STUDIES AT A MOLECULAR LEVEL OF THE ASSOCIATION BETWEEN HUMAN PAPILLOMAVIRUS DNA AND HUMAN GENITAL CARCINOMA

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BY

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To my wife, Anne

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ABBREVIATIONS USED IN THIS THESIS

bp	-	base pairs
BPV	-	bovine papillomavirus
CaCx	-	cervical carcinoma
CIN	-	cervical intraepithelial neoplasia
CN	-	cervical biopsy from acetowhite lesion
cpm	-	counts per minute
DNA	-	deoxyribonucleic acid
EV	-	epidermodysplasia verruciformis
EBV	-	Epstein-Barr virus
E1 to E8	-	early regions of the papillomavirus genome (nos 1 to 8) comprising putative open reading frames
HBS	-	hepes buffered saline
HCMV	-	human cytomegalovirus
Hist.	-	histological diagnosis
HIV	-	human immunodeficiency virus
HPV	-	human papillomavirus
HSV	-	Herpes simplex virus
kb	-	kilobase
L1 & L2	-	late regions of the papillomavirus genome (nos 1 and 2) comprising putative open reading frames
LB	-	L-broth
mRNA	-	messenger RNA
MTR	-	morphological transformation zone
MW	-	molecular weight

NCWVI	-	non condylomatous wart virus infection
NS	-	non-stringent (hybridization) conditions
NTB	-	nick translation buffer
OD	-	optical density
ORF	-	open reading frame
PV	-	papillomavirus
PVAg	-	papillomavirus antigen
RE	-	rat embryo
RNA	-	ribonucleic acid
S	-	stringent (hybridization) conditions
TBS	-	tris buffered saline
Tm	-	melting temperature
TPA	-	12-0-tetradecanoylphorbol-13-acetate
UV	-	ultraviolet
VAIN	-	vaginal intraepithelial neoplasia
VIN	-	vulval intraepithelial neoplasia
VN	-	premalignant vulval lesion
VU	-	vulval carcinoma

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SUMMARY

There is now considerable evidence for a close association between human papillomavirus (HPV) infection and genital neoplasia. These data are based upon epidemiological, colposcopic, cytological, histological, immunohistochemical and DNA hybridization studies. This thesis aims to study six aspects of this association.

The first approach was to establish the prevalence of HPV 6, 11, 16 and 18 DNA sequences in human female genital premalignancy and malignancy in the West of Scotland. Three tissue sources were used; namely biopsies from abnormal tissue (vulval and cervical premalignant and malignant tissue), internal control tissues from histologically normal adjacent tissue and external control tissue from the cervices of cytologically normal women. HPV 6/11 DNA sequences were detected in only one premalignant lesion on the cervix (1.5%) and two external control tissues (6.7%). HPV 16 & 18 DNA was detected in 69% of cervical premalignant lesions, 80% of vulval premalignant lesions, 58% of cervical carcinomas and 60% of vulval carcinomas. HPV 16 was also detected in 2 external control tissues (6.7%). The prevalence of HPV 16 and 18 DNA is comparable with other studies but the prevalence of HPV 6/11 DNA is low suggesting a geographic

variation in the prevalence of HPV DNA subtypes. This study confirms that HPV 16 is the subtype most frequently detected in these lesions (51% overall). HPV 16 DNA is however frequently detected in histologically normal tissue adjacent to neoplastic lesions (38.5% overall). Alternatively, HPV 18 DNA is less often detected than HPV 16 DNA (19% overall) but is more specific to histologically abnormal tissue [only one internal control biopsy hybridized to HPV 18 DNA, (1.5%)].

The second aspect of the association between HPV infection and genital neoplasia studied was the relationship between the different diagnostic methods used to determine the presence of HPV DNA on the cervix i.e. colposcopy, cytology, histology and immunohistochemistry. The sensitivity and specificity of Southern blot hybridisation allows this to be used as a "gold standard" for comparison with these other methods. The study showed that colposcopy was an insensitive method of identifying tissues which harboured HPV DNA as was immunohistochemistry. Cytology and histology suffered from a lack of specificity. These findings have implications for the assessment of patients and the structure of prospective study of the natural history of HPV infection which are discussed fully later.

An association between HPV infection and advancing age has been suggested. In this thesis, no association between the age of patients and the detection of HPV DNA sequences was established.

The high prevalence of HPV DNA subtypes in cervical neoplasia and the increasing data from cell line and cancer cell studies which suggest a mechanism for cell transformation by HPV DNA sequences has led to calls for the introduction of a screening test for HPV DNA. One such test utilizes dot blot hybridization. This study has compared Southern blot hybridization and slot (dot) blot hybridization. Slot blot hybridization has been shown to offer poor specificity, especially at low viral DNA copy numbers. Furthermore, this thesis shows that one third of patients who undergo laser ablation of the cervical transformation zone demonstrate persistence of the HPV genome following treatment. These two data, along with the facts that up to one third of the female population may harbour HPV DNA on their cervices, the prevalence of HPV DNA on the male penis is unknown, and a causal relationship between HPV DNA has not been established, suggest that screening for HPV DNA is not indicated at present in this country.

The fifth aspect of the association between HPV DNA

infection and cervical neoplasia to be studied was the influence of local immunosurveillance as assessed by the presence of Langerhans' cells on cervical premalignancy. An association between HPV 18 DNA and HPV 16 DNA at high copy numbers and local immunosuppression was identified. It seems li kely that smoking is associated with depression of Langerhans' cell activity and this study has demonstrated a possible relationship between these two epidemiologically important cofactors.

Finally, a possible mechanism of cell transformation by HPV DNA comprises the amplification or upregulation of cellular oncogenes. In this thesis hybridization to the proto-oncogene c-myc was studied but no evidence of amplification of that oncogene was detected in association with hybridization to HPV DNA sequences. In two cases, however, there was some evidence to tentatively suggest a rearrangement of the c-myc oncogene in association with HPV 16 DNA detection. This aspect requires further study.

The significance and implications of the results are discussed in the context of the work itself and its relation to the data reported by other workers. The implications for patient management are outlined and ideas for future work based on a tentative hypothesis are presented.

THESIS INTRODUCTION

A. REVIEW OF THE LITERATURE

1. Cervical Neoplasia

1.1 General Considerations

Cancer of the uterine cervix is a common human female genital malignancy. The world standardised incidence rate in England and Wales is 11.7/100,000 women. The reported world standardised incidence varies markedly around the world, for example, it is 86.2/100,000 women in Recife, Brazil but only 4.0/100,000 in Israel. The rate also varies within countries between ethnic groups. In New Zealand, the incidence is 11.8/100,000 Caucasian women but in Pacific Polynesian Islanders it is 64.4/100,000 women (World Health Organisation, 1987).

In the West of Scotland in 1986 the annual crude incidence rate was 16.2/100,000 and world standardised rate was 12.4/100,000. This incidence has changed minimally in the last decade. It is the seventh commonest cancer and the second commonest genital cancer in women in this area (personal communication, West of Scotland Cancer Surveillance Unit).

There are two broad histological types of cervical malignancy. Squamous carcinomata arise from the squamous

epithelium and account for 90-95% of cases. Adenocarcinomata arise from columnar epithelium and account for 4-8% of cases. In the West of Scotland adenocarcinomata account for 7.8% of cervical cancers (personal communication, West of Scotland Cancer Surveillance Unit). The remaining cases comprise sarcomas, lymphomas and melanomas (Anderson, M.C., 1985).

Whereas the relatively unusual adenocarcinoma arises from the columnar epithelium within the cervical canal, squamous carcinomata almost invariably arise from the cervical transformation zone. The transformation zone is the area over which the junction between the keratinised stratified squamous epithelium of the ectocervix and the columnar epithelium of the endocervix - the squamo-columnar junction - migrates throughout life. The transformation zone is bounded within the cervical canal by native columnar epithelium and caudally, on the ectocervix, by native squamous epithelium. The squamo-columnar junction migrates by the process of squamous metaplasia. Migration of the squamo-columnar junction is largely influenced by oestrogen stimulation and squamous metaplasia is influenced by the acid vaginal environment (Singer, A., 1975). Thus, in utero, maternal circulating oestrogen crosses the placenta and promotes migration of the squamo-columnar junction onto the

ectocervix. After birth this stimulus is withdrawn and the squamo-columnar junction migrates back into the cervical canal. At puberty, oestrogen levels rise again and the squamo-columnar junction reappears at the ectocervix. Thereafter, the squamo-columnar junction migrates back and forth in response to the oestrogen levels which may be influenced by pregnancy and oral contraception; conditions on the cervix which are recognised clinically as "ectopy" or "pill erosion". At the climacteric, oestrogen levels fall, the squamo-columnar junction retreats within the cervical canal, and the typical appearance of the post-climacteric cervix results.

The transformation zone is thus an area of increased cellular mitotic activity and there are considerable epidemiological data to be reviewed later in the text which suggest that one or more sexually transmitted agents act on this region to produce malignant transformation of cells.

Pre-invasive cervical neoplasia which may have malignant potential was initially described as dysplasia (Reagan, J.W., 1953) and was divided into mild, moderate and severe dysplasia plus carcinoma-in-situ. Poulsen, H.E., et al, (1975) defined dysplasia as "a lesion in which part

of the thickness of the epithelium is replaced by cells showing varying degrees of atypia". This nomenclature lacks precision and differentiation between severe dysplasia and carcinoma-in-situ has little prognostic relevance so the term cervical intraepithelial neoplasia (CIN) (Richart, R.M., 1967) has recently been preferred. CIN 1 corresponds to mild dysplasia, CIN 2 corresponds to moderate dysplasia and CIN 3 corresponds to severe dysplasia and carcinoma-in-situ.

The pre-malignant status of CIN. particularly CIN 3, is widely accepted. Scott, R.B., and Ballard, L.A., (1962), using cytology and biopsy, found a progression rate of 5.8 percent and a regression rate of 49.8 percent. More recently, McIndoe, W.A., et al (1984) reported on 300 women with evidence of carcinoma-in-situ who were left untreated. After 10 years, 18 percent of the women had developed invasive disease. This rose to 36 percent after 20 years. The reasons for this apparent discrepancy are probably that, firstly, the taking of a biopsy sample may remove small lesions completely or alter the local immune response to residual disease (McKay, D.G., et al, 1959) and, secondly, the clinical, colposcopic, cytological and histological assessment of premalignant cervical lesions remains subjective to a great extent (Kirkup, W., et al, 1982). It is likely, however that the

study of McIndoe, et al, is the more accurate assessment of malignant potential in CIN 3 lesions. Richart, R.M., and Barron, B.A., (1969) examined the natural history of mild, moderate and severe dysplasia by prospectively following-up women who were assessed cytologically and colpo-microscopically. No biopsies were taken. They estimated median transit times to development of carcinoma-in-situ of 86 months for mild dysplasia and 12 months for severe dysplasia. The validity of these estimates must be questioned on two counts however. Firstly, biopsies were not performed and colpomicroscopy and cytology were relied upon to establish a histological diagnosis. Walker, E.M., et al (1986) showed that 82% of women referred to a colposcopy clinic with cytology suggestive of mild atypia (equivalent to mild dysplasia) did in fact have CIN 2 or 3 (equivalent to moderate dysplasia and severe dysplasia/carcinoma-in-situ respectively) on histological assessment of biopsies. Thus progression to carcinoma-in-situ in Richart and Barron's study may have in fact been a change from cytologically and colposcopically occult carcinoma-in-situ to cytologically and colposcopically overt carcinoma-in-situ. Secondly, the histological differentiation between severe dysplasia and carcinoma-in-situ is so subjective and variable

(Cocker, J., et al, 1968) that cytological and colposcopic differentiation between these lesions is artificial. Progression to invasive disease is thought to pass through a micro-invasive stage. There is no internationally accepted definition of this stage. In the United Kingdom, the Royal College of Obstetricians and Gynaecologists Study Group on Preclinical Neoplasia of the Cervix (Jordan, J.A., et al, 1982) defined two groups which comprised early stromal invasion with a depth of invasion of less than 1mm, and measureable lesions with a depth of invasion of less than 5mm and diameter of less than 10mm. It is assumed that micro-invasive disease does not regress to normal although ethically this hypothesis will never be tested.

As CIN is an asymptomatic precursor of invasive cancer, it is ideally suited for a screening programme. Papanicolau G.N., and Traut, H.F., (1943) demonstrated that exfoliative cytology could detect early epithelial abnormalities. These early epithelial abnormalities comprise cell changes which are intermediate between normality and malignancy and are known as dyskaryosis. Dyskaryosis refers to cells of which the nucleus displays some, but not all, of the features of malignancy. The nucleus is enlarged with increased chromatin which may form clumps. The nuclear border is irregular,

multinucleation is common and cytoplasmic eosinophilia is seen. There is an altered nuclear-cytoplasmic ratio (review by Attwood M.E., 1976). Ayre, J.E., (1949) designed a spatula which gained wide acceptance as a screening tool. Recent refinements such as the Aylesbury spatula (Wolfendale, M.R., et al, 1987) may further improve the sensitivity of cytological screening. Cervical cytology screening programmes are relatively cheap and are acceptable to patient and practitioner. Studies in British Columbia have shown that a properly instituted programme with effective follow-up reduces the incidence of invasve disease. In a review of their findings Boyes, D.A., and Worth, A.J., (1976) showed that the incidence of and mortality from carcinoma of the cervix had declined over the twenty year period of cytology screening whereas this change had not been noted in countries in which screening was not employed. Recently this screening programme has been reviewed after 30 years (Anderson G.H., et al, 1988). At present 85% of women at risk from cancer of the cervix have been screened at least once. The incidence of clinically invasive cervical cancer has fallen by 78% and mortality from cervical cancer has fallen by 72%.

These data suggest that the reduction in morbidity and mortality from cancer of the cervix is attributable to

effective cervical screening. There is no satisfactory formula for quantifying the cost effectiveness of cervical screening but this study has shown a reduction in invasive cancer and a simultaneous rise in reported incidence of premalignant lesions. Premalignant lesions themselves have no morbidity or mortality and their treatment has low morbidity and mortality. Similarly, the detection of early malignant disease is associated with higher 5 year survival rates than late malignant disease.

Thus the early identification and treatment of cervical neoplasia seems to be the optimal method of reducing the morbidity and mortality associated with advanced cancer of the cervix.

It is difficult to compare cancer statistics in different parts of the world due to differences in data retrieval, data recording and differences in the availability and quality of health care. In the United Kingdom, the overall mortality from carcinoma of the cervix has declined in the last 20 years although some two thousand women continue to die of the disease per annum (Macgregor, J.E., and Teper, S., 1978). These workers have shown that, in areas such as Grampian and Tayside in which effective screening programmes have been operated, the reduction in mortality has been greatest. This

suggests that the experience of British Columbia could be repeated in the United Kingdom if adequate screening was instituted.

The failure of cervical screening in the United Kingdom has been attributed to a number of factors comprising: failure to screen the at risk population, laboratory errors, failure to follow up cases and failure to irradicate premalignant lesions adequately (Chamberlain, J., 1984). Additionally, the lack of a unified, simple screening strategy has led to administrative chaos and increased cost (Editorial, Lancet, 1986).

Successful screening strategies also require a treatment for the condition which is effective and associated with low morbidity. Initially cone biopsy of the cervix which was effective but caused significant morbidity was the only available local response to an abnormal cervical smear. The widespread use of colposcopy has reduced the requirements for cone biopsy (Donahue, L.R., & Meriwether W., 1972). The development of local destructive techniques for selected cases such as laser ablation (Evans A.S., and Monaghan, J.M., 1983), electro-diathermy (Nan Schuurmans, S., et al, 1984),"cold" coagulation (Staland, B., 1978) and to a lesser extent cryocautery (Townsend D.E., and R.M. Richart, 1983) have in recent years fulfilled these

requirements of a successful screening programme.

1.2 Aetiology

Numerous aetiological factors have been implicated in cervical carcinogenesis. The most recently described and currently most intensively studied agent is human papillomavirus (HPV). The structure of HPV and the data implicating this virus in cervical malignancy will be reviewed in detail (Introduction, 3 & 4).

Epidemiological studies on women developing cervical neoplasia.

The evidence for various aetiological factors in cervical cancer was derived from epidemiological studies of demographic and sexual characteristics of cases. Similarly, initial studies of sexually transmitted agents which might be implicated in carcinogenesis were epidemiological. These studies will be described in the subsections related to individual agents.

Sexual intercourse is an essential factor in the development of this condition. This original observation by Rigoni-Stern, D., (1842) has been confirmed in more recent times (Gagnon, F., 1950). Towne, J.E., (1955) explored the possibility that pregnancy and cervical trauma at child-birth, as suggested by Gagnon, might be an aetiological factor. Her findings that 6 cases of

squamous cervical cancer developed in a group of 13,083 virginal women suggested that pregnancy per se was not a factor. It is interesting to note that she considered the terms "virginal" and "nulliparous" to be interchangeable and cautioned that virgins were not free from carcinoma of the cervix. It may be that Towne's celibate population was different to Gagnon's 13,000 celibate women living in religious seclusion.

Wynder, E.L., et al, (1954) studied environmental factors in different racial and ethnic groups in the United States and in India. They recorded a low incidence among Jewesses, Fijians and Moslems with a higher incidence among Indians and Negroes. They found that cervical cancer patients commenced coitus at a significantly earlier age than controls. They attributed the racial differences observed to age at first coitus, number of marriages and circumcisional status of the partner. The circumcisional state of the partner was not thought to be important by Stewart, H.L., et al, (1967) however, and this has been confirmed recently by Baram, A., and Schachter, A., (1982) who reported an increase in cervical cancer in Israel which was related to the different sexual practices of recent Jewish immigrants who nevertheless maintained the tradition of circumcision.

Alternatively the importance of early age of intercourse was confirmed by Boyd, J.T., and Doll, R., (1964) and Rotkin, D., (1967). Rotkin reguarded this as the single most important epidemiological risk factor. Blythe Smith, D., and Jenkins, R.S., (1969) reported on a cluster of cases of cervical cancer found in a small housing estate populated by women of low socio-economic status who had an early age of first coitus and multiple sexual partners.

The association with social class was also noted by Beral, V., (1974) who found that mortality rates from cervical cancer in women from social class I were only 35% as high as the rates for all married women whereas the women from social class 5 experienced a rate of 181% of those all married women.

The epidemiological characteristics of patients with pre-invasive disease of the cervix were explored by Harris, R.W.C., et al (1980). They found that multiple sexual partners had an independent relationship with CIN. Their findings failed to support suggestions that adolescence is a period when the cervix is most vulnerable to the effects of sexual behaviour. They also detected an association with smoking. This relationship was confirmed by Buckley, J.D., et al (1981) who found a relative risk for smokers of 7.0 when compared to non-smokers. This risk

was similar to the relative risk for multiple sexual partners (7.8 for 15 or more partners). Trevanthan, E., et al (1983) reported a case controlled study of this association in American black women which showed that cumulative exposure to cigarette smoking was strongly related to all grades of cervical dysplasia. Cigarette smoking is thus second only to genital HPV infection in the league of associated epidemiological features.

Harris, R.W.C., et al, (1980) also noted an independent association with use of the oral contraceptive pill. An earlier retrospective study (Boyce, J.G., et al, 1977) on 689 patients with cervical cancer and controls showed no relationship between the oral contraceptive and cancer of the cervix with short term use. Alternatively, Vessey, M.P., et al (1983), in a prospective study, recorded a relative risk of developing cervical pre-malignancy or malignancy of 2.2 compared to controls with oral contraceptive use in excess of 8 years. This study controlled for cigarette smoking but did not control for age at first coitus. A W.H.O. Collaborative Study of Neoplasia and Steroid Contraception (1985) reported a relative risk of 1.19 for oral contraceptive users in a large multicentre retrospective study. This finding must be viewed cautiously, however, because the quality of data collection in these third world countries may have been

variable; control for the confounding effect of sexual variables was incomplete; no consideration of smoking was included; and oral contraceptives of a higher strength than commonly prescribed in the West were frequently used. In Britain, a prospective study of 47,000 women was reported on the association between genital tract malignancies and oral contraceptive use (Beral, V., et al, 1988). The other important epidemiological variables were controlled for in this study. An increased incidence of genital tract neoplasia was noted in "ever users" compared to "never users". Overall, the excess incidence of neoplasia was 37/100000 patients in the "ever users" group. Most of this increase comprised CIN3 lesions and the excess for invasive cancer of the cervix was only 8/100000 women. Additionally, standardised mortality rates were equivalent in the "ever users" and "never users" groups.

Recently, Pater, M.M. et al, (1988) suggested a possible mechanism for the association between oral contraceptives and cervical neoplasia. They reported that primary baby rat kidney cells were morphologically transformed in the presence of HPV 16 DNA and dexamethasone. They suggested that the transforming activity of HPV 16 DNA was due to the glucocorticoid-dependent expression of a gene specifically present in the HPV 16 genome. This effect

was not noted in the presence of HPV 11 DNA. They inferred that progestogens in the oral contraceptive pill might have similar effects and that this required to be investigated urgently. McCance, D.J. (1988) pointed out, however, that Pater et al did not carry out the point mutation studies necessary to show that the viral sequences which are responsive to dexamethasone are actually required for cell transformation. Furthermore, the pharmacological properties of dexamethasone and progestogens are quite different so extrapolation of these data to the effect of oral contraceptives on cervical cells should be viewed cautiously. In view of the great social benefits gained by oral contraception and the relatively low risk of developing cervical cancer, it seems unwise to condemn the pill as extremely dangerous in this respect. Vessey's advice that long term users of oral contraceptives should have regular cytological examination is appropriate, as rigorous cytological follow-up of pill users would be expected to identify the majority of CIN3 lesions for treatment and reduce their risk of developing cervical cancer further.

Epidemiological studies on the sexual partners of women developing cervical neoplasia

The key role of sexual activity in the development of cervical neoplasia led to more detailed assessment of the

epidemiological characteristics of sexual partners of women. Wakefield, J., et al (1973), studying the relationship between both pre-invasive and invasive disease and social class determined by the husbands' occupation, and Beral, V., (1974), studying the relationship between invasive disease alone and social class, agreed that the disease was more common in lower social class. Both authors noted that the relationship varied within social classes depending on occupation. Thus in social class IV, the wives of sailors were 3-4 times more likely to die of the disease than shopkeepers' wives. The inference is that the itinerant lifestyle of the sailors was associated with sexual promiscuity in the men and, possibly, in the wives who stayed at home. Kessler, I.I., (1977) expanded on these observations by detecting marital clusters of women developing cervical neoplasia who shared the same husband. Buckley, J.D., et al (1981) defined the relative risk to wives of promiscuous men as 7.8 if their husbands had more than 15 sexual partners outwith marriage and Singer, A., et al, (1976), reviewing the data, hypothesised that certain males were at high risk of inducing cervical neoplasia in their sexual partners.

The relationship between penile cancer and cervical neoplasia has been explored by several workers. Graham.

S., et al (1979) in New York State and Smith, P.G., et al (1980) in England and Wales both detected statistically significant associations between the two diseases which were independent of other variables. Sorahan, T., & Crombie, I.K., (1981) reviewed the age standardised rates of both diseased in North America and Europe and found a highly significant association in the black population. An apparent lack of a significant association in the white population disappeared when control for population size was introduced. It is possible that both diseases share a common aetiology and the large difference in prevalence of cancer of the cervix and cancer of the penis depends on differing suscepibility of the host tissues to malignant transformation.

The human sperm as an aetiological agent

The most obvious sexually transmitted agent at coitus is the sperm which led Reid, B.L. (1964) to study the bahaviour of human sperm in an *in vitro* system of cultured fragments of the human cervix. He noted that sperms only penetrated metaplastic epithelium and did not penetrate mature squamous epithelium. Later, he suggested that integration of sperm DNA into cervical cells may produce neoplasia but was unable to develop a model to prove this (Reid, B.L., 1985).

Interest turned to the effect of sperm basic proteins on cervical cells (reviewed by Singer, A., et al, 1976) which, it was suggested, can alter the morphology of cultured cervical cells. Reid, B.L., et al (1978) developed the theory by identifying two sperm basic proteins, a histone and a protamine. They stated that mean values for these proteins in men of lower social class were higher than those of men in higher social class. Reid, B.L., (1985) drew the data together and suggested that these proteins along with polymerised argenine might alter the intracellular mechanisms of the target cells and act as co-factors with oncogenic viruses. This proposition was based on the detection of profound disturbance of the cytoplasmic and cell surface filaments of cultured cervical cells following contact with proteins purified from the sperm head. These disturbances resulted in alteration of the shape of the cells, an inceased sensitivity to lectins and increased production of mannase and galactase. Such changes are also found when susceptible cultured cells are exposed to the oncogenic virus, Rous sarcoma virus. In experiments on cultured cells exposed to antiserum raised against papillomavirus antigen in rabbits the effect of polymerised arginine on these cell filaments was enhanced sixfold. Reid postulated from these <u>in vitro</u> data that sperm proteins

might somehow promote the oncogenic effect of HPV. However there is no in vivo evidence for this mechanism.

The effect of vasectomy on the cancer risk of female partners might give indirect evidence for a role of human sperms. The data are conflicting however. Swan, J.H., and Brown, W.L., (1979) suggested that vasectomy was protective for the female sexual partner but Harris, R.W., and Scott, W.A., (1979) criticised this study as it did not control for other sexual variables. When the number of sexual partners was controlled for, Harris & Scott showed that the apparent difference between cases and controls disappeared. The aetiological role of sperms in cervical neoplasia is therefore unproven. The possible role of other components of the ejaculate e.g. prostatic fluid which would be unaffected by vasectomy have not been studied.

Herpes simplex virus (HSV)

HSV is a DNA virus which infects the lower genital tract. There are two serotypes, HSV-1 and HSV-2; the latter more commonly infecting the genital tract than the former (Smith, I.W., et al, 1976). The evidence for an aetiological role of HSV in cervical carcinogenesis is based on cell line studies, animal studies, serological data, immunohistochemical studies and the detection of

virus specific nucleic acid in cancer specimens and derived cell lines.

Morphological transformation of hamster embryo cells by inactivated HSV-1 was demonstrated by Duff, R., & Rapp, F., (1973). Three regions of the HSV genome were shown to be associated with morphological transformation of cultured cells MTR1, MTRII and MTRIII (see Macnab, J.C.M., 1987, for review). Experiments on mice have shown that it is possible to induce cervical cancers in these rodents by application of HSV-2 DNA. This oncogenic effect can be reduced if the mice are previously immunised with HSV vaccine (Chen M., et al, 1986).

Naib, Z.M., et al (1966) first noted an increased incidence of CIN in women who also had cytologically detectable HSV infections. Serological evidence of an increased incidence of HSV infection in patients with CIN and invasive disease was provided by Rawls, W.E., et al (1968) and Nahmias, A.J., et al (1974).

HSV specific antibodies were detected in 90% of patients and only 10% of controls in one study (Aurelian, L., et al, 1973) . HSV DNA was detected in 60% to 70% of CIN lesions but infrequently in invasive cancers although HSV specific mRNA was detected in invasive tumours [McDougall, J.K., et al, (1980); Eglin, R.P., et al (1981)]. The

study by Eglin et al also included normal control tissue and the results suggested that the viral RNA probed for was specific to tumour cells. The specificity of the radiolabelled probes was however questioned by Peden, K., et al (1982), who demonstrated homology between fragments of the viral genome and host cell DNA. Park, M., et al (1983) studied 8 human cervical cancers for evidence of HSV-2 DNA by Southern blot analysis using 70% of the HSV-2 genome as a probe. Tumour and control tissue DNAs were hybridized with five different restriction fragment probes. Hybridization to HSV DNA sequences was observed in only one tumour, an adenocarcinoma, with only one probe, Bgl11n. This demonstrated unambiguously that authentic viral sequences were detected in this tumour. The Bgl11n region of the HSV-2 DNA is associated with morphological transformation of rodent cells but is not retained within these cells suggesting that the DNA need not be retained within the cell to maintain the oncogenic phenotype [Galloway, D.A., & McDougall J.K. (1981), Cameron, I.R., et al (1985)].

The inability to consistently identify HSV DNA in tumour tissues has led some workers [Skinner, G.R.B., (1976); Galloway, D.A., and McDougall, J.K., (1983)] to suggest a "hit and run" mechanism of carcinogenesis for HSV. Zur Hausen, H., (1982) hypothesised that HSV acts as an

initiator which works synergistically with HPV and other co-carcinogens. Zur Hausen's model reconciles the conflicting evidence of the low detection of HSV DNA in human tumours and the other experimental evidence for a role of HSV in a complex process of carcinogenesis.

Human cytomegalovirus (HCMV)

HCMV commonly infects the cervix and its detection is associated with sexual promiscuity (Jordan, M.C., et al, 1973). Its oncogenic potential has been demonstrated *in vitro* (Albrecht, T., and Rapp, F., 1973). Dysplasias and carcinomas of the mouse cervix have been induced by treatment with U-V inactivated HCMV (Heggie ,A.D., et al, 1986). Melnick, J.L., et al (1978) isolated HCMV from cell cultures derived from two out of ten cervical cancer biopsies. Fletcher, K., et al (1986) detected sequences that hybridized to HCMV DNA in 2 out of 43 biopsies from patients with CIN. They suggest that HCMV may act as a promotor of co-carcinogens in the neoplastic process. Thus, like HSV, there is indirect evidence for a role of HCMV in tumourogenesis in some cases although the possible role of this virus *in vivo* has not been clarified.

Epstein-Barr Virus (EBV)

The association between EBV and Burkitt's lymphoma

(Burkitt, D., 1958) is well recognised as is infection of the oropharynx producing the clinically recognised condition of infectious mononucleosis (Henle, G., et al, 1968). Sixby, J.W., et al (1986) recently detected shedding of EBV from the cervix and suggested a venereal mode of spread. The possible role of this potentially oncogenic virus on the cervix awaits further study.

Chlamydia and other sexually transmitted diseases

In a study of 383 women with cervical neoplasia and 500 controls, Schachter, J., et al (1982) detected an excess of chlamydial antibodies in patients when other epidemiological factors were controlled for. Apart from this sero-epidemiological data, however, there is little evidence of a causal relationship with cervical cancer. Similarly, evidence for a causal relationship between the notifiable venereal diseases and cervical cancer remains epidemiological only [Beral, V., (1974), Kessler, I.I., (1977)]. It may be that these agents are merely markers of sexual promiscuity and a consequent greater risk of exposure to more likely agents such as HPV, HSV or HCMV.

