

THE LOCAL RESPONSE TO HUMAN PAPILLOMAVIRUS INFECTION

AND NEOPLASTIC DISEASE OF THE UTERINE CERVIX

Rhona Grace Hughes

Presented for the Degree of Doctor of Medicine

University of Edinburgh

1988



DEDICATION

This thesis is dedicated to Tommy. Without him it would not have been possible.

This thesis has been composed by me and the work contained within it is entirely my own.

ACKNOWLEDGEMENTS

I would like to thank the Melville Trust for making it possible for me to carry out this work, and Professor D.T. Baird, who encouraged me to do so. I am very grateful to Dr Mary Norval for her invaluable help and support and to Dr G.E. Smart for his unflinching enthusiasm. I thank Mr Bill Neill, Dr Sarah Howie and Mrs Muriel Murray for their assistance and Dr Stewart Fletcher and Mrs Eva McVittie for helpful discussions. Particular thanks are due to Dr Mohammed Alloub for his assistance in recruitment and examination of control patients. I am indebted to Drs K. Guy, I. Trowbridge and A. Ziegler for the gifts of monoclonal antibodies and to Sister Pankhurst and staff at the Lothian Area Colposcopy Clinic for their co-operation. I thank Drs A. Parker and C. Ludlam for allowing me to study patients under their care and Dr M. Colquhoun for her assistance with cervical cytology. I am grateful to Mr J. Paul and all the staff of the Edinburgh University Medical Illustration Department for help with photography and art work and wish to thank Mrs Marilyn Cole for typing this so well.

ABSTRACT

The association between squamous cervical carcinoma and sexual intercourse has been recognised for over a century leading investigators to postulate that the disease is caused by a sexually transmitted agent. Many possible candidates have been considered but since the mid 1970s interest has centred upon the human papillomavirus (HPV). The lesions caused by papillomaviruses tend to be persistent and are characterised by increased proliferative activity. HPV can be identified in the majority of cases of invasive and pre-invasive cervical cancers. These features, as well as the results of animal studies, make the viruses attractive candidates for playing a part in human carcinogenesis. The work described in this thesis was designed to examine some of the local effects of HPV infection on squamous cervical epithelium, which might contribute to neoplastic change.

Patients with cervical HPV infection and with pre-invasive or invasive cervical carcinoma were studied. Their reproductive, contraceptive, sexual, smoking and cervical smear histories were compared with those of normal controls, and colposcopically directed cervical punch biopsies and/or brushings obtained.

Cryostat sections were cut from punch biopsies and stained using monoclonal antibodies to Langerhans cell (LC) and T cell markers and to major histocompatibility (MHC) class II antigens (HLA-DP, -DQ and -DR). In HPV infected epithelium and in cervical intraepithelial neoplasia (CIN) grades II and III LC were significantly reduced in number and altered in morphology and distribution. HLA-DR expression by LC was significantly increased

in HPV infection and in CIN I and II, while HLA-DQ expression was significantly increased in CIN I-III.

Cervical biopsy and brush specimens and pelvic lymph node biopsies from patients with pre-invasive and invasive lesions were prepared for flow cytometry. They were assayed for DNA content, number of dividing cells and for c-myc oncogene protein and papillomavirus antigen expression. Samples from women with CIN and HPV lesions contained significantly more dividing cells than those from controls. The proportion of DNA aneuploid samples in the HPV group did not differ significantly from the group with CIN III. More specimens from normal cervical epithelium were c-myc antigen positive than specimens from CIN II or III or invasive cancer. Papillomavirus antigens were detected in pelvic lymph nodes free from metastatic tumour.

Patients with lymphomas were compared with normal controls. Significantly more lymphoma patients than controls had CIN II or III, with the increase being particularly marked in patients with Hodgkin's disease treated with chemotherapy. Only half the cases of CIN in lymphoma patients were detected by cervical cytology.

These results show that there is a localised disturbance in immune function in HPV infected cervical epithelium and that HPV infection stimulates cellular proliferation and is associated with the development of aneuploidy. The relationship of these findings to the proposed link between HPV infection and cervical neoplasia is discussed, and other possible contributory factors, such as immunosuppression, smoking, oral contraceptive pill use and infection with other agents are considered.

CONTENTS

	Page number
Declaration	i
Acknowledgements	ii
Abstract	iii
<u>CHAPTER 1</u>	
INTRODUCTION	
Cervical carcinoma : historical aspects	1
The cervical transformation zone.	4
Nomenclature of precancerous cervical lesions	8
The epidemiology of squamous carcinoma of the uterine cervix	9
The aetiology of squamous carcinoma of the uterine cervix	11
Possible aetiological agents	11
1. Smegma	11
2. Spermatozoa	12
3. Infectious agents	12
a) Herpes simplex virus	12
b) Human papillomavirus	14
c) Other viruses	25
d) Other infectious agents	25
4. Smoking	26
5. The combined oral contraceptive pill	27
Intraepithelial immune interactions	29
a) General	29
b) Major histocompatibility class II antigens	30
c) The Langerhans cell	34
d) The T lymphocyte	40
The immune response to tumours	42
The effects of smoking and oral contraceptive use on the immune system	44
The immune response to the human papillomavirus	45
a) General	45
b) The immune response to human papillomavirus in patients with genital warts and cervical intraepithelial neoplasia	48
c) The local immune response to the human papillomavirus	49

DNA analysis of invasive and preinvasive lesions	52
Introduction	52
Chromosomal analysis	52
Static cytometry	53
Flow cytometry	53
a) DNA aneuploidy	56
b) Cell cycle analysis	58
Oncogenes and human tumours	59
a) Phosphorylation	61
b) Initiation of DNA synthesis	61
c) Regulation of transcription	61
d) The myc oncogene	62
<u>PURPOSES OF THE PRESENT STUDY</u>	65
<u>CHAPTER 2</u>	68
PATIENTS AND METHODS	68
Patient groups	68
Colposcopy	72
Cervical cytology	73
a) Spatula samples	73
b) Cervical brush specimens	74
Cervical biopsy	76
Histopathology	76
Major histocompatibility class II antigen, Langerhans cell and T cell staining	76
Preparation of nuclei for flow cytometry	79
Staining of nuclei for flow cytometry	80
1. Nuclear antigens	80
2. Nuclear DNA	80
Flow cytometer	83
<u>CHAPTER 3</u>	88
EPIDEMIOLOGICAL DATA	88
Introduction	88
Results	88
Discussion	93
<u>CHAPTER 4</u>	97
LOCAL IMMUNE INTERACTIONS AND MAJOR HISTOCOMAPTIBILITY CLASS II EXPRESSION IN THE CERVICAL TRANSFORMATION ZONE	97

	Page number
Introduction	97
Results	98
Discussion	110
<u>CHAPTER 5</u>	118
FLOW CYTOMETRIC ANALYSIS OF DNA CONTENT, C-MYC PROTEIN EXPRESSION AND PAPILLOMAVIRUS ANTIGEN EXPRESSION IN CERVICAL EPITHELIUM	118
Introduction	118
Results	118
Discussion	131
<u>CHAPTER 6</u>	141
CERVICAL INTRAEPITHELIAL NEOPLASIA IN LYMPHOMA PATIENTS	141
Introduction	141
Results	142
Discussion	151
<u>CHAPTER 7</u>	155
CONCLUSIONS	155
<u>BIBLIOGRAPHY</u>	164
<u>APPENDIX 1</u>	
Published papers	
<u>APPENDIX 2</u>	
List of abbreviations	
<u>APPENDIX 3</u>	
List of figures	
<u>APPENDIX 4</u>	
List of tables	

CHAPTER I

CHAPTER 1INTRODUCTIONCervical carcinoma: historical aspects

Population data on the frequency of cancer of various organs cannot be considered reliable prior to 1950. Nevertheless, there appears to be little doubt that carcinoma of the uterine cervix has been one of the leading causes of cancer mortality among adult women for many centuries (Koss, 1981). It was not until 1930 that Robert Meyer clearly separated carcinoma of the uterine cervix from carcinoma of the corpus. He also distinguished 'solid' carcinomas, comprising epidermoid or squamous cancers and their keratinising variants, from adenocarcinomas of endocervical origin, thus formulating a classification which is still used today. This thesis is concerned with invasive and pre-invasive squamous carcinoma, and not with adenocarcinoma. Where the term 'cervical carcinoma' is used below it refers to squamous and not adenocarcinoma.

An understanding of the sequence of morphological events in the genesis of epidermoid carcinoma was greatly enhanced by Schauenstein who, in 1908, published a paper on atypical surface epithelium of the cervix. He clearly identified the surface changes as histologically identical to those observed in invasive cervical cancer and suggested that the abnormal surface epithelium was the source of invasive cancer. The term 'carcinoma in situ' (CIS) with reference to surface changes was introduced by Schottlaender and Kermauner in 1912 and was subsequently

popularised by Broders in 1932.

Rubin (1910) and Schiller (1927, 1928) confirmed Schauenstein's observations and, like him, considered the surface abnormalities to be manifestations of cervical cancer. This belief was shaken by a study of 18 patients with cervical CIS by Scipiades and Stevens (1938). Three of these patients received no treatment and although two of them developed invasive cancer, one remained free of disease. This led the great cancer pathologist, James Ewing (1940) to the conclusion: "It thus appears that while some of the lesions must progress and become true cancer, others may be reversible and undergo spontaneous regression, so that a positive diagnosis of cancer may not be given".

Ewing's statement opened the door to a controversy which has still not been resolved. The problem became more acute with the introduction of cytological mass screening (Papanicolaou and Marchetti, 1943) which brought to light an unexpectedly large number of intraepithelial abnormalities of uncertain prognosis in asymptomatic healthy women.

It is generally accepted that invasive cervical carcinoma develops from a pre-invasive state in which the malignant cells are confined to the epithelium. Epidemiological studies (discussed below) have shown that the risk factors for the invasive and the pre-invasive conditions are identical and prevalence studies have shown that the age distribution of the two lesions is consistent with the progression of pre-invasion to invasion (Coleman et al., 1986). The strongest evidence to link the pre-invasive and invasive forms of the disease is derived from studies similar to that of Scipiades and Stevenson (1938) in which patients with

intraepithelial lesions are left untreated. There are many studies purporting to show the frequency with which lesions (dysplasia, cervical intraepithelial neoplasia or CIS) regress or progress, some of which are listed by Patten (1978). A detailed analysis is not particularly revealing because of wide variations in the use of the terms "dysplasia" and "carcinoma in-situ" and the fact that most observations have been of lesions followed by punch biopsy, which certainly interferes with the course of the disease (Richart, 1966).

Several studies have been published (Peterson, 1956; Kinlen and Spriggs, 1978; McIndoe et al, 1984) reporting progression rates (from CIS to invasion) of 19-30%. There are difficulties in interpreting this data. In some patients (Kinlen and Spriggs, 1978) the diagnosis of pre-invasive disease was made by cytology without biopsy. It is well recognised that cytology is an inaccurate method of distinguishing CIS from microinvasion (Hoffken and Soost, 1981) so that some of the so called "progressors" may well have had undiagnosed invasive disease initially. On the other hand, biopsy or the subsequent healing process may reduce the progression rate by entirely removing small lesions.

Almost the only studies in which biopsy was avoided are those confined to "dysplasia" which was not deliberately allowed to progress beyond the stage of CIS. These studies show that a significant proportion of lesions classified cytologically as "dysplasia" are capable of spontaneous regression. Estimates of this occurrence vary widely; Stern and Neely (1964) estimated that dysplasias regress at a rate of 35% per annum, while Richart and Barron (1969) found that regression was uncommon and only occurred in the mildest cases. Campion et al (1986) reported a regression

rate to normal of only 7% in cervical intraepithelial neoplasia grade I (CIN I) while 25% of cases of CIN I progressed to CIN III within 20 months. These differences may reflect differences in terminology. Peterson (1956) found that no prognostic guidance could be obtained from the histological appearance of lesions. More recently Spriggs and Boddington (1980) have suggested that it may be possible to predict invasion on the basis of the presence of parabasal dyskaryosis with poor differentiation in cytological specimens but this view is not generally accepted.

The cervical transformation zone

It is known that in at least 90% of patients the initial events in the development of cervical carcinoma take place within the cervical transformation zone (TZ) (Kolstad and Stafl, 1982). The TZ owes its existence to the increase in cervical bulk which occurs during puberty and more markedly during pregnancy, due to the influence of endogenous oestrogens. This increase in bulk leads to an "unfolding" of the cervix with passive eversion of the distal endocervical columnar epithelium on to what is anatomically the ectocervix [see figures 1 and 2]. The presence of columnar epithelium on the ectocervix is known as ectopy. The pH of vaginal secretions is lower than that of endocervical mucus and, in response to this, columnar epithelium is replaced by stratified squamous epithelium by a process known as squamous metaplasia. Some degree of squamous metaplasia is virtually universal during the reproductive years. The TZ is the area between the original squamo-columnar junction, now on the ectocervix, and the new squamo-columnar junction, somewhere in the vicinity of the external cervical os [see figure 2].

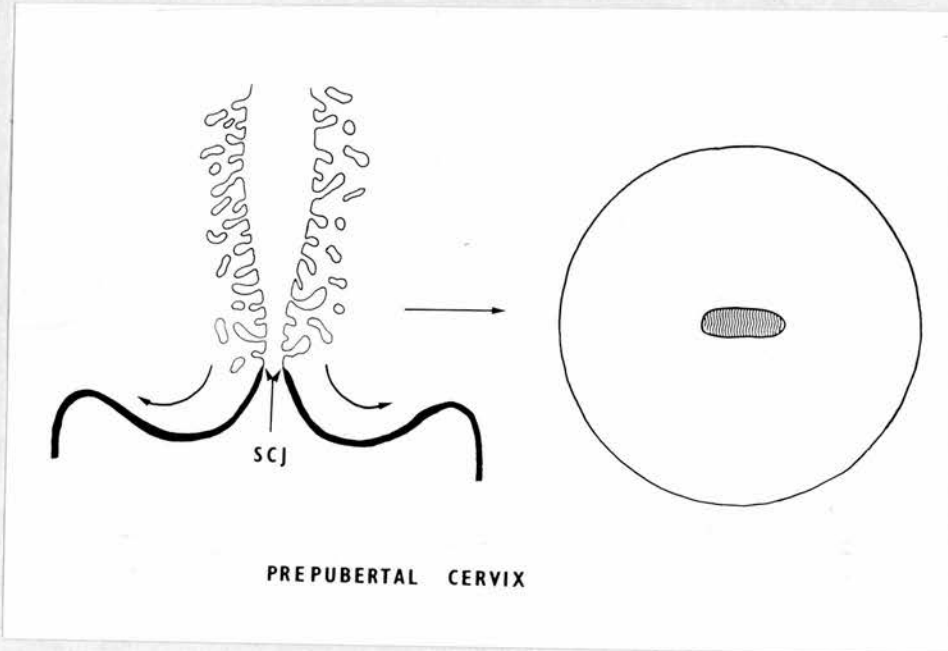


Figure 1 SCJ = squamocolumnar junction

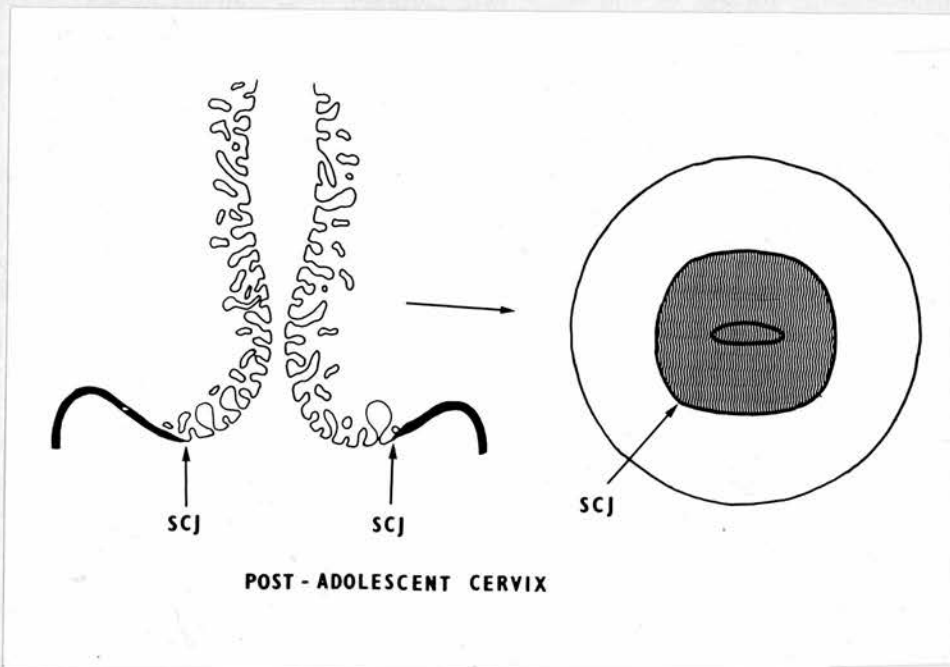


Figure 2 SCJ = squamocolumnar junction

In the early stages of the metaplastic process immature squamous cells can be seen below, and pushing up, columnar cells in a process known as reserve cell hyperplasia. These squamous cells are believed to arise from undifferentiated "reserve cells" between the columnar epithelium and the basement membrane (Christopherson et al, 1979) or may possibly be derived from underlying stromal cells (Fox and Buckley, 1982). The overlying columnar cells then degenerate and reserve cells differentiate into immature and then mature squamous epithelium. Mature squamous epithelium can be distinguished from immature by the presence of surface differentiation.

It is thought that the process of squamous metaplasia takes approximately two to three weeks to be completed, and that during this time the changing epithelium is especially vulnerable to oncogenic agents (Coppleson and Reid, 1968).

The TZ is easily accessible to visual inspection. In the 1940s it was discovered that preclinical lesions could be detected by taking a smear or scrape from the cervical TZ (Papanicolau and Marchetti, 1943; Ayre, 1947) and examining the exfoliated cells obtained under a microscope. Cervical cytology is still employed as a screening technique, generally using an adapted form of the spatula designed by Ayre (1947) [Figure 3].

Hinselmann (1933) is credited with the discovery that preclinical lesions could also be visualised by the use of a colposcope which provides a magnified view of the cervix and lower genital tract. Abnormal vasculature may be identified and areas of intraepithelial neoplasia delineated with the aid of solutions such as acetic acid and Lugol's iodine.

