STUDIES INTO THE ROLE OF HERPES SIMPLEX VIRUS AND HUMAN PAPILLOMAVIRUS IN CERVICAL AND VULVAL CANCER

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SUMMARY

DNA extracted from tumour biopsies obtained from 14 women undergoing radical surgery for cervical cancer, from 10 women undergoing surgery for vulval cancer and from internally matched control tissue from the myometrium in 8 cases of cervical cancer and from abdominal skin in 2 cases of vulval cancer was examined for the presence of HSV-2, HPV-6, 11, 16 and 18 DNA using Southern blot transfer and DNA-DNA hybridisation.

Sequences homologous with the MTRII region of herpes simplex virus type 2 were detected in one of nine invasive cervical cancers and in one of ten invasive vulval cancers. No tumour or normal tissue showed evidence of hybridisation to HPV-6 or 11 DNA and HPV-18 DNA was detected in only 2 of 22 tumours tested.

HPV-16 DNA was detected in 10 of 14 cervical tumours and in 8 of 10 vulval tumours. The copy number and integration patterns in individual tumours varied widely. HPV-16 DNA was also detected in 7 of 10 internally matched control tissue biopsies. In one case the integration pattern differed between tumour and control tissue. Long term follow up of patients demonstrated no prognostic significance in the presence or absence of viral DNA in either tumour or normal tissue.

These studies did not support a definitive role for HPV-16 in either cervical or vulval cancer.

To determine the function of viral DNA in these cancers, attempts were made using these and 14 other tumours to derive new continuous cell lines by a primary explant technique. These cell lines, where the state of viral DNA in the original tumour and in internally matched normal control tissue would be available, could be valuable model systems for examination of cell-viral interaction.

Two continuous cervical cell lines, designated Tu 22-1 and Tu 22-2 were derived from a biopsy of a squamous cancer. These cell lines differed in their requirement for epidermal growth factor, their ability for anchorage independent growth, their production of β-HCG and in their cytogenetic characteristics although they

were morphologically indistinguishable. Both cell lines contained detectable HPV-16 DNA in a similar complex pattern. The original tumour also contained HPV-16 DNA but internally matched control tissue for this tumour did not contain such DNA.

One new cell line, Tu 31, was derived from a squamous cancer of the vulva following passage through an athymic mouse. This cell line fulfilled the requirements of an established human cell line. Single insert HPV-16 DNA was detectable in this line. The original tumour biopsy also contained HPV-16 DNA but in a complex configuration. Low copy single insert HPV-16 DNA was present in internally matched normal tissue from this patient.

As it was clear from the DNA studies that the presence of viral DNA alone was not sufficient to explain malignant change, complementary clinical studies of pre-invasive cervical cancer were undertaken to establish which other factors might be involved.

A cross-sectional study of 200 women with cervical intra-epithelial neoplasia (CIN), 36 women with cervical HPV infection and 100 normal control women from the same geographical area as the DNA study, demonstrated a relative risk for CIN of 2.0 for ever use of oral contraceptives and 2.57 for cigarette smoking with a trend towards a dosage effect and a possible relationship between number of cigarettes smoked and severity of histological change. Previous exposure to human cytomegalovirus imparted a small risk (1.57) for CIN in this population. A smaller prospective study of women presenting with overt genital warts confirmed that smoking was associated with an increased risk of histological cervical abnormality but contained insufficient numbers to determine if any one factor was associated with progression following exposure to HPV infection.

These studies urge caution in the assumption that HPV infection plays a major role in lower genital tract cancer and emphasise that other factors may be as important. Aetiological factors implicated in cervical and vulval cancer are reviewed and the evidence for a role for HPV critically assessed.

CHAPTER 1

THE AETIOLOGY OF CERVICAL AND VULVAL CANCER

Section 1 : Epidemiological considerations

The observation that cervical cancer might be related to sexual behaviour was first made by Rigoni-Stern in 1842 [1]. In a study of mortality records, he concluded that uterine cancer was found more often in married women than unmarried women, and that it was almost absent in certain orders of nuns. He also observed an inverse incidence relationship of cancer of the breast to cancer of the uterus in these groups. Although no differentiation between cervical and endometrial cancer was made, it is likely that records referred to clinically overt tumours, more likely to be cervical in origin. However little attention was paid to this epidemiological information at the time.

Much of the epidemiological work in the early years of this century examined the effect of social class and race on cervical cancer. Kennaway (1948) [2] reviewed the existing epidemiological information on carcinoma of the cervix, crediting Braithwaite in 1909 as being amongst the first to document the relative rarity of cervical cancer in Jewish women. Other groups confirmed an approximately three fold difference in cervical cancer rates between Jewish and Gentile populations. Similar differences were evident between Moslem and Hindu women, irrespective of social class. There was much discussion but no conclusion on the role of ritual behaviour related to vaginal bleeding and circumcision as potential aetiological factors.

He credited Theilhaber in 1909 in Bavaria as the first to show a consistent social class gradient in cervical cancer, with only 3.7% of a group of women with cervical cancer from the highest three social class groups. He also suggested that low social class conferred a poorer prognosis for each stage of cancer. Later data from the Registrar General's standardised mortality ratios for cervical cancer in the period 1930-2, showed a clear

social class gradient in the incidence of cervical cancer in both the 35-65 and the over 65 age groups. Detailed studies from Denmark and the U.S. demonstrated similar social class gradients [3,4]. Stocks (1955) [4] proposed a possible male factor based on an analysis of social and geographical distributions.

In 1950, Gagnon [5] in Canada repeated Rigoni-Stern's study of the incidence of cervical cancer in nuns. In a survey of 13,000 nuns over a 20 year period there were no cases of cervical cancer. A further review of 140,000 pathology reports of malignant uterine tumours only identified 3 cases of cervical cancer as nuns. Independent confirmation of the paucity of cervical cancer in nuns was provided by two other large studies [6,7].

The first specific studies to examine sexual behaviour in women with cervical cancer appeared in the 1950s. Lombard and Potter (1950) [8], using case control data, found a significant excess of divorced or separated women, women who had married under 20 years of age and who had had their last child whilst under 25 in the cervical cancer group. The large study by Wynder et al (1954) [9] examined the effect of marriage in both U.S. and Indian populations. They found that multiple marriages, early age at first marriage and early age at first coitus were significant factors. The relative risk of cervical cancer was 1.9-2.2 if first coitus was under 19 with one marriage, but was 3.1-5.3 if more than 2 marriages, suggesting a risk both with early intercourse and with increasing numbers of partners. All racial differences were explainable by differences in age at coitus and remarriage rates though much was made of the differences in circumcision rates between the case and control groups, as this was thought to link with the findings in Jewish and Moslem populations. Similar findings implicating early age at 1st marriage and 1st coitus, frequency of marriage and extramarital relationships were demonstrated by many groups in different populations [4,10-13]. The effects of race and social class tended to be dependent on these differences in sexual behaviour [10,12,14].

The case for early onset of coital activity was conclusively shown in the work of Rotkin [15-17]. In a series of case-control studies specifically designed to examine early coitus, he found a highly significant association with coitus before 20 years. This association was much stronger than that of early age at 1st marriage, and applied in both invasive disease and in carcinoma-in-situ. In the later studies he demonstrated that the total number of partners operated as an independent risk variable in both invasive and pre-invasive disease. Terris et al (1967) [18] independently demonstrated the association with 1st coitus and with multiple partners.

Although Terris and Dolman (1960) [11] had suggested an effect of frequency of coitus, later studies by that group were unable to confirm this [18]. Most other groups could not demonstrate such an effect [10,12,15].

By the late 1960s it was clear that early coitus and coitus with multiple consorts were major aetiological risk factors in cervical cancer irrespective of race or geography. These factors applied equally to invasive cancer and carcinoma-in-situ [13,19,20]. There was possibly some protection afforded by barrier contraception [11-13,16] although this was controversial [9,14].

Most workers had concluded that some transmissible factor was involved. Martin (1967) [14] summed up the qualities of any proposed agent. The carrier should be largely asymptomatic and carriage should be possible by both sexes. It should primarily be transmitted by coitus. There would be an interval between infection and malignancy. To this we might add that other extraneous factors might modify the course of the progression from infection to malignancy. The most likely early candidates were smegma and syphilis, despite the often conflicting data.

Section 2 : Early candidates for sexual transmission of cervical cancer

Syphilis had long been linked with a range of cancers, in particular oral cancer. A number of workers, however, had noted

that, although the exact proportion of cervical cancer patients with positive serology varied, in general the rate was three times that of the overall cancer population (see Røjel 1953)[21]. In these studies there had been little attempt to control for any likely confounding variables such as social class or occupation. Nevertheless, both Levin (1942)[22] and Lombard and Potter (1950) [8] had shown an excess of syphilitic cases over controls. Wynder et al (1954) [9] also indicated that syphilis might be a risk factor, but were unable to comment further due to the inadequacy of the data. Jones et al (1958) [10] demonstrated a small but significant difference in the seropositivity rates for syphilis between cases and controls. Røjel (1953)[21], using the Danish Cancer Registry, specifically investigated this problem. He found a positive diagnosis of syphilis in 13.2% of cervical cancer women and a rate of 4.1% in controls matched for age, domicile and social level. He also found an increase in the proportion of prostitutes in the cancer group, though not of sufficient magnitude to alter his original conclusions. The demonstration that the syphilis rates in prostitutes were the same, whether they had cancer of the cervix or not, led him to propose that it was a factor other than syphilis that was responsible for cervical cancer.

Terris and Dolman (1960) [11] were unable to demonstrate any differences in syphilis serology and later studies controlling for social class and coital behaviour, have conclusively shown that syphilis is not an independent risk factor in cervical cancer.

The case for smegma as a carcinogen in cervical cancer had as its base the rarity of the tumour in circumcised races such as Jews or Moslems. A number of the early studies, in particular Wynder et al (1954)[9], suggested differences in circumcision practice in the partners of cervical cancer patients. Experimental work with mice [23,24] demonstrated that prolonged exposure to human smegma could produce cervical cancer in these animals at low rates. However a number of different tumour types were produced and much of the effect was thought to be due to chronic inflammatory damage. The difficulty in obtaining reliable data on the circumcision state of

partners made it difficult to sustain the theory. Dunn and Buell (1959)[25], using a questionnaire approach, were unable to demonstrate any differences in circumcision rates between cases and controls. Martin (1967)[14], whilst admitting the presence of seeming low incidence groups, described a study of Jewish women with cervical cancer compared with Jewish controls. He found that the same variables of age at 1st coitus, age at 1st marriage, multiple partners and marital infidelity in either partner were significantly different between these 2 groups as they were in all non-Jewish studies. In the same year Abou-Daoud (1967)[26] in the Lebanon showed that the cervical cancer rates in Lebanese Moslem and Christian populations were the same and that the same coital and marital differences were present in cancer patients irrespective of religion or circumcision practice.

It therefore became necessary to examine the role of other sexually transmitted diseases in cervical cancer. In the early 1970s genital infection with herpes simplex virus became a major problem and seemed to co-incide with the increasing recognition of pre-malignant cervical disease.

Section 3 : Sero-epidemiological evidence implicating Herpes simplex virus (HSV) in cervical cancer.

3.1 : Early studies

Although Lewis et al (1965)[27] were unable to demonstrate differences in complement fixing antibodies to HSV in women with cervical cancer and age, race matched controls, the following year Naib and colleagues [28] demonstrated that 10 of 32 women with cytological evidence of HSV infection had histological features of cervical preneoplastic disease. Since that time, numerous studies have demonstrated an excess of previous HSV-2 infection in women with cervical neoplastic disease compared with control women.

Rawls et al (1968) [29] initially showed an increase in seropositivity in women with invasive cervical cancer over age and race matched control women and confirmed this relationship in the following year [30] for invasive disease but not for preinvasive lesions. Naib et al (1969) [31] extended their series of biopsy data on women with cytological evidence of HSV to study the timing of infection relative to the diagnosis of cervical disease. They could not demonstrate any temporal relationship between infection with HSV and subsequent cervical neoplasia and questioned whether the association between HSV was due to differences in sexual epidemiology or whether factors other than HSV were involved.

Nevertheless the next few years saw a large accumulation of retrospective case-control data which concluded that previous exposure to HSV-2 was significantly more common in women with cervical neoplasia than in matched control women [32-35]. Of the early studies, only Royston and Aurelian [35] made any attempt to control for differences in sexual behaviour. The data supported a role for HSV-2 in cervical carcinoma but they commented that equally it could represent an independent relationship of HSV-2 and cervical cancer to promiscuity or preferential growth of HSV-2 in pre-malignant or malignant tissue.

Other early work was less convincingly supportive [36-38]. Adam et al (1971) [36] matched new cases of invasive cervical cancer with controls matched for age, race, class, parity, age at 1st intercourse and age at 1st pregnancy. They demonstrated elegantly that if matching was done only for age, race and class, as in previous studies, then there was an increase in HSV-2 seropositivity in the cancer group, but, if matching included the sexual parameters then there were no significant differences between the groups.

The first prospective study was reported in 1973 [39], following 871 women with serological, cytological or virological evidence of HSV-2 infection and 562 control women matched for age and race. There was a consistent excess of cervical cancer cases in the

HSV-2 group compared with the controls. This study, though not controlling for sexual variables did suggest strongly that previous exposure to HSV-2 was a major risk factor in the subsequent development of cervical neoplasia.

Later case-control studies indicated that the risk imparted by HSV also applied to pre-invasive disease [40-43]. Nahmias et al (1974) [41] controlled for age at 1st coitus, age at 1st pregnancy, oral contraceptive usage and number of sexual partners. 70% of women with carcinoma-in-situ had evidence of previous HSV-2 infection compared with 34.5% of matched controls. The relative risk of in-situ cancer in the studies of Thomas and Rawls (1978) [43] was 2.28 and there was an attempt to control for sexual behaviour.

It seemed that by the end of the 1970s reasonable evidence existed that women previously exposed to HSV-2 were at increased risk of all types of cervical neoplasia. However, there were three serious reservations. The first was that very little of the evidence was prospective in nature although what was available supported the hypothesis. The second was that the data in general was poorly controlled for those parameters of sexual behaviour which had clearly been esta blished in the previous decade. Many studies which did control for sexual parameters were inconclusive.

The other major problem concerned the basis of distinguishing seropositivity to HSV-2 from that of HSV-1. In the 1970s it was believed that only 5-10% of genital herpesvirus infection was due to HSV-1 [44]. No specific antibodies existed for routine use and the diagnosis of HSV-2 infection relied on complex relationships and differential reactivity of human serum with antigen raised from both viral types (see ref 45). All relied on determination of 'cut-off' ratios. The commonest test was a microneutralisation technique. Basically a comparison was made between the ability of the patient's serum to neutralise HSV-1 and HSV-2. The theory was that women with HSV-1 antibodies would neutralise HSV-1 and to a large extent HSV-2 because of cross-reactivity of antibodies. The relative ratio of HSV titres would therefore be low. In

women with HSV-2 antibodies then HSV-2 virus would be neutralised but as anti-HSV-2 antibody is much less cross-reactive with HSV-1 then these women would neutralise a much lower titre of HSV-1. The resulting ratio of neutralised titres would be high. By comparing the ratios a K value was determined. In vitro work determined a value above which the ratio indicated likely HSV-2 infection (see refs 31, 33 and 36 for details about exact determination of 'cut-off' values). Alteration in the K value chosen would alter the apparent incidence of HSV-2 infection in the populations studied. As this was a very non-specific assay, many urged caution in the interpretation of data [43,45].

It was therefore felt that more detailed prospective studies with appropriate controls remained to be carried out and that studies looking at specific herpesvirus products in women with cervical cancer might be more appropriate.

3.2 Recent population studies

Larger case control studies have been reported recently [46-48]. Graham et al (1982) [46] used a specific radioimmunoassay of HSV-2 antibody to study 187 cases of invasive cancer or CIN 3 and 130 controls matched for the appropriate sexual parameters. They concluded that the group of women with low number of sexual partners were at increased risk if HSV-2 positive. However the data is unclear and could be interpreted as merely showing an increase in HSV-2 positivity with increasing numbers of partners. Rawls et al (1986) [47] looked at infection with HSV-2 in cases and controls in a number of different populations and found variation in HSV-2 seropositivity. In their own data the relative risk attributable to prior HSV-2 infection was 1.38, compared with a relative risk of 1.21 for early age at 1st coitus and 1.33 for multiple sexual partners. This still suggested a role for HSV-2. Luthra et al (1987) [48] described 7 year data in an Indian population. They used non-specific antibody tests but looked at 650 cases and 606 controls. As this was a less promiscuous population, age at consumation of marriage was taken as the

controllable sexual variable. The relative risk for CIN was 1.3 and for invasive disease, 2.9, suggesting that exposure to HSV-2 might be required for the progression of CIN to invasive disease.

The largest and most detailed epidemiological data are from Prague [49-51]. This study involved the screening initially of a population to acquire groups of women who had disease at enrolment, who would acquire disease during the study, or who would remain disease free at 4 years. Different antibody tests were performed and different 'cut-off' values examined. Cases were compared with disease free women at 2 and 4 years. Matching was for demographic and all recognised sexual variables. Initial analysis of the data prior to commencing prospective evaluation and at the time of matching confirmed that age at 1st coitus and number of sexual partners were significantly different in women with CIN compared with demographically matched control women. Interestingly the proportion of women smoking more than 10 cigarettes per day increased from 15.4% in the control women to 37.4% in women with or developing CIN 3. Smoking was therefore used as an additional control variable. No differences in oral contraceptive usage were demonstrable between the groups. In the main prospective study [50] no significant differences were found in seropositivity between women with CIN at enrolment and women remaining disease free at 4 years using 2 different microneutralisation cut-off values or using HSV-2 type-specific antibody. Similar results were obtained comparing women developing CIN with disease free women over the 4 year period. This large population based study would seem to conclusively refute the hypothesis that prior exposure to HSV-2 increases the risk of cervical pre-invasive disease. Further evidence from Kremar et al (1986) [51] analysed the rates of conversion to seropositivity in women developing CIN and those remaining disease free. No differences were found suggesting that seroconversion to HSV-2 was not associated with the subsequent development of CIN. This extensive study would seem to demonstrate no relationship between HSV-2 and CIN in a population.

Nevertheless, the most recent study of Kjaer et al (1988) [52] examining the population incidence of different human papillomavirus (HPV) types in areas of high and low cervical cancer incidence, found no relationship between the rates of HPV types and the cervical cancer rates, but found that there were differences in the seropositivity rates to HSV-2 infection between the high and low areas.

The precise epidemiological relationship between HSV and cervical cancer still remains unclear.

Section 4 : Scientific basis for a role for herpes simplex virus in genital cancer

4.1 Oncogenic potential of herpes simplex virus

It is not disputed that HSV is an oncogenic virus under laboratory conditions, inactivation of the normal lytic cycle resulting in persistent infection, biochemical or morphological transformation.

Following the first report of the potential of HSV-2 to morphologically transform diploid cells in vitro in 1971 [53] using UV-irradiated HSV-2, further reports indicated cell transformation by HSV-2 subjected to photodynamic inactivation [54], by high temperature [55] or by temperature sensitive viral mutants at non-permissive temperature [56]. Transformation of cell morphology and behaviour in all types was associated with HSV-2 antigen expression in the transformed cells and often detectable antibodies in the sera of tumour bearing animals. Similar transformation properties were found for HSV-1 [54,56,56].

There were differences between the two viral types. HSV-1 transformed cells tended to be epithelioid and produce adenocarcinomas in animals [58] whereas HSV-2 transformed cells were fibroblastoid and produced fibrosarcomas in animals [54,58].

To some extent this suggested different pathways or different target cell types.

4.2 Transforming regions of herpes simplex virus

Although transformation of rodent and human cells was achievable by both virus types if appropiately inactivated, the precise role of viral nucleic acid remained unclear as conflicting reports on the stability and reproducibility of retention of transforming viral sequences accumulated [59-62]. The proportion of viral DNA retained and the copy number varied in differing cell lines and even in parallel passages of the same cell line [60]. There was controversy over sequential loss of sequences in successive cell passages [59,61,62].

In the late 1970s experiments using cloned DNA fragments demonstrated the ability of specific regions of HSV DNA to mediate morphological transformation.

A single region of HSV-1 appeared to be associated with morphological transformation. Cloned DNA fragments from the XbaI f region (map units 0.29-0.45) and from the BglII i region (0.311-0.415) of HSV-1 [63,64] result in morphological transformation. This region of HSV-1 was termed the MTRI region. The situation in HSV-2 was more complex. Two separate regions were identified as having transforming ability. The MTRII region was described by a number of groups [64-66] as being associated with the induction of morphological transformation. This region mapped within the BglII n fragment (0.58-0.62). A second area within the BglII c region (0.42-0.58) of HSV-2 also appeared to result in immortalisation of cultured cells [67,68]. The conflicting data on transforming regions was studied by Jariwalla et al (1983) [68] who found that the BglII n region did not confer the ability to grow in long term culture using a serial passage assay; the BglII c region did. Detailed analysis of subclones of the region suggested that the region 0.419-0.525 caused escape from senescence and that the region 0.525-0.585 was required for the tumorigenic state. These sequences were stably retained.

Interestingly, the region 0.419-0.525 was homologous both to normal human DNA sequences which cross-hybridise with HSV DNA and with the transforming region of CMV type Towne (see below).

DNA fragments used to induce morphological transformation were however not necessarily retained in transformed cells [66,69]. Where these were retained, the pattern and amount varied widely and sequences could be lost by successive passage [65].

The other major puzzle was that fragments of viral DNA too small to code for a transforming polypeptide were capable of initiating morphological transformation [70,71].

4.3. Herpes simplex expression in transformed cells.

Early studies using intact inactivated HSV-1 or 2 to transform cells in vitro consistently demonstrated detectable immunofluorescent antigen in transformed cells using antibody raised against infected cells [53,54,56,57]. Many of the cell lines which produced tumours in susceptible rodents produced a neutralising antibody response to HSV. This demonstration of apparent viral expression in transformed cells gave support to the argument that the virus and its expression were part of the transformation process.

The majority of polypeptides coding within known transforming fragments were structural proteins and unlikely candidates for a transforming function [63,72,73]. Subsequent analysis of a number of transformed cell lines could not demonstrate any viral specific polypeptides [66]. Antibodies raised against a polypeptide derived by in vitro translation from the MTRII region did not detect this polypeptide in cells transformed by this region [74].

The most attractive candidate polypeptide was ribonucleotide reductase, consisting of two subunits, Vmw140 (ICP10) and Vmw38 [75]. The large subunit corresponded to AG4, described in human cervical carcinomas by Aurelian et al (1980) [76]. The small subunit was completely encoded within the MTRII region [77]. The large subunit was mostly encoded by the BglII c region with the

carboxy terminus in the BglII n region. However transformation experiments using DNA fragments from the MTRII region have failed to detect ribonucleotide reductase activity [66,78]. The small fragments used by Galloway et al (1983) [70] and Jones et al (1986) [71] could not code for any complete subunit. Enzyme activity was detectable in cells transformed by the BglII c region [67,79] although most cells transformed by fragments from within this region did not have such activity.

An explanation for the apparent expression of viral polypeptides in transformed cells might lie in the fact that most antisera were raised from infected cells. There was convincing evidence using monoclonal antibodies to HSV polypeptides that the polypeptides identified in these experiments were not virus-coded (see ref.80). It would appear that specific cellular polypeptides were up-regulated in infected cells and that some of these continued to be expressed at high levels in transformed cells [81] being falsely labelled as virus-specific.

4.4.Animal models

Some experimental evidence exists demonstrating that cervical cancer can be produced in animals by exposure to HSV [82-86].

Formalin or UV inactivated HSV introduced into the vagina of inbred C3H mice using soaked cotton plegets produced both cervical dysplasia and invasive disease [82,83]. There was a relatively long latent period before cytological or histological changes became apparent compared with similar experiments using chemical carcinogens. Prior immunisation of mice using HSV-1 or the HSV-2 subunit vaccine protected against the development of cancer [84,85].

A major criticism of this elegant system was the preponderance of adenocarcinomas of the uterine body and cervix produced by exposure to HSV-2. Exposure to HSV-1 appeared to result largely in squamous tumours. In Man cervical carcinoma is predominantly of squamous origin.

In an attempt to more closely mimic the human system, Palmer et al (1976) [66] demonstrated the Cebus monkeys responded to genital HSV infection in a manner close to humans. There was a very low incidence of spontaneous cervical cancer in this species and it seemed ideal for prospective exposure and follow up. Initial follow up demonstrated that 8 animals developed cervical atypia at a mean of 20 months post exposure and 13 developed cervical dyplasia at a mean of 38 months post-exposure.

4.5 Putative mechanisms for oncogenesis by herpes simplex virus

There is no doubt that specific areas of the HSV genome are capable of initiating morphological transformation and that resulting transformed cells are tumorigenic. It is also clear that viral DNA is not necessarily retained within such cells and it is unlikely that there is a specific viral-coded transforming protein. How then might HSV initiate such morphological change ? To include the phenomenon of transformation by very small DNA fragments it has been proposed that some specific DNA sequence arrangement might in itself initiate transformation or that integration or recombination of viral and cellular DNA might alter cellular gene expression to initiate change. Galloway et al (1984) [70] proposed that the 737bp sequence found to be capable of morphological transformation could form a small stem/loop structure. This resembled the P elements of Drosophila although it was not large enough to encode a transposition function. The structure did not conform to the description of an insertion sequence. Jones et al (1986) [71] in their analysis of the small fragment, BamHI e, derived from the BglII c region, suggested the presence of insertion sequence-like sequences. Computer analysis of insertion sequence-like structures in HSV-2 demonstrated the presence of such sequences within both the transformation regions and in other regions of HSV DNA (see ref.80). The significance of such DNA structures is unclear. No such structures have been proposed for HSV-1.

Morphological transformation might be achieved by alterations or disruptions in cellular control mechanisms by insertion of DNA sequences by homologous recombination. Homology between HSV-1 and HSV-2 DNA and human DNA has been consistently demonstrated [87-89] predominantly within inverted repeat regions. Such a mechanism remains theoretical.

Another possible mechanism by which HSV DNA could induce morphological transformation was by biological carcinogenesis. It has been demonstrated that mutagenesis could be effected by infection with both HSV-1 and HSV-2 [90,91]. This effect, thought to be the result of point mutations, was comparable to the effects of chemical carcinogens. Introduction of HSV-2 DNA alone produced a similar effect [92]. Shillitoe et al (1986) [93] have demonstrated that the BamHI g fragment of HSV-1 (situated within the MTRI region) increased the rate of frameshift mutations in an experimental system. Many mutagens were also carcinogens.

Similarly, gene amplification might be an essential component of oncogenic change (see ref.94). HSV could induce such gene amplification [95]. The degree of amplification seen was comparable with chemical carcinogens [96]. Most interestingly, both HSV-1 and HSV-2 induced amplification of integrated HPV-18 sequences in cervical tumour derived cell lines [97] raising the possibility of interaction between these viruses at the molecular level.

An interesting possibility was that HSV infection could produce up-regulation of specific cellular polypeptides [81]. In transformed cells this up-regulation was maintained. If such changes occur in vivo, then cells could be altered in such a way as to make them more susceptible to later insults (e.g.specific HPV infection, nitrosamines, hormonal milieu), moving them further down the pathway to permanent oncological change.

In complex, multistage carcinogenesis, it was clear that the presence or absence of detectable viral information in established cancer tissue might not necessarily reflect the role of that virus in the oncogenic process.

Section 5 : Herpes simplex virus information in human cervical cancer

5.1 Herpes specific polypeptides in human cervical cancer patients

Work on human systems has taken a similar pattern, looking initially for HSV gene products, RNA and ultimately DNA in cervical tumour tissue and sera of cancer patients.

Initial studies identified a number of polypeptides in cervical cancer subjects using antibodies raised in infected cells [98-101]. It is likely that some of these may be up-regulated cellular polypeptides equivalent to those found in transformed cells (see ref.80).

The antigen described by Aurelian's group, initially called Ag-4, is known to be equivalent to ICP 10 (Vmw140), the large subunit of ribonucleotide reductase and has been extensively studied [76]. In cervical cancer patients the rate of seropositivity appeared to be related to disease severity. It was shown to be present in 7.4% of treated cancers of the cervix and in 96% of recurrences but was also present in 7.7% of other cancers and in 11% of normal women [102]. The proportion of positive sera decreased with increasing stage of cancer. In this regard it behaved much like a number of specific tumour markers. Its expression in transformed cells including those transformed by the BglII c transforming region was controversial as ribonucleotide reductase activity has been detected in cells transformed by part of the BglII c region incapable of coding for the entire subunit [68]. It would appear that a HSV coded polypeptide was expressed in cancer cells and sera.

Other early antigens studied such as the HSV-TAA of Holinshead and associates [99] were present in a range of squamous tumours and in normal tissue [102] whereas others derived from infected cells reacted strongly with exfoliated cervical cancer cells, but the

difference was quantitative with high positivity rates in normal tissue [100,101].

Later studies used more specific antisera [103-106]. Immunocytochemical staining of dysplastic but not normal cells in biopsies of premalignant cervical tissue occurred using antibodies to purified ICP 12 and 14 polypeptides [103]. The DNA binding protein VP 143 was demonstrable in biopsies from premalignant and malignant tissue [104,105] and the staining appeared to be related to disease severity. Dreesman's study [104] also implicated the DNA binding protein ICSP 34/35. Using immunoprecipitation of control and cancer sera, Gilman et al (1981) [106] identified two polypeptides of molecular weights 38K and 118K precipitated more frequently from cancer than control sera which were colinear with polypeptides precipitated from HSV infected cells. This study attempted to control for previous exposure and other epidemiological variables. A 35K polypeptide indistinguishable from that found in transformed cells was identifiable in the human cervical carcinoma cell line C4II [107].

Whilst these data were supportive of HSV expression in cervical cancer tissue, such expression might indicate growth advantage rather than direct involvement of HSV polypeptides in the initiaton and maintainance of morphological change.

5.2 Herpes specific RNA in cervical tumour tissue

McDougall et al (1980) [108] initially described the detection of HSV homologous RNA in biopsies of cervical carcinoma using radiolabelled DNA-mRNA in situ hybridisation. In the initial work 25 of 61 biopsies of intra-epithelial neoplasia showed positive grain counts in a proportion of cells compared with 4 of 75 normal control biopsies. They could detect no hybridisation with human DNA, or with rodent or human cultured cells. Positive hybridisation was obtained in HSV infected cells and in HSV transformed cells. Analysis of the biopsies indicated that hybridisation was confined to the dyplastic cells. The same group [105] used cloned HSV-2 DNA probes with variable results. Map

regions 0.07-0.40, 0.58-0.63 and 0.82-0.85 all showed increasing grain counts in pre-invasive and invasive tissue compared with normal cervical tissue. However this was a clear qualitative difference and the proportion of cells positive by each probe varied from 8-30% for tumour tissue and from 2-8% for normal tissue.

Eglin et al (1981) [109] attempted to quantitate positivity using automated grain counting and also used normal internal control tissue in some cases. THey demonstrated a highly significant increase in grain count in pre-invasive and invasive tissue compared with normal control tissue and this was confirmed on comparison of neoplastic and non-neoplastic tissue from the same patients. Again direct examination of fixed tissue suggested that grains were situated in dysplastic cells. This group [110] also examined cloned DNA probes. Significantly increased grain counts were seen for the regions 0.07-0.11, 0.27-0.52 for CIN; and 0.07-0.21, 0.27-0.4, 0.85-0.87 and 0.93-0.98 for invasive cancer. No two individual biopsies produced the same pattern of in situ hybridisation with these probes and the MTRII region did not seem to be transcribed in any significant way.

Further doubt on the relevance of the initial findings was placed by Maitland et al (1981) [111]. They used a similar DNA-mRNA in situ hybridisation technique and tested extensively for specious hybridisation to human DNA. They found 1.5% homology between adenovirus 2 and human placental DNA, and homology between the cloned HSV-2 DNA regions 0-0.05, 0.75-0.85 and 0.95-1.0 and normal human placental RNA. In formal in situ experiments using CIN tissue, there was 7% non-specific hybridisation to phage λ, 39% hybridisation to adenovirus 2, and 67% hybridisation to HSV-2. The mean grain count was always higher for HSV-2 but all Ad2 positive biopsies were HSV-2 positive and examination of the stained sections indicated that it was the same cells or areas which were stained by both. They suggested that the finding of HSV-specific RNA in tissue biopsies was far from clear cut and suggested that hybridisation might be an unrelated phenomenon determined by the rate at which cells were dividing. This

conclusion was reinforced by Eizuru et al (1983)[112] who showed a similar qualitative difference in grain counts between normal and tumor tissue but who concluded that it was not possible to distinguish between non-specific binding to human RNA and HSV-2 specific binding.

These findings would be consistent with the homology between HSV and human cellular DNA [87-89].

5.3 Herpes simplex virus homologous DNA in cervical tumour tissue

The most convincing evidence relating HSV to genital cancer would be the identification of HSV DNA from the known transforming regions in large numbers of tumours.

Frenkel et al (1975) [113] first described the identification of HSV homologous DNA in DNA extracted from a cervical tumour, demonstrating the presence of a complex fragment probably representing 39% of the viral genome at a copy number of 1-3.5 per diploid cell. This DNA seemed to be transcribed and HSV homologous RNA equivalent to 5% of viral transcripts from early infected cells was detectable.

Two subsequent studies [114,115] looking at a total of 40 invasive tumours were unable to detect viral DNA. The study of Pagano [115] stated a sensitivity of 0.125-0.25 genome copies per cell.

Using Southern blot transfer and DNA-DNA hybridisation techniques, Park et al (1983) [116] identified the MTRII region, BglII n (0.582-0.63) in one of nine invasive cervical carcinomas. This tumour was a cervical adenocarcinoma. Similarly, Galloway and McDougall (1983) [69] demonstrated viral DNA in three of nine invasive tumours, again 2 of these were adenocarcinomas. In their study, the MTRII region was seen in one tumour, the BglII j region (0.32-0.38) in one and one tumour had both DNA fragments. This study convincingly demonstrated the presence of viral DNA by the presence of known restriction enzyme sites and by the presence of fragments of definitive size. In a slightly larger study, Prakash et al (1986) [117] found the BglII j fragment in 2 of 13 invasive

cervical cancers and in 1 of 12 normal control DNAs. Rotola et al (1986) [118] identified the BglII n fragment in five of 69 cervical tumours although Manservigi et al (1986) [119] in a similar Italian population were unable to detect HSV DNA in cervical tumour biopsies. Macnab et al (1985) [120] demonstrated the presence of sequences homologous to the MTRII region in 1 of 15 squamous cervical cancers examined.

Although infrequently demonstrated, the consistent finding is of DNA from the MTRII region in DNA from a minority of tumours. The paucity of HSV DNA clearly militates against a major role, but as discussed in the previous section, HSV DNA is rarely retained in transformed cells clearly initiated by transfection with HSV DNA. Failure to demonstrate viral DNA does not rule out a role for HSV in cervical cancer.

Section 6 : Scientific basis for a role for human papillomavirus in human genital cancer

6.1. Human papillomavirus as a sexually transmissable disease

The difficulties and controversies surrounding proof of a role for HSV in cervical cancer led to the extension of the search for an infective aetiological agent to another common genital infection, genital wart virus infection.

Genital warts are a very ancient disease, being recognised in Greco-Roman times. Initially genital warts were linked with other venereal diseases such as syphilis, and subsequently with gonorrhoea. It was believed that they were caused by vaginal discharge, poor hygiene and dirt. Waelsch (1917) [121] first demonstrated transmission of warts by inoculation of human volunteers with unfiltered penile condylomata. Typical warty lesions developed in 3 to 9 months. Negra (1924) [122] inoculated filtered penile condylomata material into volunteers. One volunteer developed a wart like lesion after 5 months.

Despite the long association with sexual activity, it took until 1954 to conclusively demonstrate that human genital warts could be transmitted venereally [123], in studies of servicemen returning from the Korean war, having acquired penile warts. Vulvar warts developed at between 4 and 6 weeks after resumption of regular sexual intercourse with their partners. Particles similar to those identified in common hand and plantar warts were first identified in genital warts by Dunn and Ogilvie (1968) [124].

Initial characterisation of HPVs was based on isolation of particles from clinical warts [125] or selective DNA extraction [126]. It was clear from the outset that different clinical lesions probably contained different viral types [126]. None of the common wart types seemed present in genital warts [127]. Gissman and zur Hausen (1980) [128] were able to isolate appropriately sized DNA from genital warts and demonstrate

hybridisation with DNAs extracted from other non-genital warts. Restriction enzyme digest indicated that this was a different HPV type, designated HPV-6.

Nevertheless, detailed work on the virus was hampered by the small and variable quantities of virus or viral DNA obtainable from clinical samples until Israel et al (1979) [129] described a reproducible method of producing large quantities of viral DNA by cloning using E.coli plasmid vectors. Since then over 50 HPV types have been cloned and characterised.

It is not in doubt that these wart virus infections are venereally transmissible. The frequency with which genital wart virus infection was seen in sexually transmissible disease clinics rose dramatically in recent years. Recent specific studies demonstrating the transmission of particular papillomavirus types between infected sexual partners confirmed sexual transmission at the molecular level [130,131].

Molecular biological methods have allowed detailed examination of viral DNA and viral products in vitro. More standard virological approaches to papillomaviruses remained hampered by the lack of a suitable in vitro culture system for propagating infectious virus. This was largely due to the fact that full expression of viral function only occurred in fully differentiated squamous epithelial cells. Productive viral DNA replication, capsid antigen production, and particle formation with full expression of late viral genes only occurred in the fully differentiated cells of the wart i.e. in cells either undergoing or which have undergone keratinisation. Vegetative DNA replication occurred in the cells of the stratum spinosum and granular layer of the epidermis. Capsid protein production and virus particle assembly occurred in the upper stratum spinosum and in the granular layer where the cells were terminally differentiated. These functions were not detected in the basal layer epithelial cells. It was thought that viral DNA was present in these cells and that specific viral gene expression was responsible for cell proliferation characteristic of a papilloma. The factors controlling the changes to allow gradual onset of productive viral replication as the epithelial

cells migrate through the epithelial layers are not yet understood.

Cells grown from papillomata appeared normal morphologically. Infectious virus was not produced. Initial attempts to insert viral genomes into normal cells [132-134] were largely unsuccessful. Viral genomes could be maintained in a vegetative state and there was some evidence of low level transcription [133]. Many of the problems in successfully establishing tissue cell lines with infectious virus were outlined by Taichman et al (1984) [134]. Recently a system by which infectious HPV-11 could be propagated in vivo, allowing more detailed direct analysis of the intact functioning virion has been constructed [135]. Analysis of the attempts to transform rodent and human cells with HPV DNA will be discussed separately.

Characterisation of viral types was therefore made on the relationships a papillomaviral DNA has with other known types. Viral DNA should be in the size range of 7 to 8 kilobase pairs, persist in tissue as extrachromosomal circularly closed DNA, show partial sequence homology with other papillomavirus DNAs and have a genomic organisation comparable with other papillomaviruses. Viral DNAs were considered to be independent types if they showed less than 50% cross hybridisation with other types using reassociation kinetics of heterologous DNAs in the liquid phase. Values obtained did not necessarily represent sequence homology at the DNA level. Sequence analysis has shown that a high level of sequence homology could exist within conserved regions even between viruses which showed no cross hybridisation under stringent conditions. Viral types were grouped according to their cross hybridisation characteristics.

6.2 Oncogenic potential of papillomaviruses

Papillomaviruses are capable of inducing benign tumours in a wide range of animal species following infection of basal keratinocytes [136]. A number of these benign papillomata will undergo malignant change in response to varying cofactors [137]. The most widely

studied animal papillomaviruses are the cottontail rabbit papillomavirus (CRPV) and bovine papillomavirus (BPV).

The ability of rabbit papillomas when experimentally transferred to undergo malignant change has been known since the 1930s [138]. There appeared to be a lag or critical period following infection before the development of carcinomas and a number of chemical carcinogens could accelerate the rate and extent of malignant transformation [139]. Viral DNA was present in malignant tumours and was maintained over a long time period in transplantable carcinoma lines [140].

BPV was associated with tumours of the alimentary tract and bladder. In the U.K. approximately one half of all cattle will have tumours associated with BPV. Six different types of BPV have been identified [141], subclassified into two major groups:- BPV-1, 2, and 5, which formed mixed fibropapillomata and could transform both epithelial and fibroblastic cells in vitro; and BPV-3, 4, and 6, which formed squamous papillomata only and transformed only epithelial cells. BPV-2 and 4 have been associated with alimentary and bladder cancer in cattle. The association between BPV-4 and alimentary cancer is complex. Viral DNA was present at high copy number in benign papillomas yet was absent from invasive lesions [142]. Malignant change was dependent on environmental chemical cofactors, associated with a particular type of bracken and likely to involve naturally occurring immunosuppressants [143,144]. Analysis of both naturally occuring and experimental bladder cancers showed the presence of multiple copies of BPV-2 DNA [144]. The finding of BPV-2 in the experimentally induced cancers was interesting as only BPV-4 was inoculated into these animals. It remained unclear whether the virus was directly associated or whether malignant bladder epithelium was merely permissive to viral DNA replication.

There was a human model for considering HPV as an aetiological agent in genital cancer, the rare skin disease epidermodysplasia verruciformis (EV). First described in 1922, it consisted of disseminated flat warty lesions and red, pigmented, or achromic

macules. Lesions were usually multiple. In about one-third of individuals squamous carcinoma developed, normally at multiple sites and in the third or fourth decade of life. The disease had a strong genetic component, with more than one sib affected in 10% of families, and with consanguineous parents in 10% of cases. Most affected individuals have impairment of cell mediated immunity. Ultraviolet light was also thought to play a significant role, as most lesions developed at sun exposed sites.

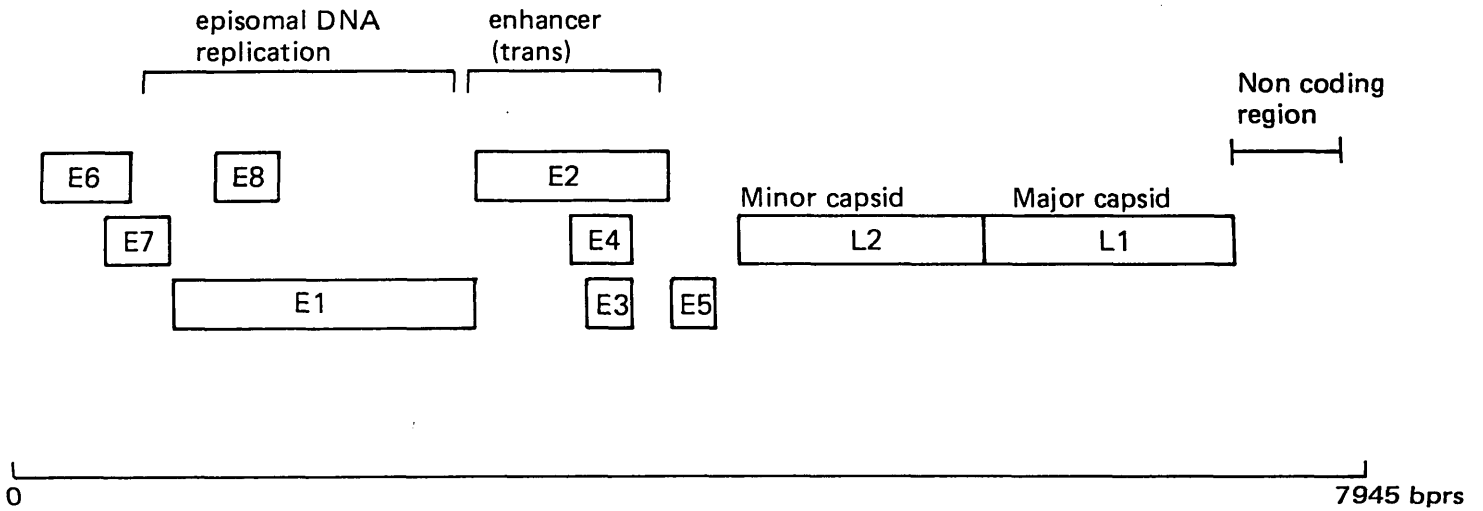
A number of papillomaviral types have been isolated from benign lesions [145], and except for types 3 and 10, all appeared to be associated exclusively with EV [146]. The most common types associated with EV were 5, 8, 17, and 20.

The onset of bowenoid carcinoma-in-situ or frank invasive squamous carcinoma occurred about 25 years after the initial onset of the benign lesions. Not all HPV types found in benign lesions were identified in malignant tissue. HPV-5 was frequently associated with skin malignancy [145,147]; HPV types 3, 8, 14, 17, 20 and 24 were identified in invasive lesions [145,148,149]. DNA was present in high copy number (100 to 300) and there was some evidence of vegetative DNA replication in some tumours [149,150]. HPV types 5 and 8 were present as free monomers or oligomers but HPV-14b was probably integrated into cellular DNA. HPV-5 and 8 could contain small deletions [150,151]. RNA transcripts from the E6/E7 open reading frames (ORFs) have been identified in HPV-5 containing tumours [146].

EV is an excellent model to study the role of HPV in carcinogenesis in man. It involves interaction between virus, genetic predisposition, and immunocompetence. The virus type seems to be important, potentially analagous with its proposed role in genital cancer. There is a long latent time between primary lesion and malignant change suggesting a multistage process through benign to premalignant to invasive disease. Many factors may therefore be involved at different stages. UV light exposure is one such important external factor.

Figure 1

Genomic organisation of BPV–1 open reading frames with putative functions



6.3 Transformation system of bovine papillomavirus type 1

The mechanisms by which papillomaviruses are capable of inducing morphological transformation are best understood in BPV 1-rodent cell systems. BPV-1 was associated with benign cutaneous fibropapillomas in cattle but would induce fibroblastic tumours in a variety of foreign hosts [136].

Its genomic organisation and the putative functions of the viral genes are shown in Fig.1

BPV-1 was initially shown to transform mouse cells in culture in 1964 [152]. Morphological transformation of rodent cells by BPV-1 was complex and appeared to be dependent on interactions between two physically separate areas of the viral genome. Transformation experiments involving cloned BPV-1 DNA fragments mapped transforming ability to the E5 region [153] and to the E6 region [154,155]. These regions could achieve morphological transformation individually [156]. It appeared that mutations affecting each of these regions had different effects on the transformation process. Mutations in the E5 region had a marked effect on the efficiency of transformation [153]. An intact E6/7 region was necessary to allow efficient tumorigenicity and anchorage independent growth [154]. One further ORF was involved in the transformed phenotype, E2. This ORF had no transforming ability in isolation. Mutations at the 5' portion of this ORF resulted in a marked decrease in the efficiency of focus formation of transformed cells compared with those transformed by the intact BPV-1 genome [154]. It was now clear that the E2 gene codes for a trans-acting factor which was required for initiation and maintainance of transformation and normal viral DNA replication [157]. This polypeptide binded to specific sites in the ncr region and elsewhere, acting as a transcriptional activator [158]. There was direct evidence that the E2 gene product was required for efficient expression of the E5 transforming gene [159]. The system was complex, requiring trans-acting E2, a cis-acting ncr region which behaved as a transcriptional enhancer and a functional promoter.

6.3a Genomic organisation and gene function in HPV

The genomic organisation of HPV is similar to that of BPV-1. There is overlap between ORFs E1 and E2, and between L1 and L2. The ORF E4 is included within the segment encoding the E2 gene product. The region between E2 and L2 is longer in genital papillomaviruses, altering the characteristics of the E5 gene product. The ncr region is shorter than in BPV-1.

Sequence analysis [1a - 4a] reveals a number of common characteristics amongst genital HPVs. These include a longer E1 ORF (by 40 amino-acids), the position of the E7 ORF immediately in front of E1, a specific coding segment in the control region and a particular structure to the promoter region upstream from E6. Phylogenetically, HPV-6 and 11 are closely related, with 58 - 92% amino-acid sequence homology, most marked in the E6/7, E1, and L1/2 regions. HPV-16 is more distantly related, the overall amino-acid homology being 25 - 69%. HPV-16 is more closely related to HPV-33 (see page 54). HPV-18 appears to link the two groups, with DNA sequence homology of 40 - 60% with HPV-6/11 and 50 - 67% with HPV-16/33.

As in BPV-1, ORFs L1 and L2 code for the major and minor capsid antigens. ORF E1 is involved in episomal DNA replication; the 3' end coding for a function involved in transient replication and the 5' end having a role as a negative modulator of viral replication[5a]. The ORF E2 has a transcriptional regulatory function [6a]. Full length E2 protein acts as a trans-activator; the carboxy terminal acts as a downstream repressor. Both bind directly to viral DNA and supress E6/E7 transcription. The function of the E4 ORF is poorly understood. It may have a role in the onset of vegetative viral replication and may therefore interact with cellular functions to alter the pathway of differentiation to favour viral replication.

The E7 ORF, as discussed in 6.4, is capable of inducing morphological transformation. The protein demonstrates homology to adenovirus E1a polypeptide and other oncogene products [7a], especially in the domains implicated in the induction of cellular DNA synthesis, cooperation with the *ras* oncogene for full oncogenic transformation and repression of transcription. It is a powerful trans-activator and, like the E1a polypeptide, binds to the retinoblastoma gene product [8a]. Its function may be to antagonise cell proteins, such as the retinoblastoma gene product, which may constrain cell proliferation.

Both ORFs implicated in transformation appeared to code for a protein product. The E5 ORF was small and appeared to code for a 44 amino-acid, very hydrophobic, membrane associated polypeptide [160]. The polypeptide encoded by the E6 ORF was detected in both nucleus and non-nuclear membrane and might have zinc binding sites [161].

The complexity of the BPV-1 transformation of rodent cells acted as a model against which transformation by other papillomaviruses could be compared.

6.4. Morphological transformation by human papillomavirus

Chesters and McCance (1985) [162] were unable to transform either NIH3T3 cells or BSC1 monkey kidney cells using HPV-16. However, Tsunokawa et al (1986) [163] initially demonstrated that HPV-16 sequences, cloned from a cervical cancer, were capable of transforming NIH3T3 cells. HPV-16 DNA was present, integrated into cellular DNA, in the transformed cell lines and RNA transcripts which hybridised with HPV-16 DNA were identified in the lines. Yasumoto et al (1986) [164] were able to transform 3T3 cells using a head-to-tail HPV-16 dimer. Six transfected lines were generated, all containing multiple copies of HPV-16 DNA, integrated into cellular DNA. Transformation efficiencies were much reduced compared with those achievable with BPV-1 and foci took considerably longer to evolve than in the bovine system, a phenomenon also noted by Morgan et al (1988) [165] in the transformation of mouse C127 cells. They noted that HPV-16 transformed cells did not exhibit the morphological alterations characteristic of transformed foci. As in earlier studies, HPV-16 DNA was integrated into cellular DNA at multiple sites in contrast to the episomal nature of BPV-1 in transformed cells. Complex integration patterns were maintained through successive passages and in derived nude mouse tumours. Tumorigenicity of transformed cells with HPV-16 could arise as a two stage event in 3T3 cells [166]. Altered growth potential with expression of a wide range of viral transcripts was seen in initially transformed cells which

were not tumorigenic in nude mice. Cells which became tumorigenic expressed quantitatively less viral RNA, although the E6 and E7 transcripts remained the predominant species.