2. Vulval Neoplasia

2.1 General considerations

The human vulva constitutes an organ which has a separate

blood and lymphatic supply from the surrounding skin. Diseases affecting the vulva can therefore be considered as separate from those affecting other areas of the integument. The classification of vulval premalignant disease has been plagued by inconsistency in the past but in the last 10 years a unified classification has been popularised (International Society for Study of Vulvar Disease, 1976). In essence this classification comprises:-

i Hyperplastic dystrophy - without atypia
- with atypia

ii Lichen sclerosus

iii Mixed dystrophy

iv Pagets disease of the vulva.

The cellular atypia associated with hyperplastic dystrophy can be mild, moderate, or severe and this corresponds to vulval intra-epithelial neoplasia (VIN) grades 1, 2 and 3. Hyperplastic dystrophy can be regarded as a pre-malignant lesion whereas Lichen sclerosus has low malignant potential. Mixed dystrophies have intermediate malignant potential. Paget's disease is a plastic change within the epithelium which is confined by the basement membrane.

Bowen's disease is a hyperplastic dystrophy and should be included in that classification.

The malignant potential of VIN is low compared to CIN on the cervix. The disease is usually multifocal and characterised by change in skin colour (commonly red and white) and pruritis, (Douglas, C.P., 1983).

Invasive cancer of the vulva is usally squamous in origin (88.8%). Other histological types of tumour comprise melanoma, basal cell carcinoma, Bartholin's gland tumour, sarcoma, and adenocarcinoma (Way, S., et al, 1982). Squamous carcinoma commonly presents as a mass or an ulcer.

The incidence of vulval premalignancy is unknown in the population. Vulval carcinoma is increasing in incidence and comprises about 5% of female genital malignant disease. In the West of Scotland the incidence is 2.4 per 100,000 women and at least some of the apparent increase is explained by increasing incidence in the older age group which may be related to the longevity of the modern population (Murdoch, J.B., & Torbet, T.E., 1986).

2.2 Aetiology

Apart from a possible role of HPV to be reviewed later (Introduction, 5) the aetiology of vulval cancer is obscure. VIN is a premalignant condition but its incidence in the population and therefore its true

malignant potential is unknown because there is no screening test for the condition and it is frequently unreported. Hay, D.M., and Cole, F.M., (1961) reported an association with tropical granulomatous disease in Jamaica. Way, S., et al (1982) considered that while toxic industrial carcinogens might play a part in isolated cases, vulval hygiene was probably an important factor in genesis of the disease. They also reported an association with lower social class but found no correlation with marital status, menstrual history or incidence of notifiable venereal disease.

3. Human Papillomavirus

3.1 Structure and organisation

Human papillomavirus (HPV) is the papillomavirus which infects man. Other papillomaviruses infect many species including horses, cattle, birds and rabbits. They are icosahedral particles approximately 45-55nm in diameter and consist of 72 capsomeres. They are members of the papovavirus family. The structure and molecular weight of HPV DNA was determined by Crawford, L.V. and Crawford, E.M. (1963). HPV DNA exists as a double stranded circular molecule and three forms are demonstrated by velocity sedimentation. These forms are supercoiled circular DNA (form 1), nicked circular DNA (form II) and linear DNA

Fig. 1

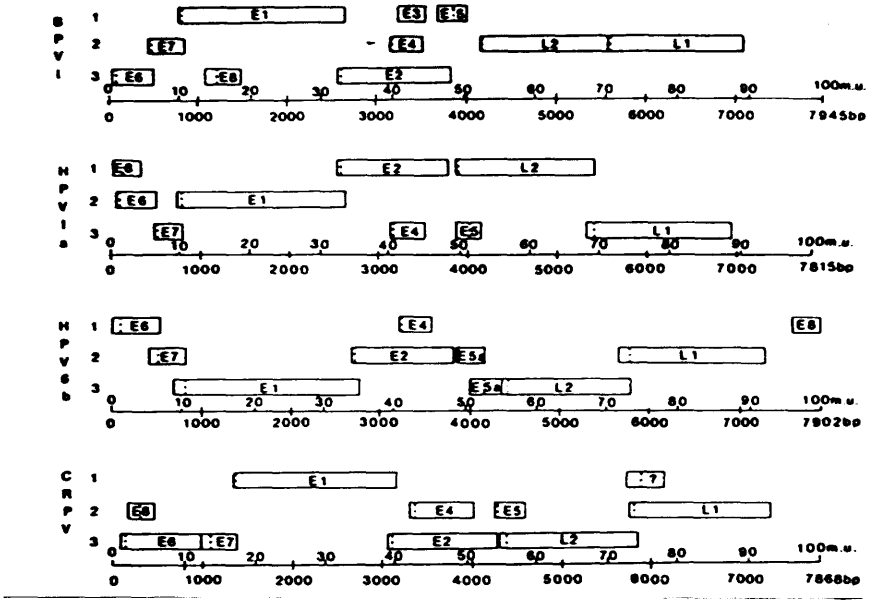


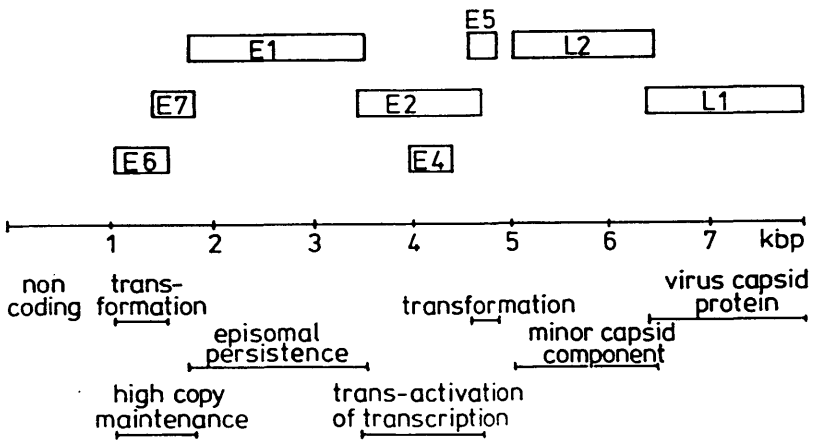
Diagram of the organization of the papillomavirus genome comparing BPV1, HPV 1a, HPV 6b & CRPV. The genome is split into early (E) and late (L) regions. There is a common organization of the ORFs between species and subtypes. From, Pfister, H. et al (1986).

(form III). The molecular weight (M.W.) is 4.9×10^6 (Gissmann, L. and Zur Hausen, H., 1976). The HPV genome is approximately 8 kilobase (kb) pairs long. HPV DNA subtypes are differentiated by nucleic acid base pair sequence homology. By convention HPV DNA subtypes with more than 50% base pair sequence homology are assigned to the same subtype; those with less than 50% base pair sequence homology are assigned to a new subtype. Currently, over 50 HPV subtypes have been reported (McCance, D.J., 1988).

Although the human and animal papillomaviruses have little sequence homology they have a common organisation of the open translational reading frames (ORFs) (Fig. 1). The genomic organisation of bovine papillomavirus type 1 (BPV1) has been extensively studied and general conclusions about other papillomaviruses can be drawn from this.

Unlike other members of the papovavirus family, the genomic organisation of papillomaviruses is such that all major ORFs are located on the same DNA strand (Danos, O., et al, 1982). Although there is no tissue culture system available for propagating papillomaviruses it has been assumed that the genes can be functionally divided into early genes (E1 to E8) which are expressed before the

Fig. 2



A functional map of the BPV 1 genome showing the putative functions of each ORF. The ORFs of the late (L) region code for capsid proteins while the ORFs of the early (E) region code for products which are involved with maintenance of the viral genome and transformation.
From, Petterson, U. et al (1986).

onset of viral replication and late genes (L1 and L2) which are expressed after viral replication commences. Lowy, D.R., et al (1980) showed that a fragment comprising 69% of the BPV1 genome transformed NIH 3T3 and C127 mouse cells and suggested that this fragment encodes for the early functions of the genome and the remainder encodes for the late functions of the genome. Of the 8 early ORFs E1, E2, E4, E6 and E7 have equivalent counterparts in all papillomavirus genomes sequenced so far (Fig. 1). A detailed functional mapping has been achieved by genetic studies of BPV1 (Fig. 2). This shows that the coding sequences in the ORFs of the early region of the viral genome are involved in the maintenance of the viral genome, genome replication and morphological cell transformation. The ORFs of the late region of the viral genome are related to production of capsid proteins.

The capsid proteins of HPV are structurally similar to histones (Pfister, H., et al, 1977). Different P.V. subtypes produce specific structural proteins which can be identified immunohistochemically (Nakai, J., et al, 1986).

Whilst viral DNA is detected in the basal layers of epithelium by <u>in situ</u> DNA-DNA hybridization (Crum, C.P., et al, 1986); late gene function and production of structural proteins are detected in the superficial layers

of terminally differentiating keratinocytes (Coleman, D.V., and Richman, P.I., 1985). Viral replication takes place in these terminally differentiating keratinocytes and the cytopathic effect of this process is recognised histologically by the presence of halo cells or koilocytes [Koss, L.G., and Durfee, G.R., (1956); Meisels, A., et al, (1981)]. As the koilocyte is a dying cell, it has been impossible to propagate the virus in vitro.

3.2 Clinical and subclinical infections with HPV

HPV infects squamous epithelium. Different subtypes are associated with lesions on different parts of the body. Thus for example, HPV 1 is associated with plantar warts, (Danos, O., et al, 1982) and HPV 11 is associated with laryngeal papillomas (Gissman, L., et al, 1982b). HPV 6 (Gissmann, L. and Zur Hausen, H., 1980); HPV 11 (Gissmann, L., et al, 1983); HPV 16 (Durst, M., et al, 1983) and HPV 18 (Boshart, M., et al, 1984) DNAs are associated with the human female genital tract. HPV 31 (Lorincz, A.T., et al, 1986) and HPV 33 (Beaudenon, S., et al, 1986) have also been detected (to a lesser extent) in female genital tissue in some parts of the world. Overall, HPV 6 and 11 tend to be more closely associated with benign lesions and HPV 16, 18, 31 and 33 are associated with premalignant and malignant lesions. The

association between HPV subtypes and neoplasia will be discussed (Introduction, 4).

On the vulva and cervix HPV infections present clinically as condylomata acuminata. These lesions are easily diagnosed and possess characteristic histological appearances (Meisels, A., et al, 1977). Ferenczy, A., et al, (1985) demonstrated that HPV DNA was detected not only in condylomata acuminata but also in clinically and histologically normal adjacent tissue. Subclinical lesions have been described on the vulva at vulvoscopy (Singer, A. and McCance, D., 1985). On the cervix, the lesion most commonly detected is the atypical condyloma (Meisels, A., et al, 1981) which Reid, R., et al (1980) named the noncondylomatous cervical wart virus infection (NCWVI). This lesion is not apparent with the naked eye and is visualised as aceto-white epithelium at colposcopy. Atypical condylomata can be difficult to diagnose cytologically or histologically because they mimic, and frequently coexist with, dysplastic changes on the cervix (Meisels, A., et al, 1981). De Villiers, E-M., et al (1987) found that 10% of women with apparently normal cervical cytology had evidence of HPV DNA infection using a dot blot hybridization technique. They suggested that this technique was insensitive and postulated that the prevalence of HPV DNA infection of the cervix in the

female population might be as high as 30%.

On the male penis HPV infection may present as condylomata acuminata or subclinically as microwarts (Campion, M.J., personal communication). Even males with apparently unaffected penises have been found to harbour HPV DNA on the glans penis (Grussendorf-Conen, E-I., et al, 1986). HPV DNA has also been detected in human semen (Ostrow, R.S., et al, 1986) indicating the presence of HPV DNA within the male urethra.

Human papillomavirus DNA infection is therefore a common finding in the genital tissues of both sexes and is associated with clinically or subclinically apparent lesions in a proportion of cases only.

4. The association between HPV and cervical cancer

4.1 Epidemiological evidence

The strong association between sexual activity and cervical cancer has been described. Genital papillomavirus infection is a common sexually transmitted disease (Report of Public Health Laboratory, Scotland 1983). The incidence of clinically obvious genital warts in both sexes has increased dramatically in recent years (Oriel, J.D., 1983). Simultaneously, the reported prevalence of CIN in younger women has also risen by some 60% (Roberts, A., 1982). Some workers (Bamford, P.N., et al, 1983) have reported that there is a subset of women under 35 years of age who are rapidly developing aggressive cervical carcinomata and Singer, A., et al, (1984) have postulated that these three factors are interrelated. Recently, Silcocks, P.B.S. & Moss, S.M. (1988) argued that this apparent increased incidence of disease in young women could be explained by trends in population structure, a general increased incidence of cervical cancer and an increased proportion of women being screened. The epidemiological evidence for a sequential association between HPV infection and the development of CIN and invasive cancer has also been challenged (Meanwell, C.A., et al, 1987). Similarly, Meanwell, C.A.,

et al (1988) have found no evidence of a poorer prognosis in young women with invasive disease. It is true, nevertheless, that these epidemiological coincidences have led many investigators to suspect that HPV is the sexually transmitted agent which causes cervical cancer (Singer, A., et al, 1984). The epidemiological case for HPV as a cervical carcinogen has been further strengthened by evidence that the "male factor" in cervical cancer might be HPV infection of the penis (Campion, M.J., et al, 1985).

Sero-epidemiological evidence for an association between HPV and cervical neoplasia was provided by Baird, P.L., (1983). He used disrupted BPV 2 virions in a solid phase enzyme linked immunosorbent assay for an IgG antibody and showed that patients with CIN and cervical cancer had significantly higher levels of serum antibody to HPV antigen than appropriate controls.

The natural history of cervical HPV infection has been studied prospectively. The major problem with prospective evaluation of patients with HPV infection lies in the criteria used for initial diagnosis. Biopsy and histological diagnosis of the nature of a colposcopically apparent aceto-white lesion is the most accurate means of differentiating HPV infection alone and HPV infection

co-existing with CIN, but biopsy itself may alter the natural history of the disease (Syrjanen, K., et al, 1985a). In an effort to avoid this problem, Syrjanen, K., and his group (1985a and 1985b) studied patients using cytology and colposcopy alone to diagnose and differentiate HPV infection and CIN. Some workers would defend the adequacy of these two subjective tests to make an accurate diagnosis [Reid, R., et al, (1984); Campion, M.J., et al, (1986); Vaeyrynen, M., et al, (1985)]. Others have shown that there is a poor correlation between cytology, colposcopy, histology and DNA-DNA hybridization analysis in this respect [Kirkup, W., et al, (1982); Walker, P.G., et al (1983); Murdoch, J.B., et al, 1988)]. Evaluation of data from prospective studies of HPV infection is difficult if the starting point is not clearly defined. What may be thought to be progression from a pure HPV lesion to CIN might, equally, be progression from HPV associated with colposcopically and cytologically occult CIN to HPV associated with colposcopically and cytologically apparent CIN. The methodological difficulties of prospective evaluation of HPV on the cervix are further demonstrated by Syrjanen, K., et al (1985a). They describe rates of progression to carcinoma-in-situ in patients with "HPV - associated CIN" as diagnosed by cytology and colposcopy and offer this as

evidence of progression from HPV infection to carcinoma-in-situ. These data could equally be used to show that CIN progresses to carcinoma-in-situ and that the presence of HPV DNA is quite fortuitous. In contrast, Cheetham, D., et al (1984) in a small controlled study showed that HPV infection diagnosed cytologically and colposcopically regressed spontaneously in 83% of cases. In addition to this, the rates of persistence of CIN lesions over the study period were unaffected by the coexistence of HPV infection. Studies of the natural history of HPV infections of the cervix are important but the design of such studies must be meticulous to allow proper evaluation (Armstrong, B., et al, 1988). Mitchell, H., et al, (1986) demonstrated an awareness of the limitations of the diagnostic performance of cytological screening in an elegant and simple study. They showed that the risk of a woman being subsequently diagnosed as having carcinoma-in-situ was 15.6 times higher if there was evidence of HPV infection at cervical cytology compared to controls. In patients under 25 years, this risk was 38.7. They, therefore, demonstrated that HPV infection on the cervix was associated with a high risk of neoplastic progression without addressing the role of HPV in that progression. This observation is clearly of great practical importance in screening for CIN and identifying

"at risk" groups. It is the most reliable available datum from prospective epidemiological studies.

4.2 Animal and human models for an oncogenic role of papillomaviruses

Several models exist in animals and more rarely in humans to support an oncogenic role for papillomaviruses.

The shope papillomavirus (Shope, R.E., 1933) which infects cottontail rabbits in certain parts of America such as Kansas and California was the first to be studied. Shope viral DNA has been demonstrated in these tumours (Stevens, J.G. & Wettstein, F.O., 1979). Bovine papillomavirus (BPV), which has been most extensively studied, has become the model for research into human papillomaviruses. BPV 4 produces papillomas in the upper alimentary tracts in cattle. Jarrett, W.F.H., et al (1978) showed that malignant transformation of these papillomata depended upon subsequent exposure to ingested bracken. It is noteworthy that although BPV appears to be necessary to induce papillomas, it is not necessary that the genome should persist for malignant transformation to occur or for it to be maintained (Campo, M.S., et al, 1985). Bracken contains several chemical carcinogens and may also be immunosuppressive. It would seem that bracken causes immunosuppression which induces the spread of papillomas

and allows their persistence beyond their average life-span in immunocompetent animals (Campo M.S., et al, 1985). The fragment of the BPV genome which is capable of inducing cell transformation is located in a BamH1/Hind 111 DNA fragment which represents 69% of the full length of the viral genome (Lowy, D.R., et al, 1980). Nakabayashi, Y., et al (1983) showed that this region represents the early region of the viral genome, and Schiller, J.T., et al (1984) demonstrated an independent cell transforming region corresponding to the E6 open reading frame of the early region of the viral genome.

Another example of papillomaviruses and a chemical cofactor being implicated in tumour formation is found in the rodent, Mastomys natalensis (Amtmann, E., et al, 1984). In this animal extrachromosomal viral DNA increases in copy number with advancing age and papillomata begin to appear. If the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is applied to the skin of young Mastomys this process is greatly accelerated compared to controls. TPA has been shown to induce replication and transcription of BPV1 DNA <u>in vitro</u> (Amtmann, E., & Sawer, G., 1982) and it may be that this effect is present <u>in vivo</u> in the Mastomys. Unfortunately Amtmann et al could not detect papillomavirus transcripts in this animal model.

In humans, outwith the female genital tract, examples of malignancy directly associated with papillomaviruses are rarer. The large condylomatous Buschke-Lowenstein tumour on the penis has been reported to undergo malignant transformation to squamous cell carcinoma (Boxer, R.J., and Skinner, D.G., 1977). The rare skin condition epidermodysplasia verruciformis (E.V.) is induced by HPV (Green, M., et al, 1982). Malignant transformation in EV is associated with HPV 5, HPV 8 and HPV 14 genomes and cancers are clustered in areas exposed to the sun, suggesting that UV light is the relevant co-carcinogen in this case [Ostrow, R.S., et al, (1982); Lutzner, M.A., et al, (1984)]. Papillomaviruses, therefore, can be regarded as oncogenic viruses. It is important to note, however, that, in the examples cited above, co-factors play a significant part in progression to malignant disease.

4.3 Morphological evidence

The atypical condyloma or "flat wart" is the most common colposcopic appearance associated with cervical HPV infection (Meisels, A., et al, 1977). Their similarity to, and confusion with, CIN lesions is well documented (Meisels, A., et al, 1981). Colposcopic appearances associated with HPV infection comprise aceto-white epithelium with mosaicism and punctation which is usually

multifocal (Meisels, A., et al, 1981).

Reid, R., et al (1984) described a weighed colposcopic index for differentiating these two lesions which in their hands accurately predicted the histological diagnosis in 96% of cases. They emphasise that HPV infection and CIN frequently coexist and regard this as evidence that there is a continuous spectrum of disease. Other workers disagree about the diagnostic reliability of colposcopy and note a poor correlation between colposcopic impression and other diagnostic criteria [Kirkup, W., et al,(1982), Walker, P.G., et al,(1983)].

4.4 Electron microscopic evidence

The presence of viral particles in human genital warts was reported in 1968 (Dun, A.E.G., and Ogilvie, M.M., 1968). The presence of viral particles was also demonstrated in atypical condylomata (Ferenczy, A., et al, 1981). Shah, K.H., et al, (1980) used papilloma group specific antiserum to detect HPV antigen in patients with mild dysplasias. They showed that cells which were found to contain virus particles at electron-microscopy also stained for papilloma virus antigen. There is therefore no doubt that HPV is present in atypical condylomata.

4.5 Cytological, histological and immunohistochemical evidence.

Koss, L.G., and Durfee, G.R., (1956) first coined the term koilocytes to describe the cells now known to be associated with HPV infection. Dun, A.E.G., and Ogilvie, M.M., (1968) showed that viral particles were present within these cells and Meisels, A., et al, (1981) defined the cytological and histological features associated with flat condylomata. The confirmation that the colposcopic, cytological and histological appearances were associated with HPV was provided by Morin, C., et al (1981) using pooled papillomavirus antigen in a peroxidase-anti-peroxidase staining technique. They showed that 60% of atypical condylomata with koilocytes present contained papillomavirus antigen. The cells which stained for papillomavirus antigen were detected in the superficial layers of the epithelium which showed that the manufacture of HPV structural proteins occurs in terminally differentiating keratinocytes. They showed that papillomavirus antigen was largely absent from areas of dysplastic change characterised by a lack of terminal differentiation of cells. These findings were confirmed in other studies [Woodruff, J.D., et al, (1980); Kurman, R.J., et al, (1981)]. Winkler, B., et al (1984) reported that the lesions which contained both abnormal mitotic

forms and papillomavirus antigen were usually characterised by aneuploidy. They suggested that this subgroup might represent a transition between flat warts and CIN.

4.6 DNA-DNA hybridization evidence

DNA-DNA hybridization studies have provided the strongest evidence for an association between HPV and genital neoplasia. The technique was first applied to HPV analysis by Favre, M., et al, (1975).

HPV 6 was the first subtype detected in female genital warts (Gissmann, L., and Zur Hausen, H., 1980). The HPV 6 genome was subsequently cloned and two subgroups HPV 6a and HPV 6b, were identified (De Villiers, E-M., et al, 1981). Gissmann, L., et al, (1982a) probed 44 samples from condylomata acuminata and detected HPV DNA in 41 (93%). They also detected HPV 6 in 3/3 Buscke-Lowenstein penile tumours but failed to detect HPV 6 DNA in 54 human female and male genital cancers. The HPV DNA detected in the condylomata acuminata was found to be in the supercoiled plasmid form and was not integrated into the host cell chromosome. The same group subsequently defined the nuclear base pair sequence of HPV 6b and showed it to be similar to HPV 1a and BPV 1 in that all the major open reading frames (ORFs) were located on one DNA strand. (See

Fig. 1; opposite p.43) Furthermore all 3 papillomaviruses comprise seven ORFs in the early region and two ORFs in the late region of the genome. Using the same clone of HPV 6 DNA, McCance, D.J., et al (1983) found HPV 6 DNA sequences in 13 out of 22 women with CIN. HPV 6 DNA was detected in all three grades of CIN. In a further study Gissmann, L., et al, (1983) probed genital and laryngeal papillomas with HPV 6 and HPV 11 DNA sequences, [HPV 11 was initially cloned from viral DNA derived from a laryngeal papilloma (Gissman, L., et al, 1982b)]. They found HPV 6 DNA in 41 out of 63 condylomata acuminata and HPV 11 DNA in 13 out of 63 condylomata acuminata. HPV 11 DNA was also detected in 2 invasive cervical cancers and 2 carcinomas-in-situ. The nucleotide sequence of HPV 11 was subsequently found to share an 82% base pair sequence homology with HPV 6 (Dartmann, K., et al, 1986). These data suggested that HPV 6 and HPV 11 had very similar DNA coding regions and were associated with benign genital lesions.

HPV 16 DNA was detected by hybridization of DNA from a cervical cancer biopsy to an HPV 11 DNA probe under low stringency conditions. The viral DNA was cloned in phage lambda and the cloned DNA was used to probe other tumours (Durst, M., et al, 1983). In this initial study, 61% of cervical cancer samples from Germany hybridized to HPV 16

DNA sequences compared to 34.8% of cervical cancer specimens from Kenya and Brazil. In contrast to HPV 6/11 only 2 out of 33 condylomata acuminata contained HPV 16 DNA sequences. These data suggested HPV 16 was more closely associated with invasive cancer of the cervix than benign lesions. Crum, C.P., et al, (1984 and 1985) studied the relationship between the presence of HPV 16 DNA and abnormal mitotic figures in precancerous lesions of the cervix. They found a strong positive correlation between the two and suggested that the presence of HPV 16 DNA was associated with an increased risk of progression to malignancy.

Cloned HPV 16 DNA supplied by the Heidelberg group (Durst, M., et al, 1983) was utilized in several studies in the United Kingdom. In London, McCance, D.J., et al (1985a) detected HPV 16 DNA in 62% of CIN and 90% of malignant lesions compared to 18% (3/17) of apparently normal control patients. They confirmed the impression that HPV 16 was associated with more severe lesions than HPV 6/11. Similar findings were recorded from East Anglia (Scholl, S.M., et al, 1985) and the West of Scotland (Millan, D.W.M., et al, 1985). The belief that HPV 16 was strongly associated with invasive disease was however challenged in one small study (Fukushima, M., et al, 1985) which detected HPV 16 DNA in only 17% of invasive cancers

compared to 83% of CIN lesions. Most studies also suggested that HPV 16 DNA was detected in its episomal form in CIN but was found integrated into the host cell chromosome in invasive cancers. The physical state of HPV 16 DNA in benign and invasive genital tumours was reported by Durst, M., et al, (1985). They probed invasive tumours and cloned virus/cell junction fragments to demonstrate integration of the viral genome into the host cell chromosome in these lesions. Di Luca, D., et al (1986) showed that integration of the viral genome was also found in CIN lesions and suggested that integration had prognostic significance of progression to malignancy.

The fourth HPV subtype associated with genital malignancy, HPV 18, was detected in 25% of malignant tumours from Africa and Brazil and 2 out of 13 cancers in Germany. There was no evidence of hybridization to condylomata or cervical dysplasias (Boshart, M., et al, 1984). This suggested that HPV 18 DNA was associated with more severe lesions and Barnes, W., et al, (1988), in a study of 30 squamous carcinomas of the cervix, reported evidence of a direct correlation between the presence of HPV 18 DNA and nodal involvement in clinically diagnosed staged 1B disease. They suggested that HPV 18 was related to more aggressive tumours than HPV 16. Alternatively, Millan, D.W.M., et al, (1986) detected HPV 18 DNA in 4 out of 26

punch biopsies from CIN lesions, 2 of which showed evidence of integration of the viral DNA.

Two other HPV subtypes are related to genital neoplasia in a minority of cases. HPV 31 is related to HPV 16 with 30-40% base pair sequence homology and has been detected in 20% of mild to moderate dysplasias and 6% of invasive tumours in one study (Lorincz, A.T., et al, 1986) and in 6% of tumours in the study of Barnes, W., et al (1988). HPV 33, also related to HPV 16, was reported in 2 of 29 CIN lesions and 1 of 53 cervical carcinomas by Beaudenon, S., et al (1986). Thirdly, HPV 35 has been isolated in 1% of anogenital intraepithelial neoplasia and in 4% of anogenital cancers in one study (Lorincz, A.T. et al, 1987). Finally, two new subtypes HPV 39 and HPV 42 have been identified in association with penile bowenoid papules and vulval papillomas respectively (Beaudenon, S., et al, 1987).

The majority of these prevalence studies, however, did not include data on normal control patients. One which did (McCance, D.J., et al, 1985a) reported an 18% incidence of HPV 16 in colposcopically and histologically apparently normal cervices. Toon, P.G., et al (1986) studied 106 patients with smears showing non specific inflammatory changes and found CIN in 12.3% of cases and HPV DNA in

22.6% of cases. It is not clear, however, what proportion of cases with CIN also exhibited HPV DNA. Furthermore in a second group of 104 controls with normal cervical cytology, 11.5% exhibited HPV DNA. In a small study in Birmingham, (Cox, M.F., et al, 1986) 38% of colposcopically and cytologically normal control cervical biopsies contained HPV DNA. Later, this same group (Meanwell, C.A., et al, 1987) detected HPV DNA sequences in 35% of a group of normal control cervices. They also reported that 66% of biopsies from invasive cancer of the cervix hybridized to HPV DNA. Interestingly, when an age discrepancy in the two groups was controlled for, there was no significant difference between cases and controls. These data suggested that the association between HPV 16 and cervical neoplasia is age mediated.

In Germany, over 9,000 women were screened for evidence of HPV DNA by a dot blot technique on cells collected by cervical scraping (De Villiers, E-M., et al, 1987). They established that the prevalence rate for HPV 16 DNA infection on the human ectocervix was 10%. Dot blot hybridization is subject to a number of methodological difficulties and the authors suggested that they may have undercalled the true prevalence by 10-20%. They further stated that it would be beneficial to compare dot-blot and Southern blot results to establish the sensitivity and

specificity of their assay.

In general, therefore, the available evidence suggests that 10%-35% of the healthy female population harbour HPV DNA on the cervix.

Studies of healthy women (external controls) are important to establish the prevalence of infection in the community. Ferenczy, A., et al (1985) examined the normal vulval skin adjacent to vulval condylomata acuminata and found HPV DNA in 45% of cases. This study emphasised the importance of two control groups when analysing the prevalence of HPV DNA in tissue samples, that is, an external control from apparently normal women and tissue from apparently normal tissue adjascent to abnormal tissue - an internal control. Macnab, J.C.M., et al (1986) reported a parallel study involving samples from invasive cancer of vulva and cervix along with internal controls. They found hybridization to HPV 16 DNA sequences in 84% of tumours but also found HPV 16 DNA sequences in 73% of histologically normal tissue within 2cm to 5cm of the tumours.

The detection of HPV DNA in the genital tract is therefore common. In patients with genital tract neoplasia the prevalence is high but the viral genome is not confined to histologically abnormal epithelium and is frequently found in adjacent normal tissue.

4.7 Evidence from studies of integration and transcription of HPV DNA in vivo and in vitro and of HPV DNA in morphological cell transformation in vitro

HPV 16 and HPV 18 have been studied to establish whether the viral genome is found in the plasmid state or integrated into the host cell chromosome in tumours and cell lines derived from cervical cancers. The transcription patterns of the viral genomes have also been studied along with the ability of HPV DNA to transform cultured cell lines.