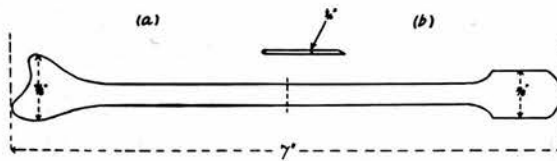


Fig. 1.—Wooden spatula for selective cytology technique. Either end may be used, depending upon the type of cervix. End (a) is best suited for nulliparous cervixes, or cervixes where the squamocolumnar junction is just outside the circle of the cervical opening. Placing the hook of the spatula in cervical os, a gentle rotation permits a light scraping of the entire squamocolumnar junction. The hook end is not suitable for cervixes with long erosions, as the scraping might miss the squamous tissue, obtaining a "surface biopsy" of glandular tissue only. In eroded, lacerated, parous cervixes, end (b) is more suitable.

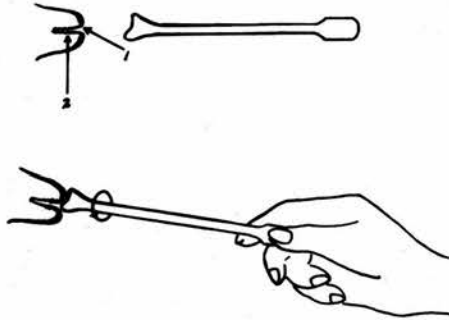


Fig. 2.—Technique for nulliparous cervix. (1) Squamocolumnar junction; (2) glandular epithelium. Method of taking selective cytology test in nulliparous cervix. Hook end (a) of spatula fits into cervix permitting gentle contact with squamocolumnar junction. By rotating spatula the entire circumference of this cancer zone may be "surface biopsied." The cells shed from this key point show the earliest indication of incipient carcinoma before any lesion may be recognizable to the naked eye.

Figure 3. The Ayre's spatula

(from Ayre, 1947)

Nomenclature of precancerous cervical lesions

It is clear from the studies cited above that reliable prognostication of intraepithelial lesions is not possible. In the 1950s, however, an attempt was made to reconcile the behaviour of these lesions with the cytological and histological patterns of the disease, leading Reagan et al (1953) to propose the separation of intraepithelial lesions into two groups: CIS and atypical hyperplasia or dysplasia. The differences were based on the cellular makeup of the epithelium: lesions composed of small, poorly differentiated cells were classed as CIS, while lesions made up of better differentiated cells, especially those with a tendency to form keratin, were classed as dysplasia. It was advocated that the two groups of lesions have a different prognosis: CIS is the malignant precursor of invasive cancer, whereas dysplasia is a benign lesion of uncertain prognosis, more likely to disappear than to progress (Christopherson, 1969).

This concept was soon found to be deficient. It was clearly shown that invasive carcinoma may originate from well-differentiated variants of epithelial abnormalities, including those with keratinised surfaces (Bangle et al, 1963). The follow-up studies, some of which are cited above, have shown that the histological patterns of precancerous abnormalities are of no prognostic significance. Studies of DNA content (Wilbanks et al, 1967), chromosomal abnormalities (Granberg, 1971) and behaviour in tissue culture (Richart et al, 1967) failed to disclose any basic differences that could be related to tissue pattern or prognosis of these lesions. For these reasons, Richart (1967) suggested that the term cervical intraepithelial neoplasia (CIN) be used for the

entire morphological spectrum of squamous precancerous lesions of the uterine cervix. CIN is divided into three grades - CIN I corresponding to mild dysplasia, CIN II to moderate dysplasia and CIN III encompassing severe dysplasia and carcinoma in situ (Buckley et al, 1982). In all cases of CIN the full thickness of the epithelium is occupied by neoplastic cells with nuclear abnormalities, but in CIN I and II there is cytoplasmic differentiation in the upper two thirds and one-third of the epithelium respectively.

The epidemiology of squamous carcinoma of the uterine cervix

The epidemiology of uterine cancer was first investigated in 1842 by Rigoni-Stern, an Italian physician at the University of Padua. He found that uterine cancer was relatively common in married women, unusual in unmarried women and virtually absent in nuns. The age distribution in Rigoni-Stern's statistics, showing that "uterine cancer" occurred first in women between 30 and 40 years of age and that it reached a peak in women between 40 and 60 years of age, strongly suggests that he was dealing primarily with squamous cervical carcinoma. Studies performed in Canada (Gagnon, 1950) and the USA (Towne, 1955) confirm Rigoni-Stern's findings. Since then, failure of squamous cell carcinoma of the cervix to be reported in confirmed virgins (with one exception reported by Mogaji, 1973) has led to an emphasis being placed upon marriage and sexual behaviour as factors contributing to the increased risk of developing this disease. It has emerged from a large number of epidemiological studies that the younger a woman is when she first experiences sexual intercourse, and the more sexual partners she has, the greater are her chances of developing squamous carcinoma

of the uterine cervix (see Coppleson 1969; Rotkin 1973; Spriggs 1984 for reviews of these studies). More recent work has shown that the same risk factors apply for the development of CIN as for invasive carcinoma (Vessey et al, 1983; Harris et al, 1986; Zaninetti et al, 1986; Greenberg et al, 1985; La Vecchia, 1985).

It has been suggested that age at coitarche is of crucial importance because the adolescent cervix is particularly vulnerable to oncogenic agents due to the metaplastic process taking place at that time (Coppleson 1969; Sebastian and Leeb 1978). It is not, however, necessary to implicate such a mechanism as a multistage model of carcinogenesis predicts that the incidence of epithelial carcinomas is a function of a power (usually ranging from 4 to 6) of the duration of exposure (Day and Brown, 1980) so that a few years difference in age at first exposure to an oncogenic agent can bring about a substantial increase in risk later in life.

More recent studies (Harris et al, 1986; Hellberg et al, 1986; Zaninetti et al, 1986) have found that the number of sexual partners is of more significance in relation to risk than age at coitarche. The sexual history of the husbands and other consorts of patients is also important. Pridan and Lilienfeld (1971) found that Israeli men with multiple sexual partners conferred an increased risk of cervical cancer upon their partners. Similarly, a British group studied the husbands of patients with cervical neoplasia who claimed only one partner and found that these men had had significantly more sexual partners than the husbands of healthy controls (Buckley et al, 1981). This finding was confirmed recently by American workers (Zunzunegui et al, 1986). Kessler (1977) described 29 "marital clusters" of cervical neoplasia in which two

women married (at different times) to the same man developed the disease. Colombia has the highest incidence of cervical carcinoma in the world (Waterhouse et al, 1976). Surprisingly, however, Colombian female students have fewer sexual partners (Alzate, 1978) than their North American and European counterparts (Luckey and Nass, 1969). Male students in Colombia, on the other hand, are much more likely to have had sexual intercourse with a prostitute (Alzate, 1977) than are their counterparts in North America and Western Europe.

The above data strongly suggest that some men may act as a "reservoir" for a carcinogenic agent. These men have been described as "high risk males" (Singer et al, 1976).

The aetiology of squamous carcinoma of the uterine cervix

Introduction

The epidemiological evidence cited above has led workers over the last three decades to investigate the oncogenic potential of any agent transmitted by sexual intercourse. These investigations are discussed below.

Futher epidemiological studies have implicated cigarette smoking and oral contraceptive use as possible risk factors in the development of cervical cancer. These studies are also discussed below.

Possible aetiological agents

1. Smegma

Smegma was believed to be relevant because of the apparent association between non-circumcision of the male partner and cervical cancer. This association has now been found to be spurious (Terris et al, 1973).

2. Spermatozoa

It has been shown that spermatozoa may enter cells in vitro and alter the genome and biochemical activity of cells in culture (Bendich et al, 1976) and recent work has demonstrated that sperm protamines may be able to transform cervical cells in culture (French et al, 1987). It is known that the amount of protamine in sperm varies between individual males (Reid et al, 1978) and it is possible that men with a high sperm protamine content are the so-called "high risk males" (Singer et al, 1976).

3. Infectious agents

a) Herpes simplex virus

Herpes simplex virus type 2 (HSV 2) came under suspicion when cervical cytologists noticed evidence of cervical HSV infection coexisting with dyskaryosis (the cytological equivalent of dysplasia) more frequently than anticipated (Naib et al, 1966). One study demonstrated a six-fold increase in atypical cervical smears amongst patients with genital HSV infection (Nahmias et al, 1971) and it was then found that patients with cervical neoplasia were more likely to have antibodies to HSV 2 than were age matched controls (Rawls et al, 1973; Aurelian et al, 1973). These studies can be criticised on a number of grounds, some of which are discussed by Rotkin (1976). First, the sexual history of patients and controls was not obtained. The more sexual partners a woman has, the more likely she is to be exposed to HSV 2 infection. The studies cited above (Rawls et al, 1973; Aurelian et al, 1973) showed that a higher percentage of women with cervical cancer had anti-HSV 2 antibodies than did the controls but this may simply mean that the women with cancer had had a greater number of sexual

partners than had the controls. It is of interest that a recent Czechoslovakian study of over 10,000 women found no significant difference in levels of anti HSV 2 antibodies between patients with CIN or cervical carcinoma and healthy controls when controls and patients were matched for age at coitarche (Vonka et al, 1984). Secondly, there was great variability in the percentage of cervical carcinoma patients with anti-HSV 2 antibodies, even in tests carried out by the same researchers on patients in different socioeconomic groups (zur Hausen 1983). Finally, the fact that cervical cancer patients are more likely to have anti-HSV 2 antibodies than are controls means that they are more likely to have acquired HSV 2 infection but does not mean that there is a direct causal link between the infection and cervical cancer.

Exhaustive attempts have been made to obtain more direct proof that HSV 2 plays a carcinogenic role but these have been largely unsuccessful. Infectious HSV 2 has been isolated from cervical tumour cells in vitro on only one occasion (Aurelian, 1971). In 1972 Frenkel et al reported the isolation of 3.5 copies per cell of a fragment of HSV-2 DNA equivalent to 40% of the total viral genome in one individual cervical carcinoma. Other workers (zur Hausen et al, 1974; Pagano et al, 1975; Cassai et al, 1981) have been unable to repeat this. HSV 2 RNA has occasionally been isolated from cervical carcinomas but no particular sequence has been isolated on a consistent basis (Maitland et al, 1981). Recent reports of cross hybridisation between HSV DNA and human cellular nucleic acids (Maitland et al 1981 and Peden et al 1983) make the significance of the results reported above somewhat uncertain.

It is known that HSV is an efficient inducer of chromosomal

aberrations, that it can cause selective gene amplification and can transform rodent cells in culture (zur Hausen, 1983). Possibly the virus initiates malignant change by a "hit and run" mechanism, as proposed by Galloway and McDougall (1983). This would explain the absence of viral DNA within malignant cells. Zur Hausen (1982) has postulated that HSV 2 and HPV may act as co-carcinogens while Winkelstein et al (1984) have suggested that cigarette smoking and HSV 2 infection may be jointly responsible for inducing malignant change, together with other possibly unknown agents.

b) The human papillomavirus (HPV)

i) General

The papillomaviruses belong to the Papovaviridae family and contain double stranded, circularly closed DNA within an icosahedral capsid. HPVs cause proliferative lesions of squamous epithelium, including that of the genital tract. So far 45 HPV types have been described, on the basis of DNA homology. Types are considered independent if there is less than 50% cross-hybridisation, and the types generally associated with genital tract lesions are HPV 6,11, 16,18 and 30. The other types listed in table 1 are involved in only a small minority of genital lesions.

Standard virological approaches to the study of HPV have been limited by the lack of a tissue culture system for viral propagation in vitro. Thus, in order to obtain viral DNA for use as a probe, DNA must be purified from biopsy material and cloning techniques then used to allow DNA amplification in bacteria. HPV replication is highly dependent on the differentiation of squamous epithelial cells and the productive functions of the virus

including DNA synthesis and the expression of late viral genes occur only in fully differentiated squamous epithelial cells. In skin, viral DNA synthesis has been detected by in situ hybridisation only in the cells of the stratum spinosum and of the granular layer of the epidermis, and not in the basal layer or in the underlying dermal fibroblasts. Viral capsid protein production and virus assembly occur only in the upper stratum spinosum and in the granular layer where keratinocytes are terminally differentiated. It is generally believed that the viral genome is present in a small proportion of basal keratinocytes and that the expression of specific viral genes in the basal layer and in the deeper layers of the epidermis is responsible for the proliferation of epithelial cells that is characteristic of a wart or a condyloma (Howley et al, 1986). The control of papillomavirus late gene expression, therefore, appears tightly linked to the state of differentiation of the squamous epithelial cells. The molecular basis for this control is not yet known. A similar pattern is seen within genital warts with HPV capsid antigens being detected only in the more superficial layers (Jensen et al, 1985) while viral DNA is thought to be present throughout the entire epithelial thickness.

ii) Benign cervical lesions

HPV induced cervical lesions may be exophytic in nature, and similar in appearance to common skin warts, or flat, and invisible to the naked eye (Figure 4). The exophytic lesions are known as condylomata (from the Greek "kondulos" meaning a knuckle) acuminatum (from the Latin "acuminare" meaning "to make pointed" and referring to the tapering profile of the rough surface

Table 1
HPV types associated with genital lesions

	HPV frequently associated	HPV rarely associated	HPV - single isolates
condylomatous genital lesions	6	16	1
	11	18	2
	31		7
			10
			30
bowenoid papulosis	16		
	42		3
			6
			18
			33
CIN I - III	6	45	10
	11		30
	16		
	18		
	31		
	33		
	39		
	42		
	43		
44			
invasive genital cancer	16	11	10
	18	31	
	33	45	
	35		
	39		

(taken from De Villiers, 1987)

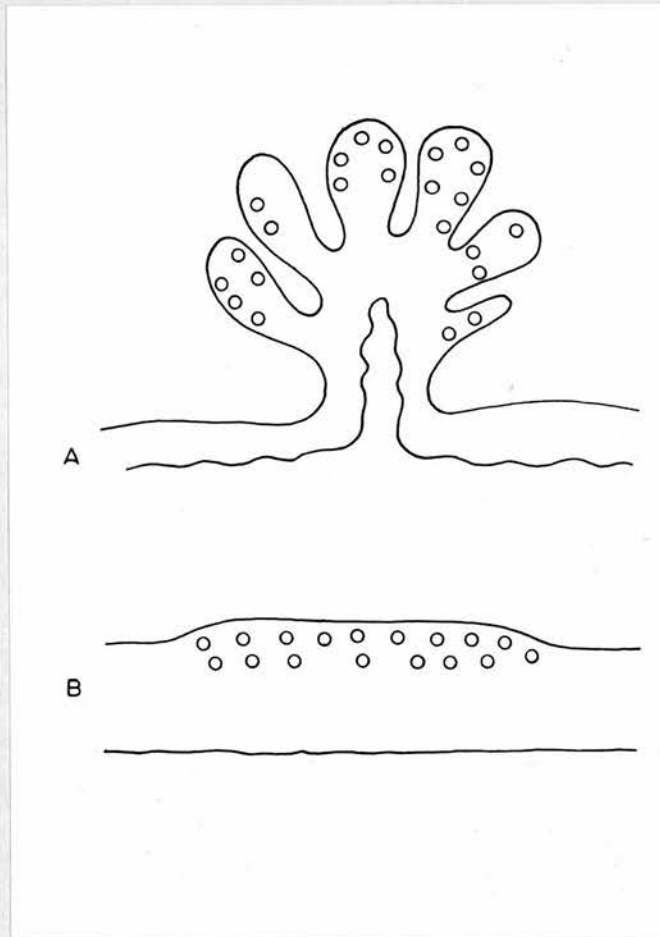


Figure 4. HPV associated lesions of the uterine cervix
A. Condyloma acuminatum
B. Flat koilocytic lesion
(reproduced with permission from an original drawing by Dr S. Fletcher).

processes). No agreement has been reached upon the correct nomenclature for the flat lesion although a recent suggestion is that it should be described as "flat koilocytosis" (Fletcher, 1983). Another form of condyloma of "endophytic nature with pseudoinvasive aspects" has also been described (Meisels et al, 1977). It is generally accepted that this appearance is due to koilocytic changes within a branching, epidermidised gland (Fletcher, 1983).

Cervical HPV infection can be identified cytologically by the presence of dyskeratotic cells, bi- or multi nucleate cells, and koilocytes among the exfoliated cells. Koilocytes (which were previously described as "halo" or "balloon" cells) possess a prominent perinuclear halo which fails to stain with eosin or periodic acid - Schiff. They were first described in vaginal smears by Papanicolau (1933) but it was not until 1960 that they were first firmly associated with condylomata acuminatum (Papanicolau, 1960). About 2% of cervical smears contain koilocytes (Meisels and Fortin, 1976). In 1968 Dunn and Ogilvie demonstrated viral particles compatible with HPV within koilocytes by electron microscopy, confirming the association, although some workers still believe that the halo may be due to non-specific cytopathic effects (Koss, 1979). Like HSV, HPV was first suspected as a possible aetiological agent in cervical neoplasia when cytologists noticed evidence of cervical HPV infection coexisting with dyskaryosis more often than would be anticipated by chance. Meisels and Fortin (1976) and Purola and Savia (1977) noted that koilocytes were present in cervical smears taken from women with CIN more frequently than in smears taken from women with

normal cervixes and this, together with reports that condylomata acuminatum could undergo malignant change (Dawson et al., 1965; Gardner and Kaufman, 1965; Kovi et al., 1974) led Meisels et al. (1977) to propose that both "flat condylomata" and condylomata acuminatum were precursors of CIN.

iii) Genital neoplasia

In CIN HPV virions have been identified by electron microscopy (Ferenczy et al., 1981) and viral antigens can be demonstrated within the more superficial epithelial cells (Kurman et al., 1981). The detection rate of viral structural antigens decreases as the lesion becomes more severe (McCance et al., 1983; Walker et al., 1983). It is thought that this is due to a loss of differentiation in the more severe lesions because, as discussed above, HPV replication is highly dependent upon the differentiation of epithelial cells.

DNA hybridisation studies have demonstrated that HPV types 6 and 11 are most commonly seen in cervical and other genital warts (Gissmann and zur Hausen, 1980), and are also associated with CIN, usually of grades I and II (Gissmann et al., 1984; McCance et al., 1985a). HPV 16 and 18 are consistently associated with the majority of invasive squamous carcinomas of the cervix with HPV 16 found in 60-90% (McCance et al., 1985a; Durst et al., 1983) and HPV 18 in 15-36% of invasive carcinomas (Boshart et al., 1984). HPV 31, found most often in North America, is associated with approximately 20% cases of CIN but is rarely associated with invasive disease (Lorincz et al., 1985). HPV 16 is also found in CIN and is isolated at an increasing rate as the disease increases in severity, with 70-75% of CIN III lesions containing HPV 16 (McCance et al., 1985a).

