa cell lines hybridized intensely with HPV 16 and 18-specific DNA probes, respectively. On the other hand, upon cross-hybridization, HeLa did not hybridize with HPV 16 DNA probe and CaSki did not hybridize with HPV 18 DNA probe. Similarly no hybridization was detected on an HPV-negative cell line or on tissue sections untreated with probe. As before, post-hybridization washes were performed at high stringency.

In the present study, examinations under light microscope revealed that HPV 16 and 18 sequences were localized in infected cells within cervical lesions and therefore, ruling out contamination from adjacent areas. This ensured that the HPV DNA detected was indeed associated with the lesions examined. It is interesting to note that while cells of some lesions seemed to have uniformly high or low HPV DNA copy numbers, cells within other HPV-positive lesions did not harbour equal amounts

of HPV DNA. This is evident from the presence of weakly stained or unstained nuclei (containing low abundance of HPV DNA copies) mixed with intensely stained ones (containing high abundance of HPV DNA copies) in some tissues. HPV 16 and 18 have similar pattern of distribution within the lesions. No specific pattern was observed that could distinguish infection between the two genotypes.

5.2.1 Distribution of HPV DNA within CIN lesions as detected by in situ hybridization

Only one out of four (25%) CIN 1 cases harboured the HPV 16 and 18 sequences. In this patient, HPV 16 and 18 occurred simultaneously at the same site within the lesion. HPV DNA was mostly detected in the proliferating basal and parabasal layers comprising the lower one third of the thickness of the epithelium. However, some HPV DNA can also detected in koilocytes located in the superficial layers. This may present an early stage of HPV infection. In contrast, Beckman et al. (1985) and Crum et al. (1986) failed to reliably detect HPV sequences in the basal and parabasal cells of lesions using biotinylated probes but showed positive signals only in cells of the upper layers. Thus, the ability to successfully detect HPV DNA in the proliferating basal cells in the present study can be attributed to the higher sensitivity of digoxigenin-labelled probes used or possibly to the presence of HPV genomes in higher copy number. Detectable amounts of DNA shown in this study may indicate that sufficient amount of HPV DNA is needed for the development of the CIN 1 lesion.

Upon entry, HPV infects the basal cells of the cutaneous or mucosal epithelium and exists as episomes (Orth et al., 1971, 1977; Bunney, 1982; Lancaster et al., 1977; Moar et al., 1981a). Initial interaction between HPV and epithelial cells results in increased basal cell proliferation. As basal cells divide, the viral episome replicate only once per cell cycle. The HPV copy number stay relatively constant for many

generations because the immature, multiplying basal cells are not permissive for independent HPV replication. As a result, HPV particles have never been observed in these cells (Wright and Richart, 1990).

HPV DNA was present in 10 out of 15 (67%) cases of CIN 2. In majority of the HPV-positive CIN 2 cases, viral sequences were detected as intense stainings in the overlying layers which showed varying degrees of cellular differentiation and koilocytotic atypia. Presence of HPV DNA in areas of lesions showing obvious maturation is indicative of a productive HPV infection. The productive infection in these non-dividing cells is a consequence of the non-productive infection of the underlying HPV-particle-free basal cells which contain only HPV DNA (zur Hausen, 1986). Differentiating and keratinizing cells are more permissive for the completion of the HPV growth cycle i. e. independent HPV DNA replication, viral particle assembly and viral maturation. Depending on the infection, viral particles in large quantities may be generated. Some cells in the areas of well-differentiated CIN have been found to contain more than 800 copies of HPV DNA (Crum et al., 1986).

HPV DNA was detectable in 17 out of 20 (85%) cases of CIN 3/CIS. Judging from the staining intensities, HPV was present in abundance in these cells. However, compared to CIN 1 and CIN 2, variations in staining intensities were more frequently encountered suggesting that HPV distribution were more heterogenous in higher grade lesions. This also indicated that there was an increase in number of cells containing low copy numbers of HPV in the more severe lesions.

Analysis by viral type reveal that the overall detection rates between HPV 16 and 18 in CIN 1 and CIN 2 were not significantly different from each other. HPV 16 and 18 were present in identical proportion of the CIN 1 lesions and in similar rates in the CIN 2 lesions. However considerable difference was noted in CIN 3/CIS lesions,

where they were 55% for HPV 16 and 85% for HPV 18. The lower HPV 16 incidences in CIN 3/CIS lesions may be due to either increased in number of lesions containing HPV 16 in low abundance or to the increase in number of lesions harbouring HPV 18.

5.2.2 Distribution of HPV DNA within invasive cervical lesions as detected by *in situ* hybridization

Of the 30 paraffin-embedded cervical cancer tissues HPV DNA was detected in 24 (80%). Cells harbouring HPV DNA were detected in abundance within the invasive lesions. Compared to the CIN lesions, there were more cases in which cells containing large amounts of HPV DNA coexist with lowly infected or uninfected ones. The reason for the variations in HPV copy numbers in different cells of the same tissue cannot be explained with the present method. Likewise it does not explain why some cells were positive for HPV DNA and morphologically identical cells immediately adjacent appeared negative. Heterogeneity in HPV DNA content suggest that there may exist microvariation in cellular differentiation which supports HPV DNA replication at varying rates. Pleomorphism which characterizes malignant cells may result in similar variation in viral DNA replication and hence detection. It is also possible that such uneven distribution of HPV DNA was caused by non-simultaneous, periodic expression of viral genes controlling the DNA replication (Lancaster and Norrild, 1989).

5.3 Prevalence of HPV 16 and 18 in cervical lesions as determined by *in situ* hybridization and PCR-Southern blot hybridization

It is widely accepted that carcinoma of the uterine cervix arises from precursor lesions. It evolves over a span of many years as an end result of a progressive multistep dysplastic epithelial changes spanning from CIN 1, CIN 2 , CIN3 and CIS.

Specific HPV types, predominantly HPV 16 and 18 have been increasingly implicated in the pathogenesis of cervical cancer and its precursors (Durst et al., 1983; Boshart et al., 1984; McCance et al., 1985; Ostrow et al., 1987; Fukushima et al., 1985). HPV 16 and 18 DNA were found to be present in 77.2% (88/114) of the preinvasive and invasive cervical lesions examined in this study.

5.3.1 Prevalence of HPV 16 and 18 in CIN

The prevalence of HPV 16 and 18 DNA increased fron CIN 1 through CIN 2 and CIN 3/CIS with frequencies of 25%, 67% and 85%, respectively. These findings are in aggreement with those of other studies (Fuchs et al., 1988; Lorincz et al., 1987; Kulski et al., 1987) which demonstrated increased incidences of these HPV types with severity of the CIN lesions. Lorincz et al. (1987) detected HPV 16 in 20% of CIN 1, 43% of CIN 2 and 50% of CIN 3 specimens obtained from the United States, Brazil and Peru. On the other hand, Fuchs et al. (1988) reported the prevalence of HPV 16 in 3% of CIN 1, 27.9% of CIN 2 and 45.7% of CIN 3.

The difference in HPV 16 and 18 incidences between CIN 3 and CIN 2 was not very significant. These two groups can be combined to become a group of high grade lesions. The prevalence of HPV 16 and 18 DNA in this group was 77%, a significant elevation from that of the low grade lesions (CIN 1). The present data

agrees with that of Lungu et al. (1992) who noted the increase of high risk HPV in 29% of women with CIN 1 to 88% of those with CIN 2 and 3.

As with Lungu et al. (1992), the present findings support the hypothesis which divides the cervical intraepithelial series of lesions into 2 discrete and histologically distinguishable categories labelled as "low grade" and "high grade" CIN based on virologic observations (Richart, 1990). According to this hypothesis, the low grade CIN which is equivalent to the histological CIN 1 is associated with the presence of the HPV types 16, 18 or 33 in less than 30% of the cases. The high grade CIN on the other hand corresponds to the histological CIN 2 and CIN 3 and frequently associates with the oncogenic viruses in nearly 90% of the time.

A possible role of HPV 16 and 18 in the development of premalignant lesions were suggested by in vitro studies on organotypic or raft culture system (Rheinwald and Beckett, 1986). Following transfection with HPV 16 DNA primary keratinocytes fron neonatal foreskin and skin carcinoma cells failed to differentiate normally but demonstrated histological changes and altered differentiation pattern resembling those seen in vivo, in genital intraepithelial neoplasia. In other studies, transplantation of HPV 16 and 18-immortalized cell lines into athymic mice had resulted in the development of histologically dysplastic epithelium (Barnes et al., 1990; Woodworth et al., 1990).

The initial published associations between HPV types and various cervical lesions implicated that the clinical course of an intraepithelial lesion may to a certain extent be influenced by the HPV type it contains (Fuchs et al., 1988; Nuovo et al., 1991). Infection with HPV 16 or 18 results in lesions with a strong tendency of progressing to higher grade dysplasia or invasive cervical carcinoma. So, the significantly lower prevalence of HPV 16 and 18 DNA in the lower grade lesions as

seen here implicate that only a small portion of these lesions will progress to severity. The likelihood of the lower grade lesions which lack HPV 16 or 18 DNA progressing to higher grade CIN is small. The distribution of HPV types in high grade lesions are generally limited to the high risk HPV 16 and 18. This is evident from the present study where a high proportion of these lesions were positive for HPV 16 and/or 18 DNA. The high prevalence of these HPV types in high grade lesions may explain the predictable nature of these lesions. Majority of the high grade CIN will progress to invasive cancer if left untreated (McIndoe et al., 1984). In one study, Fuch et al (1988) noted that 75% of HPV 16-associated CIN 2 lesions either persisted or progressed to disease. In contrast 50% of lesions harbouring non-high risk HPV types or with no evidence of HPV infection regressed.