When HPV 18 was characterised it was found that the viral genome was integrated into the host cell chromosome in invasive cancers and in cell lines derived from cancer of the cervix (Boshart, M., et al, 1984). Further analysis revealed that HPV 18 sequences from the E6/E7/E1 region were consistently preserved and that deletion of the E2 to L2 region occurred in some cases (Schwarz, E., et al, 1985). Pater, M.M., and Pater, A., (1985) analysed 8 cell lines derived from squamous carcinomas of the cervix and confirmed that the E1, E6 and E7 open reading frames (ORFs) were consistently preserved. It is possible that integration into the E2 and L2 ORFs disrupts the viral genome in such a way that enhanced expression of the E6 and E7 ORFs occurs which may have a role in malignant transformation of cells (Schneider Maunorury, S., et al,

1987). The consistent pattern of integration which is also found with HPV 16 allows transcription of not only the E6, E7 and E1 viral ORFs but also allows transcription of flanking cellular sequences and it may be that these viral-cell transcripts indicate that integration of the viral DNA into the host cell chromosome may be important in a multi-step mechanism of carcinogenesis (El Awady, M.K., et al, 1987). Integration of the HPV genome into the cell chromosome is consistent, while the site of integration in the cell chromosome appears to be randomly selected (Mincheva, A., et al, 1987).

In addition to identifying integration of the viral genome, Schwarz, E., et al, (1985) also detected mRNA sequences derived from the E6, E7 and E1 ORFs of HPV 18 in 3 cell lines. Lehn, H., et al, (1985) however, was able to identify mRNA in only one of four cervical carcinomas which exhibited integrated HPV 16 sequences. Smotkin, D., and Wettstein, F.O., (1986) also reported transcription of the E6 and E7 ORFs of HPV 16 in a cancer cell line (CaSki) and in a cervical cancer. Thereafter, the consistent expression of the E6 and E7 ORFs of HPV 16 and HPV 18 in cancer cell lines was confirmed [Schneider-Gadicke, A., and Schwartz, E., (1986); Seedorf, K., et al, (1987); and Androphy, E.J., et al, (1987)]. In contrast to the E6 and E7 ORFs the E2 to E5 ORFs are not expressed due to the

integration in the E1/E2 region of the viral genome. Such integration separates the E2-E5 ORFs from the viral promotors in the control or regulatory region of the genome. The E5 ORF has a transforming role in BPV 1 (See fig. 2, opposite p.44) so Bubb, V., et al (1988) have sequenced the E5 ORF of HPV 16 and predicted that the E5 protein is probably functionally associated with the cell membrane. As the E5 protein is not expressed when HPV DNA is integrated into the host genome it is probably not required for maintenance of the transformed phenotype. Bubb and co-workers therefore postulated that suppression of production of the E5 protein might be important to avoid a host immune response against it. Thus both expression of some ORFs and suppression of others may be implicated in a complex process of oncogenesis.

A distinct mechanism of cell transformation by HPV 18 DNA was suggested by Swift, F.V., et al, (1987) who characterised a long-range acting enhancer sequence GA1 in the Hela genome. GA1 is located within the integrated genome of HPV 18. This enhancer sequence surprisingly shows narrow cell type specificity being active in some cervical cancer cell lines but inactive in tumours derived from other cell types.

The ability of cloned HPV DNA to cause morphological

transformation of mouse C127 cells was reported by Watts, S.L., et al (1984) using cloned HPV 1 and HPV 5 DNA. These transformed cells could produce tumours in athymic nude mice. Morphological transformation with papillomavirus from condylomata acuminata of human uterine cervix cells in an *in vivo* system using athymic nude mice has been described (Kreider, J.W., et al, 1985). HPV 16 DNA produces morphological transformation in NIH 3T3 cells which are tumorogenic in athymic nude mice (Yasumoto, S., et al, 1986) and the E6/E7 region alone from HPV 18 DNA is sufficient for transformation of NIH 3T3 and Rat-1 cells (Bedell, M.A., et al, 1987). However, transfection of human keratinocytes from the foreskin with HPV 16 did not result in tumorogenic cell lines although it did extend the lifespan of these cells (Pirisi, L., et al, 1987). HPV 16 DNA E6 and E7 ORFs resemble immortalising oncogenes such as c-*myc*, and as such will cooperate with transforming oncogenes such as the EJ-*ras* gene in producing cell transformation (Matlashewki, G., et al, 1987). In addition, enhancement of HPV 18 E6 and E7 transcription is accompanied by cell proliferation whereas inhibition of E6 and E7 transcription by antisense RNA leads to decreased cell growth (Knebel-Doeberitz, M., et al, in press, cited by Rosl, F., et al, 1988). Using 5-azacytidine, a potent

demethylating agent, Rosl et al provided evidence that this enhancement of the E6 and E7 transcription might be controlled by host genes at a cellular level in non-tumorogenic hybrid cell lines.

These data provide compelling evidence of a role for HPV in the oncogenic process in vitro and in immunocompromised laboratory animals. There is, however, no in vitro system which mimics the early stages of cervical dysplasia nor is there direct evidence for a role of these HPV gene produces in human genital carcinogenesis in vivo.

5. The association between HPV and vulval cancer

Genital warts has been recognised as a sexually transmitted disease since ancient times. The epidemiology of the infection parallels that of gonorrhoea. Oriel, J.D., (1971) reviewed the natural history of genital warts and stated that untreated warts sometimes remained for long periods but spontaneous regression was documented. He considered malignant transformation to be a rare complication. There are, however, numerous case reports in the literature of malilgnant transformation of genital warts and Zur Hausen, H., (1977) estimated that about 5% of vulval carcinomata arise "within persisting genital warts". Some authors have claimed that genital warts are, in fact, premalignant conditions (Charlewood, G.P., and

Shippell, S., 1953).

Immunohistochemical and DNA-DNA hybridization data on the detection of HPV in condylomata acuminata and vulval premalignancy and malignancy has tended to be reported along with data about more common cervical lesions. Kurman, R.J., et al (1981), reported positive staining for papillomavirus antigen in 50% of cases of vulval condylomata. Crum, et al, (1982) used immunohistochemistry to study 68 VIN lesions for evidence of papillomavirus antigen. Thirty nine of these lesions were also subjected to microspectrophotometry. They found a negative correlation between aneuploidy and detection of papillomavirus antigen and reasoned that HPV required maturation of epithelial cells for its full reproductive cycle. They then surmised that, as condylomata acuminata were polyploid lesions and VIN lesions were aneuploid, an as yet undiscovered cofactor mediated conversion of one to the other. They did not point out that their data could equally suggest that VIN and condylomata acuminata were two quite separate lesions which shared the same site. HPV 6 DNA was characterised from biopsies of condylomata acuminata (Gissman, L. and Zur Hausen, H., 1980), the viral DNA was cloned (De Villiers E-M., et al, 1981) and the HPV 6 DNA was used to probe other wart biopsies to show that 93% of vulval condylomata acuminata DNA

hybridized to HPV 6 (Gissman, L., et al, 1982a). This group failed to demonstrate HPV 6 DNA in 2 vulval carcinomas but later showed hybridization of HPV 16 DNA to DNA from 2/7 (18.6%) of vulval carcinomata (Durst, M., et al, 1983). Similarly, Singer, A. and McCance, D., (1985) mentioned the detection of integrated HPV 16 DNA sequences in 6 of 9 vulval carcinomata. HPV 33 DNA has recently been found in association with a minority of cases of Bowen's disease (hyperplastic vulval dystrophy) (Beaudenon, S., et al, 1986).

These studies did not include control tissues, however, but Di Luca, D., et al, (1986) detected HPV 16 DNA in 2/10 vulval carcinomata, 5/5 VIN 3 lesions, 0/6 condylomata acuminata and 0/6 internal control biopsies. In contrast, Ferenczy, A., et al, (1985), detected HPV DNA sequences in 9/20 (45%) of internal control tissues adjacent to vulval condylomata. Similarly, Macnab, J.C.M., et al, (1986) detected HPV DNA sequences in 9/11 vulval carcinomas as well as in all of the 3 internal control samples which were available. Evidence for a role of HPV in the genesis of vulval neoplasia is therefore scantier than its very much more common counterpart on the cervix. The presence of HPV on the vulva seems to be a common event but vulval cancer is rare. The studies of Ferenczy and Macnab demonstrate the importance of adequate control tissues.

6. The role of oncogenes in carcinogenesis

All vertebrate cells harbour a number of genes which have the potential to become transforming genes or oncogenes. The genes which have the potential to become oncogenes are called proto-oncogenes (c-onc genes).

The first group of oncogenes were identified in retroviruses. This ability of DNA sequences to cause malignant change in cells was first demonstrated by Rous in 1910, who showed that the retrovirus, Rous sarcoma virus (RSV), caused sarcomata in chickens. Over 60 years later it was shown that cell transformation was caused by the RSV v-onc gene named v-src (Stehelin, D., et al, 1976). The prefix "v" indicates that the oncogene is of viral origin whereas c-onc has the prefix "c" indicating an oncogene of cellular origin. The majority of known oncogenes are v-onc genes. Most c-onc genes show homology between themselves and retroviral sequences suggesting an overlap between viral and cellular oncogenes. An example of homology between v-onc and c-onc genes is that found between the T24 bladder oncogene and the v-bas oncogene (Santos, E., et, 1982).

Cellular oncogenes are highly conserved throughout evolution and are thought to perform functions essential to the normal cell in the control of cell growth and

development which, when disturbed, contribute to the malignant phenotype (Bishop, J.M., 1981). There are now well over 20 proto-oncogenes recognised, most of which represent single copy sequences of cellular DNA. The ras gene is an exception being a constituent of a multigene family.

Proto-oncogenes can be activated in a number of ways:-

a. A proto-oncogene may undergo a mutation which would result in an abnormal oncogenic gene product. An example of this is the point mutation substituting a guanosine base for a thymidine base which is responsible for the activation of the T24 bladder carcinoma oncogene (Reddy, E.P., et al, 1982).

b. A proto-oncogene may become oncogenic when its products are expressed at higher concentrations than normal. A mutation could produce a tandem duplication of a proto-oncogene. Gene amplification could then occur by successive unequal sister chromatid exchanges in different cell cycles until sufficient protein is produced to transform the cell (Pall, M. L., 1981).
Proto-oncogenes can also be upregulated by promotor insertion (Hayward, K. S., et al, 1981) or enhancer insertion (Weber, F., & Schaffner, W., 1985).
Translocations can result in proto-oncogene upregulation.

Taub, R., et (1982) demonstrated that the c-myc oncogene has been translocated into the immunoglobulin heavy chain locus in some Burkitt's lymphomas. This brings c-myc under the influence of enhancer elements in the heavy chain locus (Boss, M.A., 1983).

c. A proto-oncogene may be expressed at an inappropriate time in the cell cycle. The c-myc gene is tightly regulated in the cell cycle being undetectable in resting cells but expressed at high levels in the G1 phase (Kelly, K., et al, 1983). In Burkitt's lymphomas this mechanism may be augmented by gene translocation as the c-myc gene is no longer switched off during B-cell differentiation in its new locus (Leder, P., et al, 1983).

d. A proto-oncogene might be expressed in an inappropriate cell type leading to unmasking of its oncogenicity. Evidence for this mechanism is difficult to find as the individual patterns of proto-oncogene expression in different cell types are not well defined and some proto-oncogene products like c-src, c-myc and c-ras are almost ubiquitous (Hunter, T., 1984).

The c-myc oncogene appears to have a close association with the the development of certain tumour types. The abnormalities associated with it include: chromosomal translocation in Burkitt's Lymphoma (b & c above);

proviral insertion adjacent to c-myc in chicken B-cell lymphomas and c-myc amplification in some human cancers, eg. colon cancer, promyelocytic leukaemia, large and small cell lung cancers and breast cancer (Rabbitts, T.H.,1987).

The c-myc and c-Ha-ras proto-oncogenes have recently been studied in connection with neoplasia of the uterine cervix. There is a correlation between the number of c-myc transcripts and the overall rate of cellular proliferation in epithelial cells which raises the possibility of a role of this oncogene in epithelial neoplasia (Stewart, T.A., et al, 1984). Riou, G.F., et al (1985) investigated 37 stage I and II cervical carcinomas for the presence of HPV DNA and the oncogenes c-myc and c-Ha-ras. HPV DNA sequences were detected in 33 samples and upregulation of both c-myc and c-Ha-ras correlated strongly with aggressive disease. They postulated that HPV DNA and activated cellular oncogenes are involved in tumour progression. Later, the same group studied expression of the c-myc gene in 72 specimens of stage I and II squamous cancer of the cervix. They identified over-expression of the c-myc gene in 25 and showed a highly significant correlation between over-expression and early relapse of disease (Riou, G.F., et al, 1987). The level of c-myc expression could thus be a significant

prognostic factor in invasive disease and this finding lends support to a putative role of this oncogene in cervical carcinogenesis. In contrast, a study of 27 cervical carcinomas and 8 control tissue samples failed to reveal any evidence of upregulation of the c-myc oncogene (Ikenberg, H., et al, 1987). In a similar study Gariglio, P., et al (1987) described 35 late cervical cancers (Stage III and IV). They found HPV 16 DNA sequences in 31%. Amplification of c-myc was detected in 32 samples. Rearrangement of c-myc alone was seen in 2 cases and both amplification and rearrangement of c-myc was detected in 15 cases. The authors describe the absence of HPV 16 DNA and alteration of the c-myc oncogene in normal control tissues but do not define the nature of these control tissues. Furthermore, from the data presented there seems to be a poor correlation between hybridization to HPV 16 DNA sequences and amplification and/or rearrangement of the c-myc oncogene.

The results of Riou et al do, however, raise the possibility that HPV DNA and its transforming gene products may act in concert with the c-myc oncogene in malignant transformation of cervical cells and this association deserves further study.

7. The role of host immune systems

The host immune system is a major defence mechanism against cancer. Patients with preinvasive and invasive cervical cancer have enhanced cell mediated immune responses as measured *in vitro* by leucocyte cytotoxicity testing (Dini, M.M., et al, 1980). Patients who are immunosuppressed to prevent allograft rejection are 10-100 times more likely to develop a malignancy (Penn, I., 1974). Cordiner, J.W., et al (1980) reported an increased incidence of CIN on the cervices of immunosuppressed renal transplant women and emphasised the need for close follow-up in such cases. Sillman, F., et al (1984) studied a group of 20 immunosuppressed women with CIN for evidence of HPV by immunohistochemistry. All 20 had HPV infections and 12 of the patients had persistent or recurrent CIN at follow-up. HPV infection and CIN are therefore common findings in immunosuppressed women.

Cigarette smoking is second only to HPV infection as a specific risk factor for the development of CIN. Interestingly, a reversible alteration in the immunoregulatory T cells OKT3+, OKT4+ and OKT8+ has been recognised in smokers compared to control non-smokers (Miller, L.G., et al, 1982). This raises the possibility that the mechanism of smoking as a co-factor in the

development of cervical malignancy might be mediated via alterations in local immunosurveillance.

In recent years nature has provided an interesting and growing group of women who are immunosuppressed by human immunodeficiency virus (HIV). Bradbeer, C., (1987) assessed a group of 11 women attending an S.T.D. clinic with HIV infection and found evidence of CIN in 9 cases. The mode of transmission of HIV seems to be important as Spurrett, B., et al (1988) reported evidence of CIN in only 1 out of 6 women who acquired their HIV infection non-sexually. This would suggest that a sexually transmitted agent, possibly HPV, is also required (Crocchiolo, et al, 1988).

In addition to evidence that the systemic immune status of the patient may be relevant to the development of malignancy, recent attention has centred on the possible role of local immunocompetent cells on the cervix. Langerhans' cells are antigen presenting cells which stimulate a T cell response to the neoplastic process and are widely detected in cervical squamous epithelium (Morris, H.H.B., et al, 1983a). Morris, H.H.B., et al (1983b) also studied the distribution of Langerhans' cells and T lymphocytes in HPV infection and CIN by an immunohistochemical technique using monoclonal

antibodies.

They found that tissue associated with HPV infection had depleted or absent Langerhans' cells compared to normal tissue. The authors suggested that HPV may have a direct local immunosuppressive effect and thereby increase the risk of malignant transformation. It seems that if there is a defect in the local immune response which contributes to the generation and progression of CIN, it is likely to be in the afferent limb of that response.

Similar findings and conclusions were reported by McArdle, J.P., & Muller, H.K., (1986) using an antibody to the S100 protein of Langerhans' cells. Langerhans' cells which stain for the S100 protein comprise 35% of the total Langerhans' cell population on the cervix and appear to be depleted to a greater extent than other subpopulations in HPV infection (Tay, S.K., et al, 1987a). In both this study and a second study of the numbers of the T4+ and T8+ subsets in HPV infection and related CIN, (Tay, S.K., et al, 1987b) this group used as control tissue biopsies from women who had "normal" cervices. These external controls numbered 20 in the first study but only 6 in the second study. No attempt was made to study internal control biopsies in normal tissue adjacent to acetowhite epithelium.

B. THE PRESENT STUDY

The present study aimed to utilize DNA-DNA hybridization to:-

1. Determine the relationship between HPV DNA detection and cytological, histological, colposcopic and immunohistochemical detection of HPV infection;

2. Determine the prevalence of HPV 6, 11, 16 and 18 genomes present in DNA extracted from abnormal punch biopsies and internal controls from women attending the colposcopy clinic for treatment for abnormal cervical cytology along with the prevalence of HPV subtypes in DNA extracted from the cervices of normal external control women;

3. Determine the prevalence of HPV 6, 11, 16 and 18 DNA in biopsies from patients with invasive carcinoma of the cervix and matched control biopsies in the West of Scotland;

4. Determine the prevalence of HPV 6, 11, 16 and 18 DNA in biopsies of patients with preinvasive and invasive disease of the vulva and matched control biopsies in the West of Scotland;

5. Determine the relationship between the age of patients, the detection of HPV DNA in the genital tract, and the histological diagnosis;

6. Determine the relationship between the presence of HPV DNA and local immunosurveillance assessed by Langerhans' cell content in CIN biopsies;

7. Determine the relationship between hybridization of biopsy DNA to the c-myc proto-oncogene, the histology of the biopsy, and hybridization of biopsy DNA to HPV DNA;

8. Determine the effectiveness of laser ablation of the transformation zone in removing HPV DNA from the cervix in cases of histologically proven CIN;

9. Determine the sensitivity and specificity of dot blot hybridization as a method for mass screening of the population for cervical HPV infection and, in the light of Study 8 above, the value of such a screening programme.

These approaches and their background will now be described.

1. The relationship between different methods of detection of HPV infection

The histological diagnosis of HPV infection has been

defined in detail (Fletcher, S., 1983). The prognostic significance of the cytological diagnosis of HPV infection of the cervix is clearly important for patient follow-up (Mitchell, H., et al, 1986).

The ability of colposcopy to differentiate between HPV infection and CIN remains controversial [Reid, R., et al (1984); Kirkup, W., et al (1982)]. Sixty percent of condylomata acuminata stain for PV antigen by immunohistochemistry, whereas 48% of cervical dysplasias stain for PV antigen (Kurman, R.J., et al, 1981). HPV DNA is detected in 60%-90% of condylomata acuminata, CIN and invasive cancer (Singer, A. and McCance, D., 1985). Given the high cost and methodological difficulties with DNA-DNA hybridization it is useful to determine the inter-relationship between these diagnostic methods in the same tissues. If a histopathologist or cytopathologist could consistently diagnose HPV infection on the cervix, there would be no need to resort to DNA-DNA hybridization analysis to diagnose cervical HPV infection. Thus studies of the natural history of HPV infection or population screening for HPV infection would not require DNA studies. DNA-DNA hybridisation analysis would, of course, still be required for differentiating viral subtypes and for studies of the physical state and transcriptional activity of HPV DNA.

This work was done in collaboration with Dr Laura Cassidy who performed the immunohistochemical assays, and the histopathology department of the Royal Infirmary, Glasgow, who provided histological diagnoses.

2. Incidence of HPV DNA in colposcopically abnormal tissues matched internal control tissues and external control tissue of the uterine cervix.

The prevalence of HPV DNA in premalignant disease of the cervix has been discussed in the literature review. Few of these workers have studied control tissues. Ferenczy, A., et al (1985) and Macnab, J.C.M., et al (1986) have clearly shown that HPV DNA can be detected in apparently normal tissue adjacent to condylomata acuminata and invasive genital cancer respectively. The key-stone of the relationship between HPV and invasive disease of the genitalia has been the detection of HPV DNA in malignant tissue. In order to assess the significance of that association two control biopsies are required i.e. internal and external controls.

Secondly, HPV type 6 and 11 have been associated with condylomata acuminata and mild CIN lesions (Gissman, L., et al, 1983) whereas HPV 16 and 18 are associated with severe CIN and invasive cancer (Crum, C.P., et al, 1985).

A geographic variation in HPV DNA subtype exists. The

prevalence of HPV 16 DNA in genital cancer in Germany is twice that found in Kenya and Brazil (Durst, M., et al, 1983). HPV 6 was detected in 28% of CIN lesions of all grades in England (McCance, D.J., et al, 1985). It is relevant, therefore to determine whether the apparent prognostic significance of HPV DNA subtypes can be explained by geographic variation in the prevalence of each HPV subtype.

This work was done in collaboration with Dr Laura Cassidy who collected some of the specimens and the histopathology departments of the Royal Infirmary and Western Infirmary, Glasgow.

3. and 4. The aims of sections 3 and 4 coincide with that of section 2 and will not be discussed further.

5. The relationship between age of the patient and HPV DNA detection

Much epidemiological evidence exists to suggest a sequential relationship between HPV infection, development of CIN and progression to invasive cancer of the cervix (Singer, A., McCance, D., 1985). Meanwell, C.A., et al (1987) suggest that this relationship is less clear and that HPV infection increases in prevalence with the patients' ages as, of course, does cervical cancer. They further suggest that the epidemiological evidence for

young age being an adverse prognostic factor in invasive disease may be misleading (Meanwell, C.A., et al, 1988). Clearly, age must be controlled for in prevalence studies of HPV infection. In this study the age of the patients were recorded so that this factor could be related to the presence of HPV DNA and histological diagnosis.

6. The relationship between Langerhans' cell numbers and HPV DNA sequence detection

The relationship between systemic or local immunosuppression and cervical neoplasia has been described (Introduction, A,4.7). Morris, H.H.B., et al (1983) further described a difference in the host immune responses between CIN and HPV lesions on the cervix as assessed by immunohistochemistry. The relationship between Langerhans' cell number in normal and abnormal tissues of the cervix has not been assessed nor has the association between Langerhans' cell numbers and HPV DNA subtypes. These experiments aimed to elucidate this relationship and explore the possibility that the proposed oncogenic effect of HPV DNA may be mediated by changes in the afferent loop of the local immuno-regulatory system. This work was done in collaboration with Dr Robert Hawthorn who assayed the Langerhans' cell number and made the histological diagnoses and with Dr R. Burnett who independently assessed the histological diagnoses.

7. The relationship between hybridization to the c-myc proto-oncogene and detection of HPV DNA sequences

There are many experimental data to suggest a role for oncogenes in carcinogenesis (Introduction, A,6). Riou, G., et al (1987) demonstrated a correlation between c-myc oncogene expression and carcinoma of the cervix. The association between HPV and genital tract malignancy is established. It is necessary to establish a mechanism whereby HPV might cause cancer. One mechanism might be related to up-regulation of cellular oncogenes by HPV DNA gene products, the most likely being the E6, E7 and E1 proteins. This study aimed to explore the relationship between the oncogene, c-myc, HPV DNA detection and histological grading of biopsies.

8. Treatment of HPV infection on the cervix by laser ablation

Laser ablation of the transformation zone effectively treats CIN lesions at the first attempt in 90% of cases in the Western Infirmary, Glasgow (J.W., Cordiner, personal communication). Treatment of HPV infection in the absence of CIN has been advocated (Singer, A., et al, 1984). The success of such a policy may depend on the presence of latent HPV DNA outwith the treatment area (Ferenczy, A., et al, 1985). HPV DNA is widely detected in the male and

female genital tracts (Introduction, A,4.6). Any screening programme for detection of HPV DNA in the female genital tract must have an effective available treatment response to positive results. It is essential, therefore, to assess the efficiency of treatment in achieving the eradication of the viral genome from the affected tissues.

9. Dot blot hybridization screening for HPV DNA

Wickenden, C., et al, (1985) described a technique of dot blot hybridization of DNA retrieved from cells harvested by cervical scraping as a method of screening for wart virus infection. This technique was used to study the prevalence of HPV infection on the cervix in Germany (De Villiers, E-M., et al, Lancet, 1987). Southern blot hybridization is the most sensitive and specific method of HPV DNA detection but it is expensive in materials and man-hours. Dot blot hybridization is a quicker, less expensive method of screening but suffers from problems of sensitivity and specificity due to background hybridization to non-viral vector and cellular sequences. Any acceptable screening test must be sensitive and specific. These experiments were designed to explore these features of dot blot hybridization.

MATERIALS

1. Cloned viral DNA, c-myc DNA and plasmid vectors

The viruses used in this study were the 8kb genomes of Human Papillomavirus (HPV) subtypes 6, 11, 16 and 18 kindly supplied by Dr Lutz Gissman.

HPV 6, 11 and 16 genomes were inserted into the Bam HI site of the E.Coli plasmid pBR322.

HPV 18 was inserted into the ECoRI site of pBR322.

The whole human c-myc gene has been shown to lie within an 8kb HindIII-EcoR1 DNA restriction fragment (Gazin, C. et al, 1984). The probe used in this study comprised the 3' end of the c-myc oncogene from exon 3 to include 1kb of the cellular flanking sequence cloned into the Cla1 and EcoR1 sites of pBR322. This probe was kindly supplied by Dr J. Neil.

2. Bacteria culture media

Bacteria were propagated in L-Broth (LB) which consisted of 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 0.17M NaCl at pH 7.5.

3. Standard buffer solutions

Triton lysis buffer	62.5mM EDTA, 50mM Tris-Hcl (pH 8.0) 2% Triton-X-100
HBS	(Hepes Buffered Saline) 140mM NaCl, 0.75mM Na2 HPO4, 25mM Hepes (N-2-hydroxyethylpiperazine N-2 ethane sulphonic acid) pH 7.1
TNE	5mM EDTA, 50mM Tris HCl(pH 7.4), 150mM NaCl
TE	1mM EDTA, 10mM Tris HCl
1 X TBE	89mM Tris-borate, 89mM Boric Acid
SSC	150mM NaCl, 15mM Trisodium citrate, pH 7.4
1 X E	30mM NaH2 PO4, 1mM EDTA, 36mM Tris-HCl (pH 7.8)
Dye Ficoll	150mM Na2HPO4, 10mM EDTA, 180mM Tris (pH 7.8)
Cell Lysis buffer	50mM Tris-HCl, 10mM EDTA, 100mM NaCl, 0.4% SDS, pH 8.0.
1 X Electro-elution buffer	5mM NaAc, 1mM EDTA, 40mM Tris HCl pH 7.5.
NTB	Nick translation buffer, 50mM Tris-HCl (pH 7.8). 5mM MgCl2, 1mM dithiothreitol (DTT).
Denhardts Solution	0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrollidone, 0.02% bovine serum albumin (BSA)

DNase dilution buffer	50% glycerol, 10mg/ml BSA, 5M NaCl, 1M Tris-HCl (ph 7.5), 250mM EDTA, 1M DTT.

4. Chemicals

Agarose, salmon sperm DNA, bovine serum albumin, polyvinylpyrollidone and Triton-X-100, were obtained from Sigma (London) Chemical Company Ltd., Kingston-upon-Thames, London, U.K.

Tricloroacetic acid (T.C.A.), and caesium chloride were purchased from Koch-Light Laboratories Ltd., Colinbrook, Bucks, U.K.

Ficoll (average molecular weight 400,000) and Sephadex G50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Bacto-tryptone and Difco yeast extract were purchased from Difco Laboratories, Detroit, Michigan, U.S.A.

Ethanol (analytical grade) was purchased from James Burroughs Ltd., London, U.K.

All other chemicals were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of analytical or reagent grade.

5. Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. The specific activities of the 5'-alpha-32p deoxyribonucleoside triphosphates were 3,000 Ci/mmol.

6. Enzymes

All restriction endonucleases and DNA polymerase I were purchased from Bethesda Research Laboratories, Rockville, Maryland, U.S.A.

Deoxyribonuclease (bovine pancreas) was obtained from Sigma (London) Chemical Company Ltd., Kingston-upon-Thames, London, U.K.

Proteinase K and Klenau were purchased from Boeringer Mannheim, GmbH, West Germany.

7. Miscellaneous Material

Kodirex X ray film, X-omat S film, Dx80 developer:- Kodak Ltd., London U.K.
Amfix:- May and Baker Ltd., Dagenham, U.K.
Dupont Cronex Lighting Plus Intensifying Screens:- Dupont, Newton, Connecticut, U.S.A.
Plastic centrifuge tubes:- Falcon Incorporated, Oxhard, California, USA.

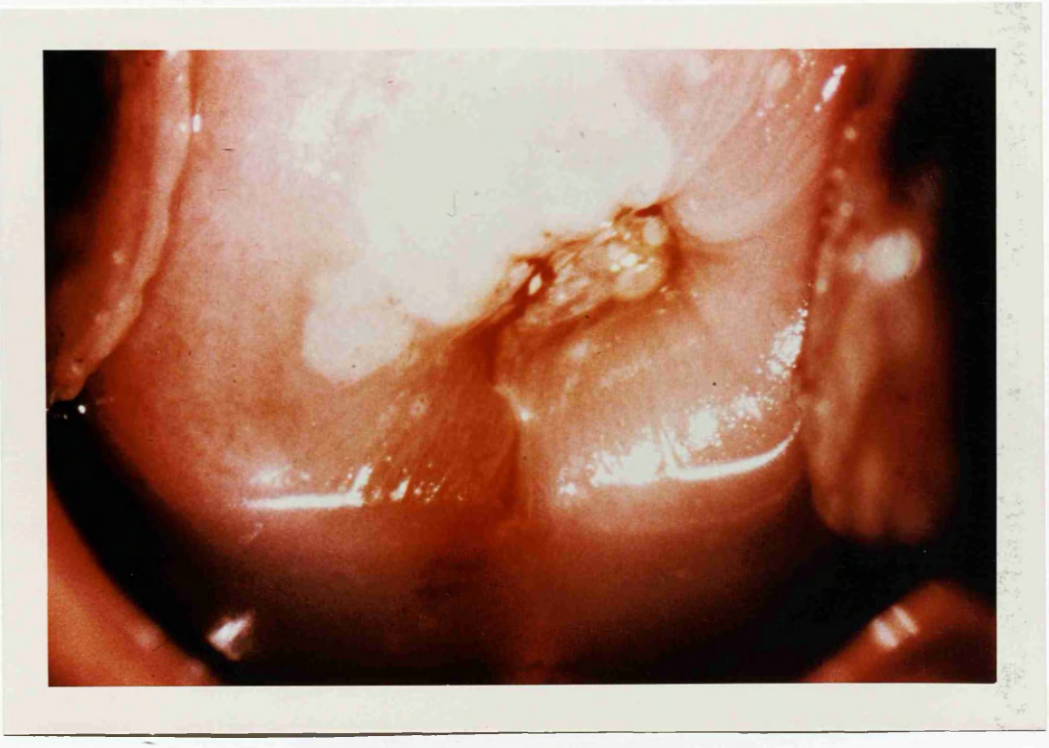
Visking dialysis membrane:- Medical International Ltd., London, U.K.