Primary rodent cells can be transformed by HPV-16 linked to the EJ-ras oncogene [167] to a fully transformed tumorigenic phenotype. This ability appeared to be linked to the E6/E7 ORF. Confirmation that transforming ability was restricted to this region came from Bedell et al (1987) [168] using different cloned viral expression plasmids. Transformation was achievable using only the E6/7 region in both 3T3 and Rat-1 cells. The transforming region was further limited by Kanda et al (1988) [169] who transformed rat 3Y1 cells using only the E7 ORF. Cells were morphologically different and were anchorage independent but were not tumorigenic.

Rodent cells were therefore capable of oncogenic transformation by HPV-16 although this was more difficult to achieve than using BPV. Transforming ability appeared to lie in the E6/7 ORF and possibly within the E7 ORF alone. In some instances, using primary cells, a fully oncogenic phenotype was not achieved.

Successful transformation of human cells has now been reported [170-172] using similar techniques. A number of differences are apparent between rodent and human transformants. Use of HPV-16 in an appropriate vector resulted only in immortalisation of human fibroblasts and keratinocytes. These cells were not oncogenically transformed in the way which BPV-1 or HPV-16 transformed rodent cells were transformed. Viral transcripts were identifiable in transformed cells. The molecular weight of the major transcript was similar to that identified in cell lines transformed by Yasumoto et al (1987) [166] and considered by them to be the E6/7 transcript. Using the oncogene EJ-ras linked to HPV-16, Matleshewski et al (1988) [172] did achieve transformation of primary fibroblasts to cells which were highly aneuploid and exhibited reduced serum growth requirements. The cells were not tumorigenic in immunocompromised rodents. These transformed cells expressed E6 polypeptide.

The tissue specificity of the human papillomavirus group has made extrapolation of findings in rodent and non-genital keratinocytes to genital tumours difficult. Woodworth et al (1988) [173] have immortalised keratinocytes derived from human exocervical epithelium using both HPV-16 and 18. In the case of HPV-16, a head-to-tail dimer was used, and in the case of HPV-18, single copy DNA was used. Immortalised cells continued to produce keratins. Viral DNA was present in transformed cells, integrated at multiple sites in the cellular genome, both as intact DNA and as rearranged DNA. This multiple integration arrangement occurred in the cell line derived from a single colony. HPV transcripts have been identified, including that associated with the E6/7 ORFs in transformed rodent lines. These cell lines did not produce tumours in nude mice demonstrating that the presence of integrated viral DNA and viral transcription did not alone confer a malignant phenotype on human cervical cells.

It is apparent from rodent, human non-genital and cervical epithelial transformation experiments that HPV-16 alone is not capable of inducing the full transformed phenotype. Co-operation with heterologous promoters is necessary even for immortalisation. Further co-operation with oncogenes may result in cells closer to a fully oncogenic phenotype, but other, as yet uncharacterised, extraneous factors must be involved in this complex multistep process. It is also clear that the system must differ from that of BPV, in that E5 and E2 ORFs are not involved in HPV mediated transformation. The story is further complicated by the finding that the E6 ORF of HPV-8, the putative oncogenic virus in EV, is alone sufficient to transform rodent cells [174] with E7 having perhaps a subsidiary role. Thus no two groups of papillomaviruses, despite their close genomic organisational similarities, appear to have the same major transforming region.

Section 7 : Human papillomavirus in human genital cancer tissue

7.1 Cytological, histological and immunological evidence

The possibility of involvement of HPV in cervical cancer stemmed directly from the observations of Meisels and Fortin (1976) [175] and Purola and Savia (1977) [176]. Independently, they described epithelial changes in cytology and histology specimens of women reported to have cervical dysplasia identical to the cytological changes seen in condylomata acuminata. The most striking finding was of cells known as koilocytes, a term first used by Koss and Dufree (1956) [177]. These cells were superficial or intermediate squamous cells with an enlarged, hyperchromatic nucleus, surrounded by a clear cytoplasmic halo. At the external margins of the halo, the cytoplasm was condensed and showed an ambophilic staining pattern. Cells could be bi- or multi-nucleated. These cells have been recognised for some time, and as early as 1960, it had been suggested that this type of cellular change might be a manifestation of viral infection.

The presence of papillomavirus particles and antigens in koilocytic cells has been confirmed by a number of ultrastructural [178,179] and immunohistochemical studies [180-182]. Initially in the absence of specific antigen for genital papillomaviruses, immunoperoxidase-Pap techniques using antisera raised against pooled plantar warts were used to identify expression of virion polypeptides in genital warts and in koilocytic cells. There was marked cross-reactivity amongst the capsid antigens of the different papillomavirus types (confirmed by the sequence conservation observed for the putative capsid protein in those papillomaviruses subjected to analysis thus far).

Both initial groups suggested that many women with 'dysplasia' probably had papillomavirus infection. Re-examination of cervical cytological and histological specimens [183,184] indicated that a high proportion of women with cervical intra-epithelial neoplasia

had co-existent evidence of HPV infection. Since those initial reports, many have reported the co-existence of pre-malignant histological change and cytological and histological changes of HPV infection [185-188]. The frequency with which HPV infection was associated with CIN varies from 35-40% [188] to over 90% [187]. Viral capsid antigen expression has been demonstrated in areas of koilocytic change within all grades of CIN [180,188-192]. Many other studies did not specify precise histology when reporting antigen positivity in CIN, but antigen expression in CIN lesions without evidence of histological HPV infection was uncommon [192].

There is no controversy over the co-existence of HPV infection with pre-malignant cervical or vulval neoplasia. Whether this association is causal or reflects the sexual behaviour of this group of women remains in dispute.

7.2 Human papillomavirus DNA in cervical cancer tissue

Attention moved to trying to identify HPV DNA in genital cancer tissue and to determine the state of this DNA within the tumour cell.

Initial studies attempting to identify HPV types 6 and 11 were disappointing. Having established that the vast majority of genital warts contained HPV type 6 or 11 DNA, Gissman et al (1982, 1983) [193,194] were unable to identify HPV-6 in any of 54 invasive cervical cancers examined. Four of 24 cervical cancers (invasive and carcinoma-in-situ) had evidence of HPV-11 DNA. Green et al (1982) [148] screened a number of genital tumours using a papillomaviral DNA derived from an EV lesion, HPV-10, and identified this DNA type in 2 of 31 invasive squamous cancers of the cervix.

In their screening of malignant tumours with HPV-11, Durst et al (1983) [195] identified DNA from one tumour biopsy which only hybridised under non-stringent conditions. Following isolation and cloning, stringent cross-hybridisation experiments with the available HPV types showed less than 0.1% hybridisation with all types except HPV-11, with which it demonstrated less than 50%

hybridisation. This DNA was designated HPV-16. Eleven of 18 invasive cervical cancers from Germany and 8 of 23 from Kenya and Brazil contained DNA homologous with HPV-16. Four of 29 premalignant cervical lesions also contained HPV-16 type DNA. Using stringent conditions, only 2 of 33 condylomata contained this DNA. This data opened the door to realistic speculation on the aetiological role of the papillomaviruses in invasive genital cancer.

The following year Boshart et (1984) [196], in the same laboratory, described a further HPV DNA isolated from malignant genital tissue. Hybridisation of this DNA with cloned DNA of other virus types revealed some relatedness with types 1-6 and 8-17 under low stringency conditions, with some hybridisation to HPV-2 even under high stringency. This new HPV DNA was designated HPV-18. The restriction digest data suggested a colinear DNA arrangement compatible with other HPVs. Analysis of tumour biopsy DNA indicated that 2 of 13 invasive cervical lesions from Germany and 9 of 36 tumours from Africa and Brazil contained homologous DNA to the new HPV-18. No condylomata contained HPV-18 homologous DNA.

A large number of studies then followed outlining the frequency with which HPV DNA was detectable in cervical neoplasia.

HPV-6/11 in cervical cancer

McCance et al (1983) [190] extracted DNA from cervical biopsies from 22 women referred with abnormal cervical cytology, 19 of whom had histological abnormalities on directed colposcopic biopsy. DNA homologous to HPV type 6 DNA was identifiable in 12 of these biopsies. Fifteen of the biopsies had histological evidence of papillomavirus infection and it could be inferred that viral DNA, detectable in 10 of these, was a result of active infection. However, 2 of 4 biopsies without histological evidence of HPV infection also had detectable HPV-6 DNA, including 1 case of CIN 3. Crum and colleagues (1984) [197] examined 26 biopsy specimens from the cervix, all of which had koilocytic atypia. Of the 13

biopsies with evidence of condylomata, 5 had detectable HPV-6/11 DNA and 1 had HPV 16-DNA. Of 10 biopsies with abnormal mitotic figures, identified by this group as being at high risk of progression, 7 had detectable HPV-16 DNA and none had HPV-6/11 DNA.

HPV-6/11 was rarely identified in invasive cervical cancer [198,199]. It was frequently identified in CIN [131,199-208] although HPV-6/11 was rarely identified in pre-malignant tissue from the West of Scotland [209]. The proportion of positive biopsies varied widely in the above studies and the frequency at which HPV-6 was detectable in CIN was generally higher in low grade CIN 1/2 biopsies than in CIN 3 [131,200,201,206-208].

HPV-16 in cervical cancer

HPV-16 was infrequently identified in genital warts [117,131,201,205,206,210-212] but was detectable in a high proportion of both CIN and invasive cervical cancer.

The frequency at which HPV-16 homologous DNA was detectable in CIN was largely dependent on the grade of CIN; it was more frequently identified in CIN 3 [131,201,204,207,208,213,214] although some studies did not demonstrate this [202,206,209,215]. The proportion of CIN biopsies having detectable HPV-16 DNA also varied geographically [195,209].

The precise significance of HPV-16 homologous DNA in CIN was unclear as many of these lesions had co-existing histological HPV infection (see ref.216). Although a number of studies gave data on the prevalence of actual HPV infection in their study populations [204,205,217-220], only in a few studies [205,209,218] was it possible to deduce that HPV-16 type DNA was present in the absence of active HPV infection.

More detailed information was available from in situ hybridisation studies. Crum et al (1986) [221] using biotinylated probes examined 10 cases of CIN, demonstrating, except for 2

instances, positive hybridisation only in areas with evidence histologically of HPV infection. They concluded that extrachromosomal viral replication was preserved even within high grade CIN. Gupta et al (1987) [222] disputed this in a study of both CIN and VIN. Viral DNA localisation, if HPV-16, was within neoplastic cells. Schneider et al (1987) [223] suggested that the highest concentration of viral DNA was in areas of koilocytic atypia but that viral DNA content varied from cell to cell. Viral DNA was detectable in basal cells showing no histological evidence of viral infection. In CIN 3 biopsies, viral DNA appeared to be uniformly distributed. This could reflect the inability of the virus to replicate in undifferentiated cells. From these studies it was not clear exactly where viral DNA resides within a given pre-neoplastic biopsy and there were data suggesting that the majority of viral DNA in these tissues might lie within recognisable infected cells. The assigment of an aetiological role for HPV in pre-invasive disease was not appropriate on this data alone.

Many groups have found that DNA extracted from squamous cervical cancer frequently contained DNA homologous to HPV-16 type DNA [117,131,199,201,207,208,213,224-230]. The frequency with which HPV-16 homologous DNA was detectable varies from 11% [202] to over 90% [201]. There appeared to be geographical differences in the prevalence of HPV-16 in invasive cancer. Studies from the U.K. generally reported high proportions of HPV-16 positive invasive lesions [201,225,227,229] with 66-90% of invasive tumours having detectable HPV-16 DNA. Reports from the U.S. tended to show intermediate levels of detection, around 40-60% [131,199,208,226]. European studies showed a variable proportion of positive tumours [195,196,207,217] although the rate of positivity was generally higher than in the U.S. The lowest rates of HPV-16 in cervical malignant disease were found in Japanese studies. Fukushima et al (1985) [202] only found 1 of 9 invasive cancers contained HPV-16 homologous DNA. Other Japanese studies have confirmed similar low levels [213,224].

Interestingly, HPV-16 DNA was identified in 72% of cervical carcinoma samples from inland China [231]. Such geographical variation was evident in the initial report of HPV-16, with differences in the rates of HPV-16 identification evident between samples from Europe, Africa and Brazil [195]. There was no relationship between HPV-16 rates and cancer incidence, elegantly demonstrated by Kjaer et al (1988)[52] in Scandinavian populations, and no obvious differences in tumour biology to account for the wide variety in detection rates.

HPV-16 type DNA was infrequently found in non-squamous cervical cancer. In those studies which describe the detailed histology HPV-16 DNA was detectable in only 9 of 27 such tumours [199,202,217,225,229,229,232]. Only Tase et al (1988) [233] addressed the question directly. HPV-16 DNA was detectable in only 1 of 40 cases of adenocarcinoma of the cervix and in 8 of 44 cases of adenosquamous carcinoma.

HPV-16 homologous DNA has been detected in secondary tumour deposits [199,207,226,232]. Lancaster et al (1986) [226] described 7 cases of squamous cervical cancer where pelvic lymph nodes were available for DNA analysis. In 6 of the 7, HPV DNA with a restriction enzyme profile identical to that of the HPV DNA found in the primary tumour was detected. Sutton et al (1987) [199] described one case where the tumour contained HPV DNA types 6, 16 and 18 and pelvic node contained HPV type 16 DNA. Six tumours were analysed in this way by Fuchs et al (1988)[207]. One tumour contained both HPV-6 and 16 DNA. The pelvic node also contained type 6 and 16 type DNA. All of the other 5 tumours and nodes contained HPV-16 DNA and in 3 where there was sufficient DNA for restriction enzyme analysis, the profiles were identical. In Smotkin's study [232], two tumours positive for HPV-16 DNA had omental and pulmonary tumour deposits available for DNA analysis. Both contained HPV type 16 DNA.

The evidence supporting a role for HPV-16 in squamous cervical cancer is therefore substantial. It appears that this type is very frequently identified in high grade pre-invasive and invasive

disease. It is identified in distant metastatic tissue suggesting that it is present within malignant cells.

HPV-18 in cervical cancer

In the initial report of Boshart et al (1985) [196] a much lesser proportion of cervical tumours contained HPV-18 homologous DNA. This was generally confirmed by other studies.

Sutton et al (1987) [199] described HPV-18 homologous DNA as present in vulval warts and HPV-18 DNA was described in cervical wart virus infection without CIN [205,218]. Reid et al (1987) [131] and Schneider et al (1987) [206] described genital warts which contained DNA homologous to a combined HPV-16/18 DNA probe.

HPV-18 was detected in CIN [206-209,214,228]. Toon et al (1987) [203] were unable to detect HPV-18 in 27 cases of CIN. No study using HPV-18 DNA as probe has described more than 20% of CIN biopsies positive for HPV-18. There did not appear to be the same relationship of increasing prevalence with increasing severity of CIN with HPV-18 as in HPV-16 [207,208,214].

In most studies invasive squamous cancers contained HPV-18 homologous DNA infrequently [199,207,224,227,228]. Reid et al (1987) [131] found this type in 25% of their tumours and 13 of 58 invasive cancers examined by Kurman et al (1988) [208] contained HPV-18 DNA. This group [234] suggested an association between poorly differentiated carcinomas and HPV-18 although histochemical staining to exclude glandular elements was not performed [235]. A number of studies were unable to detect HPV-18 DNA in any squamous tumours [232,233].

HPV-18 was found in non-squamous cancers of the cervix [202,224,232]. In the study of Tase et al (1988) [233] HPV-18 was found in 24 of 84 adeno and adeno-squamous cervical carcinomas.

HPV-18 is less frequently identified in squamous cervical cancer and its aetiological role is therefore less clear. The finding of this type in adenocarcinomas of the cervix in higher frequency warrants further investigation of this cancer to determine differences between HPV-16 and 18 in different target cells.

Other HPV types in cervical cancer

Two other viral types, 31 and 33, identified in human neoplastic cervical tissue, could be involved in cervical carcinogenesis.

Lorincz and co-workers (1986) [236] noted in a number of ano-genital neoplasms screened by hybridisation to HPV-6/11, a consistent but different Pst 1 restriction enzyme digest pattern. DNA of this type was cloned from one lesion. Detailed analysis indicated some cross hybridisation at low stringency with a number of HPV types but no cross-hybridisation at high stringency. Genome organisation was similar to other papillomaviruses and initial saturation hybridisation suggested 35-40% sequence homology with HPV-16, with limited sequence analysis showing 70% homology in the L1 region and 50% in the E6. This DNA has been designated HPV-31. In their series this DNA was detectable in 9 of 44 CIN biopsies, in 2 of 62 invasive cancers but in none of 6 genital warts. Subsequently, this type was identified in 7-15% of CIN biopsies and in 5% of invasive biopsies [131,207,208,226]. Stoler and Broker (1986) [237] using single strand RNA in situ hybridisation detected HPV-31 in malignant cells from a cervical cancer.

Beaudenon et al (1986) [228] identified DNA in one invasive cervical cancer which only hybridised to HPV-16 DNA under low stringency conditions. This DNA has been designated HPV-33. It showed 7% sequence homology with HPV-16 at high stringency. Evaluation of genomic organisation and sequencing [238] have shown a structure similar to other HPVs. There was a close relationship with HPV-16, with between 65 and 75% sequence homology in the E1,2,6,7, L1 and L2 regions, and 50% homology in the E4 and 5 regions. Allowing for conservative substitutions, L1 polypeptides of these two types showed around 90% homology, suggesting possible sharing of antigenic properties. The original study demonstrated this type in 2 of 29 CIN biopsies, and in 1 of 53 invasive biopsies but in none of 33 genital warts. A similar low frequency for this viral type in CIN and malignant tumours was confirmed by Fuchs et al (1988) [207].

Section 8 : Other sexually transmitted diseases implicated in the aetiology of cervical cancer

8.1 Human Cytomegalovirus (CMV)

Cytomegalovirus is the largest member of the Herpesviridae family, the genome being $150x10^6$ daltons. It is known to exist as a persistent infection of the genital tract [239] and has been isolated from the cervix and from semen [240,241]. It can be sexually transmitted [242] and causes cervicitis and urethritis [243]. It is frequently isolated at genito-urinary medicine clinics, in some cases more frequently than HSV [244].

Human CMV will induce morphological change in cells in vitro [245,246]. Albrecht and Rapp (1973) [247] demonstrated the presence of CMV specific antigens in the cytoplasm and cell membrane of transformed cells and in sera from tumour bearing animals.

Two regions of the CMV genome will bring about morphological transformation. A 481bp fragment of the Hind III E region of the strain AD169 will transform both rat embryo and NIH 3T3 cells [248]. Transformation of Rat-1 cells or hamster embryo cells was localised to the BamHI M and J fragments of the XbaI E region [249]; these two regions being adjacent on the genome. Viral DNA was not retained in AD169 transformed cells but was retained in cells transformed by the strain Towne [250].

The precise mechanism of transformation was unclear but proposals similar to those for HSV have been suggested. There were some structural similarities between the transforming regions of CMV and HSV-2 [248,249]. Galloway et al (1984) [70] proposed that the small transforming fragment might form a stem/loop structure similar to that proposed for HSV-2. It has also been proposed that these sequences acted as enhancer sequences of specific cellular genes. CMV codes for a protein kinase [251] compatible with putative oncogene products. A gene coding for a phosphorylated protein was mapped to the long unique region of the CMV genome [252]. CMV exhibited homology with human DNA [87].

The association between CMV and genital cancer has not been as extensively examined as that of HSV. Melnick et al (1978) [253] described the isolation of infectious CMV from explant cultures of cervical tumours. Sero-epidemiological studies of CMV in cervical cancer have been contradictory. Vestergaard et al (1972) [254] reported significantly higher levels of complement fixing antibodies in cervical cancer patients compared with controls matched for age and social status. However the seropositivity rate in the control group was 65%. Pasca et al (1975) [255] detected neutralising antibodies more frequently in women with cervical atypia than in controls but the control group was 10 years younger. Kumar et al (1980) [256] in a better matched study, although studying both invasive and pre-invasive disease, found a significant increase in seropositivity using complement fixing antibodies. Stoian et al (1982) [257] found that 20% of invasive cervical cancer patients had detectable complement fixing antibodies to CMV.

In contrast, better matched studies have demonstrated no difference in seropositivity between women with cervical intra-epithelial neoplasia and matched control populations [258,259], although Walkinshaw et al (1987) [260] did find increased seropositivity in women with CIN compared with a well matched control group.

There has been little investigation of the presence of CMV homologous DNA in cervical cancer, the major problem being the size of the viral genome. zur Hausen (1975) [261] failed to detect viral DNA in transformed cells at a sensitivity of 0.2 genome copies per diploid cell but this would miss viral DNA of $20\text{-}25 \times 10^6$. Huang et al (1984) [262] reported the detection of CMV DNA in normal and neoplastic cervical tissue using DNA-DNA reassociation kinetics. Fletcher et al (1986) [263] detected DNA homologous to the Hind III E region of CMV in 2 of 43 biopsies of cervical intra-epithelial neoplasia. In both of these biopsies the DNA was thought to be integrated into cellular DNA. This DNA region includes the known transformation region of this viral strain.

There is therefore some evidence for the presence of the virus in cervical disease, but its role - causative or casual - is not clear.

8.2 Chlamydia Trachomatis

C. trachomatis is a common isolate from the cervix [264], particularly in younger women [265]. It is often asymptomatic.

Suggestions that C. trachomatis might be involved in cervical intra-epithelial neoplasia have been present for some time. Schachter et al (1975) [266] isolated the organism from 4.1% of women with CIN compared with an overall 0.8% in their cervical screening programme. Paavonen et al (1979) [267] isolated chlamydia from 17% of women with dysplasia and 25% of women with invasive cancer. This group also described significantly higher titres of antibody compared with a control group, although there was no matching for sexual activity or other factors known to be associated with cervical cancer. However the restrictive age range of chlamydial isolation shown by Roberts et al (1987) [265] suggests that age matching would be a critical variable in such comparisons.

The strongest evidence supporting a role for chlamydial infection in cervical cancer came from the study of Schachter et al (1982) [268]. In a matched pair analysis of 149 patients with CIN, there were no differences in the seropositivity to HSV but a significant excess of seropositivity to chlamydia using the micro-immunofluorescent technique. These analyses were controlled for those sexual variables known to be involved in cervical cancer. The relative risk was 2 for previous infection, rising to 3.37 in the group where there were few other risk factors. More recently, Allerding et al (1985) [269], examining the previous cytological histories of women developing in-situ or invasive cancer, found a rate of past chlamydial infection on cytology as often as previous evidence of HPV infection.

Although some cases of intra-epithelial neoplasia might be influenced by chlamydial infection, there was no experimental

evidence suggesting any oncogenic potential for this organism. However, studies into the aetiology of CIN must take infection with chlamydia into account as a confounding variable.

Section 9 : Non-infective factors associated with cervical cancer

Current concepts of human carcinogenesis make it unlikely that single agents promote cancer. In the case of papillomavirus in particular, animal model systems where the virus is involved in malignant change and in the model of epidermodysplasia verruciformis, external co-factors play an essential role [143,145,270]. It is therefore important to examine the evidence that non-virological factors may play a part in the multistep hypothesis of genital cancer.

9.1 Oral contraceptive steroids

The theory that hormones contained in oral contraceptive steroids could have a role to play in cervical carcinogenesis was not new. Overholser and Allen (1935) [271] showed that squamous metaplasia could be produced in experimental rodents by injection of oestrogen when combined with cervical trauma over a varying time scale. Loeb et al (1936) [272] were able to produce invasive carcinomas in the vagina and cervix of experimental mice by direct application of oestrogens, and Hisaw and Lendrum (1936) [273] demonstrated that these changes could be prevented by addition of progestins.

Experimental evidence that oestrogens might actually directly cause cervical cancer in laboratory animals came from Allen and Gardner (1941) [274]. Continuous oestrogen administration resulted in an observed increase in in-situ and invasive cancer in the treated group. In 1959, Gardner [275] was able to produce cervical

and vaginal malignant tumours by local application of oestrogens to the cervix and vagina in mice.

The possibility of an interaction between oestrogens and other carcinogens was raised by Murphy (1961) [276]. Mice whose cervices had been exposed to the chemical carcinogen, 3-methylcholanthrene, were then given escalating doses of diethylstilboestrol (DES). The incidence of tumour formation and the time of development of tumours were both found to be related to the dose of DES. DES used alone was also associated with an increase in the rate of tumour formation. Similar results were obtained by Kaminetsky (1966) [277]. This study was the first to propose that progesterone alone was capable of producing an increase in cervical dysplasia, mainly mild. This was at odds with the accepted protective effect of progesterone in oestrogen induced tumorigenesis [278].

Early work on the effect of steroids used in oral contraceptive pills confirmed the known effect of oestrogens on the cervix of experimental rodents [278,279]. Again it was felt that progesterones (dihydroprogesterone, norethisterone) prevented the development of oestrogen induced genital tumours [278].

Oral contraceptive steroids produced marked morphological effects on the cervix which could potentially predispose the critical cervical transformation zone to infection or alter the characteristics of that infection. They produced oedema, softening, increase in Nabothian follicles, increase in secretion and eversion. Histologically they produced increased vascularity in the squamous epithelium, increased numbers of endocervical glands with marked epidermidisation, stromal oedema, and increased neutrophils and plasma cells [280]. Reserve cell hyperplasia was frequently described [280,281]. Apart from these fairly general changes, squamous metaplasia with persistence of the mucin secreting endocervical glands commonly occurred [281]. A more specific abnormality has been noted. Taylor et al (1967) [282] first described a form of atypical endocervical hyperplasia

associated with pill usage. Others have described a microglandular hyperplasia in which glands arose and proliferated within metaplastic epithelium [281,283].

The first clinical and epidemiological data suggesting a role for oral contraceptive steroids was the study of Melamed and colleagues (1969) [284]. In a large study comparing the incidence of cervical disease in women using oral contraception with those using barrier contraception they found a small but significant increase in the rate of cervical carcinoma-in-situ. Early studies into the role of oral contraceptive steroids in cervical cancer were conflicting and many failed to control for the appropriate variables in sexual behaviour known to be factors in the epidemiology of cervical cancer (see ref.285). Thomas (1972) [285] concluded, in his own case-control study, that short term use was not associated with an increased risk of premalignant or malignant disease. Similarly Worth and Boyes (1972) [286], controlling for some of the sexual parameters, were unable to demonstrate any significant difference in contraceptive usage or duration of oral contraceptive use in a case-control study. The large case-control study of Boyce (1977) [287], controlling for most of the sexual variables, found no differences in either overall use or duration of use between women with cervical cancer and control women.

Interesting studies by Stern et al (1970) [288] and by Ory et al (1976) [289] suggested that much of the increased risk attributed to oral contraceptives was a function of preselection. Both groups found that women commencing oral contraceptives were more likely to have abnormal cervical cytology at commencement than women choosing other forms of contraception. Thus women on oral contraceptives had an increased incidence of cervical premalignant disease prior to exposure to the pill.

However, prospective studies have shown an increased relative risk of cervical cancer associated with contraceptive pill use. Peritz (1977) [290] followed women who were either pill users or never users prospectively and found an increased relative risk

for cervical cancer in pill users which seemed related to duration of use, although there was no attempt to control for sexual behaviour. Swan and Brown (1981) [291] later analysed a small subset of women used in this study with case controls for a number of sexual variables. They were able to show that there were significant differences between cases and controls for age at first intercourse, age at regular intercourse, and number of sexual partners. They concluded that the relative risk findings from the original study could be accounted for by the observed differences in sexual behaviour. Stern et al (1977) [292] suggested that long term usage (more than 7 years) was associated with an increased risk of progression from mild to severe pre-invasive disease but found no increase in relative risk of developing CIN de novo in pill users. Harris and colleagues (1980) [293], in a well controlled study, indicated that pill usage for over 5 years was associated with a relative risk of 2 for the development of cervical precancer, independent of other variables.

Much of the recent concern over the role of the pill in cervical cancer stemmed from the large bank of data from the Oxford Family Planning study. This group [294] demonstrated an increased risk associated with use in excess of 4 years for premalignant cervical disease. There was no formal attempt to control for sexual behaviour but a small subgroup were compared for age at first intercourse and number of partners. Patterns for this large study cannot be inferred from this subgroup. Kay (1983) [295] also indicated an increased risk but felt that the confounding variables were likely to result in an overestimate of pill risk. Andolsek et al (1983) [296] showed a small increase in risk for malignant disease but with numbers similar to the Oxford group, were unable to show any substantive difference for CIN 3. Further concern arose following the preliminary results of the WHO collaborative study [297] examining invasive cancer only. there appeared to be little risk (relative risk 1.11) attributable to pill use when other confounding factors such as age at first intercourse and number of partners were taken into

account. However, even controlling for such factors, a relative risk of 1.37 was demonstrable with 5 or more years of use.

The precise role of oral contraceptive steroids remains obscure but there is sufficient theoretical and epidemiological evidence to consider that such steroids may have a substantive cofactor role.

9.2 Cigarette smoking

A link between tobacco smoking and cervical cancer was initially hypothesised by Winkelstein in 1977 [298], based on the observation of a high correlation in the geographic incidence of squamous carcinoma of the lung and cancer of the uterine cervix seen in the Third National Cancer Survey [299]. Extrapolating from the data collated in this extensive survey, he argued that major human cancers of squamous origin, in particular lung and bladder cancers, seemed to show cigarette smoking as a major independent risk factor.

In fact, a number of authors had previously drawn attention to smoking as a potential risk factor in cervical cancer. Naguib et al (1966) [300] initially demonstrated a positive, graded association in relative risk of cervical cancer between non-smokers, ex-smokers and current smokers, although the authors felt that socio-economic confounding might account for this risk. Tokuhata (1967) [301], in a case-control study of invasive cervical cancer, indicated that there was increased tobacco consumption among cases compared with controls, although this seemed restricted to tobacco consumed by means other than inhalation. Thomas (1973) [302] in a complex study of factors associated with cervical carcinoma-in-situ and cervical squamous cell dysplasia, showed an increased relative risk in smokers for carcinoma-in-situ which was weakly statistically significant. In a large follow up study between 1963 and 1972 in Sweden, Cederlof (quoted in ref.298) indicated a 7 year incidence of cervical cancer of 28.7 per 1000 in women smoking more than 15 cigarettes per day compared with a rate of 4 per 1000 for non-smokers, a

relative risk of 7.2. In the same study, the relative risk for lung cancer was 8.5, and for bladder cancer 2.2.

Detailed analysis of the Third National Cancer Survey by Williams and Horn (1977) [303], in which lifetime exposure to cigarettes was calculated, showed that there was a strong positive correlation between exposure to cigarettes and carcinoma of the cervix even after multiple regression analysis had controlled for a number of potential confounding social variables. The relative odds ratio also seemed to increase with increasing cigarette exposure, suggesting a dosage phenomenon. Preliminary data from the long term surveillance of the Oxford Family Planning Study [304], unexpectedly indicated a small but independent risk associated with smoking. This was the only study in which some account of the sexual behaviour variables had been taken.

Some dissension from the general view was proposed by Stellman et al (1980) [305] who argued that almost all of the increased relative risk associated with smoking could be accounted by confounding of socio-economic factors. However, even this data showed an increased risk associated with smoking over 10 cigarettes per day after adjusting for the various socio-economic factors.

Since then better controlled data have emerged. Wigle and Mao (1980) [306] demonstrated relative risks of 3.8 for smokers for carcinoma-in-situ and 2.0 for invasive cancer. The risk for ex-smokers was similar to non-smokers, a situation analogous to the risks of ex-smokers in lung cancer. They were able to show an almost linear increase in relative risk with increasing exposure in both premalignant and malignant disease. Harris et al (1980) [293], showed a clear increased relative risk for cervical dysplasia in smokers after controlling for sexual behaviour, this risk being just over 2. They also indicated that there seemed to be a dosage relationship between total exposure to cigarettes and risk. A similar relative risk was demonstrated by Clarke et al (1982) [307]. In this study the relative risk was also related to the amount of smoking.

In 1983 two studies were published which were specifically designed to test the hypothesis that cigarette smoking was a major independent risk factor in cervical cancer. Lyon et al (1983) [308] demonstrated a relative risk of 3 for carcinoma-in-situ in women who smoked. Their analysis controlled for known confounding variables such as social class and sexual behaviour. No dosage phenomenon was demonstrated. Trevathan et al (1983) [309], allowing for socio-economic status, oral contraceptive use, number of sexual partners, and age at first intercourse, clearly showed an increased relative risk of 2.4-3.6 for the various grades of cervical premalignant disease. Cumulative exposure to cigarettes was strongly related to risk. Women with 12 or more pack-years exposure had a relative risk of 12.7 for carcinoma-in-situ and 4.3 for mild dysplasia.

There could be little doubt that cigarette consumption was a major independent risk factor in cervical cancer and the possibility existed of a dosage phenomenon. How this might come about was less clear.

The cervix was remote from the source of inhaled mutagens. However inhaled mutagens were known to be concentrated in distal body fluids such as breast milk, urine and saliva [310-312]. Sasson et al (1985) [313] demonstrated that nicotine and cotidine were concentrated in the cervical mucus of smokers to a much greater extent than in any other body fluid. Similar concentration of these products has been demonstrated in mucus obtained from the cervices of women with CIN [314]. There are no studies comparing the women with and without CIN under standard conditions. The study of Hellberg et al (1988) [314] raises a further problem, that of passive smoking. Non-smokers had detectable nicotine and cotidine in cervical mucus though not in serum. If this was confirmed then it could suggest that epidemiological data has underestimated the risks of smoking.

Nicotine itself is not carcinogenic although it has been shown to result in a reduction in local immune response if present in high concentrations in cervical mucus [313,315]. Other tobacco products could be present in cervical mucus. One group [316] have

shown that the cervical mucus of smokers was highly mutagenic under experimental conditions. Polyaromatic hydrocarbons found in tobacco were carcinogenic to the cervix of experimental animals. Nitrosamines are the major carcinogen present in tobacco smoke. There are no data on their presence in cervical mucus although they are concentrated in urine [314].

There is therefore considerable epidemiological and experimental data which suggest a direct or indirect role for cigarette smoking in cervical cancer.

Section 10 : Aetiology of vulval cancer

10.1 Epidemiological considerations

Vulval cancer accounts for around 5% of female genital malignancy. It differs from cancer of the cervix in being largely a tumour associated with old age. The mean age at diagnosis is generally reported as over 60 years and a substantial proportion of cases are over 70. The vast majority of tumours are squamous in origin, with the documented existence of a pre-invasive intra-epithelial lesion, including carcinoma-in-situ, the mean age of which precedes the invasive form by 15 to 25 years [317,318].

Much of the early epidemiological data centred on the relationship between vulval dystrophies, in particular leucoplakia, and invasive cancer. Bonney was one of the earliest authorities suggesting such a relatonship, although he attributed Sir Henry Morris in the 1880s as being the first to demonstrate an association between vulval dystrophy and carcinoma on the same site [319]. Taussig (1940) [320] reported that almost 50% of women with invasive disease had a history of leukoplakic vulvitis. On this basis, he recommended aggressive surgical management of established leukoplakia. Although others [321,322] confirmed the high proportion of pre-existing leukoplakia in women with vulval cancer, McAdam and Kistner's (1958) [321] detailed histological study of vulval dystrophies, in which women with such lesions were followed closely, demonstrated that there was minimal risk attached to most vulval dermatoses. Jeffcoate (1966) [323] in prospective studies indicated that women with leukoplakia rarely progressed to malignancy, and gave a risk of 4-5% progression for leukoplakia, increasing to 10% if hyperplasia and atypia were present. Similar risk estimates were argued by Underwood and Hester (1971) [324] in prospective data.

Early studies had also highlighted a number of other interesting findings. Women with vulval cancer were more likely to be

nulliparous and more likely to have undergone early menopause [317,322]. There was an apparent association with co-existent medical diseases such as diabetes, hypertension, atherosclerosis and obesity [317,318,322] but this data was not well controlled. Others [325,326] were unable to substantiate these findings and it would appear that these associations were more related to the age of the patient population than to the epidemiology of the cancer.

A number of studies have looked at the role of specific local lesions in vulval cancer. Taussig (1940) [320] commented on the frequent finding of syphilitic or post-syphilitic ulcers in women with invasive vulval cancer. Hahn (1956) [325] found 8 of 131 invasive tumours had positive serology for syphylis. This excess of previous exposure to syphilis has been noted by others [318,322,327]. Green et al (1958) [322] reported that women with positive serology were younger and had poorly differentiated tumours, a finding confirmed by Franklin and Rutledge (1972) [317]. Age differences were also described in post-granulomatous cancers [328,329]. One of these studies [329] identified two distinct groups. The first group had co-existing carcinoma-in-situ. These women rarely developed secondary spread and had a high 5 year survival rate. The second group had no evidence of pre-malignant disease and had tumours characterised by rapid growth and metastasis. No obvious conventional histological differences were evident between these tumours. As all these tumours were of squamous origin, this argued for a subset of at risk women exposed to particular sexually transmissable agents who were younger and had more aggressive disease. Japaze et al (1977) [318] found a syphylis seropositivity rate of 26% in their group of invasive and in-situ cancers.

These granulomatous lesions have been observed to undergo malignant transformation [324].

The other major observation of the early studies was the marked excess of other lower genital cancers in women with vulval cancer [317,318,322,330], the reported incidence being as high as 65%

[318]. These initial impressions have been subsequently confirmed and applied to both pre-invasive and invasive vulval cancer [326,331-334]. The concept of multicentric disease, where women with vulval carcinoma were found to have or have had cervical or vaginal carcinoma, was fully established in the late 1960s and there had been speculation on a common aetiology.

In the 1970s the numbers of women with detectable pre-invasive disease of the vulva appeared to rise and the age of detection fell [318]. Woodruff et al (1973) [332] calculated that only 20% of the vulval tumours seen in the period 1936-50 had been in-situ cancer, compared with 47% in the period 1966-72. 41% of these women were under 40 years of age. Buscema et al (1980) [326] indicated that the proportion of in-situ lesions in the 21-40 age group increased from 28.5% to 37.2% between 1958 and 1978 and Friedrich et al (1980) [333] stated the modal age at diagnosis of vulval carcinoma-in-situ to be 30.

The association with sexually transmissible granulomatous disease, the apparent link with cervical carcinoma which had been established as a likely sexually linked cancer and the increasing numbers of young women with in-situ carcinoma suggested a possible infective aetiology for vulval cancer, despite the generally elderly age group to which the vast majority of vulval cancer patients still belonged.

It was therefore not unrealistic to attempt to link genital herpesvirus infection with vulval cancer, especially as the vulva was the commonest site of infection with this virus..

10.2 Herpes simplex virus in vulval cancer

The rising incidence of genital herpes infection seemed co-incident with the rising incidence of vulval carcinoma-in-situ in younger women. Freidrich et al (1980) [333] found that 5 of 53 women with in-situ carcinoma had a history of previous HSV infection, including a previously described case of a young girl who developed in-situ cancer of the vulva following severe

herpetic vulvitis in pregnancy. This lesion regressed spontaneously. The relative rarity of vulval carcinoma made it virtually impossible to mount meaningful sero-epidemiological studies.

Kaufman et al (1981) [335] expanded their group's work on the expression of HSV specific polypeptides in cervical tumour biopsies to examine a number of vulval biopsies. Seven of 8 vulval carcinoma-in-situ biopsies examined had immunohistological evidence of expression of ICSP 34/35 protein, 5 of 8 had evidence of ICP 11/12, and 6 of 8 evidence of VP 143 protein. Two additional vulval dysplasias had evidence of expression of both ICSP 34/35 and VP 143. In 4 of the cases of in-situ carcinoma, adjacent histologically normal tissue was examined and found not to express any of these specific products. Detailed examination of the tissue biopsies suggested expression of HSV related polypeptides in the superficial layers, in contrast to the cervical biopsy results. Reaction was exclusively cytoplasmic, despite the demonstration that these polypeptides are associated with the nuclei of infected cells. A more recent larger study [336] demonstrated the presence of the HSV-2 related antigen, ICSP 34/35, in 50% of biopsies from vulval carcinoma-in-situ by immunocytochemical techniques. In a similar study, Cabral et al (1982) [337] used indirect tissue immunoperoxidase staining to demonstrate the presence of the HSV-2 antigen designated VP 143 in 1 of 3 severe vulval dysplasias, three of 7 in-situ carcinomas, and 1 of 3 invasive tumours. Staining was confined to the basal cell layers in the non-malignant tissue. Internally matched normal vulval tissue showed no evidence of staining. Staining for the glycoprotein VP 119 was also in positive biopsies. In this study, staining occurred in the perinuclear area as was seen in infected cells.

There was little work on the detection of HSV specific nucleic acid in vulval cancer. McDougall et al (1980) [108] did detect HSV specific RNA in a biopsy from a pre-neoplastic vulval biopsy by DNA-RNA in situ hybridisation and Eglin et al (1984) [338] described HSV RNA in biopsies from malignant vulval tissue. Only

2 studies were described which examined the detectability of HSV DNA in vulval cancer tissue in any detail. Macnab et al (1985) [120] examined 11 invasive tumours of the vulva and found DNA homologous with the HindIII a region of HSV-2 in DNA extracted from one tumour. Manservigi et al (1986) [119] examined 4 invasive vulval tumours and 2 in-situ carcinomas for HSV sequences. DNA homologous to BglII n and o regions was detected in 2 of the 4 invasive tumours, in a rearranged fashion. One of the in situ cancers also contained detectable BglII n DNA and a fragment of the BglII o area. DNA from the MTRII region of HSV-2 had therefore been identified in both malignant and pre-malignant vulval tissue.

There was therefore some evidence that HSV information is present in vulval neoplastic tissue, although there were insufficient data from which to draw any meaningful conclusion.

10.3 Human papillomavirus in vulval cancer

Genital condylomata had long been associated with vulval malignancy. A number of studies had shown a striking proportion of vulval cancers co-existing with large condylomata, with rates of 7-20% being quoted [317,326,332,333]. Franklin and Rutledge (1972) [317] stated that in a number of cases, histological evidence of condyloma, in-situ cancer and invasive cancer could be found in the same specimen. There were many case reports of malignant transformation of genital warts [324,328,339-342].

Viral particles were identified in Bowenoid papulosis, a variant of in-situ carcinoma [343] and histological koilocytic atypia, characteristic of HPV infection, could be found in 69% of cases of in-situ carcinoma [344]. However, evidence of viral antigen expression in in-situ cancer has been conflicting [222,344,345].

HPV-6/11

Genital warts were commonly due to HPV types 6 or 11 [194,346]. HPV-6 could be identified in giant condylomata of Buschke-

Lowenstein, which were slow growing locally invasive tumours which could affect the vulva [193]. An unusual HPV type 6 was also described in an unusually aggressive vulval verrucous carcinoma [347,348]. HPV-6 was identified in pre-neoplastic vulval disease [131,194,336,349]. McCance et al (1985) [350] found HPV-6 frequently in association with HPV-16 in all grades of vulval intra-epithelial neoplasia (VIN) although a number of biopsies had histological evidence of HPV infection. Sutton et al (1987) [199] similarly identified HPV-6 in in situ lesions in higher copy number than HPV-16. In the largest study [336], HPV-6 or 11 was identified as the sole viral type in 26% of cases and in association with other types in 11% of cases.

The finding of HPV-6 in invasive cancer varied. Some groups had been unable to demonstrate HPV-6 DNA in invasive vulval cancer [131,194,227]. However Sutton et al (1987) [199] indicated that HPV-6 was the sole HPV DNA identified in 4 of 9 invasive lesions and was found in association with other types in 2 others. HPV-6 DNA was also identified in DNA extracted from lymph node metastases. HPV-6 was present as frequently as HPV-16 in condylomatous cancers [351] and was identified as the sole DNA type in 1 of 5 HPV positive squamous cancers by Carson et al (1988) [352].

Other HPV types

HPV-10 was implicated in some vulval lesions. Green et al (1982) [148] identified HPV-10 in a vulval carcinoma-in-situ and in 2 other invasive cancers of the vulva. They also showed its presence in 2 genital condylomata. Zachow et al (1982) [349] identified similar viral DNA in bowenoid papulosis, verrucous carcinoma and carcinoma-in-situ of the vulva. In both these studies the viral DNA was present in a free, unintegrated form.

HPV-31 was not detected in 21 biopsies of VIN or in 5 invasive specimens [131] but was identified in 9% of in situ lesions by Kaufman et al (1988) [336]. Beaudenon et al (1986) [228] found HPV-33 DNA in 4 of 54 VIN biopsies.

HPV-16

HPV-16 was identified infrequently in genital condylomata [117,131,199,201,205,206,210-212,346]. It has been identified more frequently in pre-invasive disease [211,350,353], though often in association with HPV-6 [211,350], and often where there was histological evidence of viral infection [217]. Both Beaudenon et al (1986) [228] and Reid et al (1987) [131] described the identification of HPV-16 DNA as sole viral DNA in over 2/3 of VIN biopsies but Kaufman et al (1988) [336] found HPV-16 as the sole type in only 11% of biopsies. No histological information on the presence of koilocytic atypia was presented in these studies. Only Gupta et al (1987) [222], using in situ hybridisation techniques, have convincingly demonstrated the presence of HPV-16 DNA in vulval in situ cancer in the absence of histological wart infection.

The frequency with which HPV-16 was identified in invasive disease also varied widely [131,199,217,227,352] with this type being present in 15-90% of samples.

HPV-18

In the initial studies of Boshart et al (1984) [196], describing HPV-18, this viral type was not found in any condylomata or vulval cancer. HPV-18 DNA has been identified rarely in vulval condylomata [131,199] and in vulval carcinoma in situ [227] by most groups. However the large study of in situ vulval cancer by Kaufman et al (1988) [336] found HPV-18 to be common in carcinoma-in-situ of the vulva (11/46).

HPV-18 DNA has only been identified infrequently (2/31 tested) in invasive tumours [131,196,199,227] and in both cases in association with HPV-6 or 16. HPV-18 was present in a lymph node secondary deposit in one tumour [199] but in association with both HPV-6 and 16.

Viral DNA has been found in a substantial proportion of both pre-invasive and invasive vulval cancers. The position is less clear

cut than in cervical cancer and there is considerably more variability in the viral type found. HPV-6/11, rarely found in invasive cervical cancer, is frequently associated with invasive vulval cancer. Although it is attractive to suggest that viruses of similar oncogenic potential, present in the genital tract, are closely involved in two related malignancies, the natural histories of the two cancers urge caution to this approach.

CHAPTER 2

HUMAN PAPILLOMAVIRUS AND HERPES SIMPLEX VIRUS IN INVASIVE GENITAL MALIGNANCY AND MATCHED CONTROL TISSUE

Section 1 : Introduction

Although there are considerable data on the prevalence of HPV DNA and HSV DNA in genital malignancy, few studies systematically examine a single group of cervical and vulval cancers for both HPV and HSV DNA [117,119,336]. No group has examined a range of invasive genital tumours with all probes from both viruses implicated in their aetiology.

There are now data on the presence of HPV DNA in histologically normal tissue [131,203,207,212,229,230,354,355]. However there is little information on the presence or absence of HPV DNA in normal tissue from women with invasive genital malignancy containing detectable HPV DNA [217,356,357].

To examine these two problems the DNA extracted from 15 invasive cervical cancers and 11 invasive vulval cancers was probed for the presence of HPV-6, 11, 16 or 18 and for HSV-2 Hind III a, e, h, and i DNA. DNA extracted from internally matched normal tissue from 8 cervical and 3 vulval tumours was similarly probed for the presence of sequences homologous to these DNAs. By this approach it should be possible to determine the prevalence of each viral DNA in malignant tissue, the possible presence of more than one viral type in tumour DNA, and the frequency with which viral DNA was present in normal tissue in women with malignant disease.

It was hoped that such detailed analysis would lay a foundation for tissue culture and cell line studies of tumours found to contain DNA homologous to viral DNA and that it would be possible to clone such homologous sequences from tumour or cell line DNAs for analysis.

TABLE 1

SOURCE OF TUMOURS USED IN DNA ANALYSES

Cervical tumour	Age	Source	Histology
1	36	RHND	Poorly differentiated squamous
2	34	RHND	Squamous
5	-	RHND	Squamous
8	44	RHND	Poorly differentiated squamous
16	42	Staging biopsy	Squamous
17	32	RHND	Poorly differentiated squamous
20	32	RHND	Squamous
22	30	RHND	Moderate/poorly diff. squamous
23	50	RHND	Well differentiated squamous
24	26	RHND	Moderately well differentiated squamous
29	35	RHND	Poorly differentiated squamous
30	25	RHND	Squamous
35	37	RHND	Squamous
38	46	RHND	Squamous
39	32	RHND	Papillary adenocarcinoma
Vulval tumour			
3	70	Radical vulvectomy	Well differentiated squamous
9	29	Radical vulvectomy	Moderately well diff. squamous
12	87	Excision biopsy	Squamous
15	66	Radical vulvectomy	Squamous
18	92	Simple vulvectomy	Squamous
19	90	Excision recurrence	Squamous
21	81	Radical vulvectomy	Squamous
27	60	Radical vulvectomy	Well differentiated squamous
28	75	Simple vulvectomy	Carcinoma-in-situ
32	73	Excision recurrence	Poorly differentiated squamous
34	76	Radical vulvectomy	Squamous
Endometrial tumour			
6	60	TAH	Adenocarcinoma
7	27	TAH	Adenocarcinoma
Vaginal tumour			
13	60	Anterior exenteration	Well differentiated squamous
Nude mouse tumour			
41	54	Staging biopsy	Well differentiated squamous

RHND Radical hysterectomy and node dissection
TAH Total abdominal hysterectomy

Section 2 : Materials and methods

2.1 Collection of tissue specimens

Normal control tissue

Histologically normal ectocervical tissue was obtained from 9 patients undergoing hysterectomy for benign uterine pathology. All had normal cervical cytology in the preceding 12 months and had no history of cervical surgery or history of malignant disease at other sites. The age range of these women was 39 to 77 years of age.

Following removal of the uterus, strips of ectocervical epithelium were dissected under sterile conditions in the operating theatre. These were placed on ice in tissue culture medium (see Chapter 3, section 2.2) and transported to the laboratory for DNA extraction. Strips were taken at intervals from the cervix and histology of the adjacent areas obtained. In all specimens used as normal controls, histology was normal. It was assumed that these control biopsies were histologically similar.

Histologically normal vulval skin was obtained from 2 women undergoing autopsy for non-malignant disease. Neither of these women had evidence of vulval pathology and adjacent skin was reported as histologically normal. These skin biopsies were stored on ice in culture medium and extracted immediately.

Cervical tumour tissue

Tumour tissue was obtained from 14 radical hysterectomy and 1 wedge biopsy specimen of invasive cancer of the cervix for DNA extraction.

The clinical details of these women are shown in Table 1.