The increased prevalence of HPV 16 and 18 DNA from CIN 1 to CIN 2 and CIN3/CIS suggest that HPV-associated cervical carcinoma develop in a stepwise fashion through increasing grades of CIN. It simultaneously support the traditional belief that cervical carcinoma develops in a stepwise manner from precursor lesions through increasing degrees of abnormality and severity.

The prevalence of HPV 18 was found to be similar to that of HPV 16 in the CIN lesions. These findings contradict those of many studies carried out worldwide (Boshart et al., Fuchs et al., 1988; Bergeron et al., 1992; Lorincz et al 1987; Kurman et al 1988). These studies demonstrated preponderance of HPV 18 in the advanced lesions of the cervix. They implied that HPV 18 produced precursors which may enter the spectrum of intraepithelial lesions at a more advanced phase (CIN 2 or CIN3). Lorincz et al. (1987) failed to detect HPV 18 in CIN 1 and 2 lesions from Brazil and the United States but demonstrated its presence in 12.5% of the CIN3 lesions. On the other hand, Bergeron et al. (1992) detected HPV 18 in a total of only 3% of

CIN lesions. Similarly, Kurman (1988) demonstrated HPV 18 positivity in only 3% of the CIN compared to 22% of the invasive cervical lesions while HPV 16 was observed in 37% and 41% of the CIN and cancer, respectively. HPV 18 DNA has also been detected exclusively in malignant cervical disease (Boshart et al., 1984).

These present findings do not dispute evidence which suggested that HPV 18 is a more aggressive virus compared to HPV 16 and also that HPV 18-associated lesions have poorer prognoses than do HPV 16-associated lesions. While HPV 18 has been successfully detected in lesions of all CIN grades, their behavour may not be the same to those associated with HPV 16. Since HPV 18 has been suggested to be more aggressive, such precursor lesions may progress more rapidly than those containing HPV 16. Nevertheless, presence in tissues of HPV 18 does not indicate a degree of aggressiveness. Due to the lack of follow-up data, it was not possible with the present findings to investigate the differences in prognosis of HPV 18-associated lesions compared with that of HPV 16. Future prospective case-studies are certainly warranted to address this question.

Variations in aggression between HPV 16 and 18 have been demonstrated in vitro (Barnes et al., 1990; Barbosa and Schlegel, 1989). Barnes et al. (1990) observed dysplastic changes in athymic mice after transplantation with early as well as late passage HPV 18-immortalized cell lines. On the other hand, dysplastic changes were not seen in mice transplanted with early passage HPV 16-immortalized cell lines. These changes were only demonstrated in those grafted with late passage HPV 16 cell lines. HPV 18 has also been found to immortalize keratinocytes five-fold more efficiently than HPV 16. This has been attributed to the production of higher levels of HPV 18 transforming proteins E6 and E7 compared to those of HPV 16 (Barbosa and Schlegel, 1989).

5.3.2 Prevalence of HPV 16 and 18 DNA in invasive carcinoma

In this study HPV 16 and 18 DNA occurred in similar proportions of 58% and 62.6%, respectively among patients with cervical carcinoma. Such similar proportions were generally not seen by investigators in other countries where HPV 16 has been found to be the more predominant type in cancerous lesions, followed by HPV 18 (Durst et al., 1983; Hsieh et al., 1988; Boshart et al., 1984; Kulski et al., 1987) but geographical variations exist and in Japan, HPV 52 and 58 were found at higher frequencies in cancer as compared to HPV 16 and 18 (Yajima et al., 1988; Matsukura and Sugase, 1990).

The two techniques used for the detection and typing of HPV 16 and 18 in cervical cancer tissues are compared. PCR-Southern blot hybridization was performed on fresh frozen biopsies of cervical carcinoma while in situ hybridization was carried out on paraffin-embedded cervical cancer sections. Table shows that the detection rates of HPV 16 and 18 in cancerous lesions by PCR-Southern blot hybridization and in situ hybridization are identical (80%).

The frequencies of HPV 16 and 18 in invasive lesions were slightly lower than in high grade CIN lesions. HPV 16 was detected in 58% of cervical cancer cases and in 60% of high grade CIN lesions. HPV 18 was seen in 61% of the cancer cases and 71% of the high grade CIN lesions. The slight drop in HPV detection rates may be due to the very low copy numbers of HPV genome in many of the invasive lesions.

Studies carried out in many different countries on the prevalence of HPV 16 and 18 in cervical cancer have shown great geographical variation. HPV 16 DNA was detected in 65% of invasive cancer in Western Australia (Kulski et al., 1987), 61% in Germany (Durst et al., 1983), 60% in Panama (Prakash et al., 1985), 57% in Austria

(Fuchs et al., 1988), 45% in Taiwan and China (Hsieh et al., 1988), 40% in Italy (DiLuca et al., 1986), 38% in East Anglia (Scholl et al., 1985), 35% in Kenya and Brazil (Durst et al., 1983), 27 to 34% in Japan (Saito et al., 1987; Yoshikawa et al., 1985) and 18% in the United States (Fukushima et al., 1985). On the other hand, HPV 18 was detected in 25% of the cancer cases in Kenya and Brazil (Durst et al., 1983), 18% in Taiwan and China (Hsieh et al., 1988), 15% in Germany (Boshart et al., 1984), 9% in Austria (Fuchs et al., 1988), 7% in Western Australia (Kulski et al., 1987) and 6% in Japan (Saito et al., 1987; Yoshikawa et al., 1985).

Variations in HPV reportings may have several implications. The variability might indicate actual difference in the prevalence of HPV worldwide (Low et al., 1990; Boshart et al., 1984). The predominance of different HPV types in these different geographical locations may reflect different etiologies of cervical carcinoma (Young et al., 1989). Cervical carcinogenesis is a multistep process which involves the interplay of HPV infection, genetic events and environmental factors. Different genetic and environmental events may have different contributions to tumour induction by different HPV types. In short, these variations may reflect the differing predispositions and susceptibility of females to infection by different HPV types (Kulski et al., 1987).

Difference in sensitivity and specificity of detection techniques used may be a factor contributing to the variations in the HPV detection rates (Munoz et al., 1988). Earlier epidemiological studies on small number of samples and using less sensitive detection techniques may have resulted in underestimation of true incidences of HPV. de Villiers et al. (1987) under-reported the HPV infections in women in Germany by a factor of two to three using filter in situ hybridization technique. Interlaboratory variation was demonstrated by Bradsma et al (1989). Identical specimen which was sent to four different experienced laboratories for the detection of HPV by Southern

blot hybridization yielded significantly different results. Other factors which may result in discrepancy in HPV reportings are sampling variations, sample selection and misdiagnoses of samples selected for study (Lancaster and Norrild, 1989).

5.3.3 HPV genotype and cell patterns

SCC was most commonly seen in this study and comprised of 93.3% (70/75) of the invasive carcinomas. ADC on the other hand was represented by only 6.7% (5/75) of the invasive carcinoma lesions.

The relationship between HPV genotypes and cell patterns was also investigated in this study. HPV 16 and 18 occurred in identical proportions among patients with SCC. This observation disaggree with those of the previous reports which demonstrated a higher prevalence of HPV 16 compared to HPV 18 in SCC of the cervix (Young et al., 1989; Lorincz et al., 1987; Kulski et al., 1987; zur Hausen, 1989). In contrast, HPV 18 clearly predominates in ADC by being positive in 60% of the cases compared to only 20% by HPV 16. This aggrees with other studies which reported higher proportion of the HPV 18-associated glandular cancer of the cervix (Arends et al., 1993; Wilczynski et al., 1988).

The pattern of HPV prevalence seen in this study supports that there may be an association between HPV type and cancer cell differentiation (Arends et al., 1993; Smotkin et al., 1980; Christopherson et al., 1979). Compared to HPV 18, HPV 16 preferentially associate with cervical cancer showing squamous differentiation. HPV 18 on the other hand was shown in this study to have similar association with SCC and ADC. One possible explanation can be put forth as an attempt to clarify the difference in HPV prevalence in SCC and ADC. Both squamous and columnar neoplasia may originate from the same precursor stem cells in the cervix

(Christopherson et al., 1979). HPV 16 infection of an uncommitted stem cells in the basal layer together with the necessary genetic and environmental events as cofactors may be more inclined to induce squamous differentiation of subsequently formed cancer. On the other hand, HPV 18 infection may have equal tendency of inducing either squamous or glandular differentiation and the respective carcinoma.

5.3.4 Occurence of mixed infections in cervical lesions

Mixed infections with HPV 16 and 18 as detected by PCR-Southern hybridization techniques were present in all histological grades (Tables 4.2 and 4.3). They occurred in 44.7% (51/114) of the total cervical lesions, 42.8% (30/70) of SCC, 20% (1/5) of ADC, 55% (11/20) of CIN3/CIS, 53.3% (8/15) of CIN 2 and 25% (1/4) of CIN 1. These results indicate that simultaneous infection with HPV 16 and 18 is a relatively common phenomenon. Except for ADC, they occurred more frequently than single infections of either type.

Mixed infection with HPV 16 and 18 were recorded by Sebelov et al. (1991) with Southern blot hybridization in 1.4% of cervical cancer cases in Norway, by Fuchs et al. (1988) with Southern blot hybridization in 2.3% of CIN 2, 0.7% of CIN 3 and none in cervical cancer cases in Austria, by Kulski et al. (1987) with filter in situ hybridization in 4% of SCC cases in Australia, by Burnett et al. (1994) with PCR in 50% of cervical cancer cases in the United States and by Griffin et al. (1990) with PCR in 57% of paraffin-embedded SCC cases in England.