Whatman chromatography paper, 3mm sheets and 2.5cm circles:- Whatman Ltd., Maidstone, England.

U.K. Gene Screen Plus (TM) Hybridization transfer membrane sheets NEF-976:- New England Nuclear, Dupont UK Ltd., NEN Products Division, Stevenage, Herts, U.K.

METHODS

Fig. 3



A colpophotograph of a cervix showing acetowhite epithelium on the anterior lip. There is no obvious abnormal vascular pattern. Paired biopsies were taken from such lesions. Internal control biopsies were taken from apparently normal areas of the transformation zone eg. the posterior lip in this case.

1. Tissue sample collection

All samples were taken from the lower genital tracts (cervix, vagina or vulva) of patients with their prior informed consent. Approval was gained for the study from the Ethical Committee of the Western Infirmary, Glasgow.

a. Cervical Intra-epithelial Neoplasia (CIN) samples

Biopsies were taken from patients referred to the colposcopy clinic with abnormal cervical cytology and with colposcopically apparent cervical abnormalities (acetowhite epithelium with or without punctate or mosaic vascular patterns).

Paired colposcopically directed punch biopsies were taken from adjacent areas of colposcopically apparently abnormal epithelium and from adjacent colposcopically apparently normal areas of epithelium in the cervical transformation zone (Fig. 3)

Separate biopsy forceps were used to collect samples from the abnormal and normal tissues to avoid cross-contamination. One biopsy of each pair (one abnormal biopsy and one normal internal control biopsy) was snap frozen at -70 deg.C. in liquid nitrogen to await DNA extraction. The other biopsy of each pair was processed for histological diagnosis and analysis for the

presence of papillomavirus antigen.

A second subset of CIN samples was analysed for hybridization to HPV DNA only.

b. Invasive cervical cancer (Ca Cx) samples

These biopsies were obtained either at the time of examination under anaesthesia prior to radiotherapy or at the time of radical hysterectomy. Biopsies from radiotherapy patients were punch biopsies. Biopsies of healthy vaginal epithelium were used as internal controls. Biopsies from hysterectomy patients were taken from fresh specimens. Again, samples of healthy vaginal epithelium were used as internal controls. All samples were snap frozen in liquid nitrogen.

c. Vulval intra-epithelial neoplasia (VIN) and vulval carcinoma (Vu Ca) samples

These samples were obtained at the time of excision biopsy (VIN samples) or radical vulvectomy (VuCa samples). Internal control tissue was from histologically normal adjacent vulval epithelium. All samples were snap frozen in liquid nitrogen.

d. External control samples

These samples were taken by punch biopsy from the

transformation zone of the cervix in women who were undergoing laparoscopic sterilization. All the women gave no history of cervical disease, gave no history of genital warts in themselves or their sexual partners, had never had an abnormal smear and had negative cervical cytology within three months of the biopsies being taken. All samples were snap frozen in liquid nitrogen.

2. Extraction of Cellular DNA

Biopsies were finely chopped with a fresh scalpel blade and digested with 200ug/ml proteinase K in cell lysis buffer for 2 hours at 37 deg.C. on a rotator. The preparation was extracted twice with TE saturated phenol and chloroform to remove unwanted cellular proteins and debris and dialysed overnight at 4 deg.C against 0.1 x SSC. The preparation was precipitated in ethanol in the presence of 0.2M sodium acetate at -20 deg.C overnight. To reduce the number of manipulations and consequent loss of DNA because the yield of nucleic acids was low (5 - 300ug DNA per sample depending on biopsy size) the samples were not treated with RNAse. RNA, which migrates ahead of DNA on gel electrophoresis, was cut from the gel prior to blotting.

3. Preparation of denatured salmon sperm DNA

Salmon sperm DNA was dissolved in sterile distilled water by gentle rotation at room temperature for several hours. After phenol and chloroform extractions, the DNA was ethanol precipitated and resuspended in 1 X TE buffer to give a final concentration of 10mg/ml. The DNA was boiled for 10 minutes and sonicated using a sonic probe to ensure denaturation. Small aliquots were stored at -20 deg.C.

4. Estimation of DNA concentration

The concentration of high molecular weight (MW) cellular DNA was rapidly estimated by agarose gel electrophoresis in mini-gels. Small samples of DNA were electrophoresed with known standard concentrations of lambda DNA on 0.6% (W/V) agarose gels in TBE electrophoresis buffer containing ethidium bromide. The DNA was visualised by UV transillumination of the gels and the concentration determined by visual comparison of the sample fluorescence with that of the standards.

The concentration of low MW DNA e.g. carrier salmon sperm DNA was estimated by optical density (OD) measurements at 260nm. The absorption at 260nm of various dilutions of the DNA sample was measured using quartz microcuvettes in a Perkin Elmer double beam R4 spectrophotometer. The

concentration was estimated assuming that 1 OD unit is equal to 40ug/ml of single stranded DNA. The purity of the DNA could be assessed by calculating the ratio between the readings at 260nm and 280nm (OD 260/OD 280). For pure preparations of DNA this ratio is 1.8.

5. Restriction Endonuclease Digestion of DNA

All restriction endonuclease digestions were performed in the buffers recommended by the suppliers for each enzyme. Digestion of high MW cellular DNA was accomplished with an estimated 4 fold excess of enzyme overnight at 37 deg.C. 10ug of cellular DNA was digested from each sample.

6. Agarose gel electrophoresis

Method (a)

Agarose gel concentrations of 0.6% (W/V) were employed in this study. The agarose was dissolved by boiling, cooled to about 50 deg.C. and 0.5ug/ml ethidium bromide was added before pouring. Horizontal gels run in E buffer were routinely used on glass plates 16.5cm x 26.5cm. The digested DNA samples were loaded with 1/10 volume Dye-Ficoll and electrophoresed at room temperature at about 2V/cm for 2-3 days to ensure tight bands of DNA fragments. In addition to sample DNA, gels were loaded with appropriate HPV DNA reconstruction samples as

positive controls.

HPV DNA reconstructions equivalent to 10 copies per cell of HPV 6, 11, 16 and 18 subtypes were used on each gel. Lack of space on gels prevented use of further reconstructions, so, reconstructions containing 0.2, 1, 10 and 100 copies per cell of each subtype were electrophoresed and blotted separately to be included in the relevant hybridization experiments.

Reconstructions of 10ug of salmon sperm DNA were used with the addition of appropriate quantities of HPV DNA as calculated by the equation:-

$$\frac{\text{Mol wt of cellular DNA genome}}{\text{Mol wt of HPV DNA genome}} = \frac{10\text{ug}}{x}$$

$$\Rightarrow \frac{4 \times 10^{12}}{5 \times 10^{6}} = \frac{10\text{ug}}{x}$$

$$\Rightarrow \quad x = 1.25 \times 10^{-5}\ \text{ug}$$

(Where x = wt of HPV DNA equivalent to 1 copy of HPV DNA per copy of total cellular DNA).

A digest of salmon sperm DNA alone was used as a negative control track. Gels were photographed under either long wave or short wave UV transillumination using Polaroid type 665 or 667 film.

Method (b)

As the majority of digests performed on cellular DNA used the restriction enzyme Bam HI the purpose of electrophoresis was primarily to separate the 8kb HPV DNA fragment and the 4kb pBR322 vector fragments from each other and from other cellular DNA fragments. This resulted in few bands (usually 2) per track when blots were subsequently probed with radiolabelled HPV DNA. This simplicity was exploited to reduce the time required to process samples by preparing 0.6% agarose in TBE and making 3 "minigels" per 16.5cm x 26.5cm plate. Three well combs were placed evenly along the plate before pouring the gels. The higher voltages used and the shorter distance of DNA migration involved allowed three times as many samples to be processed per gel in 6-8 hours without penalty. Once the DNA had migrated the required distance the gels were cut into 3 easily handled "minigels".

7. Southern blot transfer

Two modifications of Southern blotting (Southern, E.M., 1975) were used and compared.

(i) Dupont method

This is the method recommended by Dupont in the use of

Gene Screen Plus synthetic transfer membrane. High MW DNA was digested and electrophoresed with control tracks in E buffer as described. The photographed agarose gels were cut to the appropriate size. The gels were submerged in 0.2M HCl at room temperature and agitated gently for 30 minutes to depurinate them. They were then washed in distilled water and the DNA was denatured by gentle shaking in a solution of 0.4M NaOH and 0.6M NaCl for 30 minutes. The gels were then neutralised in 1.5M NaCl and 0.5M Tris (pH 7.5) for 30 minutes.

Each gel was transferred to a sheet of Whatman 3mm paper on a glass plate supported in a tray of 10 X SSC. A sheet of Gene Screen Plus, presoaked for 15 minutes in 10 X SSC was placed "B" side down over the gel followed by 6 pieces of Whatman 3mm filter paper cut 2mm smaller than the gel size. A weighted stack of dry paper towels was placed on top. The blot was left for 16-24 hours at room temperature to allow complete transfer of the single stranded DNA to the membrane.
After transfer the blots were immersed in an excess of 0.4M NaOH for 30-60 seconds to ensure complete denaturation of immobilized DNA. The blots were then placed in a neutralizing solution of excess 0.2M Tris HCl (pH 7.5) and 2 X SSC. Subsequently the membranes were dried at room temperature on filter paper. This process

was used for gels prepared by electrophoresis method (a).

(ii) Alkaline transfer

This combined a method described by Chomczynski, P. and P.K. Qasba (1984) for alkaline conditions of transfer and a method described by Landers T. (1982) of blotting minigels.

Samples were loaded and electrophoresed in agarose minigels in TBE buffer. After the minigels were cut and prepared they were gently agitated in 0.2M HCl at room temperature for fifteen minutes. The minigels were then washed in distilled water and placed in an alkaline transfer solution of 0.4M NaOH and 0.6M NaCl with gentle agitation for 30 minutes. Then the minigels were placed on a stack of 8 sheets of Whatman 3mm paper cut to the same size as the minigel and pre-saturated in transfer solution. Gene Screen Plus was then placed "B" side down on the minigels. 1 piece of dry Whatman 3mm paper was placed on the membrane followed by a 3cm stack of cut paper towels. A beaker with 250ml of water was used as a weight and transfer was complete after 90 minutes. The miniblots were neutralized in 0.5M Tris HCl (pH 7.0) and 1M NaCl. The acid treatment was employed because it improved transfer of high MW DNA in method (i). Comparison of transfer by the alkaline method with and

without acid treatment, however, showed that this step was unnecessary.

8. Slot blot transfer

This method used a commercial slot blot apparatus (Schleicher and Schuell) to rapidly probe undigested cellular DNA for the presence of HPV DNA. It is equivalent to the more familiar dot blot process using lozenge shaped wells instead of circular wells.

A sheet of Whatman's 3mm paper was presoaked in 10 X SCC and placed on the base-plate of the slot blot apparatus. A piece of Gene Screen Plus similarly presoaked in 10 X SCC was placed "B" side up on the Whatmans paper and the top plate of the apparatus with the wells in it was then positioned to complete the assembly.

Three micrograms of undigested cellular DNA was diluted to 20ul in TE buffer and inserted into the wells with gentle suction applied across the membrane. Rat embryo DNA negative controls, pBR322 plasmid controls and positive control reconstructions containing 0.1, 1 10 and 100 copies of HPV DNA per cell of HPV 6, 11, 16 and 18 were applied to each blot.

Slot blots were washed in 0.4M NaOH and 0.6M NaCl for 20 minutes to denature the DNA and then neutralised in 0.5M

Tris HCl (pH 7.5) and 1M NaCl for 20 minutes. Following hybridization and autoradiography, the copy number of HPV DNA per sample was estimated using automated densitometry. However there was little improvement in accuracy of copy number estimation by this method over simple visual estimation of copy number.

9. Preparation of plasmid DNA

This method is based on that described by Clewell and Helinski (1970).

An overnight culture was prepared from seed stocks and used to innoculate 2 litres of LB plus 100ug/ml ampicillin. The bacteria were grown with vigorous shaking to an OD of 0.8 at 630mm. Chloramphenicol was then added to a final concentration of 100ug/ml (to inhibit bacterial multiplication and increase the number of plasmids per cell). The cultures were reincubated for 16-24 hours at 37 deg.C. The bacterial cells were pelleted at 8000rpm for 15 minutes at 4 deg.C and resuspended in 16ml of cold sucrose-tris (25% sucrose in 50mM Tris-HCl pH 8.0). The bacterial suspension was distributed into 4 tubes chilled to 4 deg.C. Each tube was treated with 2ml of lysozyme (5mg/ml) for 5 minutes at 4 deg.C., followed by 3ml of 250mM EDTA (pH 8.0) for 5 minutes at 4 deg.C. and then 9ml of Triton lysis mix for 20 minutes at 4 deg.C. Cell

debris was removed by centrifugation at 15,000rpm at 4 deg.C. The lysate was extracted once with phenol saturated with TE buffer (pH 8.0) and chloroform and dialysed against TE buffer at 4 deg.C. with at least 2 changes. The nucleic acids were precipitated with isopropanol at room temperature in 0.3M NaAc and the precipitate resuspended in TE buffer. The plasmid DNA was purified by centrifugation to equilibrium in caesium chloride/ethidium bromide gradients. Gradients were prepared to give a density of 1.55g caesium chloride per ml with 500ug/ml ethidium bromide and centrifuged in a Sorval TV865B rotor on an OTD 50 ultracentrifuge for a minimum of 16 hrs at 15 deg.C. The DNA bands were visualised by transillumination in long wave UV light and the plasmid DNA collected by tube puncture. Ethidium bromide and caesium chloride were removed by extracting three times with isopropanol saturated with caesium chloride solution followed by extensive dialysis against TE buffer at 4 deg.C. The DNA was ethanol precipitated and resuspended in TE buffer to give a concentration of 1mg/ml.

10. Recovery of DNA from agarose gels

Cloned HPV DNA sequences were excised from vector sequences and purified twice by agarose gel

electrophoresis. After the appropriate restriction enzyme digestion, the fragments were separated on a 1% (W/V) agarose slab gel containing 0.5ug/ml ethidium bromide and then visualised by long wave UV transillumination. The required DNA band was cut from the gel and the DNA electroeluted using a microelution kit (ISCO Ltd). The agarose containing the DNA was cut into small fragments and added to the sample well. The DNA was electroeluted in electroelution buffer at 100V until all the DNA was concentrated on the dialysis membrane over the smaller collection well. The DNA was collected in a small volume, re-electrophoresed through a 1% (W/V) agarose gel and electroeluted a second time. The ethidium bromide was removed from the DNA by passing it through a Dovex AG-50w x 8 column suspended in TNE and the collected DNA precipitated with two volumes of ethanol and resuspended in TE to a concentration of 1mg/ml.

11. Labelling of DNA probes by nick translation

DNA probes were labelled in vitro by nick translation essentially as described by Rigby, P.W.J., et al (1977). HPV DNA is A-T rich and was labelled with [alpha 32P] d ATP and [alpha 32P] d TTP. 0.25ug DNA were nick translated in a final volume of 50ul containing 0.01% BSA, 1 X NTB, 0.04mM cold d GTP and 0.04mM cold d CTP. 2×10^{-5}

mg/ml of DNase was added, the tube was incubated for a few minutes at 37 deg.C. and then placed on ice. 30uCi of each [alpha 32P] deoxynucleoside triphosphate and 2 units of DNA polymerase 1 were added and the mixture incubated at 15 deg.C. for about 90 minutes.

The same method was used for radiolabelling the c-myc probe.

Isotope incorporation was measured by spotting duplicate filter papers with 1ul of the reaction mixture. The DNA was precipitated and the unbound counts removed from one filter paper disc by three 5 minute washes in 5% (W/V) TCA at 4 deg.C.

The disc was then rinsed with ethanol and dried. Both the washed and unwashed discs were counted for 32P in scintillant fluid (5g PPO per litre of toluene) and the percentage isotope incorporation calculated.
Unincorporated deoxynucleoside triphosphates were removed by a spun column technique. A 1ml syringe was packed with a glass wool plug and a Sephadex G50 column. The 50ul probe was made up to a volume of 100ul with TE buffer, added to the column and spun at 1000rpm for 90 seconds. Incorporated deoxynucleoside triphosphates were collected in an eppendorf tube. 1ul of the DNA probe was added to 500ul of water and counted for 32P. The probe

efficiency was calculated. Probes with efficiencies of 1-5 x 10^9 cpm/ug DNA were used for blot hybridization.

Radiolabelled probes, single stranded salmon sperm DNA (0.5ug per ul) and unlabelled plasmid pBR322 (1ug) were boiled to denature them.

12. HPV DNA blot hybridizations

Nucleic acid hybridisation has been used for many years to determine the degree of homology between DNA sequences. The hybridisation rate is maximal at approximately 25deg. below the hybrid melting temperature (Tm). Tm is related to salt concentration and the proportion of the nucleic acids guanosine and cytosine in the DNA sequence (Schildkraut, C. & Lifson, S. 1965). The Tm is also affected by the presence of formamide and is reduced by approximately 0.6deg. per 1% formamide. The Tm is decreased by approximately 1deg. per 1% base pair mismatch.

These physical properties are utilised in the hybridisation and washing conditions to determine the stringency of the experiment. Low stringency conditions will allow hybridisation between DNA sequences which are similar but have less than 50% base pair sequence homology. High stringency conditions will allow

hybridisation between similar DNA sequences which have more than 50% base pair homology. Thus the HPV DNA subtypes can be differentiated.

a. Non-stringent hybridization

The blots were prehybridized in 20% formamide, 5 X SSC, 5 X Denhardt's solution, 0.08M HEPES, 0.5% SDS and 0.5mg/ml of denatured salmon sperm DNA for 4 hours at 42 deg.C. Hybridization was carried out for 3 days at 42 deg.C. in a similar solution, in which the denatured salmon sperm DNA was replaced by the denatured radiolabelled probe. Prehybridization and hybridization were carried out in a total volume of 10ml in a sealed polythene bag submerged in a plastic box in a shaking water bath.
In the case of blots prepared by the alkaline transfer method, the hybridization protocol was modified by reducing the pre-hybridization time to 15-30 minutes and the hybridization time to 6-8 hours with no loss of efficiency. After hybridization, blots were washed in 2 X SSC and 0.1% SDS 3 times for 30 minutes at 48 deg.C. (HPV Tm -38deg.)

b. Stringent hybridization

The method described for non stringent hybridization was used except 50% formamide was used in both

pre-hybridization and hybridization mixes. After hybridization the blots were washed in 2 X SSC and 0.1% SDS 3 times for 30 minutes at 68 deg.C.(HPV Tm -18deg.)

All hybridization experiments involving the c-myc probe were carried out under stringent conditions as described for HPV DNA hybridizations except the washing temperature was 65deg. C. (Tm -8deg).

After washing, the membranes were never allowed to dry out to avoid irreversible binding of the DNA probe to the blot. The blots were placed between 2 layers of cling film and autoradiographed by exposure at -70 deg.C to flashed and non-flashed Kodak X-Omat H film used in conjunction with a Dupont phototungstate intensifier screen. Before rehybridization, probes were removed by washing the membrane in 0.4M NaOH for 30 minutes at 42 deg.C. The membrane was then transferred to 0.1 X SCC, 0.1% SDS, 0.2M Tris-HCl (pH 7.5) for 30 minutes at 42 deg.C. Thereafter blots were re-autoradiographed to ensure that the probe had been removed prior to reprobing.

13. Histological diagnosis of tissue samples

Biopsies from adjacent areas of tissue were processed for routine histological examination using haematoxylin and eosin staining in the pathology departments of the Western

Infirmary, Royal Infirmary and Stobhill Hospital, Glasgow.

14. Papilloma Virus Antigen Studies

A subgroup of 27 patients with colposcopically apparent cervical abnormalities had biopsies from colposcopically apparently normal and colposcopically apparently abnormal tissue analysed for presence of papilloma virus antigen by Dr Laura Cassidy. The antigen studies were performed with an indirect peroxidase-antiperoxidase technique (Gupta, J.W., et al, 1981). Each specimen was fixed in Bouins' solution, embedded in paraffin, and examined histologically. Non-stained sections were dewaxed in xylene and dehydrated in serial alcohol solutions. Endogenous peroxidase was quenched with 0.5% hydrogen peroxide in methanol. The sections were incubated in normal swine serum to reduce non-specific background staining. Group specific bovine papillomavirus antiserum (Dako) was added in dilutions of 1/250 to 1/1000 and incubated overnight. Bridging antibody (swine antirabbit 1gG) was applied for 30 minutes followed by the addition of peroxidase - antiperoxidase complex at 1/20 dilution for 10 minutes. The peroxidase reaction was developed with diaminobenzoic acid giving a dark brown intranuclear precipitate in cells in which the HPV antigen was

expressed. Haematoxylin was used as a counter stain.

15. Langerhans' Cell Studies

A subgroup of 23 patients, having been referred to the colposcopy clinic with abnormal cervical cytology, had biopsies taken from colposcopically abnormal and colposcopically normal areas of the cervical transformation zone as previously described (Methods, 1.a). These biopsies were analysed for evidence of local immunodeficiency by Dr Robert Hawthorn (Hawthorn, R., et al, 1988). Biopsies were examined histologically and abnormal biopsies were available for hybridization to HPV DNA sequences.

For Langerhans' cell studies seven micron frozen sections were cut on a Bright Microtome, collected on gelatinised slides and stored at -20 deg.C. until use. Every fifth section was stained with haemotoxylin and eosin to confirm the histological diagnosis. Immunohistochemical staining was performed within on week of collection.
An indirect immunoperoxidase method using a mouse monoclonal antibody to T-6 antigen (NA1/34 SERALAB), and to MHC class 1 (W6/32 SERALAB) and class 11 antigens (DA6/231 MRC EDINBURGH) was utilised. The frozen sections were air dried at room temperature, fixed in acetone, washed in Tris Buffer Saline (TBS pH 7.4) and treated with

3% hydrogen peroxide. Normal rabbit serum (diluted 1.5:1 in Tris Buffer pH 7.4) was overlaid. Sections were then incubated with the monoclonal antibody (diluted 1:100 in TRIS Buffer) for 1 hour after the normal rabbit serum had been poured off. Incubation with 1:20 peroxidase congugated rabbit-anti-mouse antibody (P260 Dakopatts) (diluted in 1:1 Tris Buffer : normal human serum) for 45 minutes followed. A 10 minute incubation with 3'3' diaminobenzidene (0.5 mg/ml) to which one drop of 100% H2O2 had been added was used to develop the reaction. Appropriate control sections were run in parallel. Washes in Tris Buffered Saline (pH 7.4) with 3 changes were performed before and after incubation with the primary antisera and after incubation with the second antibody. All incubations with antisera were in moist chambers at room temperature.

Sections were counterstained with blued haemotoxylin, dehydrated and mounted in Histomount for microscopy.

The distribution and morphological detail of the Langerhans' cells were carefully recorded for both the normal and abnormal biopsy in each case. Langerhans' cell counts per 100 basal cells were made from representative areas of each biopsy using an Olympus microscope at 250 times magnification.

RESULTS

1. Experimental Conditions

1.1 The sensitivity of Southern Blotting

HPV DNA may be present at very low copy numbers in both preneoplastic and neoplastic tissues in the female genital tract so that the sensitivity of the test required to be maximised. The experiments were performed in a manner which routinely detected viral DNA at a level of over 0.2 copies per cell. A sensitivity as low as 0.1 copies per cell was achieved in some experiments.

Viral DNA was more easily detected by transferring larger amounts of cellular DNA for probing. The small size of some biopsies limited the amount of DNA available for experiments. A successful compromise between these conflicting requirements was achieved by transferring 10ug cellular DNA per sample for Southern blots and 3ug cellular DNA per sample for slot blots.

To increase the efficiency of transfer of high MW fragments the DNA was "acid nicked" or depurinated by washing gels in 0.2M HCl prior to denaturing in the first method used (method i, p.100). Experiments with and without "acid nicking" using alkaline transfer (method ii, p.102) revealed that this step was not necessary as

transfer of 8-9kb DNA fragments was unaffected by omitting "acid nicking". The effect of "acid nicking" is to partially cleave the high MW DNA into smaller fragments which bind more efficiently to the membrane.

The hydrolysis reaction is not allowed to proceed too far to produce fragments of less than 300bp which are too short to bind efficiently to the membrane. The experimental sensitivity was further increased by labelling the cloned DNA fragments used as probes in the hybridization reactions to high specific activities with alpha 32P deoxynucleoside triphosphates. Probes were used with a minimum specific activity of 1×10^9 cpm/ug DNA.

1.2 <u>The speed of processing of samples</u>

The original method of processing prepared cellular DNA comprised:-

a) an overnight restriction enzyme digest
b) 2-3 days gel electrophoresis
c) an overnight Southern blot
d) prehybridization for 6 hours
e) hybridization for 3 days

Thus an experiment would take 7-8 days to prepare for autoradiography. A maximum of two 12 track gels could be

comfortable processed at one time with 7 samples and 5 control tracks on each gel. The modifications introduced which comprised large gels using TBE buffer and 3 gel combs spaced along the gel; alkaline transfer: and rapid hybridization resulted in the following time scale:-

a) an overnight digest

b) 6 hour minigel electrophoresis

c) 30 minutes minigel denaturation

d) 90 minutes alkaline transfer

e) 30 minutes prehybridization

f) an overnight hybridization (16 hours)

This experiment could be efficiently completed in 40 hours. Six 12 track minigels could be processed with 7 samples and 5 control tracks per minigel. The differences in these labour intensive experiments resulted in considerable saving of man-hours and costly materials. As the blots were to be reprobed with each of the HPV DNA subtypes (HPV 6, 11, 16 and 18) positive control reconstructions of each viral DNA subtype were required on each gel. Full reconstructions comprising 0.1, 1, 10 and 100 viral DNA copies per cell would have left no room for sample DNA. Therefore representative 10 copy per cell reconstructions of HPV 6, 11, 16 and 18 were used on each gel. In order to demonstrate detection of viral DNA at low copy numbers, separate strips of membrane containing

full reconstructions of the relevant viral DNA subtype were included in the sealed polythene bags in hybridization experiments. In the case of blots which were reprobed with the c-myc probe, separate c-myc reconstructions comprising 100, 10, 1 and 0.1pg of DNA were used as positive control tracks.

1.3 Selection of hybridization transfer membranes

Punch biopsies provide only small amounts of tissue from which limited amounts of DNA could be extracted. The smallest punch biopsies were only a few millimetres in diameter and provided as little as 10ug total DNA although most provided 50ug to 100ug. Only one biopsy of abnormal or control tissue was available from each patient. The study hoped to provide data on all four relevant HPV DNA subtypes. This was accomplished using reprobable nylon-based transfer membranes for Southern and slot blot analysis. These membranes are durable, withstand washing at high temperatures and can be successfully reprobed a number of times. Gene Screen plus (TM) hybridization transfer membranes were used because they have the above qualities and they are designed to increase the efficiency of transfer of high MW DNA fragments from electrophoresis gels.

1.4 Method of DNA transfer

Both Southern and slot blot transfer methods were used and compared. Southern blots have the advantage that sample DNA can be digested by restriction enzymes and the fragments separated by gel electrophoresis before transfer. This allows analysis of the physical state of the viral DNA (either existing as a plasmid or integrated into the host cell genome). Secondly, electrophoresis allows detection of a true 8kb band and also allows further digestion to prove that the band is a particular HPV subtype. Every effort was made to purify the viral sequences from the vector by twofold electrophoretic separation in preparation of the probes but Macnab, J.C.M., et al, (1986), Cameron, I.R., et al, (1985) and Park, M., et al, (1983) have shown that some contamination with plasmid vector is inevitable. The main drawbacks of Southern transfer are that it is a relatively long process compared to slot blots and that larger quantities (10ug) of scarce cellular DNA are required. Slot blots, however, are rapid, easy to perform, and require only 3ug of cellular DNA. The drawbacks of slot blots are that they give no information on the physical state of the viral DNA and background hybridization of non-viral, pBR322 plasmid vector sequences, which cannot be differentiated from hybridization of viral sequences occurs. Furthermore, they

cannot be used to prove that the viral subtype probed for has a characteristic restriction pattern.

1.5 Conditions for Southern blot hybridizations

The stringency of hybridization of radiolabelled DNA probes to sample DNA is controlled by the salt concentration, the percentage of formamide in the hybridization mix, and by the hybridization and washing temperatures. The presence of 1% formamide in the hybridization solution reduces the Tm (melting temperature of DNA) by 0.6 deg.C. thus promoting dissolution of the bonds between the single stranded DNA probe and homologous single stranded cellular DNA. Low stringency conditions of 20% formamide with blots being washed at 48 deg.C. (Tm-38deg) allows hybridization of DNA with low base pair homology. High stringency conditions of 50% formamide and washing at 68 deg.C. (Tm-18deg.) allows hybridization of DNA with very high base pair homology only. Cross-hybridization of a probe from one viral subtype with another viral subtype in the sample can therefore be differentiated from true hybridization of DNA of the same viral subtype. HPV 6 and 11 share approximately 80% base pair homology (Dartmann, K., et al, 1986) and cross-hybridization between these related subtypes occurs. This feature was exploited using low and

high stringency washes to reduce the amount of probing required. If a sample was negative for HPV 11 DNA at low stringency it was inevitably negative for HPV 6 DNA also and probing with HPV 6 was unnecessary. HPV 6/11 DNA, HPV 16 DNA and HPV 18 DNA were more readily differentiated as they have low base pair homology.