HPV 16 DNA has been detected in one cervical adenocarcinoma (Smotkin et al, 1986) but it is possible that the DNA originated in an adjacent area of CIN, as in a significant proportion of patients cervical adenocarcinoma is reported to coexist with CIN (Luesley et al, 1987).

HPV 16 DNA is also found in vaginal and vulvar intra-epithelial neoplasia (McCance et al, 1985b), in invasive vulvar carcinoma (McCance, 1986) and in bowenoid papulosis, a generally benign in situ lesion found on the vulva and penis (Ikenberg et al, 1983; Gross et al, 1985).

Penile carcinoma is rare in Europe and North America and tends to occur in old age (over 60 years) but is much more common in certain African countries and in central and South America (McCance, 1986). In one study of penile carcinomas in patients from Brazil, HPV 16 was found in 50% of lesions (McCance et al, 1986a). The patients were much younger (33-55 years) than in countries where there is a low incidence of penile carcinoma. Other factors must contribute to this high incidence, since penile cancer is rare in other countries where female genital cancers are common.

Other areas such as the anal canal have been shown to be infected with HPVs. HPV16 has been found in anal intraepithelial neoplasias (McCance et al, 1985b) and in invasive diseases in this area (Beckman et al, 1985). Although HPV 16 is associated with premalignant lesions throughout the female genital tract, malignancy occurs most often on the cervix. This may be due to the particular susceptibility of the cervical TZ, as discussed above.

The papillomavirus genome is in a free and unintegrated state in benign condylomata and in premalignant lesions of the cervix, vulva and penis (McCance, 1986). In malignant lesions of the cervix, vulva and penis, however, the HPV genome appears to be integrated in the majority of cases. Junction fragments containing HPV 16 and human DNA sequences have been cloned to prove that the viral DNA is integrated into chromosomes (Durst et al, 1985). Integration occurs at several random sites in the host chromosome either as single copies or as random repeats (Durst et al, 1985; Lehn et al, 1985). The pattern of random integration in tumours suggests that the site of integration is not important. There does, however, appear to be some specificity as to the break site in the HPV 16 genome, with interruptions occurring in 4 of 5 tumours in one series in the E1 or E2 open reading frames (ORF) (Lehn et al, 1985).

Some tumours contain free episomal DNA in addition to integrated DNA, and at least one cervical tumour has been found to contain only free DNA with no detectable integrated sequences (McCance et al, 1985a).

Several cell lines grown from cervical cancers harbour either integrated HPV 16 or HPV 18 DNA sequences (Schwarz et al, 1985; Yee et al, 1985) suggesting that integration is more than a coincidental event. Most of the cell lines contain multiple copies of either HPV16 or HPV 18 and in three lines, HeLa, C4-1 and SW 756, the breakpoint in the viral genome for integration occurs in the region of the E1/E2 ORFs (Schwarz et al, 1985). These three cell lines also contain transcripts from the E6/E7 ORFs and a product of the HPV 18 E6 region has been identified in HeLa and

SiHa cells (Banks et al, 1987). It may be relevant that the E6 ORF of bovine papillomavirus (BPV) type 1 is important in the transformation of mouse cells (Yang et al, 1985).

These data suggest that HPV may have a more important role in the aetiology of cervical carcinoma than HSV. There have been recent reports, however, of the isolation of HPV6 from between 11% (Wickenden et al, 1985) and 50% (Schneider et al, 1987) and of HPV 16 from between 9% (Toon et al, 1986) and 38% (Cox et al, 1986) of apparently normal cervixes suggesting that HPV may be a very common commensal. Cox et al (1986) found no evidence of HPV DNA integration in normal cervixes and this may be significant.

iv) Extra-genital neoplasia

In man, malignant transformation of HPV induced lesions can be seen in the rare inherited condition, epidermodysplasia verruciformis (EV). Most patients suffering from this condition have a defect in cell mediated immunity (Praver et al, 1977). They suffer from multiple skin warts caused by EV specific HPVs (types 5,8,9,12,14, 15,17 and 19 to 25) as well as HPVs 3 and 20 which induce plane warts in the general population (Jablonska and Orth, 1985). Approximately one-third of EV patients develop multiple skin cancers which are usually invasive squamous cell carcinomas and which usually occur at light exposed sites. Hybridisation experiments have demonstrated the extrachromosomal persistence of HPV5 and HPV5 related (HPV 8 and 14) DNA in carcinomas (Ostrow et al, 1982). This contrasts with the greater variety of HPV types found in the benign lesions of the same patients, suggesting that HPV 5 related types have a higher oncogenic potential than other EV specific HPVs, or than the HPV

types which are common to EV and to the general population.

Renal transplant recipients are immunosuppressed with agents such as prednisolone and azathioprine and experience skin warts more frequently than the general population (Van der Leest et al, 1987). Like EV patients, they harbour HPVs of a much wider range of types than is generally found (Rudlinger et al, 1986; Van der Leest et al, 1987). They also have a higher than expected incidence of skin tumours, especially squamous cell carcinoma, and it is thought that some of these tumours are due to the malignant transformation of HPV induced lesions (Rudlinger et al, 1986). HPV DNA, including HPV 5 DNA which was previously thought to be found only in EV, has been isolated from malignant skin tumours in these patients (Lutzner et al, 1983).

The cancer incidence in renal transplant recipients is proportional to the duration of immunosuppression (Hardie et al, 1980) and to the duration of sunlight exposure (Boyle et al, 1984), suggesting that there are similarities between this group of patients and those suffering from EV.

Squamous cell carcinomas of the skin from normal individuals have been negative for HPV DNA when tested with known types (McCance, 1986)

HPV causes laryngeal papillomatosis in infants and young children (Mounts and Kashima, 1984). Children probably acquire the infection from the maternal genital tract during vaginal delivery while accumulating evidence suggests that some adult laryngeal warts are transmitted by oro-genital sexual practices (Anon, 1984). The lesions are benign but malignant conversion occurs at a high frequency in patients treated with radiation

(Gissman et al, 1983). Smoking is also believed to be a possible co-factor in the malignant conversion of the lesions (Brescia et al, 1986). Carcinomas of the oral cavity including the tongue (Ostrow et al, 1987; Lookingbill et al, 1987) and buccal mucosa are associated with HPV2, 11 and 16 while hyperplasia of the buccal mucosa (the Heck lesion) is associated with HPV 6 and 13 (Pfister et al, 1983; De Villiers et al, 1986). HPV DNA has been isolated from oesophageal carcinomas (Hille et al, 1986) and from carcinomas of the lung and caecum (Ostrow et al, 1987). A recent publication from Maitland et al (1987), however, reports the isolation of HPV 16 DNA from 5/12 (42%) specimens of normal oral mucosa including one specimen in which the viral DNA was integrated. A similar proportion (46%) of oral tumours analysed contained HPV 16 DNA. This data suggests that HPV 16 may be a normal commensal in the mouth and that integration may not be as significant an event as had hitherto been believed.

Thus, HPVs have been associated with benign and malignant disease at various epithelial sites. For cancers apart from genital and laryngeal lesions, however, the number of HPV positive tumours is small.

v) Animal models

It is known that other papillomavirus induced lesions, under certain circumstances, undergo malignant change. The bovine papilloma virus (BPV) type 4 induces oesophageal papillomas in cattle. In cattle which eat bracken fern (which contains carcinogens and immunosuppressants) the papillomas sometimes progress to squamous cell carcinomas (Campo and Jarrett, 1987). The sequence of events involved in the conversion of a papilloma to

a carcinoma can also be examined in domestic rabbits experimentally infected with the Shope or cotton-tail rabbit papillomavirus (SPV) (Kreider and Bartlett, 1985). It is not known whether the SPV genome plays an essential role in malignant transformation but it is present in carcinomas (Stevens and Wettstein, 1979) as are SPV-encoded antigens (Mellors, 1960) and it is known that the probability of carcinomatous change is directly proportional to the dose of SPV used to initiate the papillomas (Rous et al, 1936).

c) Other viruses

Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are herpes viruses which are associated with human malignancies, and which are shed from the female genital tract. So far there is no firm evidence to implicate CMV in cervical tumorigenesis. The literature concerning CMV and carcinogenesis is reviewed by Rapp (1984). EBV has only recently attracted attention as a possible aetiological agent in cervical cancer as its shedding from the cervix was not previously appreciated. Its possible role is discussed in a recent Lancet editorial (Anon, 1986).

d) Other infectious agents

Chlamydia, *Trichomonas vaginalis* and *Gardnerella vaginalis* are all known to be transmitted by sexual intercourse and have therefore been investigated as possible carcinogenic agents. A recent study found no evidence to support the hypothesis that Chlamydia is important either alone or in combination with HPV in causing CIN (Syrjanen et al, 1986). There is little recent work on *Trichomonas vaginalis* but studies published suggest that it is not an important factor (Thomas and Anderson, 1974). *Gardnerella vaginalis*, like EBV, has attracted attention only recently (see

Gonzalez-Falco et al, 1986).

3. Smoking

Cigarette smoking is known to be a risk factor for the development of malignant tumours not only in the lung, but elsewhere in the body. It was discovered recently that nicotine and other mutagenic derivatives of cigarette smoke can be isolated from the cervical mucus of smokers (Sasson et al, 1985) and it is possible that these derivatives have a direct carcinogenic effect upon epithelial cells within the cervical transformation zone. In addition, smokers are known to have a variety of alterations in immune function (see below) so that they may be less efficient at detecting malignant cells and at mounting an immune response.

Epidemiological studies performed over the last 15 years appear to show a clear link between smoking and cervical neoplasia. Some of these studies are reviewed by Winkelstein et al (1984) who include four studies in which the association persists after adjustment for various potentially confounding factors such as sexual history. Since Winkelstein's review four further studies have been published which confirm the association between smoking and cervical neoplasia (Clarke et al, 1985; Greenberg et al, 1985; La Vecchia et al, 1986; Hellberg et al, 1986). Apart from Greenberg et al (1985) all investigators took a sexual history from patients and controls, and all found that smoking remained a significant risk factor after adjustment for this. It is true that it is easier to publish work demonstrating a positive association than work showing no association but nevertheless epidemiological studies would appear to indicate that smoking is a significant factor in the aetiology of cervical neoplasia.

4. The combined oral contraceptive pill (OCP)

The role of the OCP in the aetiology of cervical neoplasia is, like that of smoking, still a matter for debate. In the UK deaths from cervical cancer among women under the age of 35 have increased over the last 20 years, while there has been a progressive decrease in mortality from this disease in women over 50 (Doll, 1985). This increase in mortality in young women has coincided with the introduction of the OCP as a popular contraceptive method and some authors argue that the two observations are causally related (Doll, 1985).

It is known that the OCP can induce cervical cancer in mice (Thomas, 1972) and the reported association of various degrees of cervical neoplasia with endometrial hyperplasia has suggested for some time that cervical cancer may be oestrogen dependent. Like smokers OCP users are known to have alterations in immune function (see below) and it has been suggested that the OCP may be a risk factor for the development of cervical neoplasia by virtue of its effects on the immune system (MacDonald, 1982). Pater et al (1988) have shown that addition of glucocorticosteroids to culture medium increases the transforming ability of HPV 16 in cooperation with an activated ras oncogene, and suggest that this evidence implies a causal role for oral contraceptives in the aetiology of cervical neoplasia.

There have been two major reviews of studies investigating the role of the OCP in cervical neoplasia published recently (Francheschi et al, 1986; Piper, 1985). Francheschi et al (1986) examined epidemiological evidence concerning the OCP through pooled computations of relative risks emerging from 21 studies of three categories: studies based on routine cytological screening programmes, case control investigations and prospective studies. The pooled estimates of relative risk for ever versus never use of the OCP were broadly similar and slightly above unity (1.4 from screening programmes, 1.1 from case control and 1.4 from

prospective studies). The risk increased with duration of use. When allowance was made for indicators of sexual habits a noticeable decrease in the excess risk was evident. Francheschi et al (1986) stress the point made in the last section in relation to smoking, i.e. that it is easier to publish positive studies than investigations showing no association. There are several reasons why the OCP may appear to confer higher risk than is really the case. OCP users tend to visit a doctor more frequently than non-users, thus making opportunistic screening, and detection of asymptomatic cases, more likely. If women using barrier methods of contraception are included in the control group the rate of cervical neoplasia in this group will be brought down, as these methods may confer specific protection against cervical malignancies (Richardson and Lyon, 1981; Vessey et al, 1983). Similarly if women using no contraception are included as controls and no adjustment is made for sexual history the cervical neoplasia rate in the control group will be artificially low, as these women are less likely to be sexually active than OCP users.

Two recent studies, not reviewed by Francheschi et al, have questioned the role of the OCP in the aetiology of cervical neoplasia. Hellberg et al (1986) found that the association between long term (over 5 years) OCP use and CIN disappeared when adjustment was made for smoking and sexual history, and if users of barrier methods were excluded from the control group. Clarke et al (1985) also adjusted for smoking and sexual history and again found that the association between OCP use and CIN then disappeared.

Stern et al (1977) suggested that the OCP may be implicated not as an initiator but as a promoter and may accelerate

progression from pre-invasive to invasive disease. Two recent prospective studies (Vessey et al, 1983 and Andolsek et al, 1983) lend support to this theory. Vessey et al compared 6838 OCP users with 3154 women using an intrauterine contraceptive device (IUCD). A sexual history was taken from a sub-section from each group and the two groups were found to be very similar with regard to sexual variables. Thirteen cases of invasive cervical carcinoma were identified during the trial, and these were all in OCP users. Andolsek et al (1983) report that all the cases of invasive carcinoma identified in their trial were also in OCP users.

The evidence concerning the OCP is therefore much less persuasive than that concerning smoking. Further prospective trials in which women using modern low dose OCPs are compared with IUCD users and both groups are questioned about sexual factors and about smoking are necessary before we can draw any firm conclusions. The findings of Vessey et al (1983) and Andolsek et al (1983) concerning invasive carcinoma are worrying, however. Perhaps clinicians should consider advising women who are thought to be at high risk of developing invasive disease (because of previously treated CIN or other factors) to consider using other contraceptive methods.

Intra-epithelial immune interactions

a) General

Until recently the skin and other epithelia were considered immunologically inert, only able to protect the body from infection and other environmental hazards by providing a physical barrier. Recent advances in technology including the development of monoclonal antibodies (Mabs) have permitted detailed examination of

the immunology of skin and have revealed that lymphocytes and antigen presenting cells (APC) are continuously circulating between the epithelial surface and local lymph nodes. Streilein (1978) has proposed that the entire unit be named SALT (skin associated lymphoid tissue) in analogy with GALT (gut associated lymphoid tissue). Bos et al (1987) have argued that the two systems are not analogous, because the evidence for co-operation between lymphocytes and keratinocytes is less strong than the evidence for co-operation between lymphocytes and epithelial cells lining the gastrointestinal tract. It is generally accepted, however, that foreign antigen breaching the epithelial surface is picked up by APC and presented to T lymphocytes within the epithelium, or in the draining node. As a result T helper cell proliferation occurs with the possible generation of cytotoxic T cells, specific for that antigen. Alternatively free antigen may pass straight to the node to be picked up by APCs there, leading to a similar immune response.

b) Major histocompatibility complex (MHC) class II antigens

The genes of the MHC are found on chromosome 6 in humans and code for three classes of antigens (see figure 5). They control the expression of alloantigens present on the surface of all nucleated cells. These are the human leucocyte antigen (HLA)-A and -B or class I antigens associated with transplant rejection (Svejgaard et al, 1979). The second series of genes of the human MHC controls the expression of alloantigens with a restricted tissue distribution. These are the HLA-D or class II antigens and were originally defined by the mixed lymphocyte reaction (MLR) which is the ability to induce a proliferative lymphocyte response and is brought about by lymphoid cells bearing these alloantigens.

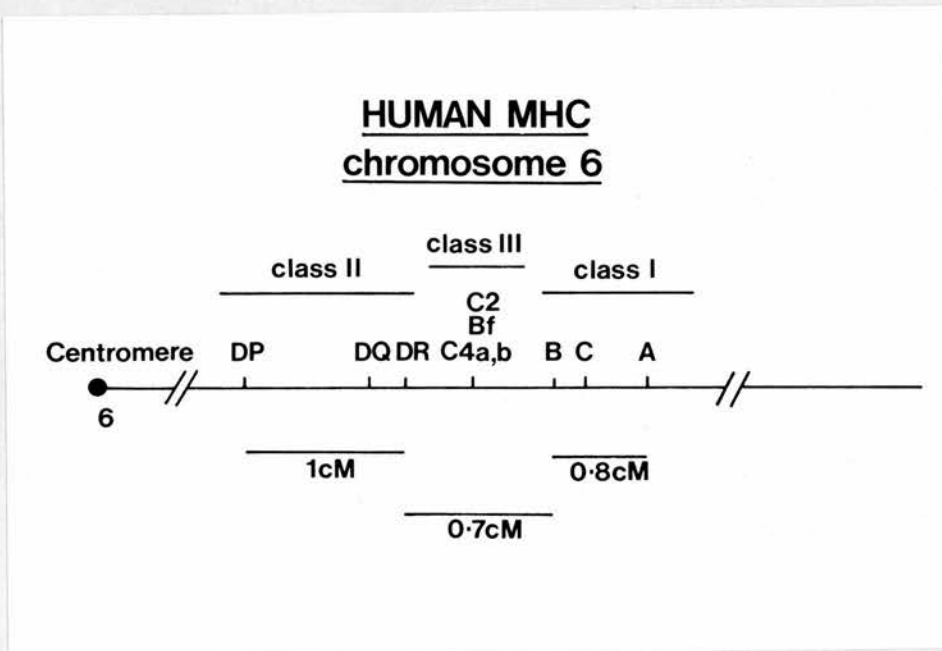


Figure 5

Reproduced with the kind permission of Dr K. Guy.

The final locus in the MHC (HLA-C or class III) controls the production of certain serum complement factors.