The relatively higher prevalence of mixed infections with HPV 16 and 18 in cervical lesions examined in this study as compared to those observed by other studies may reflect population differences or due to the higher sensitivity of the detection system used. Since high stringency conditions were employed in the

Southern blot hybridization and in situ hybridization, cross-reactions between closely related HPV types can be ruled out. Thus, mixed infection addressed here truly represent simultaneous infections of HPV 16 and 18 within the lesions. In most of these tissues the hybridization signal or band of one probe was accompanied by a weaker signal or band with another probe. In contrast, other tissues gave signals of similar intensities with both probes. Thus, how one HPV type could affect another in a mixed infection remains unexplained by the present data and requires detailed molecular investigations. It is possible that coexisting HPV types interact synergistically in contributing to neoplasia (Morrison et al., 1991). Alternatively, these different HPV types may exist independently of each other, exerting independent oncogenic effects or that only one of the HPV types may be responsible for the viral lesion while the other exists as an innocent passenger or as cofactors.

In situ hybridization detected 52% of mixed infections in cervical lesions embedded in paraffin. Since the in situ hybridization can only detect HPV genomes present in high copy numbers, the 52% of mixed infections detectable by this method may represent a good estimate of the actual incidence of active, proliferating infections of both HPV types. However, the same cannot be said for the 35% of mixed infections in fresh biopsies of cervical cancer investigated by PCR. This because the extreme sensitivity of PCR is capable of detecting latent as well as active infections.

The frequencies of mixed infections increased from CIN 1 to CIN 2 and CIN 3 but dropped slightly in invasive carcinomas. Observations revealed that simultaneous infections with HPV 16 and 18 occurred more frequently in benign lesions. However, further analyses suggest that severity of a lesion or cancer occurance was not

significantly influenced by the nature of infection by HPV 16 and 18 (either singly or as mixed infection).

5.3.5 HPV-negative invasive carcinoma

A small number of cervical carcinoma have been reported without persisting HPV DNA (Riou et al., 1990). In this study, 15% (6/40) of the fresh frozen cervical biopsies were found to be HPV-negative after PCR-Southern blot hybridization. On the other hand, 20% (7/35) of the paraffin-embedded cervical cancer sections were found to be negative for HPV 16 and 18 DNA. One possible explanation for the non-detection in these cases could be that they were associated with HPV types sufficiently distinct from HPV 16 or 18 and therefore undetectable under the present experimental conditions. It is also conceivable that specific oncogenic HPV may have been present in the initial stages of the tumour development but were lost in the course of the disease. In these exeptional cases, the continual presence of the HPV DNA genome may not be necessary for the maintenance of the malignant phenotype (Ostrow et al., 1987). Perhaps what was observed in these tumours was the later stage of carcinogenesis. Nevertheless there is a possibility that the development of these carcinomas was indeed HPV-independent and that other factors might be involved in the initiation of the tumours. In HPV-negative cancers it is believed that cellular genes may have been altered and the dysregulation causes transformation (Riou et al., 1990).

5.3.6 Age of patients at diagnosis and histologic diagnosis of cervical lesions

The mean age of the study population was analysed. In general, women with CIN 1 and CIN 2 were younger than women with CIN 3/CIS who in turn were younger than those with cervical cancer. The mean age of the 75 women with cervical cancer seen here was 49.6 years old, 9.3 years older than those with benign lesions. These findings obviously agree that CIN generally occur in younger age group than cervical cancer (Brown and Wells, 1986; Ostor et al, 1984; Griffin et al., 1991).

5.3.7 Prevalence of HPV 16 and 18 in relation to the age of patients

The distribution of HPV 16 and 18 infection in cervical lesions was further analysed in relation age of the patients. It was found that the mean age of HPV 16-positive patients was 47.8 years, almost similar to the mean of HPV 18-positive patients which was 48.4 years. Despite the increase from age group 25-34 to 35-44 the frequency of HPV 16 did not show further increase in the older patients. Thus, unlike Meanwell et al. (1987) who observed an increased HPV 16 positivity with increasing age, the prevalence HPV 16 seen here did not change with age. Similarly and as found by Yoshikawa et al. (1985) the frequency of HPV 18 positivity in cervical lesions was not affected by age. The data therefore suggest that although the mean ages of patients with CIN 1, CIN 2, CIN3/CIS and cervical cancer examined here increased from 29.5 years to 38.14 years to 44 years to 49.6 years, respectively, the apparent rise in frequency of HPV 16 and 18 DNA positivity with severity of the lesions was not the effect of increasing age as claimed previously by Meanwell et al. (1987) but may simply be due to the increasing involvement of these viruses in tumour progression. These observations are also in line with those of de Villiers et al. (1987)

who reported that the prevalence of HPV infection was high and age-independent in women with CIN and cervical cancer.

5.3.8 Detection of HPV 16 and 18 DNA in normal cervical scrapes

This study also allowed the evaluation of the rate of HPV 16 and 18 infection in normal cervical scrapes. PCR-Southern blot hybridization showed that 26% (8/30) of women with normal cervices examined are carriers of HPV 16 and 18. The remaining two which were positive for HPV DNA but did not hybridize with HPV 16 or 18 probes may contain other HPV types. Early reports have indicated that HPV 16 DNA was rarely found in normal cervical epithelium (Schneider et al., 1985). Through the years however, the high risk, oncogenic viruses were increasingly demonstrated in cytologically normal cervices. The increased detection rates may be attributed to the more reliable and sensitive detection methods used. Sensitivity of PCR has improved the detection of HPV in normal cervices (Young et al., 1989; Tay et al., 1989).

In agreement with others, the present findings indicate that HPV 16 and 18 are ubiquitous. Thus far the detection rates of HPV 16 in normal specimens varied from 0 to 84%. HPV 16 was detected by Meanwell et al. (1987) in 35%, by Murdoch et al. (1988) in 60% and by Griffin et al. (1990) in 30% of the normal cervical epithelium. HPV 16 and 18 were also identified in 43% of the normal controls in a pilot study in Latin America (Reeves et al., 1989) and in 6 to13% of those in Denmark and Greenland (Kjaer et al., 1988).

Most latent infections were due to HPV types strongly associated to cervical cancer (de Sanjose et al., 1992). The detection rates presented here may indeed reflect the true frequencies of latent infections with these HPV types or may possibly be an underestimation of the viral presence. HPV are known to have a long latency

period during which they exist as non-replicating DNA deep in the basal layers of the epithelium (Lorincz et al., 1986). Since in this study, analysis was made on surface exfoliated cells of cervical scrapes, the cells which harbour the latent infection by HPV 16 and 18 might be missed. Thus, it seems likely that the analysis of the full thickness biopsy specimens might be more reliable than analysis of cervical smears (Meanwell et al., 1987). However, biopsy of normal cervices for routine screening may not be ethically possible.

It is clear from the findings here that the mere presence of HPV 16 or 18 genomes does not necessarily imply that the host tissue is diseased. Instead, they indicate that HPV 16 and/or 18 is necessary but not sufficient to convert an abnormal cell to malignancy. However with the increasing evidence associating HPV16 and 18 with cervical carcinogenesis, the detection of these viruses suggest that the host tissue is predisposed to malignant transformation (Meanwell et al., 1987). However, more prospective studies should be carried out to determine the future risk of cervical neoplasia among women who have latent cervical HPV infection. Should HPV 16 and 18 be conclusively implicated to have a role in cervical carcinogenesis, HPV screening and typing will identify women at high risk of developing cervical cancer. They can then be observed prospectively with a view of early detection and treatment of precursor lesions. Persistence of HPV 16 and 18 detection is mainly associated with persistence of abnormalities whereas regression has been found to associate with HPV-negativity, HPV DNA clearing or fluctuations (Meijer et al., 1992).

The clinical course of HPV infections be it latent, subclinical or overt is dependent upon the complex interplay between the virus, the infected epithelium, the host immune surveillance system and exogenous factors (Meijer et al., 1992). Thus far, the nature of the interaction between these factors have been poorly understood.

In order to account for the large number of womenwith latent HPV infection, zur Hausen (1986b) suggested a mechanism of failing intracellular control of persisting viral genomes in proliferating cells which results in the development of tumour. The intracellular surveillance system is regarded as a defence mechanism which protects the host at the cellular level against any possible lethat effects of coevolving persisting viruses.

This model proposes that a humoral factor is required to activate the cellular control genes, the CIF genes. The trans-acting interfering factor (CIF) transcribed from the CIF genes negatively regulates HPV expression by affecting the upstream regulatory region of HPV DNA. During differentiation, the cellular genes are switched off, allowing viral transcription, replication and maturation to take place. The expression of the controlling functions in these differentiating cells which allows the replication and maturation of the existing papillomavirus results in the balance of virus-host interactions. Modifications involving the CIF or their binding sites within the viral genome will result in malignant growth.

The current concept of carcinogenesis spells the importance of exogenous cofactors which could disturb the intracellular control system and increasing the risk for malignant conversion of HPV-containing cells. Different exposures to different cofactors may account for varying responses to HPV infections among infected individuals. Several risk factors which have been proposed are chemical or physical carcinogens (zur Hausen, 1982; Schmitt et al., 1989), heavy and prolonged smoking (zur Hausen, 1989; Brinton et al., 1986b; Brinton, 1992), coinfection with other viruses such as human herpes virus type 6 (Chen et al., 1994) and exposure to hormones such as progesterone (zur Hausen, 1991) and glucocorticoids (Pater et al., 1988).