1.6 Cloned DNA fragments used in hybridization reactions

All samples were probed with HPV 6, 11, 16 and 18 DNA. The DNA was inserted into the E.Coli plasmid pBR322. These viral subtypes were used as probes because DNA from female genital tract premalignancy and malignancy have been found to hybridize to them with great frequency (Introduction, A, 4.6 & 5) Selected samples were probed with c-myc. This oncogene was used because it has been reported to be involved in other human neoplasia. Additionally, there are conflicting reports of c-myc rearrangement and amplification in cervical cancers (Introduction, A, 6).

2. Hybridization Experiments on Tissue Biopsies

2.1 Human Female Genital Tract Tissue and Controls

Biopsies were taken from patients as described (Methods, 1) at the Western Infirmary, Royal Infirmary, Stobhill Hospital and the Royal Beatson Memorial Hospital, Glasgow.

One subgroup of CIN biopsies with internal controls were examined histologically and analysed for the presence of papillomavirus antigen by Dr Laura Cassidy (Results, 2.2) prior to hybridization studies. Other subgroups of CIN (Results, 2.3), cervical carcinoma (Results, 2.5), VIN (Results, 2.6), vulval carcinoma (Results 2.7), and external control biopsies (Results, 2.4) were analysed for the histological diagnosis and presence of HPV DNA sequences only. A selection of samples were analysed by both slot blot and Southern blot (Results, 2.11) to compare these methods. A third subgroup of biopsies from CIN patients were analysed for evidence of HPV DNA both before and after laser therapy for CIN lesions (Results, 2.10). A fourth subgroup of CIN patients had biopsies analysed for histological diagnosis, HPV DNA by slot blot hybridization, and Langerhans' cell status (Dr Robert Hawthorn) (Results, 2.9). Finally HPV DNA hybridization data for the whole study with patients' ages and histological diagnoses were collated (Results, 2.8). Four types of control tissue were used. Two were experimental controls comprising rat embryo (RE) DNA, which was extracted from approximately 17 day old embryos from an inbred Hooded Lister rat colony maintained in this Institute, as a negative control; and reconstructions of HPV DNA types 6, 11, 16, 18 and the c-myc probe previously

Table 1a

Hist(A)	Virus	P.V.Ag (A/N)	HPV 16(S) (A/N)	HPV 18(NS) (A/N)
Virus only (n=6)	6	0/0	3/3	0/0
CIN 1 (n=6)	4	2/1	4/3	1/0
CIN 2 (n=10)	10	3/1	6/4	1/0
CIN 3 (n=5)	2	1/1	4/3	2/0
total (n=27)	22	6/3	17/13	4/0
100%	81%	22%/11%	63%/48%	15%/0%

Table 1b

Hist (N)	HPV 16 (S)
Normal (n=15)	7 (47%)
Virus only (n=9)	5 (56%)
CIN 1 (n=3)	1 (33%)
total (n=27)	13 (48%)

Tables 1a & 1b

Data collated from Appendix 1.

Hybridization to HPV 16 and 18 was detected. There was poor correlation between colposcopic appearances, histological diagnosis of virus infection, detection of papillomavirus antigen and hybridization to HPV DNA.

Key

Hist (A) = Histological diagnosis of colposcopically abnormal tissue.
Hist (N) = Histological diagnosis of colposcopically normal tissue.
Virus = Histological or cytological evidence of virus infection.
P.V. Ag (A/N) = Biopsies with evidence papillomavirus antigen (abnormal biopsies/normal biopsies)
HPV 16(S) (A/N) = Hybridization to HPV 16 DNA (stringent conditions) (abnormal biopsies/normal biopsies)
HPV 18(NS) (A/N) = Hybridization to HPV 18 DNA (non stringent conditions) (abnormal biopsies/normal biopsies)

described as positive controls. The other two controls were tissue controls comprising internal controls from apparently normal tissue adjacent to genital premalignancy or malignancy and external control tissue from cervices of cytologically normal women.

2.2 Study comparing colposcopic appearances, histological diagnosis, papillomavirus antigen status and hybridization to HPV DNA sequences.

The data derived from this group of 27 patients is presented in Appendix 1 and tables 1a and 1b. The histological diagnosis of colposcopically abnormal tissue ranged from evidence of virus only in the absence of dysplasia [that is, koilocytotic atypia, individual cell keratinisation, multinucleation, parakeratosis, acanthosis and papillomatosis; (Anderson, M.C., 1985)] to CIN 3, (tables 1a and 1b and fig 4). Twelve samples of colposcopically apparently normal tissue were reported as showing histological features of virus infection and three of these were classified as CIN 1 plus virus. Altogether 22 of the 27 (81%) women with colposcopic abnormalities were reported as having histological and/or cytological evidence of viral infection (Table 1a).

Six (22.0%) of abnormal biopsies were positive for papillomavirus antigen (95% confidence interval 6.4% to 37.6%) compared with three (11%) of normal specimens

Fig.4

Histological section of CIN with marked viral changes. The basement membrane is arrowed (bm). Koilocytes (K) abound in the superficial layers. (H & E) (Courtesy of D. Millan).

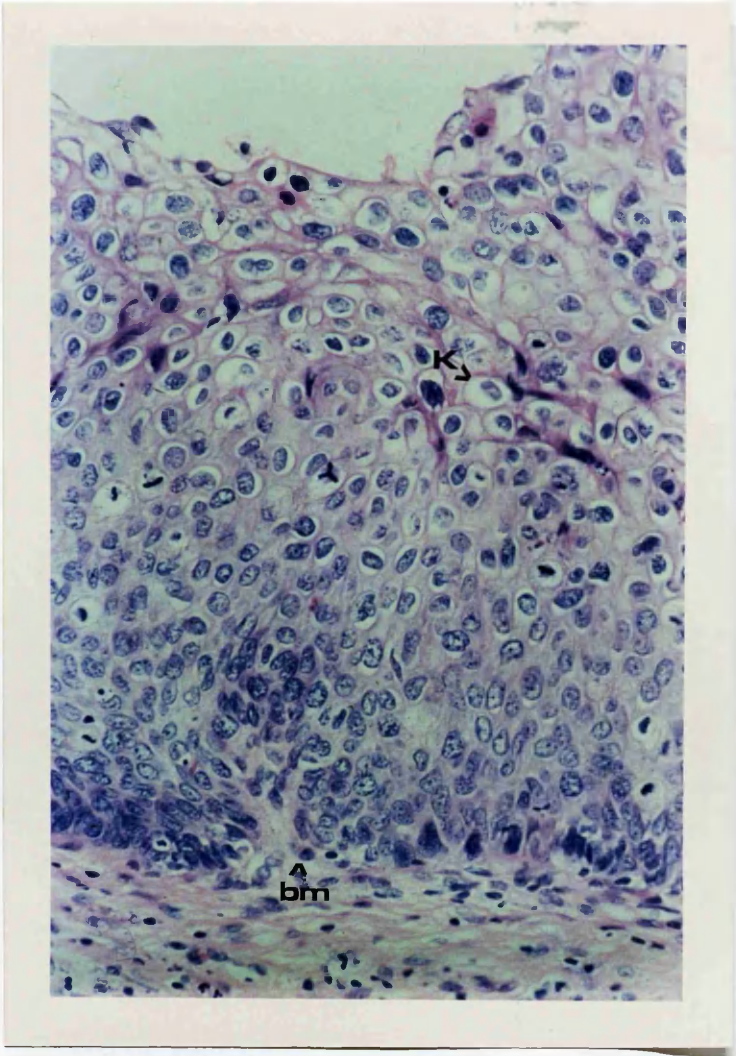
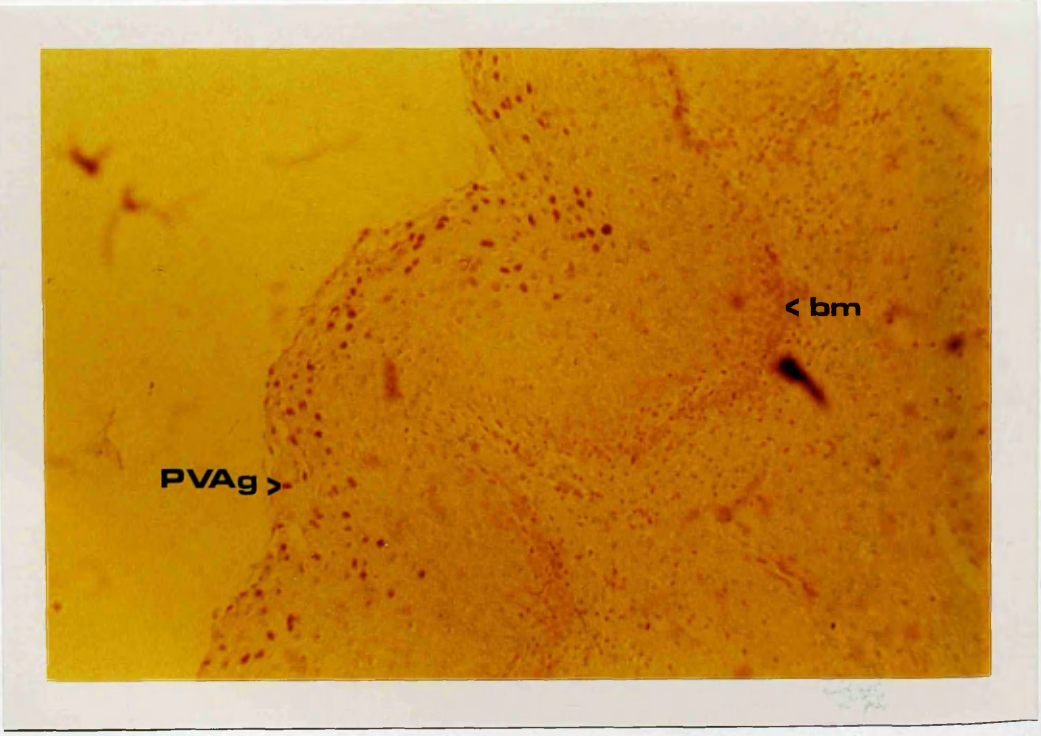


Fig. 5 Histological section of CIN stained for papillomavirus antigen (PV Ag). PV Ag stains dark brown and is found near the surface away from the basement membrane (bm). (Courtesy of L. Cassidy)



(95% confidence interval -0.8% to 22.8%) (table 1a) (fig 5).

Hybridization to HPV 16 DNA sequences under stringent conditions was found in 17 (63%) abnormal biopsies (95% confidence interval 44.8% to 81.2%) and 13 (48%) colposcopically normal internal control specimens (95% confidence interval 29.2% to 66.8%)(Tables 1a, & 1b) (fig.6). The different rates of detection in abnormal and normal biopsies is not statistically significant (chi squared = 0.675; $0.5>p>0.1$, Yates correction).

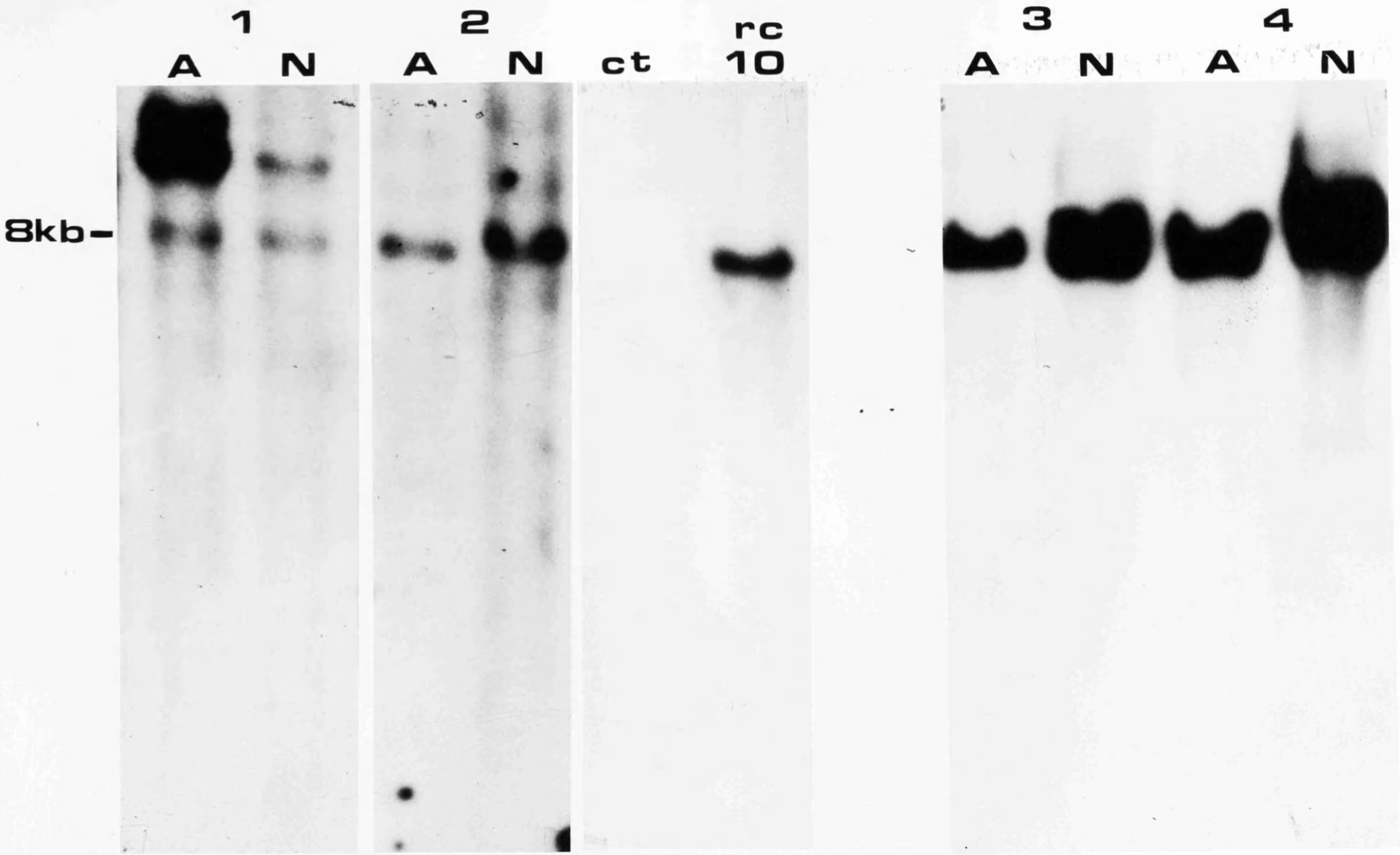
In three cases in which hybridization to HPV 16 was not detected under stringent conditions, hybridization to HPV 18 was detected under non-stringent conditions only. In one further case in which hybridization to HPV 16 was detected under stringent conditions, HPV 18 was also detected under non-stringent conditions only. This may represent a mixed HPV-16/HPV-18 infection or cross hybridization between the HPV-18 probe and HPV 16 in the sample or it may represent cross hybridization to another HPV subtype.

No colposcopically normal biopsy hybridized to HPV 18.

The difference in hybridization to HPV 18 DNA in abnormal and normal biopsies is not statistically significant

Fig 6

Autoradiograph of Bam H1 digested DNA extracted from CIN and normal tissues. Panels 1-4 show 10ug DNA (digested with BAM H1) extracted from histologically confirmed lesions of CIN (A = abnormal) and from histologically confirmed normal adjacent internal control tissue (N = normal) from four different patients. The HPV 16 radiolabelled probe hybridizes to inserts in CIN and normal samples under stringent conditions. Specimens 1A, 1N and 2N contain HPV DNA potentially integrated into different sites in the cell genome. In specimen 1 the genome copy number in the abnormal tissue exceeds that in the normal tissue. In specimens 2, 3 and 4 the reverse is the case. Track ct is negative control rat embryo DNA and rc10 is a positive control track comprising 10 copies of HPV DNA present in control DNA per cell. The 8kb molecular weight marker is indicated.



124.a

(chi squared = 2.43; 0.5>p>0.1, Yates correction). HPV 11 DNA failed to hybridize to any samples in this group. The 80% base pair homology shared by HPV 6 and HPV 11 means that HPV 6 was also not present in this group. In total 20 (74.1% : 95% confidence interval 57.6% to 90.6%) of the colposcopically abnormal samples from this group hybridized to HPV 16 or HPV 18 or both probes. The difference between hybridization to all HPV subtypes in normal and abnormal biopsies is not statistically significant (chi squared = 2.80; 0.1>p>0.05, Yates correction).

Six (30.0% : 95% confidence interval 10.0% to 50.0%) of the 20 HPV DNA positive cervices were detected by staining for papillomavirus capsid antigen. This indicates that the entire genome may not be transcribed in all cells with HPV DNA and that this is an insensitive method of detecting HPV infection. It is somewhat surprising that papillomavirus antigen was detected in abnormal biopsies more frequently than in normal biopsies. Viral replication occurs in terminally differentiating squamous epithelium and capsid production is a function of the late region of the viral genome. CIN is characterised by a lack of differentiation of epithelial cells and one would expect that the late region of the viral genome would be less often expressed

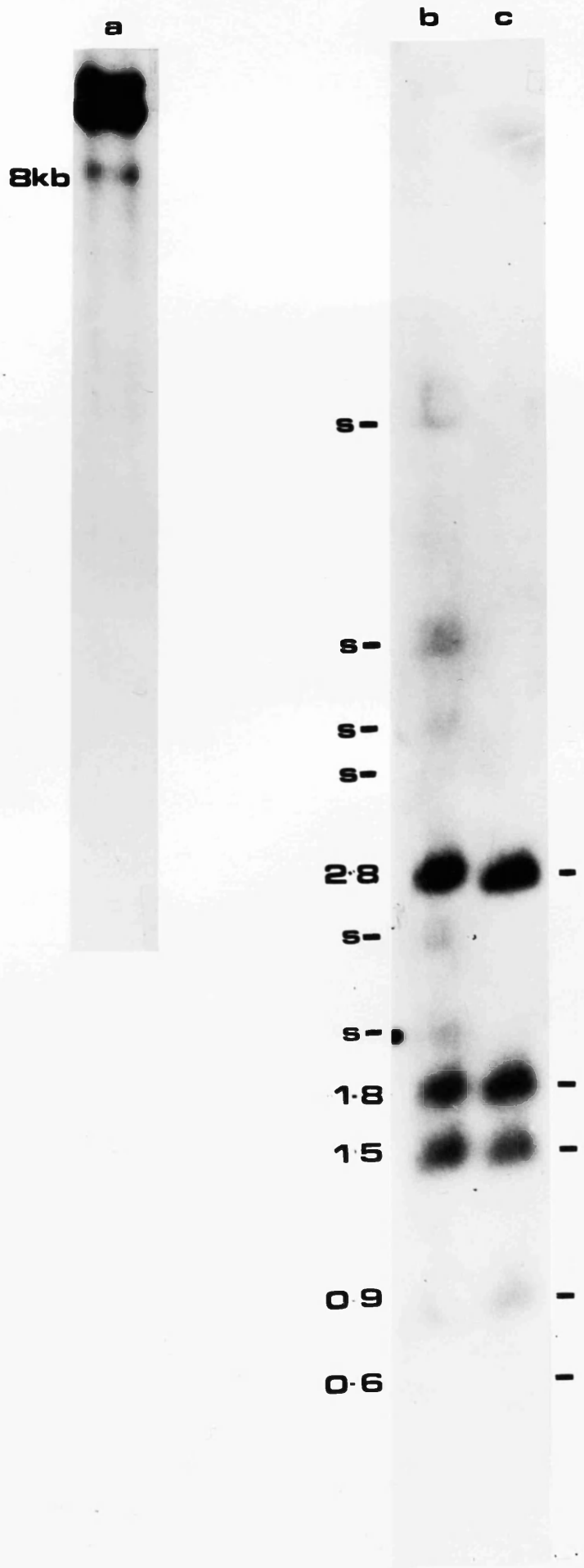
in such de-differentiated tissues compared to normal tissue.

Samples which hybridised to the HPV 16 DNA probe under stringent conditions sometimes produced one to three bands on digestion, even with a fourfold excess of the restriction enzyme Bam HI (fig 6). This suggests that the HPV DNA is integrated at multiple sites in the host cell chromosome in both CIN and normal tissue. Durst, M., et al (1983) reported integration of HPV 16 DNA into cell DNA in invasive cancer by cloning virus-cell junction fragments. Also, by digesting cell DNA samples with both a restriction enzyme which cuts HPV and a combination of cut and non-cut enzymes, they detected submolar bands representing virus-cell junction fragments. Insufficient DNA precluded detailed study of integration in all the samples, but in one case (case 1 Sample A; fig. 6) a sample of cell DNA was digested with the restriction enzyme Pst 1 alone (HPV contains recognition sites for Pst 1) and a second sample was digested with the restriction enzymes Pst 1 plus Xho 1, Sst 1 and Hind III (HPV DNA does not contain recognition sites for Xho 1, Sst 1 and Hind III). Six submolar bands were identified suggesting that the HPV genome was inserted into three sites in the host chromosome in this sample of CIN (fig. 7). In some cases - for example case 1 (fig 6) -

Fig 7

Autoradiograph of further restriction digests of Sample 1A (fig 6). All three tracks are hybridized to radiolabelled HPV 16 DNA under stringent conditions.

Track (a) is the Bam H1 digested sample (fig 6). Track (b) is sample DNA from the same lesion digested with Pst 1. This shows presence of six submolar bands. Track (c) is the sample DNA digested with Pst 1 plus the non-cut enzymes Hind 111 Sst 1 and Xh01 showing loss of the submolar bands. This indicates integration of the HPV DNA into three sites on the host cell chromosome. Size markers are to the left of the tracks.



12.6a

Table 2

Histology	Virus	Hybridization to HPV DNA sequences			
		6/11	16	18	Total
Virus only (n=2)	2	0	0	2(100%)	2(100%)
CIN 1 (n=1)	1	0	0	0	0
CIN 2 (n=4)	2	0	2(50%)	0	2(50%)
CIN 3 (n=14)	10	1(7%)	8(57%)	3(21%)	12(86%)
total (n=21)	15	1(4.8%)	10(48%)	5(24%)	16(76%)
Normal Internal controls (n=11)	0	0	5(45%)	0	5(45%)

HPV DNA hybridization data collated from the patients listed in Appendix 2. All subtypes were detected under stringent conditions. All internal controls were histologically normal. Virus = histological evidence of virus infection.

the genome copy number in the abnormal biopsy specimen exceeded that in the normal specimen. By contrast, however, other patients showed a higher HPV copy number in control tissues than in colposcopically abnormal tissue - for example, case 2 (fig 6). Thus the site on the cervix of maximum colposcopic abnormality is not necessarily the site at which the maximum viral genome copy number is found. Maximum copy number may reflect replicating DNA.

2.3 Study comparing colposcopic appearances, histological diagnoses and detection of HPV DNA sequences.

As with the study of the first 27 patients, this group of 21 patients had paired punch biopsies taken for histological examination and HPV DNA probing. It was possible to take internal control biopsies from the transformation zone in 11 of these, all of which proved to be histologically normal (table 2).

HPV 6/11 was detected in one (4.8%) case (95% confidence interval - 4.3% to 13.9%). Hybridization to HPV 18 DNA was also detected under stringent conditions in this patient. Cross-hybridization is not likely between HPV 6/11 and HPV 18 under these conditions and it is probable that a mixed infection is present. This patient (CN 48A)

is interesting in that she was the recipient of a renal transplant and had recurrent CIN 3 and widespread vulval warts.

HPV 18 DNA was detected in 5 (23.8%) cases (95% confidence intervals 5.6% to 42.0%) of which 2 were cases which were histologically virus only and the other three were CIN 3. HPV 18 DNA was not detected in histologically normal tissue. The difference in detection between abnormal and normal biopsies is not statistically significant (chi squared 1.56, $0.5>p>0.1$, Yates correction).

HPV 16 DNA was again the predominant HPV subtype detected, being found in 10 (47.6%) CIN biopsies (95% confidence intervals 26.3% to 68.9%) and 5 (45.5%) histologically normal biopsies (95% confidence intervals 16.1% to 74.9%). In total therefore, HPV DNA was detected in 16/21 (76.2%; 95% confidence interval 58.0% to 98.4%) abnormal biopsies and 5/11 (45.5%; 95% confidence interval 16.1% to 74.9%) normal biopsies. This difference in HPV DNA detection in normal and abnormal biopsies is not statistically significant (chi squared = 1.56, $0.5>p>0.1$, Yates correction).

2.4 Study of HPV DNA hybridization to clinically and cytologically normal external control patients

Thirty patients had biopsies taken at the time of laparoscopic sterilization. None of these women gave a history of abnormal cervical cytology and all had negative cervical cytology within three months of biopsy. None of them gave a history of genital warts in themselves or their sexual partners. The patients were not examined colposcopically nor were their biopsies examined histologically. They, therefore, comprise an unselected external control group representing the apparently normal, sexually active female population as defined by present, cytological, screening methods. The mean age of the group was 38.2 years with an age range of 26-50 years. Two samples (6.6%; 95% confidence intervals -2.3% to 15.5%) were found to hybridize to HPV 6 DNA under stringent conditions. Two further samples (6.6%; 95% confidence intervals -2.3% to 15.5%) were found to hybridize to HPV DNA under stringent conditions. Thus, in total, 4/30 samples (13.3% ; 95% confidence intervals 1.2% to 25.4%) hybridized to HPV DNA sequences in the study group.

Hybridization to HPV 18 was not detected.

Table 3

Tissue	Hybridization to HPV DNA		
	16	18	total
Abnormal (n=31)	16(52%)	2(6.5%)	18(58%)
Normal (n=18)	4(22%)	1(5.6%)	5(28%)

Hybridization of HPV 16 and 18 DNA to invasive cervical carcinomata and vaginal epithelial internal control biopsies under stringent conditions. HPV 6/11 were not detected.

2.5 Study of biopsies from cases of invasive carcinoma of the cervix along with internal control biopsies from vaginal epithelium comparing histological diagnosis and hybridization to HPV DNA sequences.

Patients undergoing radiotherapy or radical hysterectomy for histologically diagnosed invasive cancer of the cervix had paired biopsies removed in the same fashion as previously described. Biopsies were obtained from 31 cases of invasive cancer. Internal control biopsies from vaginal epithelium were available in 18 cases. All but one of the 31 patients had squamous carcinomas, the thirty second being an adenocarcinoma (table 3). Sixteen (51.6%) of the cancers hybridized to HPV 16 DNA sequences (95% confidence intervals 34.0% to 69.2%) and 4 (22.2% ; 95% confidence intervals 3.0% to 41.4%) vaginal internal controls also hybridized to HPV 16 DNA. Two (6.5%) cancers hybridized to HPV 18 DNA (95% confidence intervals -2.2% to 15.2%) and one (5.6%) internal control hybridized to HPV 18 DNA (95% confidence intervals -5.0% to 16.2%). Overall, the different rates of hybridization to HPV DNA sequences are not statistically significant (chi squared = 3.07, $0.5>p>0.1$, Yates correction).

Table 4

Case	Age	Histology	Hybridization to HPV 16 (S)
VNO1 A	52	VIN I	+
VNO1 N		n	–
VNO2 A	27	VIN II/III	+
VNO2 N		n	–
VNO3 A	41	Bowen's disease	+
VNO3 N		n	+
VNO4 A	N/K	VIN III	–
VNO4 N		n	–
VNO5 A	59	VIN II/III	+
VNO5 N		n	–

HPV DNA hybridization data on biopsies taken from clinically abnormal and clinically normal areas of patients with vulval premalignant lesions. Hybridization to HPV 16 only (stringent conditions) was detected.

Prefix VN = patient with pre malignant vulval lesions
Suffix A = biopsy from clinically abnormal tissue
Suffix N = biopsy from clinically normal tissue
VIN = vulval intra-epithelial neoplasia

2.6 Study of HPV DNA hybridization to biopsies from non metastatic vulval epithelial abnormalities and histologically normal adjacent vulval epithelium.

Biopsies from 5 patients were available for study. The histological diagnosis ranged from Bowen's disease to VIN 3 (Table 4). Histologically normal vulval epithelium was available for use as an internal control in each case. Hybridization to HPV 16 DNA was detected in 4/5 (80%) of abnormal biopsies (95% confidence interval 44.9% to 115.1%) and in 1/5 (20%) of normal biopsies (95% confidence interval -15.1% to 55.1%). Hybridization occurred under stringent conditions. Hybridization to HPV 6/11 and 18 DNA was not detected in this group. The mean age of the group was 44.8 years with a range of 27 to 59 years.

2.7 Study of hybridization of HPV DNA to biopsies from cases of invasive squamous carcinoma of the vulva along with internal control tissue from histologically normal adjacent vulval epithelium.

Biopsies were collected from specimens at radical vulvectomy. Abnormal biopsies were taken from histologically diagnosed lesions and control tissue was taken from the skin edge of the specimen. In one case, Vu03, an exophytic wart in the specimen was also biopsied and in Vu05 control tissue was not collected.