Viable tumour tissue was dissected in theatre from the radical hysterectomy specimen. Care was taken to avoid necrotic areas or

stromal tissue. Tissue was washed in culture medium and then either placed in culture medium on ice for immediate extraction or snap frozen in liquid nitrogen for late extraction.

Vulval tumour tissue

Tumour tissue was obtained from 7 radical vulvectomy specimens, 1 simple vulvectomy and 3 vulval biopsy specimens. The patient details of these tumours are shown in Table 1.

Tumour tissue from viable tumour areas was dissected from the surgical specimens in theatre. It was washed in culture medium and either placed in medium on ice or was snap frozen in liquid nitrogen. The decision to snap freeze tumour tissue was dependent on both the original biopsy histology and the time to DNA extraction, with those tumours where a clear histological diagnosis was not available being frozen until it was established that further samples were not required. Those tumours from the Regional Gynaecological Oncology Unit, Queen Elizabeth Hospital, Gateshead were frozen as the minimum transit time was 4 hours.

Matched control tissue

Paired matched internal control tissue was obtained in 8 cases of carcinoma of the cervix and 3 cases of carcinoma of the vulva.

Using a fresh scalpel, a block of tissue was dissected from the uterine fundus of radical hysterectomy specimens. Tissue was largely myometrium with serosal tissue. Care was taken not to enter the endometrial cavity to avoid potential contamination with tumour tissue. This tissue was snap frozen in liquid nitrogen. Tissue taken was in the order of 1 cm^3 in volume.

In 3 women undergoing radical vulvectomy, a strip of abdominal skin immediately superior to the tranverse abdominal incision of the radical procedure was excised and snap frozen in liquid nitrogen. The skin samples were at least 2cm. from the nearest tumour margin on gross inspection. A fresh scalpel was used for this dissection.

Samples were stored until full histological examination of the surgical specimen was completed. Samples were only regarded as histologically normal if the tissue immediately adjacent to the matched control tissue was reported as histologically normal. DNA was then extracted.

Other tumour tissue

Tissue was dissected from 2 hysterectomy specimens from women with endometrial adenocarcinoma following bisection of the uterus in the operating theatre. Tissue was obtained from a single case of squamous carcinoma of the vagina. Tissue was obtained from a subcutaneous tumour produced in a athymic nude mouse by injection of a suspension of cells obtained from a biopsy of a squamous carcinoma of the cervix prior to radiotherapy. Details are shown in Table 1.

2.2 DNA extraction

Fresh tissue to be extracted was finely minced using a sterile scalpel or scissors. Total cellular DNA was isolated by proteinase K digestion and phenol extraction [358]. Minced samples were incubated for 3 to 24 hours at 37°C in 50mM trometamol-HCl (TRIS-HCl), 10mM edetic acid (EDTA), 100mM NaCl, and 0.4% sodium dodecyl sulphate (SDS) lysis buffer containing 200µg/ml proteinase K (Boehringer, Mannheim), all at pH 8.0. Nucleic acids were extracted twice with phenol equilibrated with TE buffer (0.001M edetic acid, 0.01M TRIS-HCl pH 8.0), once with chloroform (to remove the phenol), dialysed extensively against 1xSSC (150mM NaCl, 15mM sodium citrate, pH 7.2) and precipitated in absolute alcohol containing 0.2M sodium acetate at -20°C overnight. The nucleic acid pellet was collected by centrifugation, washed in absolute alcohol, re-pelleted, and air dried. The total nucleic acid pellet was redigested with 100µg/ml RNase A (Sigma, Poole, England) in 10mM TRIS-HCl, 10mM EDTA pH 7.5 for 3 hours at 37°C. This digest was re-extracted,

twice in phenol and once in chloroform. The resultant DNA solution was extensively dialysed against 1 x SSC at 4°C, precipitated in Burrough's absolute alcohol and resuspended at appropriate concentration in TE buffer.

Prior to usage the DNA concentration of each sample was determined by comparison of band intensities visualised by ethidium bromide with those of standard λ DNA (Gibco, Paisley, Scotland) on a short gel.

Snap frozen samples were extracted in a similar manner except that the samples were crushed in dry ice prior to digestion.

2.3 Gel electrophoresis and Southern blot transfer

Restriction enzyme digest

The relevant DNAs for each Southern blot transfer were first digested with the appropriate restriction enzyme; Hind III, Bam HI, or Pst I (BRL, Glasgow, Scotland). Restriction digests were carried out to the manufacturers specifications using 30 μg rat embryo DNA for reconstruction tracks and 10-20μg total cellular DNA. Digests were carried out using excess enzyme, normally 40 units for 20-30μg DNA. Digests were carried out at 37°C for a minimum of 18 hours. The reaction was stopped by freezing on stop buffer containing bromophenol blue dye.

Gel electrophoresis

Products of digestion were separated on full length 0.6% agarose gels. Agarose was dissolved in electrophoresis buffer (one litre of buffer contained 43.625g TRIS, 46.875g sodium dihydrophosphate and 3.7g EDTA; 0.05ml of ethidium bromide stock solution was added to each litre). DNAs were run in buffer at 10V, minimum current for 6-8 hours and at 20V, minimum current for 24-36 hours.

Gels were examined under UV light to confirm adequate fragment separation and were trimmed to minimum size.

Relationship of cloned HSV-2 DNA probes to morphological transforming regions of HSV and to DNA identified in genital tumours

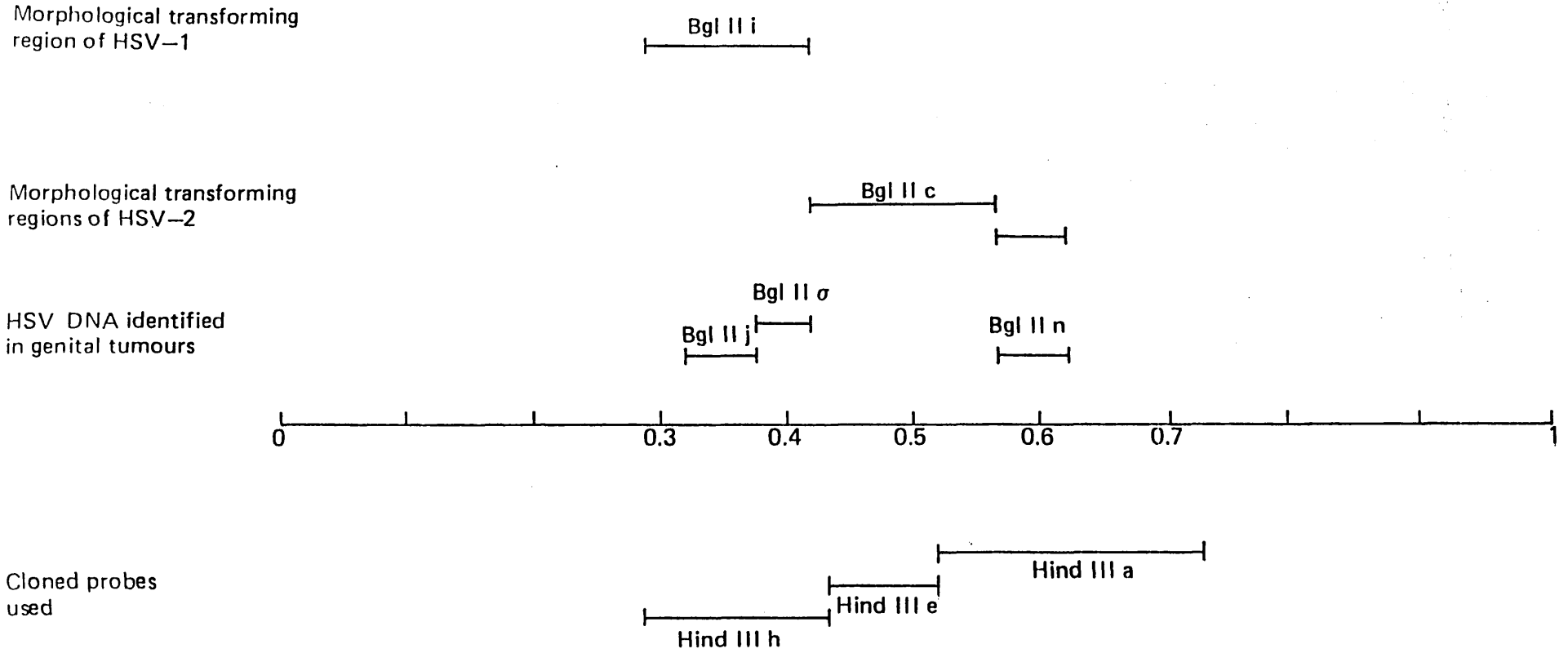


Figure 2

Southern blot transfer

DNA fragments were transferred onto nitrocellulose membranes (Schleicher and Schuell) using the method of Southern (1975) [359] or onto nylon membranes (Biodyne, Pall; Genescreen plus, New England Nuclear) using the manufacturers methods.

Gels were washed in 0.2M HCl for 30 minutes; in 0.2M NaOH, 0.6M NaCl for 30 minutes and in 1M TRIS pH 7.5, 0.6M NaCl for 30 minutes. Transfer was acheived in 10 x SSC overnight.

The transfer membrane was washed twice in 2 x SSC for 30 minutes, air dried, and baked at 70-80°C in a vacuum oven to immobilise the DNA fragments.

2.4. Preparation of viral DNA probes

HSV-2 probes were derived from the HSV-2 strain HG52, cloned into the plasmid vector pAT153 [360]. The probes used and the genomic map positions are shown in Fig 2. These probes cover 42% of the HSV-2 genome. Viral DNA was released from the vector by appropriate restriction endonuclease digestion and purification twice by agarose gel electrophoresis.

HPV probes were kindly provided by Drs. zur Hausen and Gissmann and colleagues. HPV-6, 11 and 16 are cloned into the Bam HI site of pBR322, and HPV-18 is cloned into the EcoRI site of the same vector. Purification of the viral probes was carried out as described above.

Radiolabelling of viral probes

Nick translation of viral probes was carried out using the technique described by Rigby et al (1977) [361]. Probes were labelled with ^{32}P-orthophosphates (Amersham International, England); α-deoxy-[^{32}P]-adenosine triphosphate and α-deoxy-[^{32}P]-thymidine triphosphate for HPV and with α-deoxy-[^{32}P]-cytosine triphosphate and α-deoxy-[^{32}P]-guanosine triphosphate for HSV. The difference in labelling reflected the differing nucleotide

proportions in each viral probe and allowed maximalisation of the specific activities of the probes.

For HSV, 0.25μg of probe DNA in buffer solution [50mM TRIS-HCl pH 8.0, 5mM magnesium chloride, 50μg/ml bovine serum albumin (BSA), and 1mM DDT], 0.02mM Adenosine triphosphate, 0.02mM thymidine triphosphate, and 1% BSA was digested with a $1x10^{-5}$ dilution of DNase for 2 minutes at 37°C. Radioactive triphosphates were then added and 1 unit of DNA polymerase-1 added to the solution . This was incubated at 15°C for 90 minutes, with counting to check incorporation at 30 minutes, and the reaction stopped on ice.

Incorporation was checked by spotting 1μl of the reaction mix onto two filter papers and comparing the scintillation counts following washing of one filter. Incorporations of between 25 and 40% were accepted.

Labelled probe was purified from non-specific label by passing the remaining solution down a Sephadex column (Sephadex in TE buffer) and collecting the maximal fraction peak only. Only nick translations which achieved a specific activity of labelling of greater than $1x10^{8}$cpm/μg DNA were used in hybridisations.

2.5 Hybridisation procedure

Prehybridisation

Filters to be probed with HSV probes were prehybridised with 3 x SSC, 50μg salmon sperm DNA/ml., 0.2% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.1% SDS in aqueous solution at 72°C overnight. Filters to be probed with HPV probes were prehybridised with 50% formamide (Fluka), 5 x SSC, 50mM Hepes pH 7.0, 0.01% Ficoll, 0.01% polyvinylpyrrolidone, 0.01% BSA, 50μg/ml. salmon sperm DNA at 42°C for 4 to 24 hours.

Conditions of stringency

Hybridisations were carried out under stringent or non-stringent

conditions. Stringency was determined by the relationship of the hybridisation conditions to the normal melting temperature of the probe DNA, i.e. the temperature at which exact complementary DNA strands will separate. Under conditions close to the melting temperature, DNA will re-anneal with DNA containing a high proportion of sequence homology. These were high stringency conditions indicating a high likelihood of sequence homology between probe and source DNA. The further from the melting temperature, the more likely probe DNA was to re-anneal with DNA with less sequence homology. It has usually been assumed that positive hybridisation under stringent conditions indicated that the source DNA contains the probe DNA sequences.

Melting temperature (T_m) was calculated using the formula of Schildkraut and Lifson (1965) [362]. This temperature is dependent on the relative proportions of purine and pyrimidine bases in each virus and was influenced by the salt concentration. Hybridisation with HSV probes was always carried out under stringent conditions (T_m-10). Hybridisation with HPV probes was carried out at non-stringent conditions (T_m- 40) and the filters washed at stringent or non-stringent temperatures. By exposing filters for autoradiography at each stage cellular sequences showing some homology with the HPV probe could be detected at non-stringent washing. Specific DNA homology could be confirmed at high stringency washing.

Hybridisation procedure

HSV DNA probes were denatured in 0.2M NaOH on a heating block, neutralised and the filters hybridised in 6 x SSC, 0.02% respectively of Ficoll, polyvinylpyrrolidone (PVP), and BSA, 0.1% SDS, and 10% dextran sulphate in aqueous solution for 20 hours at 72°C. Filters were then washed in stepwise decreasing washes of 3 x -0.2 x SSC plus 0.1% SDS and 10mM sodium pyrophosphate. Filters were air dried and exposed for autoradiography (Kodak) with Dupont lighting plus intensifier screens for 2-7 days.

HPV probes were similarly heat and alkali denatured before hybridisation in 50% formamide, 5 x SSC, 50μg/ml salmon sperm DNA, 0.01% Ficoll, PVP and BSA in Hepes buffer pH 7.0 at 42°C for 48 hours. For stringent conditions the filters were washed at 68°C and for non-stringent conditions filters were washed at 48°C prior to exposure for autoradiography. The use of formamide allowed hybridisations to be carried out at lower temperatures (1% formamide allows reduction by 0.75°C).

Biodyne filters could be re-probed following washing of hybridised DNA from the filter. This was achieved by washing in 50% formamide and 10mM sodium pyrophosphate buffered to pH 6.5 for 1 hour at 65°C, followed by vigorous washing at room temperature in 2 x SSC and 0.1% SDS for 15 minutes. Washing at this temperature in formamide was effectively above the T_m for HPV and should remove all hybridised sequences. Filters were air dried and re-exposed for autoradiography for 21 days. Providing no hybridisation was evident after this time span, the filters were re-probed as appropriate.

Hybridisation with undigested DNA

To determine the state of the viral DNA in the tumour DNA, undigested DNA was hybridised with HPV probes under non-stringent conditions. The procedure, other than the absence of restriction enzyme digestion, was identical to that described above.

Reconstruction (positive control) experiments

All gels examined contained a standard reconstruction assay containing known quantities of the viral DNA to be probed. The amount of viral DNA required for each reconstruction was expressed in genome copies per diploid cell. This was calculated from the relative weights of viral and human cellular DNA. These tracks acted as positive control markers for each experiment, and as size markers for the viral DNA. RC1 would denote 1 genome

TABLE 2

DNA PROBES IN INVASIVE TUMOUR BIOPSIES

Cervical tumour	HindIII a	HindIII h	HindIII e	HindIII l	HPV 6/11	HPV 16	HPV 18
1	-	-	-	-	-	+	+
2	+	-	-	-	+	+	-
5	+	-	-	-	-	+	+
8	+	-	-	-	+	+	-
16	+	+	+	+	-	-	-
17	+	+	+	-	+	+	+
20	-	-	-	-	+	+	+
22	+	+	+	+	+	+	+
23	-	-	-	-	+	+	+
24	+	-	-	-	+	+	+
29	-	-	-	-	+	+	-
30	+	-	+	+	+	+	-
35	-	-	-	-	+	+	+
38	+	-	-	-	+	+	+
39	-	-	+	-	+	+	+
Vulval tumour							
3	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+
18	-	+	+	+	+	+	+
19	+	-	-	-	-	-	-
21	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+
Other tumours							
6	-	-	-	-	+	+	+
7	-	-	-	-	+	+	+
13	+	+	-	-	-	+	+
41	+	-	-	-	+	+	+

copy of viral DNA per diploid cell and would act as a sensitivity marker for that hybridisation.

Where the viral probe has not been excised from the plasmid vector, plasmid DNA was included in the gel run as control against non-specific hybridisation to vector sequences.

Figure 3, a - c and figure 4 demonstrate typical reconstruction experiments for Hind a, e and h, and HPV-16, showing the characteristic fragment pattern on digestion of these probe DNAs with Bam HI. Fragment size markers are shown.

Section 3 : Results

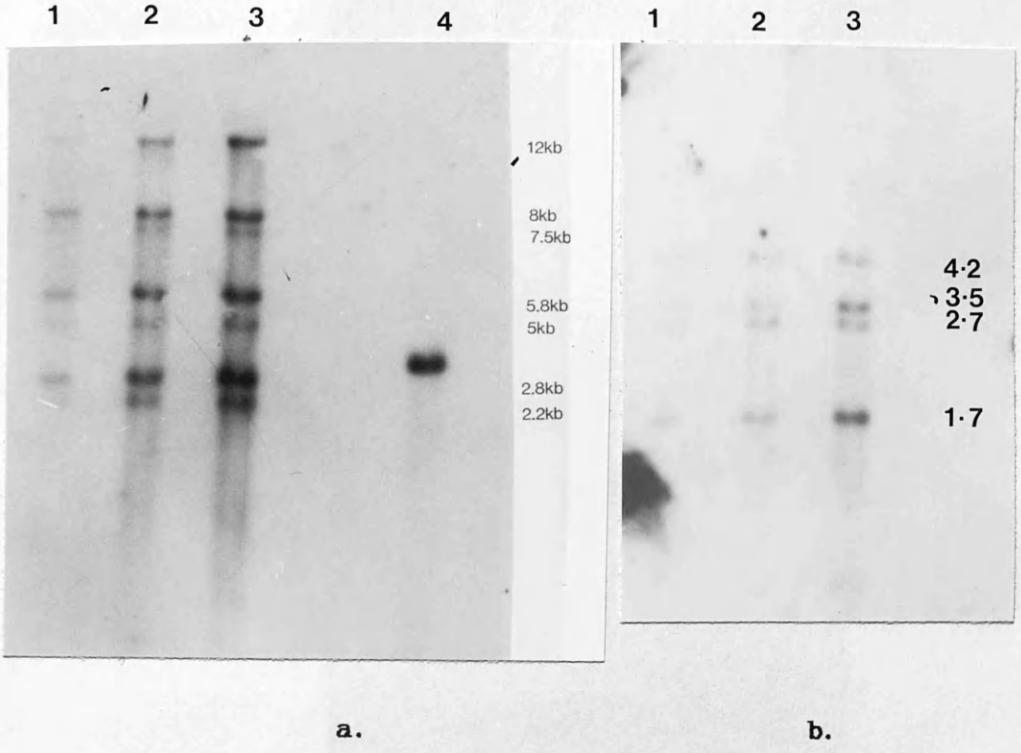
3.1. Hybridisation with herpes simplex type 2 probes

Table 2 lists the DNA probes used for each tumour biopsy.

Two tumour DNAs showed evidence of hybridisation to the HindIII a probe under stringent conditions. Figure 5 shows a Bam HI digest of DNA extracted from the cervical tumours 8 and 17, the endometrial cancer 7, and the vulval cancers 9, 18 and 19, probed with HindIII a (map units 0.512-718). Hybridisation was seen in the track containing Tu 8, a poorly differentiated squamous carcinoma of the cervix, with a fragment at around 8kb, the copy number being about 10 genome copies per cell. This band was not colinear with HSV-2 virion DNA, and might represent HSV-2 homologous sequences integrated into cellular DNA.

No other cervical tumour sample showed evidence of hybridisation to HindIII a or any other HSV cloned probe.

Figure 6 is a Bam HI digest of DNA extracted from cervical tumours 2 and 5, and vulval tumour 3, probed with HindIII a DNA. Hybridisation to a fragment, approximately 10kb in size, was seen in vulval Tu 3. This did not co-migrate with virion DNA and was presumed to represent HSV-DNA integrated into high molecular weight cellular DNA. No hybridisation of any other vulval tumour DNAs was seen with HindIII a or any other HSV probe.



a. b.

FIGURE 3

a. Reconstruction hybridisation for HindIII a. Lanes 1, 2 and 3 contain 1, 5 and 10 copies per cell HindIII a DNA, digested with Bam HI, and probed with HindIII a. The characterisitic hybridisation pattern of 7 bands is seen, due to the cleavage pattern of Bam HI with this DNA. Size markers are shown on the right and lane 4 contains plasmid vector DNA as probe DNA was not excised from its vector in this case.

b. Reconstruction hybridisation for Hind III e. Lanes 1, 2 and 3 contain 1, 5 and 10 copies per cell HindIII e DNA, digested with Bam HI, and probed with HindIII e. Size markers are on the right. The hybridisation pattern shows 4 bands, typical of this digest. Two other bands, one of high molecular weight, and one of lower molecular weight are not demonstrated on this autoradiograph.

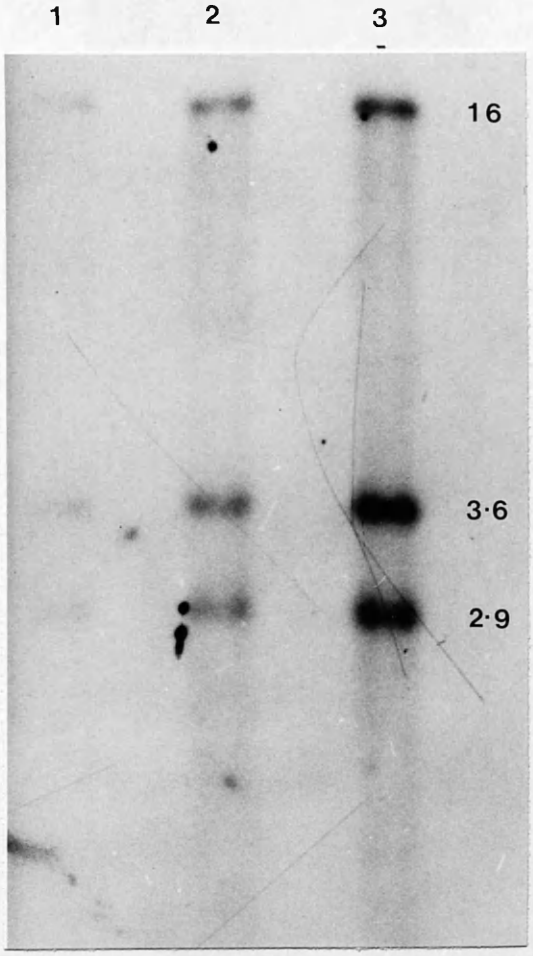


FIGURE 3c

Reconstruction hybridisation of HindIII h DNA. Lanes 1, 2 and 3 contain 1, 5 and 10 copies per cell HindIII h DNA, digested by Bam HI, and probed with HindIII h. A typical 3 band digest pattern is seen, the size markers indicated on the right.

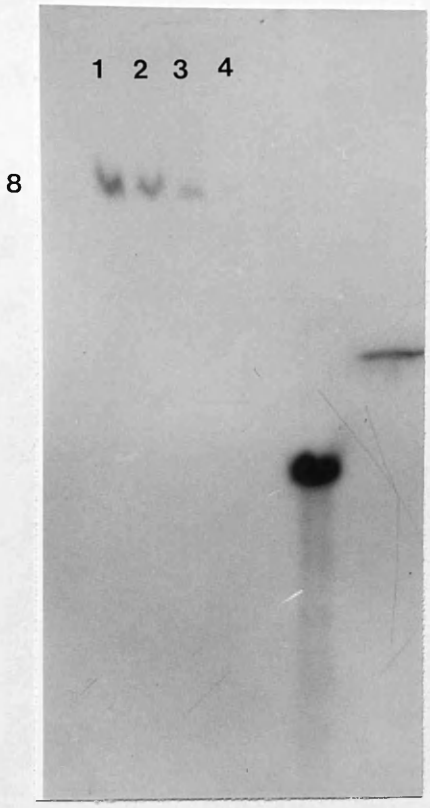


FIGURE 4

Reconstruction hybridisation of HPV-16 DNA. Lanes 1, 2, 3, and 4 contain 20, 10, 5 and 1 copies per cell HPV-16 DNA, digested with Bam HI and probed with HPV-16 DNA under stringent conditions. A typical single band at 8kb is seen in lanes 1-3, and faintly in lane 4. Lane 5 contains plasmid DNA

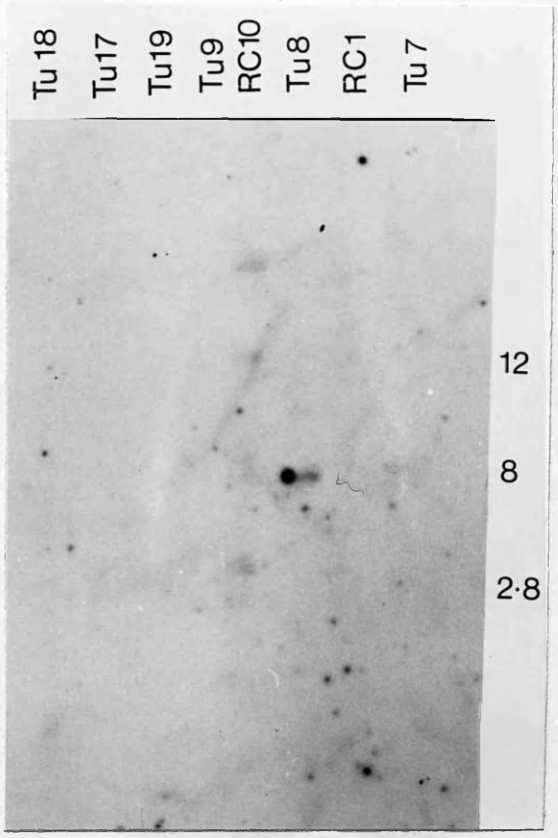


FIGURE 5

Bam HI digest of tumour DNAs probed with HSV-2 cloned probe HindIII a under stringent conditions.
Lanes 1, 3 and 4 contain DNA from vulval tumours 18, 19 and 9; Lanes 2 and 6 DNA from cervical tumours 17 and 8; lane 8 DNA from the endometrial cancer Tu 7. The reconstruction tracks are in lanes 5 and 7, showing 10 and 1 copy per cell respectively. The size markers are indicated on the right. Hybridisation is demonstrated in lane 6 (cervical tumour 8) with a single band of approximately 10 copies per cell at around 8kb. The RC1 track in lane 7 is faintly seen.

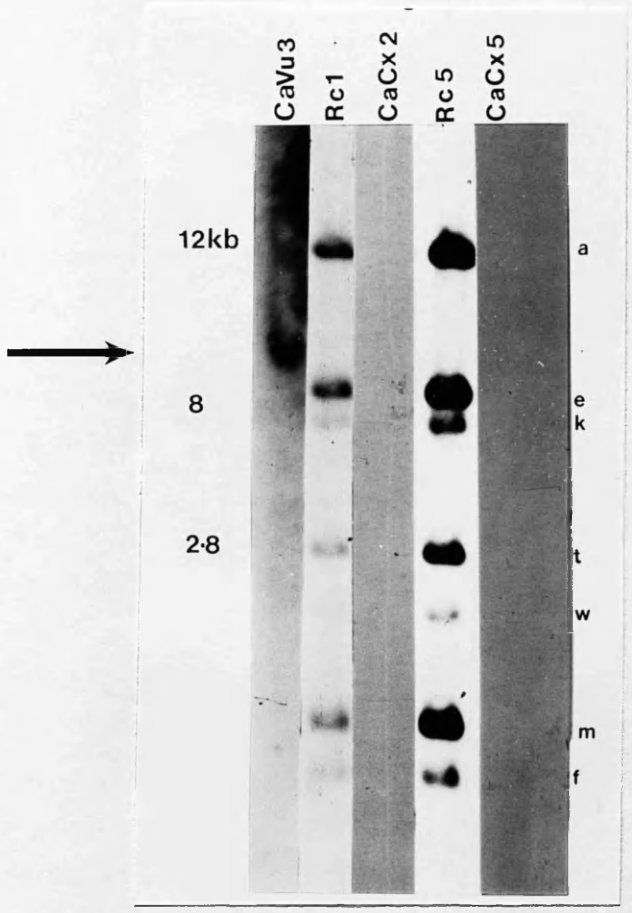


FIGURE 6

Bam HI digest of tumour DNAs hybridised with cloned probe HindIII a.

Lane 1 contains DNA from vulval tumour 3, lanes 3 and 5 DNA from cervical tumours 2 and 5. The reconstruction tracks, one and 5 copies per cell, are shown in lanes 2 and 4. Size markers are shown on the left and the band nomenclature on the right. Hybridisation is seen in both reconstruction tracks. In addition hybridisation is seen to a band in lane 1 (vulval tumour 3) at approximately 10kb (as indicated).

TABLE 3

HYBRIDISATION TO HPV 16 DNA OF TUMOUR AND MATCHED CONTROL TISSUE DNA

	Tumour		Control	
	Stringent	Non-stringent	Stringent	Non-stringent
Tu 20	+	+	+	+
Tu 22	+	+	–	–
Tu 23	+	+	+	+
Tu 24	+	+	–	–
Tu 29	+	+	+	+
Tu 30	+	+	–	–
Tu 38	+	+	+	+
Tu 39	+	+	+	+
Tu 27	+	+	+	+
Tu 34	+	+	+	+

Hybridisation to HindIII a DNA was therefore seen in 1 of 9 cervical and 1 of 10 vulval tumours tested.

3.2 Hybridisation with human papillomavirus types 6 and 11 probes

No evidence of hybridisation to these probes was seen under stringent conditions to any tumour DNA although even under stringent conditions, cross-hybridisation between HPV-11 and HPV-16 DNA was noted.

Figure 7 demonstrates cross-hybridisation under non-stringent conditions. This experiment shows DNA from vulval tumour 27 and its derived cell line Tu 31 hybridised with HPV-11. Tracks 1 and 2 contain HPV-16 reconstructions at 1 and 10copy per cell; tracks 5 and 6 HPV-11 reconstructions. Clear hybridisation of HPV-11 probe DNA to linear 8kb HPV-16 DNA is seen.

3.3 Hybridisation with human papillomavirus type 16 probe

Tumours and matched control tissue

The results from those tumours from which matched control tissue was available are shown in Table 3. All cervical and vulval tumours tested demonstrated the presence of DNA homologous to HPV-16 DNA under stringent hybridisation conditions. Figure 8 represents a Bam HI digest of tumours 24 and 39, probed with HPV-16 DNA under stringent conditions. Tumour 39 showed hybridisation of a single band, migrating at less than 8kb, suggesting that only part of the viral genome was present in this tumour. Tumour 24 demonstrated hybridisation to 3 fragments, one of which was colinear with the reconstruction tracks, representing a linear insert of intact viral DNA. The additional non-colinear fragments were presumed to represent viral DNA integrated into different sites within cellular DNA.

Figure 9 represents undigested tumour and matched control DNA probed with HPV-16 under stringent conditions. The tumour and control pairs 20, 23, 24, and 39 are shown. In addition internal

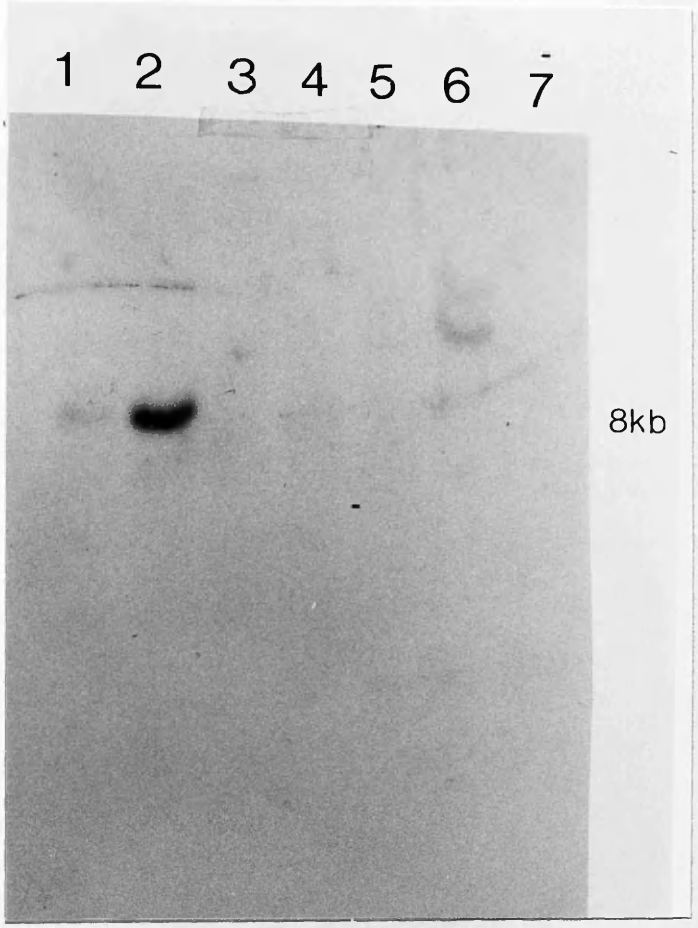


FIGURE 7

Bam HI digest probed with HPV-11 under non-stringent conditions. Lanes 1 and 2 contain HPV-16 DNA at 1 and 10 copies per cell. Lanes 3 and 4 contain DNA from vulval tumour 27 and vulval cell line 31 respectively. Lanes 5 and 6 contain HPV-11 DNA at 1 and 5 copies per cell, and lane 7 contains plasmid DNA. Hybridisation is seen with the HPV-11 reconstruction in lane 6. Cross-hybridisation is also clearly seen to unit length HPV-16 DNA in both lanes 1 and 2. There is no hybridisation to either tumour or plasmid DNA.

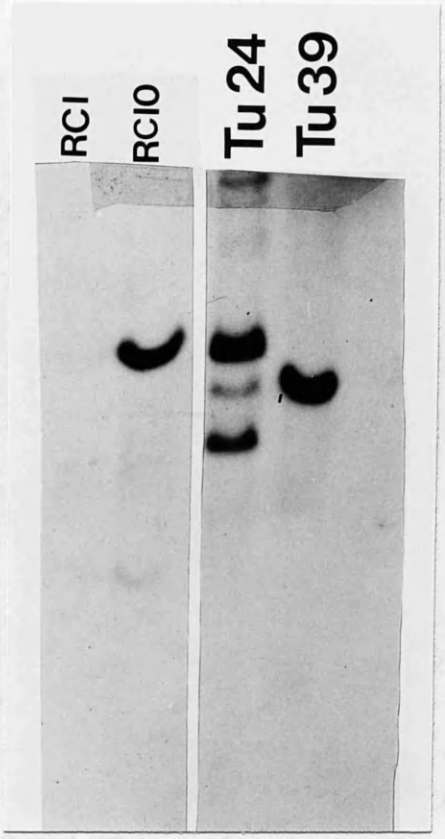


FIGURE 8

Bam HI digest of cervical tumour DNA probed with HPV-16 under stringent conditions.

The reconstruction experiments are in lanes 1 and 2 at 1 and 10 copies per cell. Lane 3 contains DNA from cervical tumour 24 and lane 4 DNA from cervical tumour 39.

Hybridisation to the reconstruction tracks is seen at one copy per cell (lane 1). Both tumour DNAs hybridise to HPV-16 DNA at a level of about 10 copies per cell. Tumour 24 exhibits 3 bands, one of which is colinear with the reconstruction band in lane 2. Additional, lower molecular weight bands are also seen. Tumour 39 exhibits a single band, not comigrating with the reconstruction track DNA.

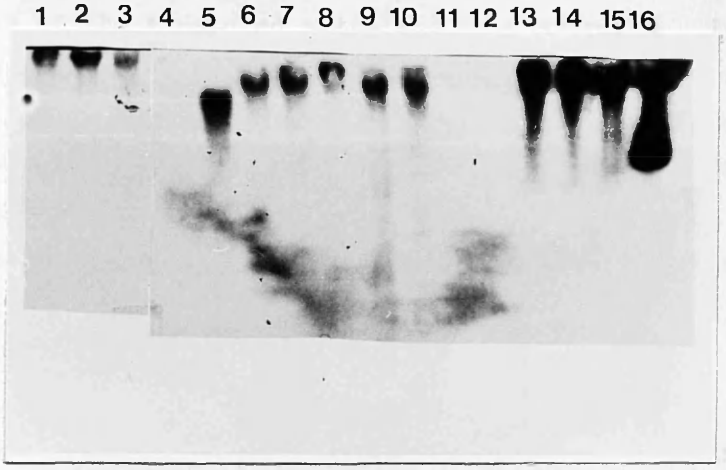


FIGURE 9

Undigested tumour and internal control DNA probed with HPV-16 DNA under stringent conditions.
DNA from tumour and control pairs 23, 24, 39, and 20 are shown in lanes 1+2, 3+4, 6+7, and 10+9 respectively. Control DNA only from tumours 27, 29 and 22 are also demonstrated in lanes 5, 8 and 12. Lane 11 contains DNA extracted from a normal external control cervix and lanes 13-15 contain the reconstruction tracks with 1, 5 and 10 copies per cell. Lane 16 contains plasmid DNA. Hybridisation to all samples except controls 22 and 24 (lanes 12 and 4) is seen under stringent conditions. No hybridisation to external control cervix is seen in this sample.

controls 27, 29 and 22, and a normal external control cervix are demonstrated. Hybridisation of matched control tissue was clearly seen in 3 of the 4 matched pairs and in control 29. In all, 5 of 8 cervical control tissues demonstrated hybridisation under stringent conditions. Both vulval control tissues showed evidence of hybridisation. Hybridisation of Bam HI digested DNA from vulval Tu 34 and its matched control normal skin is shown in Figure 10. Tu 34 had a single 8kb band, present at approximately 5 copies per cell, which co-migrated with the reconstruction track. The matched control tissue also demonstrated a single comigrating band, at lower copy number. Comparison of Bam HI digests of the other vulval tumour, Tu 27 and its matched control will be described more fully in Chapter 4.

Hybridisable sequences comigrated with high molecular weight cellular DNA in both tumour and control DNAs, suggesting that HPV DNA was present either in concatemeric form or integrated into cellular DNA. The complex patterns seen, for example in Tu 24 and 27, suggest that integration was more likely.

Unmatched tumours

Cervical tumours 1, 2, 5, 8, 17 and 35 were probed with HPV-16 DNA. There was insufficient DNA for analysis of tumour 16. Figure 11 shows Bam HI digested DNA from the cervical tumours 2, 17, 23 and 35 and the vulval tumour 28 probed with HPV-16 under stringent conditions. No hybridisation was demonstrable in Tu 2. Tu 23 DNA demonstrated faint bands on this exposure which did not co-migrate with virion DNA. Tu 17 DNA clearly demonstrated an 8kb band, at more than 10 copies per cell and Tu 35 had a single band at similar copy number, but migrating more slowly than the 8kb virion band. Neither Tu 1, 5 (see figure 12) or 8 showed evidence of hybridisation with HPV-16 under stringent or non-stringent conditions. The nude mouse tumour 41, derived from a squamous carcinoma of the cervix, did show evidence of hybridisation to an 8kb fragment on long autoradiographic exposures.

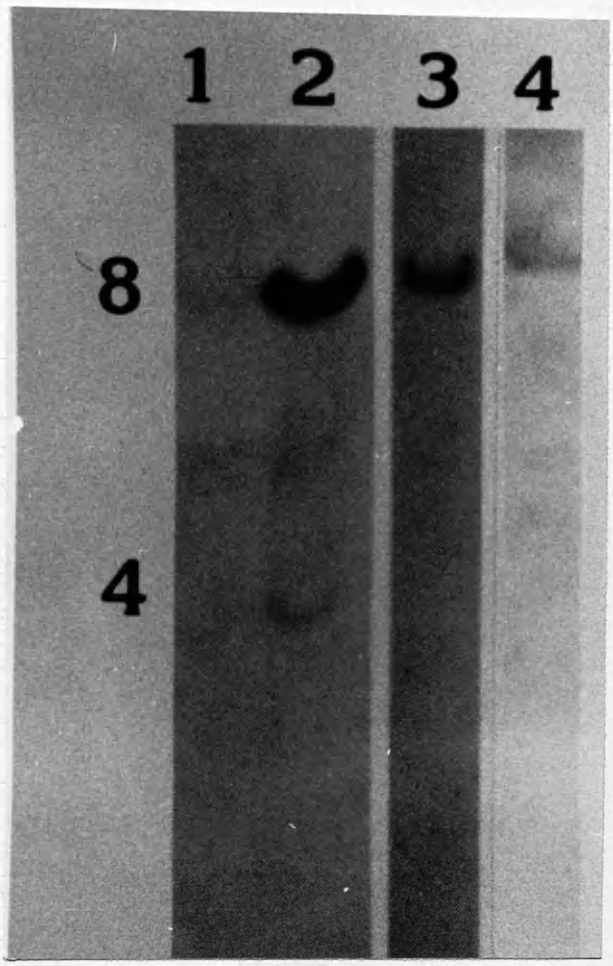


FIGURE 10

Hybridisation of Bam HI digest of vulval tumour 34 and internal control tissue with HPV-16.
Lanes 1 and 2 contain HPV-16 DNA reconstructions at 1 and 10 copies per cell. Lane 3 contains DNA from vulval tumour 34 and lane 4, DNA from control skin from this patient. Size markers (kb) are indicated on the left.
Hybridisation to the reconstruction tracks is seen, faintly on this exposure at a level of one copy per cell. There is hybridisation to vulval tumour 34 DNA at a level of 5 copies per cell which comigrates with the reconstruction tracks at 8kb, suggesting the presence of unit length HPV-16 DNA in this tumour. In lane 4 a faint band is seen at a level of 1 - 2 copies per cell, also comigrating with the reconstruction track DNA. This suggest that the control DNA also contains unit length HPV-16 DNA.

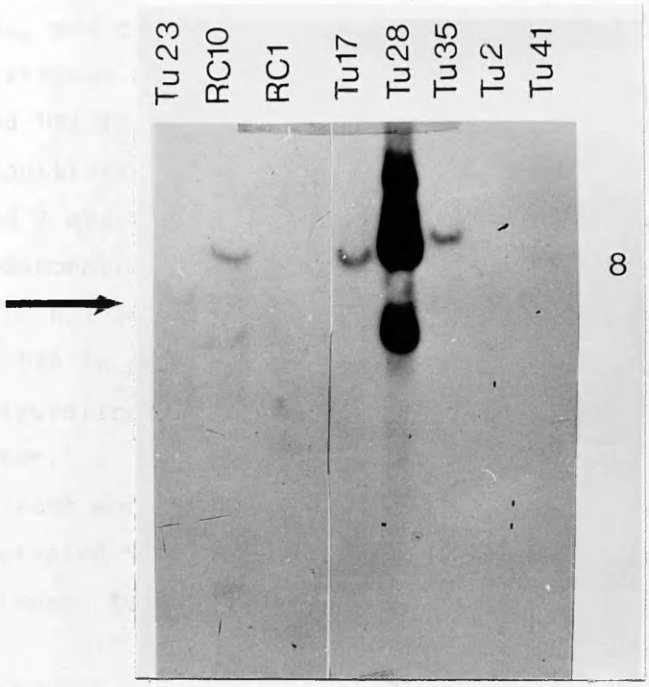


FIGURE 11

Bam HI digest of tumour DNAs probed with HPV-16 under stringent conditions.

The reconstruction tracks, at 10 and 1 copies per cell, are shown in lanes 2 and 3. DNA from cervical tumours 23, 17, 35 and 2 is present in lanes 1, 4, 6 and 7 respectively. Lane 5 contains DNA from the vulval carcinoma-in-situ 28 and lane 8 contains DNA from the nude mouse tumour 41, derived following injection of cells from a cervical squamous carcinoma. In this exposure hybridisation at 10 copies is clearly seen (lane 2). Tumour 17 (lane 4) shows hybridisation which comigrates with the reconstruction track at 8kb. Tumour 41 (lane 8) also has a faint band at 8kb on this exposure, confirmed on longer exposures. Tumours 23 and 35 (lanes 1 and 6) also show hybridisation to single but differing bands (the band in lane 1 is indicated). Tumour 2 (lane 7) does not hybridise to HPV-16 DNA. The vulval tumour 28 (lane 5) has multiple bands at high copy number (see text for details).

Vulval tumours 3, 9, 12, 15, 18, 21, 28,and 32 were probed with HPV-16. Only tumour 3, which contained DNA homologous to HSV-2 HindIII a DNA, and tumour 18 did not show hybridisation to HPV-16 DNA under stringent conditions. Figure 12 shows HPV-16 probing of undigested DNA from tumours 9, 18, 21, 27 and 34 under stringent conditions. Cervical tumour 5 and the two endometrial tumours 6 and 7 are also shown on this figure. Figure 11 has already demonstrated the in situ carcinoma, Tu 28, hybridising to HPV-16 DNA. This tumour contained very high copy number viral DNA in an apparent complex integration pattern. The precise configuration of the viral DNA in this tumour will be discussed later.

Analysis of both endometrial carcinomas under stringent conditions revealed that HPV-16 homologous DNA was present. The vaginal carcinoma, tumour 13 did not contain such sequences.

Normal control tissue (unmatched)

Nine samples of normal ectocervix and 2 samples of normal vulval skin were probed with HPV-16 DNA. An example of normal cervical tissue is included in Figure 9 and an example of vulval skin is shown in chapter 4. One of the cervical samples did show evidence of hybridisation to HPV-16 DNA under stringent conditions.

3.4. Hybridisation with human papillomavirus type 18 probe

The tumour DNAs probed with HPV-18 DNA are listed in Table 2. None of the matched tumour-control pairs exhibited evidence of hybridisation to HPV-18. Two tumours, vulval tumour 28 and cervical tumour 35, did hybridise to HPV-18 DNA under stringent conditions. Fig 13a shows these 2 tumours probed with HPV-18 after Bam HI digestion. Both tumours also hybridised to HPV-16. Reconstruction experiments indicated that even under stringent hybridisation conditions there was some weak cross-hybridisation between HPV-16 and 18. Figure 13b shows HPV-16 DNA

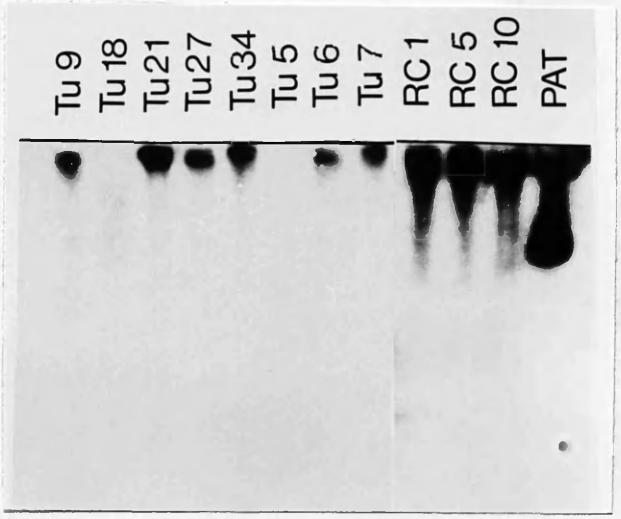
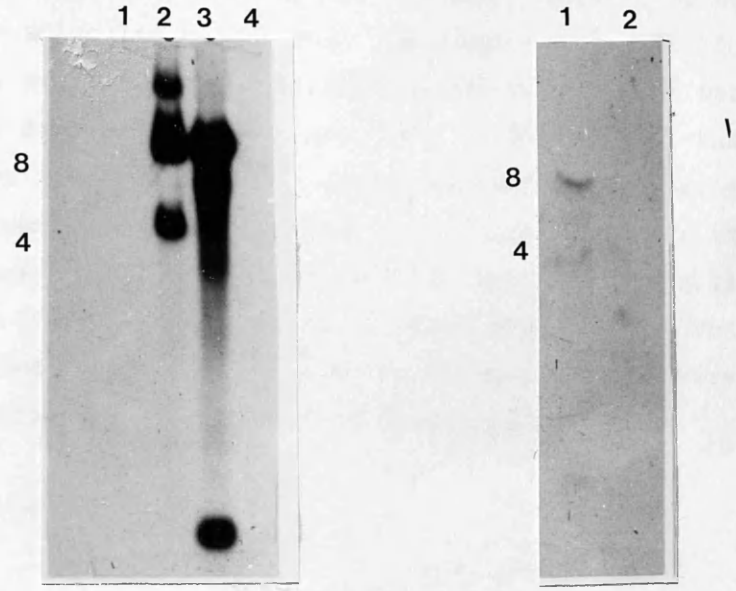


FIGURE 12

Undigested tumour DNAs probed with HPV-16 under stringent conditions.
Lanes 1 to 5 contain DNA from the vulval tumours 9, 18, 21, 27 and 34 respectively. Lane 6 contains DNA from cervical tumour 5 and lanes 7 and 8, DNA from the endometrial tumours 6 and 7. Lanes 9 to 11 contain reconstructions at 1, 5 and 10 copies per cell. Lane 12 contains plasmid DNA.
Hybridisation is seen to all samples except vulval tumour 18 (lane 2) and cervical tumour 5 (lane 6).



a. b.

FIGURE 13

a. Bam HI digest of tumour DNAs probed with HPV-18 under stringent conditions. Lanes 1, 3 and 4 contain the cervical tumours 2, 35 and 17. Lane 2 contains DNA from the vulval carcinoma-in-situ 28. Size markers (kb) are shown on the left. No hybridisation is seen in lanes 1 and 4. Tumour 35 (lane 3) shows hybridisation at high copy number, with multiple bands. The low molecular weight band in this lane is consistent with hybridisation to HPV-18, confirmed by the hybridisation pattern seen on Pst I digest of this tumour DNA (see text for details). The hybridisation seen in lane 2 (tumour 28) also shows multiple bands. The band pattern is however similar to that seen on probing of this tumour with HPV-16 (see lane 5, figure 11) and could represent cross-hybridisation at high copy number.

b. Bam HI digest of HPV-16 DNA probed with HPV-18 DNA under stringent conditions. Lane 1 contains 10 copies per cell and lane 2, 1 copy per cell. Hybridisation is seen in lane 1.

reconstructions probed by HPV-18 DNA under stringent conditions, demonstrating the degree of cross hybridisation evident. Tumour 35 showed the characteristic Bam HI digestion pattern of HPV-18 with the low molecular weight band, obtained as the HPV-18 genome is cut twice by this enzyme. Tumour 28 did not exhibit this band. Pst I digest of these tumour DNAs confirmed that the HPV-18 homologous DNA in tumour 35 had the characteristic pattern of HPV-18, whereas the relative digestion pattern of tumour 28, although showing hybridisation to HPV-18, was similar to that of HPV-16. The precise significance of these findings was unclear. It could be due to cross-hybridisation between the two viral types when viral DNA was present in high copy number.