5.4 Immunohistochemical detection of HPV antigens.

In order to evaluate further the relationship of HPV 16 and 18 infection to CIN and invasive carcinoma, an immunohistochemical study was undertaken aimed at determining the precise location of HPV 16 LI and HPV 18 E6 proteins within specific cell population of CIN, CIS and invasive carcinoma. Using specific immunoglobulins, the distribution patterns of HPV 16 L1 and HPV 18 E6 antigens were correlated with the presence of the respective viral sequences which was earlier determined by in situ hybridization. Localization of viral-associated antigens in vivo may be of great value in understanding their biological significance to the development of the lesion.

The expression of the HPV 16 L1 and HPV 18 E6 antigens varied widely in intensities as well as in topographical distribution within the lesions. This variability in nuclear staining between cells within the same area of a lesion, be it CIN, CIS or invasive carcinoma may reflect the varying number of the HPV genome copies harboured by these cells.

5.4.1 Detection HPV 18 E6 antigen in cervical lesions

In the current work, the role of HPV 18 protein expression in the progression of HPV 18-associated neoplasia was evaluated by studying the correlation between HPV 18 E6 protein expression and the various histological types of cervical lesions. The expression of E6 protein was analysed not only in patients who were positive for HPV 18 DNA but also in patients who did not show HPV 18 DNA positivity by in situ hybridization.

Specificity of the anti-E6 monoclonal antibody utilized was attested by the following observations. Characteristic E6 protein expression was not visible in all negative controls which comprised of HPV 18-negative cell line, CaSki as well as

tissue sections incubated with water. In contrast, HPV 18-containing cell line HeLa demonstrated the expression of the HPV 18 E6 protein. This supports the findings of others (Androphy et al., 1986b; Seedorf et al., 1987) that E6 ORF is retained and actively transcribed in human cervical cancer cell lines. In most tissue sections examined, the E6 protein was confined in cells within the lesions and not found in cells of adjacent areas such as the stroma. Occasionally faint, diffuse unspecific staining was seen but was easily distinguished from the characteristic staining of the protein in tumour cells.

5.4.1.1 Distribution of HPV 18 E6 protein in CIN

E6 protein was not detected in any of the CIN 1 cases although one have been confirmed to harbour HPV 18 sequences. The one case may be an early stage of HPV infection where gene expression occurred at low level. Thus the inability to detect the E6 protein here may not indicate total negativity but merely that it occurred at a level too low to be detected using immunohistochemical techniques used here. Though too low for detection, the E6 protein expression was obviously high enough to contribute to the development of CIN 1.

The E6 protein was visible in 53% (8/15) of the CIN 2 cases. In contrast to the distribution of the HPV 18 DNA where most cells containing the sequences were predominantly situated in the layers overlying the basal cells, the E6 protein appeared to be more widely distributed. In most cases, the protein was found scattered throughout the entire thickness of the epithelium extending from the upper basal layers to the more differentiated layers. CIN 2 is a relatively more advanced lesion compared to CIN 1 . In this stage, HPV infection becomes more productive in the middle and upper layers. In these lesions, viral replication probably occur at a higher

rate than in CIN 1, resulting in higher copy number of the viral genome which in turn enable the E6 protein to be expressed at relatively higher levels. The present observation contrasts those of Crum et al. (1987, 1988). These studies indicated that early HPV genes are expressed principally in the upper and more mature strata and very rarely in the basal layers. Thus, the ability to detect the expression of the E6 protein within the basal cells of CIN 2 cases examined in this study reflect the sensitivity of the immunohistochemical test used.

Further increase in the number of positivities for the E6 protein was demonstrated in CIN 3/CIS cases. The protein was detectable in 90% (18/20) cases. Cells positive for the protein were present in abundance throughout the entire thickness of the dysplastic epithelium and were comparatively more numerous and more intensely stained than those of CIN 2. The distribution pattern seen here again confirms that the E6 protein can be expressed in all layers of the epithelium and that it is not dependent on maturation or state of differentiation of infected cells.

Comparisons of the E6 expression pattern between low grade and high grade CIN have been previously carried out by Stoler et al. (1990) and repeated by another team (Durst et al., 1991). As with these investigators the present study observed a shift in the E6 protein expression from low grade to high grade CIN lesions. The higher level of E6 protein expression in CIN 3/CIS cases examined here reflect higher E6 transforming activity in these lesions. The present observation also reveals the presence of the antigen in the same areas which hybridized for HPV 18 DNA in in situ hybridization.

5.4.1.2 Distribution of HPV 18 E6 protein in cervical cancer

The E6 protein was detected in 85.7% (30/35) of cervical cancer cases. The cells exhibiting the protein were numerous, uniformly distributed and intensely stained. This is parallel to the findings of others (Schwarz et al., 1985; Banks et al., 1987; Smotkin and Wettstein, 1986) which revealed that a vast majority of HPV-positive cervical cancer actively transcribe the E6 (and E7) regions of the persisting HPV DNA.

The presence of the E6 protein did not always correlate with the in situ hybridization findings. The absence of HPV 18 sequences in 11 E6-positive cases ranging from CIN 1 to cervical cancer may be attributed to low copy number of the viral genome harboured by these lesions or due to the destruction of the viral sequences during fixation. On the other hand, the E6 protein was not detectable in 4 lesions which contained HPV 18 DNA. Again, the formalin used in fixation may have diminished the reactivitiy of the E6 protein. Another possible reason is the periodic expression of the viral antigen by infected cells. Since only a single sample was available from each patient, these episodes of periodic expression may have been missed. The same was suggested by a recent study of 102 recurrent laryngeal papillomas (Lack et al., 1980). When only one laryngeal papilloma sample for each patient was tested, only 48% showed positivity for HPV antigen. However, the detection rate increased when at least 4 consecutive laryngeal biopsies were examined.

The ratio (R) correlating the HPV 18 E6 protein positivity with HPV 18 DNA positivity (Figure) increased from 0 in CIN 1 to 1 in CIN 2, 1.05 in CIN 3/CIS and 1.2 in cervical cancer. One probable explanation for the increase in the abovementioned ratio with severity of the lesions is that in higher lesions the number of cells harbouring

HPV DNA in low copy number increases and may be too low to be detectable by in situ hybridization.

The E6 is localized in the nucleus and membrane fractions of an infected cell (Androphy et al., 1985). A specimen is considered positive for the E6 protein if it displays the characteristic intranuclear or cytoplasmic or both intranuclear and cytoplasmic stainings. Careful analysis of the proportion of each type of staining was carried out. Stainings in both nucleus and cytoplasm were found in similar proportions in CIN 2, CIN 3/CIS and cervical cancer. Staining in only the cytoplasmic region was seen the most in CIN 2 and decreased significantly in CIN 3/CIS and cervical cancer. On the other hand, cells exhibiting only intranuclear staining were limited to CIN 3/CIS and invasive cancer. The basis for the variations in localization of E6 protein in different histological grades of cervical lesions and whether or not it is significant to tumour progression is at present unknown.

As seen in the present investigation, the relative expression of the E6 protein increased with severity of CIN and continued to be highly expressed even after malignant transformation. The E6 protein were also regularly demonstrated in the HeLa cell line which have been cultivated for many years. The presence of the HPV 18 genetic information and E6 protein expression in preinvasive and invasive cervical lesions as well as derived cell lines suggest that the persistence of the E6 oncoprotein is critical for the development and maintenance of the cervical neoplastic state (Schwarz et al., 1985; Liang et al., 1993; Hudson et al., 1990). The present observations also strengthen the support for the hypothesis that the spectrum of CIN stages may reflect the progressive stages of cervical cancer development. The strict localization of the E6 oncoprotein directly within the lesions demonstrates the direct relationship of HPV 18 to these lesions.

E6 is one of the two oncoproteins actively produced in HPV-associated high grade CIN lesions and cervical cancer. Although E7 appears to be the major transforming protein, E6 and E7 act synergistically and combination of both are required for efficient immortalization and high frequency transformation of human epithelial cells (Hudson et al., 1990; Bedell et al., 1987; Hawley-Nelson et al., 1989). The E6 or E7 protein expressed by itself was shown to be less effective or sufficient to immortalize primary human keratinocytes (Hudson et al., 1990).

The regular high expression of the E6 protein seen in the present series of CIN 3/CIS and cervical cancer is a consequence of integration of the HPV 18 genome into host sequences. An important event in tumorigenesis, integration is a selective advantage which leads to the over-expression of E6 (and E7) oncoproteins and subsequent uncontrolled cellular proliferation (Choo et al., 1988, Schwarz et al., 1985). Overexpression of E6 (and E7) protein is thought to induce the shift from high grade CIN to invasive carcinoma (zur Hausen, 1991). The obligatory retention of the E6 (and E7) gene expression in high grade CIN and cancer suggest that cells which lack it may simply stop proliferating (Bedell et al., 1987).

It has been hypothesized that the E6 protein encoded by high risk viruses mediate cell transformation by forming complexes with the wild-type cellular tumour suppressor protein, p53 (Werness et al., 1990). When functioning normally the p53 protein negatively regulates cell growth. Complex formation of the E6 protein with p53 stimulates rapid degradation of the latter, abbrogates its function in the nucleus leading to uncontrolled cellular proliferation, chromosomal instability and aneuploidy (Scheffner et al., 1990). Studies have demonstrated that the cellular levels of wild-type p53 were sharply reduced in several cervical cancer cell lines and HPV-immortalized squamous epithelial cells (Matlashewski et al., 1986).

5.4.2 Detection of HPV 16 L1 protein in cervical lesions

The expression of the L1 protein was analysed not only in patients who were positive for the HPV 16 DNA but also in patients who did not show HPV 16 DNA positivity by in situ hybridization. A total of 52.7% (39/74) of the cervical lesions examined exhibited the presence of the L1 protein.