Table 5

Case	Age	Hybridization to HPV 16 DNA (S)
VU01 A	70	–
VU01 N		–
VU02 A	65	–
VU02 N		–
VU03 A	62	+
VU03 N		+
VU03 V		+
VU04 A	66	+
VU04 N		+
VU05 A	55	+

HPV DNA hybridization data on biopsies taken from histologically diagnosed squamous carcinoma of the vulva with paired internal control biopsies taken from histologically normal epithelium at the edge of specimens produced at radical vulvectomy. HPV 16 DNA only was detected (stringent conditions)

Prefix VU = squamous crcinoma of vulva
Suffix A = histologically abnormal biopsy
Suffix N = histologically normal biopsy
Suffix V = histologically diagnosed exophytic wart

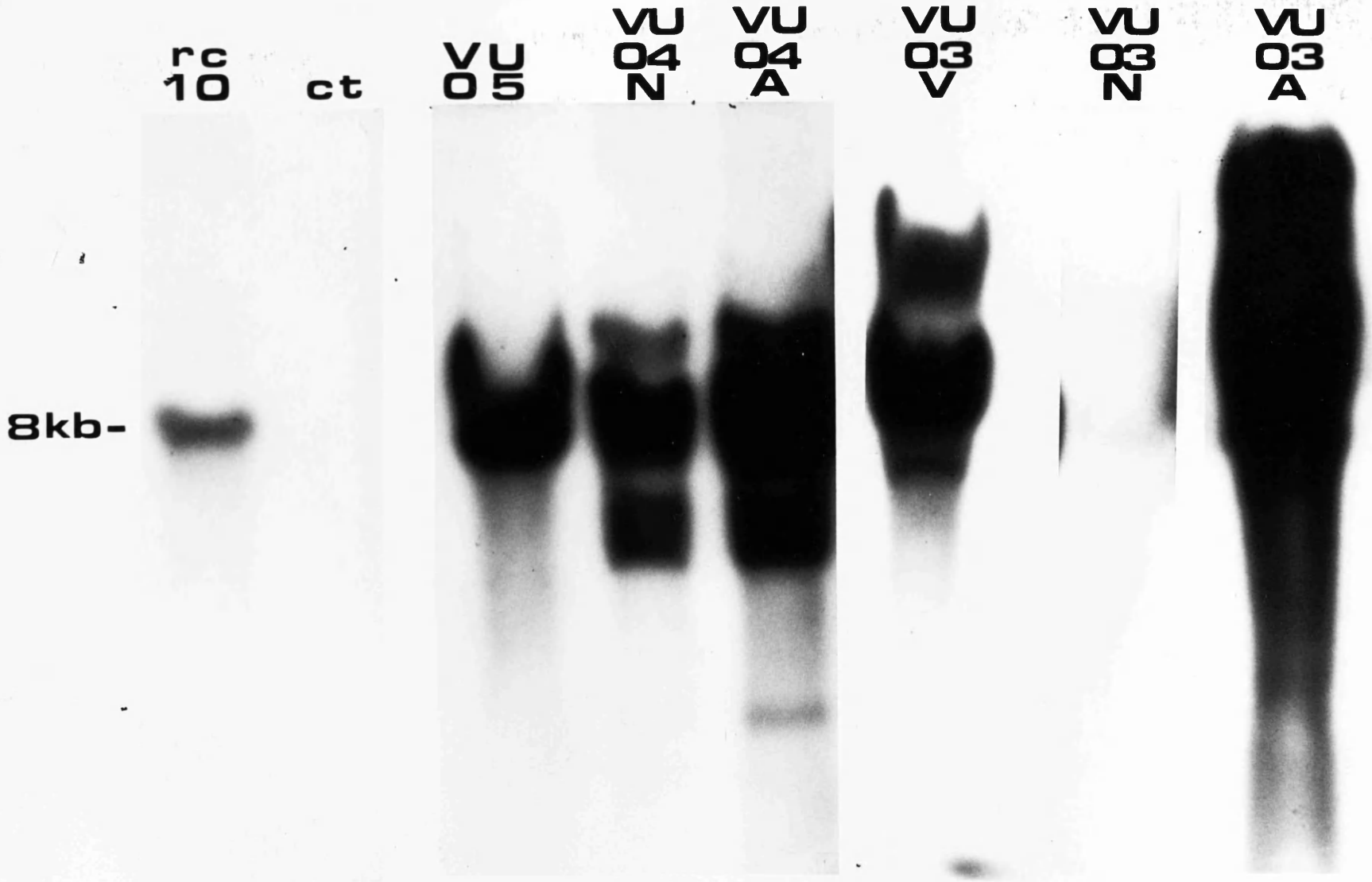
Hybridization to HPV 16 DNA was detected under stringent conditions in 3/5 (60% ; 95% confidence intervals 17.1% to 102.9%) of abnormal biopsies and in 2/4 (50% ; 95% confidence intervals 1.0% to 99.0%) of internal controls. The difference in hybridisation to HPV DNA sequences in abnormal and normal tissues is not statistically significant (chi squared = 0.14, $p>0.5$, Yates correction). HPV 16 DNA was detected in the exophytic wart (Vu03V) (Table 5)(fig 8). Hybridization to HPV 6/11 and 18 DNA was not detected in this group. The mean age was 63.6 years and range was 55-70 years.

A detailed analysis of such small numbers is ,of course, of limited value. However, it is interesting to note that samples Vu03 and Vu04 produced multiple bands on hybridization to HPV 16 DNA. This restriction pattern is compatible with integration of the viral genome into the host cell chromosome. In the case of Vu04 the same pattern is found in the sample and control tracks. In the case of Vu03, a single 8kb band is detected at low copy number in the internal control biopsy but multiple bands are detected in the tumour and exophytic wart biopsies. This exophytic wart is therefore associated with integrated HPV16 DNA and not episomal HPV6/11 DNA and may represent a case of vulval cancer arising from a vulval wart as suggested by Zur Hausen, H. (1977).

Fig 8

Three cases of invasive squamous cancer of the vulva (Vu 03, 04 and 05). The cell DNA has been digested with Bam H1 and the samples probed with radiolabelled HPV 16 DNA under stringent conditions. Vu03A (abnormal biopsy) is over-exposed but contains several bands. Vu03V (exophytic wart) demonstrates a similar restriction pattern. Vu03N (histologically normal internal control) contains a single 8kb band at very low copy number. There are also multiple bands in Vu04A and Vu04N but only one 8kb band in Vu05.

Track ct comprises negative control rat embryo DNA. Track rc10 comprises 10 copies of HPV DNA present in control DNA per cell. The 8kb molecular weight marker is indicated.



132a

Table 6

Histology of Abnormal Biopsy	Age Range	Age Mean	Hybridization to HPV DNA sequences (S + NS) 6/11	16	18	Total
v (n=8)	21-42	28.3	0	3	2	5 (63%)
CIN (n=21)	24-46	32.2	0	10	4	13* (62%)
CIN+V (n=38)	19-52	30.0	1	19	11	28* (74%)
total (n=67)	19-52	30.5	1	32	16	46 (69%)
CaCx (n=31)	26-86	54.6	0	16	2	18 (58%)
VN# (n=5)	27-59	44.8	0	4	0	4 (80%)
VuCa (n=5)	55-70	63.6	0	3	0	3 (60%)
External Controls (n=30)	26-50	38.2	2	2	0	4 (13%)

* 4 biopsies hybridized to more than one HPV DNA subtype.

VN = all vulval non-invasive abnormalities

Summarised results comparing age (range and mean), histological grade in biopsies from colposcopically or macroscopically abnormal tissue and hybridization to HPV DNA sequences (stringent and non-stringent).

2.8 Study of the relationship between the age of the patients, the histological diagnosis and HPV DNA detection.

The data described in this section are derived from the patients in Appendix 1, Appendix 2, Appendix 3, Appendix 4 and table 4, and table 5. These data comprise the total study group of female patients (table 6) bar one case of vaginal intra-epithelial neoplasia (Results, 2.13). A total of 138 patients were studied comprising the full range of cervical premalignancies and malignancies, vulval premalignancies and malignancies; and external control patients. Internal control biopsies were available for study in selected cases as described (Table 6).

Of the non-invasive cervical biopsies a total of 46/67 (69%; 95% confidence interval 58.0% to 80.0%) and 18/38 of the internal control biopsies (47.4%; 95% confidence interval 31.5% to 63.3%) hybridized to HPV DNA sequences under stringent and non stringent conditions. The different rates of detection in these two groups of biopsies is not statistically significant (chi squared = 3.77, $0.1>p>0.05$, Yates correction). However, the difference between HPV DNA detection in non-invasive cervical lesions and external controls [4/30 (13.3%; 95% confidence intervals 1.1% to 25.5%)] is statistically significant (chi squared = 23.4, $p<0.001$, Yates

Correction). Furthermore, the study group were significantly younger than the control group ($p<0.001$, student t test).

Similarly, as has been described (Results, 2.5) there is no statistically significant difference between hybridization to HPV DNA in invasive cervical cancer biopsies and internal controls (chi squared = 3.07, $0.5>p>0.1$, Yates correction). There are, however, significantly higher rates of HPV DNA detection in cervical cancer biopsies compared to external controls (chi squared = 11.4, $0.01>p>0.001$ Yates correction). In these two groups of patients, the study group was significantly older than the control group ($p<0.001$ student t test). In contrast to the study of Meanwell, C.A. et al (1987) this suggests that HPV DNA detection is not related to the age of the patient but to the presence or absence of the neoplastic process.

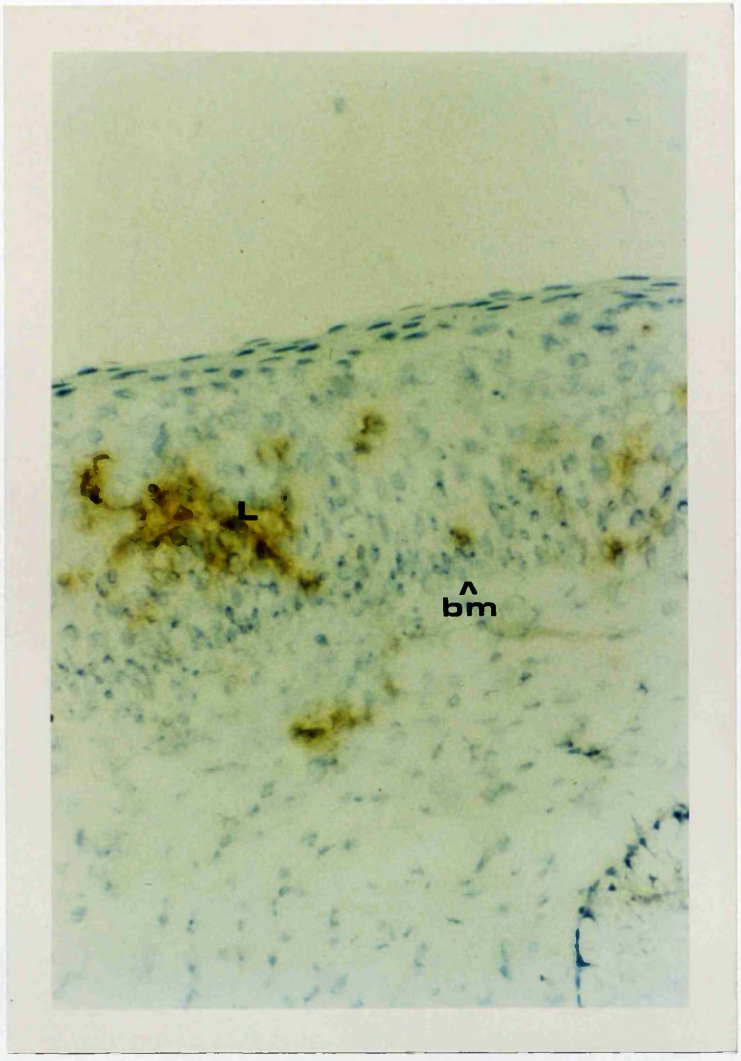
When comparing HPV DNA detection in each of the three groups, i.e. mild premalignant abnormalities, severe premalignant abnormalities, and invasive cancer; no statistically significant difference was detected (chi squared = 1.518, DF = 2, $p>0.5$). Secondly, if the premalignant lesions are separated into CIN without HPV DNA and CIN with HPV DNA no significant age difference is

detected ($p>0.5$, student t test). Thirdly, if the invasive cervical lesions are separated into those which do hybridize to HPV DNA and those which do not, no statistically significant age difference is manifest ($0.5>p>0.1$, student t test).

From these data it can be concluded that HPV DNA is detected equally in all grades of cervical lesion and there is no increased prevalence of HPV DNA associated with increasing age in this study. Similarly, there is no evidence that HPV DNA detection is associated with a prognosis of progression to invasive cancer at an earlier age. The prevalence of HPV DNA detection in cases compared to external controls does, however, confirm the association between HPV DNA and cervical neoplastic disease.

It is well recognised that invasive cancer of the cervix affects an older age group than does pre-invasive disease of the cervix. Thus it is not surprising that the patients with invasive disease were significantly older than those with pre-invasive disease ($p<0.001$, student t test). However, there is no such age difference between patients with mild premalignant abnormalities (virus only and CIN 1) compared to those with severe premalignant abnormalities ($p<0.5$, student t test). This is entirely

Fig. 9



Histological section of CIN stained for Langerhans' cells (L) which stain dark brown. Blued haematoxylin was used as a counterstain.
bm = basement membrane.
(Courtesy of R. Hawthorn.)

consistent with the concept of progression of disease at least from CIN 2 or 3 to invasive cancer.

An assessment of the ability of a routine diagnostic histology service to diagnose HPV infection (against the gold standard of hybridization to HPV DNA) in non-malignant cervical lesions can be made from the data in table 6. Of the 67 lesions studied the histologist reported evidence of viral infection in 46 cases. HPV DNA was detected in 45 cases. Histological assessment offers a sensitivity of 72%, a specificity of 40% a positive predictive value of 74% and a negative predictive value of 38%.

2.9 Study comparing histological diagnosis, local immunocompetent cells (Langerhans' cells) and hybridization to HPV 16 and 18 DNA sequences

This subgroup of 23 patients, referred to the colposcopy clinic with abnormal cervical cytology, had biopsies taken from colposcopically and histologically abnormal transformation zone and normal ectocervical tissue, for analysis of Langerhans' cell numbers quantified per 100 basal cells (Dr R. Hawthorn) (fig 9). Abnormal biopsies were stored at -20 deg.C. for later HPV DNA studies. Because of the very small size of the biopsies and possibly because of the storage conditions, only small amounts of total cellular DNA (5 - 30ug) were

Table 7a

Histology	n	Hybridization to HPV sequences 16 (s)	18 (s)	total
2	1	0	0	0
2+v	7	3	4	7 (100%)
3	10	2	3	5 (50%)
3+v	5	4	1	5 (100%)
total	23	9 (39%)	8 (35%)	17 (74%)

(2= CIN2 ;3= CIN3 ;+v= virus infection).

Data collated from Appendix 4 comparing histology and HPV DNA hybridization data.

Table 7b

Subtype	Copy No	Wilcoxon Paired Sign Test	95% Confidence Intervals (%)
HPV 16	10-50	p=0.500	18.4 to 78.4
HPV 16	>50	p=0.054	4.60 to 54.4
HPV 18	10-50	p=0.006	19.7 to 50.5
HPV 18	>50	p=0.054	13.2 to 66.9
Negative		p=0.023	-79.4 to -5.20
HPV 16	>10	p=0.500	10.8 to 57.0
HPV 18	>10	p=0.006	27.5 to 46.4
HPV 16+18	>10	p=0.054	-38.7 to 46.4

Analysis of Langerhans' cell numbers (normal biopsy minus abnormal biopsy) and HPV DNA subtype and copy number detected by DNA hybridization in the abnormal biopsy. A highly significant correlation exists between HPV 18 DNA and reduced Langerhans' cell numbers. A significant correlation exists between high copy numbers of HPV 16 DNA and reduced Langerhans' cell numbers.

retrieved from these biopsies. Southern blot analysis was not possible so slot blot analysis was used. Hybridization between total cellular DNA and HPV DNA sequences at levels greater than 10 copies per cell only were accepted as positive results because of the poor specificity of slot blots at levels below 10 copies per cell. Data on individual patients is recorded in Appendix 4. The histological diagnosis and hybridization to HPV DNA sequences are compared in the Table 7a; and a statistical analysis of the Langerhans' cell number in the abnormal biopsy (expressed as an increase or decrease with respect to the normal internal control biopsy) compared with the HPV DNA hybridization data is found in Table 7b. There is a good correlation between histological diagnosis and detection of HPV DNA sequences (100%) but HPV DNA is also detected in 50% of biopsies which showed no histological evidence of virus infection. (table 7a).

There is a significant ($p = 0.059$) association between hybridization to HPV 16 DNA at >50 copies per cell and reduced Langerhans' cell number in the abnormal biopsy compared to the normal biopsy. There is also a highly significant ($p<0.001$) association between hybridization to HPV 18 DNA at >10 copies per cell and reduced Langerhans' cell number in the abnormal biopsy compared

Table 8.

Colposcopic appearance of cervix	Hybridization to HPV 16 DNA (stringent)
Abnormal (CIN)	12/15 (80%)
Normal (internal control)	9/15 (53%)
Normal (post-laser)	5/15 (33%)

Pre and post laser biopsies.
Hybridization data from Appendix 5 collated.

to the normal biopsy. (Table 7b).

2.10 Study of hybridization between cervical cell DNA and HPV DNA sequences before and after laser therapy for histologically proven CIN lesions.

Fifteen patients from the groups detailed in Appendices 1 and 2 were available for repeat biopsy four months following laser therapy for histologically diagnosed CIN. The relevant patients details are recorded in Appendix 5. Biopsies were taken from colposcopically normal and abnormal tissue prior to laser therapy. The whole transformation zone was vapourised to a depth of at least 7mm. At four months the patients were found to be colposcopically and cytologically normal. Biopsies were taken from the same area of the cervix in which the CIN lesion originally lay. The results are found in Table 8.

Five biopsies (33%) hybridized to HPV 16 DNA. Four of them came from the cervices of patients whose original abnormal biopsies hybridized to HPV 16 DNA. The fifth came from a cervix in which no hybridization to HPV 16 DNA was detected in the abnormal biopsy but hybridization was detected in the normal biopsy. These positive post-laser biopsies may represent infection from genital tissue outwith the treatment area or they may represent re-infection from infected sexual partners after intercourse following laser therapy.

Table 9a

	Positive by:	
Copy No	Slot Blot	Miniblot
>100	7	7 (100%)*
10-99	23	22 (95%)
1-9	27	15 (56%)
0.1-0.9	29	10 (34%)
Negative	27	1#
total	113	55 (49%)

* As % of slot blot positives
# HPV DNA detected at 10-99 copies per cell as a result of operator error (wrong DNA sample loaded)

Comparison between slot blot and miniblot showing true positive rate of slot blots over range of copy numbers (stringent conditions). 27 samples were negative for HPV DNA on slot blot analysis of which one was a false negative on miniblot testing#.

Table 9b

Copy No. (SB)	Sensitivity %	Specificity %	PPV%	NPV%
>100 (n=7)	100	100	100	100
> 10 (n=30)	97	96	97	96
> 1 (n=57)	100	67	67	96
>0.1 (n=86)	100	43	63	66

Sensitivity and specificity of slot blots compared to "gold standard" miniblots (stringent conditions)

2.11 Study comparing the specificity and sensitivity of slot-blot analysis with Southern blot analysis in detecting hybridization between cellular DNA and HPV DNA sequences.

One hundred and thirteen biopsies from preneoplastic, neoplastic, internal control and external control tissue in the study groups yielded sufficient DNA for comparison of the sensitivity of slot blot and Southern blot analysis. Total cellular DNA was digested with Bam H1 and transferred to Genescreen plus by the minigel/miniblot method. All results are for hybridization detected under stringent conditions. The sensitivity and specificity of the miniblots was established by using reconstructions consisting of 0.1, 1, 10 and 100 copies of the HPV genome together with normal cervical DNA, CIN and cancer DNA samples containing known amounts of the HPV genome and negatives with no evidence of the HPV genome. An experimental sensitivity which regularly detected 1 copy per cell and often detected 0.1 copies per cell was achieved for miniblots making them a valid "gold standard" for assessment of the performance of slot blots.

The results show (Tables 9a and 9b) that at high copy number the sensitivity and specificity of slot blots is good but at copy numbers below 10 copies per cell [56/86 (65%) of slot blot positive biopsies] the specificity is

low (45%) as is the positive predictive value (45%). The false positive results probably arise from cross hybridization between amplified cell DNA and vector pBR322 sequences, with homology between virus and cell DNA sequences and with inability to purify a virus insert completely (fig. 10) (Macnab, J.C.M., et al, 1986, Cameron, I.R. et al, 1985 and Park, M. et al, 1983).

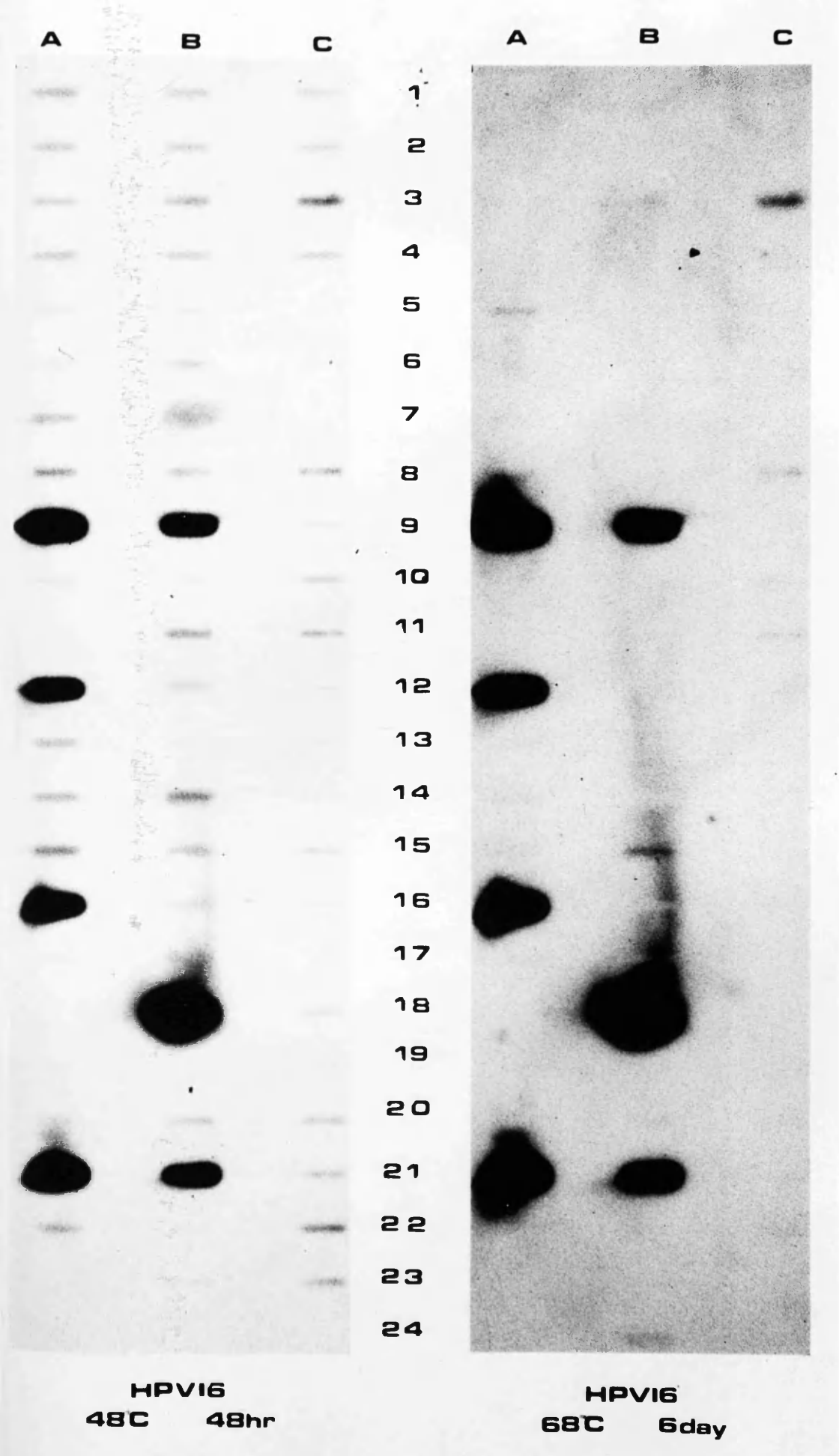
The specificity and positive predictive values at low copy numbers (0.1 to 9 copies per cell) which are frequently found in CIN lesions are poor (65% of slot blot positive biopsies in this study in which the specificity = 45% and the positive predictive value = 45%).

2.12 Study of hybridization between the c-myc oncogene probe and invasive genital cancers with internal and external control biopsies

Biopsies from 22 invasive cancers of the cervix (stage IIb to stage IV) from the group reported in Results, Section 2.5 were available for study. In 8 cases histologically normal vaginal epithelium was available to provide internal controls. Three vulval cancers from the group reported in Results, Section 2.7 were studied with internal control biopsies available in two cases. Eighteen external control biopsies from the group

Fig. 10

Autoradiographs of a slot blot probed with radiolabelled HPV 16 DNA sequences under non-stringent (48 deg. C) and stringent (68 deg. C) conditions. Row 3 comprises HPV 16 DNA reconstructions of 0.1, 1, and 10 copies per cell (A1, A2 & A3 respectively). Rows 1, 2 & 4 comprise similarly arranged HPV 6, 11 and 18 DNA reconstructions respectively. At low stringency, there is substantial cross-hybridization between viral subtypes in the reconstruction slots and non-specific hybridization to background sequences in the sample slots (rows 5 -24). At high stringency, samples which are positive at high copy number (eg. sample A9) are easily identified. Positivity at low copy number is, however, much more difficult to determine (eg. sample C10). Slot A24 comprises a rat embryo DNA control track.


A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
A
B
C
HPV16
48C 48hr
HPV16
68C 6day

reported in Results, Section 2.4 were also probed with the c-myc probe.

All the biopsies were previously analysed for the presence of HPV 6/11, 16 and 18 DNA sequences. Only HPV 16 and 18 DNA sequences were detected.

HPV 16 DNA sequences were detected in 11/22 cervical cancers (50%; 95% confidence interval 29.9% to 70.1%) and HPV 18 DNA sequences were detected in a further 2/22 cases (9.1%; 95% confidence intervals -2.9% to 21.1%). HPV 16 DNA sequences were detected in 1/8 internal control vaginal biopsies (12.5%; 95% confidence interval -10.4% to 35.4%). HPV 18 DNA was not detected in any of these biopsies. HPV 16 DNA sequences were detected in all three vulval cancers and in both internal control biopsies. HPV 18 DNA sequences were not detected in any vulval tissue.

Neither HPV 16 nor HPV 18 DNA sequences were detected in any of the external control biopsies.

The criteria of a 4 fold amplification of c-myc in the cancer biopsies compared to control tissues was used (Riou, G.F., 1987). The copy number was estimated using automated densitometry. This was possible because the cancer and control samples were processed on the same

autoradiography plate so that there was no problem comparing band densities. There was no evidence of amplification of the c-myc oncogene in the tumour or control biopsies (internal and external) irrespective of whether or not HPV DNA sequences were detected (fig. 11).

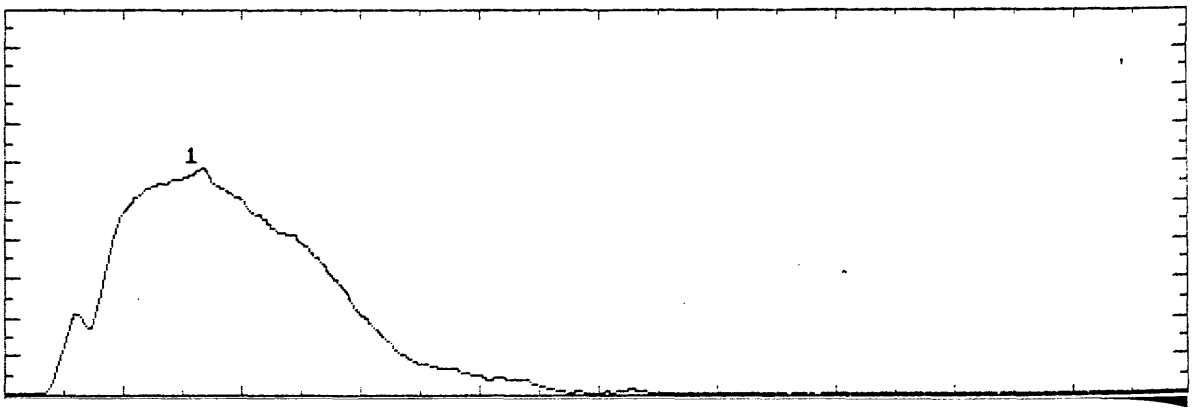
In two cases of invasive cancer of the cervix an extra band of approximately 8kb size was detected on hybridization with the c-myc probe (fig. 11). In one of these cases, a corresponding internal control biopsy was analysed and no extra band was detected. There was no evidence of this band in any of the external control specimens, the internal control specimens or any other tumour samples.

It is possible that this represents rearrangement of the c-myc oncogene. Further detailed restriction digests would be required to clarify this point. Alternative explanations include non-specific hybridization to vector pBR322 sequences or residual hybridization of radiolabelled HPV 16 DNA which did not wash off the blot prior to re-probing with the c-myc probe. The first explanation is unlikely because the bands in question co-migrate with the 8kb HPV 16 DNA band and are therefore not the same size as the 4kb vector and, additionally, an excess of unlabelled pBR322 was

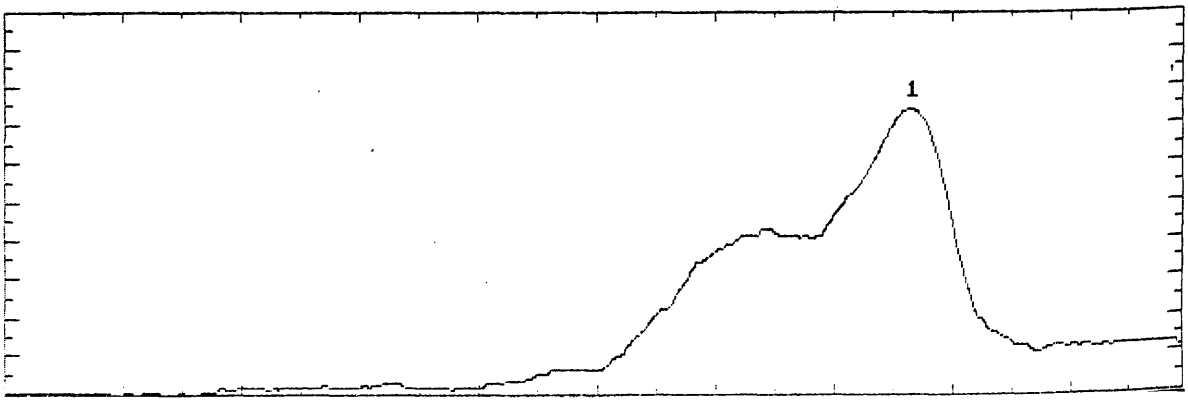
Fig. 11

Autoradiographs of tissue DNA samples hybridized to radiolabelled HPV 16 DNA and radiolabelled c-myc DNA under stringent conditions. All sample DNA was digested with Bam H1. Sample CO is a biopsy from the cervix of an external control patient who showed no evidence of hybridization to HPV DNA. Sample 3 is a biopsy from an invasive cervical cancer which also hybridized to HPV 16 DNA. Automated densitometry failed to demonstrate a four-fold amplification of the c-myc oncogene in tumour DNA versus external control DNA (below). Samples 1 and 2 hybridized to HPV 16 DNA sequences at high copy number and also displayed an unusual restriction pattern on probing with the c-myc DNA sequences indicating a possible re-arrangement of the c-myc oncogene in these tumours. The negative control track rat embryo DNA is designated ct. Rc10 comprises an HPV 16 DNA reconstruction at 10 copies per cell. C-mycs recs comprises positive control c-myc reconstructions of 100, 10, 1 and 0.1 pg c-myc DNA respectively.