Studies on the MHC have been carried out extensively in mice (McDevitt et al, 1976) where inbred and congenic strains are available. The murine equivalent of the human HLA-D region is the Ir (immune response) region. It controls several important immune phenomena in addition to the MLR and including the ability to respond to certain types of antigen, the graft versus host reaction, T-B cell co-operation, T cell-macrophage co-operation and antigen presentation (Rowden, 1981). Human HLA-D or class II antigens are heterodimeric glycoproteins (Klareskog et al, 1977) expressed on the surface of B lymphocytes, macrophages, Langerhans cells, and, under certain circumstances, on activated T lymphocytes and some epithelial cells (Wiman et al, 1978). The number of different class II antigens expressed by a single cell varies, depending on whether the cell is homozygous or heterozygous as regards the HLA-D region. All class II molecules are composed of one α and one β chain, which have very similar structures and are believed to share a common ancestor. Most, if not all, class II genes appear to be localised to three well defined loci known as HLA-DP, -DQ and -DR.

Expression of MHC class II products can be modulated by T cell products. It was originally demonstrated that low levels of class II antigen expression could be augmented by T cell factors present in crude lymphokine preparations (Steeg et al, 1982). The inducing factor has been shown subsequently to be gamma interferon and the observations have been extended to human monocytes (Gonwa and Stobo, 1984) and to Langerhans cells (Berman et al, 1985).

Expression of MHC class II antigens is not, however, confined to traditional cellular components of the immune system. Initially it was noted that several human tumour cell lines of non-bone marrow origin also expressed class II antigens (notably melanoma cells) and that this expression could be increased by gamma interferon (Basham and Merigan, 1983). It was then shown that normal cultured human endothelial cells, as well as human dermal fibroblasts, could be induced to express HLA-DR, and that this effect was mediated by activated T cell products or by gamma interferon (Poher et al, 1983a; Collins et al, 1984).

In fact recent studies have demonstrated that cultured cells from virtually every organ and every embryonic germ layer can be induced to express HLA-DR in response to gamma interferon, although a few tumour cell lines such as teratocarcinomas and neuroblastomas are exceptions (Houghton et al, 1984).

The expression of MHC class II antigens by normal and diseased tissue has been extensively reviewed (Natali et al, 1981; Daar et al, 1984; Forsum et al, 1985). It has been proposed that in inflammatory conditions class II expression is induced by lymphokines produced by infiltrating lymphocytes (Lampert, 1984; Aubock et al., 1986) but the mechanisms controlling class II expression by healthy tissue (Morris et al, 1985; Carr et al, 1986) are unclear. It has been suggested that class II expression may be under the influence of hormones including sex hormones (Klareskog et al, 1980; Tabibzadeh et al, 1986a) and thyroid stimulating hormone (Todd et al, 1986).

The role of class II expression by non-lymphoid cells is unresolved. Comparison of HLA-DR antigens of autologous melanoma

and lymphoblastoid cell lines reveals differences in glycosylation, but not in protein structure (Alexander et al, 1984). The ability of HLA-DR positive melanoma and other non-lymphoid cells to function as accessory cells in immune responses may be limited by the nature of the induced class II antigens, as well as by their ability to produce interleukin 1 which is required to maintain (or support) T cell responses. It is of interest that it has been shown that HLA-DR positive cultured melanoma cells from early but not late disease are able to stimulate autologous lymphocytes (Guerry et al, 1984).

It has also been shown that the HLA-DR antigen induced on human umbilical vein endothelium (HUVE) and human dermal fibroblasts (HDF) does function in immune responses in a variety of ways. HLA-DR antigens on HUVE and HDF are recognised by HLA-DR restricted cytotoxic T cells with subsequent target cell cytolysis (Pober et al, 1983b) and HLA-DR positive human thyroid cells can present nominal antigen to cloned T cells (Londei et al, 1984).

c) The Langerhans cell

The major APC within squamous epithelium is the Langerhans cell (LC) (figure 6). It was first described by Paul Langerhans in 1868 who believed it to be an intraepidermal sensory element, because of its long dendritic processes and staining properties. It is now known that LC belong to the macrophage/monocyte lineage (although they are poorly phagocytic) (Wolff and Schriener, 1970), possess Fc receptors and C3 receptors (Stingl et al, 1977) and express MHC class II antigens (Rowden et al, 1977). LC are present in all stratified squamous epithelium possessing the ability to keratinise and appear in epithelium undergoing squamous

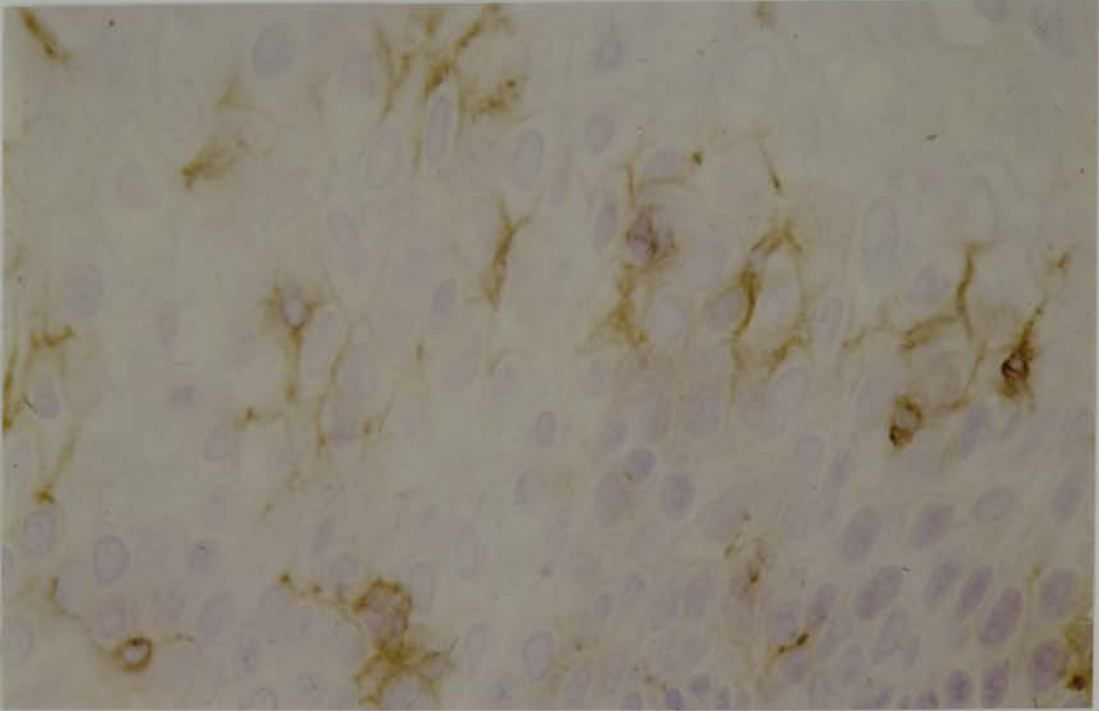


Figure 6 DAKO-T6 staining of Langerhans cells in normal cervical transformation zone epithelium (magnification x 250)

metaplasia (Rowden, 1981). They were first demonstrated in human cervix in 1968 (Hackermann et al, 1968; Younes et al, 1968). In skin the cells are usually seen in the epidermis in a suprabasal position (Hunter, 1984) and can be seen crossing the dermal-epidermal junction, in the superficial dermis and within dermal lymphatic vessels (Wolff and Stingl, 1983; Rowden, 1981). Some dendritic cells, usually seen in the basal layer of the epidermis, have been called indeterminate cells because they appear similar to LC and melanocytes but contain neither the granules characteristic of LC nor melanosomes. The study of serial sections and surface antigens (Rowden et al, 1979) of these cells suggests that most are probably LC precursors.

i) Methods of identification

LC can be identified by electron microscopy (EM) where they are characterised by a variety of histochemical and immuno-histochemical means. The cell is characterised on EM by the possession of Birbeck granules, the purpose of which is unknown (Birbeck et al, 1961).

Histochemical stains include gold (Juhlin and Shelley, 1977), osmium (Rodriguez and Caorsi, 1978) and adenosine triphosphate (Wolff and Winkelmann, 1967). These stains have not proved sufficiently specific for light microscopy in the hands of most workers and will not be discussed further.

The most commonly used marker for LC is currently T6, thymocyte antigen. Anti T6 reacts with 70% of thymocytes but not with peripheral T lymphocytes (Bhan et al, 1980; Reinherz et al, 1980) and has been shown by an immunoperoxidase EM technique to label intraepidermal LC (Murphy et al, 1981). Indeterminate cells

within the epidermis are also T6 positive (Murphy et al, 1982). T6 antigen on the surface of thymocytes has been partially characterised. It is a 49,000 dalton glycoprotein and is associated with microglobulin (Terhorst et al, 1981). The character of the antigen on LC is unknown at present.

Mabs to MHC class II antigens can also be used to identify LCs. However, MHC class II antigen expression by LC is known to be variable (Dezutter-Dambuyant et al, 1985) in response to such stimuli as ultra-violet irradiation (Aberer et al, 1981) so that T6 positive LC may sometimes appear MHC class II negative (Muhlbauer et al, 1982 and Harrist et al, 1983). There are also other MHC class II positive cells within squamous epithelium which may be confused with LC. These include B lymphocytes, activated T lymphocytes and, in some cases, keratinocytes (Carr et al, 1986) as discussed above.

ii) Functional properties

In 1976 Shelley and Juhlin found that LC had an affinity for common external contact sensitisers including metal ions. Stingl et al (1980a) then demonstrated that guinea pig epidermal LC could replace macrophages in their capacity to induce antigen specific T cell activation. Braathen and Thorsby (1980) were able to show the same phenomenon in man.

There is now considerable evidence that specially functioning cells play an essential role in the presentation of antigens to effector lymphocytes in many immune responses such as antibody production, antigen and mitogen induced T cell proliferation and cytotoxic T cell induction (for review see Katz et al, 1985) and recent studies implicate dendritic cells rather than macrophages

Klinkert et al, 1982). It has been shown with respect to at least one antigen (purified protein derivative) that LC are superior to blood derived dendritic cells at inducing antigen specific T cell proliferation (Bjercke et al, 1985). LC are believed to play an important role in immunosurveillance against tumours and viral infections of squamous epithelium. These functions will be discussed below.

For T cell responses to be induced the antigen presenting cell (APC) and T cell must be histocompatible, sharing the same MHC class II molecules (Braathen and Thorsby, 1982). Considerable knowledge has gathered with regard to the mechanism(s) by which APC "process" and present antigen to T cells. Studies from various laboratories demonstrate that antigen presentation by macrophages and B lymphocyte tumour cells can be inhibited by chemicals interfering with lysosomal transport and function (Katz et al, 1985). These studies indicate that large molecules (i.e. ovalbumin, cytochrome and myoglobin) must be digested so that the smaller proteolytically cleaved molecules may be presented to T cells (Streicher et al, 1984). Glutaraldehyde fixed APC which are unable to present intact ovalbumin to T cells are able to present an immunogenic 17 residue peptide, thus bypassing the lysosomal "processing" step. In skin it is not known whether LC process antigen as well as presenting it or whether keratinocytes process it before presentation by LC.

There are two hypotheses concerning the way in which T cells recognise antigen and MHC products on cell membranes (Roitt, 1985) [see figure 7].

The dual receptor hypothesis suggests that the MHC product and

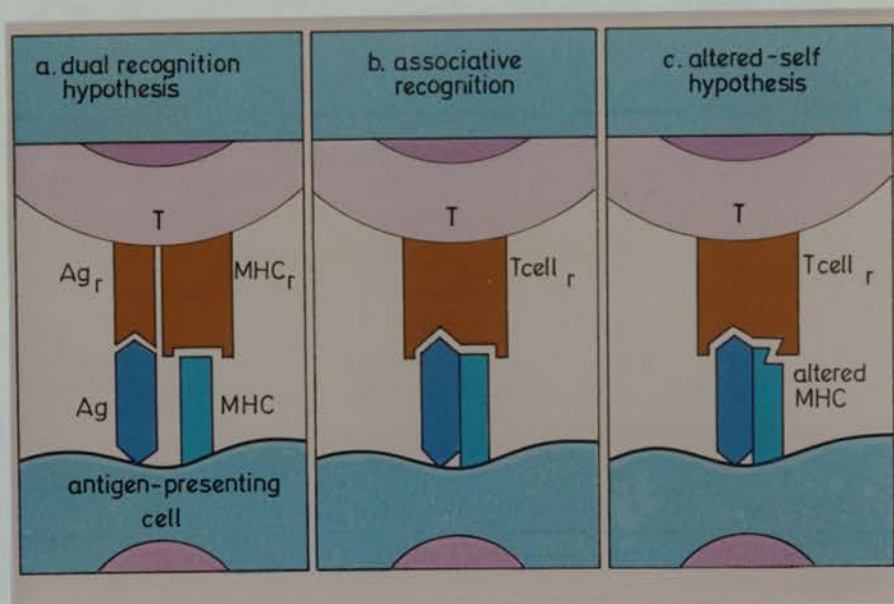


Figure 7 Antigen presentation : 3 models
(modified from Roitt *et al.*, 1985)

the antigen are recognised by separate receptor molecules on the lymphocyte surface.

The associative recognition hypothesis (which is currently more popular) proposes that antigen, possibly modified by antigen-presenting cells, associates with the MHC product, and is recognised by a single T cell receptor. This hypothesis has been modified to give the altered self hypothesis according to which the self-MHC on the APC is somehow modified by the presence of antigen. It is proposed that a single T cell receptor recognises both antigen and altered self. The activation of T cells is thought to occur in three phases. Following binding of the T cell to the APC an unknown factor from the T cell induces the APC to produce interleukin-1. This, in association with antigen stimulation, induces interleukin-2 receptors on the T cells and stimulates T cells to release interleukin-2 which drives antigen-activated cells into proliferation.

d) The T lymphocyte

In normal squamous epithelium T lymphocytes exceed B lymphocytes in number. Normal skin (Bos et al, 1987), gastrointestinal mucosa (Selby et al, 1983) and epithelium lining the male genital tract (Eldemiry et al, 1985) contain predominantly T suppressor (CD8 positive) cells. It is argued that a predominance of suppressor cells within the gastrointestinal mucosa leads to a state of tolerance of oral antigens (Mowat, 1987) while in the male genital tract the presence of these cells is believed to prevent the development of an immune response to autoantigens present on spermatozoa (Eldemiry et al, 1985). In the skin it is believed that CD8 positive cytotoxic/suppressor cells may recognise altered

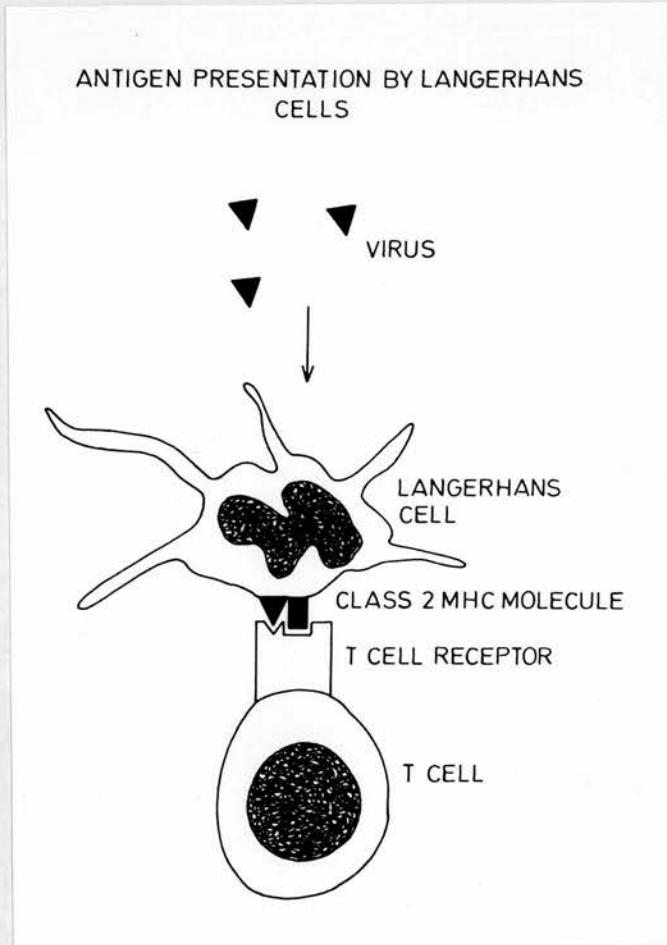


Figure 8.

self and destroy potentially malignant cells (Streilein, 1978).

The immune response to tumours

It has been believed for many years that one function of the immune system was to provide "immunosurveillance" against tumours (Thomas, 1959; Burnett, 1970). This was first challenged in the 1970s (Stutman, 1978) when it was discovered that T lymphocytes had another role. It was then argued that the increased incidence of malignant disease in patients with inherited disorders of immunity might be due to some other genetic abnormality rather than to their immunodeficiency (Kirchner, 1984). In addition it has been argued that the increased incidence of malignancy in patients with secondary immunosuppression such as lymphoma patients may be due to the mutagenic effects of the chemotherapeutic agents employed in their treatment (Anon, 1985). A collaborative UK-Australasian study (Kinlen et al, 1979) of 3823 renal transplant recipients and 1349 others who had received azathioprine, cyclophosphamide or chlorambucil found that only lymphoreticular and cutaneous malignancies were increased in incidence. Three squamous cell carcinomas of vulva were included in the "cutaneous malignancy" category and cervical CIS was excluded. The authors argue that a mutagenic effect of the agents used is unlikely as there was no dose effect and a constant risk was observed from the time of first exposure. It has been argued on the basis of this survey that immunosurveillance against tumours may be confined to the skin and to other components of the immune system (Mitchison and Kinlen, 1980).

Iochim (1976) investigated the stromal reaction in CIS of the cervix, bronchus, vagina and breast and found large collections of

lymphocytes just below the basement membrane. He noted a greater cellular reaction in precancerous and early cancerous lesions than in advanced metastatic lesions and argued that this was evidence of an immune response to tumour cells.

Husby et al (1976) and Ferguson et al (1985) have similarly demonstrated increased numbers of T lymphocytes around and within malignant tumours. Viac et al (1977) extracted lymphocytes from basal cell carcinomas (described as "weakly malignant") and superficial spreading melanomas (described as "highly malignant"). Basal cell carcinomas contained more T lymphocytes and plasma cells than did melanomas.

Ferguson et al (1985) examined tumours of the female genital tract and found that infiltrating T cells were predominantly CD8 positive. Bussy-Fernandez et al (1983) reported a similar predominance in malignant skin tumours, whereas Gatter et al (1984) reported that T cells surrounding malignant skin tumours were predominantly of the CD4 (helper/inducer) phenotype. It would be of interest to discover the subsequent outcome in these patients. It is possible that the predominance of a particular T cell subset within a tumour infiltrate may be related to the prognosis.