Specificity of the anti-L1 monoclonal antibody used in this study was attested by the following observations. Characteristic L1 protein expression was not visible in tissue sections incubated with water (negative controls). In the tissue sections examined, HPV 16 L1 protein was confined in cells within the lesions and was not found in cells of adjacent areas such as the stroma.

5.4.2.1 Detection of HPV 16 L1 protein in CIN

The presence of L1 protein was not observed in the one case of CIN 1 which was previously shown to harbour HPV 16 sequences. The absence of the capsid L1 protein in the one HPV 16-positive case may be due to the early stage of HPV infection which may be minimally productive. In this case, the amount of the capsid protein produced may still be too low to be detected by the present method. The remaining three cases of CIN 1 which was negative for HPV 16 DNA was also negative for the L1 protein.

Only 26.6% (4/15) of the CIN 2 cases demonstrated the presence of the L1 protein. Cells displaying the protein were not abundant. Corresponding to the distribution of HPV DNA and consistent with the findings of others (Meisels et al., 1977; Kurman et al., 1983; Winkler et al., 1984), the protein was mostly located in upper layers of the epithelia. The distribution pattern observed here support the

concept that virus replication and production is dependent on the differentiation and maturation of the viral-harbouring keratinocyte. In general, the late (capsid) proteins are synthesized and assembled to form mature viral capsids simultaneously or after the onset of the HPV DNA replication and is most prevalent in the upper epithelial layers (Shah and Gissmann, 1989; Schneider and Koutsky, 1992).

Previous observations indicate that the viral capsid protein was not normally seen within nuclei of cells deep in the basal layer but can occasionally be detected in the parabasal cells (Kurman et al., 1983). However in several isolated cases observed in the present study, positive staining was not confined to cells of the middle and upper strata. In these cases, a few L1-positive cells were also found within the basal and parabasal layers. This may be due to the presence of isolated, patchy areas of premature cell differentiation which in turn provide a permissive environment for HPV replication (Crum et al., 1986).

Up to 50% (10/20) of the CIN 3/CIS cases were positive for the L1 protein. In contrast to findings by Kurman et al. (1983), the present series showed that the L1-positive cells were localized directly within the high grade lesions. L1-positive cells were found scattered, mixed with the apparently L1-negative cells and occupied regions from the upper two thirds to the entire thickness of the epithelium. However in comparison, Kurman et al. (1983) found that only two cases of CIN 3 and none of CIS contained HPV capsid protein directly within the lesions. Even in the two CIN 3 cases, the protein was found localized within the most superficial layers of the epithelium. They further demonstrated that the remaining positive cases of high grade lesions harboured the capsid proteins not directly within the specified lesions but in adjacent epithelial areas. Similarly, (Shah and Gissmann, 1989) located the capsid protein in less severely affected areas surrounding the CIS lesions.

5.4.2.2 Detection of HPV 16 L1 protein in invasive carcinoma

HPV 16 L1 protein was detected in 71.4% (25/35) of the total cervical cancer cases. The protein was localized within the nuclei of the malignant cells. Cells showing L1-positivities were found scattered within the lesions and mixed with cells not exhibiting the positive signal. However in general, the staining was more intense in these invasive lesions as compared to the CIN lesions.

Obvious in the present investigation, the frequency of L1 protein expression increased with severity of the cervical lesions and continued to be highly expressed in cervical cancer. A significant increase of the L1 protein expression from the CIN lesions to cervical cancer was recorded. This is very puzzling as it clearly contradicts the data from many different labs which consistently show that the capsid protein prevalence declines with the increasing degree of neoplasia and drops to zero in invasive cancer (Shah and Gissmann, 1989; Sato et al., 1987; Shah et al., 1980; Sterret et al., 1987;Kurman et al., 1983). These studies suggest that the failure to detect the capsid protein in cases of severe dysplasia and cancer may be attributed to the disturbance in virus assembly associated with neoplastic transformation. The present data does not necessarily dispute this well-accepted concept.

The detection of the L1 protein in the benign lesions seen here may have different implications than the detection of the same in invasive lesions. HPV exists as episomes in benign lesions where all ORFs including the L1 ORF are intact and available for transcription into functional RNAs which in turn are subsequently translated into functional proteins. L1 proteins are believed to be expressed only in productive viral infections (Firzlaff et al., 1988). Thus as mentioned earlier and apparent from the L1 distribution pattern in CIN 2, the stained nuclei in these benign

lesions are sites of virus production. Since the benign lesions still retain some degree of differentiating epithelia necessary for the completion of the HPV growth cycle, the prevalence of the capsid protein was expected to occur at a higher rate than the more severe lesions. However, it is not true here. This may be because HPV genomes which exist as episomes in these lower lesions can be lost during cell division, probably due to unequal distribution of copies ultimately resulting in the lower frequency of L1 antigen positivities.

The increased detection of the L1 protein in CIN 3/CIS and cancer may be an indicator of a more stable existence of the virus due to integration into the host genome and not indicative of productive infections or actual capsid synthesis as in lower lesions. Integration into the host genome often results in the disruption and deletions of large sectors of the viral genome. Late genes are usually either lost or partially disrupted along with parts of the early region, leaving E6 and E7 as the only two ORFs consistently retained in cancer (Schwarz et al., 1985; Yee et al., 1985; Pater and Pater, 1985; zur Hausen, 1989). The results of this study suggest that deletion due to integration into the host genome did not extend into the L1 ORF. Seventy one percent of the paraffin, embedded cervical cancer tissues and 57.5% of the fresh frozen cervical cancer tissues retained at least a portion of the HPV 16 L1 DNA region. This region which was represented by the HPV 16 DNA probe used in in situ hybridization and Southern blot hybridization was obviously not deleted. In one other study (Kaur and McDougall, 1989) a single integrated copy of HPV 16 DNA extracted from a cervical tumour was cloned and shown to retain partial L1 ORF together with partial E1, complete E6 and E7 ORFs and the NCR.

The unusual expression of the L1 antigen in CIN 3/CIS and cervical cancer seen in this study is probably attributed to the arrangement of the viral DNA in relation

to the host genome. The samples considered here probably have multiple copies of HPV 16 DNA integrated mainly in the form of head-to-tail repeat units. If the viral genome is arranged as head-to-tail repeats, all ORFs present are in continuous arrangement as if in a circular molecule. There is a possibility that when the early genes are transcribed, the retained region of the L1 ORF is transcribed as well (Gissmann and Schwarz, 1987). Thus the L1 antigen detected here may have been translated from the early region transcripts which read through into the L1 ORF. The L1 protein detected here may not represent the entire major capsid protein but rather a portion of it that is not functional.

There was no evidence of the L1 protein expression in the CaSki or SiHa cell lines. The same was observed by Baker et al. (1987). As a result, these cell lines were not able to be utilized as positive controls for the detection of HPV 16 L1 protein. In both cell lines the HPV 16 L1 ORF was not completely deleted in the integrative event. This was demonstrated earlier by hybridizing with a probe specific for the HPV 16 L1 region. Regardless of the physical state of the late genes, the expressions of the L1 and L2 are totally suppressed in cultured cells (Schwarz et al., 1985; Smotkin and Wettstein, 1986).

The L1 protein of HPV 16 is a polypeptide of 58kDa which is exclusively localized in the nucleus of infected cells (Browne et al., 1988; Zhou et al., 1991). The nuclear localization of the L1 protein is determined by two nuclear localization amino acid sequences. These sequences facilitate the L1 rapid selective entry after synthesis in the cytoplasm through the nuclear pore complex into the nucleus (Zhou et al., 1991).

Apart from specific nuclear reactivity, diffuse cytoplasmic staining was also seen in one case (25%) of CIN 1, 11 cases (73%) of CIN 2 and 11 cases (55%) of CIN

3/CIS. When present, the cytoplasmic staining was presented in the keratinocytes of the intermediate and superficial layers of the affected epithelia which retained some degree of differentiation and maturation. Other reports (Viac et al., 1978; Jenson et al., 1980; Lack et al., 1980; Woodruff et al., 1980) have documented similar staining of the keratinocyte cytoplasm in superficial epithelial layers. It is interesting to note that none of the malignant cells of the cervical cancer cases or cell lines SiHa and CaSki demonstrated such staining. At the present time it cannot be determined if the cytoplasmic staining is a consequence of fragmentation of infected nuclei during fixation or sample preparation resulting in the release of viral particles and viral-associated proteins into the cytoplasm. Neither can it be unequivocally decided if it is otherwise due to the nonspecific crossreaction of the anti-HPV 16 L1 antibodies with cellular proteins. The specific L1 staining pattern was easily distinguished from the cytoplasmic staining of the cells. Thus due to the inability to explain specifically why cytoplasmic staining occurred, lesions which did not exhibit demonstrable L1 protein in the nuclear region were regarded as L1-negative.

As with HPV 18 E6 antigen, the presence of the HPV 16 L1 protein did not always correlate with that of HPV 16 DNA. In 10 cases ranging from CIN to cervical cancer, the L1 protein occurred in the absence of any detectable HPV 16 sequences. On the other hand, 20 cases showed only the presence of the viral sequences. The ratio (R) correlating the expression of the L1 protein with the presence of the HPV 16 DNA (Figure) increased from 0.25 in CIN 1 to 0.399 in CIN 2, 0.91 in CIN 3/CIS and 1.22 in cervical cancer. Such increase reflect the higher number of cells in the more severe lesions harbouring HPV 16 DNA in low copies.