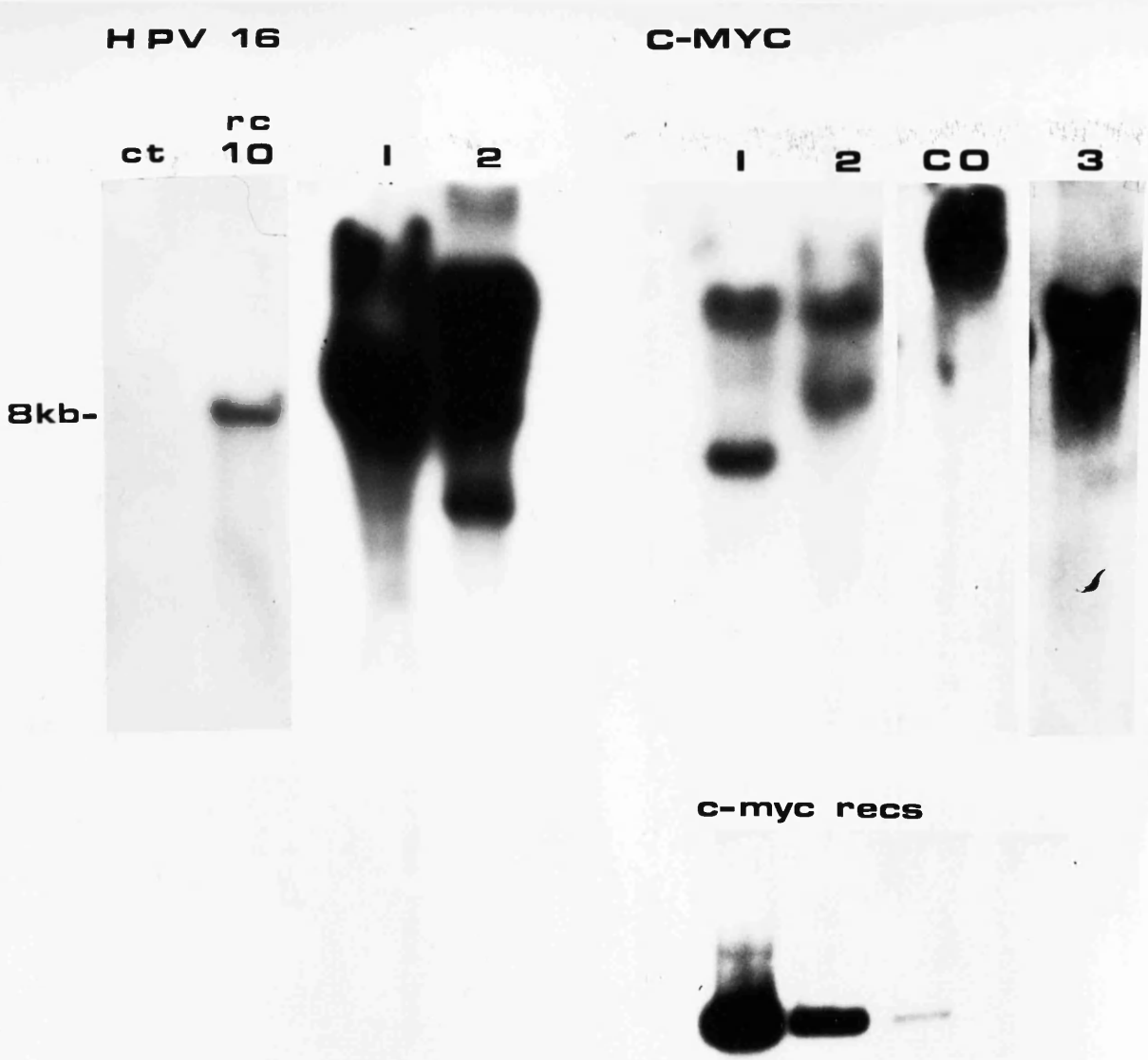
Automated Densitometry



Sample CO - area under curve = 48104 units.



Sample 3 - area under curve = 39482 units.



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used in the hybridization experiments to block unwanted hybridization of radiolabelled probe to vector sequences. Furthermore, there was no evidence of detectable quantities of residual vector sequences in the separate c-myc reconstructions which were hybridised in the same polythene bags as the sample blots. The second explanation is also unlikely because the 8kb bands in the blot hybridized to HPV 16 DNA are minor elements of the very strong signal demonstrated in the tracks. It would therefore be expected that if the problem lay with incomplete washing of the blots to remove the HPV 16 probe then these stronger elements of the signal would also remain. Furthermore, all blots were subjected to check autoradiography after probes were washed off to ensure that no residual probe remained.

2.13 Other tissues studied for evidence of hybridization to HPV DNA sequences.

Biopsies from two other patients were available for study. One case of vaginal intra-epithelial neoplasia grade 3 (VAIN 3) occurred in a 61 year old lady who had undergone cone biopsy of cervix for CIN 3 one year previously. The CIN 3 lesion was incompletely excised.

Hybridization to HPV 16 DNA was detected at low copy number under stringent conditions in the histologically

normal vaginal epithelium control but not in the VAIN 3 biopsy. The second case was a biopsy from a squamous carcinoma of the penis kindly supplied by Dr P Walker, Royal Northern Hospital, London. Despite multiple attempts, it proved impossible to adequately purify the total cellular DNA for successful restriction enzyme analysis.

Slot blot analysis under stringent conditions, however, showed hybridization to HPV 16 DNA at a level of several hundred copies per cell.

DISCUSSION

This study has used Southern Blot analysis to study the relationship between HPV and the neoplastic process in the human female genital tract.

The methodology of Southern Blot analysis has been studied and improvements in efficiency have been identified and tested. I have studied the sensitivity and specificity of other methods of diagnosis of HPV infection and related these findings to the clinical management of patients, the feasibility of screening for and treatment of HPV infection of the genital tract, and the difficulties of prospective study of the natural history of genital HPV infection. Through the use of appropriate tissue controls and the study of all grades of neoplasia, the relationship between detection of HPV DNA in normal tissue and different stages of the neoplastic process have been more closely defined.

The remaining experimental work has studied the relationship between HPV DNA and other cellular mechanisms which may be involved in cell transformation, i.e. local immuno-surveillance and activation of a cellular oncogene.

These data will be discussed in order. A discussion of the possible role of HPV in carcinogenesis and suggestions for the future clarification of that role will be presented.

1. The methods of identification of HPV infection in the female genital tract.

1.1 Southern blot analysis

The increasing interest in HPV in genital tract neoplasia has led to demands for rapid, reliable identification of HPV infection and accurate subtyping of the viral DNA involved. This has been stimulated by the apparently different oncogenic potential of HPV 6/11 as opposed to HPV 16 and 18, [Gissman, L., et al (1982a and 1983) ; Crum, C.P., et al, (1984 and 1985); Boshart, M.L., et al, (1984); zur Hausen, H., (1987)].

Southern blot hybridization is expensive in man hours and materials. In this study, several individual modifications have been combined to reduce the time required to process samples. This has been achieved without sacrificing the benefits of Southern blotting in the study of viral DNA. The simple pattern of restriction fragments produced by digestion with the restriction enzyme BamH1 allowed the running of 3 short length electrophoresis rows on one agarose gel as described (Methods, 6b). Thus the 8kb HPV linear genome could be identified at copy numbers down to 0.2 to 0.1 copies per cell and differentiated from cross-hybridization to non-viral fragments with a high degree of sensitivity and

specificity. This method was used as a "gold standard" for comparison with other methods.

Bam HI digestion produced several bands in a number of cases suggesting integration of the viral genome into the host cell chromosome. These cases were identified for further study if indicated. When more than one band is identified it might be suggested that the restriction pattern represents hybridization to super-coiled circular, nicked circular and linear forms of plasmidial HPV DNA (McCance, D.J., et al, 1983). The addition of a four fold excess of Bam HI enzyme minimised this risk. There was seldom sufficient cellular DNA available for detailed study but further strong evidence for integration of the viral genome into the host cell chromosome was achieved in one case of CIN 3 by digestion of the sample DNA with restriction enzymes which do, and restriction enzymes which do not, cleave the 8kb linear genome and are called "cut" and "non-cut" enzymes to demonstrate the presence of sub-molar bands corresponding to hybridization to virus-cell junction fragments. This method of identifying integration of the viral genome into the host cell chromosome was validated by Durst, M., et al (1985) who performed these experiments on genital tumours in parallel with caesium chloride/ethidium bromide gradient centrifugation, two dimensional agarose gel

electrophoresis and cloning of virus-cell junction fragments. The possible role of integration of the viral genome into the host cell chromosome will be discussed (Discussion, 7).

These studies demonstrate the ability of variations of Southern blot hybridization to accurately identify HPV DNA and to provide information about the physical state of the viral DNA in human genital tissue cells. The different subtypes of HPV DNA were identified in this study by use of the reprobable membrane Genescreen Plus (TM) and the relevant probes under conditions of high stringency.

1.2 Slot blot hybridization analysis

Whilst the studies in Section 1.1 improved the efficiency of sample analysis, they also demonstrated that Southern blot analysis remains unsuitable for large scale diagnostic or screening programmes. A second problem with Southern blots is that tissue biopsies are usually necessary to provide sufficient cell DNA for detailed analysis. As an alternative, Wickenden, C., et al (1985) suggested the use of dot blot hybridization (analogous to slot blot hybridization) as a screening test. They pointed out that sufficient DNA for analysis was collected by non-invasive cervical scrapes rather than biopsy. Their results were compared to clinical, colposcopic and

cytological findings. The validity of this comparison will be discussed later (Discussion 1.4/5). Their small study identified HPV 6 DNA in 10% of women with CIN and 10.5% of cytologically and colposcopically apparently normal women.

A numerically impressive study was conducted on 9295 smears from German women comparing dot blot hybridization to HPV DNA and cytological evidence of virus infection (De Villiers, R.M., et al, 1987). This group again detected HPV DNA in 10% of cytologically normal women but reported hybridization to HPV DNA in 35-40% of women with CIN. The authors cited the much higher rates of HPV DNA detection by Southern blot analysis in CIN and cervical cancer which have been reported in independent studies as evidence that dot blot hybridization is a relatively insensitive method of HPV DNA detection. They surmised that the true prevalence of HPV DNA in the female population may be as high as 30%. Finally the authors commented that careful validation studies using different methods of HPV analysis were needed to substantiate this possibility.

One hundred and thirteen biopsies from genital tract tissues were studied (Results 2.11) comparing slot blot hybridization with the "gold standard" Southern blot. Identically extracted DNA samples using similar radiolabelled HPV 16 DNA probes were used. The results

indicate that 55% of samples with an HPV DNA copy number between 0.1 and 9 copies per cell were slot blot positive but were scored as negative by the Southern blot technique. This represents a positive predictive value of slot blots of only 45% when screening for HPV DNA at less than 10 copies per cell. Samples with a copy number of 0.1 to 9 copies per cell comprised 65% of the slot blot positive results. Thus detection of HPV DNA by slot blots at low copy number which occurred in over half of the cases was poor, demonstrating the low specificity and positive predictive value of slot blots rather than low sensitivity. It is likely that these findings are due to problems with cross hybridization between amplified cell DNA and pBR322 vector sequences, with homology between virus and cell DNA sequences, and with inability to purify a virus insert completely.

It would seem, therefore, that, contrary to the reasoning of De Villiers et al, slot (dot) blot hybridization is as likely to overcall the true prevalence of HPV DNA on the cervix in a population as underestimate it. In addition to this, the proposed method of collection of cervical cells by cervical scraping will only yield cells from the most superficial epithelial layers. This method of sampling will not identify cases in which HPV DNA is lying latent within cells close to the basement membrane. This

will reduce the sensitivity of a screening programme. This test would appear to be inappropriate as a screening method for HPV DNA infection because of its poor performance at low viral copy numbers which are frequently present in cervical tissue.

1.3 Staining for papillomavirus antigen as a means of identifying HPV infection in cells.

In early studies of cervical condylomas and cervical dysplasias, antiserum raised in rabbits against pooled plantar warts was used to confirm that the papillomavirus was the aetiological agent associated with histologically apparent condylomata. [Woodruff, J.D., et al, (1980); Shah, K.H., et al, (1980); Kurman, R.J., et al, (1981); Morin, C., et al, (1981)]. These studies suggested that papillomavirus antigen could be detected in about 50% of condylomatas and mild dysplasias but less often in severe dysplasias. In the study reported in this thesis, (Results, 2.2) group specific bovine papillomavirus antiserum was used and all grades of cervical "dysplasia" were studied. Twelve patients had a histological diagnosis of virus only or CIN 1 of which only 2 (17%) stained for papillomavirus antigen. Alternatively, 4/15 (27%) of CIN 2 and CIN 3 biopsies stained for papillomavirus antigen. Additionally, twice as many abnormal biopsies stained for papillomavirus antigen as

normal internal controls (six versus three). The numbers involved are small but these data do not agree with the larger studies cited. Furthermore, the production of the two major capsid antigens is a function of the late region of the viral genome (Danos, O., et al, 1982) and this region is usually only expressed in terminally differentiating keratinocytes. This would mean that higher levels of staining for papillomavirus antigen might be expected in differentiating epithelium compared to dysplastic epithelium. The corresponding HPV DNA hybridization data suggests that HPV is frequently present in the normal tissue so it seems that the normal cycle of viral replication is inhibited here also. HPV 16 and 18 DNA was detected in this study whereas HPV 6/11 is associated with exophytic warts in which the full cycle of viral replication is prominent [Gissman, L., et al, (1980); Woodruff, J.D., et al, (1980)]. Clearly, therefore the effects of HPV 6/11 and HPV 16 and 18 on tissues differ as, possibly, do the effects of host tissues on these two groups of viruses.

The parallel study of the prevalence of papillomavirus antigen and hybridization to HPV DNA subtypes also emphasises that staining for papillomavirus antigen is an inefficient method of detecting infection with HPV DNA. Only 6/17 (35%) HPV DNA positive biopsies from the

abnormal group also stained for papillomavirus antigen. This is important. Firstly, it highlights the deficiencies of immunohistochemical studies as a means of establishing prevalence rates of HPV infection in the community. Secondly, immunohistochemistry fails to identify cases in which HPV DNA is present and transcription of the early part of the viral genome only may be occurring. The available evidence suggests that, both in the bovine and human systems, it is transcription from the early region of the genome which may be important in malignant transformation [Campo, M.S., et al, (1985); Schneider-Gadicke, A., et al, (1986)].

The late region of the viral genome comprises 31% of the total genome length and encodes for the two major capsid proteins, L1 and L2. The L1 protein appears to be highly conserved among papillomavirus subtypes whereas the L2 protein appears to be subtype specific and may be involved in host and tissue specificity (Schwarz, E., et al, 1983). However, Nakai, Y., et al, (1986) demonstrated a type specific antigenic determinent on the L1 protein of BPV1 so it is clear that this protein also plays a role in subtype specificity. Recently, Patel, D., et al (in press) have used monoclonal antibodies to identify a type specific antigen in the L1 protein of HPV 16. They suggest that, as these antibodies can be used on routine

histological sections, they will be helpful in diagnosing particular HPV infections. It, however, would appear from the arguments cited above that the value of this test will be limited.

1.4 Cytology and histology as a means of identifying HPV infection.

The early work of Meisels A., et al, (1977 and 1980) established the criteria for cytological and histological diagnosis of HPV infection. They identified the koilocyte as the pathognomonic feature of HPV infection along with other more subtle changes. In these publications the authors emphasised the difficulty of diagnosing the atypical condyloma both cytologically and histologically because it "mimics" dysplasia. In a later review (Meisels A., & Morin, C., 1985) they further emphasised the different diagnostic features of HPV infection and CIN and discussed the diagnostic difficulties posed by mixed lesions.

Other reviews (e.g. Kurman, R.J., et al, 1985) changed the emphasis of Meisel's and Morin's reports and suggested that HPV infection and dysplasia were part of a continuum of the same disease. Alternatively, Fletcher, S., (1983) in a detailed review of the histological appearances of HPV infection, cervical dysplasias and mixed lesions

emphasised:-

a. the difficulties of diagnosing HPV infection
b. the fact that these two entities could be regarded as coexisting at the same site rather than being sequential and
c. that HPV infection could make the diagnosis of the grade of dysplasia more difficult with the tendency being to assign a higher grade to the dysplasia than was actually present.

Alternatively, Pilotti, S., et al (1981) reported a tendency to underestimate the cytological "diagnosis" of CIN when koilocytosis was prominent.

These findings serve to emphasise the limitations of cytology as a "diagnostic" tool in the differentiation and grading of HPV and CIN lesions. Even histological assessment of the lesions suffer from inter-observer variation. Anderson, M.C., (1985) reviewing the histological criteria for diagnosing HPV infection stated that many of the individual features including koilocytotic atypia may not be HPV-specific and it is rather the sum of several features which allows a diagnosis of HPV infection. This necessary latitude in interpretation of histological and cytological appearances allows an inexactitude in diagnosis in the less characteristic lesions and gives rise to inter-observer

variation. Indeed the same observer may grade the same lesions differently on different occasions. Furthermore, it is the common experience of histopathologists and cytopathologists that if there is diagnostic difficulty, lesions tend to be assigned higher grades than lower ones.

This study (Results 2.2) has found that routine cytology and histology reporting in city laboratories has overcalled the prevalence of HPV infection on the cervix by 18%. Furthermore, the correlation between detection of HPV DNA and histological identification of HPV infection in individual biopsies is poor (7/16:47% of histologically normal internal control biopsies and 8/15:53% "virus only" biopsies). Similarly, in the collated data (Results 2.8, Table 6), only 32/46 (69%) of histologically diagnosed lesions with evidence of virus infection actually contained HPV DNA.

Thus, overall, as a measure of prevalence of HPV DNA, the histological assessment of a biopsy offers a sensitivity of 71%, a specificity of 36%, a positive predictive value of 70% and a negative predictive value of 38% compared to the gold standard hybridization analysis of HPV DNA. The validity of DNA-DNA hybridization as a gold standard compared to cytology and histology lies in two main areas.

Firstly, the experimental design allows identification of HPV DNA at very low copy numbers (down to 0.1 copies per cell in some experiments) under conditions of high stringency which preclude cross-hybridization with unrelated DNA sequences. Additionally, precise positive and negative controls are included at all stages. Hybridization to HPV DNA is either present or not present. Secondly, DNA-DNA hybridization identifies HPV DNA. Histology and cytology identify cellular changes associated with replicating virus which may or may not be specific to HPV and may or may not be obvious to individual observers. The proposed oncogenic potential of HPV lies in the early region of the viral genome. Thus unlike histology and cytology, DNA-DNA hybridization is precise, is not subject to observer error and identifies the most important component of the virus namely the viral genome.

1.5 Colposcopy as a means of identifying HPV lesions on the cervix.

The identification at colposcopy of aceto-white epithelium is simple. The diagnosis of the nature of the aceto-white lesion is, however, difficult and depends on the subjective opinion of the colposcopist, the value of which depends on the colposcopist's experience. Reid, R., et al (1980) described colposcopic appearances which they

considered to be associated with HPV infection and devised a "colposcopic index" for differentiating subclinical HPV infection and CIN (Reid, R., et al, 1984). In the hands of these highly specialised observers the correct approximate histological diagnosis was achieved colposcopically in 96% of cases. Despite this impressive result they stated that the index was more useful in differentiating "mild" from "severe" lesions than differentiating "warty" and "non-warty" lesions. Alternatively, Kirkup, W., et al (1982) found that over two-thirds of patients with histological "warty features" did not show the colposcopic changes referred to by Reid et al. Similarly, Walker, P.G., et al, (1983) found it impossible to distinguish between HPV infection and CIN. An explanation at the cellular level for the difficulties experienced by the latter workers was provided by Crum, C.P., et al (1986). This group studied aceto-white epithelium for evidence of HPV 16 DNA by <u>in situ</u> hybridization and demonstrated variations in hybridization from one area to another within a biopsy and variations from cell to cell within an area. These data would suggest that colposcopy cannot therefore differentiate CIN alone, from HPV alone or from CIN + HPV on the cervix such is the intimacy of the intermingling of these lesions.

In this study, biopsies were taken from 67 areas of aceto-white epithelium and 38 areas of the transformation zone which did not display aceto-white epithelium at colposcopy. HPV DNA was detected in 72% of the colposcopically abnormal and 47.4% of the colposcopically normal biopsies. The results show that as a diagnostic test for the presence of HPV DNA, the colposcopic identification of aceto-white epithelium offers a sensitivity of 71%; a specificity of 47%; a positive predictive value of 67% and a negative predictive value of 53%. These findings support the view that colposcopy is a useful method for the identification of areas on the cervix which are most likely to contain significant premalignant lesions (all cases of CIN 2 and CIN 3 were confined to the colposcopically abnormal group). The findings do not, however, support a role for colposcopy in differentiating areas of the cervix which harbour HPV DNA from those which do not.

1.6 Conclusion

The proposed role of HPV in genital carcinogenesis depends on the presence of HPV DNA and viral gene products in host cells. Southern blot hybridization remains the most sensitive and specific means of identifying and studying HPV DNA in host cells. Studies which base their findings

upon other criteria for diagnosis of HPV infection (colposcopy, cytology, histology, and immunohistochemistry) must be viewed with caution. This is particularly relevant to prospective study of the natural history of HPV infection as a precise knowledge of the start point of such a study is crucial to the accurate interpretation of results.

A rapid, reliable means of non-invasive identification of the presence of HPV DNA in host cells would be a major advance in prospective study of this condition. Slot (dot) blot hybridization of cervical scrapes is not sufficiently specific for this purpose.

2. Discussion of the role of screening for HPV infection of the cervix.

An ideal screening test for any condition should possess the following characteristics:-

a. the test should be sensitive and specific, and it should have a high positive and negative predictive value;
b. the condition screened for should be a proven precursor of more serious disease;
c. a treatment should be available which has low morbidity, has low or absent mortality, effectively treats the condition or prevents progression to more

serious disease, and is acceptable to patient and practitioner;

d. the condition should be sufficiently common to make screening cost effective;

e. the test should be cheap, easy to perform and amenable to automation.

The performances of the available methods of detection of HPV infection of the cervix have been explored in Section 1. Current methods of HPV DNA detection rely on probing samples with radiolabelled or biotin labelled DNA. Where dot blot hybridization is relatively quick, cheap and potentially suitable for automation it suffers from low specificity and a poor positive predictive value. Southern blot hybridization is sensitive and specific, it can be made more efficient to facilitate processing of larger numbers of samples but is expensive in man-hours and materials and is not amenable to automation in its present form. Screening samples for viral subtypes using subtype specific monoclonal antibodies to capsid antigen is potentially cheap and easy to perform but relies on the presence of replicating virus and will therefore not identify cases where HPV DNA is present but not replicating.

There is at present no ideal screening test for the

detection of HPV DNA in large numbers of the female population. There is little doubt that the presence of HPV DNA on the cervix is intimately associated with premalignancy and malignancy of the cervix. There is growing evidence from in vitro studies which propose a mechanism for malignant transformation by HPV DNA. Final in vivo proof that HPV DNA is a major cofactor in the development of cancer is lacking. CIN is generally accepted as a precursor of invasive cancer. The potential for preventing the majority of cervical cancer was discussed in the Introduction, Section 1.1. It would seem irrational to divert scarce economic resources from improving the existing cytological screening programme designed to identify a known precancer (CIN) to provide a screening test for a suspected precancer (HPV). It might be suggested that screening for HPV DNA could be used to identify patients at risk of eventually developing cancers at an early stage. This argument is flawed on two counts. Firstly, HPV DNA has been detected in 60% to 90% of premalignant lesions (Singer, A., et al, 1984). In this study 68% (40/59) of CIN lesions contained HPV DNA. This would mean that HPV DNA screening would fail to identify 10% to 40% of established pre-malignant lesions. Secondly, the best estimate of the prevalence of HPV DNA on the cervices of the female population suggest that 10%

to 30% of women are affected (De Villiers, E.M., et al, 1987). The relative rarity of invasive cancer of the cervix (Introduction, 1.1) indicates that the vast majority of these women are not in danger of developing cervical cancer. HPV infection involving all subtypes can enter a latent phase [Ferenczy, A., et al (1985); Macnab, J.C.M., et al (1986)]. If screening for HPV infection was introduced large sums of money would be required either to treat or intensively follow-up (with cytology, colposcopy and biopsy) these large numbers of healthy women. This would have the effect of diluting the surveillance of patients most at risk, that is, those with CIN 2 or CIN 3 to their potential detriment.

There is little value in introducing a screening test for which there is no appropriate response. Follow-up surveillance of up to one third of the female population would prove costly. This study has shown that treatment of the transformation zone with laser ablation fails to eradicate HPV DNA in one third of cases. This may be due to persistence of the viral DNA outwith the treatment area or due to reinfection from an infected male. Although high cure rates have been claimed for laser eradication of vulval and penile warts (Bellina, J.H., 1983) it would be impossible to eradicate HPV DNA from the whole female genital tract and from the penis and urethra of the male

with laser therapy. Other methods of treatment such as topical antimetabolites (Weimar, G.W., et al, 1978) or interferon (Androphy, E.J., 1986) may prove more useful.

It has been suggested that vaccination against HPV infection might be appropriate. Antibodies raised against the L1 or E1 gene products have been suggested (Crawford, L., 1986). Bubb, V. et al (1988) showed that the structure of the putative protein encoded for by the E5 region of the HPV DNA subtypes 6, 11, 16, 18, & 33 exhibits a conserved hydrophobic structure which may interact with the cell membrane. It may be that this protein will be a suitable target for a vaccine. A recombinant DNA vaccine against BPV appears to offer protection in cattle (Pilacinski, W.P., et al, 1985). The value of such a vaccine in reducing the incidence of invasive cancer of the cervix would be difficult to prove and large, long-term placebo controlled trials would be required to control for the many other variables involved. The likelihood of such trials being mounted seems low.

In summary therefore, screening for and treatment of HPV DNA infection of the human female genital tract is inappropriate at present. It is likely to provide little benefit for a large cost and result in over investigation and over treatment of large numbers of healthy women.

There is no evidence to suggest that it will reduce the incidence of invasive cancer of the cervix. It would divert resources away from improving a system of cervical cytology screening which has been shown to substantially reduce the incidence of invasive cancer when it is properly organised.

3. The value of internal and external control tissues in assessing the relationship between genital tract neoplasia.

Early studies of the prevalence of HPV DNA in the human female genital tract either did not study control biopsies or examined very few. Later studies of apparently normal external control women showed that the prevalence of HPV DNA subtypes on the cervix was 10% - 35% (see Introduction, 4.6 for review). This contrasts with a prevalence of 60%-90% in neoplastic tissues of the genital tract in published studies. In this study 70/108 (65% : 95% confidence interval 56% to 74%) biopsies from neoplastic tissue and 4/30 (13% : 95% confidence interval 1% to 25%) external control biopsies hybridized to HPV DNA subtypes (table 6). This difference is statistically significant (chi squared = 23.0, $p<0.001$, Yates correction).

The use of adequate external control biopsies is important on two counts. Firstly, it establishes the association

between human female genital neoplasia and the presence of HPV DNA. Secondly, it allows assessment of the prevalence of HPV DNA in the genital tracts of the apparently normal population. The implications of the prevalence of HPV DNA in the population for screening and treatment have been discussed (Discussion, 2).

The importance of internal control biopsies was identified by Ferenczy, A., et al, (1985) and Macnab, J.C.M., et al, (1986). These two studies showed that HPV DNA could be detected in apparently normal tissue adjacent to condylomata acuminata and malignant tissue respectively. No statistically significant difference was detected between abnormal biopsies and internal control biopsies in studies in this thesis.

The presence of HPV DNA in a tissue does not in itself indicate that a tissue will be histologically abnormal nor can HPV DNA induce neoplasia on its own. The frequent detection of HPV DNA in internal controls supports the role of other locally active cofactors in a multistage process of neoplastic transformation. Such cofactors, for example, may be operating in one area of the cervical transformation zone but not in another which results in the particular location of a premalignant lesion on the cervix. Secondly, Ferenczy, A., et al, (1985) established a relationship between the detection of latent HPV DNA and

recurrence of condylomata acuminata. It may be that the detection of HPV DNA in internal controls associated with premalignant or malignant lesions means that this tissue is susceptible to malignant transformation at a later date. This may be one mechanism of recurrence of CIN after apparently successful local ablation of existing lesions. The numbers in this study are insufficient to provide follow-up data relating the presence of HPV DNA in internal controls to subsequent recurrence of disease but such a study might be valuable in predicting which women are more susceptible to recurrence of CIN.

On the vulva, the management of vulval premalignancy poses management dilemmas because of its multifocal nature (McCance, D.J., et al, 1985b) and tendency to recur after local treatment (Douglas, C.P., 1983). It may be that the application of anti-viral agents to the vulva following local ablation of established VIN lesions would reduce the incidence of recurrent disease in cases in which hybridization to HPV DNA in abnormal and normal tissue is detected. A controlled trial involving four arms comprising two treatment arms for cases with and without hybridization to HPV DNA and two non-treatment control arms with and without HPV DNA could answer this question and provide strong evidence of a central role of HPV DNA in cell transformation in humans.

Data on the association between detection of HPV DNA in internal controls related to invasive cancer are scarce. One small study has failed to detect any relationship between the presence or absence of HPV 16 DNA in tissue distant to malignant disease of the cervix and vulva and recurrence of disease or long-term survival (Walkinshaw, S.A., et al, 1987). It is likely that if the presence of HPV DNA in control tissue does predispose to invasive cancer in adjacent, normal tissue it would act through the development of new primary disease. This effect would be easily obscured by recurrence of disease due to spread of malignant cells from the original primary tumour. By definition, such spread is not possible in premalignant disease so comparison of the prognostic implications of HPV DNA in control tissue related to premalignant and malignant disease is not appropriate.