It is postulated that LC play a role in the putative immunosurveillance system by virtue of their situation in the "front line" of the body's defences (Streilein and Bergstresser, 1980). Studies of the effects of ultraviolet (UV) irradiation on the skin would support this hypothesis. UV irradiation plays an important part in the induction of neoplasms of both keratinocytes and melanocytes (Rowden, 1981) as well as reducing the numbers of cutaneous LC (Stingl et al, 1980b), altering LC morphology and

antigen presenting function (Perry and Greene, 1982), and reducing MHC class II expression by LC (Aberer et al, 1981). It is not known whether UV irradiation destroys the LC or causes it to migrate out of the epidermis. Gurish et al (1982) have reported an increase in APC activity in draining lymph nodes following UV irradiation suggesting that LC migrate away from the epidermis, rather than being killed.

Mice exposed to UV irradiation develop T suppressor cells which prevent immunological rejection of the highly antigenic skin cancers induced by UV irradiation (Fisher and Kripke, 1982). If mice are immunised with epidermal cells that have been exposed in vitro to UV irradiation prior to their coupling with antigen the T suppressor cell pathway is activated (Kripke and Morison, 1985) suggesting that activation of the T suppressor cell pathway and consequent failure to reject tumours results from a direct effect of UV irradiation on APC.

When hamster buccal mucosa is treated with a carcinogenic agent (7,12 dimethylbenzanthracene) LC are reduced in number and their morphology is altered (Schwartz et al, 1981). This may contribute to the carcinogenic effect of this agent.

Studies of LC in human tumours have shown that LC numbers are greater in benign lesions (excluding those induced by HPV) and well differentiated tumours than in poorly differentiated tumours (Lisi, 1973; Gatter et al, 1984; Furukawa et al, 1984).

The effects of smoking and oral contraceptive use on the immune system

It has been suggested that the apparently increased risk of CIN in smokers and OCP users may be due to the effects of these

agents upon the immune system, and on viral and/or tumour immunosurveillance (MacDonald, 1982).

Smokers have been shown to have low circulating natural killer cell activity (Ferson et al, 1979 and Hughes et al, 1985) and low serum immunoglobulin levels (Ferson et al, 1979). Heavy smokers have also been shown to have a reduced percentage of circulating CD4 positive (helper) T cells and an increased percentage and total number of CD8 positive (cytotoxic/suppressor) T cells (Miller et al, 1982). Of particular interest is the recent report from Barton et al (1988) of a significant reduction in LC numbers in both normal and neoplastic cervical epithelium of smokers, when compared with non-smokers.

Progesterone has an immunosuppressive effect which is thought to be partly responsible for maternal tolerance of the fetus (Munroe, 1971). Women using the combined oral contraceptive pill have been shown to have an impairment of peripheral blood lymphocyte proliferation in response to a non-specific mitogen (phytohaemagglutinin) (Barnes et al, 1974; Pater et al, 1988).

The immune response to the human papillomavirus

a) General

The role of the systemic immune response in resistance to HPV infections and in regression of warts is not well understood. Clinical observations, however, suggest that cell-mediated immunity (CMI) is of more importance than humoral immunity. Studies of patients with primary predominantly humoral immunodeficiencies do not reveal a high incidence of warts, except in the common variable form of hypogammaglobulinaemia. One third of the patients suffering from this disease also have an alteration in T lymphocyte

function (Kienzler, 1985). In secondary humoral immunodeficiencies such as multiple myeloma the incidence of warts is not higher than in a control population (Morison, 1975a).

Some workers regard HPV antibodies to be a consequence of wart regression rather than a factor contributing towards regression. It has been shown in some studies that HPV antibodies only occur during or after treatment (Genner, 1971 and Eriksen, 1980) and it is well recognised that antibodies do not protect against reinfection (Cubie, 1972). Pyrhonen and Johansson (1975) examined a group of patients with regressing warts and identified several patients who underwent a rapid spontaneous cure without developing demonstrable antibodies.

The above data suggest that antibodies do not have a significant role in causing regression of warts or in preventing reinfection although it has been suggested that they may reduce spread of infection by neutralising HPV once liberated (Kienzler, 1985). It is important to remember that many of the early studies employed complement fixation to detect antibodies. This is a relatively insensitive method and is particularly poor at detecting IgM. It was also thought until fairly recently that all HPV-induced lesions were due to a single virus. Now that the antigenic heterogeneity of HPVs has been established it will be necessary to repeat antibody studies using distinct HPV antigens in patients with strictly defined warts, as has been reported in recent studies (Kienzler et al, 1983; Cubie and Norval, 1988).

Patients with specific CMI deficiencies on the other hand, do have an increased incidence of warts. Epidermodysplasia verruciformis has been discussed above but other examples are

patients with Hodgkin's disease (Morison, 1975a) and Fanconi's anaemia (Johansson et al, 1982). In 1974 Brodersen et al reported that children in whom skin warts develop are more likely to be anergic to purified protein derivative.

Specific anti-HPV CMI has been tested in patients with warts using in vivo and in vitro methods. Thivolet et al (1982) performed intradermal skin testing using inactivated viral particles from plantar warts and found that significantly more patients with current or past warts had a positive skin test than controls, and that a higher response was obtained in patients with regressing or past warts than in controls.

In vitro tests such as the leucocyte migration inhibition (LMI) test and lymphocyte transformation test (LTT) have been used to obtain similar results. Morison (1974) using a LMI test showed that few patients gave a positive response while they had skin warts but that many were positive just after resolution of their warts. Similarly using a LTT stimulation was greatest in those who had had warts in the recent past (Ivanyi and Morison, 1976; Lee and Eisinger, 1976). Weak or negative responses were obtained in those patients who had warts of long duration (Morison, 1975b).

Obalek et al (1980) found non-specific CMI as assessed by response to phytohaemagglutinin (PHA) to be markedly reduced in patients with flat warts as well as in EV patients, although there was only a slight defect in patients with common warts. Response was almost normal in patients with plantar and genital warts. In the same study they found that the percentage of E rosetting T lymphocytes was reduced in patients with common, flat and plantar warts while Chretien et al (1978) report a decreased percentage of

T lymphocytes and an alteration in T cell morphology in patients with previous and current warts. In a more recent study Carson et al (1986) found a reduction in the CD4+ : CD8+ T cell ratio in patients with skin warts.

b The immune response to HPV in patients with genital warts and CIN

These patients have been studied in an attempt to identify an abnormality in their immune response to HPV which might somehow permit progression to neoplasia in a manner analogous to that seen in EV. It is not currently possible to study the specific humoral or cell mediated immunity to the HPV types associated with genital lesions as, for reasons discussed above, viral antigen has to be prepared from HPV associated lesions, and genital warts contain too little viral antigen to allow the development of satisfactory serological tests (Gissmann and Gross, 1985).

Baird (1983) reported higher levels of antibody to detergent disrupted BPV antigen in women with anogenital warts, with CIN and especially with invasive cervical carcinoma than in controls but a more recent study of patients attending a Colposcopy Clinic found that anti-BPV and anti-HPV antibody levels correlated with a history of skin warts and were unrelated to the presence of CIN or of genital HPV infection (Cubie and Norval, 1988). Baird (1983) does not provide details of skin wart history.

Seski et al (1978) studied a group of women with recalcitrant genital warts and reported a significant reduction in CMI. This finding was confirmed by Carson et al, 1986. A similar study of patients with CIN (Neill, 1984) showed, however, normal responsiveness to PHA, concanavalin A and pokeweed mitogen, apart from a reduced response to PHA in patients with CIS.

c) The local immune response to HPV

Interest in the local immune response was stimulated by the observation that plane or flat warts (caused by HPV 3) regress spontaneously following inflammation (Tagami et al, 1977). Biopsies taken from regressing flat warts show a massive mononuclear infiltrate (Tagami et al, 1980) and if explants from these lesions are cultured in vitro mononuclear cells can be seen attacking the wart derived epidermal cells. Broderson and Genner (1973) and Kossard et al (1980) describe a minimal or absent inflammatory infiltrate in cutaneous common warts (caused by HPV 2) showing signs of involution. Nevertheless, HPVs cause a localised epithelial infection and the local immune response must be of central importance in the pathogenesis of these lesions. Characterisation of the cells involved in the local response has been made more feasible in recent years by the availability of monoclonal antibodies.

i) Langerhans cells in HPV induced lesions and in cervical neoplasia

The literature on LCs is very sparse in the area of their possible role as sentinels against viral infection. Nagao et al (1976) studied sites of vaccinia virus inoculation and demonstrated virus in the phagolysosomes of LCs on the developing blister roofs. Intradermal injection of cytomegalovirus also leads to an accumulation of virions in LCs, both in the epidermis overlying the injection site and in the dermis (Rowden, 1981). The observations of Oguchi et al (1981) that LC around regressing plane warts showed evidence of activation led other workers to examine LC in HPV induced lesions more closely. Most workers have

found LC to be reduced in number around skin and genital warts and have noted an alteration in LC morphology including a loss of dendritic processes and a rounding of the cell body (Morris et al, 1983; Gatter et al, 1984; Chardonnet et al, 1986; Tay et al, 1987a). Two groups, however, report an increase in LC numbers in genital although not in skin warts (Vayrynen et al, 1984; Bhawan et al, 1986). However, Vayrynen et al (1984) compared their patients with current genital HPV lesions with "controls" who had treated or regressed HPV lesions and who cannot be regarded as true normal controls. Bhawan et al (1986) report a decrease in LC numbers in skin warts but an increase in three condylomata acuminatum (exact site not given). LC numbers were assessed purely by a visual estimate which is not considered a very reliable method of enumeration (De Jong et al, 1986).

In CIN, in contrast, most workers have found LC to be increased in number within the abnormal epithelium and to display a similar morphological alteration as seen in HPV associated lesions (Morris et al, 1983; McArdle and Muller, 1986). Interestingly, Caorsi and Figueroa (1986) in an electron microscopic study found that LC were increased in number in CIN but, in contrast to other studies, found an increase in the more ramified types of LC in CIN, with the most ramified cells being more frequent in most severe lesions.

Vayrynen et al (1984) found LC numbers to be increased relative to normal in CIN I but decreased in CIN III, while Syrjanen et al (1984) in a prospective study found that LC counts were lower in patients whose disease progressed than in those whose disease regressed. Tay et al (1987a) in a recent study enumerated

LC within cervical HPV lesions and CIN using a variety of markers (adenosine triphosphatase, T6, HLA-DR and S100). Positive cells were counted per mm^2 sectional area and per mm length of epithelium. LC were reduced in number by all markers in HPV lesions and in CIN. S100 positive cells were almost completely depleted in abnormal epithelium and the authors postulate that the S100 protein may mark a histogenetically distinct subpopulation of LC, with a different functional role. The significance of this finding is, however, somewhat uncertain as S100 is also present on melanocytes (Halliday et al, 1986) and on some T suppressor cells (Takahashi et al, 1985).

Hawthorn et al (1988) studied the relationship between the number of LC and the presence of HPV types 16 and 18 in CIN. They found that the number of LC present was inversely related to the number of copies of HPV 16 DNA per cell present, and that the presence of HPV 18 DNA, even at low copy numbers, reduced the LC numbers significantly. Thus these HPV types may affect the local afferent immune response and help to explain their suggested oncogenic potential.

ii) T lymphocytes in HPV induced lesions and in CIN

Some of the above studies quantified T cell subsets, in addition to LCs, within the cervical transformation zone (TZ).

Morris et al (1983) found that CD8 positive (suppressor/cytotoxic) T cells outnumbered CD4 positive (helper/inducer) T cells in normal cervical epithelium. This corresponds to the pattern described by other workers in the skin, gut and male genital tract (see above). In HPV associated cervical lesions Morris et al noted a reduction in total numbers of T cells with CD8



positive cells still exceeding CD4 positive cells in number.

Other workers (Edwards and Morris, 1985; Tagami et al, 1983) have confirmed these findings, i.e. CD8 positive cells outnumber CD4 positive cells in normal and abnormal cervix.

In contrast, Syrjanen et al (1984) found that although CD8 positive cells exceeded CD4 positive cells in HPV lesions (with no CIN), the ratio was reversed in CIN. Tay et al (1987b) reported the reverse of this situation; i.e. CD4 positive cells were more numerous in normal cervical epithelium but CD8 positive cells were in the majority in abnormal (koilocytic and neoplastic) epithelium.

Clearly the picture regarding T cell subsets within the cervical TZ requires clarification.

DNA analysis of invasive and preinvasive lesions

Introduction

The DNA content of tumours can be analysed using one of three techniques; these are chromosome analysis, static cytometry and flow cytometry. The principles involved in chromosomal analysis and static cytometry will be outlined briefly here and flow cytometry will be described in more detail.

Chromosomal analysis

The application of this technique to the study of solid tumours has been reviewed recently (Sandberg and Turc-Carel, 1987). The technique is ideal for the study of leukaemias but solid tumours require mechanical or enzymatic disaggregation and in many cases this means that interpretable chromosome spreads cannot be obtained. Modern tissue culture techniques are helping to solve this problem.

Once a single cell suspension has been obtained it is exposed

to a mitotic inhibitor and prepared on glass slides. The interaction of alkylating fluorochrome and histochemical stains produces multiple characteristic bands which can be identified on metaphase chromosomes (Verma and Dosik, 1982).

The normal human somatic cell with 46 chromosomes is referred to as diploid (Tijo and Levan, 1956). Cells with three or more complete sets of the haploid component (i.e. multiples of 23 chromosomes in man) are known as polyploid and cells with a chromosome content differing from the diploid but not by a complete haploid set are known as aneuploid. A cell with a balanced gain or loss of chromosomes, or with structural rearrangements, that still has 46 chromosomes, is pseudo-diploid.

Static cytometry

Cellular DNA can be measured by one of two procedures. The Feulgen-Schiff technique entails removal of the purines from DNA by acid hydrolysis and the subsequent reaction of the exposed aldehyde groups of deoxyribose sugars with aldehyde specific Schiff reagents. The second method relies on the use of fluorescent dyes which bind directly to DNA in a stoichiometric fashion - that is the degree of staining is directly proportional to the amount of DNA present .

Stained single cells are then visualised by a microscope based system and the amount of DNA bound dye measured directly or by absorption or fluorimetric means (Caspersson, 1979). In contrast to chromosome analysis, this technique can be used to examine interphase cells independent of the proliferative activity of the tissue. The detection of a chromosomal abnormality by static and flow cytometry is a function of its relative frequency within the

population studied, the amount of aberrant chromosomal material involved, and the resolution of the technique used. Static cytometry is applicable to the analysis of formalin fixed tissue or fixed smear preparations from tumours, where the DNA content of morphologically identified tumour cells can be measured. Owing to the relatively slow data acquisition rates of the procedure, however, measurement of DNA content is usually restricted to several hundred cells in any one sample.

Flow cytometry

This recently developed technique permits the rapid examination of many thousands of cells and as a result has replaced static cytometry in many centres. The major disadvantage of the technique, which does not apply to static cytometry, is that specimens to be analysed must be reduced to a single cell suspension. This means that information concerning structural relationships in solid tissues is lost.

Tissue can be disaggregated and stained when fresh or after frozen storage (Stone et al, 1985) or retrieved from paraffin embedded blocks (Hedley et al, 1985). Alternatively cytological samples can be analysed in what appears to be one of the most exciting applications of the technique from a diagnostic point of view. Cervical brushings or smears (Elias-Jones et al, 1986; Linden et al, 1979), bladder washings (Melamed and Klein, 1984) pleural effusions (Czerniak et al, 1985) or semen (Evenson et al, 1982) can be studied.

Within the flow cytometer the single cell suspension encounters a laser or other light beam and information is obtained regarding the size and granularity of the cells [see figure 9]. A

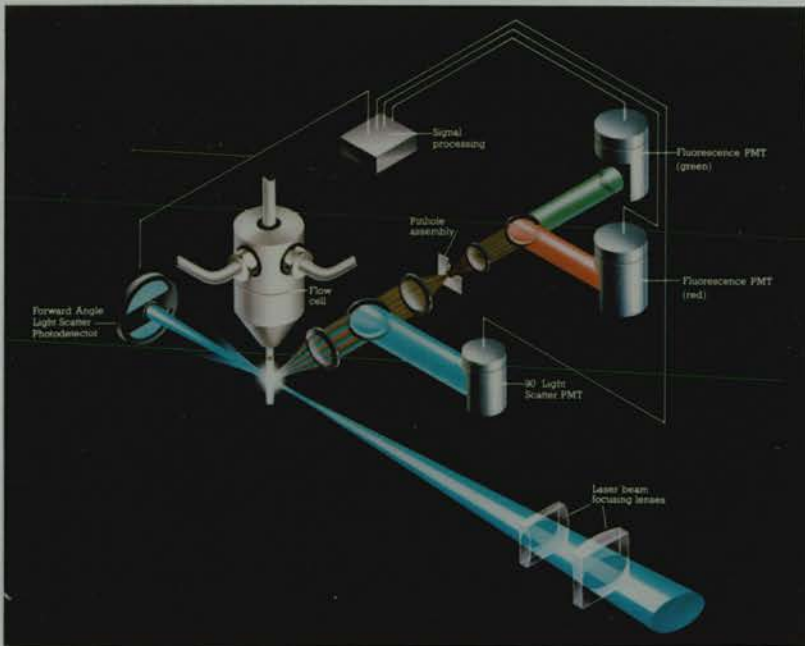


Figure 9 Schematic representation of flow cytometry. For explanation see text.

DNA specific fluorescent dye may be used to obtain a quantitative assessment of DNA content and antibodies to surface and nuclear antigens may be labelled with fluorescent stains to obtain information about the expression of these antigens. Assessment of enzyme activity (Quirke and Dyson, 1986), steroid receptors (Kute et al, 1983), and intracellular pH (Visser et al, 1979) and calcium (Quirke and Dyson, 1986) is also possible.

Cell populations of particular interest may be sorted from accompanying cells by applying an electrical charge to those of interest. The cell stream then passes between two high voltage plates and the cells are deflected according to their charge. In this way rare populations of cells such as fetal cells (Hezenberg et al, 1979) and trophoblast (Covone et al, 1984) in the maternal circulation have been concentrated. The sorting of human chromosomes (Young et al, 1981) has produced genomic libraries of cloned DNA (Davies et al, 1981) and the isolation of substantial quantities of pure single chromosomes for molecular biological studies (Collard et al, 1985).