3.5. Summary of results

HSV-2

HindIII a sequences (which span the MTRII region of HSV-2, see figure 2) were detected in one of nine invasive cervical and in one of ten invasive vulval cancers. Hybridisation to other probes spanning the putative mtr regions of the HSV genome was not seen in any specimen.

HPV-6 and 11

Hybridisation to these probes was not detected in any specimen.

HPV-16

HPV-16 DNA appeared to be ubiquitous in cervical and vulval invasive cancers in this study. Hybridisation to HPV-16 DNA under stringent hybridisation conditions occurred in 10 of 14 cervical tumours and 8 of 10 vulval tumours examined.
However, HPV-16 DNA was frequently identified in non-malignant genital tract tissue in these women. Where internally matched normal tissue was available, 7 of 10 samples demonstrated

TABLE 4

PROGNOSIS OF WOMEN WITH INVASIVE CANCER AND DNA STATUS

	Tumour tissue			Matched control		
	HSV-2	HPV 16	HPV 18	HPV 16	Months followed	Status
Cervix						
Tu 1	n/d	-	-	N/A	18	Died of disease
Tu 5	-	-	-	N/A	35	Disease free
Tu 8	+	-	n/d	N/A	12	Died of disease
Tu 16	-	n/d	n/d	N/A	34	Alive,recurrence
Tu 17	-	+	-	N/A	14	Died of disease
Tu 20	n/d	+	-	+	42	Disease free
Tu 22	-	+	-	-	48	Alive,recurrence
Tu 23	n/d	+	-	+	42	Alive,local recurrence
Tu 24	-	+	-	-	24	Died of disease
Tu 29	n/d	+	-	+	37	Disease free
Tu 30	-	+	-	-	18	Died of disease
Tu 35	-	+	+	N/A	30	Died of disease
Tu 38	-	+	-	+	30	Disease free
Tu 39	n/d	+	-	+	30	Disease free
Vulva						
Tu 3	+	-	-	N/A	48	Died of disease
Tu 9	-	+	-	N/A	48	Alive,recurrence
Tu 15	-	+	-	N/A	40	Disease free
Tu 18	-	-	-	N/A	40	Died;not tumour
Tu 21	-	+	-	N/A	19	Died of disease
Tu 27	-	+	-	+	12	Died of disease
Tu 28	-	+	+	N/A	36	Disease free
Tu 32	-	+	-	N/A	32	Disease free
Tu 34	-	+	-	+	33	Disease free

n/d, insufficient DNA for full analysis
N/A, not available
- , no detectable DNA at stringent conditions
+ , hybridisation at stringent conditions

hybridisation to HPV-16 under stringent conditions. No significant difference in the prevalence of HPV-16 genomes in malignant and non-malignant tissue in women with invasive genital cancer was demonstrable (χ^2 = 0.28, not significant).

HPV-16 DNA was also identified in 2 biopsies from endometrial adenocarcinoma.

HPV-18

HPV-18 DNA was rarely identified in this population. Of 22 tumours probed with HPV-18, only 2 demostrated the presence of HPV-18 homologous DNA. Both of these tumours also hybridised to HPV-16 DNA probes, with one tumour demonstrating the restriction pattern of HPV-16 rather than HPV-18.

Section 4 : Prognosis of women with and without detectable human papillomavirus genomes in genital cancer tissue.

The ubiquity with which HPV-16 genomes were detectable in both tumour groups make comparisons of prognosis in these small numbers difficult.

Follow up data on the outcome of 14 cervical and 9 vulval tumours were available. The outcome and DNA status is shown in Table 4.

Of three HPV-16 DNA negative cervical tumours, including the HSV-2 DNA containing tumour, two have died of secondary disease and one was disease free at 40 months post treatment.

Of ten HPV-16 DNA positive tumours reviewed, 4 have died from disease at between 14 and 30 months following surgery. Two have evidence of recurrent disease but were alive at the time of review. Four women remain alive and disease free.

The two patients whose vulval tumours did not contain HPV-16 homologous DNA have died, one from recurrent disease and one from unrelated causes. This latter patient's tumour contained HindIII

a homologous DNA. Of the 7 tumours which contained DNA homologous to HPV-16 DNA, 4 were disease free, 2 have died of recurrent disease and one was alive but had received treatment for recurrent disease. This latter patient was the youngest patient with vulval disease and had local excision of a vulval recurrence. Since then she has successfully completed a pregnancy and remained disease free at time of review.

Analysis of the outcome of patients with cervical cancer where matched control tissue distant to the primary tumour was available, showed that of the 3 women without evidence of HPV DNA distal to the tumour, 2 were dead from disease. One was alive but has had recurrent disease. This patient, from whom the cell lines Tu 22-1 and 22-2 are derived, will be described in detail in chapter 4. Of the 5 women in whom HPV-16 DNA was detected in distal tissue, 4 were disease free, and one was alive with recurrent disease. There was no apparent relationship between disease free survival and HPV-16 status in either tumour or distal tissue. Indeed it appeared that HPV-16 DNA in distal tissue imparts a better prognosis in this small group.

The overall survival for the cervical cancers in this study, considering that all but one sample was obtained from women with early disease, suitable for surgical treatment, was poor. Patient selection is discussed in the next section and the possible implications of using atypical tumours to demonstrate viral oncogenesis will be discussed more fully in chapter 4 and in the discussion in chapter 6.

Section 5 : Discussion of methodology

5.1 Selection of patients

Selection of patients used in this and in the culture studies described in chapter 3 was arbitrary. The decision to utilise a particular tumour was dependent on availability and tumour size as sufficient tumour tissue was required for DNA extraction, tissue culture and for adequate histological study. Cervical cancer patients were therefore a selected group of women with early disease, suitable for radical surgery, but with relatively large tumours. The mean age (35.8±7.4 years) of such patients was lower than that normally ascribed to cervical cancer for these reasons. Selection bias could therefore have influenced the results.

However, the prevalence of HPV-16 DNA was high in this younger population, contrary to what might have been expected based on the age progression seen by Meanwell et al (1987) [229]. It was not thought that these women represented an atypical sample for viral DNA prevalence studies. There was less selection for vulval tumours as most of these were sufficiently large and all women fit for anaesthesia underwent radical surgery. The wide age range (up to 92 years) reflected the aggressive surgical policy of the Northern Regional Gynaecological Oncology Unit and the high standard of available anaesthesia.

Both tumour groups were small and it was difficult to draw major inferences on population prevalence on small samples. Nevertheless the data from this were consistent with other U.K. studies [201,225] and were consistent with local studies which indicated that HPV-16 was present in a very high proportion of pre-malignant genital tissue [205,209].

5.2 Control tissue

In the latter half of the study, it was appreciated that there was little data on normal internal control tissue in women with malignant genital cancer. Previous work within the laboratory with in situ hybridisation using HSV probes [338] had demonstrated the value of such comparisons. Control tissue was therefore collected from 9 cervical and 5 vulval tumours. Initial difficulties with DNA extraction of large biopsies of frozen tissue resulted in very poor yields from the extraction process and insufficient quantities for analysis in 1 cervical and 3 vulval samples (tumours 19,21,32,38). Prior pulverisation of these large samples in dry ice as suggested by Green et al (1979) [363], resulted in the extraction of large quantities of control DNA.

The choice of tissue site was a clinical one. For cervical tumour specimens, a section from the uterine fundus was chosen as it represented genital tract tissue, was far distant from the primary site (at least 5cm.), and was non-epithelial tissue not thought to be permissive for HPV. Great care was taken not to include endometrial tissue in the sample and a fresh scalpel was utilised for the control sample. I was therefore confident that no contamination of the control tissue with tumour tissue occurred in the sampling process. To ensure that the tissue used was histologically normal, samples for extraction were snap frozen. DNA was not extracted until histological examination of the surgical specimen was complete. In the event, no histological evidence of tumour was found within 5cm of the control sampling area. This tissue was therefore uncontaminated internal control tissue.

The choice of control tissue for vulval cancer was more difficult. The idea of using DNA extracted from blood taken during the procedure was dismissed as impractical and possibly dangerous for older women undergoing radical surgery. It was decided to use abdominal skin from the uppermost part of the radical surgical specimen. Skin removed during radical

vulvectomy was taken as a butterfly shaped excision. The upper edge of this excision was normally several centimetres from the vulval tumour. The same sampling precautions were taken. Such tissue was less satisfactory than myometrium as control tissue as it was squamous epithelium, and was closer to the tumour. Microscopic subepithelial invasion has been described for vulval cancer, making it difficult to decide the minimum distance at which one could be confident that epithelium would be normal. Ferenczy et al (1985) [211] demonstrated the presence of viral genomes up to 15mm from the nearest histological evidence of condylomata and possibly HPV-16 DNA 15mm from one case of VIN 3. Our control tissue was a minimum of 20mm from the nearest histological evidence of tumour.

5.3 Hybridisation techniques

Southern blot hybridisation techniques were complex and time consuming. They still represented the best method for the detection of HPV DNA in human cells. The technique was sensitive, detecting down to 1 genome copies per cell. They allowed positive identification of the viral DNA involved by use of restriction enzyme pattern analysis. Thus any variants would be detectable. They were the only method which could tell whether viral DNA was free or integrated into cellular DNA. However they relied on total tissue DNA. This had two major drawbacks. Firstly, a tumour biopsy will consist of a minority of malignant cells. Viral DNA present in these cells would be diluted by normal cellular DNA. Southern blot hybridisation did not distinguish between 10 cells with 100 copies of viral DNA and 1000 cells with one copy. In histological terms, this type of analysis did not give any information on the type of cells containing viral DNA or their distribution within the tumour. Other techniques have been utilised in the analysis of viral DNA in genital tumours. Filter hybridisation bypasses the tissue digestion and DNA extraction required for Southern blot analysis [354] as cells are disrupted, DNA immobilised and rendered single

stranded on the hybridisation filter. Such a technique could more readily allow analysis of large numbers of samples as could dot blot hybridisation techniques [206,207]. These techniques might not be as sensitive as conventional hybridisation and still relied on total tissue DNA. In addition, problems with cross hybridisation of probe DNA with normal human DNA could arise. HSV DNA contains sequences which readily hybridise with human DNA [87-89] and these regions have been identified. HPV DNA cross hybridises with Alu sequences [364] and it has been suggested that part of the highly conserved E6 region of the HPV-16 and 18 types could be present and transcribed in normal human DNA [365]

In situ hybridisation of tissue sections using biotin-avidin labelling [366], tritiated thymidine [367] or ^{35}S-thionucleoside triphosphates[222] has been applied to genital tissue. This has allowed study of stored tissue and historical comparisons. It has also allowed accurate delineation of the cell type containing viral DNA. This was particularly applicable to the study of pre-malignant tissue where the presence of a limited number of cells with high copy number viral DNA allowed positive hybridisation using these techniques. Biotin-avidin hybridisation has a sensitivity of over 100 copies per cell, ^{35}S-labelling 10-30 copies per cell and tritium labelling 1-3 copies per cell. Such techniques might therefore not be sensitive enough for use in invasive tissue in which most cells contained low copy viral DNA (e.g. SiHa cell line). The application of single stranded RNA probes [237] which are as sensitive as southern blot techniques and allow cellular and intracellular localisation of DNA and RNA could prove valuable.

The recent introduction of polymerase chain reaction techniques [368], which are capable of detection of target DNA sequences at a sensitivity of 10^5 fold that of southern blotting and could therefore be applicable to small samples such as cervical smears, could allow more representative ascertainment of the prevalence of HPV both in neoplastic and in normal tissue. This technique is very sensitive and there are reservations regarding false

positivity [369] especially in view of the known cross-hybridisation with specific HPV DNA sequences [365].

Nevertheless, none of the other techniques offer any information on the pattern of viral DNA within tumour DNA or whether or not viral DNA is integrated into cellular DNA.

5.4 Cross-hybridisation

In the reconstruction experiments, cross-hybridisation between HPV-11 and HPV-16, and between HPV-16 and HPV-18 was noted. This was apparent under non-stringent conditions (as in Figure 7) and was consistent with similar cross-hybridisation described under non-stringent conditions in early studies [195, 196]. Of more concern was the cross-hybridisation noted even under stringent hybridisation conditions (as in Figure 13b for HPV-18).

No tumour DNA hybridised to HPV-11 under stringent or non-stringent conditions, and therefore in terms of the prevalence of particular viral types in tumour tissue, the cross-hybridisation noted might not be of direct relevance. However, both tumour DNAs hybridising to HPV-18 DNA also hybridised to HPV-16. In the case of Tumour 35, given the restriction patterns, it appeared likely that the tumour contained both viral types. Such mixed infections have been described by others in both premalignant and malignant tissue [199, 205]. In the case of tumour 28, it would appear that cross-hybridisation had occurred, the Pst I digestion

THe HPV-18 DNA probe was kindly supplied by Drs zur Hausen and Gissmann. Reconstruction experiments with HPV-18 indicated sensitivities of detection down to 1 genome copy per cell. Under stringent hybridisation conditions, weak cross-hybridisation was observed between HPV-18 and 16. The HPV-18 used did not cross-hybridise with HPV-11. Exposure times to demonstrate the cross-hybridisation with HPV-16 were usually in excess of 10 days. In two instances (e.g.see figure 13b) cross-hybridisation was detected on normal exposure times (2-7 days), and to a more marked degree. Contamination of the probe preparation was excluded as no hybridisation to known HPV-16 containing tumours was demonstrated in these particular experiments. The possibility of inadvertant lower stringency conditions was considered.

CHAPTER 3

CULTURE OF BIOPSY TISSUE FROM INVASIVE CANCERS OF THE LOWER GENITAL TRACT

Section 1 : Introduction

A major problem in the study of the putative role of HPV in genital cancer has been the lack of a suitable in vitro model system in which the expression of the virus can be observed under experimental conditions. Most information on viral organisation, expression and oncogenesis had until recently come from extrapolation of work carried out in bovine papillomavirus.

Viral types 16, 18 and 31, those most frequently associated with malignant genital tumours, could not be maintained in cell culture. Therefore to examine the action of these viral types in human cell systems it was necessary to either morphologically transform cells in vitro or to find a natural model system where HPV genetic information was already present in transformed cells.

Transformation of rodent cells has been achieved using transfection of HPV-16 DNA [163-169]. These cells retain HPV DNA and transcribe RNA. Such cells exhibited some oncogenic properties. As described in chapter 1, transformation of human keratinocytes by HPV-16 was feasible [170-172] and established long term epithelial cultures have been described from transfection of ectocervical cultures by HPV [173]. Such cells did not exhibit the full oncogenic range, and as such might not resemble in any way the situation in vivo.

An alternative approach would be to study those cell lines derived from genital tumours in which viral DNA was identifiable. Under these circumstances viral DNA has been introduced into cells under natural conditions and thus examination of the function of such DNA might be more representative of the true

role of HPV in these cancers. A number of studies examining old and newly established cervical cell lines have been carried out [196,358,370-374], which have identified DNA homologous to HPV-16 or 18 DNA in cell line DNA. The only vulval cell line studied is the A431 line [371] and viral DNA was not detectable. There were a number of important reservations in extrapolating such data to the clinical situation.

The relative proportions of HPV-16 and 18 in established cell lines contrasted strongly with the distribution of these viral types in tumour biopsies (see chapter 1). This dissociation made it difficult to extrapolate findings from these lines to an in vivo situation.

The role of a sexually transmissable factor in the genesis of cervical cancer and to a lesser extent vulval cancer, applied to squamous carcinomas which undergo a premalignant phase. Many of the cell lines studied were poorly classified in histological terms and could be adenocarcinomas or mixed tumours, again raising doubts regarding their usefulness in elucidating the precise nature of the role of HPV in cancer. These older lines have also been extensively passaged and this could result in cell selection or DNA rearrangements far removed from the original tumour.

Two other reservations were impossible to assess in existing cell lines. There was always selection in attempting to obtain continuous cell lines from tumour biopsies and therefore reservation on the ability of a given cell line to reflect the behaviour of tumour cells in vivo. Comparison of the state of viral information in biopsy material and in derived cell lines could allow better utilisation of the cell line. Similarly if the presence of viral information in cells of a derived continuous line was to be regarded as meaningful then it must be clear that such viral information was not present in that patient's normal tissue. These questions have not been addressed to date.

Attempts were therefore made to derive continuous cell lines where DNA analysis of the original tumours was available and

where, in a proportion of cases, normal tissue from the same patient was available as internal control. Conservation of copy number and arrangement of sequences would be strong evidence that such cell lines would be accurate tools for the study of the mechanisms of carcinogenesis in genital malignancy.

Section 2 : Tissue culture methods

2.1 Primary explant culture

Culture of tumour biopsy material was largely carried out using a primary explant technique modified from Vesterinen et al (1981) [375] which had been successfully used in cervical tumour explant culture by Kitchener (1985) [376]

Tissue was obtained from the following sources:-

: hysterectomy specimens from women without malignant disease with a recent history of normal cervical cytology

: cervical tumour biopsies obtained from either punch biopsy, wedge biopsy, or radical hysterectomy for invasive cervical cancer

: vulval tumour biopsies obtained from either excision biopsy simple or radical vulvectomy for invasive vulval cancer.

For normal cervical tissue, strips of ectocervix were dissected aseptically in the operating theatre from hysterectomy specimens. Care was taken to remove as much stromal tissue as possible at the time of the initial dissection. Tissue strips were immediately placed in culture medium, stored on ice and taken to the laboratory. For tumour tissue, areas of viable tumour were aseptically dissected in theatre from the surgical specimen,

taking care to avoid areas of stroma and necrosis. This tissue was similarly placed in culture medium and transported on ice.

Tissue was washed extensively in Hank's Balanced Salt Solution (BSS)at 4°C and minced into 2 to 3mm fragments. Four to six explant fragments were seeded onto 50mm plastic petri dishes and overlayed with 1.5 ml. of medium. Plates were incubated overnight at 37°C in 5% CO_2. The following day a further 3.5ml of medium was added and the plates reincubated. Medium was changed every 4 days and non-adherent explants removed.

2.2 Culture medium

Primary culture was carried out in a 1:1 mixture of Ham's F12 medium and Dulbecco's MEM (Flow laboratories, Irvine, Scotland) supplemented with 20% fetal calf serum (Gibco), 2mM L-glutamine, 100u/ml penicillin, 100µg/ml streptomycin and 10ng/ml epidermal growth factor (Boehringer, Mannheim).

Initial subcultures of some tumour explants were carried out in a 2:1 mixture of fresh medium and spent medium obtained from established, growing primary cultures of normal cervical epithelium. The spent medium was filtered through a 20µ Millipore filter and stored at -20°C. Prior to use each batch was thawed and an aliquot plated onto blood agar. Provided no bacterial or fungal growth ensued in 10 days, then the batch was used in explant culture. All other subcultures were carried out in fresh medium.

2.3 Subculture of explant cultures

When it was felt that there was sufficient explant growth (usually >60% of plate), then attempts were made to disaggregate and subculture the primary culture.

Medium was removed and the cultures washed twice in 0.02% EDTA in phosphate buffered saline (PBS) at room temperature. Cultures were overlayed with 1ml of 0.0625% trypsin in 0.02% EDTA for 5 minutes at room temperature and 10-15 minutes at 37°C. Plates

were directly observed during this time for evidence of disaggregation. Plates were then washed with 1-2 ml. of fresh medium and the plates inspected for completeness of disaggregation. If the majority of the cells were still adherent, the process was repeated. The resultant cell suspension was centrifuged at 2000rpm for 5 minutes at 4°C and the pellet suspended in fresh medium or a mixture of fresh and spent medium. Cell viability after this procedure in normal cells was 65-80% by trypan blue exclusion.

2.4 Management of fibroblast contamination

Fibroblastic contamination was not a major problem in normal cervical cultures, but was a persistent problem in tumour explant culture.

Great care was taken to try to dissect stromal tissue from epithelial or tumour tissue both at the time of the initial dissection and when mincing tissue into small explants. However as tumour tissue was rarely a pure cell type, fibroblasts persistently grew in epithelial cultures.

Isolated fibroblast colonies were removed under direct vision by scraping with a sterile heated Pasteur pipette at regular intervals. If this was insufficient or if the colonies of tumour and fibroblasts were too closely interspersed, then fibroblasts were removed by partial disaggregation. Medium was removed and the plate washed as above. 1.5ml of 0.02% EDTA in PBS was overlayed and the plate incubated at 37°C for 2 minutes. The plate was washed with 2ml of fresh medium. Generally this removed human fibroblasts without disaggregating epithelial tumour cells.

2.5 Culture of cervical and vulval tumours in athymic *nude* outbred mice.

The high failure rate (see results) in establishing primary growth from tumours in explant culture led to an attempt to

establish cell lines following passage of tumour biopsy material through laboratory rodents. This work was carried out in the Department of Experimental Tumour Research, Royal Hospital for Sick Children, Glasgow in collaboration with Dr. Tariq Ali.

Tumour tissue was collected as before and stored in normal medium on ice. The biopsy was then washed extensively in ice cold PBS and minced in a hand grinder. The resultant preparation was passed through a series of needles to gauge size 23 under strict aseptic conditions. The resultant suspension was checked for cell viability by trypan blue exclusion. The cell suspension was centrifuged at 2000rpm for 10 minutes and the cell pellet reconstituted with fresh PBS to give a cell concentration of approximately $1x10^6$ viable cells/ml.

Outbred athymic, *nude* mice were used as the vector for these experiments. One milliliter of cell suspension was injected either subcutaneously in the subscapular region (4 cases) or 1ml of cell suspension was injected intra-peritoneally under light general anaesthesia (1 case). The mice were observed for evidence of tumour growth or metastasis for 16 weeks.

Once the presence of a discrete tumour was noted this was excised under general anaesthesia. The animals were observed further and sacrificed at a later date.

TABLE 5

PRIMARY EXPLANT CULTURE OF CERVICAL CARCINOMA

TUMOUR	HISTOLOGY	AGE	SOURCE	DAYS IN CULTURE	SUBCULTURE	REASON FAILED
1	Poorly diff. sq.	36	RHND	0	No	Technical-incubator CO2
2	Squamous	34	RHND	0	No	Technical-incubator CO2
4a	Squamous	77	Staging biopsy	24	No	Bacterial contamination
4b	Poorly diff. sq.	42	Biopsy pre-radiotherapy	18	Yes	-
5	Squamous	-	RHND	20	No	Bacterial contamination
8	Poorly diff.sq.	44	RHND	0	No	Prior radio-therapy
10	Squamous	31	RHND	0	No	No growth
11	Moderately diff.sq.	33	RHND	0	No	Bacterial contamination
14	Squamous	46	Staging biopsy	0	No	Bacterial contamination
16	Squamous	42	Staging biopsy	41	No	Fibroblastic overgrowth
17	Poorly diff.sq.	32	RHND	34	No	Fibroblastic overgrowth
20	Squamous	32	RHND	0	No	Prior RT
22	Moderate/ poorly diff.sq.	30	RHND	28	Yes	-
23	Well diff.sq.	50	RHND	19	No	Fibroblastic overgrowth
24	Moderately diff. sq.	26	RHND	0	No	Prior radio-therapy
25	Squamous	60	Staging biopsy	0	No	Fibroblastic overgrowth
29	Poorly diff.sq.	35	RHND	36	Yes	-
30	Squamous	25	RHND	25	Yes	-
35	Squamous	37	RHND	19	No	Fibroblastic overgrowth
36	Squamous	36	RHND	0	No	Prior RT
38	Squamous	46	RHND	0	No	Prior RT
39	Papillary adeno-ca.	32	RHND	0	No	Fibroblastic overgrowth
40	Adeno-carcinoma	-	Nude mouse tumour(Tu 39)	0	No	No growth
41	Squamous	-	Nude mouse tumour	0	No	No growth

RHND-Radical Hysterectomy and pelvic lymph node dissection
sq.- squamous; diff.- differentiated; RT - radiotherapy

Section 3 : Results of explant culture

3.1 Primary explant culture of normal cervical ectothelium

Cultures were attempted from eight normal cervices. In all but one, adequate epithelial growth occurred and confluent cultures of squamous keratinocytes were obtained after 21 to 42 days. Generally, two to three explants showed evidence of outgrowth. Fibroblastic contamination was rarely a problem with normal cultures. Cells showed evidence of differentiation and keratinisation. Figure 14 demonstrates primary explant growth of normal epithelium with evidence of stratification.

Attempts were made from all cultures to subculture, but this was not successful in any case.

3.2 Primary explant culture of invasive cervical cancer

Sustained epithelial cell growth was obtained in 10 of 24 invasive cervical carcinomas. Full details of the patients, duration of primary culture, and reason for failed culture are shown in Table 5.

Early epithelial outgrowth occurred at 3-4 days. Figure 15 shows an explant of Tu 35 surrounded by radiating growth of rather pleomorphic tumour cells at around day 15. If there was reasonable growth from more than one explant then epithelial growth from these could coalesce. This is demonstrated in Tu 30 (Figure 16). Ultimately solid monolayers of epitheloid tumour cells may form as shown in Figures 17 and 18.

The problems of fibroblast contamination are demonstrated in Figures 19 and 20. Figure 19 shows a confluent monolayer of cells derived from explants of Tu 30. Epitheloid and fibroblastic cells intermingle. This culture was eventually overwhelmed by fibroblasts despite selective disaggregation. Figure 20, of a

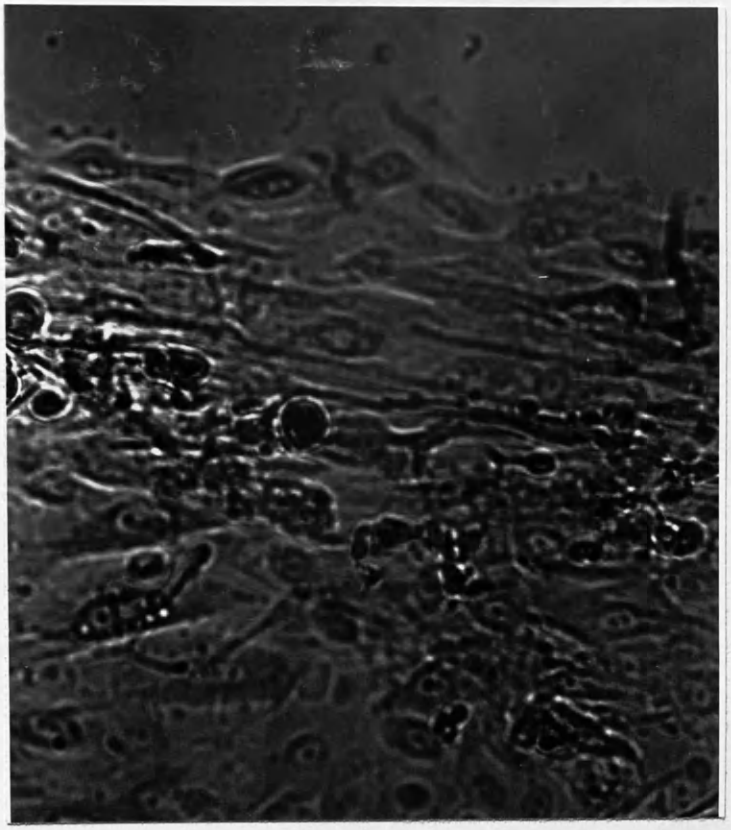


FIGURE 14

High power view of growing edge of primary explant culture of normal cervical ectothelium. Note stratified nature of cultured cells.
(x 40)

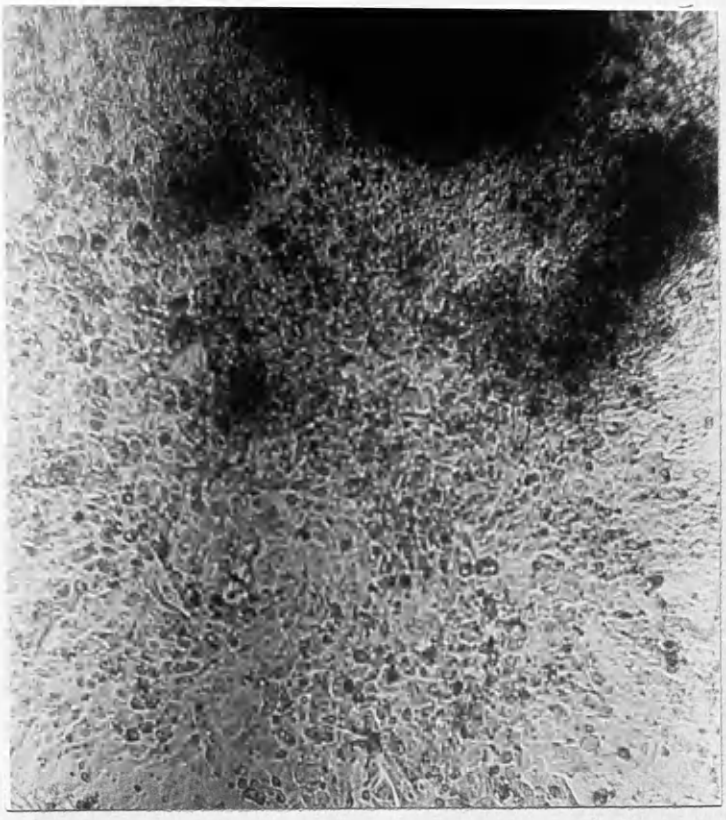


FIGURE 15

Low power view (x 25) of epithelioid cells growing from a primary explant culture of cervical tumour 35, examined at day 15.

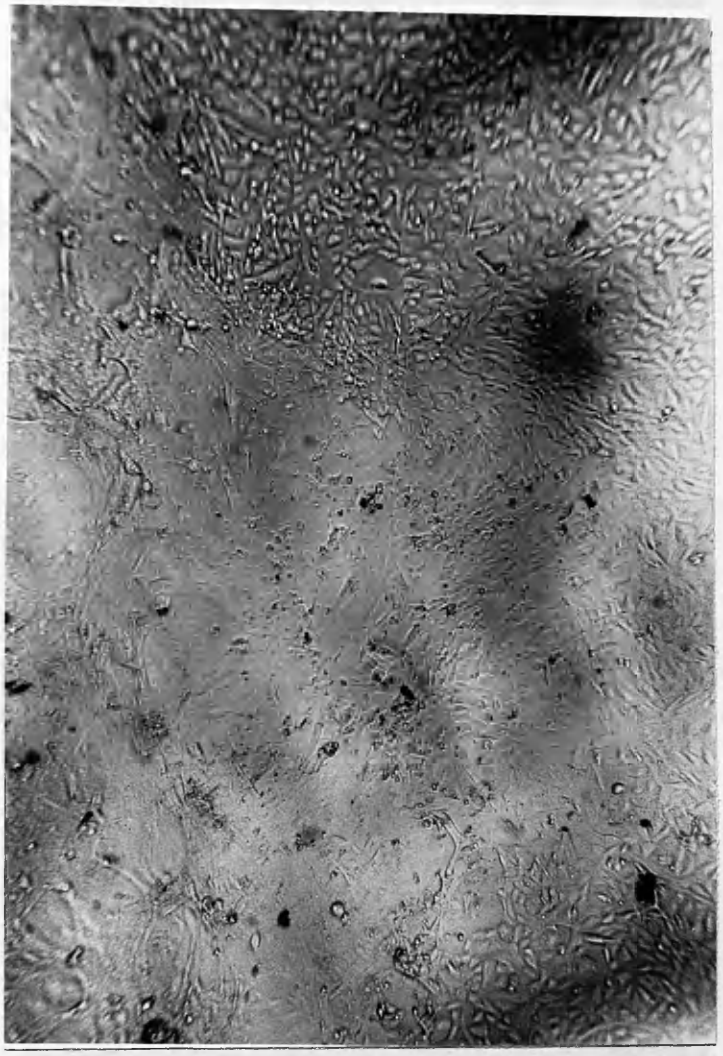


FIGURE 16

View of cells growing from 2 explants of cervical tumour 30 at day 26. Tumour cells from the two explants have almost merged on the right hand edge of the figure.
(x 25)

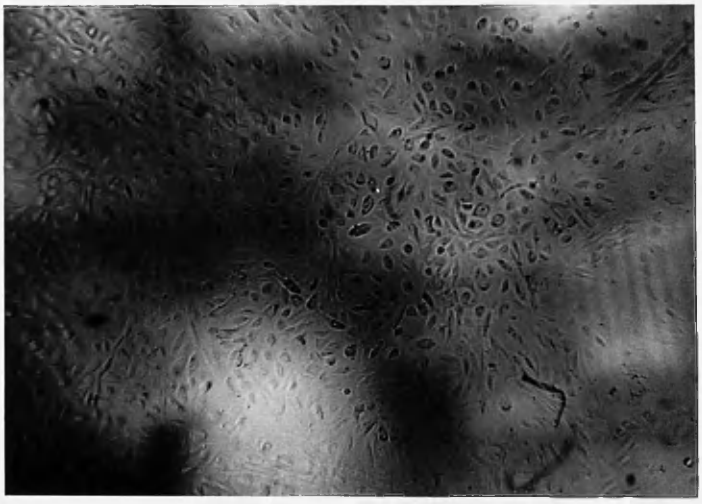


FIGURE 17

Low power view (x 25) of monolayer primary culture derived from explants of cervical tumour 29 prior to disaggregation at day 36.

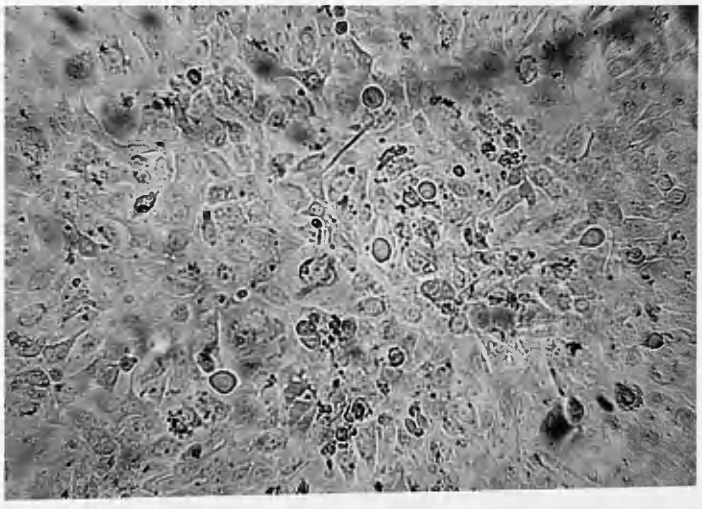


FIGURE 18

High power view (x 40) of primary monolayer culture of cells derived from cervical tumour 30.

FIGURE 19

High power (x 40) view of explant growth from a different plate of tumour 30. There is intermingling of epithelial and fibroblastic cells (at day 22), the culture being eventually overwhelmed by fibroblast contamination.

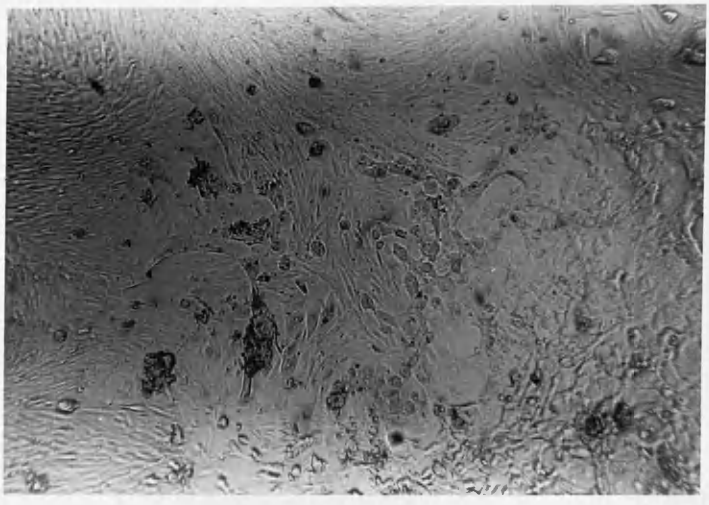


FIGURE 20

View (x 25) of primary culture of tumour 35. The culture is largely fibroblastic. Isolated tumour cells can be seen in the centre of the picture and compression of edge of the epithelial culture is seen on the right.

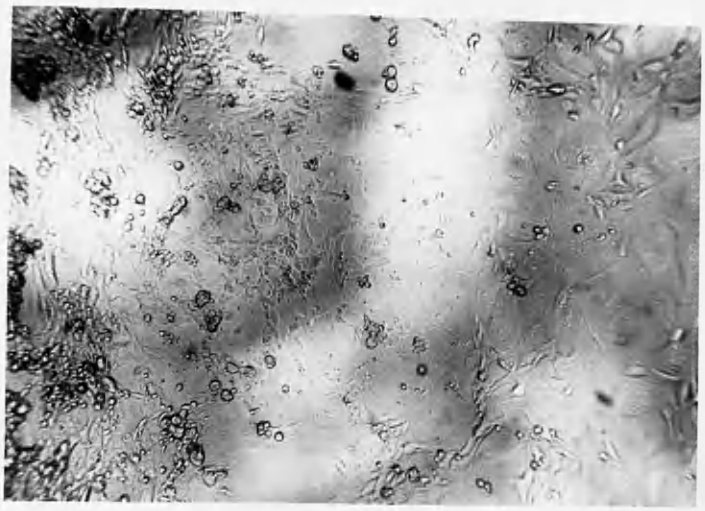


FIGURE 21

Low power view (x 25) of another primary culture of tumour 30. In the growing edge of the epithelial culture on the left there is patchy degeneration of cells.

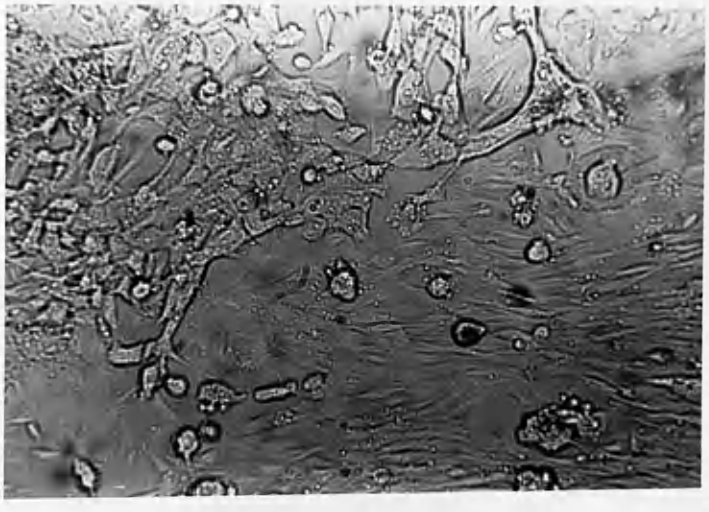


FIGURE 22

View (x 40) of edge of primary explant culture of tumour 35. Fibroblastic overgrowth is seen on the right of the picture. Some cells at the edge of the culture have become elongated with a slightly granular internal appearance.

TABLE 6

PRIMARY EXPLANT CULTURE IN VULVAL TUMOURS

TUMOUR	HISTOLOGY	AGE	SOURCE	DAYS IN CULTURE	SUBCULTURE	REASON FAILED
3	Well diff,sq,	70	Radical vulvectomy	23	No	Fibroblastic overgrowth
9	Moderately well diff,sq,	29	Radical vulvectomy	11	No	Fibroblastic overgrowth
12	Squamous	87	Excision biopsy	0	No	No growth
15	Squamous	66	Radical vulvectomy	29	Yes	-
18	Squamous	92	Simple vulvectomy	0	No	Bacterial contamination
19	Squamous	90	Excision biopsy recurrenc	26	No	Bacterial contamination
21	Squamous	81	Radical vulvectomy	0	No	Bacterial contamination
26	Squamous /VIN 3	77	Excision biopsy	21	No	Fibroblastic overgrowth
27	Well diff,sq,	60	Radical vulvectomy	0	No	Bacterial contamination
28	Carcinoma-in-situ	75	Simple vulvectomy	0	No	No growth
32	Poorly diff,sq,	73	Excision biopsy recurrence	33	No	Cell death
33	Well diff,sq,	82	Excision biopsy recurrence	19	No	Fungal contamination
34	Squamous	76	Radical vulvectomy	0	No	No growth

TABLE 7

PRIMARY EXPLANT CULTURE-OTHER TUMOURS

TUMOUR	HISTOLOGY	AGE	SOURCE	DAYS IN CULTURE	SUBCULTURE	REASON FAILED
6	Adenoca, uterine body	60	T,A,H,	18	No	Cell death
7	Adenoca, uterine body	27	T,A,H,	17	No	Cell death
13	Well diff,sq, vagina	60	Anterior exenteration	0	No	No growth

TAH-Total abdominal hysterectomy

different plate of Tu 35, demonstrates the compression of the growing epithelial edge by fibroblasts.

Uncontrollable fibroblast contamination was responsible for the failure of 4 of 10 primary cultures. Bacterial contamination resulted in the loss of 2 other established primary cultures. Other plates initially grew epithelial outbuds but these failed to continue to grow, ultimately showing patchy disintegration, as in Figure 21 of Tu 30, or elongation and vacuolation, as in Figure 22 of Tu 35.

Fourteen sets of explant cultures failed to exhibit any epithelial growth. Two did not establish due to early bacterial contamination, and 2 failed for technical problems with the CO_2 concentration in the incubator. Two others resulted in a pure fibroblast culture. The remainder did not exhibit growth of any kind despite prolonged culture.

3.3 Primary explant culture of invasive vulval cancer

Sustained epithelial growth was obtained in 7 of 13 vulval invasive cancers. Table 6 shows comparable data on histology and days in culture.

Tumour explants behaved in a similar fashion to cervical explants. Figure 23 shows early epithelial tumour growth from an explant of Tu 33 at day 5. Figure 24 demonstrates continuing epithelial growth from Tu 33. Similar problems with fibroblast contamination and cell death were encountered and are demonstrated in Fig 25, which shows a higher power view containing epithelial tumour cells, fibroblasts and degenerating cells.

Bacterial contamination was a more serious problem in vulval explant culture, being responsible for the failure of culture initiation in 3 instances and failure of growing cultures in 1. Three cultures failed due to uncontrollable fibroblast contamination.

FIGURE 23

Early epithelial outgrowth (day 5) from explant of vulval tumour 33. Growth is largely epithelial but a few fibroblastic cells can be seen.
(x 10)

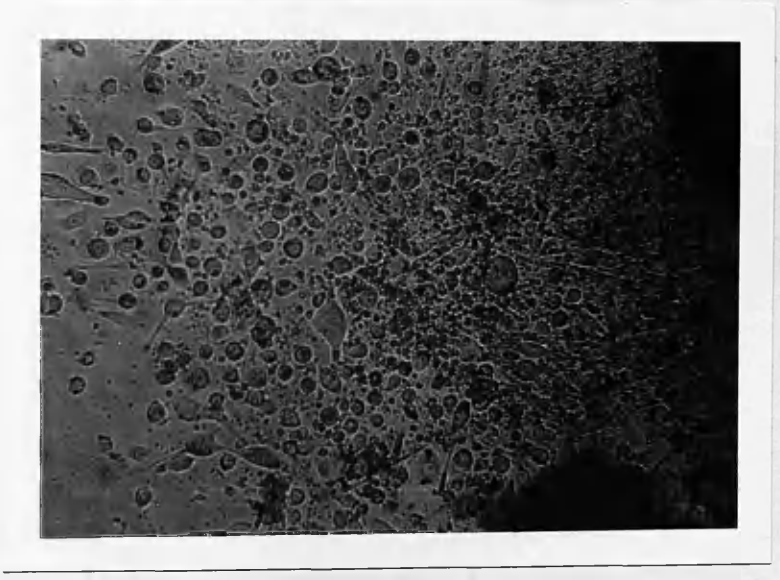


FIGURE 24

Continuing epithelial growth from explant of tumour 33 at day 10. Cells are pleomorphic and fibroblastic elements are more pronounced than in figure 23.
(x 25)

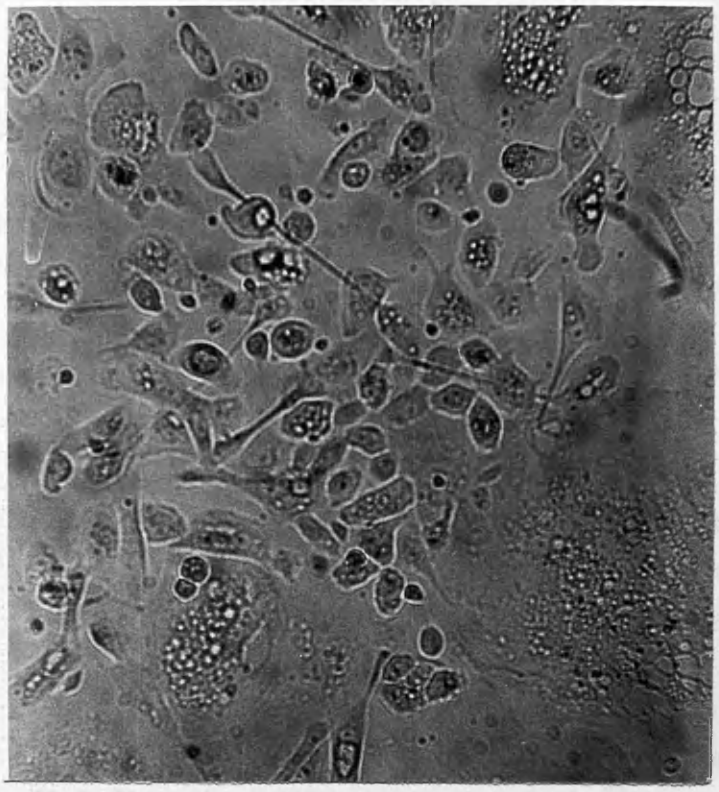


FIGURE 25

High power view (x 40) of cells from primary explant culture of tumour 32. Some cells have degenerated, and some show evidence of intracellular vesiculation which appeared to be a precursor of degeneration.

3.4 Primary explant culture of other tumours

Three other tumours were cultured as shown in Table 7. None was successfully subcultured.

3.5 Subculture of primary explant cultures

Attempted subculture was carried as described in all established primary cultures not grossly overwhelmed by fibroblasts as soon as approximately 50% of the dish contained confluent epithelial cells. This was carried out in 5 instances and 4 are described in detail below. The subculture of Tu 22 will be described in detail in chapter 4.

a. Tu 4b

Two of 3 plates obtained from this squamous carcinoma of the cervix exhibited good epithelial growth by 6 days. By day 15 epithelioid cells covered about 50% of one plate although there were numerous fibroblast colonies. These were removed by scraping and on day 18 the epithelioid cells were disaggregated as described. Cells proved very adherent and 2 treatments were required to remove most cells. The resultant cell suspension was replated into a 50ml flat flask with 10ml fresh medium.

Within 48 hours there was adherence of some pleomorphic cells and a number of fibroblasts. Despite attempts to remove these, the few epithelial colonies were overwhelmed by confluent fibroblastic growth in 96 hours.

b. Tu 29

Fifteen plates were obtained for culture from this poorly differentiated squamous carcinoma of the cervix. After 12 days one plate showed reasonable epithelial growth. By day 29 there were large epithelial colonies interspersed with fibroblasts. These were removed by treatment with EDTA and on day 36 the epithelial cells were disaggregated and re-seeded. Although small

TABLE 8

TUMOUR GROWTH IN ATHYMIC NUDE MICE

TUMOUR	HISTOLOGY	INJECTION SITE	TIME TO EXCISION	IN VITRO CULTURE
27	Well diff. sq. vulva	Intra-peritoneal/ subcutaneous	42 days	Yes
30	Sq.cervix	Subcutaneous	No	No
35	Sq.cervix	Subcutaneous	No	No
39	Adenoca. cervix	Subcutaneous	21 days	No
41	Sq.cervix	Subcutaneous	84 days	No

epithelial colonies did form they were replaced by a pure fibroblast culture by 5 days.

c. Tu 30

Nineteen plates were obtained for culture of this carcinoma. Good epithelial growth was seen in 9 (see Figures 16 and 18). On day 25 a number of large colonies were disaggregated within cloning rings and reseeded into another 50mm dish. Figure 26 shows cell growth at day 12. This plate was subcultured but fibroblast contamination was a major problem requiring repeated treatment and ultimately the culture became contaminated with bacteria.

d. Tu 15

Eight plates were obtained for Tu 15. Good epithelial growth was seen in 7 of these initially but 5 subsequently succumbed to fibroblastic over growth. By day 22, 2 plates had grown to virtual confluence and were disaggregated and reseeded into an 80ml flask with fresh medium. A mixed epithelial and fibroblast culture resulted which, despite treatment with EDTA, persisted until confluence. Subsequent subculture resulted in a predominately fibroblastic growth.

3.6 Growth of tumours in athymic *nude* mice

Five tumours were injected into laboratory rodents as described, 4 by the subcutaneous route and 1 by both subcutaneous and intra-peritoneal routes. The results are summarised in Table 8. Two tumours failed to establish detectable subcutaneous tumours after 16 weeks. Autopsy of the animals showed no evidence of tumour. Two tumours did establish discrete tumours in the animals which were excised. Explant cultures were established as for the primary tumours but there was no evidence of epithelial cell growth after 14 days observation. The tumour Tu 41 was derived from biopsy material from a patient undergoing radiotherapy. Original tumour tissue was not available.

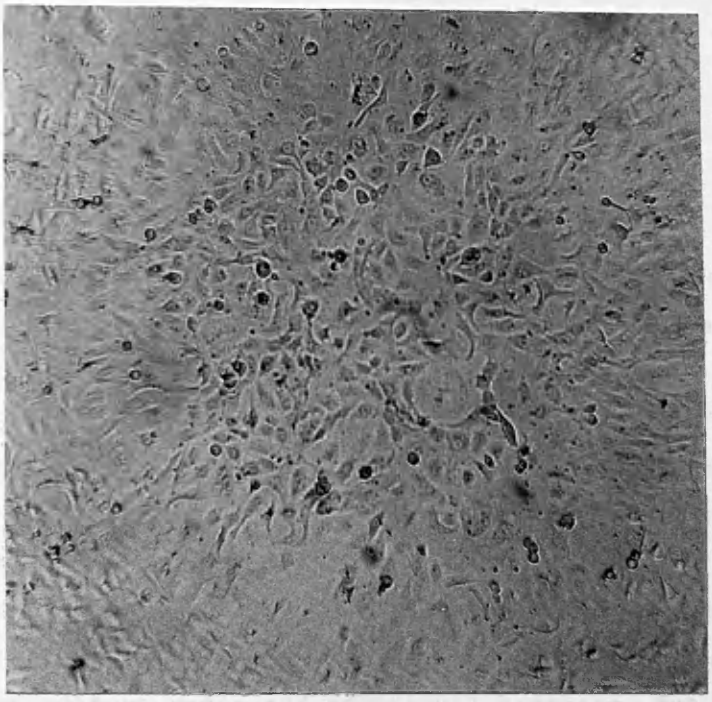


FIGURE 26

Low power view (x 25) of cells of passage 1 subculture derived from cervical tumour 30. Cells have been 12 days in culture.