5.5 Serology to HPV

In the recent years, site-directed serology with synthetic peptide-based ELISA has been widely utilized in the diagnosis of viral-associated diseases and tumours (Dillner, 1984; Dillner et al., 1989; Norrby et al., 1987a, 1987b; Moriarty et al., 1985; Smith et al., 1987). Synthetic peptides which represents only linear epitopes are the source of pure, well-defined, non-denatured viral epitopes (Galloway, 1992). In ELISA the use of purified antigens is essential because it ensures that the signal generated is the result of reaction by the antibodies to the HPV protein and not noise resulting from antibodies reacting with contaminating non-HPV protein. Synthetic peptide-based ELISA measures only antibodies directed against continuous, linear epitopes but not antibodies targeted to non-linear, discontinuous, conformational epitopes that are also present in the native proteins (Dillner et al., 1990a; Galloway, 1992). However it has been shown in some viral systems that antibody responses against only sequence-specific linear epitopes are sufficient to be used as diagnostic markers for viral infections and diseases (Dillner et al., 1984; Norrby et al., 1987a, 1987b; Moriarty et al., 1985; Smith et al., 1987).

During the last few years, a number of laboratories have reported on the detection of anti-HPV antibodies. With some exceptions, these findings were inconsistent. The discrepancies in reportings were due to one or more factors which include assay variations, different type, source and purity of antigen targets, different criteria for the subject selection, different criteria to determine antibody positivity and population differences (Dillner and Dillner, 1994). Due to these inconsistensies it is therefore difficult to compare the data from different studies (Galloway, 1992).

The present work evaluates the serologic response to HPV 16 and 18 in sera obtained from 80 cervical cancer patients, 32 healthy pregnant women and 30 healthy

university students in ELISA assays using as antigens, selected synthetic peptides based on sequences deduced from the early and late regions of HPV 16 and 18.

In the present study, necessary precautions were taken to minimize interassay variations. Predetermined experimental conditions such as for coating of solid phase (for example the concentration of reactant, time of coating, temperature and pH) were strictly adhered. Carbonate buffer (coating buffer) pH 9.6 used were optimal for most proteins and lipoproteins because satisfactory sensitization is achieved. Microplates made of polystyrene were used to give adequate, reproducible uptake of most antigen targets by passive adsorption in alkaline solution (Voller et al., 1984). Each series of incubation steps was separated by washing in PBS containing 0.05% Tween to remove unbounded reagents and prevent carry-overs from one step to another. The sera were diluted in PBS containing 10% lamb serum (Gibco). The addition of the latter was to prevent non-specific attachment to the solid phase and reduce noise to signal ratio (background). The antigen-antibody reaction in the wells were photometrically assessed. All test samples giving absorbance values of at least 0.1 above the values of the reference wells (devoid of antigen target) were considered as seropositives. Hence the greater the difference the more immunoreactive the serum. Due to the inavailability of respective cervical tissues or smears at the time the sera was collected, the serum donors were not characterized virologically. Thus at the time this study was carried out, the status of the individuals in the different groups with regard to the past or present HPV 16 or 18 infection were unknown.

5.5.1 Immune response to HPV 16 L1:13 and L1:30 peptides

Only a small portion of the cervical cancer patients develop IgA response to the two peptides deduces from sequences of the L1 ORF used in this study. For peptide L1:13, the IgA response occurred exclusively in but 13.75% of the cancer patients whereas for L1:30 it was demonstrated in a lesser portion of only 6.25% . Though occurring in minority of the cancer patients, the IgA responses against both peptides were especially strong. Anti-L1:30 IgA antibodies were very rare in pregnant women and university students.

The IgG responses against L1:13 and L1:30 were found to be more prevalent than that of the IgA. It was represented in all three groups of subjects. The prevalences of IgG reactivities to L1:13 were slightly more in the cancer patients and pregnant women as compared to university students. For L1: 30, IgG reactivities were significantly more prevalent in the cancer patients than in the university students but did not differ significantly from that of the pregnant women.

Peptide L1:13 was deduced from sequences in the internal region of the L1 protein. The L1 ORF was earlier shown by Dillner et al. (1990a) to contain several linear epitopes, spanning amino acid 167-271 (represented by peptides L1:12 thru L1:18) that were immunoreactive with IgA and IgG antibodies in the sera of HPV 16-carrying cervical neoplastic patients and to a considerable lesser extent in the sera of healthy controls. This was later confirmed in another study (Dillner et al., 1990b) where L1:13 was tested for reactivity. Both anti-L1:13 IgA and IgG responses were elevated among cervical cancer patients as compared to healthy controls. The study reported anti-L1:13 IgA reactivity in 66% and 58% of the cancer patients and controls, respectively. IgG reactivities, though less prevalent than IgA, were still higher among patients as compared to controls. The occurrance of anti-L1:13 as described in the

present study was much lower than those reported by Dillner et al. (1990a). This interlab variations may be ascribable to one of the factors described earlier.

L1:13 which was found not to be very immunoreactive with IgA and IgG antibodies in the sera of Malaysian subjects considered here was instead highly IgM reactive, unlike L1:30. Anti L1:13 IgM were seen in majority of individuals from the three groups, 85% of cancer patients, 84.4% of pregnant women and 73.3% of university students. There is no significant difference in prevalences of the reactivity between the three groups. Equally high prevalence of anti-L1:13 IgM reactivities were also observed by Dillner et al. (1990a) in both groups cervical neoplastic patients and controls. These observations suggest that L1:13 may possibly be a major IgM-epitope. Alternatively, the high IgM reactivities seen may be a result of cross-reactiom. The internal region of the L1 protein contains group specific amino acid sequences and thus have high homology between the different genital HPV types (Dillner et al., 1990a). For example, the degree of sequence homology between the L1 region of HPV 16 and 18 is close to 80% (Baker, 1987a; Cason et al., 1989). Since IgM antibodies are known to be prone to cross-reactions, the IgM reactivities seen here may be the result of extensive immunological cross-reactivities between the different HPV genotypes that may be present in the subjects. The same IgM reactivity was not seen with peptide L1:30 and may be due to tha lack of similar sequence homology within this region of the L1 protein.

5.5.2 Immune response to the L2 peptide

The L2 ORF had only a few significant immunoreactive epitope, the major one being L2:49 which is positioned in the internal region of the L2 protein. This epitope was tested in the present study for reactivity with IgA, IgG and IgM antibodies.

The present study showed that the peptide was IgA and IgG-reactive with the sera of cancer patients, pregnant women and university students. Anti-L2:49 IgA responses occurred in similar proportions and there was no significant difference in the antibody levels between the three groups. On the other hand, the IgG response which was found to be more prevalent than IgA was demonstrated in 65% of cancer patients, 62.5% of pregnant ladies and 46.7% of university students. The differences in IgG antibody levels and prevalences between the three groups were not at all significant. In agreement with the findings of Diilner et al. (1990a), IgG reactivity to peptide L2:49 is an example of a highly immunoreactive peptide that is not disease-specific.

The L2 ORF is normally deleted, when as part of carcinogenesis, the virus integrates into the host genome (Durst et al., 1987). Thus in cancer patients examined in this study, antibodies to L2:49 were very likely to occur as a response to primary HPV infection and may thus signify past exposure to the protein (Kochel et al., 1991) rather than a marker for the diseased condition. The relatively lesser prevalence of anti-L2:49 IgA positivities in all three groups as compared to that of IgG, are likely to be related to the general properties of the IgA antibodies. IgA antibodies are to a large extent induced by mucosal infection and does not persist after the antigen has been removed. In contrast, IgG antibodies may persist long after the antigen has been removed (Dillner et al., 1990a).

Peptide L2:49 does not contain epitope reactive to IgM antibodies. The only response detected was in one cervical cancer patient. The lack of IgM immunoreactivity against this peptide was also demonstrated by Diilner et al. (1990a) . It cannot be determined as yet whether L2:49 is overall less immunogenic to IgM antibodies or if the major IgM reactivity is mainly conformationally dependent.

5.5.3 Immune response to HPV 16 E7:5 peptide

HPV 16 E7:5 is a 21-residue synthetic peptide positioned at the carboxyl-terminus of the E7 protein. This peptide was examined because E7 protein derived from high risk HPV types is a mediator of HPV-induced epithelial transformation (Crook et al., 1988; Phelps et al., 1988; Bedell et al., 1989). It has been shown to have the ability to bind to the product of tumour suppressor gene, Rb and promote its degradation (Dyson et al., 1989). Cotransfection of an activated ras oncogene with E7 ORF of HPV 16, 18, 31or 33 resulted in transformation of primary epithelial cells in vitro (Storey et al., 1988). E7 is the most abundant viral protein which is consistently expressed in HPV-containing cervical cancer cells and derived cell lines (Watanabe et al., 1989; Schwartz et al., 1985). In fact, increased biological activity of HPV 16 E7 ORF is associated with cervical cancer. Owing to the possible oncogenic role of HPV 16, the prevalence of antibodies to E7 oncoprotein is expected to be of major importance as discriminating marker for cervical cancer.

Reactivities with all three classes of immunoglobulins were detected in the sera of cervical cancer patients. IgA was detected in 26.25%, IgG in 21.25% and IgM in 52.5% of the cases examined. Anti-E7:5 antibodies were also observed among the non-cervical cancer group - the pregnant women and university students.

In contrast to findings by numerous other investigators (Jochmus-Kudielka et al., 1989; Kochel et al., 1991a; Mann et al., 1990) this present study did not find anti-E7 antibodies to occur more frequently among cervical cancer patients as compared to non-cancer subjects. Jochmus-Kudielka et al. (1989) noted a 14-fold higher prevalence of anti-E7 antibodies among cervical cancer patients. Kochel et al. (1991a) reported a 5.6-fold higher occurances of seropositive cases among cervical cancer patients. On the other hand, Mann et al. (1990) saw IgG reactivity to E7

protein among 25% of cases versus 6% of healthy controls. As a result, all these studies suggested that antibodies to HPV 16-derived E7 protein are markers for cervical cancer. Unfortunately the same cannot be said here if based solely on frequencies of anti-E7:5 antibody positivities.