Flow cytometry has been used to study two aspects of the DNA content of tumours. These are: [a] DNA aneuploidy and [b] cell cycle kinetics.

a) DNA aneuploidy

A recent working party report on nomenclature in flow cytometry (Hiddemann et al., 1984) has recommended that the term aneuploidy be reserved for chromosomal studies and should be modified to DNA aneuploidy when describing flow cytometric data. DNA aneuploidy is defined as the presence of an "abnormal DNA stem line" and Hiddemann et al recommended that it should only be reported when at

least two separate G_0/G_1 peaks are demonstrated.

Aneuploidy is a well recognised feature of human tumours and until recently has been regarded as being confined to malignant or premalignant conditions. The recent identification of chromosomal abnormalities in benign tumours such as lipomas (Heim et al, 1986) and meningiomas (Zang, 1982) and of DNA aneuploidy in meningiomas (Danova et al, 1987), benign thyroid tumours (Johannessen et al, 1982), benign monoclonal gammopathies (Montecucco et al, 1984) and in cervical warty atypia (Evans and Monaghan, 1985) make it necessary to re-examine this assumption. Nonetheless, a large body of work suggests that the development of aneuploidy is a significant event in the process of carcinogenesis and has serious prognostic implications (for review see Friedlander et al, 1984a).

DNA aneuploidy has been shown to be inversely related to differentiation in bladder (Chin et al, 1985) and breast tumours (McGuire and Dressler, 1985) and in non-Hodgkin's lymphoma (Diamond et al, 1982). It can be used to predict invasive potential in preinvasive bladder carcinoma (Gustafson et al, 1982) and in borderline ovarian lesions (Friedlander et al, 1984b). Patients with DNA aneuploid rectal adenocarcinomas have a poorer chance of surviving for five years than those with tetraploid tumours (with twice the normal diploid DNA content) (Quirke and Dyson, 1986).

DNA aneuploidy has been shown to increase with increasing severity of disease in CIN (Jakobsen et al, 1983; Reid et al, 1984). This is in accordance with cytogenetic investigations which show that only 29% of cases of cervical CIS have a chromosome number in the diploid range (Spriggs et al., 1971). Nasiell et al (1979) were, however, unable to demonstrate a significant

correlation between aneuploidy and progression in CIN in a study employing static cytometry to examine cervical smears. Evans and Monaghan (1985) also used static cytometry to investigate cervical warty atypia and found that aneuploid cases did not regress, while approximately half the diploid and polyploid cases did regress over one year.

A significant correlation has been demonstrated between "DNA index" and the incidence of pelvic node metastases in squamous cervical carcinoma, with "high ploidy" (DNA index > 1.5 if diploid = 1) tumours having a higher likelihood of metastasising (Jakobsen, 1984a). A second flow cytometric study by Jakobsen (1984b) of patients with cervical carcinoma also showed that patients with low ploidy (DNA index < 1.5) had a significantly better prognosis than those with high ploidy. The distribution of ploidy was not correlated with stage, and there was a significantly higher recurrence frequency in patients with high ploidy tumours in all stages investigated. This is interesting as it has been suggested that DNA tetraploid tumours behave less aggressively than their DNA aneuploid counterparts in prostatic (Ronstrom et al, 1981) and bladder (Tribukait et al, 1982) carcinomas, tending to be of lower histological grade and stage.

These data, together with experimental findings (Isaacs et al, 1982) lend support to the suggestion that karyotypic progression in tumours occurs first by doubling the chromosome number to DNA tetraploidy with subsequent loss or gain of chromosomes producing DNA aneuploidy (Nowell, 1976). At this stage the expression of recessive oncogenes may occur (Atkin, 1985) which might explain enhanced biological aggressiveness.

b) Cell cycle analysis

There are problems inherent in the analysis of the cell cycle by flow cytometry. These stem from the fact that DNA content is measured at a single point in time, rather than over a period of time. Samples which contain a large percentage of cells in the S and G_2+M phases of the cell cycle (see figure 10) are assumed to contain a large percentage of dividing cells. It is, however, theoretically possible that these cells have longer S and G_2+M phases, and a shorter G_0/G_1 phase than other cells, and are dividing at the same rate. This possible interpretation must always be remembered but S and G_2+M phase percentages are generally assumed to reflect the rate of cell division.

Much less work has been performed in this area with respect to human tumours than in the area of DNA aneuploidy. It is reported that low S phase breast tumours are more likely to be oestrogen receptor positive (and thus to have a better prognosis) than are high S phase tumours (McGuire et al, 1985). In non-Hodgkin's lymphoma a high percentage of cycling cells is found significantly more often in high grade tumours (Braylan et al, 1980) and a high S phase fraction in low grade lymphomas may predict progression to a higher grade tumour (MacCartney et al, 1986).

Oncogenes and human tumours

Oncogenes were first isolated as part of the genomes of rare RNA tumour viruses known as acute transforming retroviruses, which efficiently transform cells in culture and cause tumours in animals. Over two dozen viral oncogenes have now been identified in the genomes of DNA and RNA viruses, encoding a wide diversity of proteins. So far there is little correlation between what is

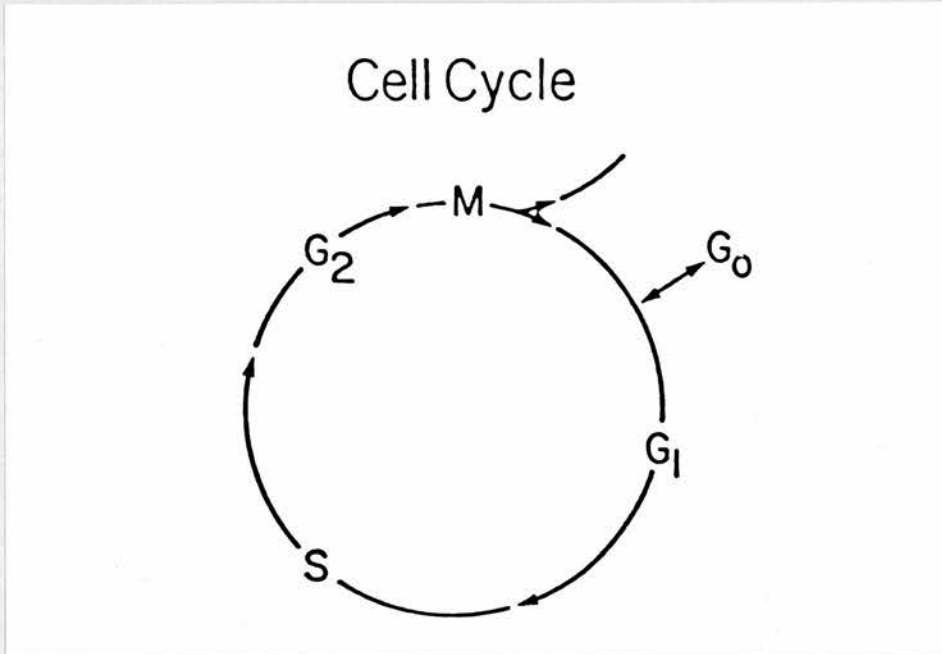


Figure 10. G₀ = resting phase
G₁ = first gap phase
S = DNA synthetic phase
G₂ = second gap phase
M = mitotic phase

known of how the proteins act and the nature of the tumours they induce.

Three biochemical strategies have been identified by which the products might act in inducing carcinogenesis. These are phosphorylation, initiation of DNA synthesis and regulation of transcription.

a) Phosphorylation

Both proteins and phospholipids are potential substrates for phosphorylation. The transforming protein may be a factor that elicits phosphorylation, such as a ligand that binds to a receptor on the cell surface; or it may be the catalytic kinase itself. Alternatively it may act on phosphorylation at a distance, by regulating adenylate cyclase and thus the activity of protein kinases controlled by cyclic AMP. The v-src and v-abl oncogene products are protein-tyrosine kinases and thus are examples of this class of protein (Bishop and Varmus, 1982).

b) Initiation of DNA synthesis

Unrestrained synthesis of DNA is an inevitable component of the neoplastic phenotype. Some transforming proteins allegedly elicit this property by acting directly to initiate DNA synthesis (e.g. the simian virus 40 large T antigen (Martin, 1981)).

c) Regulation of transcription

Several of the transforming proteins may influence transcription from cellular genes, either by stimulation or by inhibition. They may do so by interacting with other proteins, with promoters for transcription, or with enhancers (Kingston et al, 1985).

The adenovirus E1A products are believed to inhibit

transcription from MHC class I genes (Schrier et al, 1983) and the v-myc product is also believed to regulate transcription (Bishop, 1985; Studzinski et al, 1986).

Normal vertebrate cells contain similar but non-identical copies of viral oncogenes. These cellular genes have been termed cellular oncogenes (c-oncs) or proto-oncogenes. It has become clear that viral oncogenes arose as a consequence of genetic recombination between slow retroviruses and cellular oncogenes (Bishop, 1983). Unlike viral oncogenes, however, cellular oncogenes do not normally transform cells. Their expression in normal cells appears to be under tight control for they are expressed only in small quantity and only at appropriate stages of differentiation and cell division. A role for oncogenes in spontaneous transformation has been strongly suggested by the finding that tumour cells often possess proto-oncogenes that are genetically altered, compared to a normal cellular counterpart from the same individual.

The myc oncogene

The myc oncogene encodes a product which localises in the nucleus (Persson and Leder, 1984) and which appears to have some structural similarity with the E1A-transforming protein of adenoviruses (Kelly and Siebenlist, 1986). It is thought to act as a transcriptional regulator (Bishop, 1985). C-myc is ubiquitously expressed in almost all differentiated cell types, where its expression is differentially regulated in resting and activated cells. Increased c-myc expression is closely associated with the entry of a cell from G_0 into the cell cycle, while almost complete shut off of c-myc expression accompanies the inhibition of

proliferation associated with differentiation (Kelly and Siebenlist, 1986).

Structural alterations in the c-myc gene and abnormal expression of the c-myc product have been observed in numerous tumours, particularly B and T cell malignancies.

Murine plasmacytomas and human Burkitt's lymphoma are characterised by consistent reciprocal translocations involving the c-myc locus and one of the three immunoglobulin loci (Bernheim et al, 1981). It is thought that these translocations result in deregulation of c-myc transcription. All, or almost all of the c-myc RNA present originates from the translocated allele with little or no transcription originating from the germline allele (Leder et al, 1983). C-myc may be negatively regulated by a mechanism that can repress the normal gene but not the translocated allele.

Gene amplification of c-myc has been described in relation to a variety of malignancies (Kelly and Siebenlist, 1986). A causal role for c-myc amplification in contributing to the degree of malignancy has been suggested by the observation that highly malignant small cell lung carcinoma cells show c-myc gene amplification, while less malignant small cell lung carcinoma cells do not necessarily do so (Little et al, 1983). In the cervix it has been shown that stage 3 and 4 carcinomas display c-myc DNA amplification while stage 1 carcinomas do not (Riou et al, 1984; Ocadiz et al, 1987) and that stage 1 and 2 carcinomas in which c-myc overexpression (relative to normal) is detected are more likely to relapse after treatment than carcinomas in which such over-expression is not detected (Riou et al, 1987). There is little

published work on c-myc expression in CIN but it is interesting that one group report a higher level of c-myc expression by epithelial cells from normal cervix than by cells from CIN or cervical carcinoma (Hendy-Ibbs et al., 1987). The same group obtained similar results in biopsies taken from testicular teratomas where c-myc was expressed at a higher level in well differentiated tumours than in undifferentiated tumours (Watson et al., 1986).

Although it is accepted that the c-myc product acts as a transcriptional regulator, its role in tumorigenesis is not yet understood and it is not therefore possible to ascribe functional significance to these findings at present.

PURPOSES OF THE PRESENT STUDY

PURPOSES OF THE PRESENT STUDY

Epidemiological data (Chapter 3)

This section was designed to obtain and collate information concerning the reproductive, contraceptive, sexual and smoking histories of patients with cervical intraepithelial neoplasia (CIN) and invasive carcinoma and of normal controls who attended the Lothian Area Colposcopy Clinic and the Royal Infirmary, Edinburgh.

This information made it possible to compare women with cervical neoplasia in South-East Scotland with normal controls in this area and with patients and controls elsewhere and to identify any epidemiological features distinguishing patients with cervical neoplasia from normal controls.

The local immune response to the human papilloma virus (HPV) and CIN in the uterine cervix (Chapter 4)

In this section the local immune response was characterized by enumerating Langerhans cells (LC) and T lymphocytes within normal and abnormal cervical transformation zone epithelium. LC and T cells were counted, any alteration in distribution and morphology noted, and expression of MHC Class II antigens HLA-DP, -DQ, and -DR assayed. An attempt was made to relate these features to the presence or absence of HPV infection (in the form of koilocytosis), to the grade of CIN, to such factors as smoking and oral contraceptive use, and to clinical outcome in order to elucidate the role played by local immune interactions in the development of cervical neoplasia.

Flow cytometric analysis of DNA content, c-myc protein expression and papillomavirus antigen expression in cervical biopsy and brush specimens (Chapter 5)

The DNA content of biopsies and brush specimens from normal and abnormal cervical epithelium was examined to look for evidence of aneuploidy. Particular attention was paid to the DNA content of koilocytic cervical epithelium, as it was felt that the detection of aneuploidy here would provide evidence to support the theory that HPV infection can lead to neoplastic change. The rate of cell division in koilocytic and neoplastic epithelium was also examined, in order to gain further understanding of the effects of HPV infection on cervical epithelium.

The c-myc oncogene is known to be abnormally expressed in numerous human tumours (see Chapter 1, page 63) and its expression in HPV infected and neoplastic epithelium was compared with expression in normal cervical epithelium. C-myc expression is known to be closely associated with stage of the cell cycle and a double staining technique was employed to examine the relationship between c-myc expression and cell cycle kinetics.

A similar technique was employed to relate expression of papillomaviral antigens to cell division.

Cervical intraepithelial neoplasia and cervical HPV infection in patients with lymphomas (Chapter 6).

This chapter describes a clinical survey designed to investigate the effect of immunosuppression on the prevalence of cervical HPV infection and CIN. Female patients with Hodgkin's and non-Hodgkin's lymphomas underwent cytological and colposcopic screening in order to compare the prevalence of cervical

abnormalities in this group with the prevalence of abnormalities in a normal control population. The data obtained from this survey provides further understanding of the role of the immune system in the development of cervical neoplasia.

CHAPTER 2

CHAPTER 2

PATIENTS AND METHODS

Patient groups

Controls

Three groups of control patients were studied. One group of 12 patients (control group "A") were women with normal cervical cytology who were referred to the Lothian Area Colposcopy Clinic due to concern about the appearance of the cervix, or because of post coital bleeding. None of these patients had ever had a suspicious cervical smear, but six had previously had inflammatory smears. One of these women was found to have CIN II and three had evidence of cervical HPV infection in the form of koilocytosis (n=2) or presence of HPV antigen (n=1) [see page 129]. These four women were included as controls for comparison with lymphoma patients (see below) but cervical biopsies and brushings from them were not included in the "normal control" group for other experiments. No colposcopic or histological evidence of koilocytosis or CIN was found in any of the remaining patients. In three of this group, cervical biopsies and/or brushings were examined by flow cytometry for the presence of HPV antigen, and found to be antigen negative. HPV antigen was not looked for in biopsies or brushings from the other five controls in this group, and the possibility of HPV infection was therefore not excluded.

A full reproductive, contraceptive, sexual, smoking and

cervical smear history was taken from all women in this group, and, after colposcopy and histology had revealed no abnormality, they were referred back to source for routine cytological screening. Four patients were given cold coagulation treatment to the cervical transformation zone to prevent further intermenstrual bleeding, before being referred back to source.

The second group of control patients (control group "B") were ten women undergoing minor gynaecological procedures (laparoscopic sterilisation, dilatation and curettage, etc) and three women who underwent total abdominal hysterectomy. Informed consent for cervical biopsy was obtained and colposcopic examination was not performed. All 13 women had had normal cervical cytology within the preceding three years and random biopsies taken from their cervical transformation zones were reported as containing no abnormality. Cervical biopsies from two of these women contained HPV antigen [see page 129] and they were therefore transferred from the "normal" group to the "HPV infection" group. Cervical biopsies from two further women in group "B" were found to be negative for HPV antigen, while biopsies from the nine remaining controls in this group were not analysed for the presence of HPV antigen. A sexual history was not taken from these patients, and they were not used as normal controls for the purpose of comparison with the lymphoma patients (see below). The third group of control patients (control group 'C') were 69 patients undergoing minor gynaecological procedures such as dilatation and curettage or laparoscopic sterilisation. They had never had a suspicious cervical smear and had had a normal smear within the preceding three years. A full reproductive, sexual, smoking, gynaecological

and cervical smear history was taken from all group 'C' controls and informed consent was obtained for colposcopy and cervical punch biopsy from each control. Colposcopic examination was performed on all women in this group. A proportion of these examinations were performed by the author, with the remainder being performed by another trained colposcopist, Dr Mohammed Alloub. Group 'C' controls were compared with lymphoma patients, but cervical biopsies from them were not processed for immunoperoxidase staining or for flow cytometry.

Patients with cervical HPV infection or CIN

Twenty three patients with evidence of cervical HPV infection in the form of koilocytosis, and 140 patients with CIN were studied. They had been referred to the Lothian Area Colposcopy Clinic on account of one or more suspicious cervical smears, or because of concern about the appearance of the cervix. A full reproductive, contraceptive, smoking and sexual history was taken, and full colposcopic examination of the lower genital tract carried out. Colposcopically directed punch biopsies were taken from all abnormal areas visualised, and in all but 13 of the patients included in this study, one biopsy specimen was divided; half being fixed for routine histopathology and half being snap frozen as described below. Three patients with koilocytosis only and 10 with CIN had biopsies taken for routine histopathology, but the biopsy was not divided and only cervical brushings were taken for the purpose of this study.

All patients with CIN II or III received treatment either on the same day, or 2-9 weeks later. The treatment given was either local ablative therapy to the cervical TZ in the form of CO₂ laser

or cold coagulation treatment, or cone biopsy. Patients with CIN I or koilocytosis alone received either cold coagulation treatment or entered a clinical trial in which they were randomised to receive no treatment, to use condoms, or to receive CO₂ laser treatment. All patients had a cervical smear taken 6 months after treatment, and repeat colposcopic examination and cervical smear 12 months after treatment.