The tumour Tu 27 did result in successful in vitro culture and this will be described in detail in chapter 4.

Section 4 : Discussion of culture techniques

4.1 Culture techniques

There are many different methods described for culture of human cervical epithelium. The method used here was based on the primary explant technique of Vesterinen et al (1980) [375] and was used successfully in the same laboratory in primary growth of normal cervical explants and cervical carcinoma explants [376] and to develop the cervical carcinoma cell line Cx106 [374]. The technique resulted in good explant growth in almost all normal cervical explants cultured.

The culture medium used was complex, though few additional growth factors were utilised. Wilbanks (1975) [377] utilised numerous combinations of culture media and additional hormones, but concluded that the culture medium made little difference. Addition of oestrogen, progesterone or insulin did not affect growth of normal cells. Others found that high serum concentrations are necessary in primary epithelial culture [375]. Rheinwald and Green (1977) [378] described the effect of epidermal growth factor of cultured keratinocytes. They suggested that this polypeptide growth factor increased the number of cell generations and delayed senescence. Subculture of normal cells was enhanced. Stanley and Parkinson (1979) [379] found that EGF increased colony size in cervical epithelial cultures. The basic medium was therefore supplemented with EGF. Hydrocortisone has also been implicated as a necessary growth factor in cervical cell culture [375,379] but experiments with normal explant culture showed no difference in the time to confluence over a 50mm culture plate with or without hydrocortisone. I was unable to subculture normal

cervical ectothelial cells under any conditions, including cultures carried out with added hydrocortisone. This was therefore not added to the culture medium for growth of tumour explants.

Feeder fibroblast layers have been suggested as essential for culture and passage of normal cervical cells [379] but Vesterinen's method [375] demonstrated that culture and subculture were achievable without this refinement. Feeder layers were not used in these cultures.

In a number of tumour explant cultures, including that of the successful Tu 22 culture, conditioned medium from normally growing normal ectocervical explants was added. It is assumed that such conditioned medium contains small growth factors and hormones specific to squamous epithelium and that this might enhance tumour cell growth.

4.2 Success rate of explant culture

Existing cervical cell lines have been achieved using a range of culture techniques and culture media [374,380-391]. Essentially the culture methods fall into two main groups, explant technique [374,383,386,387] or minced tissue/disaggregation technique [381,382,388,390,391]. All have resulted in successful cell lines. Few data were available on the relative success rates of the different methods or what might influence this. Friedl et al (1970) [383], in establishing the SiHa cell line, stated that this was the only success in 10 attempts, although epithelial outgrowth was noted in 4 other explant cultures. Fjelde (1955) [392] demonstrated good growth in primary culture in 8 of 36 epidermoid cancers and 10 of 40 squamous cancers, although only one established cell line resulted from these cultures. Giard et al (1973) [386] established 7 cell lines from 116 carcinomas. In the same laboratory in which these studies were performed, Kitchener (1985) [376] established one cervical line from 56 primary explant cultures. Using more complex disaggregation, media supplements and

feeder layers, Kelland et al (1987) [391] established 4 cell lines from 15 cervical biopsies.

Primary cervical culture was attempted in 24 biopsies and primary growth was established in 10, a success rate of 42%. Growth was adequate enough for attempted subculture in only 4 of these. Two related cell lines (see chapter 4) resulted from these cultures.

The reasons for growth failure were outlined in the tables. Essentially, no tumour which underwent pre-operative radiotherapy established primary growth in explant culture. Other reasons for failure were either infection or fibroblast overgrowth. Despite careful dissection and aggressive removal of fibroblastic growth by scraping or disaggregation, this remained the major limiting factor in attempts to establish subcultured tumour cells. Similar limitations are described by others [376,391].

A standard antibiotic combination was used throughout. Other antibiotics, in particular cephalosporins, were used in attempts to clear infection from some plates, as were antifungal preparations such as amphotericin; all unsuccessfully. More aggressive prophylactic additives, such as aminoglycoside antibiotics, might have been useful, particularly in cultures of vulval tumours (see below).

4.3 Vulval tumour culture

The difficulty in establishing vulval tumour cells in tissue culture is exemplified by the relative sparsity of established cell lines. Only 3 have been described [386,393,394]. The line LT-2 was established from a nodal metastasis from a vulval squamous carcinoma [394]. The line A431 [386] was established by explant culture of a primary vulval epidermoid tumour. Details of the other line, SW-962, described by Fogh et al (1977) [393], are not given.

Primary vulval tumours were often gross exophytic growths, more often than not infected. It was very difficult to establish primary growth in vulval squamous tumours and infection played a larger role in these cultures than in cervical cultures. This

difficulty led us to collaborate with the existing experimental tumour group in the Department of Child Health. They had considerable experience in mouse xenografts with a range of rare childhood solid tumours. This was more successful, in terms of primary growth, but the excised tumours had similar problems with fibroblast contamination. Only the tumour injected intraperitoneally resulted in an established cell line.

CHAPTER 4

DEVELOPMENT AND CHARACTERISATION OF CONTINUOUS CELL LINES DERIVED FROM INVASIVE TUMOURS OF THE CERVIX AND VULVA

Section 1 : Introduction

The rationale behind the attempts to derive fresh cell lines, rather than rely on existing lines was described in the introduction to chapter 3.

Given the reservations regarding extrapolation of results from old established cell lines to in vivo tumours, it was felt essential to derive cell lines where the state of viral DNA in both tumour and internally matched normal tissue was known. With this information, such cell lines could offer a unique model system for the examination of viral-tumour cell interactions.

Section 2 : Establishment of continuous cell lines.

2.1 Establishment of cell lines Tu 22-1 and Tu 22-2, derived from a squamous carcinoma of the uterine cervix.

A 30 year old nulliparous woman was referred to gynaecological outpatients following the finding of a clinically suspicious cervix at routine cervical cytological screening. Malignancy was suspected at the outpatient visit and arrangements were made for examination and biopsy. A wedge biopsy of the cervix from a clinical stage 1 invasive carcinoma demonstrated moderately to poorly differentiated squamous carcinoma of the cervix (Fig. 27). She underwent radical hysterectomy and pelvic lymph node

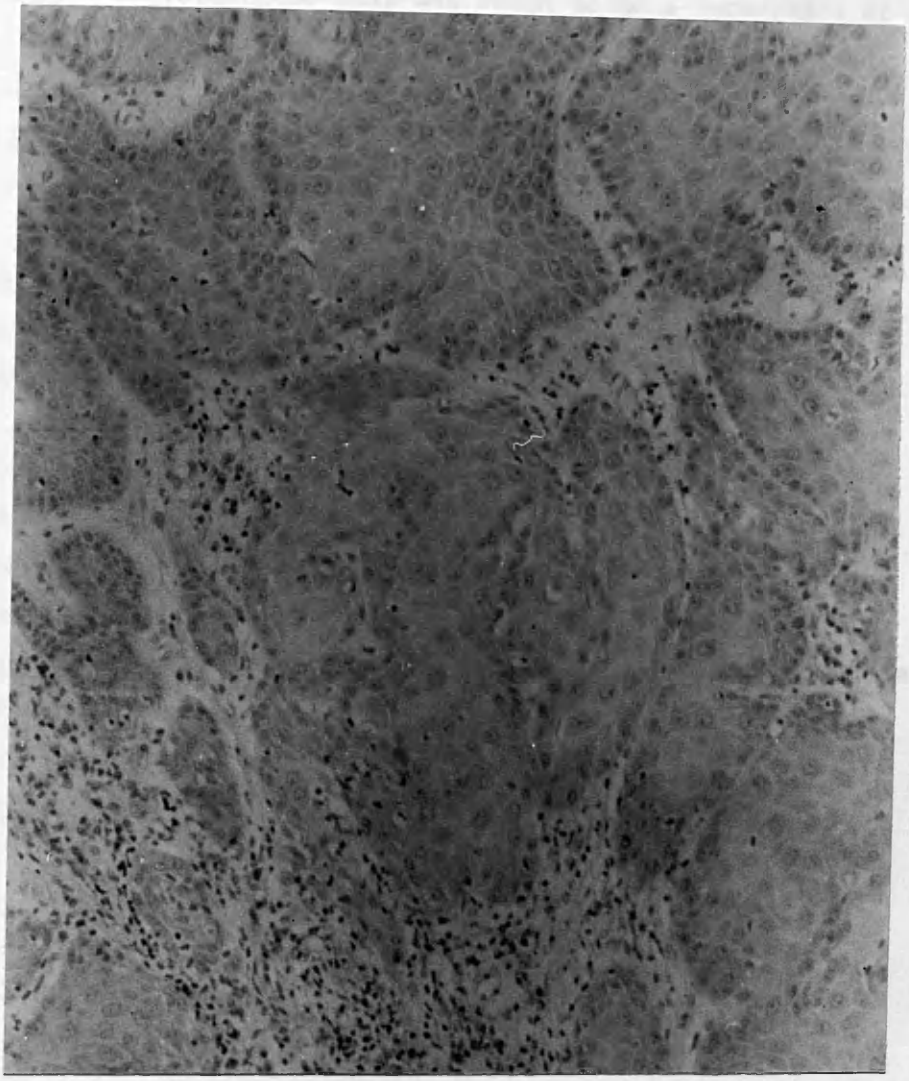


FIGURE 27

View showing histology of tumour 22. This is a moderate to poorly differentiated squamous carcinoma; there being little evidence of cell differentiation or keratin formation.
(x 50)

dissection with conservation of both ovaries on 29th April 1983. Following surgery she remained well but subsequently developed a pelvic mass. At laparotomy this was found to be a recurrence of tumour in her right ovary. Both ovaries were removed and she underwent a course of radiotherapy [395].

A segment of viable tumour tissue was dissected from the surgical specimen in theatre and placed in normal medium on ice. One piece of this biopsy was processed for DNA extraction and the remainder utilised for culture. A section of myometrial tissue was removed from the uterine fundus and snap frozen in liquid nitrogen as described in chapter 2.

Twenty plates were initially set up from this tumour, the remainder of the biopsy being utilised for DNA extraction. The medium used for these plates was a 2:1 mixture of fresh and spent medium (as described in chapter 3, section 2.2). By day 14 most plates had been overwhelmed by fibroblast overgrowth but 2 plates showed good epithelial cell growth. At day 29, cells on one plate were disaggregated and seeded into an 80 ml. flask containing fresh 2:1 medium mixture. The cells were very difficult to disaggregate and fresh medium was added to the initial plate. After a further 4 days the cells on this plate were subcultured.

Serial subculture of the first disaggregation indicated that an increasing proportion of the cells were fibroblastic and by passage 4 the majority were fibroblasts. In contrast, the cells from the second disaggregation remained epithelial in appearance. These cells were serially subcultured successfully in normal fresh medium and represent the continuous cell line Tu 22-1.

From passage 6 attempts were made with random flasks from each passage to grow cells in medium lacking EGF. One flask at passage 9 demonstrated normal growth of cells in the absence of EGF. This flask was serially subcultured in the absence of EGF and these cells are the subline Tu 22-2. Further attempts to subculture cells without EGF at later passage failed.

Section 2.2 : Establishment of the cell line Tu 31, derived from a squamous carcinoma of the vulva.

A 60 year old woman presented with an obvious vulval tumour and underwent wedge biopsy. This demonstrated a well differentiated squamous carcinoma of the vulva (Figure 28). She underwent radical vulvectomy with inguinal node dissection. Nodes were negative for tumour and she recovered well. She remained well until 9 months later when she developed a lesion on her right forearm. This was excised and proved to be squamous carcinoma indistinguishable from the parent vulval tumour. She developed recurrent disease and died from secondary groin recurrences which ulcerated through to the femoral vessels 12 months after primary surgery.

A large biopsy was removed from the vulvectomy specimen in the operating theatre and placed in culture medium. A strip of skin from the distal superior abdominal edge of the specimen was excised and snap frozen in liquid nitrogen.

Explant culture of the tumour was prepared in the standard way. All plates were grossly infected within 48 hours with Pseudomonas aeruginosa. The patient had problems with suture line breakdown also associated with Pseudomonas infection.

In vivo culture in athymic *nude* mice was attempted in this case in parallel with explant culture. A cell suspension was prepared from a small biopsy as previously described. Cell viability prior to injection was 60%. One millilitre of this suspension was injected intraperitoneally into an anaesthetised athymic *nude* mouse. The animal remained well for 4 weeks with no evidence of tumour formation, but thereafter developed increasing abdominal ascites. At 6 weeks, under general anaesthetic, a peritoneal lavage was performed with a 19 gauge needle using PBS at 37°C. The ascitic fluid obtained was centrifuged at 3000rpm for 10 minutes and the cell pellet resuspended in fresh culture medium. The total cell count was 1.8×10^6 viable cells and these were seeded at high density (4.5×10^5 cells per 50mm dish) in fresh medium.

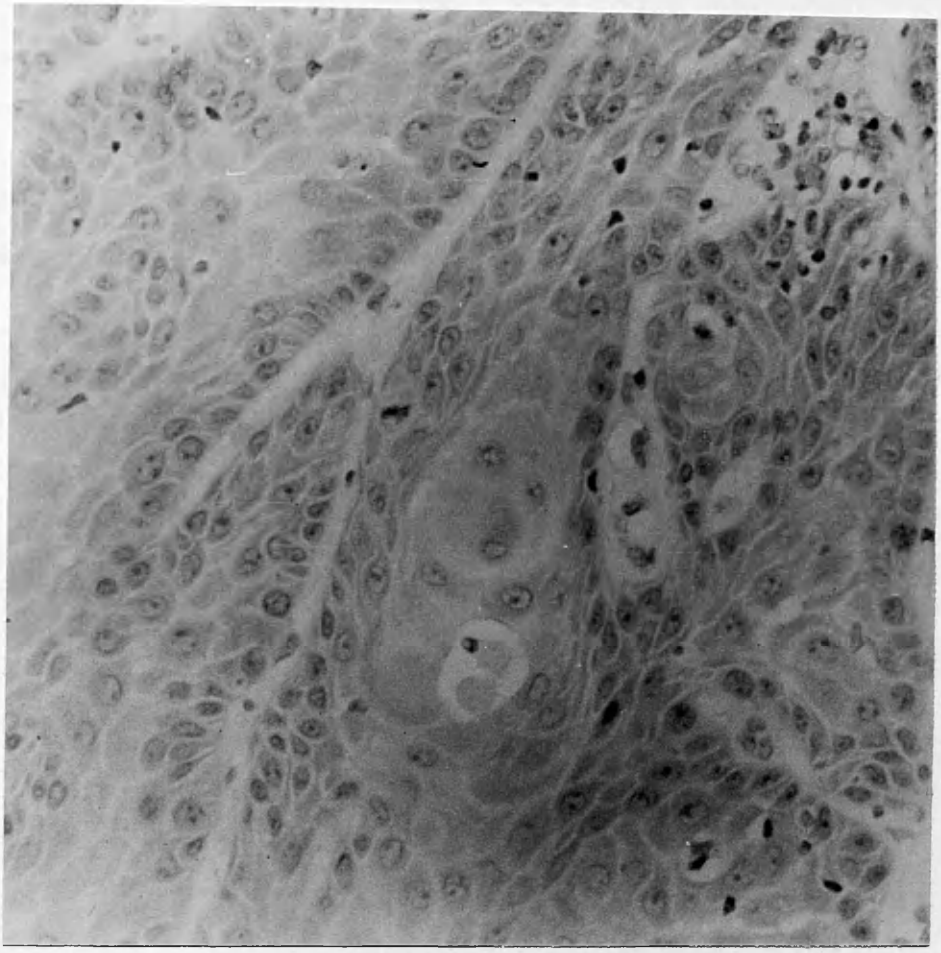


FIGURE 28

Histology of vulval tumour 27. This shows a well differentiated squamous carcinoma with evidence of keratin 'nests' (as seen centrally in this picture).
(x 50)

Confluent monolayers of apparent epithelial cells developed within 48 hours. Cells were subcultured, but by passage 3 it appeared that the predominant cell type was fibroblastic. A large number of highly pleomorphic viable cells were noted to be present in suspension. Pooled medium from all passage 4 flasks was centrifuged and the cell pellet resuspended in fresh medium without EGF. Numerous small colonies developed, becoming confluent in 14 days. Successful subculture of these cells resulted in the continuous vulval cell line Tu 31.

Section 3 : Methods for characterisation of cell lines

3.1 Storage of cells.

Subconfluent monolayers of cells were harvested as previously described. Cells were pelleted by centrifugation at 3000rpm for 10 minutes at 4°C and resuspended in fresh medium with 10% fetal calf serum and 10% glycerol. Cells were aliquoted into sterile plastic 1ml vials, placed at -20°C for 6 hours, and then transferred to storage in liquid nitrogen. Forty-eight hours after any single batch was frozen, one vial was gently thawed, 5ml fresh normal medium added and the cells replated to check that growth characteristics and viability remained normal.

3.2 Cell morphology

Subconfluent and confluent cell monolayers were grown on glass coverslips. After removal of the medium cells were fixed and stained with haematoxylin and eosin or with Masson stain. Cells were examined by standard light microscopy. Cells were also examined directly in growing cultures following removal of most of the culture medium.

Ultrastructural analysis was also carried out on cells grown on coverslips. Thereafter cells were fixed in 2% gluteraldehyde and osmic acid, dehydrated in methanol and embedded in Epon-Araldite.

Thin sections were cut and stained with uranyl acetate and lead citrate.

3.3 Growth kinetics

Cells at varying passage numbers were disaggregated, counted and cell viability checked by trypan blue exclusion. Cell suspensions were prepared and equal numbers of viable cells seeded into fresh medium in 50 mm plates. The experiment was carried out in quadruplicate. At each 24 hour time point, the cells were harvested and counted. The number of cell doublings was calculated from the cell counts at 2 time points a and b as follows :

$$D \text{ (doubling time)} = \frac{b-a \text{ (hours)}}{\log_2(\text{count at time b}) - \log_2(\text{count at time a})}.$$

The doubling times quoted in these experiments were an average of 2 separate values (i.e.using 2 sets of time points within the same experiment).

3.4 Anchorage independent growth

2 ml. of single cell suspension containing $5x10^4$, $1x10^5$, $2x10^5$ and $5x10^5$ cells/ml in complete medium containing 0.3% agar (Difco, Noble) were plated over 2 ml. complete medium containing 0.6% agar. Plates were incubated at 37°C in 5% CO_2 and 0.5 ml. of fresh medium added at 3 day intervals. Plates were inspected regularly for the presence of discrete colonies.

3.5 Tumorigenicity in athymic *nude* mice

Single cell suspensions of 1 x 10^7 viable cells/ml were prepared. 100µl of this suspension was injected into the subscapular area of oubred athymic nude mice (by Dr.Raza as described in chapter 3). At least three attempts from three different late passage numbers were undertaken for each cell line. Animals were inspected for evidence of discrete tumour growth or metastatic tumour over a 16 week period. The animals were then sacrificed and examined for evidence of tumour. Discrete tumours were excised under general anaesthesia.

3.6 Cytogenetic analysis

Subconfluent monolayers in the logarithmic growth phase were incubated with Colcemid solution at 37°C for 2 hours. Stock solution was N-deacetyl-N-methylcolchicine 100mg, 95% ethanol (5.3g), 10g propylene glycol in phosphate buffer made up to 100 ml with distilled water. Cells were incubated in a 1 in 25 dilution using Hank's BSS. Cells were disaggregated, pelleted by centrifugation and resuspended in 75mM KCl solution for 25 minutes. Cells were fixed by the addition of absolute methanol/glacial acetic acid (3:1). Slide preparations were made by droplet technique and air drying. Giemsa-trypsin banding of chromosomes preparations was carried out using the technique of Seabright (1971) [396]. Essentially the slides were passed through sequential solutions of 0.15M NaCl, 0.15M NaCl containing crude trypsin solution, NaCl, Giemsa stain in phosphate buffer and distilled water. Slides were air dried and then examined.

3.7 Assays for oncofetal products and steroid receptors.

Medium from subconfluent monolayers was assayed for the presence of a number of known oncofetal antigens; namely, α-fetoprotein (AFP), β-sub-unit of human chorionic gonadotrophin (β-HCG), carcino-embryonic antigen (CEA), human placental lactogen (HPL), and Schwangerschaftsprotein 1 (SP-1). Aliquots of medium from subconfluent monolayers were assayed using standard radio-immunoassay techniques in the routine laboratories performing these assays for clinical use. The levels of sensitivity for the assays were:- β-HCG 3u/l, CEA 10u/l, SP-1 0.3ng/ml, and HPL 0.05g/l. Immediately after removal of the aliquots for measurement, the medium was removed and measured, the cells harvested and counted. The results were expressed per million cells.

Oestrogen and progesterone receptors were assayed by the technique of Leake et al [397]. Briefly, cells were homogenised in Hepes buffer, EDTA and 0.25mM DTT pH 7.4 and the cell pellet extracted by centrifugation. The cell pellet was washed in NaCl and hepes buffer. Aliquots were incubated for 18 hours at 4°C with

^{3}H 17β-oestradiol (concentrations 1,1.5,2,4,6,8,10x10^{-10}M). Bound steroid in the cytosol fraction was calculated following addition of EDTA, hepes, dextran coated charcoal and Triton-toluene scintillant and counted at 25-30% efficiency in a liquid scintillation counter. The nuclear bound steroid was calculated following washing with normal saline and addition of toluene-PPO (5g/l) scintillant counted at 35% efficiency. Scatchard plots using at least 5 points were constructed and results compared with a known standard established by competition with known amounts of di-ethyl stiboestrol.

Approximately 10^{6} cells were required for each assay. The oestrogen receptor contents of both the cytosol and pellet fractions were determined.

Section 4 : Characterisation of the continuous cell lines Tu 22-1, Tu 22-2, and Tu 31

4.1 Storage of cells

Cells from all cell lines were frozen at regular intervals from passage 4 onwards. Successful cultures have been achieved from all batches of frozen cells.

4.2 Cell morphology

Tu 22-1 and 22-2

Cells of both lines grew to confluence to give pavement-like monolayers (Figure 29). There was no contact inhibition and cells displayed considerable overlapping and layering if culture was continued beyond confluence.

Cells of the two cervical cell lines were not distinguishable by light microscopy. Figure 30a shows direct photography of growing Tu 22-1 cells to demonstrate their regular epithelial appearance. Figure 30b shows cells of Tu 22-2, fixed in situ. Figure 31, a high power view of Tu 22-1 cells, shows large, regular epithelial cells with pleomorphic nuclei containing numerous prominent nucleoli. The cytoplasm contained many organelles and mitochodria.

Transmission electron microscopy demonstrated the presence of tight desmosomes (Figure 32a) and intracellular tonofilaments and microvilli (Figure 32b) characteristic of squamous cells in both cell lines.

Cells have been in continuous culture for up to 15 months and have been passaged in excess of 50 times.

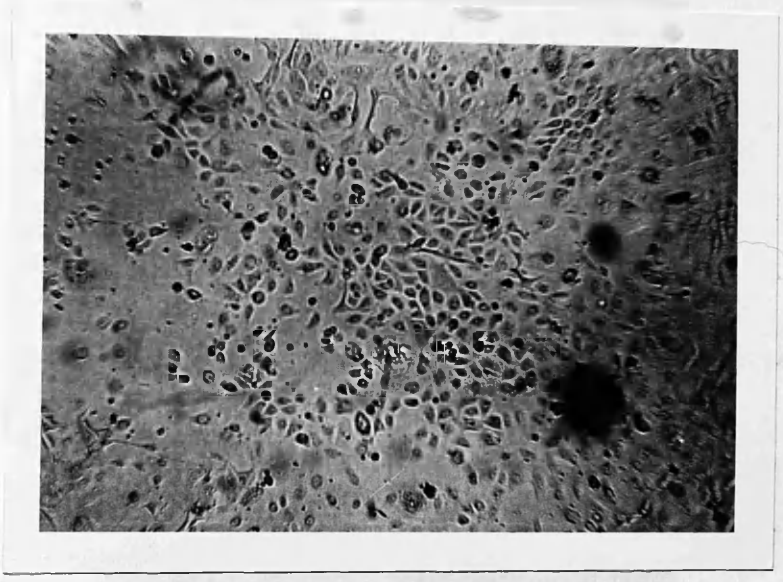


FIGURE 29

Low power view (x 25) of subconfluent monolayer of Tu 22-1 cells, passage 24, in culture showing 'pavement like' epithelial culture.

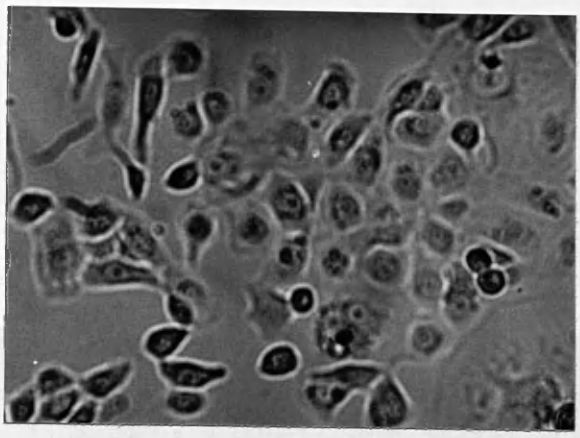


FIGURE 30a

Culture of growing Tu 22-1 cells, passage 34, showing typical epithelioid cells.
(x 40)

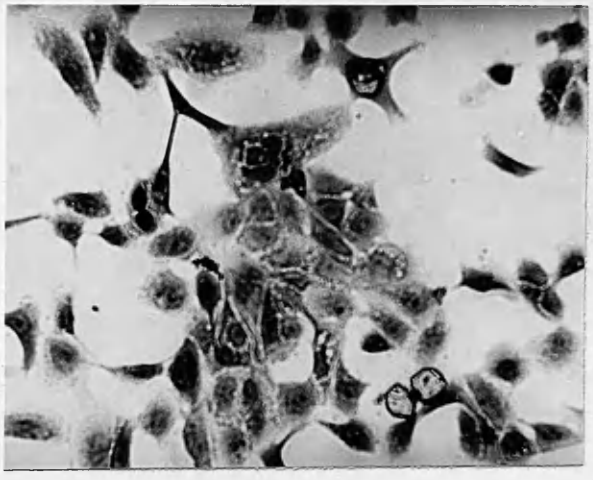


FIGURE 30b

View of Tu 22-2 cells, passage 38, fixed in situ, showing typical epithelioid cells of this line.
(x 40)

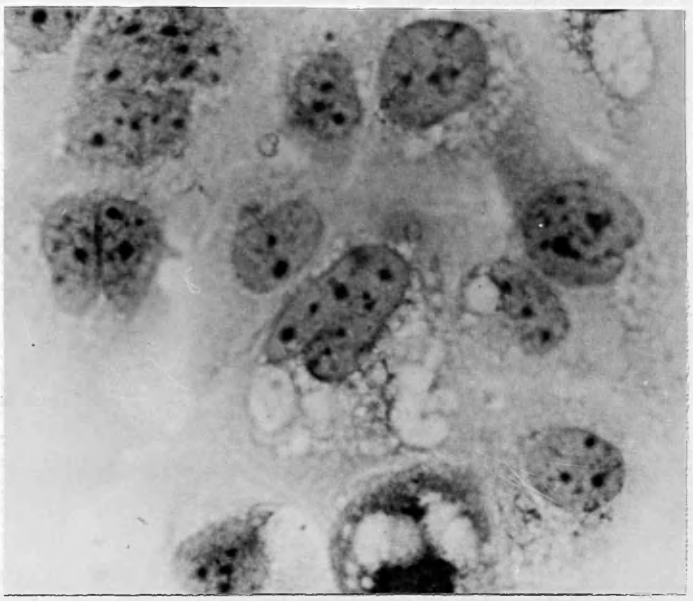


FIGURE 31

View of Tu 22-1 cells, passage 12, showing highly pleomorphic cells with multiple nucleoli.
(x 160)

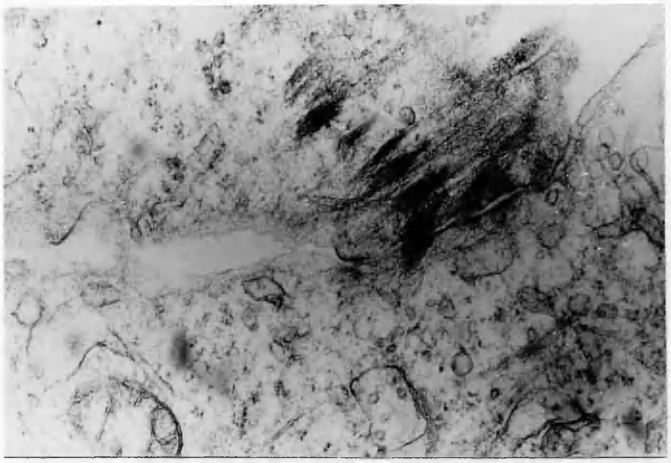


FIGURE 32a

Electron micrograph of Tu 22-1 cells, passage 16, showing tight desmosome junction between two cells.
(x 12400)

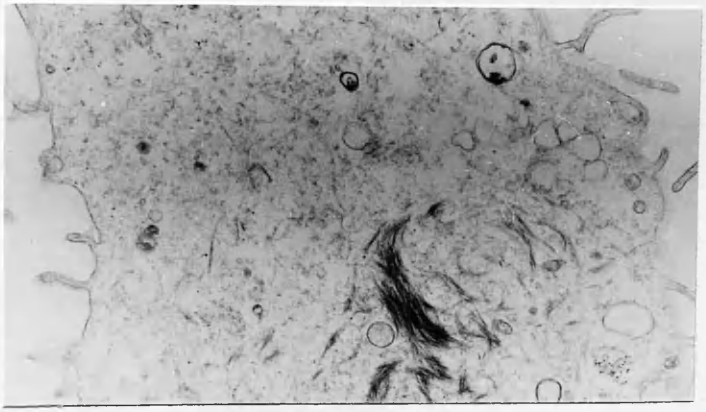


FIGURE 32b

Electron micrographs of Tu 22-1 cells, passage 16, showing intracellular tonofilaments and microvilli.
(x 12400)

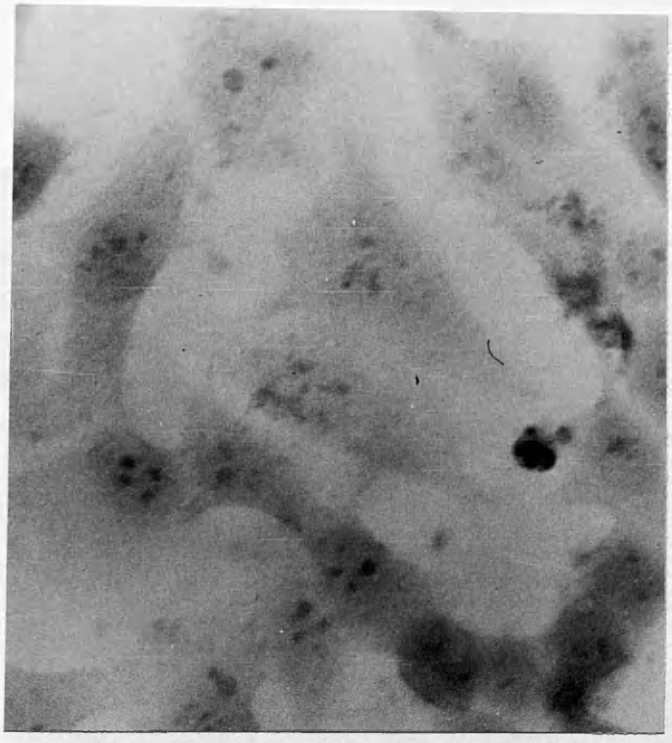


FIGURE 33

High power view of early passage (passage 6) Tu 31 cells. Cells are fibroepithelial at this stage.
(x 100)

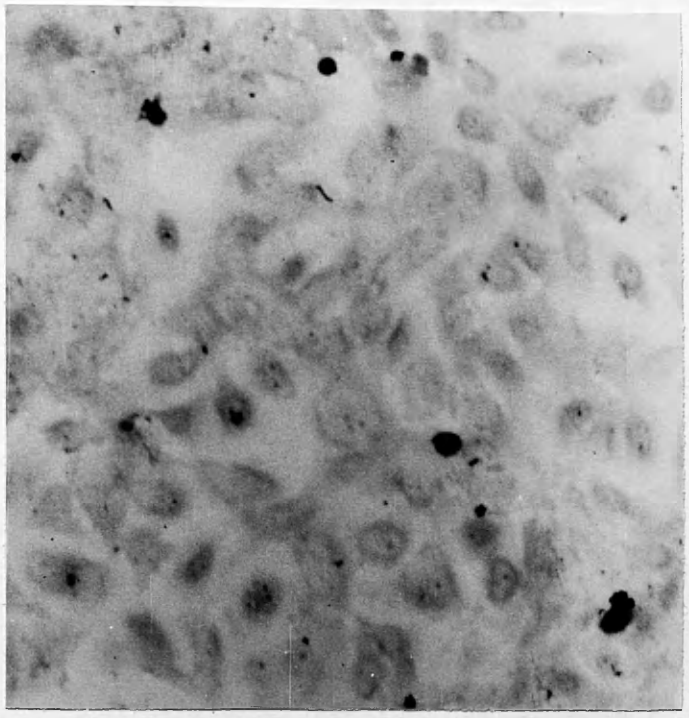


FIGURE 34

High power view of late passage (passage 46) Tu 31 cells showing now epithelial morphology.
(x 40)

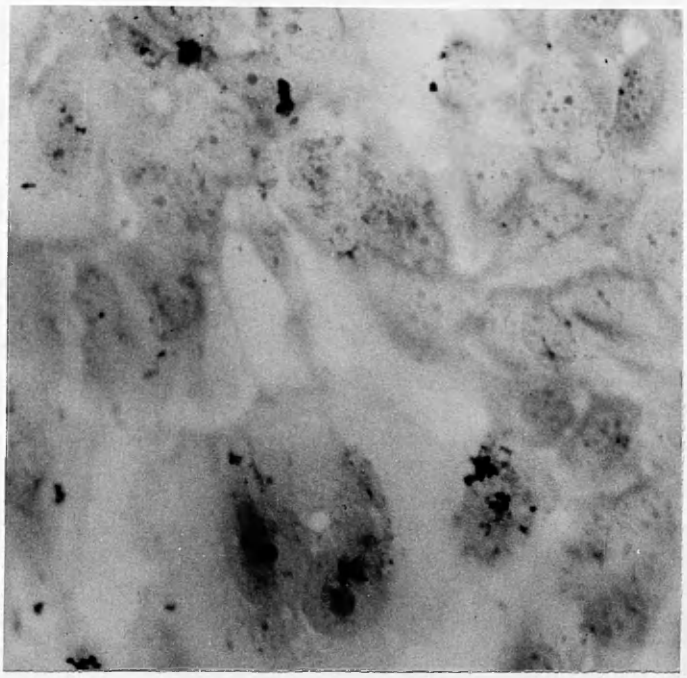


FIGURE 35

Large pleomorphic cells of Tu 31, passage 34. Multiple nucleoli are seen.
(x 100)

TABLE 9

GROWTH KINETICS OF TU 22 CELLS

TIME (HRS)	VIABLE CELL COUNT (S.D.) TU 22-1		TU 22-2		
	Normal medium	10% serum	Normal medium	10% serum	EGF+
0	3.76×10^5	4.00×10^5	4.00×10^5	4.00×10^5	4.00×10^5
24	4.37×10^5 (0.32)	3.33×10^5 (0.28)	2.72×10^5 (0.18)	1.84×10^5 (0.20)	2.12×10^5
48	6.4×10^5 (0.58)	4.31×10^5 (0.36)	2.47×10^5 (0.17)	2.78×10^5 (0.25)	3.25×105
72	1.09×10^6 (0.06)	9.11×10^5 (0.87)	4.66×10^5 (0.38)	3.69×10^5 (0.40)	2.10×10^5
96	1.66×10^6 (0.07)	1.35×10^6 (0.13)	7.08×10^5 (0.61)	6.30×10^5 (0.56)	3.99×10^5
120	-	-	1.19×10^5 (0.08)	8.40×10^5 (0.73)	7.09×10^5
144	1.60×10^6 (0.12)	1.21×10^6 (0.09)	-	1.16×10^6 (0.09)	-

TABLE 10

GROWTH KINETICS OF TU 22-1 CELLS IN EGF DEFICIENT MEDIUM

TIME(HRS)	VIABLE CELL COUNT (MEAN OF 2 COUNTS) PASSAGE 1	PASSAGE 2	PASSAGE 3
0	4.00×10^5	1.50×10^5	2.00×10^5
24	2.19×10^5	1.78×10^5	1.80×10^5
48	1.35×10^5	1.95×10^5	1.75×10^5
72	2.10×10^5	2.02×10^5	1.80×10^5
96	3.99×10^5	3.17×10^5	1.81×10^5
120	5.88×10^5	3.33×10^5	1.76×10^5
144	6.86×10^5	-	1.78×10^5

TABLE 11

GROWTH KINETICS OF TU 31 CELLS

TIME (HRS)	VIABLE CELL COUNT NORMAL (S.D.)	10% serum*	5% serum*	2% serum*
0	1×10^5	5×10^5	2.0×10^5	2.0×10^5
12	$1.1(0.08) \times 10^5$	4.7×10^5	-	
24	$1.29(0.11) \times 10^5$	5.03×10^5	1.8×10^5	1.32×10^5
48	$3.03(0.31) \times 10^5$	5.75×10^5	3.4×10^5	4.28×10^5
72	$6.42(0.60) \times 10^5$	1.24×10^6	4.74×10^5	1.02×10^6
96	$9.75(0.89) \times 10^5$	3.04×10^6	1.94×10^6	1.28×10^6
120	-	-	1.28×10^6	2.81×10^6

* mean of 2 counts

Tu 31

In contrast to the cervical cell lines, early passage Tu 31 cells retained a slightly fibro-epithelial appearance on direct microscopy (Fig. 33). Later passages however formed monolayers of large ovoid cells (Fig 34), seen in Figure 35 with large pleomorphic nuclei with many nucleoli and intracellular organelles. Cells left at confluence detached rapidly from the surface. Transmission electron microscopy showed highly active cells but no evidence of desmosome formation or intracellular tonofilaments as seen in the cervical cell lines.

The cells have been in continuous culture for up to 9 months and passaged in excess of 50 times.

4.3 Growth kinetics

Subconfluent monolayers of Tu 22-1 passage 20 and 33, and Tu 22-2 passage 32 were used to establish the growth kinetics of the 2 cervical cell lines. To observe the effect of EGF deficiency on Tu 22-1 cells, cells from this line were serially passaged 3 times in the absence of EGF with cells harvested at 24 hour intervals. This experiment was performed in duplicate. Growth of Tu 22-2 in EGF medium and the growth characteristics of both lines in medium containing only 10% fetal calf serum were also studied.

The results are shown in Tables 9 and 10. Tu 22-1 cells had a doubling time in the logarithmic growth phase of 35.8 hours. When grown in EGF deficient medium, these cells exhibited a lag phase of 48 hours followed by a doubling rate of 31.5 hours. At second passage cells had a similar lag phase before dividing very slowly with a doubling time of 97.8 hours. On a subsequent passage the cells failed to divide, eventually ballooning and detaching from the plate surface. Later attempts to achieve EGF independence of the Tu 22-1 line followed this pattern with apparently normal growth at the first passage followed by slow growth and ultimate cell death on the third passage. In 10%

fetal calf serum, cells had a doubling time similar to that of growth in standard medium, 31.2 hours.

Tu 22-2 cells had a doubling time of 33.5 hours following a 48 hour lag phase in normal medium lacking EGF. In the presence of EGF there was a longer lag phase of 72 hours followed by logarithmic division and a doubling time of 27.3 hours. Growth in 10% fetal calf serum and EGF deficiency was impaired, with a doubling time of 40.5 hours.

Cells of the cell line Tu 31 grew rapidly in normal medium in the absence of EGF. They were readily subcultured. The growth kinetics of passage 16 cells were evaluated and the results shown in Table 11. Under normal growth conditions the doubling time was 20.7 hours. Growth in reduced fetal calf serum concentrations resulted in little change in growth kinetics, with the doubling times being 20.5 hours in 10% serum, 19.1 hours in 5% serum and 30 hours in 2% serum. Tu 31 cells have never required EGF for continued growth.

4.4 Anchorage independent growth

Attempts were made to demonstrate formation of cell colonies from single cell suspension in sloppy agar at passages 21, 23, and 47 of Tu 22-1 and at passages 21, 35 and 45 of Tu 22-2.

Cells of the Tu 22-1 cell line did not form discrete colonies even at very high plating densities and observation periods of up to 8 weeks. Cells of the Tu 22-2 cell line at passage 45 did form small colonies of 20-30 cells after 7 weeks (Fig. 36). Very few colonies formed and plating efficiency was therefore not calculated. Colonies formed only at plating densities of $5x10^5$ cells per plate.

In contrast, Tu 31 cells readily formed discrete colonies from single cells in agar. Growth in sloppy agar was assessed using passage 48 cells. Numerous colonies developed at cell seeding densities of 1, 2 and $5x10^5$ cells. These developed within 21 days. Colonies were large,often exceeding 100 cells. A typical colony is shown in Figure 37.

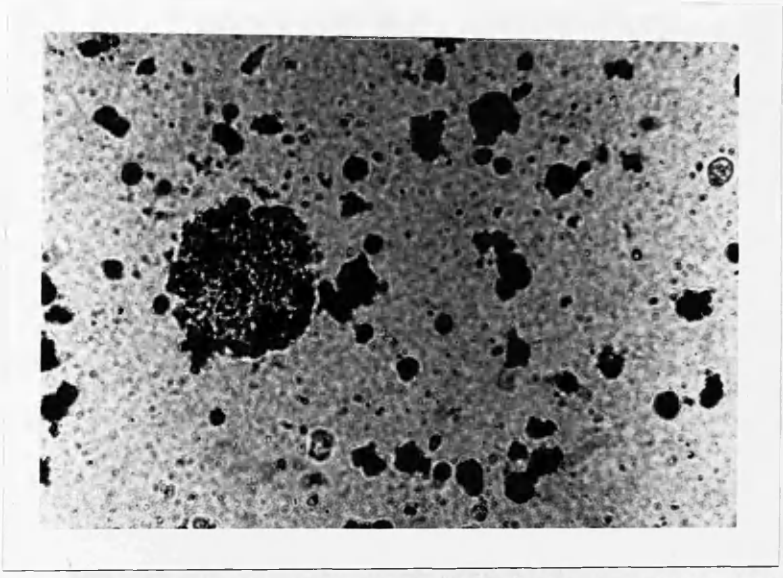


FIGURE 36

Colonies in agar derived from single cell suspension of Tu 22-2 cells, passage 45, plated at 5 x 10^5 cells per plate. One large colony and two very small colonies are shown here after 7 weeks in culture.
(x 25)

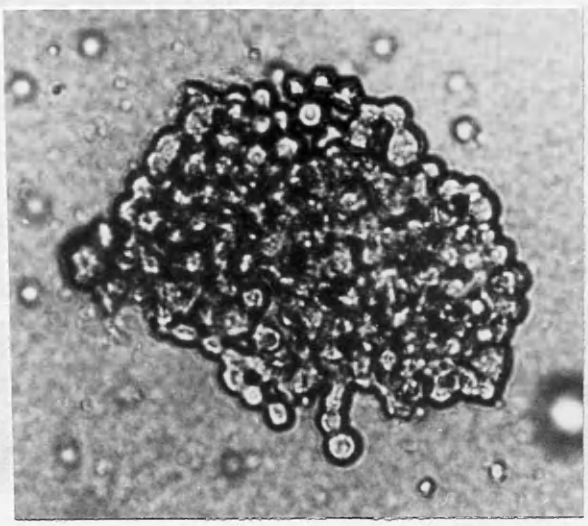


FIGURE 37

Typical colony in agar derived from single cell suspension of Tu 31 cells at passage 48, photographed on day 19.
(x 40)

4.5 Tumorigenicity in athymic *nude* mice.

Attempts were made on several occasions to produce tumours by subcutaneous injection of cells from both Tu 22-1 and Tu 22-2 cell lines into the subscapular area of laboratory rodents. Cells were from passages between 35 and 52. On no occasion was any detectable tumour produced despite observation periods of 4 months. At autopsy, no animal had any evidence of tumour.

Vulval tumour cells from Tu 31 were highly tumorigenic in mice. Cells harvested from passage 47 were suspended in PBS at a concentration of $1x10^6$ viable cells/ml. One ml. of this suspension was injected into the subscapular area of an athymic nude mouse. Within 21 days a palpable tumour of approximately 2cm in diameter was detectable. One-half of this tumour was sent for histological analysis and the other half heterotransplanted into another mouse. The histology is shown in Figure 38. The tumour was a poorly differentiated squamous carcinoma. The second mouse developed a tumour in 4 weeks.

4.6 Karyotype analysis

Cytogenetic analysis was based on cells from passage 48 for Tu 22-1, passage 51 for Tu 22-2, and passage 55 for Tu 31. Figures 39 to 41 show representative karyotypes from the 3 cell lines. Chromosome distribution is shown in Figure 42.

Tu 22-1 cells had a wide distribution of chromosome number. There was no modal number, with a range of 83-103 in 25 counts. The karyotype was essentially hypotetraploid with a large number of rearranged chromosomes. The distribution of individual chromosomes is shown in Table 12 and the putative origins of some the rearrangements in Table 13. It was not posible to assign with confidence many of the rearranged chromosomes. Tu 22-1 cells consistently contained the rearrangements B, E, F, H, I, J, N, O, Q, S, U, W, Y, Z, and the fragment 1C.

Tu 22-2 cells had a tighter chromosome distribution as shown in Fig 44. The modal number appeared to be 91-93, with a range of 86-99 in 22 counts. The karyotype was hypotriploid with a large number of rearranged chromosomes, the distribution of individual

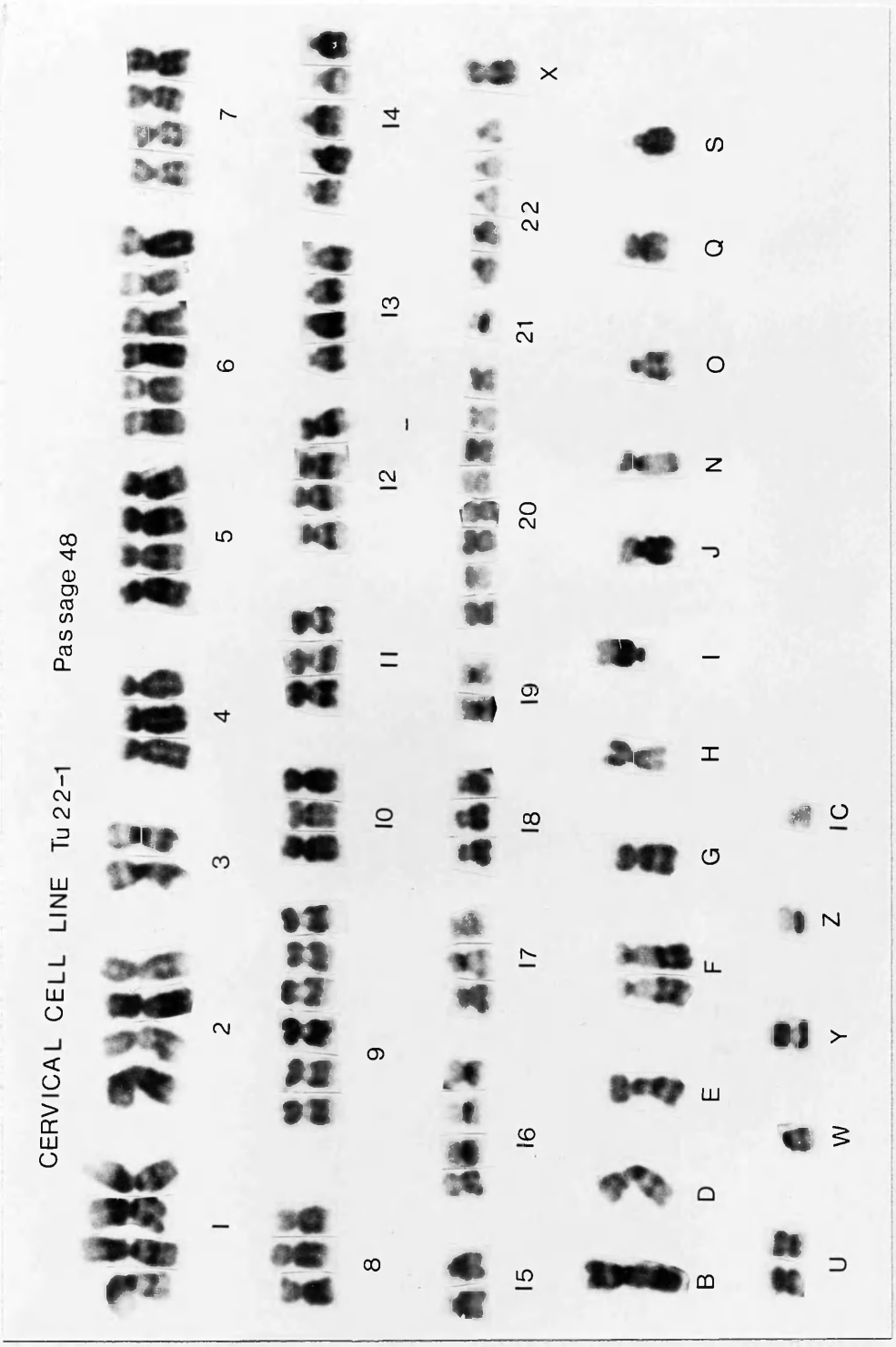


FIGURE 39

Representative karyotype of Tu 22-1 cells, passage 48. Chromosomes are trypsin-giemsa stained. Derivation of additional chromosomes is shown in Table 13.