E7 protein contains several immunoreactive epitopes (Dillner and Dillner, 1994). As with other proteins, they may be present as linear epitopes or as conformational epitopes and in addition, may have different degree of immunogenicity. Evident from the present study, the epitope represented by the E7:5 peptide is an epitope presented by the cancer cells as well as by cells of some pregnant women and university students. Thus, the epitope contained in the E7:5 peptide is clearly immunogenic with the sera of cervical cancer patients as well as pregnant women and university students. E7:5 may contain epitope different from those tested by the other investigators mentioned. The differences in target antigens may be a factor in the discrepancy in reporting between the present and previous studies. Similar frequencies of anti-E7:5 seropositivities among the three groups of women seen here suggest that humoral response to the epitope represented by the E7:5 peptide can develop at any stage of HPV infection and not necessarily late in tumour progression.

Despite not being disease-specific, the present results are in agreement with those of other findings (Mann et al., 1990; Jochmus-Kudielka et al., 1989; Muller et al., 1992) in that the E7 proteins are expressed at higher levels in cervical cancer patients as compared to women without cervical cancer. The levels of anti-E7:5 antibodies were found to be significantly higher in the cancer patients than that of pregnant women and university students. The enhanced anti-E7:5 IgA response in cancer patients may have resulted from the constant exposure of the host immune system to

the E7 protein in persistently infected cells of the cervical mucosa. This reflects the higher E7 transforming activity in cervical cancer cells and simultaneously supports the involvement of E7 protein in cervical oncogenesis.

If HPV 16 E7 protein activity is elevated in cancer cells then one would expect higher frequency of seropositives among cancer patients. However as seen here, only a proportion of patients develop measurable antibodies (26.3% for IgA and 21.3% for IgG). Bleul et al. (1991) demonstrated that only a fraction of the HPV DNA-positive women have sera which reacted with E7 peptide in ELISA. In the present study, the remaining which did not respond to the HPV 16 E7:5 peptide may instead contain antibodies reactive to other epitopes of the E7 protein. Thus the use of a combination of different epitope would be an advantage in serologic studies. One other explanation for the absence of measurable anti:E7:5 antibodies in some cancer patients is the inability of the present method to detect antibodies directed against conformational epitopes which also exist in the native E7 protein (Galloway, 1992; Dillner et al., 1990).

5.5.4 Immune response to HPV 16 and 18 E2: 245 peptide

The E2 is the most reactive of all HPV 16 ORF (Dillner et al., 1990). It contains several epitopes, two of which, peptides 245 and 9, have been extensively characterized (Dillner and Dillner, 1994). The distribution of the antibodies to the E2 protein of HPV 16 and 18 in the sera of cervical cancer patients, pregnant women and university students were investigated using peptide E2: 245. Originally described by Dillner et al (1989), the 19-residue peptide was deduced from a region close to the carboxyl terminus of HPV 16 and 18 E2 ORF.

HPV 16 E2: 245 was the first reported HPV 16 antigen that was reactive with sera from CIN and cervical cancer patients (Dillner et al., 1989). In agreement, this study observed a higher prevalence of anti-HPV16 E2:245 IgA antibodies in cervical cancer patients (46.3%) as compared to pregnant women (25%) and university students(10%). In addition, a significant elevation of the anti-HPV 16 E2: 245 IgA levels was seen among cancer patients compared to university students. While IgG antibodies were recorded in similar frequencies of 26.3% and 34.4% in cancer pateints and pregnant women, respectively, IgG reactivity was not observed among university students. Comparatively, Dillner et al. (1990) found IgA response to E2: 245 in 73% of cervical cancer cases but only in 22% of controls. Reeves et al. (1990) and Mann et al. (1990) reported an elevation of anti-245 antibodies for some patients with cervical cancer. Lehtinen et al. (1992) found that IgA seropositivity to E2:245 was 7.5 times more common among cervical cancer patients than among healthy controls. These previous studies only analysed immune response in cancer patients to compare with healthy controls. They did not consider anti-E2:245 responses in pregnant women.

Antibodies against the homologous peptide E2:245 of HPV 18 were also preferentially found among cervical cancer patients compared to pregnant women and university students. IgA antibodies were detectable in 22.5% of cancer patients and in 6.25% of pregnant women. On the other hand IgG seropositivities were prevalent in 26.25% and 12.5% of the former and the latter, respectively. However, there were no significant difference in the IgA and IgG antibody levels between the two groups. On the other hand, none of the university students developed reactivity to HPV 18 E2:245. These findings are in agreement with other studies (Lehtinen et al., 1992;

Dillner and Dillner, 1994) which also showed the association between anti-HPV 18 E2:245 antibodies with cervical cancer.

The E2 gene of the papillomavirus encodes a regulator of viral gene expression (Spalholz et al., 1985; Haugen et al., 1987). The E2 protein affects several HPV functions among which are transformation, replication and plasmid maintenance. The nuclear-binding protein regulates the HPV 16 p 97 promoter and thereby, the level of E6 and E7 expressions by binding to $ACCN_6GGT$ motifs located in the HPV 16 upstream regulatory region (Androphy et al., 1987; Lambert et al., 1987; Phelps and Howley, 1987).

The presence of anti-E2:245 IgA and IgG in some of the sera of cervical cancer patients indicate that the E2 protein is continuously being expressed and presented to the immune system of these patients. These findings contradict the hypothesis that HPV 16 and 18 E2 transcription regularly cease in cancers. According to the hypothesis of cervical carcinogenesis, integration of HPV DNA into the host genome in many late stage precancers and cancers disrupts the expression of the E2 ORF (Durst et al., 1987). Cervical cancer-derived cell line SiHa and HeLa harbour HPV genome in which the E2 region is disrupted (Schneider-Gadicke and Schwarz, 1986). It was suggested that disruption of the E2 ORF is a prerequisite for late stage tumour progression as it results in unregulation and overexpression of the transforming E6 and E7 gene transcription in cervical carcinomas (Choo et al., 1988; Schwarz et al., 1985). In a study by Smits et al. (1988), interruption of the E2 ORF increases the frequency of HPV-induced transformation of human fibroblasts.

Findings of several other studies have also refuted the claims that the E2 ORF is regularly disrupted in cancers. The E2 protein was demonstrated in vitro in two established HPV-associated cervical cancer cell lines, CaSki and C4-1 (Dillner et al.,

1989) and in two HPV-16 immortalized keratinocytes W12 and AC 89/E2 cell lines (Bouvard et al., 1994). The E2 protein is also being increasingly demonstrated in vivo, in late stage tumours (Bouvard et al., 1994).

All these observations suggest that the interruption of the E2 ORF is not a critical prerequisite for HPV-mediated transformation. Elevated response of some cervical cancer patients to HPV 16 and 18 E2:245 peptides reflect an increased E2 protein activity in them. Since the anti-E2 antibodies are more frequently found in these patients, then it is reasonable to believe that the E2 protein may be positively involved in the genesis of cervical cancer.

Earlier, Munger et al. (1989) had reported that only the E6 and the E7 genes are required for HPV-mediated immortalization. Then recently, Romanczuk et al. (1991) suggested that other components of the HPV genome, in addition to the LCR transcriptional regulatory elements and the viral transforming genes E6 and E7, may also affect the immortalization efficiencies of the genome. HPV 16 E2 protein has been shown to transactivate the E6 gene expression in vitro and in cooperation with the Ha-ras oncogene culminate in high efficiency transformation within cells. (Bouvard et al., 1994; Lees et al., 1990). Transactivation of viral transcription is clearly important and in the absence of the E2 protein the transcription efficiency from HPV promoters have been shown to decrease (Giri et al., 1985; Thierry and Yaniv, 1987). The extreme tumorigenicity the E2-containing cell lines suggest similar effects in vivo (Lees et al., 1990).

5.5.5 Immune response of pregnant women to HPV antigens

Anti-HPV 16 and 18 antibodies have been detected in the sera of pregnant women examined in this study. The presence of HPV 16 and 18 infections in these

women is not totally unexpected. This is because of the immunosuppression conditions associated with pregnancy. Maternal CMI is lowered in pregnancy (Hagen and Froland, 1972; Purtillo et al., 1972). Line of evidence which supports this include depressed maternal lymphocyte responses in vitro to the mitogen phytohemagglutinin and prolonged survival of skin homografts in pregnant women. The impaired cell mediated immunity found in pregnancy is a fail-safe mechanism which prevents the rejection of the conceptus. Various hormones including chorionic gonadotrophin corticosteroids, estrogens, progestrogens as well as placental barriers, blocking antibodies and carcinoembryonic antigen and alpha-fetoproteins found in the serum of pregnant women cummulatively contribute to the immunosupression (summarized by Purtillo et al. (1971)).

Cellular immunity is important in antiviral defence (Purtillo et al., 1971). Several studies have shown that HPV infections increased in prevalence among pregnant women (Schneider et al., 1987; Meisels, 1992; Woodruff and Peterson, 1958). Besides facilitating primary HPV infection, transient immunosupression associated with pregnancy reactivates latent HPV infections leading to increased production of viral particles (Schneider et al., 1987). Hormones such as estrogen and progestagens whose levels increase during pregnancy could directly influence viral regulatory elements (Ponta et al., 1985). Comparatively, only intermittent viral reactivation and replication or excretion are seen with immunocompetence (Melbye et al., 1990). In a study based on the detection of HPV DNA (Schneider et al., 1987), infections were 2.3 times more common in pregnant women than in non-pregnant controls. In addition, pregnant women were found to contain HPV DNA in much higher amounts.