Patients with invasive cervical carcinoma

Eleven patients undergoing extended abdominal hysterectomy for invasive squamous cervical carcinoma were studied. A reproductive, contraceptive and smoking history was taken from each patient, but sexual histories were not obtained. Biopsy specimens were taken from the tumour (n=7), from adjacent cervical epithelium (n=7) and from external and internal iliac lymph nodes (n=11) within 20 minutes of the uterus and nodes being removed from the patient. Each biopsy specimen was divided; half was fixed for routine histopathology and half was snap frozen (see below)

Lymphoma patients

A group of 45 female patients attending the Department of Haematology, Royal Infirmary, Edinburgh under the care of Dr A.C. Parker or Dr C.A. Ludlam were invited to participate in this study. They were diagnosed as suffering from Hodgkin's or non-Hodgkin's lymphomas, all had been sexually active in the past or at present, and were between the ages of 16 and 60. Twenty seven agreed to take part in the study. Ten patients did not wish to participate and eight were excluded for other reasons; five having undergone total abdominal hysterectomy, one being pregnant, and two living out of Edinburgh.

A full history (as for CIN patients) was taken from all patients and they underwent full colposcopic examination. A cervical smear was taken from all patients while cervical punch biopsies were taken only if a colposcopic abnormality was visualised.

Methods

Colposcopy

The cervix was visualised using a Cusco's bivalve speculum and mucus removed from it using a dry swab. The colposcope was then focussed on the cervix using a magnification of x10 to allow a large area around the external os to be seen in one field. Magnification was increased to x16 to permit a more detailed view of a smaller field and optimal contrast was obtained by insertion of a green filter.

The vascular pattern of cervical epithelium was examined and abnormalities such as punctation and mosaicism sought. These abnormalities may signify neoplastic change. "Atypical" vessels, i.e. terminal vessels strikingly irregular in size, shape, course and mutual arrangement are found almost exclusively in microinvasive and invasive disease. The surface contours of the cervix and any lesions seen were examined.

Three per cent acetic acid was applied to the cervix at this stage. This coagulates cytoplasmic and nuclear proteins of the epithelium making them opaque and white. In CIN III, which is characterised by cell "crowding", there are more cells and thus more protein per unit area of epithelium than in CIN I or in normal squamous epithelium. CIN III therefore appears whiter following

the application of acetic acid than CIN I or normal epithelium. Vascular changes such as punctation and mosaicism become more apparent when acetic acid has been applied, and the border between abnormal and normal epithelium can be visualised. CIN II and CIN III are characterised by sharp boundaries while CIN I and immature squamous metaplasia possess less distinct borders. Flat koilocytic lesions and condylomata acuminatum may take on the appearance of acetowhite islands surrounded by normal squamous epithelium and can thus be distinguished from areas of CIN which are found within the TZ and therefore very rarely appear to be completely surrounded by normal squamous epithelium.

Aqueous iodine solution B.P. (Ransom and Son plc) was then applied. Glycogen appears dark brown after the application of iodine; thus native squamous epithelium and mature metaplastic squamous epithelium is stained brown. Columnar epithelium, areas of immature metaplasia, some inflammatory lesions and CIN lesions contain much smaller amounts of glycogen and are stained a light yellow. After the menopause squamous epithelium becomes thinner, loses much of its glycogen content and thus also takes on a light yellow appearance after the application of iodine. Iodine application destroys the finer detail seen after the application of acetic acid but is useful in demarcating the extent of any abnormality and particularly in determining whether or not lesions extend on to the vagina. For a more detailed description see Cartier (1977).

Cervical Cytology

a) Spatula samples

An Ayre's spatula was used to obtain samples for routine

cervical cytology. The "hook" end of the spatula was placed in the cervical os and rotated through 360° (see figure 3). The exfoliated cells obtained were then spread on a glass slide and fixed immediately in methylated spirit (740P). Smears were then processed for routine cytological screening and graded according to the standard Lothian Area classification as follows:

- 0 = unsatisfactory
- 1 = normal
- 1+ = inflammatory changes bordering on mild dyskaryosis
- 2 = dyskaryosis consistent with CIN I or CIN II
- 3 = malignant cells seen, consistent with CIN III or
invasive carcinoma.

Koilocytes (Meisels and Fortin, 1976) were reported if present.

The cervical smears taken from patients with lymphomas did not go through the routine screening process but were read by Dr M. Colquhoun, cytopathologist. All previous smears from these patients that could be traced were reviewed by Dr Colquhoun.

b) Cervical brush specimens

A Cytobrush (Medscand) (Glenthoj et al, 1986) was used to obtain these specimens with the bristle end of the brush bent over at an angle of about 50° . The distal end was inserted just inside the external os and the brush rotated through 360° in order to obtain exfoliated cells from the whole of the TZ. The plastic holder was then cut in half and the brush placed in a sample tube containing 5 ml methanol or 5 ml 1% formol saline in phosphate buffered saline (PBS) [see figure 11]. The sample was stored at room temperature until processed further. Satisfactory results were obtained from samples stored for up to four months in this



Figure 11. The Cytobrush (Glenthoj et al, 1986)

fashion.

Cervical biopsy

Biopsy specimens of cervical epithelium may be obtained by punch biopsy, wedge biopsy, endocervical curettage or cone biopsy. Only punch biopsy was used in this study; the other techniques will not therefore be discussed.

Punch biopsies were taken from the TZ of patients and controls using Schumacher biopsy forceps. Those from patients and from group A and group C controls came from coloscopically abnormal areas. Cervical biopsies were not taken from patients with lymphomas if the cervix appeared entirely normal. The biopsy specimen was immediately divided; half was placed in fixative for histopathological assessment as part of the routine diagnostic process and half was snap frozen in O.C.T. compound (Mackay and Lynn) on dry ice, and stored at -70°C until processed further.

Histopathology

A cryostat section $8\ \mu\text{m}$ in thickness was cut from the centre of each biopsy specimen and placed on a slide. It was air dried, fixed in acetone at 4°C for 10 minutes and stained with eosin (5 seconds) and Mayer's haemalum (20 seconds) for routine histopathological assessment. CIN was graded according to recognised criteria (Buckley *et al*, 1982) and features suggestive of HPV infection (koilocytosis, multinucleation, individual cell keratinisation) were reported if present.

Major histocompatibility (MHC) class II antigen, Langerhans cell (LC) and T cell staining

Serial cryostat sections ($8\ \mu\text{m}$ thickness) were prepared, mounted on poly L-lysine coated slides, fixed in acetone at 4°C for

10 mins, and air dried. Sections were then stained using an indirect immunoperoxidase technique adapted from the method of Carr et al (1986). Briefly, sections were incubated in 20% normal rabbit serum (NRS) in 0.001M Tris-buffered saline (TBS) pH 7.6 for 20 mins. Excess serum was shaken off and sections then incubated for 45 minutes with mab (Table 2) at room temperature. DAKO T6 is ascitic fluid (Thomas et al, 1982) and all other mabs are culture supernatants (Watson et al, 1983; Pawelec et al, 1982; Lampson & Levy, 1980; Guy et al, 1982). The optimal dilution of antibody made in 2% NRS was determined by titration. Negative controls were prepared by substituting 2% NRS for mab. Sections were then washed twice in 2% NRS and incubated with a rabbit antiserum to mouse immunoglobulins conjugated with horseradish peroxidase (DAKOPATTS) diluted 1 in 80 in 2% NRS for 15 mins. The enzyme reaction was developed using diaminobenzidine (Sigma, Poole, Dorset) with 0.068% imidazole (BDH) and 0.02% hydrogen peroxide in Tris buffer (pH 7.4). All incubations were carried out in a moist chamber.

Quantification of LC and T cells

The squamous epithelium included in each section was photographed. If the whole area of epithelium was too large to be included in one frame a series of overlapping photographs were taken. These were printed and assembled as a composite representation. The outline of the squamous epithelium photographed was then traced on to tracing paper and the outline cut out. The tracing paper representing each sample was weighed and the surface area of the squamous epithelium calculated from this measurement. Cells possessing at least two dendrites

Table 2

Origin and specificity of monoclonal antibodies.

Monoclonal antibody	Working dilution	Origin	Specificity
DAKO-T6	1:20	DAKOPATTS (Thomas <u>et al</u> 1982)	Langerhans cells, common thymocytes
DA6.231	1:10	K. Guy (Guy <u>et al</u> 1982)	HLA-DP, HLA-DQ, HLA-DR
B7/21	neat	I. Trowbridge (Watson <u>et al</u> 1983)	HLA-DP
TU22	neat	A. Ziegler (Pawelec <u>et al</u> 1982)	HLA-DQ
L243	1:20	American Type Culture Collection (Lampson & Levy 1980)	HLA-DR
UCHT1	1:10	UNIPATH	CD3 (expressed by all T lymphocytes)
DAKO-T4	1:10	DAKOPATTS	CD4 (expressed by most helper/inducer T lymphocytes)
DAKO-T8	1:50	DAKOPATTS	CD8 (expressed by all suppressor/cytotoxic T lymphocytes)

attached to a cell body and staining positively with DAKO T6 were classified as LC. All T6 positive LC or CD3 positive T cells within the epithelium were then counted and counts expressed as cells per mm² as this has been found to be most reliable method of enumerating LC (De Jong et al, 1986).

Statistical analysis

The significance of the difference between each group and the control group was calculated using the Mann-Whitney U test.

Preparation of nuclei for flow cytometry

A single 8 µm cryostat section was taken from the centre of each cervical, prostatic and lymph node biopsy specimen to be processed for flow cytometry, and prepared for routine histopathological assessment as described above. The remainder of the specimen was thawed, minced and incubated according to a modification of the method of Hedley et al. (1983). It was incubated in 3 ml 0.5% pepsin solution (Sigma) in 0.01M phosphate buffered saline (PBS), with the pH adjusted to 1.5 with hydrochloric acid, at 37°C for 45-60 minutes. The supernatant was drawn off and centrifuged (all concentrations were at 700 x g for 10 minutes), and the resultant pellet washed in PBS and respun. Any clumps of nuclei were dispersed by syringing through a fine gauge needle (26G).

Cervical brush specimens were prepared for flow cytometry by first centrifuging the entire specimen (including the brush) at 700 x g for 10 minutes and removing the methanol or formol saline. After one wash in PBS and a further centrifugation the sample was resuspended in 5 ml pepsin solution (as described above). After incubation for 45 minutes at 37°C the brush was agitated in the

solution and then discarded. The nuclei released by the procedure were then centrifuged and the supernatant discarded. The nuclei were washed in PBS and respun.

Staining of nuclei for flow cytometry

1. Nuclear antigens

Nuclei were fixed in 1% formol saline in PBS containing 2% normal sheep serum (NSS) for 10 minutes at room temperature. They were centrifuged and resuspended in 20% NSS in PBS at a concentration of 10^6 nuclei/ml for 20 minutes, and nuclear antigens stained by a modification of the method of Elias-Jones et al (1986). Nuclei were centrifuged and resuspended in primary antibody (200 μ l per 10^6 nuclei) overnight at 4°C (see table 3). Primary and secondary antibodies were diluted in 2% NSS in PBS. Nuclei were then washed twice in 2% NSS in PBS and fluorescein isothiocyanate (FITC) conjugated secondary antibody (see table 3) added for 45 minutes at 4°C. Nuclei were then washed twice in 2% NSS in PBS and stored in 1% formol saline in the dark. Nuclei could be stored under these conditions for up to two weeks with no loss of staining. Controls were prepared by substituting 2% NSS for primary antibody. Chippings obtained from six patients at trans-urethral resection of prostate for benign prostatic hypertrophy were snap frozen and processed as described for the cervical biopsies. They were then stained with antibody to papillomavirus antigens and used as controls for these experiments.

2. Nuclear DNA

The nuclear suspension was stained with a modified method of Deitch et al (1982) (see figure 12).

Nuclear pellets were dispersed in 2 ml solution containing 50

Table 3
Origin and specificity of antibodies used for flow cytometry

Primary antibody	Specificity	Dilution	Source	Ref	Secondary antibody	Dilution	Source
Rabbit anti-bovine papillomavirus type 1	Papillomavirus genus-specific antigens	1/50	DAKOPATTS	Kurman et al 1984	Fluorescein isothiocyanate conjugated sheep anti-rabbit immunoglobulin	1/30	Wellcome
Myc 1-6 E10	p62 c-myc	1/10	G. Evan	Evan et al 1985	Fluorescein isothiocyanate conjugated sheep anti-mouse IgG F(ab') ₂ fragment	1/40	Sigma

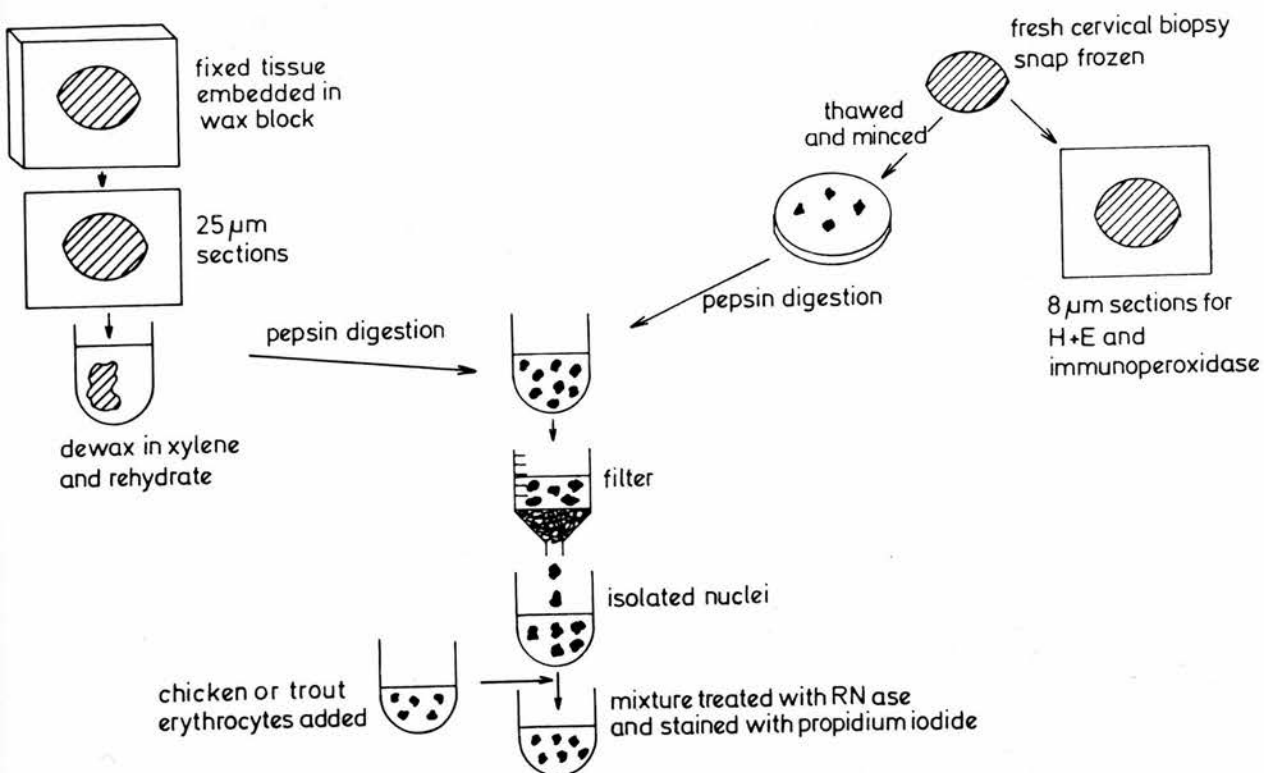


Figure 12. Method of preparing nuclei for DNA staining (see text for explanation).

μg propidium iodide (Sigma) per ml in 0.01M Tris (pH 7.0) with 5 mM magnesium chloride, 0.1% Triton X-100 and 15 μg ribonuclease (Sigma) per ml and incubated at 4^oC for 15 minutes to 2 weeks. DNA staining was carried out on unstained nuclei (immediately after pepsin digestion) or on nuclei stained with antibodies to nuclear antigens and FITC labelled conjugates.

Chicken red blood cells (CRBC) were run with each sample as an internal control, the DNA content of such cells being 35% of that of a human diploid cell (Vindelov et al, 1983) [figure 13]. They were stained with the propidium iodide solution as described above. Human peripheral blood lymphocytes were stained and run with CRBC as a further DNA control at the start of each run.

Flow cytometer

The flow cytometer employed was an EPICS 'C' (Coulter) [figure 14]. The stained nuclear suspension was injected into the flow cell [see figure 9] where it was hydrodynamically focussed into "indian file" for passage through the interrogation point. The nuclei interacted at this point with the laser beam. The laser in the EPICS 'C' flow cytometer is an argon ion laser which emits light at 488 nm and was set at an output of 100-200 mW. Light is scattered in all directions by nuclei crossing the laser beam. Light scatter in the forward direction is related to the size of the nucleus and that at 90^o to the degree of refraction of its internal structure. Excitation of propidium iodide and FITC occurred at the interrogation point with emission of light of a longer wavelength (see figure 9). This was also collected at 90^o; selected wavelengths were directed by dichroic mirrors to photomultiplier detectors and unwanted fluorescence and scattered

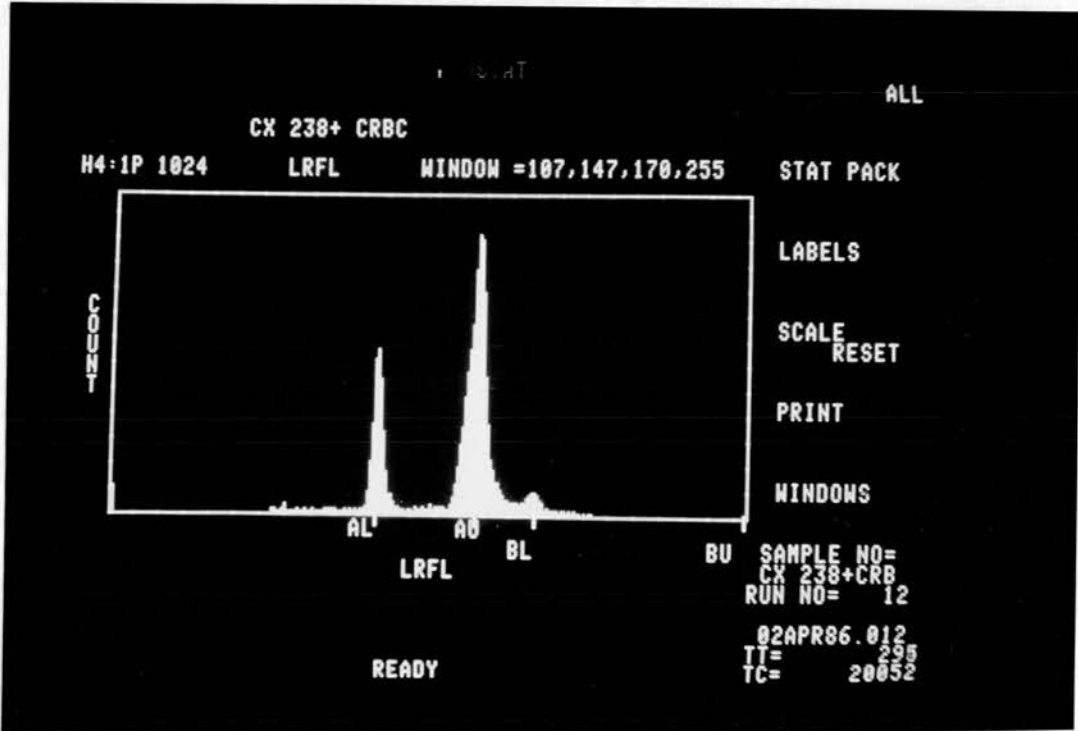


Figure 13. Representative DNA histogram showing cervical nuclei and chicken red blood cell nuclei (CRBC) (LRFL = log red fluorescence, AL = CRBC, AU = cervical nuclei in G_0/G_1 phases, BL = cervical nuclei in $G_2 + M$ phases¹).