It is interesting to note that HPV 6/11 DNA sequences were detected in only one case of CIN (in a patient who had been referred to Glasgow from the North-West of England) and two local ladies who were external controls. The lack of detection of HPV 6/11 DNA in the West of Scotland is confirmed by other studies. Fletcher, K. (reported in Murdoch, J.B. et al. 1988) detected HPV 6/11 DNA in only 2/43 CIN samples and Millan, D.W.M. et al (1986) reported detection of only one case of

hybridization to HPV 11 DNA in a study of 26 cases of acetowhite cervical epithelium. Thus in the West of Scotland the cumulative rates of detection of HPV 6/11 DNA in cases of cervical acetowhite epithelium is 3/136 (2.2%). These data are quite different to the findings of McCance, D.J. et al (1983) who reported a 28% detection rate of HPV 6 DNA in CIN lesions in the London area. This would suggest that there is a marked geographical difference in the prevalence of HPV 6/11 DNA sequences within the United Kingdom.

Finally, HPV 16 DNA has been detected widely in abnormal and normal biopsies in this study. In contrast HPV 18 DNA was detected in 2 "virus only" biopsies, 1 case of CIN 1, 5 cases of CIN 2, 9 cases of CIN 3 and 2 cases of invasive cancer of the cervix. HPV 18 DNA was detected in only one internal control related to an invasive cancer of the cervix and no external control biopsies (Results, Tables 1,2,3,7a). Thus, in total, HPV 18 DNA is detected in 19/98 abnormal biopsies (19.4%:95% confidence intervals 11.6% to 27.2%) and in 1/65 internal controls (1.5%:95% confidence intervals -1.4% to 4.4%)
(chi squared = 9.97, $0.01>p>0.001$, Yates correction). The author knows of no reports of hybridization to HPV 18 DNA in normal external control tissue, although it has been described in 1 case of "chronic inflammation" and one case

of histological "viral changes" (Millan, D.W.M., et al, 1986). This suggests that HPV 18 DNA is more closely associated with malignant transformation than even HPV 16 DNA and that its detection could have prognostic significance for the patient. This possibility is supported by a study of invasive cervical cancer (Barnes, W. et al, 1988) which also showed that, while HPV 18 DNA is detected less often than HPV 16 DNA, there is an association between the detection of HPV 18 DNA, young age of the patient, poor tumour cell differentiation and secondary nodal spread.

Thus, unlike mass screening of the normal population for HPV DNA, the selective study of specific tissues from patients conventionally diagnosed as having premalignant or malignant genital disease would contribute to their management and follow-up. This could be achieved by identifying patients who would benefit from specific antiviral therapy or closer monitoring.

4. <u>The relationship between detection of HPV DNA, histological diagnosis of a lesion and the age of the patient.</u>

In a study of 47 patients with invasive carcinomas of the cervix, Meanwell, C.A.. et al (1987) detected an association between the age of the patients and the detection of HPV 16 DNA. They suggested that patients

over 40 years were significantly more likely to have tumours which hybridized to HPV 16 DNA than those under 40 years. They postulated that the association between HPV 16 DNA and cervical cancer was age-mediated and that this did not support an aetiological role for HPV DNA. Alternatively, McCance, D.J., et al (1985) showed that HPV DNA was commonly detected in young women with CIN (mean age 29 years, range 18-39 years).

In this study no association between the age of the patient and detection of HPV DNA was noted. No significant difference was found in the detection of HPV DNA in invasive cancers between those patients under 40 years (n=5) and those patients over 40 years (n=26) (chi squared = 0.348, $p>0.5$, Yates correction). The patients with premalignant lesions of the cervix were significantly younger than the external control group ($p<0.001$, Student t test). The external control group were in turn significantly younger than the group with invasive cancer ($p<0.001$, Student t test). There was no statistically significant difference between detection of HPV DNA in patients with mild premalignant lesions ("virus only" and CIN 1); patients with severe premalignant lesions (CIN 2 and CIN 3); and patients with invasive cancer (chi squared = 1.518, DF=2, $p>0.5$). There was a statistically significant difference between HPV DNA detection in

patients with abnormal tissue (premalignant or malignant) and external control patients (chi squared = 22.9, $p<0.001$). These data show that there is no association between hybridization to HPV DNA and the age of the patient in this study. There is, however, a highly significant association between hybridization to HPV DNA and the presence of a neoplastic abnormality. These data do not support the findings of Meanwell, C.A., et al (1987). There is no evidence of a statistically significant difference in the ages of patients whose lesions show hybridization to HPV DNA and those who do not [either premalignant lesions ($p>0.5$, student t test) or malignant lesions ($0.5>p>0.1$, student t test)]. This suggests that patients with HPV plus CIN do not have a worse prognosis than those with CIN alone. If the presence of HPV DNA did result in a worse prognosis for malignant transformation of cervical cells it would be expected that patients with premalignancy and malignancy associated with HPV DNA would be younger than those without HPV DNA. Prospective randomised controlled trials would be required to prove this. The methodological difficulties of trials with an end-point of CIN 3 have been discussed (Introduction, 4.1). A prospective trial with an end-point of invasive disease would be unethical.

These data, therefore, suggest that patients with HPV plus

CIN should not be treated differently to patients with CIN alone. The arguments for improving screening of the population for CIN and not introducing a further tier of screening for HPV DNA in the population have been discussed (Discussion, 2). These results suggest that attention should continue to be focused on treating CIN irrespective of the presence of HPV DNA as CIN is the final common pathway towards invasive disease and treatment of CIN is most likely to interrupt progression of disease.

5. The relationship between detection of HPV DNA and local immmuno-supression on the cervix.

In this part of the study the detection of HPV DNA in CIN biopsies was achieved using slot blot analysis. Slot blot analysis was used because of the very small quantities of total cellular DNA available for study. This was due to the very small size of biopsies and, possibly, to the storage conditions following the histological processing for Langerhans' cell counting. The specificity of slot blots has been shown to be poor with copy numbers below 10 copies per cell (Results, 2.12) so only biopsies with HPV DNA copy numbers greater than 10 copies per cell were accepted as positive (sensitivity 97%, specificity 96% positive predictive value 97%; negative predictive value 96%).

Langerhans' cells were detected in histological biopsies using staining for surface T6 (CD1) antigen using NA1/34 (human thymocyte antigen) as this is one of the most sensitive methods for their detection in squamous epithelium. Some workers [Tay, S.K., et al, (1987); McArdle, J.P., et al, (1986)] have used the S100 antigen as a marker but this detects only 35% of Langerhans' cells. In this study Langerhans' cell counts from the histologically normal ectocervix were used for comparison. The numbers of Langerhans' cells on the ectocervix are valid internal controls because their numbers are similar to those found in the normal transformation zone [Morris, H.H.B., et al, (1983a); Edwards, J.N.T., et al (1985)].

The results from this study show that Langerhans' cells are significantly decreased in CIN compared to controls in the presence of HPV 18 DNA even at low copy numbers. In the presence of HPV 16 DNA the Langerhans' cells are reduced at high copy numbers only. This may be because the Langerhans' cells are truly decreased or because the surface antigens are being blocked or altered by the presence of HPV or its gene products. It is also possible that the Langerhans' cells are inhibited from migrating into the epithelium or are encouraged to migrate out of the epithelium more rapidly than normal. The depletion of Langerhans' cells may be a function of both the subtype

of HPV DNA present and the copy number. It may be that HPV DNA gene products have an immuno-suppressive effect which releases the neoplastic process from inhibition by Langerhans' cells. Alternatively, local immunosuppression, possibly as a result of smoking (Barton, S.E., et al, 1988) may facilitate infection by HPV DNA with subsequent neoplastic transformation by early viral gene products.

This study provides evidence for a mechanism of interaction of the two most important specific risk factors for malignant transformation on the cervix (HPV DNA and smoking via local immunosuppression). It may be that this interaction is facilitated by the nature of the transformation zone as this is an area of increased mitotic activity and cell generation. Whether or not other environmental cofactors play a part in the process remains to be shown.

6. The role of the c-myc oncogene in female genital tract cancer.

The c-myc gene product is thought to play an important role in the regulation of cell proliferation and synthesis of the c-myc protein is present throughout the cell cycle (Henriksson, M., et al, 1988). Transcription of c-myc RNA shuts off when cells stop dividing so c-myc expression

seems to be finely coupled to the differentiation and divisional state of the cell. It is not surprising that disruptions of the gene are frequently associated with cancer (Rabbitts, T.H., 1987).

There is conflicting evidence as to whether amplification and/or overexpression of the c-myc proto-oncogene is associated with genital cancer [Riou, G.F., et al (1985 and 1986); Ikenberg, H., et al (1987); Gariglio, P., et al (1987)].

The study presented here (Results 2.13) failed to show any evidence of amplification of the c-myc gene in association with cervical or vulval cancer compared to controls. No attempt was made to assay the expression of c-myc mRNA so no comment can be made about the possibility of overexpression of the c-myc gene product in these tumours. In two cases of cervical cancer an abnormal BamH1 restriction pattern was observed which raises the possiblity of a rearrangement of the c-myc oncogene. To clarify this point further detailed restriction analyses would be required. There was not sufficient time available during the course of this project to study this interesting question but further analysis of these tumour specimens is being carried out in the laboratory.

Such a rearrangement might release the c-myc oncogene from

normal cellular control allowing overexpression of the oncogene product. Alternatively, the rearrangement could bring the oncogene under the control of a foreign promotor or enhancer element. Thirdly it is possible that the rearrangements could so alter the c-myc DNA sequence as to result in abnormal mRNA transcription which results in cell transformation. It would be tempting to postulate that HPV DNA might be involved in such changes and further detailed assessment of the association between the c-myc and HPV DNA in genital tumours is warranted.

In order to explore this possible association it would be necessary to study the transcriptional activity and physical state of HPV DNA in cancer cells. Secondly, it would be necessary to confirm the presence of c-myc rearrangements in these cells with further restriction digests and to analyse the transcriptional activity of the oncogene in the presence and absence of such rearrangements. This would have to be analysed in tumour tissue, normal internal control tissue and normal external control tissue.

7. A possible role for HPV in cervical carcinogenesis.

The close association between HPV 16 and HPV 18 DNA sequences and cervical neoplasia has prompted many workers

to suspect that these two viral subtypes are causal agents in the development of cervical cancer. Powerful support for this argument has been supplied by studies of HPV DNA in cervical cancer cell lines and primary cervical cancer biopsies. These studies have shown a consistent viral integration pattern within the E1 and E2 ORF's of the viral genome (Durst, M., et al, 1985) and frequent transcription of the E6/E7 ORF's (Schwarz, E., et al, 1985).

HPV 16 DNA (Pirisi, L., et al, 1986) and 18 DNA (Bedell, M.A., et al 1987) have been shown to morphologically transform human and rodent cells and the E6/E7 ORF's appear to be the segments of the viral genome necessary for this event. A possible mechanism for cell transformation is the presence of a small intron within the E6 ORF in three cervical cancer lines (Schneider-Gadicke, A., Schwarz, E., 1986). This results in the second E6 exon being read in a different reading frame which results in a putative protein with a distant relationship to epidermal growth factor. The same splice donor and receptor sites also exist in HPV 16 and 33 DNA but are absent in HPV 6 and 11 DNA. A second mechanism is suggested by McCance, D.J., (1988) whereby integration of the viral genome into the host cell chromosome may disrupt the production of E2 ORF proteins which result in

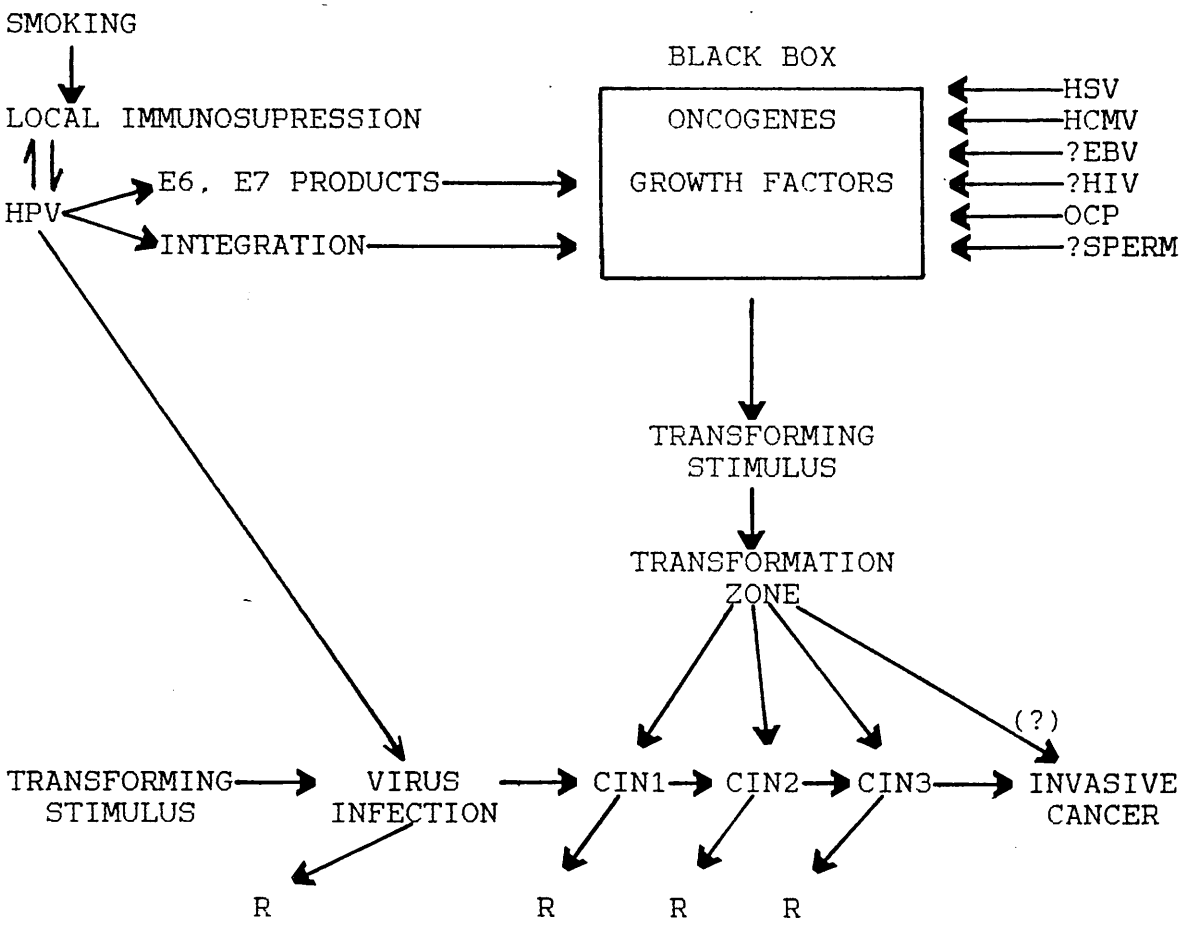
uncontrolled transcription of the E7 ORF making the cells susceptible to other events which could lead to a malignant phenotype.

It is clear from studies of the prevalence of HPV DNA on the cervix that the presence of HPV DNA is a common event with transformation of cervical cells occurring fairly infrequently. This thesis, by showing that HPV DNA sequences are frequently detected in normal tissue adjacent to neoplasia, has supported the suggestion that the presence of HPV DNA alone does not result in cell transformation. Other co-factors appear to be required as is the case in the bovine papillomavirus model.

The study reported here (Results, 2.9) demonstrates a relationship between HPV DNA sequences and impaired local immunosurveillance in the cervix. This may be the mechanism whereby smoking acts as a cofactor in cervical carcinogenesis (Barton, S.E., 1988), possibly by promoting infection with HPV subtypes which later promote cell transformation.

Pater, M.M., et al, (1988) suggest a possible mechanism whereby the oral contraceptive pill may act as a cofactor with HPV DNA although support for a significant role of the oral contraceptive on epidemiological grounds remains contentious.

Fig. 12



R = regression

Flow diagram illustrating a possible link between HPV ,other co-factors and cervical carcinogenesis

In an effort to reconcile the evidence for HSV as a significant cofactor in the genesis of cervical cancer, Zur Hausen, H., (1982) suggested that HSV possessed "initiator-like" functions and a synergism existed between HSV and the "promotor" HPV. Zur Hausen subsequently turned his attention to reconciling such features of viral oncogenesis as (a) only a small proportion of those who carry HPV eventually develop cancer; (b) the latency period between developing a viral infection and developing cancer may last for years and (c) the resulting cancers are monoclonal (Zur Hausen, H., 1986). He suggested that intracellular surveillance mechanisms may control papillomavirus infections and that cancer was a result of a failing host cell control of persisting viral genes.

8. A Hypothesis

It seems unlikely that a model for HPV as a possible cervical carcinogen would simply comprise a spectrum of cervical abnormalities ranging from koilocytotic atypia at one end to invasive cancer of the cervix at the other. Far more likely, is a multistage process for the development of malignancy in which HPV DNA plays an important role (fig. 12).

The concept is conveniently considered using an intracellular "black box" in which cellular control

mechanisms such as proto-oncogenes are influenced to a greater or lesser degree by environmental factors such as HPV DNA, smoking, oral contraception and possibly other viruses. Depending on the interplay between host and environmental factors a "transforming stimulus" develops to which the mitotically active cells of the cervical transformation zone are particularly susceptible. The fundamental difference between the incidence of cervical cancer and cancer of the vulva and vagina appears to be this susceptibility of the host cells of the cervical transformation zone to malignant transformation. Depending on the strength of that transforming stimulus lesions may develop which are recognised histologically as CIN 1, 2 or 3. It is possible that a suitable strong transforming stimulus might result in the development of aggressive tumour cells de novo which could explain the subset of women who appear to develop cancer very quickly.

Alternatively HPV infection may merely infect cells and produce koilocytotic atypia. Thereafter, the same or a different transforming stimulus could drive the particularly susceptible cells of the CIN lesion forwards towards malignancy.

9. Future work.

This thesis has provided several lines of future work which can be pursued to clarify the relationship between HPV, other possible cofactors, and cervical neoplasia.

The numerically small study of the association between the detection of HPV DNA and decreased local immunosurveillance in the cervical transformation zone should be repeated using larger numbers of patients. This study would include detailed recording of the smoking habits of the patients in an effort to confirm the findings of Barton, S.E. et al (1988). Secondly, improved handling of tissue biopsies should yield larger ammounts of total cellular DNA for study of the viral subtypes present and their physical state within cells by detailed restriction enzyme analysis. Additionally, specific analysis of the transcriptional activity of the E6/E7 ORFs of HPV genomes present in biopsies could be performed.

In this way, the association between smoking via local immunosuppression and the intracellular events thought to be involved in the proposed oncogenic function of HPV could be clarified.

Secondly, the possible role of oncogenes in cell

transformation in the presence of HPV DNA requires to be studied further. The study of the physical state of HPV DNA, transcription of the E6/E7 ORFs, and amplification and overexpression of oncogenes, especially c-myc in the same tissues would show whether or not these prosesses were intimately associated with each other and would provide a model for intracellular events leading to cell transformation.

Finally, this study has shown that adequate control tissues are essential for the study of associations between the ubiquitous HPV genome and specific tissues. In situ studies have shown that the detection of HPV DNA varies even within biopsies (Crum, C.P. et al 1986). In order to confirm the association between HPV DNA sequences and cell transformation in the cervix it is now necessary to determine which cells actually contain viral sequences and what the transcriptional activity of these sequences is. If there is no evidence of HPV DNA in dyskaryotic cells within a CIN lesion and HPV DNA is only to be found in morphologically normal cells and/or koilocytes then the association between HPV DNA and CIN is likely to be casual. If, however. transcriptionally active HPV DNA can be shown to be present in dyskaryocytes but not in normal internal and external control cells then a causal association is much more likely.

Recently, the polymerase chain reaction (PCR) has been developed which can amplify and detect a target DNA molecule present only once in a sample of 100,000 cells (Saiki, R.K. et al, 1988). This test is approximately 1.5×10^5 times more sensitive than the most sensitive assay described in this thesis. Furthermore, human DNA minisatellite alleles up to 10kb long can be amplified from single cells taken from a buccal smear (Jeffreys, A.J. et al, 1988). Young, L.S. et al (1989) have reported its use in detecting HPV DNA in cervical smears and suggest that PCR will be useful in epidemiological studies. If PCR could be used to clarify some of the questions posed above by targetting specific viral and oncogene DNA sequences in specific cell types within precancerous and cancerous lesions of the cervix, it might answer fundamental questions about the role of HPV DNA in cervical cancer.

APPENDICES

Appendix 1

Details of patients attending the colposcopy clinic with colposcopically abnormal, acetowhite, cervices (prefix CN) who had punch biopsies taken from colposcopically abnormal (suffix A) and colposcopically normal (suffix N) areas of the cervical transformation zone. The patients' ages are recorded in years. Analysis of the histological diagnosis (Hist.), cytological evidence of virus infection (Smear), staining for papillomavirus antigen (PV Ag.) and hybridization to HPV 16 and 18 DNA subtypes are presented. Hybridization results are for stringent conditions unless specified as nonstringent (NS). Hybridization to HPV 6 and 11 DNA was not detected under stringent or nonstringent conditions

(Hist: 1= CIN 1; 2=CIN 2; 3= CIN3; v= evidence of virus infection; n= histologically normal.)

Patient	Age	Hist.	Smear	PV Ag	HPV 16	DNA 18
CN01A	26	2+v	+	+	–	+(NS)
CN01N		v		–	–	–
CN02A	24	1	+	–	+	–
CN02N		v		–	+	–
CN03A	19	2+v	+	+	+	–
CN03N		1+v		+	+	–
CN04A	45	3	–	–	+	–
CN04N		n		–	+	–
CN05A	30	v	+	–	+	–
CN05N		n		–	+	–
CN06A	42	v	+	–	–	–
CN06N		v		–	–	–
CN07A	34	2+v	–	–	–	–
CN07N		n		–	–	–
CN08A	35	1+v	–	–	–	–
CN08N		n		–	–	–

Appendix 1 (cont.)

Patient	Age	Hist.	Smear	PV Ag	HPV 16	DNA 18
CN09A	23	v	–	–	–	–
CN09N		n		–	–	–
CN10A	21	v	–	–	+	–
CN10N		v		–	+	–
CN11A	30	3	–	+	+	+(NS)
CN11N		n		+	–	–
CN12A	22	1+v	–	+	–	+(NS)
CN12N		n		–	–	–
CN13A	31	v	–	–	–	–
CN13N		n		–	–	–
CN14A	35	3+v	+	–	–	+(NS)
CN14N		1+v		–	–	–
CN15A	23	2+v	–	–	–	–
CN15N		n		–	–	–
CN16A	26	2+v	+	–	+	–
CN16N		1+v		–	–	–
CN17A	30	3+v	–	–	+	–
CN17N		v		–	+	–
CN18A	30	1	–	–	+	–
CN18N		n		–	–	–
CN19A	37	1+v	–	–	+	–
CN19N		n		–	+	–
CN20A	29	2+v	–	+	+	–
CN20N		v		–	–	–
CN21A	32	2+v	–	–	+	–
CN21N		n		–	+	–
CN22A	38	2+v	–	–	–	–
CN22N		v		–	–	–
CN23A	36	1+v	–	+	+	–
CN23N		v		+	+	–

Appendix 1 (cont.)

Patient	Age	Hist.	Smear	PV Ag	HPV 16	DNA 18
CN24A	23	v	+	–	+	–
CN24N		v		–	+	–
CN25A	29	3	–	–	+	–
CN25N		n		–	+	–
CN26A	35	2+v	–	–	+	–
CN26N		n		–	+	–
CN27A	30	2+v	–	–	+	–
CN27N		n		–	+	–

Appendix 2

Details of patients attending the colposcopy clinic with colposcopically abnormal, acetowhite, cervices (prefix CN) who had punch biopsies taken from colposcopically abnormal (suffix A) and colposcopically normal (suffix N) areas of the cervical transformation zone. The patients' ages are recorded in years. Analysis of the histological diagnosis (Hist.), and hybridization to HPV 16 and 18 DNA subtypes are presented. Hybridization results are for stringent conditions only. Hybridization to HPV 6/11 DNA sequences was not detected in CN28 - CN47

Patient	Age	Hist.	HPV DNA 16	18
CN28A	34	1+v	-	-
CN29A	24	2+v	-	-
CN30A	46	2	+	-
CN30N		n	+	-
CN31A	20	3+v	+	-
CN31N		n	+	-
CN32A	38	3+v	+	-
CN32N		n	+	-
CN33A	25	3+v	+	+
CN34A	31	v	-	+
CN35A	52	3+v	-	+
CN36A	31	3+v	-	-
CN37A	25	v	-	+
CN38A	36	3+v	-	-
CN39A	34	3+v	+	-
CN39N		n	-	-
CN40A	29	3	+	-
CN40N		n	-	-
CN41A	31	3	+	-
CN41N		n	-	-

Appendix 2 (cont.)

Patient	Age	Hist.	HPV DNA 16	18
CN42A	25	3	–	–
CN43A	25	3+v	+	–
CN43N		n	–	–
CN44A	31	3+v	+	–
CN44N		n	+	–
CN45A	31	3	–	–
CN45N		n	+	–
CN46A	29	2+v	–	–
CN46N		n	–	–
CN47A	34	2	+	–
CN47N		n	–	–

Patient	Age	Hist.	HPV DNA 6/11	16	18
CN48A	25	3+v	+	–	+

Appendix 3

Details of patients undergoing pelvic irradiation or radical hysterectomy for invasive cervical carcinoma (prefix CaCx) who had biopsies taken from histologically diagnosed tumour (suffix A) and biopsies from histologically diagnosed normal vaginal epithelium (suffix N) taken within 5cm of the lesion. All tumours were squamous cell carcinomata except for CaCx18A* which was an adenocarcinoma. In CaCx10, a pelvic lymph node which contained metastatic squamous cancer deposits was available for analysis. Hybridization to HPV 6/11 DNA sequences were not detected in this group. Hybridization to HPV 16 and 18 DNA sequences were detected under stringent conditions.

Patient	Age	HPV16	Patient	Age	HPV16
CaCx01A	73	–	CaCx17A	59	+
			CaCx17N		+
CaCx02A	55	+			
			CaCx18A*	60	–
CaCx03A	32	+	CaCx18N		–
CaCx04A	60	+	CaCx19A	65	–
			CaCx19N		–
CaCx05A	65	–			
			CaCx20A	52	+
CaCx06A	62	–	CaCx20N		–
CaCx07A	44	+	CaCx21A	49	–
			CaCx21N		–
CaCx08A	74	–			
			CaCx22A	64	–
CaCx09A	43	–	CaCx22N		–
CaCx10A	35	–	CaCx23A	56	–
CaCx10(node)		–	CaCx23N		–
CaCx11A	58	+	CaCx24A	76	+
			CaCx24N		–
CaCx12A	62	–			
			CaCx25A	26	+
CaCx13A	54	+	CaCx25N		+
CaCx13N		–			
			CaCx26A	58	–
CaCx14A	27	+			
CaCx14N		+	CaCx27A	86	+
			CaCx27N		–

Appendix 3 (cont.)

Patient	Age	HPV 16
CaCx15A	28	+
CaCx15N		−
CaCx16A	60	+
CaCx16N		−
CaCx28A	64	+
CaCx28N		+
CaCx29A	56	+
CaCx29N		−

Patient	Age	HPV 18
CaCx30A	50	+
CaCx30N		+
CaCx31A	41	+
CaCx31N		−

Appendix 4

Details of patients who attended the colposcopy clinic with CIN lesions (prefix CN) who had punch biopsies taken from colposcopically and histologically abnormal and colposcopically and histologically normal areas of the cervical transformation zone. The age of the patient; the histological diagnosis (Hist.) of the abnormal biopsy; the copy number of HPV 16 and 18 DNA sequences detected in the abnormal biopsy; and the Langerhans' cell number (counted per 100 basal cells) in the abnormal and normal biopsies are recorded. HPV 16 and 18 DNA sequences were detected under stringent conditions. DNA copy numbers are banded in groups: <10; 10-50; 50-100 and >100 copies per cell. HPV 6/11 DNA sequences were not detected in this group.

Patient	Age	Hist.	HPV DNA 16	HPV DNA 18	Langerhans' cell no. Normal	Langerhans' cell no. Abn.
CN39	34	3+v	10-50	<10	7	15
CN40	26	3	10-50	<10	4	15
CN43	25	3+v	10-50	<10	8	4
CN44	31	3+v	10-50	<10	11	5
CN49	31	3	<10	<10	10	13
CN50	27	2	<10	<10	6	9
CN51	40	3	<10	<10	6	10
CN52	29	3	<10	10-50	11	5
CN53	28	2+v	<10	50-100	14	10
CN54	19	2+v	10-50	<10	6	6
CN55	26	3	<10	10-50	6	4
CN56	33	2+v	<10	>100	12	7
CN57	29	3+v	<10	50-100	12	6
CN58	23	2+v	<10	10-50	14	11
CN59	36	3	<10	10-50	7	5
CN60	34	3	<10	<10	11	21
CN61	24	2+v	<10	10-50	8	5
CN62	27	2+v	50-100	<10	7	5
CN63	33	3	<10	<10	11	14
CN64	32	2+v	50-100	<10	5	4
CN65	30	3	<10	<10	9	8
CN66	35	3+V	10-50	<10	9	12
CN67	37	3	50-100	<10	10	6

Appendix 5

Hybridization data on biopsies from colposcopically abnormal (suffix A) and colposcopically normal (suffix N) tissue prior to laser therapy for histologically confirmed CIN (prefix CN); plus biopsies taken from the same geographical area of the same cervices in which the CIN lesion originally lay 4 months post-laser therapy (suffix P).
Laser ablation of the transformation zone was performed to a depth of 7mm. All post-laser cervices were colposcopically and cytologically normal.
Hybridization to HPV 16 DNA sequences only was detected under stringent conditions.
Hybridization to HPV 6/11 and 18 DNA sequences were not detected in this group.

Patient	HPV16	Patient	HPV16
CN21A	+	CN39A	+
N	+	N	−
P	−	P	−
CN22A	−	CN40A	+
N	−	N	−
P	−	P	−
CN25A	+	CN43A	+
N	+	N	−
P	−	P	−
CN26A	+	CN44A	+
N	+	N	+
P	+	P	−
CN27A	+	CN45A	−
N	+	N	+
P	+	P	+
CN30A	+	CN46A	−
N	+	N	−
P	+	P	−
CN31A	+	CN47A	+
N	+	N	−
P	+	P	−
CN32A	+		
N	+		
P	−		

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