Antibody status after HPV reactivation in pregnancy have not been extensively studied. Despite the more frequent detection of HPV DNA in cervical smears and HPV-associated lesions, Schneider et al. (1987) failed to demonstrate higher anti-HPV 16 E4 and E7 antibodies in the sera of pregnant women. However in the present study , the higher rate and productivity of HPV infection among pregnant women is reflected in slightly higher prevalences of anti-HPV 16 and 18 antibodies as compared to non-pregnant university students. Such higher prevalences were observed for anti-HPV 16 L1:13 IgG (15.62% versus 6%), anti-HPV 16 L1:30 IgG (21.87% versus 6.6%), anti-HPV 16 L2:49 IgG (62.5% versus 46.5%), anti-HPV 16 E2:245 IgA (25% versus 10%), anti-HPV 16 E2:245 IgG (34% versus 0), anti-HPV 18 E2:245 IgG (12.5% versus 0).

The presence of antibodies to HPV 16 E7:5 and E2: 245 as well as to HPV 18 E2:245 in pregnant women indicates the expression of these proteins. As described earlier, these proteins may have an important role in the genesis of cervical cancer. The increased expression of these proteins in pregnant women is thought to have resulted from the lowered CMI. However it is important to stress here that immunosupression associated with pregnancy is only temporary. A high regression rate of HPV infections and a dramatic drop in the number of individuals positive for HPV DNA have been observed after delivery (Woodruff and Peterson, 1958; Cook et al., 1973; Garry and Jones, 1985). Therefore the expressions of the E2 and E7 proteins are expected to duly drop after delivery. Transient expression of E7 and E2 proteins in pregnancy may not be sufficent for cervical carcinogenesis because persistence in the presence of other risk factors are required for such development.

5.5.6 Immune response of healthy university students to HPV antigens

Immune responses against peptides representing the late and early regions of HPV 16 and 18 were observed among healthy university students examined in this study. The seroprevalence in these students is not surprising and provide further support that asymptomatic infections with HPV 16 and 18 are widespread among healthy females. HPV 16 and 18 DNA have been detected by many (Griffin et al., 1990; Meanwell et al 1987; Reeves et al., 1989; Kjaer et al., 1988) in histologically normal cervical epithelia. Seroprevalence has been reported for HPV 16 and 18 E7, E4, E2, L1 and L2 antigens among healthy adults (Dillner et al., 1990; Jochmus-Kudielka et al., 1989; Dillner, 1990; Kochel et al., 1991b)) as well as in children (Jenison et al., 1990; Cason et al., 1992). In fact serologic studies were claimed to have uncovered more HPV 16 and 18 infections in normal females than by nucleic acid hybridization (Kochel et al., 1991).

The presence of antibodies in these students suggest that HPV 16 and 18 late and early antigens are also immunogenic in the sera of healthy women. Considering the present findings and supported by others, it is safe to suggest that infection by HPV 16 and 18 can induce host response even in the absence of clinical lesions. The significance of such host response, whether or not it is protective of the development of disease, is at present still unclear.

At first glimpse, the similar prevalence of anti-E7:5 antibodies in students and cancer patients appear rather puzzling because E7 protein is involved in HPV-mediated cellular transformation. However although anti-E7:5 antibody have been detected among these healthy subjects, the level is significantly lower than that in the cervical cancer patients. The lower antibody levels reflect low amounts of the transforming protein produced in these students. Low levels of anti-E7 antibodies in

the sera of healthy women have also been reported elsewhere (Jochmus-Kudielka et al., 1989; Mann et al., 1990; Kochel et al., 1991a, 1991b; Jenison et al., 1990).

There is a possibility that antibodies detected in these healthy students are produced by HPV infections at other body sites. Infections by these genital HPV types in squamous epithelia at nongenital sites are common. For example, HPV 16 DNA has been frequently found in histologically normal oral mucosa (Maitland et al., 1987). The status of the students with regard to any past or present HPV 16 and 18 infection is unknown. Antibodies once induced by an infection may persist for a long time. In this context, serology allows not only prospective but retrospective studies as well. Thus, antibodies detected in these healthy students may well be a serologic indicator for the fact that these individuals has come into contact with HPV 16 and/or 18 during their lifetime.

CHAPTER 6

CONCLUSION

The techniques used in this study has been successfully applied to study the association of HPV 16 and 18 with cervical cancer as well as the occurance of these viruses in women without cervical cancer. PCR-based HPV diagnosis and *in situ* hybridization using digoxigenin-labelled HPV probe detected and identified HPV 16 and 18 DNA in routine pathological specimens. The latter technique also was seen to be sensitive enough to detect HPV DNA in all layers of the cervical epithelium including the lower basal layers. Immunohistochemistry using HPV-specific monoclonal antibodies was an effective method for studying HPV 18 E6 and HPV 16 L1 protein expression in clinical materials as well as in tissue culture systems.

The synthetic-peptide based ELISA is a reliable and sensitive quantitative test useful for the evaluation of anti-HPV antibody levels in the sera in cervical cancer patients, pregnant ladies and university students. The test is very simple and therefore have the potential to be useful in large scale studies. The antibodies detected by the present ELISA shows that synthetic peptide can to a certain extent mimic the linear antigenic sites present in the native HPV protein.

Our findings imply that the pattern of immunoreactivity is different for different peptides. Anti-HPV 16 E2:245 IgA, anti-HPV 18 E2:245 IgA and IgG antibodies seemed to have specificity for cervical cancer and thus have the potential to be used as a discriminatory marker for the disease. None of the antibodies to the other peptides examined (L1:13, L1:30, L2:49 and E7:5) seemed to be sufficiently disease-specific to be used as markers. However it is possible that an assay based on

combination of several peptides derived from a variety of HPV 16 and 18 protein could provide an immunoassay of high sensitivity and specificity for HPV-associated cervical cancer. Thus, depending on which peptide is being used or which antigenic determinant is being analysed in ELISA, serology can be useful in either epidemiology of HPV infection or as marker for cervical cancer.

The available data clearly demonstrates that HPV antigens are immunogenic and are targets for immune surveillance mechanism. The presence of anti-HPV 16 and 18 antibodies in the sera of healthy individuals show that anti-HPV antibodies can develop at any stage of HPV infection and not necessarily late in tumour progression.

This study support the involvement of HPV 16 and 18 as major etiological factors in cervical carcinogenesis in Malaysian women. Unlike other findings where HPV 16 has been found to be the more predominant HPV type in cancerous lesions of the uterine cervix, followed by HPV 18 (Durst et al., 1983; Hsieh et al., 1988; Boshart et al., 1984; Kulski et al., 1987) the present data showed equal dominance of HPV 16 and 18. Dual infection with HPV 16 and 18 was very common. The significance of such infection is at the present moment still unknown but observations suggest that infection with one type did not interfere with infection by the other HPV. DNA studies further implies progressive involvement of these viruses with tumour progression and offers support that HPV 16 and 18-associated cervical cancer develops in a stepwise manner from low grade to high grade CIN and and invasive cancer.

The demonstration of HPV 16 and/or 18 genetic material and activity in premalignant and malignant cervical lesions indicate that the virus does not exist as an innocent passenger in the host epithelium of Malaysian women. It has been reported that E6 and E7 oncoproteins have a role in HPV-mediated transformation. Increased expression of the E6 protein from low grade to high grade CIN and invasive

cancer indicates the presence of higher E6 protein activity in severe lesions. Likewise, elevated anti-E7 antibodies suggest that E7 protein is present in higher amounts in cervical cancer pateints as compared to women without the disease. Higher levels of anti-HPV antibodies indicate that cancer patients have active virus-carrying disease. Anti-E7 antibodies were present in lower amounts in healthy subjects. These studies support that persistent and high level E6 and E7 transforming activity is important for the development and maintenance of the cervical neoplastic state.

In addition to E6 and E7, E2 protein also appeared to be positively involved in the genesis of cervical cancer. Elevated levels of anti-E2:245 antibodies in majority of sera from cervical cancer patients reflect high E2 protein activity in these patients. These observations suggest that the loss or interruption of E2 ORF is not absolutely critical for HPV-mediated transformation.

HPV L1 ORF is not totally disrupted in cancer. The present findings suggest that it may be integrated along with the HPV early genes into the host genome. Relatively higher presence of L1 DNA and L1 protein expression in the more severe CIN lesions and cervical cancer as compared to the milder CIN lesions does not signify productive infections but may instead indicate a more stable existence of HPV 16 due to integration into the human genome.

The present findings indicate that HPV 16 and 18 infections are widespread among healthy Malaysian females. HPV 16 and 18 DNA were detected in cytologically normal cervices. In addition anti-HPV 16 and anti-HPV 18 antibodies were seen in women without CIN and cervical cancer. Anti-HPV antibody reponses were also successfully studied in healthy pregnant ladies. Antibodies such as anti-HPV 16L1:13 IgG, anti-HPV 16 L1:30 IgG, anti-HPV 16 L2:49 IgG, anti-HPV 16

E2:245 IgA and IgG as well as anti-HPV 18 E2:245 IgA and IgG were found in higher prevalences as compared to healthy non-pregnant university students. The present observations support that immunosuppression associated with pregnancy lead to increased HPV infections. This study indicate that immune response can be induced even in the absence of clinical lesions. It can therefore be deduced that an infection with HPV 16 or 18 infection per se may not represent a sufficient single etiological component for cervical cancer development. The role of possible cofactors have already been discussed. However, these healthy women in whom HPV 16 and 18 DNA and antibodies are detected may require close surveillance to determine the future risk of cervical cancer.