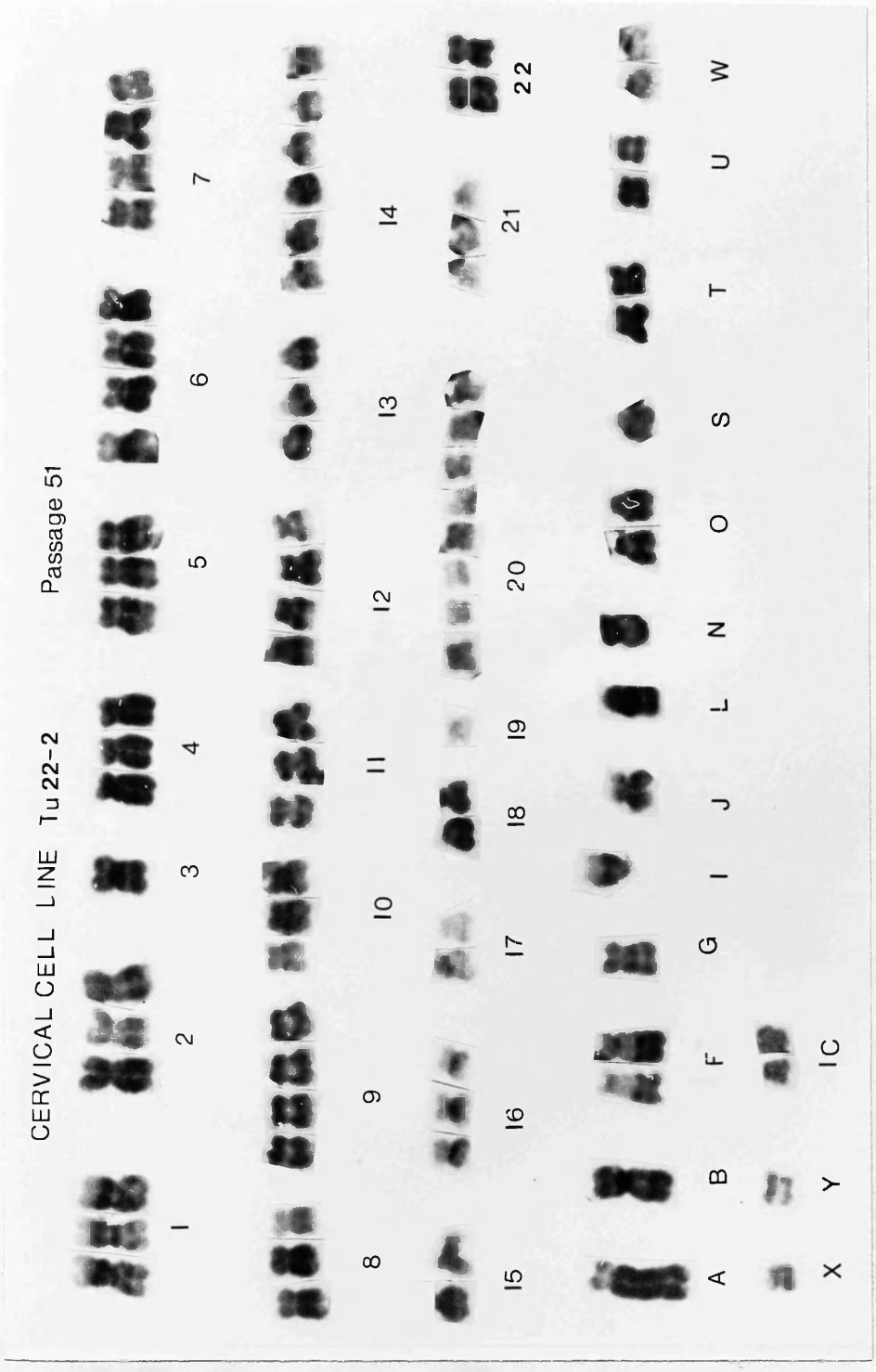


FIGURE 40

Representative karyotype of Tu 22-2 cells, passage 51. Chromosomes are trypsin-giemsa stained. Derivation of additional chromosomes is shown in table 13.

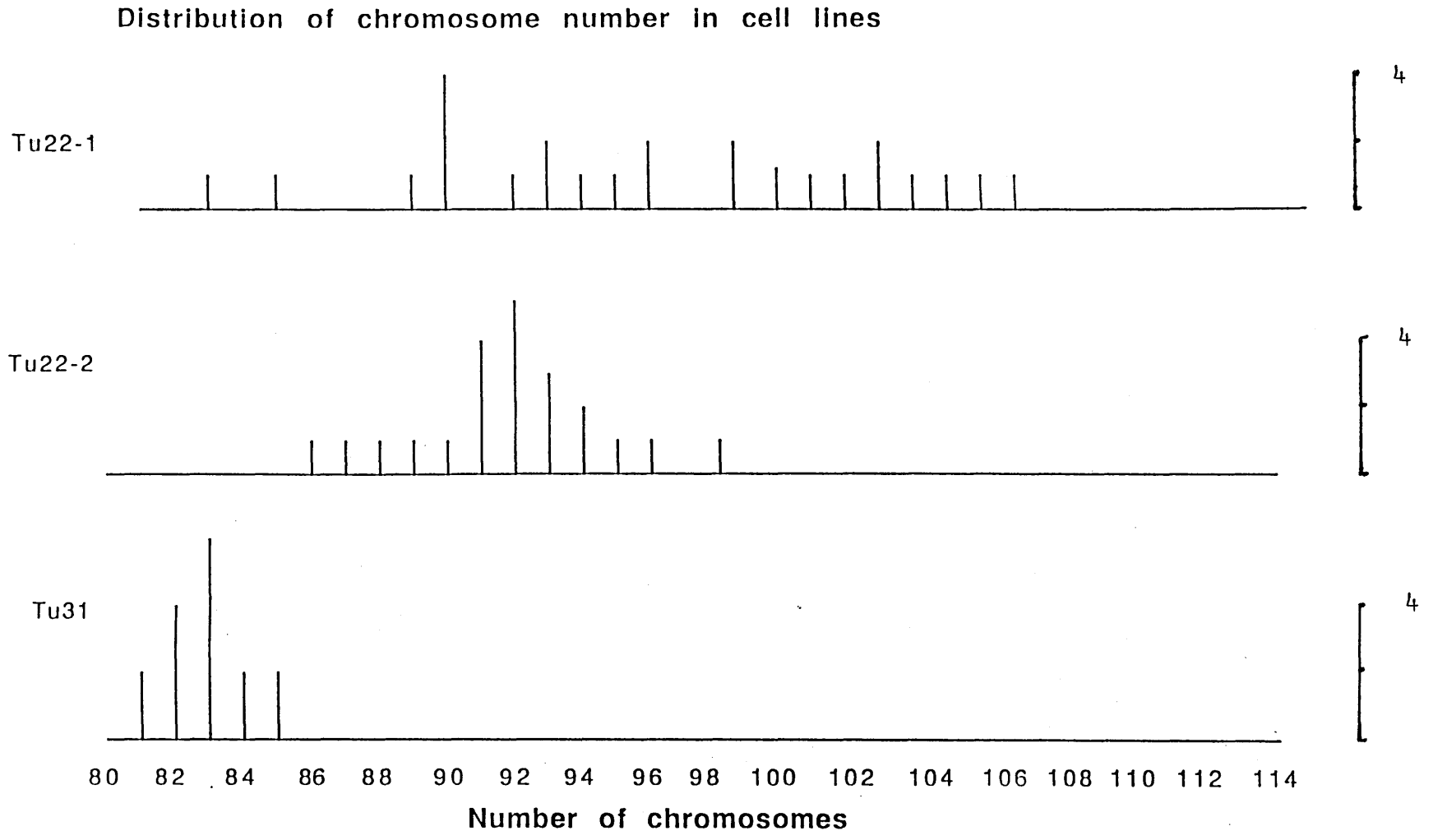

Distribution of chromosome number in cell lines
Tu22-1
Tu22-2
Tu31
4
4
4
80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114
Number of chromosomes

Figure 42

TABLE 12

INDIVIDUAL CHROMOSOME DISTRIBUTION IN TUMOUR CELLS

CHROMOSOME	TIMES REPRESENTED		
	TU 22-1	TU 22-2	TU 31
1	4	3	1-3
2	4	3	1-3
3	2	1	0-1
4	3	3	2,3
5	3,4	3,6	2,3
6	6	4	3,4
7	3,4	2,4	2,3
8	3	2,3	2,3
9	4-6	2,4	3
10	3,4	2,3	3
11	3	3	2
12	4	3,4	3
13	3,4	3	3
14	5	6	3
15	2	1,2	3,4
16	3,4	3	3
17	3	2	4
18	3	2,3	2,3
19	2	1,2	1,2
20	8,9	8	2,3
21	1	1-3	2
22	2-5	2,3	1,2
X	1,2	0-4	3

TABLE 13

PUTATIVE ORIGIN OF REARRANGED CHROMOSOMES IN TU 22 CELLS

A unknown, may be >2 chromosomes
B Chromosome 3 + unknown material added to long arm
C Chromosome 1 with partial deletion long arm
D possible isochromosome 1q
F Chromosome 20 + 7q
H Translocation 14q:15q
I Chromosome 1, deletion q11→qter
K 3p
O 7q
S D or G derivative
U Isochromosome 21q
V Isochromosome 11p

Rearrangements designated E, G, J, L, M, N, P, Q, R, T, W, X, Y, Z, and 1A-C not possible to characterise.

TABLE 14

ORIGIN OF CHROMOSOME REARRANGEMENTS IN TU 31 CELLS

A Most of chromosome 1 plus undetermined material on long arm.
B Most of chromosome 2 plus additional material on long arm.
C Translocation 3p:1q
D Translocation unknown:3q
E Translocation 3p:unknown
F Chromosome 1 with 1p deletion
H Translocation 1p:9q
I Translocation 13:14
J 5p-
K 7p-
L 6q-
P Derived from chromosome 11
R Isochromosome, origin unclear
S Translocation unknown:21
V D or G group derivative
X 21p-

Rearrangements designated G, M, N, O, T, U, and W cannot be clearly assigned.

chromosomes being shown in Table 12. Cells consistently contained the rearranged chromosomes A, B, F, I, J, L, N, O, S, U, X, Y, 2 copies of W, and the fragment 1C.

In both cervical cell lines there was consistent overrepresentation of chromosomes 6, 14, and 20. Relative to Tu 22-1, Tu 22-2 cells consistently had fewer representatives of chromosomes 1, 2, 3, 6, 9, 10, 17, and 22. Tu 22-2 cells had more representatives of chromosomes 14 and 21.

A number of rearrangements were common to both lines, namely B, F, I, O, S, U, the unclassified chromosomes J, N ,W, Y, Z, and the fragment 1C. Tu 22-2 cells contained the very distinctive chromosome A, which had material from more than 2 chromosomes and the rearrangements L and X not found in the parent line.

Tu 31 cells had a narrow chromosome distribution, 81 to 85 in 17 counts. The modal number was 83. The distribution is shown in Figure 42. Cells were essentially hypotriploid with 22 or 23 rearranged chromosomes, as shown in Table 12. The proposed origins of the rearrangements for this cell line are given in Table 14.

The majority of chromosomes were present in a triploid state. Chromosome 3 was consistently under-represented and frequently absent. Chromosomes 1, 2, 11, 19, 21 and 22 were consistently under-represented. Chromosomes 6, 15, and 17 were usually over-represented. All cells studied were triple X. A high proportion of the identifiable rearrangements contained chromosome 1 or 3 material. All cells studied contained 5 representatives of an unidentified isochromosome R.

4.7 Expression of oncofetal products and steroid receptors.

Medium from subconfluent monolayers of all cell lines was assayed.

The results are summarised in Table 15. Fresh cell-free medium containing EGF contained 21 iu/ml of measurable AFP but no other detectable oncofetal products. The amount of measurable AFP in medium taken from growing cells was therefore not significantly

TABLE 15

EXPRESSION OF ONCOFETAL PRODUCTS AND STEROID RECEPTORS IN CELL LINES

CELL LINE	AFP	β-HCG	CEA	HPL	SP-1	Cytosol oestrogen receptor	Cytosol progesterone receptor	Nuclear oestrogen receptor
Tu 22-1	40	520	<17	<90	<5	n.d	n.d	n.d
Tu 22-2	64	67	<17	<90	<5	n.d.	n.d.	n.d.
Tu 22-2 (+EGF)	48	69	<17	<90	<5	n.d.	n.d.	n.d.
Tu 31	65	68	<17	<90	<5	n.d.	n.d	n.d.
TU 31 (+EGF)	66	61	<17	<90	<5	n.d	n.d	n.d.

Results expressed as per 10^6 cells except receptor assays which are expressed as fmol/ng protein for cytosol receptors and fmol/mg DNA for nuclear receptors.

n.d. not detectable

different from this. Tu 22-1 cells produced an 8-fold excess of β-HCG compared with Tu 22-2 cells and Tu 31 cells. Tu 31 cells were similar to the EGF independent sub-line Tu 22-2 in producing detectable β-HCG only. Growth of Tu 22-2 or Tu 31 cells serially in the presence of EGF did not increase the production of β-HCG. No cell line had detectable oestrogen or progesterone receptors under normal growth conditions.

Section 5 : Analysis of cell line, tumour and control DNA

DNA was extracted, as described in chapter 2, from the tumour biopsies Tu 22 and Tu 27, from internal control myometrium Co 22 and internal control vulval skin Co 27, and from harvested cells from cell lines Tu 22-1, passage 48, Tu 22-2, passage 36, and Tu 31, passage 18.

5.1 Herpes simplex virus DNA probes

All DNAs were probed under stringent hybridisation conditions for the presence of HSV-2 DNA, cloned restriction fragments HindIII a, e and h spanning the map units 0.28-0.72 which includes the regions BglII j, c, and n, associated with morphological transformation in vitro (see Figure 2, page 79). Figure 43 shows a Bam HI digest of cervical cell lines Tu 22-1 and Tu 22-2, original tumour Tu 22 and control DNAs probed with HSV-2 HindIII a DNA. The reconstruction tracks demonstrate the typical pattern of Bam HI digestion of the HindIII a region and size markers in kilobases are indicated. No hybridisation is demonstrable. The reconstruction at one copy per cell is poorly visualised in this exposure, but prolonged exposure confirmed no hybridisation at a level of one copy per cell. Figure 44 shows a similar digest probed with HindIII e DNA. There was no

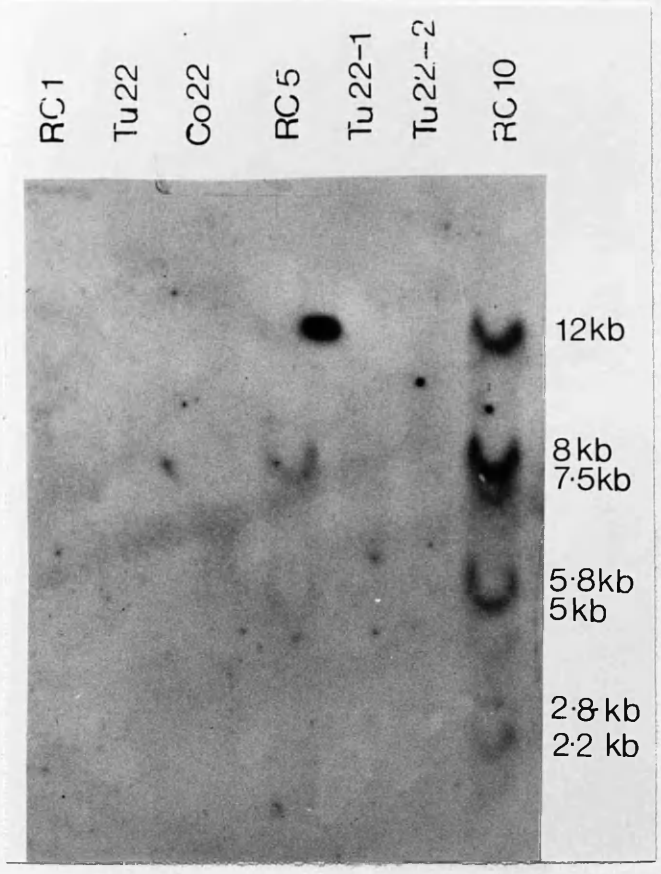


FIGURE 43

Bam HI digest of tumour, control and cell line DNAs probed with HindIII a under stringent conditions.
The reconstruction tracks are lanes 1, 4 and 7 with 1, 5 and 10 copies per cell respectively. Lane 2 contains DNA fron the original tumour 22, lane 3 DNA from internal control myometrium, and lanes 5 and 6, DNA from the cell lines Tu 22-1 and 22-2.
Size markers are shown on the right.
Hybridisation to the reconstruction lanes is seen at 5 copies per cell, and faintly on this exposure at 1 copy per cell. There is no hybridisation to tumour, control or cell line DNA.

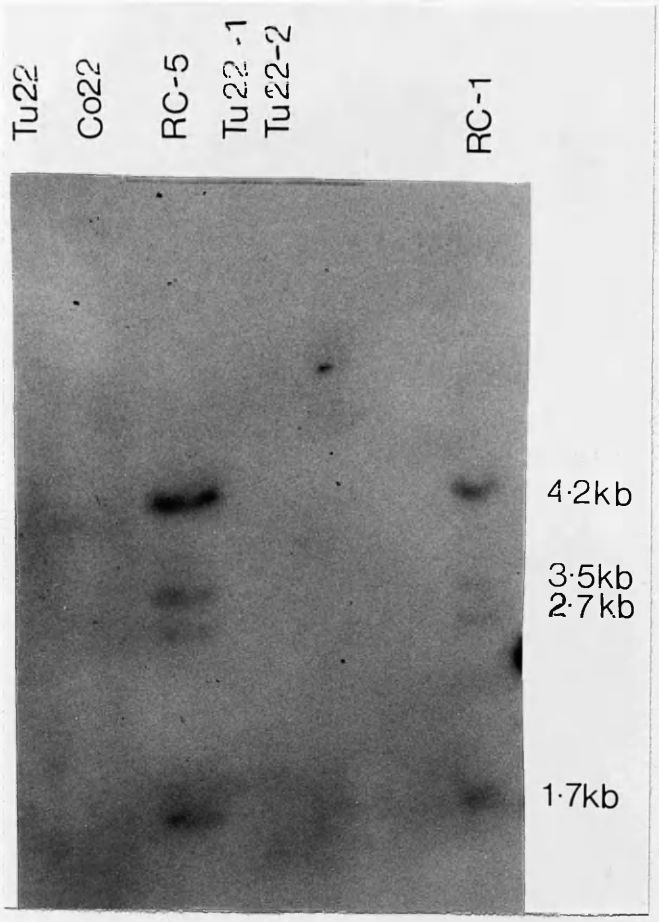


FIGURE 44

Bam HI digest of DNAs from cervical tumour, control and cell lines probed with HindIII e under stringent conditions.
Lanes 3 and 6 are reconstruction at 5 and 1 copy per cell respectively. Lane 1 contains DNA from the original tumour 22, lane 2 DNA from internal control myometrium, and lanes 4 and 5, DNA from cell lines Tu 22-1 and 22-2. Size markers are shown on the right.
Hybridisation at a level of 1 copy per cell is seen in lane 6 but there is no evidence of hybridisation to tumour, control or cell line DNAs.

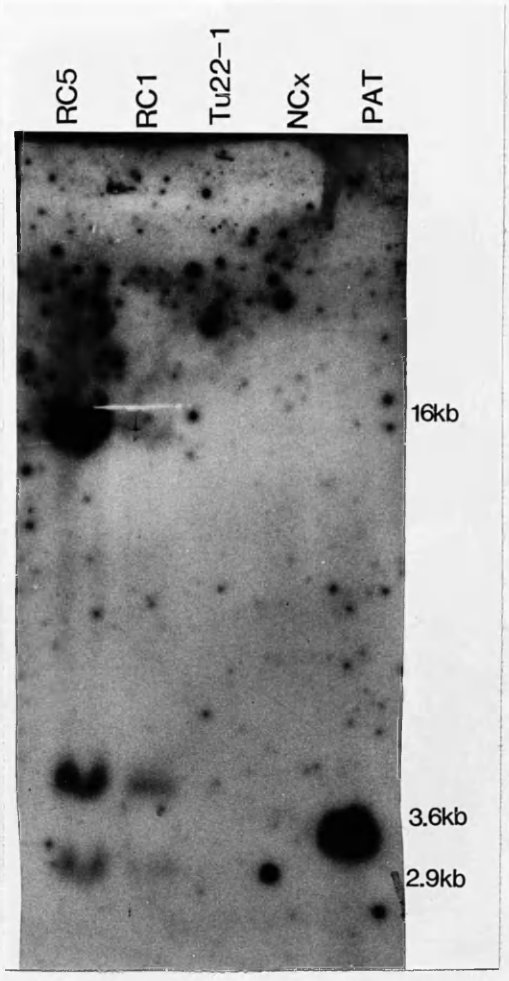


FIGURE 45

Bam HI digest of cell line and control DNAs probed with HindIII h under stringent conditions.

Lanes 1 and 2 are reconstructions with 5 and 1 copy per cell. Lane 3 contains DNA from cell line Tu 22-1. Lane 4 contains DNA from an external control normal cervix and lane 5 plasmid DNA, included as on this occasion probe DNA was not excised from its vector. Size markers (kb) are on the right. Hybridisation at a level of 1 copy per cell is seen in the reconstruction in lane 2 and there is hybridisation to plasmid DNA. No hybridisation is seen with cell line DNA or external control DNA.

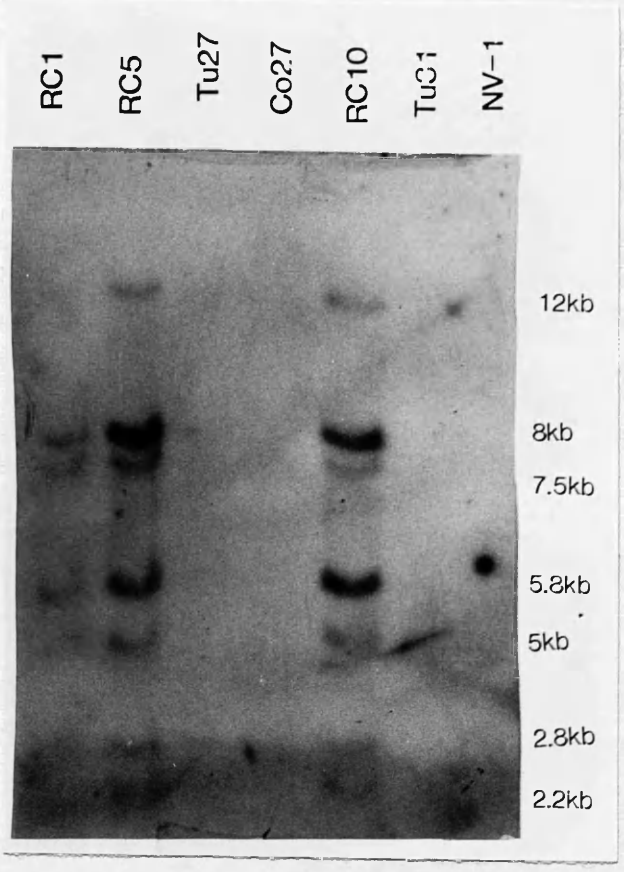


FIGURE 46

Bam HI digest of vulval tumour, control and cell line DNA probed with HindIII a under stringent conditions.
Reconstructions are in lanes 1, 2 and 5 at 1, 5 and 10 copies respectively. Size markers are on the right.
DNA from vulval tumour 27 DNA is in lane 3, from internal control skin 27 in lane 4 and from cell line 31 in lane 6. Lane 7 contains DNA from an external control vulval skin biopsy.
Hybridisation at a level of 1 copy per cell is seen in the reconstruction track in lane 1. No hybridisation is evident to tumour, control or cell line DNA.

demonstrable hybridisation to any tumour DNA at a sensitivity of less than one copy per cell. Figure 45 shows a digest of Tu 22-1 DNA only probed with HindIII h DNA. The reconstruction tracks show the pattern for a Bam HI digest of the HindIII h region. Track 5 contains plasmid DNA as on this occasion probe DNA was not excised from the vector. No hybridisation is seen in the cell line track or to external control normal cervix (NCx).

Similar hybridisations with cloned HSV probes were carried out on the vulval DNAs. Figure 46 shows a Bam HI digest of cell line Tu 31, original tumour 27 and control DNA, probed with HindIII a DNA. The other track (lane 7) contains NV-1, DNA extracted from one of the normal vulval skin biopsies at postmortem. No evidence of hybridisation was detected in the vulval cell line or tumour with any of the HSV probes.

5.2 Probing of cell lines with human papillomavirus DNA

The DNAs above were probed under stringent and non-stringent conditions (see chapter 2, section 2) with cloned HPV-6, 11, 16 and 18 DNA.

No hybridisation was seen with HPV-6, 11 or 18. Cross-hybridisation between HPV-11 and HPV-16, and between HPV-16 and 18 reconstruction tracks was noted in these experiments as previously discussed in Chapter 2. Figure 7 (page 84) demonstrated cross-hybridisation between HPV-11 and 16 in an experiment involving Tu 27 and Tu 31 cell line DNA, but even under non-stringent conditions there was no evidence of hybridisation to HPV-11.

Fig. 47 demonstrates the results of hybridisation experiments with HPV-16 DNA under stringent conditions for cell lines Tu 22-1, 22-2 and Co 22. Lanes 1 and 2 represented reconstructions at one and 5 copies per cell respectively. Lanes 3 and 4 contained Tu 22-1 and Tu 22-2 respectively and lane 5 contained Co 22 myometrium. Both cell lines contained viral DNA in a similar configuration, with three distinct bands of different molecular

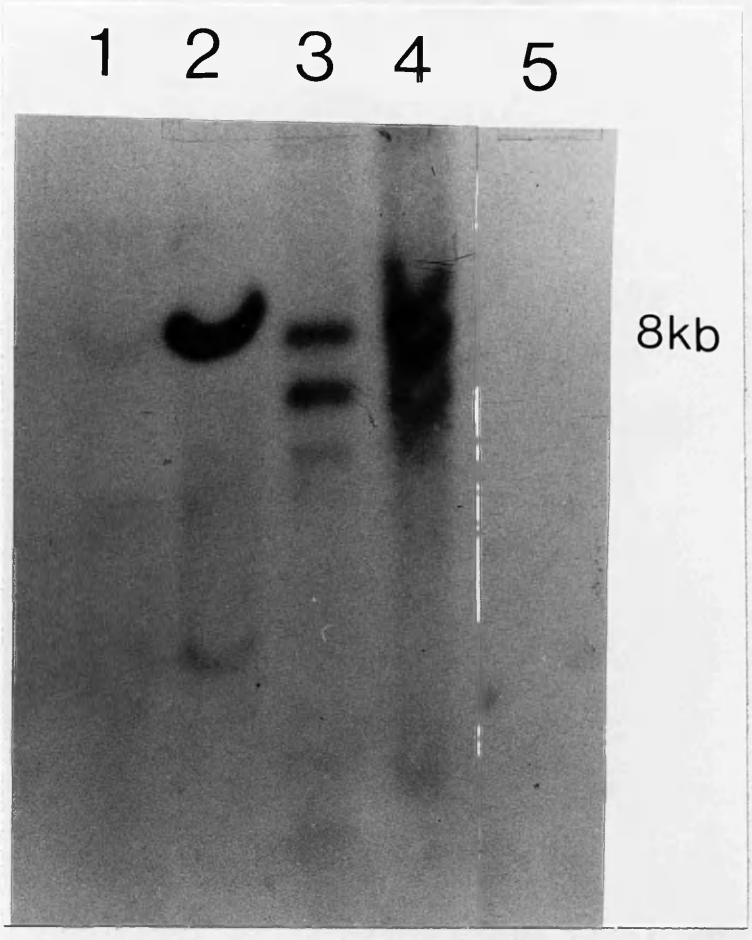


FIGURE 47

Bam HI digest of cell line and control DNAs probed with HPV-16 under stringent conditions.

Reconstruction tracks are lanes 1 and 2, at 1 and 5 copies per cell. Lane 3 contains DNA from cell line Tu 22-1, lane 4 DNA from Tu 22-2 and lane 5 DNA from internal control myometrium 22. The 8kb marker is shown on the right.

Hybridisation at 1 copy per cell is seen in lane 1. Both cell line DNAs hybridise to HPV-16 DNA in a similar configuration, with 3 major bands and one fainter low molecular weight band. One of these major bands appears to comigrate with the 8kb reconstruction band.

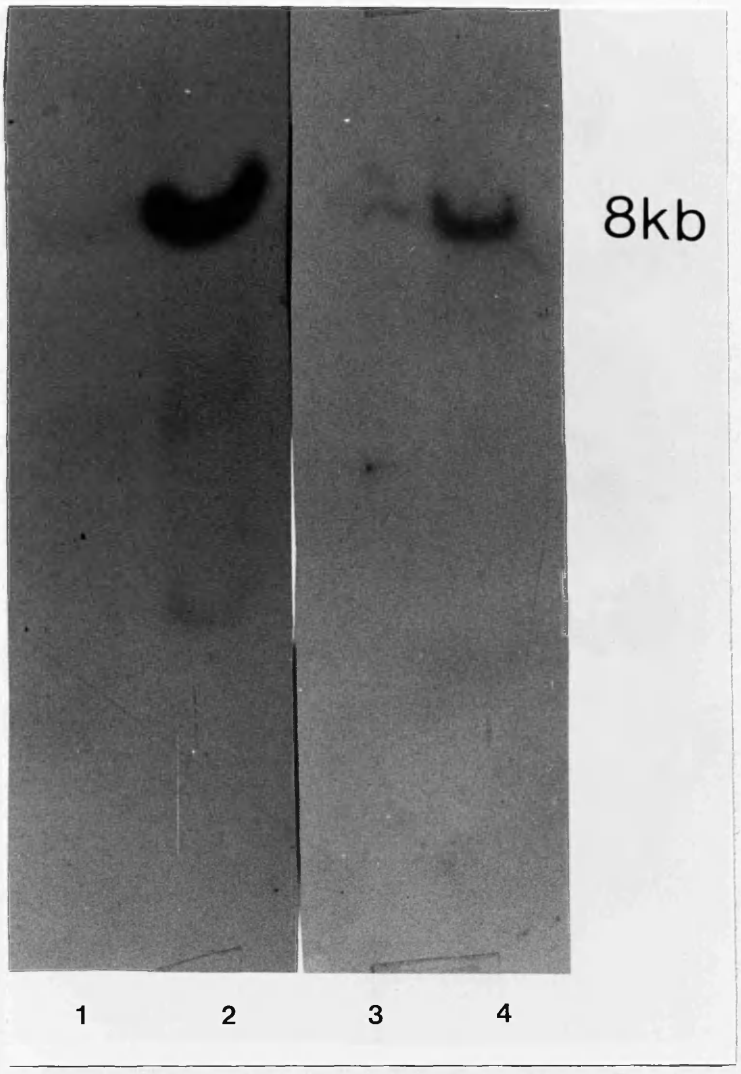


FIGURE 48

Bam HI digest of vulval cell line and control DNA probed with HPV-16 under stringent conditions.
Reconstructions are in lanes 1 and 2, at 1 and 5 copies per cell. Lane 3 contains DNA from internal control skin 27 and lane 4 DNA from vulval cell line Tu 31. The 8kb size marker is shown on the right. Hybridisation at 5 copies per cell is clearly seen in the reconstruction in lane 2. Tu 31 DNA hybridises at a level of 3 copies per cell, with a single band colinear with the 8kb reconstruction DNA, suggesting a single linear insert. A faint band is seen in lane 3, comigrating with the 8kb band, suggesting a linear insert of HPV-16 DNA in control skin. The presence of HPV-16 DNA in control 27 is more clearly demonstrated in lane 5, figure 9.

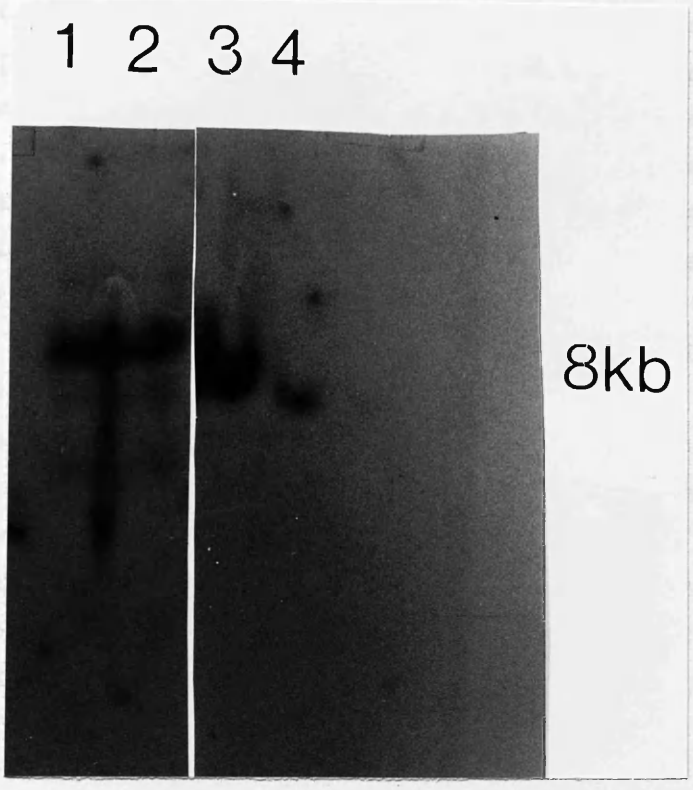


FIGURE 49

Bam HI digest of vulval tumour 27 probed with HPV-16 DNA under stringent conditions.
The reconstruction tracks are lanes 3 and 4, with 10 and 1 copy per cell.
Lanes 1 and 2 are a double loaded track containing Tu 27 DNA.
The 8kb marker is on the right.
Hybridisation at a level of 1 copy per cell is seen in lane 4.
Tu 27 DNA hybridises to HPV-16 DNA at between 5 and 10 copies per cell. The pattern is of 3 bands, not colinear with the 8kb reconstruction.

weight being identifiable. On much longer exposures, a fourth band of lower molecular weight, barely visible in this photograph in both tracks 3 and 4, was noted. One of these bands comigrated with the 8kb virion DNA. This configuration suggested multiple insertion sites for viral DNA within tumour cell DNA and that this configuration had been conserved down both cell lines. As selection occurred at passage 8, it was likely that this pattern was present in the first subcultures. Unfortunately, DNA extracted from the original tumour 22 was extensively tested against all HSV probes and against HPV-6 and 11 in the first instance in the early part of the study, prior to the availability of HPV-16 DNA. Sufficient DNA remained to screen undigested DNA with HPV-16 under stringent conditions, confirming the presence of HPV-16 sequences comigrating with high molecular weight cellular DNA. There was insufficient DNA for detailed analysis. It seemed likely that this configuration of inserts was present in the original tumour, but it cannot be confirmed. No HPV sequences were detected in internally matched control tissue for these cell lines. HPV-16 DNA is present only in malignant cervical cells.

The vulval DNAs were similarly probed under stringent and non-stringent conditions with cloned HPV-6, 11, 16 and 18 DNA. Hybridisation was only seen with HPV-16. Figure 48 shows cell line Tu 31 and matched control DNA Co 27, digested with Bam HI and probed with HPV-16 under stringent conditions. Tu 31 DNA contained unit length HPV-16 homologous DNA which co-migrated with the reconstruction tracks. Viral DNA was present at around 3 genome copies per diploid cell. Normal control DNA also contained unit length HPV-16 homologous DNA at a level of 0.1 - 0.3 genome copies; seen here as a faint band at 8kb but present in longer exposures. Confirmation of the hybridisation of normal Co 27 DNA to HPV-16 DNA was shown in Track 5, Figure 9 using undigested DNA.

Fig. 49 shows parent tumour Tu 27, loaded as a double track, and reconstruction tracks, digested with Bam HI and probed with

HPV-16 DNA under stringent conditions. Tu 27 DNA contained HPV-16 homologous DNA. This DNA was present as three distinct bands of different molecular weight. Undigested Tu 27 DNA demonstrates hybridisation to HPV-16 DNA which co-migrated with high molecular weight cellular DNA (see track 4, figure 12). The complex pattern seen in the original biopsy differed substantially from that of both the derived cell line and internally matched control tissue. This pattern suggested multiple integration sites.

5.3 Other cervical cell lines probed with human papillomavirus DNA

For comparison DNA extracted from cells from three established cell lines derived from cervical carcinomas were probed with HPV-16 DNA.

HeLa cells [380] of strains RE and WS were grown under standard conditions. Cells from the lines C4-1 and C33-1 [381], stocks of which were kindly provided by Dr L.Crawford, were subcultured under recommended conditions and DNA extracted as previously described.

DNA from the two HeLa cell strains showed no detectable hybridisation with HPV-16 DNA under stringent hybridisation conditions. Similar findings were reported by Boshart et al (1984) [196] who described HeLa cells as containing HPV-18 homologous DNA.

Figure 50 shows the results for C4-1 and C33-1 cell DNAs. Reconstruction tracks for HPV-16 are in lanes 1 and 2, and for HPV-11 in lanes 5 and 6, again demonstrating cross-hybridisation even under stringent conditions. Lane 3 contained C4-1 DNA, and a faint band was visible (as indicated) at around 9kb at less than one copy per cell. C4-1 cells have subsequently been shown to contain HPV-18 sequences [370] and this could represent cross hybridisation between HPV-16 and 18, as previously noted in Chapter 2. C33-1 cells (in lane 4) did show a faint signal on prolonged exposure but it was not certain if this was

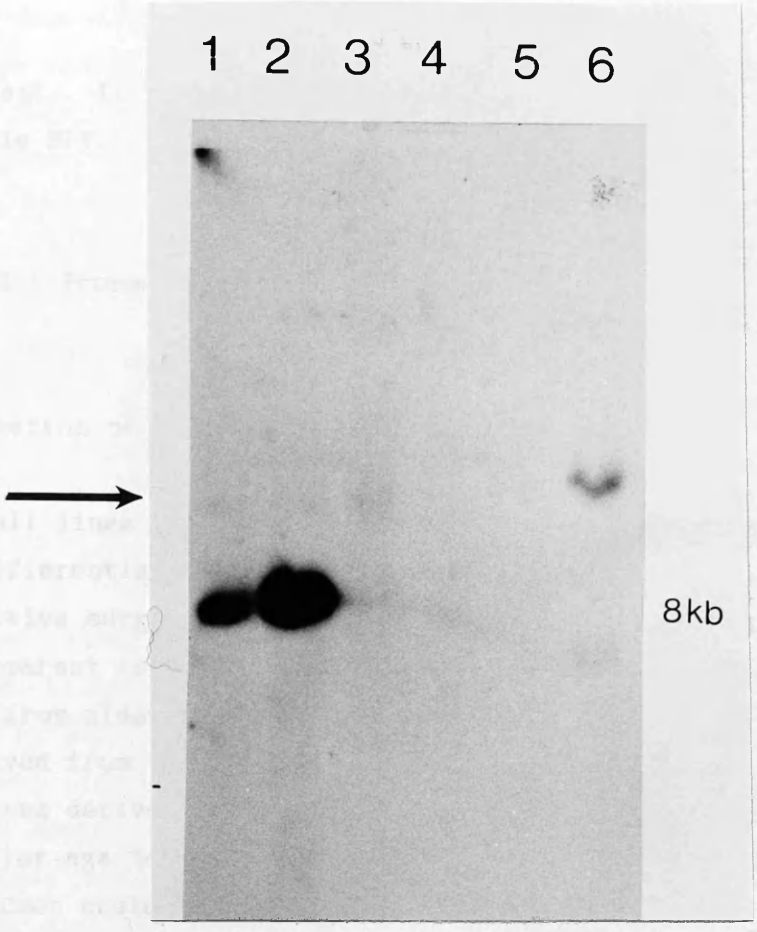


FIGURE 50

Bam HI digest of cervical cell line DNAs probed with HPV-16 under stringent conditions.

Lanes 1 and 2 are reconstruction experiments containing 1 and 10 copies per cell HPV-16. Lanes 5 and 6 are reconstruction tracks containing 1 and 10 copies per cell HPV-11. Lanes 3 and 4 contain DNA from cell lines C4-1 and C33-1. The 8kb marker is on the right. Hybridisation to HPV-16 at 1 copy per cell is shown in lane 1. Hybridisation is also evident to HPV-11, lane 6 confirming cross-hybridisation between HPV-16 and 11 under stringent conditions. There is no evidence of hybridisation in lane 4 (C33-1) but a faint band is seen at around 9kb in lane 3 (C4-1), as indicated.

significant. It is thought that this cell line does not contain detectable HPV.

Section 6 : Properties of cell lines

6.1 Derivation of cell lines Tu 22-1 and 22-2

These cell lines derived from a single biopsy from a moderate to poorly differentiated squamous carcinoma in a young woman prior to definitive surgery. Most existing cell lines, derived prior to the apparent increase in genital wart virus infection, were obtained from older women although CaSki [387] and SKG-III [390] were derived from women aged 40 and 38 years respectively. Two of the lines derived by Kelland et al (1987) [391] were from women of a similar age to those described here. Cell lines from younger women could be important as it has been suggested that such tumours could behave differently from those in older age groups [398] although recent data has disputed this [399].

Cell selection plays an important role in the establishment of continuous cell lines. It was interesting to note the behaviour of the tumour in this woman. Radical hysterectomy with pelvic lymphadenectomy was carried out in view of the localised nature of the tumour and the young age of the patient. Such surgery allowed conservation of ovarian function, ovarian metastases being rare in cervical carcinoma. The tumour from which the cell line was derived, however, did result in a large ovarian metastasis within a relatively short space of time [395]. The tumour was to some extent atypical. Few reports of other lines gave sufficient detail of patient outcome. The CaSki cell line [387] derived from a mesenteric metastasis 1 year after primary treatment. ME-180 [384] was similarly derived from an omental metastasis and the patient died within 3 months. The line EC-50 [388] was derived from ascitic fluid obtained shortly before death in a woman

who presented with advanced disease but who deteriorated rapidly despite radiotherapy. The patient from whom one of the earliest cell lines, OG [382], was derived, presented with terminal disease four years later. Only the patients from whom Kelland et al (1987) [391] derived their cell lines remained well although stated follow up time was short. Continuous cell lines might not necessarily be repesentative of the biology of the majority of cervical cancers and this must be taken into account in the interpretation and extrapolation of experimental data from established cell lines.

6.2 Tu 22-1 and 22-2 are new continuous cell lines

The cell lines were derived by primary explant culture similar to SiHa, CaSki and Cx 106 [374,383,387]. They retained the characteristics of squamous cells on electron microscopy. Cells from both lines have been subcultured through over 50 passages over 12 months and were rescuable from frozen stocks. On this basis they would appear to be established continuous cell lines. However, despite repeated attempts it was not possible to produce tumours in immunocompromised rodents, unlike any of the other cell lines. Fogh et al (1977) [393] have suggested that the production of tumours in nu/nu mice was the only sure way of differentiating that the cultured cells were derived from tumour cells and not from non-malignant cells within the tumour biopsy. Neither cell line would classify as a new established cell line under these criteria.

Normal cervical squamous cells have not been passaged to this degree, with or without feeder layers [375,379]. These cells were undoubtedly squamous cells, were aneuploid and behaved otherwise as tumour cells. Tu 22-2 cells were capable of anchorage independent growth. They also retained the viral DNA present only in the parent tumour.

This behaviour was comparable with in vitro transformation data. HPV-16 transformed human cells were not tumorigenic although exhibiting anchorage independent growth and aneuploidy [170-172].

Human ectocervical cells transformed by HPV-16 or 18 were immortalised but were not tumorigenic [173]. In rodent cell transformation by HPV-16, it is thought that development of the full oncogenic phenotype could be a progressive process[166,400]. A similar multistep pathway to full tumorigenicity was noted in HSV-2 transformation systems [68]. These in vitro studies have been supported by the work of Schneider-Maunory et al (1987)[401], who have developed a cell line derived from a vulval carcinoma in situ. Initial cultures contained high copy episomal HPV-16 DNA and were not tumorigenic in mice. At passage 20, amplification of a cellular region containing an integrated single copy of HPV-16 DNA was noted and these cells were tumorigenic in mice.

It is attractive to propose that Tu 22 cells demonstrate this phenotype. However, these cell lines were derived from an invasive tumour which has subsequently demonstrated a capacity for metastatic spread. As Tu 22 cells were immortalised cells, aneuploid and as the subline formed colonies in agar, it was therefore felt appropriate to define these cultures as established cervical cell lines.

6.3 Comparison of properties of Tu 22-1 and 22-2

Both cell lines derived from the same explant culture plate. Attempts were made to wean early passage cells from EGF for purely fiscal reasons. This was possible at a critical passage number and later attempts to grow established Tu 22-1 cells in the absence of EGF resulted in the growth patterns seen in Table 10. The cells were otherwise indistinguishable morphologically. They did exhibit interesting differences in their behaviour, in their chromosome distribution and in the secretion of β-HCG sub-unit.

The EGF independent sub-line produced small colonies from single cell suspensions in agar. Repeated attempts with Tu 22-1, even at late passage and at high seeding densities, failed. The inability of Tu 22-1 cells to form colonies in agar and tumours in athymic mice could be related to its EGF dependence. However,

EGF, in cervical cultures, has been shown to increase plating efficiency and to result in increases in colony size [378,379]. Continued supplies of EGF were required to maintain the ability of this line to subculture although cell replication could occur in its absence. This difference in the 2 lines might be of value in the investigation of the role of EGF in the growth of cervical cells and tumours.

The karyotypic differences were inversely related to the degree of differentiation of the 2 lines. The EGF independent Tu 22-2 cells had a much tighter chromosome distribution at late passage compared with the parent line which retained a wide scatter of chromosome number. Both lines exhibited a tighter chromosome distribution than other recent cell lines [390,391].

The quantitative difference in β-HCG production demonstrated could reflect subtle changes in the cells of each line. This difference was not due to the presence of EGF as it was not abolished by growth in EGF of Tu 22-2 cells. A similar range of production was seen in both EGF independent cell lines, Tu 22-2 and Tu 31. The coding sequences for β-HCG are located on chromosome 16 but there were no dosage differences in this chromosome between the 2 lines. A number of other cervical cell lines have been reported as producing β-HCG [387,390]. Patillo et al (1977) [387] demonstrated the presence of β-HCG in the serum of the patient. Nozawa et al (1983) [390] also demonstrated the presence of SP-1 in the SKG-IIIa cell line (a possible adenocarcinoma cell line). No evidence of SP-1, CEA or AFP secretion was detectable in the Tu 22 cell lines. The differential production of β-HCG in cell lines derived from the same tumour might allow examination of this hormone in cervical cancer.

6.4 Properties of the vulval cell line Tu 31

The cell line Tu 31 was derived from a well differentiated squamous cancer of the vulva. Cell selection occurred at two levels. Cells were initially passed through an athymic *nude*

mouse. Intraperitoneal injection was performed as the primary tumour appeared infected at the time of surgery, confirmed by gross Pseudomonas contamination of the primary explant cultures within 48 hours. Ascitic fluid from the animal provided the first source of cells, but secondary selection occurred by replating epithelial cells growing in suspension. The characteristics of the Tu 31 cells could reflect this selection process.

The parent tumour itself behaved in an atypical manner. Normally, the prognosis for a locally invasive node negative tumour would be expected to be excellent. This patient died of recurrent disease within 12 months. The LT-2 vulval cell line [394] derived from a groin node secondary deposit. Although the tumour was more advanced clinically than that presented here, and treatment was by radiotherapy, the patient died within 15 months of presentation. No details of the clinical course of the tumour from which the A431 line [386] derived, other than age (85 years), were available. Results from cell lines derived from tumours whose biological behaviour is at the poor prognostic end of the clinical spectrum might not be representative of that tumour, and extrapolation of results to clinical practice might not necessarily be valid.

The Tu 31 cell line exhibited all the characteristics of a fully oncogenic continuous cell line. The cells were rapidly growing (doubling time = 20 hours), aneuploid cells which showed anchorage independent growth and were tumorigenic in athymic mice. Morphologically they retained an epithelial appearance although the electron microscopic characteristics of squamous epithelial cells have been lost. This could be a reflection of the complex selection process, in particular the selection of cells capable of viability in suspension.

The karyotype of this cell line again exhibited a tighter chromosome distribution than other vulval lines [394]. Oncofetal products produced were similar to the EGF independent Tu 22-2 cell line. LT-2 cells also produced significant quantities of β-

HCG [394] and produced CEA in early passage. Early passage Tu 31 cells have not been examined for CEA production.

Tu 31 represents a new continuous cell line derived from a squamous carcinoma of the vulva.

CHAPTER 5

INTERACTION OF VIRAL AND ENVIRONMENTAL FACTORS IN THE PROGRESSION OF GENITAL WART VIRUS INFECTION TO CERVICAL INTRA-EPITHELIAL NEOPLASIA

Section 1 : Introduction

The evidence implicating human papillomavirus in cervical carcinogenesis, described in Chapter 1, does not answer the question of what may influence the development of cervical intra-epithelial neoplasia in some women with genital wart virus infection but not in others.

The reported incidence of overt genital (mainly vulval) warts increased dramatically in recent years (PHLS 1986) and although the incidence of cervical pre-malignant disease also increased, the majority of women with genital warts did not develop CIN. Other factors must therefore be involved in cervical HPV infection and the progression of such infection to pre-malignant disease. The clinical studies were aimed at approaching this problem in pre-invasive disease in the same geographical area as the invasive cancer studies.

Colposcopic examination of women presenting with genital warts revealed that 30% will already have established histological evidence of CIN at first assessment [402,403]. However the precise risk to the other 70% who did not have CIN at initial screening and which other factors could modify any risk were not known. In Walker et al's study [402] 3 of 22 women, initially normal, developed colposcopic abnormalities within 6 months, although it was not clear whether this was CIN or cervical wart infection. Franchesci et al (1983) [404] retrospectively examined women with genital warts and normal smears. Ten percent of these

women, in whom subsequent cervical cytology was available, had an abnormal smear at a mean of 3 to 4 years later.

There are clearer data on the subsequent risk of CIN in women with established cervical HPV infection. Short follow up studies have indicated that colposcopic HPV progressed to CIN within 6 months in some women [403]. Longer term studies have now clearly established this progression. Mitchell et al (1986) [405] undertook a 6 year follow up of women with cytological evidence of HPV infection only. 13.4% of such women developed CIN, confirmed by histology. Syrjanen and colleagues have undertaken longitudinal studies of a large group of women with cervical HPV infection, histologically confirmed [216]. These women were followed for a mean of 25 months and assessment has involved colposcopy, cytology, DNA studies and assessment of HSV and chlamydial status. 14.1% progressed, 11.9% to carcinoma-in-situ. Sixty percent of lesions persisted in this study. Nevertheless, a significant proportion of women with cervical HPV infection either did not develop CIN or the viral infection resolved without treatment. Extraneous factors influencing these events are poorly understood.

zur Hausen (1984) [406] has proposed synergism between HSV and HPV, with HSV being an initiator of the chain of events leading to malignant transformation and HPV having a promotor role. As discussed in the introduction other viruses, such as CMV, could be involved. Potential environmental agents were oral contraceptive steroids and cigarette smoking. There was substantial evidence suggesting a role for these two factors in cervical cancer and it might be that their role was in association with HPV.

Viral type itself could be an important factor. It has been conventionally accepted that HPV-16/18 represented a high risk viral group in comparison to HPV-6/11 and there was evidence that lesions containing HPV-16 were more likely to progress [204,219]. The relationship between HPV and cervical cancer is therefore likely to be complex. At any stage other factors, such as local immunity, hormonal milieu, other viral infection or other carcinogens may accelerate or reverse the progression. If

factors are identifiable which allow clinicians to determine which women are at significant risk of CIN following viral infection of the vulva or cervix then this will allow a more rational approach to screening, treatment or immunisation.

As the previous studies had identified that both cervical and vulval invasive disease contained a high prevalence of HPV-16 in our geographical area and an invasive model system has been evolved for both these tumours, it seemed appropriate to examine groups of women with papillomavirus infection and pre-invasive disease. Others [205] later have indicated that CIN lesions of all grades in this area contain a very high proportion of HPV-16 and 18.

Clinical studies were designed to compare the occurrence of known associated factors in women with vulval HPV infection, cervical HPV infection and CIN. The first study examined cross sectional data to elucidate differences in associated cofactors in women with normal cervices, cervical HPV infection and CIN. It was felt that this might determine which of these factors were important in influencing the progression from cervical HPV infection to CIN. In the second study women with vulval warts with colposcopically normal cervices were studied prospectively to determine factors influencing progression of disease after primary treatment for clinical warts and to identify differences between women with existing CIN at initial assessment and those without. These studies could firstly establish whether there was any clinical evidence of potential interaction between HPV and other factors and secondly at which stage in the progression these factors operated.

Section 2 : Study methods

2.1 Cross sectional study

Criteria for entry

Women referred to the Colposcopy Clinic, Western Infirmary, Glasgow for assessment of abnormal cervical cytology were eligible for inclusion into the study groups. Cervical cytology was classified as normal, suspicious, or malignant only and was not expressed in classes nor was there any attempt to correlate the cytology with possible histology. Women with suspicious cervical cytology would include those with histological mild inflammatory atypia through to CIN 3.

Data on age, parity, socio-economic class, current contraceptive usage, and current smoking habit were obtained.

Microbiological data

Bacteriology swabs were obtained from the urethra, posterior vaginal fornix and endocervix. These were transported in Stuart's medium (Oxoid) to the laboratory and plated onto blood agar (aerobic and anaerobic), sensitivity blood agar, MacConkey agar, Thayer and Martin medium and Robertson's meat broth fluid medium, with subculturing if necessary. Endocervical swabs were obtained for chlamydial culture. These were transported in 2 SP medium and inoculated into monolayer culture of McCoy cells without prior feeding as described by Harper (Harper et al 1982) [407]. Lugol's iodine was used to stain the cultures 3 days after inoculation. Endocervical swabs were taken for isolation of herpes simplex virus. These were transported in BHK 21 medium (Gibco Europe Ltd) and cultured in BHK C13 cells in transport medium supplemented by 10% fetal calf serum. Cultures were examined for the typical cytopathic plaques of HSV types 1 or 2.

A diagnosis of infection with Trichomona vaginalis was made by direct visualisation on cervical cytology.

Venous blood was withdrawn for estimation of complement fixing antibodies to CMV and HSV and for estimation of antibodies to C. trachomatis. Chlamydial antibodies were estimated using the method of Thomas [408].

Colposcopic assessment

All women were assessed colposcopically. With the patient in the lithotomy position, the cervix was visualised with a Cusco's speculum. A cervical smear was taken using an Ayre's spatula and spread and fixed immediately. The cervix and upper vagina were inspected directly. Five percent acetic acid was then applied to the cervix and the cervix again inspected. Colposcopically abnormal areas were directly biopsied using Eppendorf biopsy forceps. Women with unsatifactory colposcopy or suspicious findings underwent knife cone biopsy of the cervix at another time.

Study groups

From the referral population, three groups were delineated based on the histological diagnosis:

Group 1a 116 women with histological evidence of CIN alone,
Group 1b 84 women with evidence of CIN and HPV infection,
Group 2 36 women with histological evidence of HPV infection alone.

Histological criteria for HPV infection were based on those of Dyson et al (1984)[409]. Diagnosis and grading of CIN was based on the criteria of Buckley et al (1982) [410].

Control group

A group of women with normal cervical cytology were recruited from gynaecological inpatients and outpatients over the same time

period. These women had normal cervical smears, no history of previous treatment to the cervix and were undergoing treatment or investigation for non-malignant disease. One hundred women were recruited, matched as a group for age, parity, social class, and current genital infection rate with C.trachomatis and T.vaginalis with women with CIN. Twenty-four of these women consented to undergo colposcopic examination. All were normal.

Women with histologically proven CIN and some women with HPV infection alone underwent local ablative treatment of the cervical transformation zone when appropriate with the carbon dioxide laser and were reviewed colposcopically at 4 and 12 months.

2.2 Prospective study

Criteria for entry

Fifty-nine women with overt vulval warts referred from Gynaecology, Dermatology, and Genito-urinary Medicine clinics for assessment and treatment of their warts, agreed to participate in a prospective study into the natural history of wart infection after treatment. They gave fully informed consent to colposcopic examination and to cervical punch biopsy.

A full history was obtained to include data on age, parity, marital status, contraceptive practice, smoking habit, age at first coitus, and number of sexual partners.

Colposcopic examination

All women underwent colposcopic examination. A cervical smear was obtained under direct vision using a modified Ayre's spatula and immediately fixed. The cervix was visualised and any overt papillomata on the cervix or vagina noted. Three percent acetic acid was then applied to the cervix and upper vagina. If a

colposcopic abnormality was visualised, then a directed biopsy was taken using Epppendorf biopsy forceps. If no colposcopic lesion was visible, then 2 biopsies were taken from the centre of the cervical transform ation zone on the upper and lower cervical lip.

The vulva was inspected to assess the extent of the overt warts. Representative papillomata were removed by avulsion or excision after injection of local anaesthetic agent (1% plain lignocaine). This tissue was snap frozen in liquid nitrogen after appropriate labelling.

Treatment and follow up

All women were offered treatment of the warts with podophyllin solution, Efudex (5-fluoro-uracil) cream, or electro-diathermy under general anaesthesia if necessary. The cervix of women with histological evidence of CIN [410] or cervical HPV infection [409] was, in addition, treated by local ablation using the Semm Cold Coagulator [411]. All women with cervical histological abnormality were followed annually at the Cervix clinic, Ninewells Hospital and underwent repeat colposcopy at that visit.

Women with normal findings were advised about seeking examination of their regular partners, and about barrier contraception if their partners were unwilling to seek medical advice. They were invited to attend for a repeat assessment at 1 and 2 years.

2.3 Viral DNA analysis

DNA was extracted from snap frozen vulval wart tissue as described in chapter 2 except that high volume lysis buffer (30 ml. per sample) was used, digestion times were up to 24 hours and the RNAse treatment was omitted.

The suspended DNA was sent in a single batch to Dr. D. McCance, Dept of Microbiology, United Medical and Dental School, Guy's

Campus, Guy's Hospital. The DNAs were numbered and the cervical histology was not available at the time of DNA analysis.

The DNA was probed for the presence of HPV-6, 11 and 16 using the technique described by McCance et al (1983) [190]. One-third of each sample was digested with the bacterial restriction endonuclease Pst 1. The reaction was stopped with edetic acid and the DNA fragments precipitated at -70°C with 0.1 volume 5mmol/l ammonium acetate and 1 volume of isopropanol. Samples were redissolved in loading buffer (10ml TRIS HCl pH 7.5; 1mmol/l EDTA; 50% sucrose and bromocresol green) and run on a horizontal 0.8% agarose gel in trometamol-acetate buffer (40mmol/l TRIS HCl pH 7.5; 5mmol/l sodium acetate; 1mmol/l EDTA) for 16-18 hours at 1 volt/cm. The gels were washed in 0.5mol/l NaOH and 1mol/l NaCl for 1 hour to denature the DNA and then neutralised by washing for 1 hour in 1mol/l TRIS, 3 mol/l NaCl. The DNA was then transferred to nitrocellulose filters by the method of Southern [359].

The filters were baked at 80°C under vacuum and then washed for 2-4 hours at 68°C in 6 x SSC, 0.2% ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin. The filters were transferred to hybridisation solution containing the above and 400µg/ml single stranded salmon sperm DNA, 0.1% SDS and nick translated ^{32}P labelled papillomavirus DNA. The method of nick translation was that of Rigby [361] and was described in chapter 2. The DNA was labelled to specific activity of 1-3 x 10^8 cpm/µg DNA. The hybridisation was carried out at low stringency (T_m-40°C), the filters washed 3 times at room temperature for one hour in 0.1 x SSC, and 0.1% SDS then washed at low stringency and again at room temperature before exposure to Fuji X ray film for 5-7 days. The filter was then washed at high stringency (T_m-10°C) and at room temperature and re-exposed.

2.4 Statistical methods

Continuous variables were compared using Student's t test. Numerical groups were compared using a χ^2 test with Yates correction where appropriate. Social class distributions were compared using Wilcoxon-Mann-Whitney non-parametric testing.

Crude relative risks were obtained from odds ratios calculated for unmatched case-control studies and the confidence intervals calculated using the logit method [412].

Section 3 : Results from cross sectional study

3.1 Characteristics of study and control groups

There were no statistically significant differences in age, parity, or social class between the study groups or between the study groups and the control group. The data are shown in Table 16. The relatively low proportion of women with CIN 3 reflected the referral pattern to the clinic. Women could be referred with only one atypical cervical smear.

Bacterial culture results are shown in Table 17. There were no differences in Chlamydia or T. vaginalis, the isolation rates being as high in cytologically normal women as in the study groups with abnormal cervical histology. Herpes simplex virus was not isolated from any patient or control. There were some minor differences in the bacterial flora isolated in the study groups. Women with CIN had a higher rate of colonisation with Gram-negative entero-bacteria and enterococci than the control normal group (all Coliforms/enterococci $\chi^2=9.497$ $p<0.01$, CIN compared with controls; $\chi^2=3.948$ $p<0.05$, CIN+HPV compared with controls). Yeasts were not isolated in any case where HPV infection of the cervix was present without CIN.

TABLE 16

DEMOGRAPHIC CHARACTERISTICS IN CROSS SECTIONAL STUDY

		NORMAL CONTROLS	CIN ONLY	CIN+HPV	HPV ONLY
AGE (YRS)		29.6	29.8	28.8	29.8
S.D.		±8.05	±6.4	±6.05	±9.2
PARITY		1.9	1.7	1.8	1.2
S.D.		±1.6	±1.6	±1.6	±1.6
SOCIAL CLASS	1	3	3	0	0
	2	22	26	17	5
	3	36	36	26	10
	4	13	23	12	13
	5	10	8	4	1
	6	16	20	25	7
CIN 1			27	18	
CIN 2			50	47	
CIN 3			39	19	

Social class 6 = never worked, unemployed >3years.

TABLE 17

GENITAL TRACT ISOLATES IN CROSS-SECTIONAL STUDY

	NORMAL CONTROLS	CIN ONLY	CIN+HPV	HPV ONLY
Chlamydia trachomatis	10	12(10%)	12(14%)	4(11%)
Herpes simplex virus	0	0	0	0
T. vaginalis	12	10(8%)	4(5%)	1
N.gonococcus	0	0	1	0
Group β streptococci	2	2	2	0
Streptococci (other)	8	5(4%)	4(5%)	1
Gardnerella vaginalis	2	4(3%)	1	1
Yeasts	11	8(7%)	5(6%)	0
Coliforms	2	11(9%)	5(6%)	5(14%)
Enterococci	6	14(12%)	8(10%)	5(14%)
E.Coli	1	4(3%)	3(4%)	0
Bacteroides sp.	1	2	3(4%)	0
S.albus	2	10(8%)	5(6%)	3(8%)
S.aureus	2	2	1	0
Mixed anaerobes	1	1	2	0

TABLE 18

SEROPOSITIVITY IN STUDY GROUPS

	CONTROL NORMAL	CIN ONLY	CIN+HPV	HPV ONLY
ALL HSV POSITIVE (%)	63(63)	84(72)	63(73)	26(72)
ALL CMV POSITIVE (%)	32(32)	49(42)	36(42)	18(50)
ALL CHLAMYDIA POSITIVE (%)	13(13)	24(21)	18(21)	9(25)
HSV ONLY	32	36	28	7
CMV ONLY	7	5	5	1
CHLAMYDIA ONLY	3	6	4	1
HSV+CMV POSITIVE	20	30	20	12
ALL POSITIVE	5	12	11	4

Evidence of past infection was sought using serology. As up to 20% of genital isolates may be HSV type 1 [413] and HSV type 1 is capable of morphological transformation, type common antibody to HSV was determined. Women were scored for the presence or absence of significant antibody titres using the normal 'cut-off' values for the individual laboratories. A 'positive' HSV titre was above 1 in 8, a 'positive' CMV titre above 1 in 8, and a 'positive' chlamydia titre above 1 in 16. The data are given in Table 18. No differences were apparent in past infection with HSV or Chlamydia between the study groups or between the study groups and the normal group. Previous CMV infection was significantly more common in both the HPV only (χ^2=3.492, $p<0.1$) and total CIN group (χ^2=5.296, $p<0.05$) than in the normal control group, but there were no significant differences between the study groups. The crude relative risk of CIN for previous CMV infection was 1.57 (95% C.I. 0.8-2.6).

3.2 Contraception

Contraceptive usage is shown in Table 19. No information was obtained for duration of use of contraceptive steroids or type of preparation used. Women were asked if they had ever used oral contraception in the past if they were not current users. There were significantly less users of barrier contraception in the CIN+HPV group compared with the normal group (χ^2=6.297, $p<0.02$) although the numbers in each group were small.

A significantly greater proportion of women with CIN were ever users of oral contraceptives compared with the normal group (χ^2=7.5, $p<0.01$, all CIN cf controls; Odds ratio 2.0, 95% C.I. 1.54-2.60 for CIN for ever pill use). The proportion of ever users in the HPV group was higher than the control group, though this did not reach statistical significance. Although the odds ratio for HPV infection for ever users was 1.7, the confidence interval was 0.76-3.83. It would appear that ever use of oral contraceptives imparted an increased risk of CIN. It could also impart an increased risk for HPV infection.

TABLE 19

CONTRACEPTIVE USAGE

	CONTROL NORMAL	CIN ONLY	CIN+ HPV	HPV ONLY
BARRIER METHODS	14	10	2	3
NEVER PILL	51	45	33	15
EVER PILL	35	61*	49†	18

* = $p < 0.05$
† = $p = 0.02$

TABLE 20

PATIENT CHARACTERISTICS BY PILL USAGE

	CIN(ALL)		CONTROL NORMAL		HPV ONLY	
	EVER PILL	NEVER PILL	EVER PILL	NEVER PILL	EVER PILL	NEVER PILL
AGE(±S.D)	27.8 ±5.6	31.8 ±7.0	29.7 ±6.8	30.2 ± 9.2	27.8 ±7.7	31.4 ±11.4
PARITY (±S.D.)	1.6 ±1.6	2.2 ±2.1	1.4 ±1.2	2.1 ±1.7	0.8 ±1.2	1.5 ±2.0
CIN 1	23	19				
2	57	35				
3	30	24				
HSV +ve	75	59	20	33	13	12
CMV +ve	44	33	16	15	7	10
Chlamydia +VE	28	14	4	6	4	6
SMOKING						
NONE	29	30	18	26	8	3
<10/day	18	7	4	4	5	5
>11/day	63	41	13	21	5	7

Excludes barrier contraceptive users

Table 20 compares the characteristics of women with CIN, CIN+HPV, HPV, and the normal group by use of oral contraceptive steroids. Women using barrier methods were excluded from this analysis. Women with CIN exposed to oral contraceptive steroids tended to be younger ($t=4.444$, $p<0.001$) and have had less pregnancies ($t=2.239$, $p<0.05$) than women who have never used this form of contraception. The effect of parity was seen in the control group, but the effect of age was not. There was a trend in the HPV group but this never achieved any statistical relevance. No differences in serology, severity of CIN, or smoking habit were seen within the histological groups.

The data could suggest that oral contraceptive usage resulted in the development of CIN at an earlier age than would occur in the absence of such exposure although severity was not affected. The increase in parity in the never users could be an age effect, or it could be speculated that these women had different sexual behaviour, exposing them to other risk factors (e.g. different viral types). The lack of any significant difference in contraceptive habit between women with CIN and those with cervical HPV alone suggested that oral contraceptives did not influence the progression from HPV to CIN. If oral contraceptives have a role, as suggested here, then it was in the transition from normal to abnormal.

3.3 Smoking

Smoking habit is shown in Table 21. There were highly significant differences in smoking habit between the normal cytology group and all other groups, the normal group having a higher proportion of non-smokers. The crude relative risk for CIN (all CIN) for smokers was 2.57 (95% C.I. 1.47-4.49). The crude relative risk attributable to smoking appeared to be relatively constant in both CIN groups and in the HPV alone group (2.51, 2.66, 2.56 respectively for CIN, CIN+HPV, HPV). For CIN, the relative risk appeared higher for the heavier smokers (2.75 compared with 1.97) although the confidence intervals were wide,

TABLE 21

SMOKING HABIT

	CONTROL NORMAL	CIN ONLY	CIN+HPV	HPV ONLY	ALL CIN
NON-SMOKERS	53	36†	25**	11*	71
SMOKE <10/day	11	13	12	11	25
SMOKE>11/day	36	67	47	14	114

* = p < 0.02
** = p < 0.01
† = p < 0.001

TABLE 22

PATIENT CHARACTERISTICS BY SMOKING HABIT

	NORMAL CONTROL			ALL CIN			HPV ONLY		
	NON-	<10	>11	NON-	<10	>11	NON-	<10	>11
AGE(YRS)	31.2	25.2	26.7	30.9	27.2	29.8	27.4	33.1	26.1
(±S.D.)	±8.5	±2.0	±7.5	±7.3	±5.3	±5.9	±10.7	±9.7	±5.5
PARITY									
(±S.D.)	1.7	2.7	1.7	2.2	2.1	1.8	0.9	2.8	0.5
	±1.5	±2.0	±1.8	±2.1	±2.1	±1.7	±1.6	±2.2	±0.9
HSV +VE	31	8	25	45	19	81	6	8	11
CMV +VE	15	5	12	25	8	49	2	5	9
Chlamydia +VE	6	2	5	9	6	26	2	2	6
EVER PILL	17	4	13	28	15	61	8	6	6
NEVER PILL	36	7	23	33	10	53	3	5	8
CIN 1				16	7	20			
2				28	14	55			
3				17	4	39			

suggesting a possible dose effect. This difference was not apparent in the group with HPV alone.

Examination of differences between women with CIN and those with HPV alone demonstrated a highly significant difference in smoking habit between the CIN only group and the HPV only group (χ^2=7.275, p<0.02). There seemed to be a similar proportion of non-smokers (31% cf 30.5%) in each group but amongst those who smoke, women with CIN were heavier smokers than women with HPV alone. There were similar differences between the HPV group and the CIN+HPV group, although these did not reach statistical significance. The general similarities between the two CIN groups in all parameters studied suggested that they behaved as a homogeneous group. Comparison of the all CIN group with HPV confirmed the difference in smoking habit. The odds ratio for CIN compared to HPV only for smoking >11/day was 1.47 (95% C.I. 0.52-4.12). This could suggest that smoking imparted a risk of abnormality, but the severity was dependent on the extent of cigarette consumption. Unfortunately, no data were available on total life consumption. Smoking could therefore act at two separate levels.

A detailed analysis of the smoking groups by histological diagnosis is shown in Table 22. Within each histological group there were few differences. Control women who were non-smokers tended to be older compared with those who smoke but there were no differences in parity or serology. In particular there were no significant differences in pill usage associated with smoking. Within the CIN group the moderate smoking group were younger than both control and heavy smoking groups. There were no other differences and no difference in the severity of CIN with increasing cigarette consumption. These findings were likely to be a statistical aberration. A similar phenomenon was seen with parity in the HPV group, the difference in age not achieving statistical significance.

This analysis suggested that the differences in smoking habit between control and study groups and between CIN and HPV groups were independent of other confounding factors in the data set and

reflected an effect of cigarette smoking in the aetiology of CIN. The differences between CIN and HPV groups were particularly interesting and could suggest that smoking plays a role in promoting the development of CIN once cervical HPV infection is established.

Section 4 : Results of prospective study

4.1 Patient characteristics

The patient data are presented in Tables 23 to 25. The women were grouped by initial histological diagnosis. Of the 59 women referred, 17 already had established histological CIN. Seven women had CIN 1, 7 had CIN 2 and 3 had CIN 3. Twelve women had histological evidence of cervical HPV infection in addition to overt vulval warts. Thirty women had no cytological, colposcopic or histological abnormality of the cervix at initial assessment. Five of the 12 women with cervical HPV infection were reported to have normal cervical cytology at the time of diagnosis. Seven of the 17 women with CIN, including 2 of the 3 with CIN 3, were reported to have normal cervical cytology at initial diagnosis. Comparison of the 3 histological groups is shown in Table 26. There were no differences in age or parity between the groups although the women with CIN were slightly younger than both the HPV and normal groups. No differences in sexual behaviour were demonstrable between the groups.

The majority of women in all groups took oral contraceptive steroids. Women with an initial diagnosis of CIN used oral contraceptives for a significantly shorter time than either the HPV group ($t=2.548$, $p<0.02$) or the normal group ($t=2.067$, $p<0.05$). As only 8 of the 59 women however did not have exposure to oral contraceptive steroids, the significance of the differences in duration of use were difficult to interpret.

TABLE 23

CHARACTERISTICS OF WOMEN WITH CIN AT FIRST ASSESSMENT

	AGE	PARITY	AGE 1ST COITUS	NO.PARTNERS	SMOKING (PACK YRS)	PILL (yrs)	OTHER CONTRACEPTION	HPV TYPE	HISTOLOGY
1.	26	1+1	17	<5	1	2	IUCD	6b	CIN2 + HPV
2.	21	0+0	17	<5	none	3	-	6d	CIN2
3.	18	0+0	16	<5	2,5	-	-	-	CIN1 + HPV
4.	24	0+1	18	<5	4	-	-	6d	CIN2 + HPV
5.	17	1+0	15	<5	2,25	-	-	-	CIN2
6.	21	0+0	16	<5	none	1	-	6d	CIN3
7.	20	1+0	16	>5	4	2	IUCD	11	CIN1 + HPV
8.	19	0+0	18	<5	none	2	-	n/s	CIN2
9	17	0+0	16	<5	none	1	-	-	CIN1 + HPV
10.	37	0+0	18	<5	none	2	-	6d	CIN1 + HPV
11.	21	0+0	18	<5	none	3	-	6d	CIN + HPV
12.	23	3+2	16	<5	7	-	IUCD	-	CIN2
13.	38	1+7	21	<5	none	5	-	-	CIN + HPV
14.	22	0+1	19	<5	none	3	-	-	CIN1 + HPV
15.	19	0+1	16	<5	2,25	1	-	6d	CIN3
16.	22	1+0	16	<5	3	2	-	-	CIN3 + HPV
17.	21	0+0	19	<5	none	2	-	-	CIN2

TABLE 24

CHARACTERISTICS OF WOMEN WITH CERVICAL HPV AT FIRST ASSESSMENT

	AGE	PARITY	AGE 1ST COITUS	NO.OF PARTNERS	SMOKING (PACK YRS)	PILL (YRS)	OTHER CONTRACEPTION	HPV TYPE
18.	23	0+0	18	>5	6	6	-	6d
19.	21	0+0	16	<5	3,75	6	-	-
20.	25	0+0	16	<5	7,5	9	-	6d
21.	21	0+0	18	<5	3,75	4	-	n/s
22.	21	1+1	14	>5	3,75	2	-	-
23.	19	0+0	16	<5	2	6	-	-
24.	24	0+0	19	<5	none	5	-	6a
25.	28	4+1	18	<5	10	1	Tubal lig.	-
26.	33	2+2	18	<5	none	-	IUCD	6d
27.	26	2+0	18	<5	none	3	IUCD	6d
28.	19	0+0	16	<5	3	2	-	-
29.	24	3+0	16	<5	3	2	-	6d

n/s insufficient for DNA extraction

TABLE 25

CHARACTERISTICS OF WOMEN WITH NORMAL CERVIX AT FIRST ASSESSMENT

	AGE	PARITY	AGE 1st COITUS	NO.OF PARTNERS	SMOKING (PACK YRS)	PILL (YRS)	OTHER CONTRA-CEPTION	HPV TYPE
30.	21	0+0	20	<5	none	2	-	6d
31.	36	3+2	17	>5	6	2	Tubal lig	n/s
32.	20	1+0	17	<5	none	1	-	-
33.	20	0+0	16	<5	7	6	-	-
34.	42	1+1	19	<5	40	5	Tubal lig	-
35	18	0+0	18	<5	none	-	-	-
36	44	2+0	20	<5	30	2	Tubal lig	6d
37	17	0+0	16	<5	none	2	-	6a
38	37	1+0	18	<5	10	10	-	6d
39	20	0+0	18	<5	3,75	3	-	-
40	32	2+0	20	<5	16	6	Barrier	6d
41	19	0+0	17	<5	none	1	Barrier	-
42	47	2+0	18	<5	none	-	-	-
43	22	0+1	18	<5	none	5	-	-
44	20	0+0	19	<5	none	1	-	6d
45	24	0+0	18	<5	3,75	13	-	-
46	21	0+0	18	<5	none	3	-	-
47	24	0+0	19	<5	0,6	5	-	-
48	20	0+0	18	>5	none	2	-	6d
49	17	0+1	16	<5	1,5	1	-	6d
50	21	0+1	19	<5	none	2	-	6d
51	23	0+0	21	<5	none	-	Barrier	-
52	25	3+0	17	<5	4	1	-	-
53	42	3+1	21	<5	10	4	Tubal lig	-
54	33	1+1	16	<5	none	4	-	6d
55	18	1+1	15	<5	none	1	-	6d
56	23	0+0	21	<5	none	2	-	-
57	21	1+0	19	<5	none	1	-	-
58	20	0+0	18	<5	none	2	-	6d
59	20	0+0	17	<5	3	3	-	6d

TABLE 26

DEMOGRAPHIC DATA BY HISTOLOGICAL GROUP

	CIN (n=17)	HPV (n=12)	NORMAL (n=30)
AGE(YRS)±S.D.	22.7±6.04	23.7±4.0	25.7±9.1
PARITY (±S.D.)	1.1±2.2	1.3±1.8	1.1±1.3
AGE 1ST COITUS (±S.D.)	17.1±1.5	16.9±1.5	18.1±1.6
SEXUAL PARTNERS			
<5	16	10	28
>5	1	2	2
DURATION PILL USE(YRS)	1.7±1.4	3.8±2.6	3.0±2.9
SMOKING			
MEAN PACK YRS	1.5±2.1	3.6±3.1	4.5±9.3
NON-SMOKERS	9	3	18
SMOKERS	8	9	12

The smoking data highlighted the difficulties in defining risk in this complex situation. Women with CIN appeared to be the least exposed to cigarettes yet had a higher proportion of smokers than the normal group. Examination of the control group revealed that those who smoked were very heavy smokers. The wide range of cigarette consumption resulted in there being no statistical difference in the mean pack years consumed between the 3 groups. If the data were analysed to give a relative risk of cervical abnormality attributable to smoking, then this was 2.13 although the confidence limits were very large (0.4-6.0).

It was not clear how such data should be interpreted, other than with caution. It was more supportive of risk being a function of smoking or non-smoking, rather than a dosage related phenomenon.

No clear picture emerged on differences between those histologically normal or abnormal at initial diagnosis and larger numbers would be necessary to unravel these relationships.

4.2 DNA analysis

Sufficient DNA was extracted for analysis in 56 of the 59 samples. The distribution of viral type is shown in Table 27. There were no differences in the proportion of HPV positive samples in the different groups. No DNA sample had detectable HPV-16.

The DNA labelled HPV-6d was a probable variant of HPV-6. Samples containing this DNA hybridised with HPV-6 DNA under stringent conditions. However, the pattern of DNA fragments produced after restriction enzyme digestion with the enzyme Pst 1 differed from the profile of the known HPV-6 subtypes. Figure 51 demonstrate samples of vulval wart DNA digested with Pst 1 and hybridised against HPV-6 under stringent conditions. Lane 3 showed the typical pattern of HPV-6a. Lanes 2, 4, and 5 contained no hybridisable sequences. Lanes 1 and 6 demonstrated another pattern not corresponding with any known pattern of HPV-6 subtypes. This particular pattern was noted in a large number of samples.

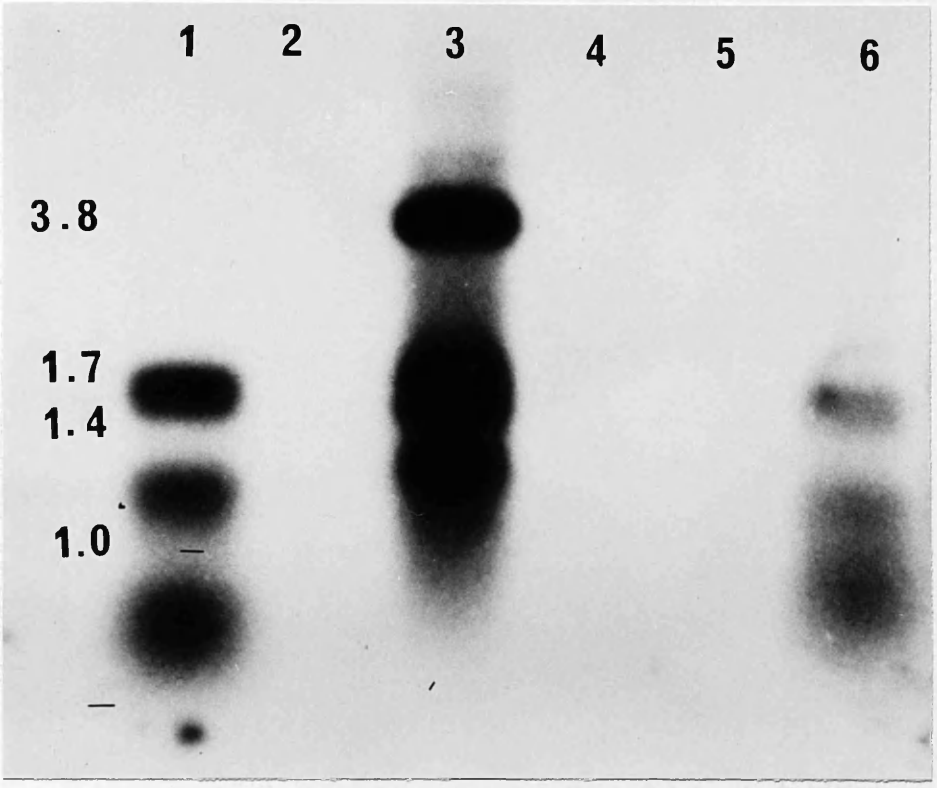


FIGURE 51

PstI digest of vulval wart DNAs probed with HPV-6 under stringent conditions.

Lanes 1 to 6 represent DNAs extracted from 6 different wart samples. Size markers are shown on the left.

No hybridisation with samples 2, 4 and 5 is seen. The sample in lane 3 shows a pattern consistent with the presence of HPV-6a. The samples in lanes 1 and 6 show a different pattern, not consistent with known HPV-6 types. This pattern has been labelled HPV-Dundee and was seen in other samples.

TABLE 27

DETECTION OF VIRAL DNA - TYPE BY INITIAL HISTOLOGY

	CIN	HPV	NORMAL
HPV-6a	0	1	1
HPV-6b	1	0	0
HPV-6d	6	5	12
HPV-11	1	0	0
HPV-16	0	0	0
NO DETECTABLE DNA	8	5	16
INSUFFICIENT DNA	1	1	1

HPV-6d see text

It is possible that some of these alterations could be due to degradation of DNA samples, as extraction from wart tissue was on occasions difficult with long digestion periods. However, this could not account for the consistent pattern seen in lanes 1 and 6 of figure 51, and which was found in other samples. It was felt that this was likely to be a geographical variant of HPV-6 (HPV-6 Dundee).

4.3 Follow up studies

All women diagnosed as having histological evidence of CIN, whatever the grade, were treated by local ablative therapy using the Semm cold coagulator. They were seen annually thereafter. One woman treated for CIN 1 was found to have CIN 3 at 1 year follow up and was re-treated. She was normal at further follow up.

Of the 12 women with cervical HPV at first visit, 8 were seen at 1 year. Six were normal, 1 had persistent cervical HPV and 1 woman had an abnormal cervical smear but no colposcopic abnormality. Of these 8 women, 4 were seen at 2 years. In addition 2 who did not attend at 1 year, attended at 2 years. Two women did not attend for any follow up. Of the 6 women seen at 2 years, 2 had developed CIN 1 and the woman with abnormal cytology had a persistently abnormal smear but again normal colposcopy.

In the normal group, 18 attended for 1 year follow up. One had developed CIN 1 and 1 cervical HPV. Ten of the 18 were seen at 2 years and 4 women who had not attended at 1 year attended at 2 years. Of this group, 1 had developed CIN 1 and 1 CIN 3. One other woman had abnormal cytology but normal colposcopy. Full data on follow up is shown in figure 52. In all 8 women received no follow up at 1 or 2 years.

Thus 3 women with initially normal cervices who had treatment for vulval warts developed CIN within 2 years of presentation.

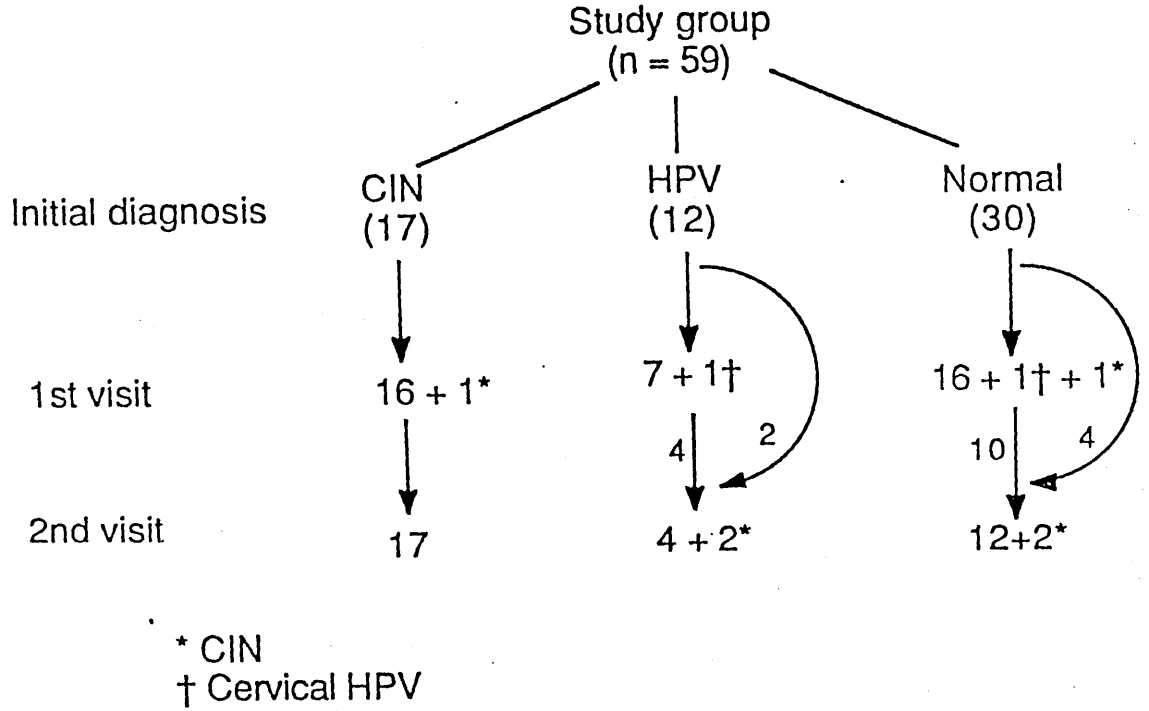


Figure 52
Follow up of women with vulval warts

Section 5 : Discussion of methodological problems

5.1 Cross-sectional study

The major methodological problem in the first study was the lack of detailed data on sexual variables. As discussed in Chapter 1, controlling for differences in sexual behaviour could dramatically alter the conclusions of any study on the aetiology of cervical cancer.

The decision not to collect this data was a conscious one. Data were being obtained from women attending for investigation for abnormal cervical cytology. The normal management protocols were in operation. Sequential visits for diagnosis, treatment and review were required, to ensure optimal management. Such a clinic therefore relied heavily on patient trust in the clinician. It was felt that potentially embarassing questioning on aspects of sexual behaviour might result in poor compliance with follow up. For this reason it was elected not to collect this type of data.

It was felt that indirect evidence of sexual behaviour could, in part, be obtained by bacteriology and viral serology. Exposure to chlamydia has been suggested as a potent indirect method of assessing sexual behaviour [264]. Positive virological assessment of 2 sexually transmissible viruses could add to this indirect assessment.

No substantive differences in bacteriological flora isolation rates were demonstratedbetween the CIN and control groups. Although no differences in prior infection with HSV or chlamydia were demonstred statistically, the prior infection rates with chlamydia in the study groups (25% for HPV only, 21% for all CIN) were sufficiently different from the control population (13%) to urge caution in comparing the study with the control group in the field of sexual behaviour. This caution was reinforced by the findings for CMV exposure.

The data proposing a role for this virus in cervical cancer were discussed in chapter 1. Although the data from this study suggested a relative risk of 1.57 for CIN in women exposed to CMV, the confidence interval was large. The finding could be spurious. The rate of seropositivity for the control group was lower than expected. Using ELISA assays, Hart et al [259] found seropositivity rates of 51 - 67% in the age range 18 - 41 years. The comparable findings using complement fixing antibody were 58% in a large series of pregnant women[414], using a similar 1 in 8 titre cut-off value. The mean ages of women in these two studies were comparable. There was no clear explanation for the low values obtained in this study, but it did cast some doubt on the 'normality' of the control group.

Validating the 'normality' of this group was a major problem. Cytological and colposcopic normality did not necessarily equate with absence of intraepithelial neoplasia of HPV infection histologically. Biopsy was not performed, although in the prospective study 30 colposcopically and cytologically normal women were biopsied; none having CIN or HPV on histology. Others have described similar negative results in larger patient groups[203] although Cox et al [355] found that 6 of 17 cytologically and colposcopically normal cervices had histlogical CIN at biopsy. There must therefore remain some uncertainty regarding the absence of pathology from the control group.

There was less reservation in interpreting differences between the CIN and HPV groups. Although the same difficulties arose with the lack of sexual parameters, it was likely that these groups were comparable. Bacterial isolation rates and prior exposure rates were comparable between these two groups. Others [415] have established that women with cervical HPV infection without established CIN were indistinguishable from those with CIN in terms of sexual behaviour. Therefore comparisons between these groups in the cross-sectional data were valid.

The problems of appropriate serological assessment of prior HSV infection in women with cervical cancer have been discussed. In this study complement fixing antibodies to HSV were measured.

This method did not distinguish between HSV-1 and HSV-2 infection. Therefore considering the high background rate of HSV-1 infection in local populations (88%), even a study of this size might not necessarily show a significant difference. There was justification for such an approach. Early reports [41] suggested that genital infection with HSV-1 was rare and that methods which differentiated the 2 viral types would distinguish genital from oro-facial infection. Data from Edinburgh [413], geographically close to the area from which our patients were drawn, suggested a rate of up to 40% for genital HSV-1 infection. It was felt unjustified to limit serology to one viral type.

The data were cross-sectional. This limited the interpretation of both pill and smoking data as no data were collected on duration of use or extent of exposure. This was a major flaw but the published literature was not consistent on the effect of either of these two variables. Duration of pill use was consistently reported as a factor [290,291,293,294] but the threshold duration at which the risk becomes significant varied. For smoking, the early studies [293,303,306] suggested a dosage effect. Of the 2 major recent studies, Lyon et al (1983)[308] were unable to demonstrate any dosage effect whereas Trevathan et al (1983)[309] did demonstrate a linear response. There were wide variations in the actual relationship between dosage and relative risk. The precise relationships of prolonged low exposure and short high exposure have not been adequately studied. In this study it was elected to differentiate current smoking behaviour on the arbitrary basis of heaviness of current smoking habit rather than on averaged pack years. Further difficulties in interpreting average smoking and pill exposure are discussed below.

5.2 Prospective study

The major problems with this study related to study size. Patients were self selected on the basis of initially referring for treatment of their lesions and secondary self selection then

ensued on the basis of consent to colposcopy and biopsy. Although 59 women were recruited to the study, this reflected only a small proportion of women attending the various clinics with genital warts. Colposcopy was an invasive procedure and recruitment was difficult. The sample size made any statistical comparisons difficult and follow up data were confounded by the high drop out rate. This loss was to be expected in the study of sexually transmissible disease.

In contrast to the first study, it was felt justifiable in women presenting with genital warts to ask specific questions regarding sexual behaviour as advice regarding sexual behaviour and treatment of partner were offered. Data on duration of pill use and total cigarette exposure were also collected in this study. There were thus more detailed data in this smaller study. The data demonstrated no differences in sexual behaviour in the different histological groupings.

Another criticism was the lack of DNA analysis of the cervical tissue. It could be argued that DNA analysis of vulval warts did not necessarily correlate with DNA type in the cervix. This detracted from the major aims of the study which were to determine the differences between women with vulval warts who had cervical disease and to follow the course of those women who did not have cervical disease at presentation. The study had a practical aim, that of determining whether there were any criteria by which a clinical decision on the management of women with vulval warts could be individualised rather than the blanket approach suggested by Singer et al (1985) [416]. There were data from a number of studies which have looked at DNA types within individuals to suggest that the viral type in the external genital region would be that found in the cervix [131,223,350]. It was felt that viral typing of the vulval lesions would reflect viral type on the cervix. The role of viral types will be discussed more fully in chapter 6.

Section 6 : Risk to women with treated vulval warts.

The second study confirmed the risk of CIN in women presenting with vulval warts and reinforced the argument for screening these women at presentation. The mode of screening is controversial. To screen all women presenting with vulval warts by colposcopy would require considerable resource input for clinics in genito-urinary medicine. Logistically it would be easier to screen all by cervical cytology. However this study demonstrated a substantial false negative rate in women having smears of known abnormality under colposcopic control. Others have voiced concern over the false negative rate for cervical cytology and it could be argued that in such an obviouly high risk group, colposcopy would be more effective. Nevertheless, colposcopy is a traumatic experience for women [417]. Many of the lesions of the cervix will be mild. There has been some recent disquiet[418] over a blunderbuss approach to mild atypia and CIN 1 [416]. The decision on which screening procedure will ultimately be based on local, financial and political factors.

And what of those women treated for vulval warts and who have no cervical abnormality at presentation ? There are no guidelines. In the second study, it was alarming to find that 3 women cured of their vulval lesions, given specific advice about partners and who were aware of the potential risks, still developed CIN within 2 years. Even if all non-attenders were assumed to have remained normal, 10% of this group developed CIN in a very short time span. The numbers were inadequate to draw conclusions on particular risk factors which might identify those at particular risk. On the basis of such results annual cytological screening, similar to that recommended for women with treated CIN, would seem appropriate. Larger studies of such women, probably based on genito-urinary medicine clinics with colposcopy facilities, are urgently required to determine follow up policies.

CHAPTER 6

DISCUSSION : THE ROLE OF VIRUS IN GENITAL CANCER

Section 1 : The significance of human papillomavirus sequences in cervical carcinoma

1.1 Significance of viral type

HPV-16 DNA sequences are almost invariably found in invasive squamous cervical cancers (10/14) whereas HPV-18 is present in only one tumour. The ubiquity of HPV-16 in this geographical region is confirmed by Millan et al (1986) [205], who found evidence of HPV-16 DNA in 7 of 16 biopsies from CIN 2/3, including 1 case without histological evidence of HPV infection. Three biopsies from this group contain HPV-18 DNA. Subsequently, Murdoch et al (1988) [209] describe the presence of HPV-16 DNA in 14 of 21 biopsies of CIN. Only 1 case of CIN in these two studies contains HPV-6/11. Histological or cytological evidence of HPV infection is present in most of the CIN samples in both studies, and Cassidy et al (1988) [419] describe the presence of HPV antigens in a proportion of CIN biopsies with histological evidence of HPV infection. HPV-16 appears to be predominant in our geographical area in premalignant and malignant cervical tissue with little evidence of HPV-6/11. HPV-18 is rarely identified in invasive tissue: no more frequently than HSV-2 DNA. A significant role for this HPV type in our area cannot be substantiated on this data.

Other U.K. studies investigating malignant cervical disease show a high prevalence of HPV-16 (ref.225 5/6; ref.201 12/13; ref.229 27/42) in squamous cell carcinoma of the cervix. Similarly, although McCance et al (1983) [190] initially describe a number of cases of CIN with HPV-6 DNA present, other

studies from different areas of the U.K. [201,203] have infrequently identified HPV-6/11 in biopsies from CIN of all grades.

In two of these areas [190,201,420] the predominant wart virus infecting the vulva remained HPV-6.

The HPV types 16 and 18 are associated with malignant change and progression from mild to severe premalignant disease appears to be more likely if these viral types are present in the initial mild lesion [204,219]. Despite the high frequencies of high risk viral types, there is no evidence that cervical precancer or malignant disease behaves atypically in this country. The mortality from cervical cancer is comparable with other countries. There do not appear to be differences in age or stage at presentation. The cure rates from local ablative therapy in premalignant disease are comparable with other geographical regions [411,421].

Such data raises some questions on the role of particular viral types in the genesis of genital neoplasia. It is agreed that women with vulval warts are at considerable risk of CIN [402,403] and the data presented in chapter 5 confirms this. Vulval warts generally contain HPV-6 or 11 [190,194,201,420] although HPV type 16 is being increasingly recognised in overt condylomata [117,131,199,205,210-212]. It is argued that the viral type in vulval lesions may not reflect that of the cervical lesion or that mixed infections are common. Thus the cervix will be more susceptible to the more oncogenic viral types. Mixed infection does occur in a proportion of cases [201,206] of cervical HPV and in CIN.

There are marked differences in the proportions of the different HPV types found in cervical wart virus infection. HPV-16 is present in up to 40% of cervical HPV infection in the area from which the first study groups are drawn [205,209]. Other studies looking at DNA types in cervical HPV infection indicate that 35 - 63% of these lesions contain HPV-6 or 11 [131,206,216]. Only in Schneider's study [206] is the frequency with which HPV-16 is present in such lesions greater than that

of HPV-6/11. Studies examining DNA type within individuals with cervical and vulval lesions find a 60-90% concordance in viral type [131,206]. The majority of non-concordant samples contain mixed types. It therefore appears that a DNA type identified in vulval wart will be reflected in any pre-malignant lesion higher in the lower genital tract.

There is little doubt that HPV-16 and 18 predominate in CIN 3 [131,201,204,207,208,214] although this is not the case in premalignant tissue locally [205,209]. Nevertheless, in many studies a significant proportion of biopsies from CIN contain HPV-6/11 [202,206,218] including those from women with CIN 3. HPV-6/11 has been identified in malignant cervical tissue [194,198,199,422]. Progression and persistence of CIN is more likely if the original lesion contains HPV type 16 or 18 [204,218] although a significant proportion of HPV-6/11 containing lesions do progress [215,218,219]. Syrjanen's group, who have the longest experience in the follow up of cervical HPV infection, emphasise that HPV-6/11 identification in mild lesions does not mean that these lesions are to be regarded as low risk [215]. Local experience suggests that despite the very high prevalence of 'high risk' HPV types in both premalignant and invasive tissue, the cure rates and recurrence rates are comparable with other clinical centres. Follow up of women with invasive disease for up to 4 years does not demonstrate any differences in prognosis imparted by the presence or absence of viral DNA in tumours (see chapter 2, section 4). The significance of 'high risk' types remains 'not proven'.

From experimental studies, it was initially felt that HPV-6 or 11 could not bring about in vitro morphological change, either as whole virus or using only the E6/7 ORFs [423,424] even in the presence of activated oncogenes. This may be related to differences in RNA processing as suggested by Scheider-Gadicke et al (1986) [425] or differences in behaviour within the lcr [426]. However, Kasher and Roma (1988) [427] were able to transform rodent cells using an HPV-6b variant cloned from an

invasive vulval cancer. HPV-6 is then capable of in vitro transformation.

Even if a proportion of the geographic variation is due to interlaboratory variation [428], the inconsistency of these data and the geographic variation in viral types identified in viral infected, pre-malignant and malignant cervical tissue despite the relatively consistent biological behaviour argues against a specific role for particular viral types in the genesis of cervical squamous cancer.

The putative role of viral type is further complicated by recent data on adenocarcinoma. Traditionally thought to be uncommon, it now appears to be occurring with increasing frequency [429] and tumours of mixed squamous and adenomatous elements are probably underdiagnosed [235]. CIN is associated with these lesions [430]. There is no evidence of any link between sexual behaviour or sexually transmissible disease and these tumour types. Oestrogens may however be implicated [431] as may oral contraceptive steroids [432].

In the study of viral type, only one adenocarcinoma is included, and it contains HPV-16 DNA. Others also describe HPV-16 in adenocarcinoma [217,229]. However, other groups suggest that non-squamous tumours contain a higher proportion of HPV-18 [224,232,233]. Tase et al (1987) [233], examining a large group of non-squamous tumours using in situ hybridisation, demonstrate that, of 17 adenocarcinomas containing HPV DNA, only one contained HPV-16, the remainder having HPV-18. Of the 16 adenosquamous tumours positive for HPV DNA, 50% contain HPV-18 DNA. None of the squamous carcinomas examined by this group contained HPV-18 DNA. It therefore seems that histology plays a role in the relative frequencies with which HPV types are found.

The presence of HPV DNA in tumours of non-squamous origin itself requires explanation. HPV conventionally requires differentiating squamous cells to allow viral replication although vegetative virus may exist in basal layers. Its presence in columnar epithelial cells and in cancer subtypes

thought to have no relationship with sexual transmission raises many questions regarding the role of HPV in carcinogenesis and in the ability of the virus to infect different tissues. The finding of apparently integrated HPV-16 DNA in both endometrial adenocarcinomas studied is interesting. Although not confirmed in a subsequent study by Bergeron et al (1988) [433], de Villiers et al (1986) [434] did identify HPV DNA in endometrial tissue. Endometrial adenocarcinoma is not related to sexual behaviour, indeed the opposite is true, the tumour being more common in nulliparous women. There is no explanation for these findings and further work is needed to interpret the significance of HPV DNA in non-squamous malignant tissue.

The findings of this study, as in others, that HPV type 16 DNA is detectable in a high proportion of invasive cervical tissue, does not per se assign an aetiological role for this viral type. The weight of evidence, both in vivo and in vitro, is highly suggestive of such a role. There are, however, a number of epidemiological, biological and molecular biological contradictions which require more critical appraisal before a definitive causal relationship can be established. There is insufficient evidence to attribute higher risk to particular HPV DNA types and extreme caution is necessary in predicting the clinical course of pre-invasive disease from molecular biological data.

1.2 Significance of the state of viral DNA within tumour cells

In contrast to the conflicting studies into the cellular localisation of HPV DNA in pre-neoplastic biopsies, it is reasonably clear, using combined histology and in situ hybridisation with tritiated probes, that HPV genomes reside in neoplastic cells within invasive tissue [233,367]. As discussed earlier, the demonstration of

concordant HPV DNA in secondary deposits and primary tumour biopsies[207,226,232] re-inforces the likelihood that tumour cells contain the detected sequences. It remains unclear whether the state of the viral DNA itself within the cell is a significant factor in oncogenesis.

Wide variation in copy number and DNA pattern are reported by all groups studying the prevalence of HPV in genital cancer. It is conventionally thought that viral sequences are maintained as free episomes in pre-invasive tissue and that integration occurs in invasive tissue. Thus as HPV-6 and 11 are rarely integrated into cellular DNA, they represent a lesser risk of malignant progression.

This overview is controversial; the statement initially being based on the analysis of HPV-16 DNA in 2 mild dysplasias and 2 bowenoid papuloses of the vulva [436]. Invasive tumours contain only integrated sequences and the original sequences from which HPV-16 derives existed as 50% integrated and 50% oligomeric sequences. Although some groups confirm the impression that HPV-16 sequences exist in episomal form in CIN and integrated in invasive cancers [201,215,356] other groups are less clear [117]. Others [213,217] detect integration in all grades of pre-invasive disease and the presence of episomal DNA in invasive lesions. Meanwell et al (1987) [229] describes the vast majority of invasive tumours as containing free episomal DNA whereas Choo et al (1987) [436] suggest that all invasive tumours have integrated DNA. Smotkin and Wettstein (1986) [437] demonstrated the presence of HPV transcripts from a primary tumour and its nodal metastasis containing episomal HPV-16 DNA. Local data [205,220] is strongly suggestive that both HPV16 and 18 DNA can exist as integrated sequences in pre-invasive cervical biopsies.

In this study, hybridisation with HPV-16 under stringent conditions using undigested DNA (e.g.Figures 9 and 12) indicates that hybridisable sequences comigrate with high molecular weight cellular DNA. This suggests that HPV DNA is present either in concatemeric form or is integrated into host cell DNA. Analysis

of digested DNAs indicated that many specimens contained unit length 8kb viral DNA of varying copy number (e.g. Figure 10, Tu 34). It is presumed that as these sequences comigrate with cellular DNA in undigested hybridisations, they represent integration of tandem full length viral DNA into cell DNA. Other tumours (e.g. Figure 8, Tu 24; Figure 49, Tu 27) contain additional fragments not colinear with cloned linear HPV-16 DNA, some of which are larger than intact form III linear viral DNA. It is thought that these sequences represent integrated viral sequences at varying locations within cellular DNA. High molecular weight fragments probably indicate viral DNA and associated junction fragments. The copy number in this study varied from 1 to over 100 (Tu 28). Only one tumour in this series has undergone detailed analysis of a genomic library, the vulval carcinoma in situ Tu 28 [436]. Viral DNA in this tumour appears to be present as a head-to-tail dimer in an episomal configuration. Cloned inserts are 16kb, suggesting that exactly two genomes are present. Detailed restriction enzyme analysis also suggests that there may be a small deletion within the non-coding region (see Figure 1). This region is known to contain the origin of DNA replication and transcriptional control sequences.

The presence of complex patterns, not colinear with virion DNA following restriction endonuclease digestion, in conjunction with migration of hybridisation with high molecular weight cellular DNA following probing of undigested DNA, does not necessarily infer that sequences are integrated into cellular DNA.

It therefore appears that viral DNA sequences can be integrated or episomal in both pre-invasive and invasive tumour biopsies, and that episomal DNA present in invasive biopsies can be transcribed. It seems unlikely, on existing data, that integration is a necessary prerequisite of malignant change. Other papillomaviruses are known to function as autonomous episomes within transformed and tumour cells [145]. It may be that small defects within these episomes may result in alterations in function or expression sufficient to promote

cellular transformation. The deletion in the lcr of the HPV-16 episomes detected in Tu 28 may be interesting in this regard. Others describe alterations in the lcr in HPV-6 isolates from atypically aggressive tumours [347,348,439] and the HPV-6b used in the transformation of NIH 3T3 cells [427] had insertion sequences within the lcr region.

More detail of the precise state of viral DNA is required from both malignant and pre-malignant biopsies. It is possible that switch on of viral transforming genes could be achieved either by integration or by alterations in the lcr region in episomal viral DNA. Detailed analysis of invasive lesions with non-integrated HPV DNA might clarify this controversial issue.

1.3 Human papillomavirus DNA in histologically normal tissue

The previous discussion suggests that neither the type of HPV nor the state of viral DNA within the neoplastic cell are necessarily important factors in the genesis of neoplastic change in genital cancer. Is then the presence of papillomavirus alone in genital tissue necessary for malignant transformation ? To address this aspect it is necessary to examine the frequency with which HPV DNA is present in neoplastic and appropriately controlled non-neoplastic tissue.

HPV-16 sequences were present in one sample from a colposcopically and histologically normal cervix. These sequences comigrated with high molecular weight cellular DNA in a manner similar to that found in tumour DNA.

Such findings are consistent with other early studies [117,201,440] although Boshart et al (1984) [196] did not detect viral DNA in 15 normal samples tested. More recent larger studies using southern blot hybridisation confirm the impression that around 10% of normal cervices harbour detectable HPV DNA sequences [203,207,355]. The availabilty of filter in situ hybridisation [354] allows studies of larger populations. Schneider et al (1985) [200] indicate 2.7% of normal cervical

smears as harbouring HPV-16 sequences. With more detailed cytological and colposcopic data Toon et al (1986) [203] describe HPV-16 DNA in 9 of 104 normal samples. Other small studies tend to show similar findings [212]. The largest population study to date [230] describes HPV DNA in 9% of women with normal cervical cytology.

The significance of such findings is unclear. DNA positive women may represent a high risk sub-group within the normal population. Even so, if 9-10% of all women smeared harbour HPV genomes, only a small percentage of these will develop CIN. This would suggest that other factors may be as important in determining progression to malignancy. Long term surveillance of these apparently normal women will be required to answer this question. Alternatively, the finding of viral genomes in normal tissue may simply represent the false positive rate of hybridisation techniques. This is an aspect of the circumstantial evidence which has not been rigorously tested. In many studies, including this study, the numbers of tumours available for testing does not warrant statistical testing to exclude random chance association. Only the studies of De Villiers (1987) [230] and Schneider et al (1985) [200] lend themselves to such analysis.

Few studies of invasive disease systematically attempt to compare the frequency of HPV detection in women with invasive cervical cancer and normal women in sufficient number to warrant statistical comparison. Meanwell et al (1987) [229] found that 66% of tumours to contain HPV-16 DNA. 30% of cytologically, colposcopically and histologically normal control samples also contained HPV-16 DNA. Statistical analysis suggests that this does not represent a significant difference. If the groups are corrected for age then there are no differences in the HPV-16 DNA detection rates. Such data seriously questions the signifance of HPV DNA in cervical cancer tissue. Fuchs et al (1988) [207] found HPV-16 DNA in 9 of 102 normal biopsies, 1 of 33 biopsies of CIN 1, 12 of 43 biopsies of CIN 2, 64 of 140 biopsies of CIN 3 and 21

of 44 invasive carcinomas. There is no difference between the prevalence of HPV-16 in normal and CIN 1 tissue. There is a statistical difference in the prevalence between CIN 2 and normal tissue, although the 95% confidence interval of this difference includes an odds ratio for infection with HPV-16 of only 1.5. Much has been made of the poor control for sexual behaviour and other demographic variables in epidemiological studies of cervical cancer and HSV-2. There are no studies where the frequency of detection of HPV DNA in pre-malignant or malignant tissue has been compared with that of an appropriately matched normal control group. Recent data using the highly sensitive polymerase chain reaction to detect HPV sequences in cytological specimens from women with CIN and normal women, has suggested that 70 to 84% of entirely normal women have detectable HPV-16 DNA in cervical cells [441,442]. This is at a level which would argue that HPV was a commensal organism rather than a pathogenic virus. If this is confirmed, then the role of HPV will require complete re-assessment.

It has always been difficult to recruit appropriate control women in studies where matching for sexual behaviour is necessary. One alternative is to examine tumour and normal tissue within patients. The patient then controls for all confounding variables. Few studies examine this approach in genital cancer. Lehn et al (1985) [356] state that non-malignant tissue from 2 cervical tumours, positive for HPV-16 DNA, do not contain viral sequences. Tsunokawa et al (1986) [357] collected internal control tissue for cervical tumours comparable with this study. Three tumours contain HPV-16 sequences. One of the control samples also contains HPV-16 sequences. The marked difference in copy number between tumour and control tissue led them to conclude that this might represent contamination. In the current study, collection criteria are more rigid, avoiding potential contamination. In some instances, internal control tissue from tumours having low copy number DNA are positive. This makes contamination unlikely. In one instance (Tu 27 and

Co27), the restriction pattern of viral sequences differ. The most comparable study is that of Di Luca et al (1986) [217] who examined both cervical and vulval tumours. No details are given as to the precise site of the internal control tissue, although it appears to have been normal tissue adjacent to the tumour. Internal control tissue is available from only 2 cervical tumours but from 6 vulval tumours. None have detectable viral DNA.

The data presented in the current study clearly demonstrates that although HPV-16 DNA is frequently present in invasive cervical and vulval cancer tissue, it is present in normal tissue from all but three patients. It can only be concluded that HPV-16 DNA is more likely to be present in DNA extracted from any tissue in women with cervical or vulval cancer than in women without cancer. HPV-16 DNA is detectable as frequently in non-malignant tissue as in malignant tissue. An aetiological role for HPV-16 in genital cancer is not substantiated by this study. Even if all studies of invasive disease where internal control tissue is available are grouped together, no statistical difference can be demonstrated between the detection rate of HPV-16 DNA in malignant and non-malignant tissue in the same women. The data suggests only that women with lower genital tract malignany are more likely to harbour HPV DNA in their tissues than unmatched women without such disease.

More information is available from pre-invasive disease as it is simpler to collect adjacent normal tissue for comparison. Ferenczy et al (1985) [211] demonstrate that recurrence of condylomata is more likely if viral genomes are detectable in histologically normal tissue adjacent to the treatment margins. Such DNA is detectable in 45% of 20 cases. These findings raise many questions regarding treatment of papillomavirus lesions. Follow up of women in this study does not detect any increase in tumour recurrence amongst those with viral sequences distal to the primary tumour.

Nevertheless other groups [131,336] demonstrate that a high proportion of histologically normal tissue adjacent to both CIN

and VIN contain HPV DNA. In neither of these studies are the samples strictly paired for comparison. Murdoch et al (1988) [209], describe paired biopsies from colposcopically normal and abnormal areas of the cervix. Although only 15 of the 27 'control' biopsies are histologically normal, 9 of these contain HPV-16 DNA. It is not possible to precisely pair these results but HPV-16 DNA is detected in 17 of the 27 colposcopically and histologically abnormal tissues.

It therefore appears that viral DNA sequences are as commonly found in tissue adjacent to and distal to pre-malignant and malignant tissue as in the neoplastic tissue itself. Viral infection alone is insufficient to evoke morphological change. Such changes may require focal breakdown of local immune response within a field of viral infection as proposed by Reid et al (1987) [131]. Abnormalities in local immmune response have been noted within pre-invasive cervical disease [443,444] and recent data proposes that exposure to cigarette smoking may be an aetiological factor in these alterations [315].

The high frequency with which HPV genomes are detected in tissue distal to invasive disease, especially when such tissue is thought not to be permissive for papillomavirus infection, is however, not explainable by such a hypothesis. The conclusion from this study does not support a specific role for HPV-16 in invasive genital cancer. It was almost 20 years before appropriately controlled sero-epidemiological studies finally demonstrated that women with cervical neoplasia were not exposed to HSV-2 infection more commonly than normal women [49]. Appropriately controlled studies of the prevalence of HPV DNA in women with pre-invasive and invasive disease and women with histologically normal cervices are still awaited some time after the initial demonstration of an apparent link between HPV and cervical cancer. Experimental data at molecular level, discussed further in section 3, however detailed, does not necessarily confer aetiological status to HPV. There is a wealth of molecular data on the oncogenicity of HSV in culture systems but

it appears unlikely that it is a significant factor in the aetiology of cervical cancer.

Section 2 : The significance of human papillomavirus DNA sequences in vulval cancer.

80% (8/10) of vulval tumours demonstrate hybridisation to HPV-16 DNA. Seven of these are invasive squamous cancers, the eighth an in-situ cancer. This latter tumour also shows evidence of hybridisation to HPV-18 DNA. There are no comparable local data of pre-malignant vulval disease and no other studies of invasive vulval carcinoma in the U.K. McCance et al (1985) [350] in their analysis of 5 cases of multifocal genital neoplasia, describe combined infection with both HPV-6 and 16 in all areas of vulval intra-epithelial neoplasia positive for viral DNA. These data suggest that HPV-16 is present in high frequency in vulval tumours in the U.K.

The data on prevalence of viral type in vulval cancer is more varied than that for cervical cancer. European studies [119,195,196,217] find HPV-16 DNA infrequently in invasive vulval cancer and HPV-18 DNA is absent. HPV-16 DNA is however frequently identified in pre-invasive biopsies [217,228,353]. Previously, only Beaudenon et al (1986)[228] have reported the isolation of HPV-16 DNA in invasive vulval cancer in Europe in any frequency (63%). The current study reports the highest prevalence of HPV-16 in invasive vulval cancer.

North American studies have given more conflicting results. It appears that HPV-16 predominates in pre-invasive disease [131,211,222]. HPV-18 has been rarely identified as the sole HPV type in either pre-malignant or malignant tissue except in the large study of Kaufman et al (1988) [336]. More importantly, HPV-6 is identified frequently in high grade pre-malignant and in invasive tissue [199,351,352]. Variant HPV-6 DNAs have been cloned from atypically invasive vulval tumours [348,427,439] and

one of these [427] is capable of oncogenic transformation of rodent cells.

Reference has already been made to the state of viral DNA in genital cancer. There is little data on this for vulval lesions. It appears that hybridisation to vulval tumour DNA migrates with total cellular DNA (see Figure 12) in this study, suggesting integration. As previously discussed, detailed analysis of the in situ cancer, Tu 28, indicates that viral DNA is in episomal form.

All internally controlled tissue studied contains HPV sequences. Reference has already been made to the frequency with which normal tissue adjacent to pre-invasive vulval neoplasia is found to contain HPV sequences [131,211,336].

The same problems in interpretation of the significance of HPV DNA sequences elucidated in cervical cancer, namely viral type, state of viral DNA, and presence of sequences in internal control tissue, apply to vulval cancer. It does appear at least that the HPV type commonly associated with vulval warts, can be found with reasonable frequency in neoplastic vulval tissue, although this is not the finding of this study.

It is attractive to speculate, in light of the finding of similar high prevalences of HPV-16 in cervical and vulval cancer within one geographical area, of common mechanisms bringing about morphological change. Vulval cancer has a different epidemiology from cervical cancer; it is not thought to be sexually transmitted, although there are associations with sexually transmissible factors (see chapter 1, section 10.1). The differing epidemiology and the differing distributions of HPV types found in vulval neoplasia advise caution in the interpretation and extrapolation of molecular data obtained from studies involving cervical carcinoma. The age distribution in vulval cancer strongly suggests that other and possibly different cofactors may act with HPV of varying types to produce premalignant and malignant change. In one interesting experimental model [445] in the rodent *Mastomys natalensis*, papillomaviral genomes persist, untranscribed, in various

tissues. The genome copy number increases with age. Invasive tumours of the skin appear with ageing and this correlates with the onset of viral transcription. Chronic treatment with tumour promoter accelerates this process. Such a system is a close model for vulval cancer but must involve different mechanisms to those interacting with HPV in the genesis of cervical cancer. It may be that HPV is capable of prolonged persistence in vulval skin and that varying tumour promoters, possibly including HSV, may either activate E6/7 transcription directly or cause alterations in the non-coding control region such that cellular suppression of viral expression is overcome.

TABLE 28

HPV INFORMATION IN CERVICAL CELL LINES

NAME	TYPE	CHROMOSOME SITE	DNA ARRANGEMENT/ INTEGRATION SITE	RNA TRANSCRIPTS	PROTEIN SYNTHESIS
HeLa	18	5,8,9,22	Deletion E2/4/L2	Multiple,E6/7/L1	E6,E6*, E1,E7
756	18	12	Probable genome E2 integration	Multiple,E6/7/L1	E6,E6* E1,E7
C4-1/II	18	8	Deletion E2/4/L2	Multiple,E6/7/L1	E6,E6* E1,E7
Me180	18	-	<1 copy per cell	None detectable	-
MS751	18	-	Deletion E2/4/5/ L1/2	Single 1.5kb	-
SKG-I	18	-	Probable entire	Lcr,E6/7,L1/E1	-
SKG-II			Integration E2/4/5		
HT-3	none detected				
C33-1	none detected				
CaSki	16	8,12,20	Tandem repeats Entire genome Integration E2	E2,E6/7 +others	E4,E6,E7
SiHa	16	13	Head to tail, IntegrationE2/4 E2 deleted	E6/7+others	E4,E6,E7
SKG-III	16	-	Deletion E2/E4 E5/L2/E1	Mainly E6	-
QG-U	16	-	Entire genome Integration E2/4/5	Multiple	-
QG-H	16	-	Entire genome IntegrationE4/5/L2	Multiple	-
Cx106	16	-	-	-	
Hx151c	16	-		Multiple,E6/7	-
Hx155c	16		Deletion E1/2	Multiple,E6/7	-
Hx156c	16	-	Complex pattern	Multiple,E6/7	-
Hx160c	16		Complex pattern	Multiple,E6/7	E6

Section 3 : Human papillomaviral sequences in cell lines derived from genital cancers.

3.1 Viral DNA in continuous cell lines

Much of the difficulty in establishing the role of HPV in genital cancer might be alleviated by examination of the interplay between viral sequences and cellular function in model systems.

Many of the existing cervical cell lines have been examined for the presence of HPV DNA [196,358,370-374]. Table 28 summarises the main findings. In all, only 2 cell lines, 33-1 and HT-3 [371] do not contain identifiable HPV DNA, although it has been suggested [370] that C33-1 cell DNA does faintly hybridise with a combined HPV-16/18 probe. In these studies, on long exposures there is a similar suggestion of faint hybridisation to HPV-16 (see Figure 50). This cell line may contain an unrelated HPV type. Of 19 other cervical carcinoma cell lines, 11 have identifiable HPV-16 DNA and 8 HPV-18 DNA. In all cases DNA is thought to be integrated into cellular DNA. The quantity of viral genomes present in these lines varies from 1-2000 genome copies per diploid cell. The integration pattern of viral DNA varies from single copy intact inserts (e.g.CaSki, ref. 370) to multiple insertions on different chromosomes (e.g.Hela, refs. 372,446).

Only the A431 vulval cell line has been examined for the presence of viral sequences and none were found [371]. A keratinocyte cell line derived from a bowenoid papulosis, a premalignant vulval lesion, which is tumorigenic in *nude* mice is described as containing integrated HPV 16 sequences and these are expressed [401]. Many of these cell lines are being used extensively to determine the function of viral DNA in genital malignancy.

As outlined in chapter 4, there are several potential difficulties in the use of existing cell lines and further, better defined lines were derived.

Both the derived cervical and vulval cell lines described contain DNA homologous to HPV-16 DNA. Tu 22-1 and 22-2 cells contain viral DNA in an similar complex pattern with bands at molecular weights greater than intact viral DNA, suggesting viral-cellular fragments. These cell lines are derived from the same biopsy and it is interesting that the pattern of viral integration is so similar in each. Two other cell line groups are derived from a single biopsy, the C4-1 and 2 lines [381] and the SKG 111a and b lines [390]. The C4 cell lines contain HPV-18 DNA in an identical pattern and the SKG 111 lines, though morphologically quite different, contain HPV-16 DNA in identical configuration. Evidence of hybridisation of HPV-16 to DNA extracted from the C4-1 cell line is seen (see Figure 50). In the main study some cross hybridisation of HPV-16 and 18 occurred even under stringent conditions (see figure 13b). This may be the explanation in this instance.

The significance of viral DNA in established cell lines requires confirmation before data obtained from these are extrapolated to intact tumours. No existing cell line has parent tumour tissue or normal tissue from that patient for comparison. The cervical tumour (Tu 22) contains HPV-16 sequences but unfortunately the amount of DNA extracted is insufficient for detailed analysis of the integration pattern. However HPV-16 DNA is present only in malignant tissue in this case as no detectable viral DNA is present in normal tissue from this woman. Expression of viral function in the Tu 22 cell lines may be more confidently assumed to be related to carcinogenesis than in existing cell lines even if of low passage [373].

Viral DNA in the vulval line Tu 31 appears to be as a single insert of intact DNA. This configuration differs from that in the parent tumour DNA where three molecular weight bands are seen. The significance of this difference is unclear. Matlashewski et al (1987) [447] describes a similar phenomenon in

3T3 cells transformed by HPV-16 DNA linked to a heterologous retroviral promoter. The pattern of viral DNA integration in the initial transformed lines and tumours produced by injection of these cells into *nude* mice differs. The cell lines contain integrated single original insert DNA plus rearranged viral-cellular DNA fragments of varying molecular weights. Tumours produced from the cells contain only original single insert DNA. However, Morgan et al (1988) [165] in transformation experiments with rodent C127 fibroblasts demonstrate similar complexity of integration pattern in cell line and tumour DNA. Some type of cell selection will have occurred in vitro but it appears that selection or rearrangement of viral sequences has also occurred during the selection process. Such selection in viral DNA configuration may suggest that the analysis of the sites of integration and the inferences from the configuration of ORFs in cell line DNA may not necessarily be applicable to an in vivo tumour and that results so obtained must be regarded with caution.

3.2 Integration of viral DNA in cell lines

Detailed restriction fragment analysis of integrated viral DNA has suggested that common to all cell lines studied, DNA fragments containing the intact open reading frames (ORFs) of the E6 and E7 genes, the non-coding long control region (lcr) and usually the complete ORF of the L1 region are conserved [370-372,448-450]. This applies to both HPV-16 and 18 containing cell lines. In cell lines where the site of integration has been determined, disruption of the E1, E2 or E4 ORFs is consistently reported [370,374,401,425,437,448].

Similar analysis of intact tumour tissue is less conclusive. Lehn et al (1985) [356] examined the site of viral integration in 4 HPV-16 containing tumours. The opening site for viral integration was different in all 4 tumours (namely E1;E4/5;L2;E5), suggesting random integration. Matsukura et al (1986)[364] cloned viral DNA from a cervical cancer biopsy.

Restriction fragment analysis demonstrates that cellular DNA interupted the viral DNA within the E1 ORF and that E2,4,5 and part of L2 are deleted. Smotkin and Wettstein (1986) [437] describe gene expression in a tumour biopsy, but viral DNA is not integrated in this tumour. The significance of viral products in this case remains unclear. Entire genomic HPV-16 DNA is found in a number of tumours [451]. Detailed analysis of 6 tumours containing HPV-16 DNA [436] demonstrates that, although integration patterns were all different, 11 of 14 integration sites lay within the E1/2/4/5 ORFs. The E2 region is invariably lost. The E6/7 and LCR regions are invariably retained.

No consistency has been demonstrated in the site of integration into the cellular genome. Integration of HPV-18 in HeLa cells appears to occur in chromosomes 5, 8, 9, and 22 [446] whereas it occurs at a single site on chromosome 12 in SW756 and on chromosome 8 in C4-1 cells [452,453]. The chromosome location of HPV-16 in SiHa cells is 13 [449,453] whereas CaSki cells have multiple sites on chromosomes 8, 12 and 20 [453]. Interestingly, human fibroblasts with a specific deletion of the short arm of chromosome 11, can be transformed to anchorage independence by HPV-16 alone, without retroviral promoters [454]. This reinforces the possibility of gene products from this chromosome being involved in genital malignancy as acquisition of a single normal copy of chromosome 11 is known to suppress the malignant phenotype of HeLa/fibroblast hybrids [455]. This suggests that at least one of the cellular control mechanisms may be located on this chromosome. Karyotypic analysis of the cervical cell lines Tu 22-1 and 2 shows trisomy of this chromosome and the normal complement is present in Tu 31 cells. The integration pattern is not known.

It therefore appears that random integration into a number of chromosomal sites may occur in both HPV-16 and 18 containing cell lines. Some of these sites in some cell lines, notably the HPV-18 containing HeLa and C4-1 lines, are related to known proto-oncogene positions in the genome [453]. It has been shown that there is elevation of c-myc transcription in these cell lines

compared to those where the integration site is on different chromosomes. Overexpression of c-myc in cervical cancer has been linked to poorer prognosis [456] although there is no apparent relationship between c-myc expression and either HPV type or copy number in tumour biopsies [457]. It is interesting to speculate, in view of the co-operative effect of proto-oncogenes and HPV in transformation experiments, that chromosomal sites of integration may not necessarily be as random as they appear.

The relative uniformity of viral integration site, and the almost invariable retention of the E6/7 ORFs and lcr does suggest that particular configurations may be required to allow expression of the E6/7 transforming region or to allow alterations in its control by cellular or extraneous factors.

3.3 Viral expression in continuous cell lines

In all cell lines examined except ME180 line, irrespective of the type of viral DNA present, RNA is transcribed. Transcipt size varies although commonly includes a 1.5kb transcript. Analysis of RNAs produced has shown that the major transcripts derive from the E6, E7, and L1 genes [372,373,425,437,448,450,451] with E7 transcripts being quantitatively greatest. Other ORFs are transcribed in some lines. Again data from tumour biopsies is less convincing. Lehn et al (1985) [356] detected HPV-16 transcripts in only one of four tumours. Smotkin and Wettstein (1986) [437] were able to detect 2 major transcripts in a cervical cancer recurrence which coded for the E2, E6 and E7 ORFs although the transcripts do not co-migrate with the transcripts from the CaSki cell line. Takebe et al (1987) [451] has shown that E6 transcripts are present in cancer tissue but other regions are transcribed. Tsunokawa et al (1986) [357] detected poly-A transcripts in 2 of 4 HPV positive tumours although they state that RNA degradation may have been a problem in the 2 negative tumours. Di Luca and colleagues [458] could not detect E6 polypeptide in invasive or preinvasive lesions.

The major polypeptides coded by the putative transforming region have been characterised. Smotkin and Wettstein (1986) [437] identified the E7 protein of HPV-16, a cytoplasmic phosphorylated protein of short half-life frequently associated with other proteins [459], suggesting a regulatory function. This protein is detectable by immunoprecipitation from both the CaSki and SiHa cell lines, known to contain HPV-16 sequences, and not from the HeLa cell line. These polypeptides are also present in cervical biopsy material. The E6 protein of HPV-16 [161] is detectable in the same two lines and in an HPV-16 transformed line [460]. Banks et al (1987) [461] describe the presence of an HPV-18 specific E6 protein in HeLa cells and of an antigenically distinct HPV-16 derived E6 protein in SiHa cells. A number of studies have now confirmed the presence of E6 and/or E7 in HPV-16 containing cell lines [373,462,463] and the presence of E6 protein in HPV-18 containing lines [365,462]. Some other polypeptides have been identified in some lines [462].

Despite the similarities in DNA sequence, it is clear that the putative transforming proteins of HPV-16 and 18 are different. It has been suggested that the E6 and E7 proteins of the varying papillomaviruses have evolved from a common 33 amino-acid peptide by duplication [464]. Throughout the sequence there are regular repeats of a Cys-X-Cys-X-Cys tetrapeptide. This and other structural considerations has led these workers to conclude that these different proteins may form nucleic-acid binding domains. Grossman et al (1988) [465] have subsequently demonstrated that the E6 protein of HPV-18 is a small lysine-arginine rich polypeptide capable of DNA binding. This binding is non-specific and they note the poor conservation of this protein in different HPV types. It is therefore likely that there may be a group of proteins of similar properties linked to transformation, and this may explain the differing ORFs involved in transformation by BPV, HPV-8, and HPV-16 and 18.

Recent studies have demonstrated that specific inhibition or down-regulation of HPV transcription in cancer cells will alter cellular growth [466,467]. Inhibition of transcription of the

E6/7 ORFs reduces the detectable E7 protein in the HPV-18 positive C4-1 cell line, using transfection with plasmids containing antisense E6/7 RNA, and is associated with a reduction in cellular proliferation and clonal growth in agar. This suggests a direct effect of a viral protein in driving cell proliferation in a long established cell line. Suppression of HPV-18 transcription in non-tumorigenic HeLa/keratinocyte hybrids using DNA methylation inhibition, results in cessation of cellular growth. Similar cessation of growth is seen in keratinocytes immortalised by HPV-16. However, no effect of this treatment is noted in established SiHa cells or tumorigenic HeLa hybrids. There is therefore direct evidence that alterations in HPV transforming functions will affect cell growth in genital cancer cells.

3.4 Summary of evidence

In summary, examination of existing continuous cell lines has shown that virtually all contain HPV DNA sequences. These are transcribed and the predominant RNA species appears to be that of the E6/E7 region. Polypeptides coded by these sequences have been identified in cell lines.

There appears to be consistency regarding the viral break point at which sequences are integrated into cellular DNA although the site of integration in cellular DNA varies widely. Sequences equivalent to the E6/E7 and lcr regions appear to be retained.

Finally, selective alteration in expression of HPV transcripts in HPV DNA containing cell lines is associated with alterations in cellular growth and cell division. The case for a direct aetiological role at the molecular level would seem to be watertight.

Nevertheless, there are a number of inconsistencies which make such claims currently uncertain.

The proportions of cell lines containing HPV-16 and 18 differ substantially from those found in biopsy specimens. As discussed previously, HPV-18 is associated more closely with adenocarcinoma

of the cervix than with squamous carcinoma and a relationship of this tumour type with sexual behaviour sufficient to implicate a sexually transmissible virus in its aetiology remains to be shown. Conclusions extrapolated from data on HPV-18 containing cell lines may not necessarily apply to squamous carcinoma in vivo.

Equivalent data from tumour biopsy material is less conclusive for integration site, viral break points and viral expression and it cannot be assumed that cell line conclusions can be directly transferred to a clinical situation.

The atypical biological behaviour of tumours from which cell lines are derived (discussed in chapter 4) further restricts the confidence with which in vitro results can be translated to in vivo.

Much is made of integration site and common break points in viral DNA. However there is no consensus that such integration is a prerequisite for oncogenic change, as discussed in section 1 of this chapter. Many invasive lesions contain episomal DNA and preinvasive lesions integrated DNA [217,229,437].

Finally, there remains a lack of appropriate control data. The studies presented in chapter 4 represent the only attempt to date to derive cell lines where the state of viral DNA is known in both normal and tumour tissue in the parent individual. Under these circumstances the demonstration of different integration patterns in Tu 31 cells compared with Tu 27 tumour biopsy serves to emphasise the problems in interpretation of cell line data.

Section 4 : Herpes simplex virus and human cytomegalovirus in genital cancer

This study confirms the paucity with which HSV DNA sequences are identified in cervical cancer using southern blot methods [69,113-117]. The sequences identified are similar to those previously noted as the HindIII a region includes the BglII n sequences. The frequency of such sequences is consistent with the larger study of Rotola et al (1986) [118] who identified the BglII n fragment in 6 of 64 cervical tumours. Subsequently, Di Luca et al (1989) [458] have described the detection of sequences homologous to the BjlII n or BamHI e region in 29% of invasive cervical cancers.

This study is the first to describe the finding of HSV specific DNA sequences in vulval cancer [120], again identifying the region HindIII a (0.512-0.718). Subsequently, Manservigi et al (1986) [119] have demonstrated the presence of the BglII n and BglII o (0.38-0.42) regions in 2 of 4 invasive cancers of the vulva. These two regions appear to be linked within the same cellular DNA fragment. This group also detected DNA homologous to BglII n and part of the BglII o region in one of two vulval dysplasias.

Although rarely found in either malignancy, sequences identified consistently correspond to those associated with malignant transformation of rodent cells in vitro [64-67]. Viral antigens encoded within the BglII o and j regions have been identified in a number of genital tumours [104-106,335,337,458]. Where comparative study of polypeptide expression by HPV and HSV has been carried out [458], the ICP10 protein (encoded within the MTRIII region, 0.42 - 0.58) is identified more frequently than either HPV-16 capsid antigen or E6 polypeptide. However, the 38K polypeptide identified within the MTRII region, and which represents the small subunit of ribonucleotide reductase, has not been identified in tumour biopsy material using immunoperoxidase staining [105]. A polypeptide of this molecular weight has been

immunoprecipitated from the sera of women with cervical cancer [70]. This polypeptide, however, is not the small subunit previously identified. In situ hybridisation detects RNA sequences consistent with the BglII o and j regions in the majority of biopsies where hybridisation is detected [105,109,111]. Hybridisation to sequences from the BglII n region is infrequently seen and when present is at very low levels [109].

Transformation of rodent cells in vitro is not associated with retention of detectable viral DNA at high copy number [64] and in some instances viral DNA is not detectable by DNA-DNA hybridisation [66] although intact virus may be recovered by superinfection with temperature sensitive mutants [464]. Nevertheless these transformed cells form invasive and metastatic tumours which are fatal to laboratory rodents. Although it is attractive to find that a particular virus transforms a normal cell, is retained within the transformed cell and is functionally required to maintain the transformed phenotype as is the case for SV40 and Ad2, such a system is not invariable. The highly oncogenic virus adenovirus 12 does not necessarily maintain its DNA in tumour cells [469]. BPV-4 is required for oncogenic change in the bovine alimentary tract yet is absent from malignant tissue [142].

The development of pre-malignant and malignant change is the end result of complex actions and interactions. All cervical and vulval cancers are not necessarily the result of infection with a single virus family.

The effect of each viral type on different genital sites must be different as the epidemiology of the two tumours is markedly different. The metaplastic epithelium of the cervical transformation zone may be susceptible to malignant change by any sexually transmissible virus which is capable of transforming cells. Thus although infection with HPV may be the predominant event in the majority of tumours, a similar end point could be reached by infection by HSV-2 or by HCMV. Although we did not identify HPV and HSV DNA within the same tumour biopsy,

suggesting a role for HSV perhaps in HPV-free tumours, others [458] have identified both DNA types within single tumours. Similarly in vulval carcinoma in-situ, Kaufman et al (1988) [336] demonstrated the presence of putative HSV polypeptide and HPV DNA in the same biopsies, although there is no DNA data on HSV available.

Serological studies in pre-malignant disease suggest a slight risk attributable to previous CMV infection. It is interesting that the finding of DNA from the putative transforming region of CMV in CIN biopsies reported by Fletcher et al (1986) [263] should occur in women attending the same colposcopy clinic in which a small relative risk of CIN due to previous CMV infection was seen. Despite the paucity of support for CMV as an aetiological agent in cervical cancer described in chapter 1, section 8.1, a role for this virus cannot be excluded within the population presented in the current studies.

The possible mechanisms by which herpesviruses may bring about oncogenic change are described fully in chapter 1, section 4.2. The potential effects of herpesvirus infection on cellular control mechanisms by way of biological mutagenesis, amplification of cellular DNA sequences, and quantitative alterations in host protein synthesis may in themselves promote oncogenic change, or produce a cellular milieu in which other carcinogens, viral or environmental, may finally produce uncontrolled cellular proliferation. These effects may differ in different epithelial sites.

Although viral nucleic acid is infrequently retained in invasive genital cancers, and prospective sero-epidemiological studies have shown no association between previous infection with herpesvirus and the development of cervical cancer, a place in the cascade of events required to transform benign squamous epithelial cells into invasive malignancies cannot be excluded. Recent molecular biological data [458] and epidemiological data [52] demand that it is not excluded from the scene.

Section 5 : Non-viral factors in genital cancer

It is clear that not all women infected by HPV of whatever type develop genital cancer. Even at the experimental level, HPV-16 and 18 alone are insufficient to induce full oncogenic change in human cells. Other factors are therefore likely to be of equal importance.

5.1 Oral contraceptive usage

Differences in oral contraceptive usage are apparent in the cross sectional study. Both CIN groups have a higher proportion of ever users than the control group, with the HPV group occupying an intermediate position. The relative risk of CIN for ever pill users is 2.0 compared with never users. This is similar to the risk described by Peritz et al (1977) [290] who also used previous genital infection as an indirect assessment of sexual behaviour. It is also comparable to the relative risk stated by Harris et al (1980) [293], 1.75, for exposure of 5-9 years and this study controlled for a number of sexual variables. No data are available in the cross-sectional study for duration of pill use and sexual behaviour has been inferred from indirect measures.

Although the proportion of ever users in the HPV group does not differ significantly from either the CIN or normal groups, the relative risk of cervical HPV for ever users is 1.7. The odds ratio comparing pill use and CIN or HPV is virtually unity (1.17). Oral contraceptive usage therefore imparts risk of histological cervical abnormality, both established CIN and HPV infection. The association is not strong given the confidence intervals but the lack of difference between HPV and CIN groups in the study would argue that these steroids are more likely to be involved in promoting or establishing cervical infection with HPV rather than affecting the progression from HPV to CIN.

This speculation is partly supported by examination of the age/pill associations. Detailed assessment of pill usage demonstrated an age difference between women with either CIN or HPV infection who are ever users and those who were never users. This age difference does not occur in the control group. There are similar differences in parity but this difference also occurs in the control group. There are no other differences and in particular no differences in the distribution of severity of CIN. Histological change occurs in many women never exposed to oral contraceptives. It may be that such exposure might allow earlier establishment or persistence of HPV in the cervices of young women.

With a working hypothesis that oral contraceptive steroids may predispose the cervix to HPV infection, the second study, analysing in more detail the differences between women with and without histological change in the cervix after known exposure to external HPV, may be expected to demonstrate marked differences in pill usage. The contrary is true. Those women with established CIN at first assessment have the least exposure to oral contraceptive steroids in terms of mean duration of use. Even if all women with histological change are combined, there is no difference in the mean duration of pill use (3.1 cf 3.0 years). Precise conclusions from this information are difficult. Of the 59 women enrolled into the study, only 8 had never used oral contraceptives. These are evenly distributed between the three histological groups. Women with abnormal histology are significantly younger in this study compared with the initial study. Although there is a wide standard deviation in age results, there is a 3 year age difference between women with CIN and those with a normal cervix at initial examination. Between all histological abnormalities and the normal group there is a 2 year difference (mean 23.8). Thus the mean age at which oral contraceptive steroids were commenced may be different in the abnormal and normal groups. The small size of the study precludes any meaningful conclusion, but such a difference in the mean age at start of exposure may be critical. The cervix may be

more susceptible to the changes brought about by oral contraceptives at a younger age. Similar durations of use commencing at different ages may result in different changes or time course of changes, thus subtly altering the ability of the cervix to deal with HPV infection once established.

How such interaction occurs is speculative. Older experimental data suggests that oestrogens alone could induce cervical changes. Morphological changes induced by oral contraceptive steroids may produce a situation conducive to HPV infection.

At the molecular level, there are now some data to support interaction between steroid hormones and HPV DNA. The lcr region of HPV-16 contains sequences with a large degree of homology to the consensus glucocorticoid-responsive element sequence [470]. These sequences are responsive to dexamethasone. Subsequently, Crook et al (1988) [423] demonstrated that the combination of HPV-16, its own promoter region and the activated oncogene, EJ-ras, can only achieve oncogenic transformation of primary rodent cells in the presence of either dexamethasone or progesterone. Continued cell growth is possible only in the continued presence of these hormones. These results have been confirmed separately by Pater et al (1988) [424] who were unable to transform under similar conditions using HPV-11, which has similar functional hormone responsive sequences.

A role for progesterone has not been proposed in cervical cancer although Kaminetsky (1966) [277] has suggested that progesterones can cause the development of cervical tumours in mice pre-treated with chemical carcinogens. Most early studies suggest a protective effect in experimental animals [278]. Neither of the cervical cell lines described in these studies contain detectable progesterone receptors.

Notwithstanding the molecular studies, epidemiological data remains unconvincing. Early work is plagued by poor control for differences in sexual behaviour between the groups (see ref.285) and those studies controlling partly or fully for such variables are unable to demonstrate any significant differences [285-287]. Recent studies [293,294] suggest a dosage effect of the pill in

CIN and the most recent analysis of the long term RCGP oral contraceptive study [471] supports this hypothesis.

One other possible relationship requires further investigation. It appears that adenocarcinoma of the cervix in increasing in frequency [429], especially in the younger age groups [432]. There have been suggestions that this is pill related [472]. HPV-18 appears to be more strongly associated with cervical adenocarcinoma [233] and it may be that this viral type interacts with these steroids in a predictable fashion.

The analyses presented here serve to demonstrate the difficulties in assessing exposure to oral contraceptives, although suggesting that its action may be in allowing establishment of HPV infection. The relationship between oral contraceptive steroids and CIN, if it exists, is likely to be complex and mean age at exposure as well as gross exposure may need to be taken into consideration.

Further detailed work at both epidemiological and molecular level is required to clarify these issues. Epidemiological studies must take cogniscence of morphological effects of these steroids and the possible effect of exposure by age. Further studies into the role of progesterone and oestrogen in the modulation of HPV gene function will be required to complement those epidemiological studies proposing a role for oral contraceptive steroids.

5.2 Cigarette smoking

There are significant differences in the relative proportions of non-smokers and smokers in the CIN and HPV groups in the cross-sectional data compared with women with normal cervical cytology. 53% of women with normal cytology are non-smokers compared with just over 30% of the other groups. Although there are no differences in the proportions of smokers and non-smokers between the two main abnormal groups, women with CIN who smoke are significantly heavier smokers than those with HPV alone who smoke.

Non-smokers tend to be older than smokers, except within the HPV alone group. No differ ences are evident in serology, pill usage or severity of CIN between smokers and non-smokers within groups.

The crude relative risk of CIN attributable to smoking in the cross sectional study is 2.57. There is some suggestion of a dosage effect, with the risk for heavier smokers being 2.75 and for light smokers, 1.97. This order of risk is confirmed in the initial data collected during the prospective study, with the crude relative risk of abnormal histology in smokers being 2.13 for women presenting with vulval warts. These data are consistent with the relative risk stated by Harris et al (1980)[293], of 2 for smoking over 15/day. No real difference in the relative risk occurred in heavier smokers. In the two other important studies, Trevatham et al (1983) [309] indicated a higher relative risk, of the order of 2.4-3.6 and did demonstrate a dosage effect with the relative risk of carcinoma-in-situ being 2.4 with 1- 4 pack years exposure and 12.7 with 12 or more pack years exposure using a logistic regression model. Lyon et al (1983) [308] indicated a relative risk of 3 for CIN 3 but could not show any dose relationship. All of these studies controlled for sexual behaviour and the first two for oral contraceptive usage. This study has used indirect indices of sexual behaviour to control this aspect.

There are no available data on the relative risk of cervical HPV in smokers. This study suggests that smoking is a risk factor for cervical HPV without CIN (odds ratio = 2.56) and this is comparable with the risk of CIN. The differences in smoking habit between the HPV and CIN groups are interesting, with the CIN group being heavier smokers, although the relative risk of CIN compared with HPV for smokers is only 1.47 (C.I. 0.52-4.12). However it suggests that smoking may act at two levels, allowing establishment of HPV infection and in the promotion of HPV infection to preclinical neoplasia.

These relationships are complex, as indicated by the two methods of analysis of the second study. With strict comparison of mean

exposure, in terms of pack years, women with histological abnormality at initial assessment are less exposed to cigarettes than those with a normal cervix. But there is wide variation in smoking habit in these small numbers with women with normal cervical findings amongst the heaviest individual smokers. Comparison of relative risks give the opposite result as a higher proportion of the abnormal group smoke. It is impossible to speculate on the differences to the cervix in women smoking 5/day for 10 years compared to 20/day for 2½ years, both of whom will have an exposure of 2.5 pack years. All three women who progressed to CIN were non-smokers.

These studies support the view that it is the presence or absence of exposure that is critical rather than the level of exposure. A dosage threshold cannot be ruled out, although if present it may be at a fairly low level.

The difficulties in unravelling the relationships between the factors involved in cervical cancer are highlighted in the recent RCGP update on oral contraceptives and cancer[471]. Close analysis of the data shows that the relative risk of CIN 3 decreases with increasing cigarette consumption in pill users, from a relative risk of 4.8 in non-smokers to only 1.3 in those smoking over 15 per day.

It is not surprising that these two factors interact. Smoking has been associated with oestrogen deficiency states such as early menopause and osteoporosis and it appears that menstrual disturbances and impaired fertility are more common in smokers [473]. There is biochemical evidence that nicotine may increase 2-hydroxylation of circulating oestradiol, resulting in reduced bio-availability of active oestrogens [474]. From this information, combined with the known concentration of nicotine and cotidine in cervical mucus from normal and pre-neoplastic cervices [313,314], direct interaction at cellular level is possible. It can be speculated that reduction in the availability of oestradiol due to smoking may result in potentiation of the effects of other local hormones such as progesterone on cells containing HPV, thus linking with molecular

biological evidence of a role for this hormone in transformation experiments.

Cigarette smoking may therefore have three possible modes of action. It may interact with sex steroid hormones at a cellular level as discussed above, it may be directly carcinogenic as suggested by the description of mutagenic cervical mucus [316], or as others have proposed, it may alter local immune response.

There is evidence both, that impaired immune response, as occurs in immunosuppressed women, results in an increased incidence of cervical precancer, and that smoking can induce alterations in immune response [475]. Nicotine has been shown to result in a reduction in local immune response if present in high concentrations in cervical mucus [313,315]. Local populations of Langerhans' cells, an antigen presenting cell, are depleted in both normal and preneoplastic cervical epithelium in current smokers [315]. Furthermore, these cells appear to be depleted in HPV-16 or 18 containing CIN tissue compared with internally controlled paired normal tissue [444]. Therefore there are potential mechanisms which may allow infection by, persistence of or abberant response to HPV, mediated by cigarette smoking.

How this can result in oncogenic transformation is still unknown, but it may provide one step on the pathway. There is experimental evidence that tobacco products might alter the ability of DNA viruses to undergo normal lytic infection allowing persistence of non-replicating virus [476]. This group studied the effect of tobacco products on the lytic cycle of HSV-1. Addition of nicotine or n-nitrosamines to infective cell cultures of HSV-1 results in persistence of the virus in a non-replicating form. Inhibition of the normal lytic cycle is a prerequisite of cellular transformation by HSV in rodent cell systems. Thus it is conceivable that similar effects could be brought about in HPV.

There is therefore considerable epidemiological and experimental data which might suggest a direct or indirect role for cigarette smoking in cervical cancer. The studies presented support such a role and suggest that this role, although likely to act at the

time of cervical HPV infection, could act at two separate points in the carcinogenic cascade.

Section 6 : Conclusions

6.1 Summary of results

1. HPV-16 DNA is frequently identified by Southern blot DNA-DNA hybridisation in DNA extracted from biopsies of invasive carcinoma of the cervix and vulva.

2. Other viral types, HPV-18 and HSV-2, are infrequently identified by these techniques in both tumour types.

3. Normal tissue from women with cervical and vulval malignant disease frequently contains identifiable HPV-16 DNA. No significant difference is demonstrable between the frequency of this viral type in malignant and non-malignant tissue.

4. HPV-16 DNA is identified in DNA extracted from endometrial carcinoma biopsies.

5. Immortalised cervical cell lines have been derived from a squamous carcinoma of the cervix. HPV-16 sequences are present in the original tumour biopsy but not in internally matched tissue. Both cell lines contain detectable HPV-16 DNA in a similar pattern.

6. A continuous cell line has been derived from a squamous carcinoma of the vulva. HPV-16 sequences are present in the original tumour biopsy and are present in low copy number and in a different pattern in internally matched control tissue. The cell line contains HPV-16 DNA as a single insert, a different configuration from that seen in the tumour biopsy.

7. Clinically based studies carried out within the same geographical area suggest a role for both oral contraceptive steroids and cigarette smoking in the development of pre-invasive cervical cancer. Cigarette smoking may confer a risk of both pre-invasive disease and cervical HPV infection. There may be a dosage effect in the progression from cervical HPV to cervical intra-epithelial neoplasia. Oral contraceptive steroids confer a risk of cervical intra-epithelial neoplasia only. There may be a relationship between age at exposure to oral contraceptives and risk of histological abnormality.

8. A small risk for cervical intra-epithelial neoplasia is demonstrable for previous exposure to human CMV but not to HSV.

9. No relationship between the presence of HPV-16 sequences in invasive or normal tissue and subsequent prognosis is seen. No relationship is demonstrable between the presence of detectable HPV sequences in genital warts and the presence or development of cervical histological abnormality.

10. A novel subtype of HPV-6 is present in the majority of vulval warts in the Tayside region of Scotland.

6.2 Does genital virus infection cause malignancy ?

The DNA studies presented do not support a specific role for HPV in genital tract malignancy. Although a single HPV type, HPV-16, is present in the majority of cancers, it is as likely to be present in internally matched control tissue. The presence of viral DNA sequences alone does not appear to confer malignant potential. The other major HPV type, HPV-18, implicated by others in genital malignany, is rarely identified in these studies, HSV-2 DNA being as frequently identified.

The DNA studies emphasise the importance of appropriate control tissue in molecular biological studies. Studies which have examined internally matched tissue for viral nucleic acid support the conclusion of this study, that the presence of viral DNA does not alone result in malignant change. Its presence in both myometrial and endometrial tissue, as described here, strengthens this statement.

If HPV alone does not cause morphological change, then which other factors interact with it to bring about such change ? Epidemiological evidence implicates both smoking and oral contraceptive steroids and there is now increasing experimental evidence of how the effects of these may be mediated. The studies here support this view. Nevertheless, caution is required in interpreting epidemiological data. There is no clear consensus on the importance of duration of contraceptive use or amount of smoking. The studies presented here serve to emphasise the difficulties in interpreting simple statements of pill years and pack years. More attention to the age at which both commence is needed and to the precise relationship between duration of smoking and numbers consumed daily is needed. It has not really been appreciated within the oncological literature that smoking has an effect on sex steroid metabolism, and the relationships between virus, steroids and smoking may be even more complex than predicted.

It is felt that many of these questions and inter-relationships can only be examined at cellular or molecular level. Continuous cell lines, in which viral DNA has associated with tumour cells in vivo, are attractive model systems for examining the role of HPV in cancer. Much information has already been derived from these studies. Reservations on the usefulness of this data have already been discussed and the new cervical cell lines described in this study may offer a sounder base for such studies. The new vulval cell line will allow similar studies to be carried out for vulval cancer.

It is also clear that neither the presence of HPV in tissue, the viral type, nor integration of viral DNA into cells is sufficient to induce oncogenic change in human cervical or vulval cells. Data on integration and viral expresssion obtained from continuous cell lines may therefore not be relevant to tumours in vivo.

Different factors may result in the same end point in different populations. HSV may play a role in some tumours or may interact with HPV. The studies presented here in conjunction with DNA hybridisation data suggest that CMV may have a role within this population.

As stated earlier, over a decade passed before appropriate epidemiological studies demonstrated that previous exposure to herpes simplex virus was not a risk factor in cervical cancer. It is now essential that studies are undertaken into the prevalence of HPV in women with cervical neoplasia and control populations matched for those confounding variables known to be important in this cancer. The data becoming available from sensitive PCR techniques on the prevalence of HPV DNA in normal tissue emphasises the need for such studies. Only by this approach can both the relative importance of HPV as an aetiological factor be assessed, and the molecular mechanisms clarified.

Separate studies will be required to examine vulval cancer as data obtained in vivo or in vitro cannot be translated between two such clinically and epidemiologically diverse cancers.

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