Figure 14. EPICS 'C' flow cytometer

laser light were blocked by optical filters. Electrical signals were generated for each particle and converted to digital signals for processing by computer software in order to generate one parameter or two parameter correlated histograms.

Flow cytometry - nuclear antigen staining

A total of 40,000 nuclei were analysed from each sample at a flow rate of 400-500 nuclei/second. The nuclear fraction was selected by gateing on a one-parameter histogram measuring forward angle light scatter (FLS) or log FLS (LFLS) and the percentage of nuclei showing fluorescence greater than the background level was recorded. Fluorescent signals were simultaneously acquired from a two-parameter histogram measuring FLS against 90° light scatter, using a bit map around the nuclear population. The results from both histograms differed by less than 2%, and all results presented are those acquired by gateing on LFLS signals.

Flow cytometry - nuclear DNA staining

Nuclei were run at a rate of 150/second until 20,000 stained nuclei had been counted. The data obtained were subjected to parametric analysis with the EPICS PARA 1 program, which determined the proportion of nuclei in each phase of the cell cycle [see figure 15].

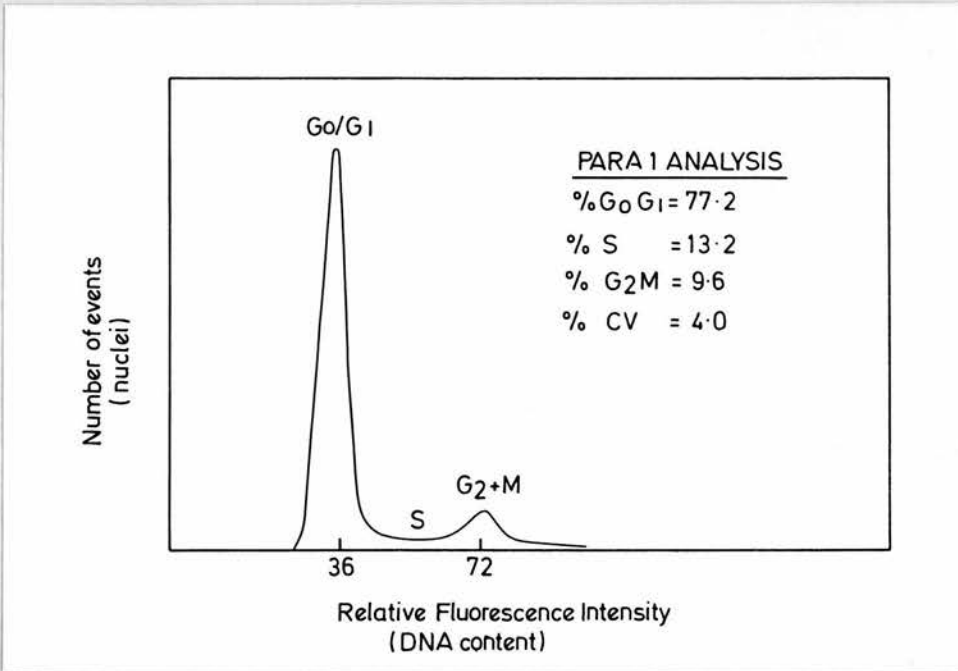


Figure 15. Representative DNA histogram.

CHAPTER 3

CHAPTER 3
EPIDEMIOLOGY

Introduction

The subjects of this thesis were compared to patients with CIN studied elsewhere, and any differences in their sexual, contraceptive or smoking habits identified. Patients with papillomavirus infected or neoplastic cervical epithelium were compared to normal controls, in order to discover whether the risk factors described by other authors (see Chapter 1, "The epidemiology of squamous carcinoma of the uterine cervix") also apply to women in South-East Scotland.

Results

The information obtained from the three groups of normal controls and from patients with HPV infection, CIN and invasive carcinoma is shown in table 4. It can be seen that the age range of patients in all groups was very similar, except for group C controls who were slightly older. The percentage of parous patients and the percentage of women using the oral contraceptive and barrier methods of contraception was similar in all groups while the percentage of patients with CIN III and with invasive disease who smoked was significantly higher than the percentage of women in the control groups who smoked ($\chi^2 = 10.87$, $P < 0.01$ if patients with CIN III and invasive disease are compared with Group C controls alone; $\chi^2 = 8.49$, $P < 0.01$ if these patients are compared with Group A + C controls and $\chi^2 = 6.48$, $p < 0.02$ if they are compared with Group A,B and C controls). The difference between patients and controls is not significant ($\chi^2 = 1.48$ $P >$

Table 4
General Clinical Data

Cervical Histology	mean age (range)	Number (%) parous	Coitarche (range)	Median no. sexual partners (range)	Number (%) smokers	Current OCP use (%)	Number (%) using barrier methods
normal* (group A) (n = 8)	29 (19-40)	7 (88)	18 (15-21)	3 (1-7)	6 (75)	3 (38)	2 (25)
normal* (group B) (n = 11)	32 (26-43)	9 (69)	N.K.	N.K.	8 (73)	2 (18)	2 (18)
normal* (group C) (n = 48)	41 (24-61)	41 (85)	19 (15-24)	2 (1-3)	20 (42)	9 (19)	6 (13)
HPV infection alone (n = 26)	30 (17-45)	21 (81)	18 (14-22)	2 (1-7)	15 (58)	8 (31)	6 (23)
CIN I (n = 38)	30 (18-44)	23 (61)	18 (16-22)	4 (1-12)	18 (47)	11 (29)	9 (24)
CIN II (n = 39)	29 (17-37)	29 (74)	18 (16-22)	2 (1-12)	19 (49)	13 (33)	4 (10)
CIN III (n = 63)	31 (21-47)	53 (84)	18 (18-22)	3 (1-25)	45 (71)	18 (29)	5 (8)
Invasive carcinoma (n = 11)	34 (28-54)	9 (81)	N.K.	N.K.	8 (73)	3 (27)	2 (18)

* See text. All controls with CIN, koilocytosis or with positive staining for HPV antigen have been excluded from control groups A, E and C.

** This group includes patients with histological evidence of cervical HPV infection, or whose cervical biopsies contained HPV antigen.

n = number of patients

N.K. = not known

0.1) if CIN I and II patients are included with CIN III and invasive disease for comparison with Group A,B and C controls. A sexual history was not obtained from group "B" normal controls or from patients with invasive carcinoma but in all other groups age at coitarche and median number of sexual partners was very similar, and did not vary significantly between groups.

Tables 5 and 6 contain information concerning number of sexual partners and age at coitarche of the patients and controls included in the present study, and of patients with CIN and controls studied as part of four recent epidemiological surveys conducted elsewhere in Europe. La Vecchia et al (1984) and Zaninetti et al (1986) studied women in Milan, Italy, Vonka et al (1984) women in Prague, Czechoslovakia and Hellberg et al (1986) women in Falu, Sweden. The women studied by Zaninetti et al were all younger than 20.

It can be seen from table 5 that the number of sexual partners reported varied greatly from country to country, and that there were larger differences between controls in different studies than between controls and cases within some studies. Thus 9% of the controls, and 23% of patients with CIN in the group studied by La Vecchia et al reported three or more sexual partners while the corresponding figures from Hellberg et al are 62% and 82%.

All investigators, however, found that patients with CIN reported, on average, more sexual partners than did the normal controls. The difference was not always statistically significant.

It can be seen from table 6 that, again, cases and controls within each group studied tend to be more similar than cases or controls from different groups, in terms of age at coitarche. Thus 55% of controls and 70% of patients with CIN questioned by Hellberg

Table 5

A comparison of the results of 5 epidemiological studies of patients with CIN and normal controls
a) Number of sexual partners

No. of sexual partners	La Vecchia (1984)		Zaninetti et al (1986)		Vonka et al (1984)		Hughes (1988)		Hellberg et al (1986)	
	controls (n=183) %	CIN (n=183) %	controls (n=1914) %	CIN (n=126) %	controls (n=2,778) %	CIN (n=1386) %	controls (n=56) %	CIN (n=119) %	controls (n=265) %	CIN (n=133) %
< 1	82	65	64	41	33	26	41	26	20	8
2	8	10	16	24	20	20	25	21	16	8
3-5	[[[[27	29	25	39	32	28
6-10	9	23	20	35	20	25	9	9	21	36
> 10]]]]]]	-	6	9	18

significance (CIN patients vs controls) p < 0.01

$\chi^2 = 19.7$
p < 0.01

significant difference between CIN I and II and controls but not between CIN III and controls (χ^2 test)

N.S.
(Mann-Whitney U test)

$\chi^2 = 26.3$
p < 0.001

n = number of patients

Table 6

A comparison of the results of 5 epidemiological studies of patients with CIN and normal controls
b) Age at coitarche

Study	Age at coitarche	% controls (n=183)	% patients with CIN (n=183)	significance (controls vs CIN patients)
La Vecchia <u>et al</u> (1984)	≤ 17	9	21	N.S.* when adjusted for number of partners
	18-22	48	49	
	> 23	43	31	
Zaninetti <u>et al</u> (1986)	≤ 14	9	14	N.S.
	15	20	21	
	16	27	27	
	17	27	19	
	18-19	20	19	
Vonka <u>et al</u> (1984)	≤ 17	23	30	p < 0.05 (χ^2 test)
	18	28	28	
	> 18	49	42	
Hughes (1988)	≤ 14	-	3	N.S. (t.test)
	15	5	4	
	16	2	12	
	17	17	27	
	18	26	20	
	19	17	15	
	20	14	8	
	21	12	4	
	22	-	7	
	23	5	-	
	24	2	-	
Hellberg <u>et al</u> (1986)	≤ 14	14	22	p < 0.01 (t.test)
	15	41	48	
	16-19	36	23	
	> 20	9	7	

* N.S. = not significant
n = number of patients

et al were 15 or younger when they started to have sexual intercourse while only 9% of controls and 21% of CIN patients in the La Vecchia et al study were 17 or younger at the commencement of sexual activity. There was a trend (corresponding to that seen in relation to number of partners) for CIN patients to have been younger than control patients at coitarche, but this was significant in only two of the studies.

Table 7 contains data concerning smoking from four of the five studies. Again, considerable differences can be seen between countries.

Clinical data obtained from lymphoma patients is contained in Chapter 6.

Discussion

The data presented here was collected in order to discover whether or not women with CIN living in South-East Scotland share epidemiological characteristics with patients with CIN living elsewhere, and to see whether it was possible to separate these women from "normal" controls on the basis of epidemiological features. As discussed earlier in this chapter, women with CIN in Italy, Sweden, Czechslovakia and Scotland differ considerably from one another in terms of sexual behaviour. It is clear, therefore, that epidemiological data acquired from one country cannot be used to calculate the relative risk of CIN elsewhere and that epidemiological information including age at coitarche and number of sexual partners should be collected from controls and cases drawn from the same population.

It is interesting, however, to note that the incidence of cervical carcinoma in Sweden in the 1970s was almost twice as high

Table 7

A comparison of the results of 4 epidemiological studies
of patients with CIN and normal controls
c) smoking history

Study	Controls (%)	current smoking CIN patients (%)
Zaninetti <u>et al</u> (1986)	55	49
Vonka <u>et al</u> (1984)	15	25
Hughes (1988)	51	59
Hellberg <u>et al</u> (1986)	41	69

as in Scotland (annual incidence per 100,000 females 17.7 in Sweden and 10.1 in Scotland - Waterhouse et al, 1976. The equivalent Italian figure is not available) and to note that Hellberg et al (1986) reported that normal controls in Sweden had had more sexual partners and were younger when they became sexually active than the Scottish patients with CIN in this study. It is tempting to speculate that this difference in sexual behaviour is responsible for the higher incidence of cervical carcinoma in Sweden. Of course, other factors such as screening policies must also be considered. Sweden does, however, have a well organised cervical screening programme which was in existence in 1973 (Laara et al, 1987) and which covers a larger proportion of the population than is covered in Scotland (Laara et al, 1987 and Scottish Health Statistics, Advance Table, 1987) so that poor screening cannot be blamed for the higher incidence of cervical carcinoma in Sweden.

Information concerning the male partner's sexual history was not obtained for this study, or for the other surveys discussed above. This is an important gap, as evidenced by the high rate of cervical cancer in South America where the majority of women have only one sexual partner (see Chapter 1 "The epidemiology of squamous carcinoma of the uterine cervix").

It should be noted that in the present study no significant difference was detected between the normal controls and the patients with cervical HPV infections and cervical neoplasia, in terms of parity and sexual parameters. The controls in group A cannot be regarded as entirely normal as there had been sufficient clinical concern about all of them (because their cervix appeared abnormal, or because of intermenstrual bleeding) to warrant

referral to the Colposcopy Clinic. The group 'C' controls can, however be regarded as normal controls, and it is interesting that they did not differ significantly from patients with CIN, in terms of sexual history. As discussed in the Results section of this chapter, the difference in number of sexual partners reported by cases and controls in the four other studies cited was often relatively small and would not have achieved statistical significance if the numbers studied had been smaller, while the difference in age at coitarche was not significant in two of the studies. Significantly more patients with CIN III and invasive disease than control were cigarette smokers. No attempt has been made here to adjust for sexual history. Nevertheless, this finding adds further support to the increasingly popular contention that cigarette smoking plays a significant role in the aetiology of cervical neoplasia (see p. 26 and p. 44).

CHAPTER 4

CHAPTER 4**LOCAL IMMUNE INTERACTIONS AND MAJOR HISTOCOMPATIBILITY CLASS II
EXPRESSION IN THE CERVICAL TRANSFORMATION ZONE.**Introduction

The nature of an effective immune response to papillomavirus infections is not well understood, although cell-mediated immunity is thought to be more important than humoral immunity (Thivolet et al, 1982). It is possible that a deficient immune response to human papillomavirus (HPV) contributes towards the development of neoplasia. The Langerhans cell (LC) is the predominant antigen presenting cell (APC) in cervical epithelium and koilocytic and neoplastic cervical epithelium has been shown by some authors to contain fewer LC than normal cervical epithelium (see Chapter 1 "Langerhans cells in HPV induced lesions and in cervical neoplasia"). The ability of the LC to present foreign antigen to T-lymphocytes is dependent on the expression of surface MHC class II antigens (Pehamberger et al, 1983), the level of which is known to be variable (Berman et al, 1985) and which reflects the state of "activation" of the cell.

The purpose of the experiments outlined in this chapter was to relate the number, distribution and morphology of LC and their expression of MHC Class II antigens to the presence of HPV infection and of CIN I-III. The number and phenotype of T cells were examined in relation to these epithelial abnormalities.

Results

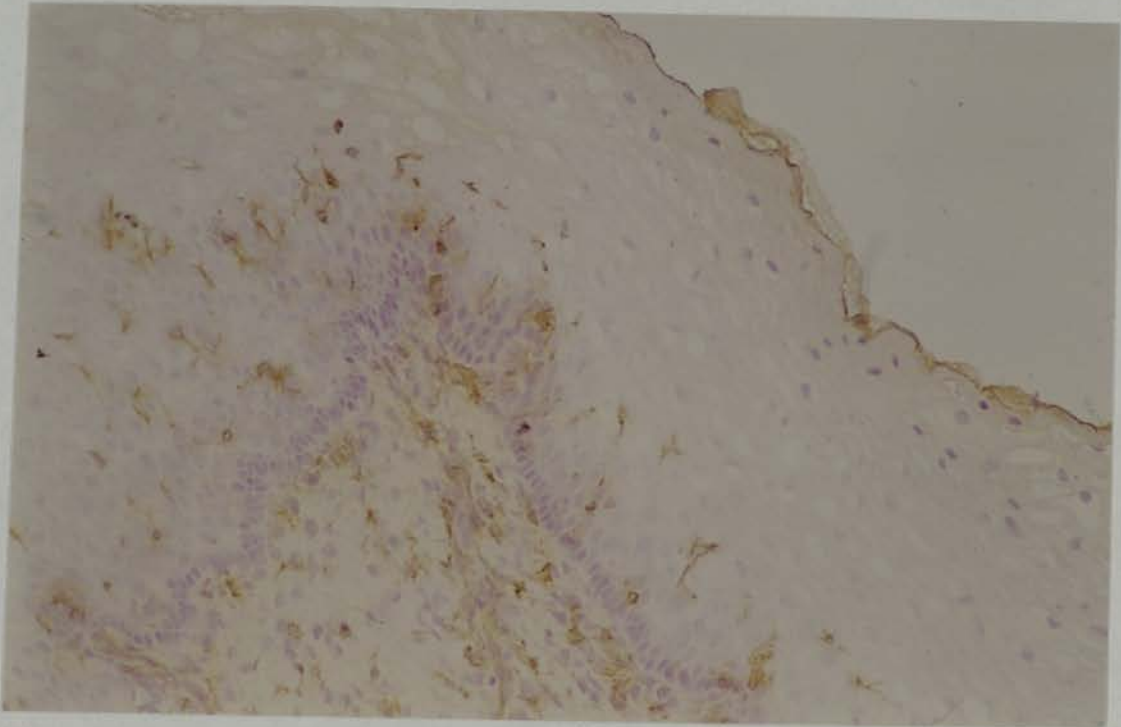
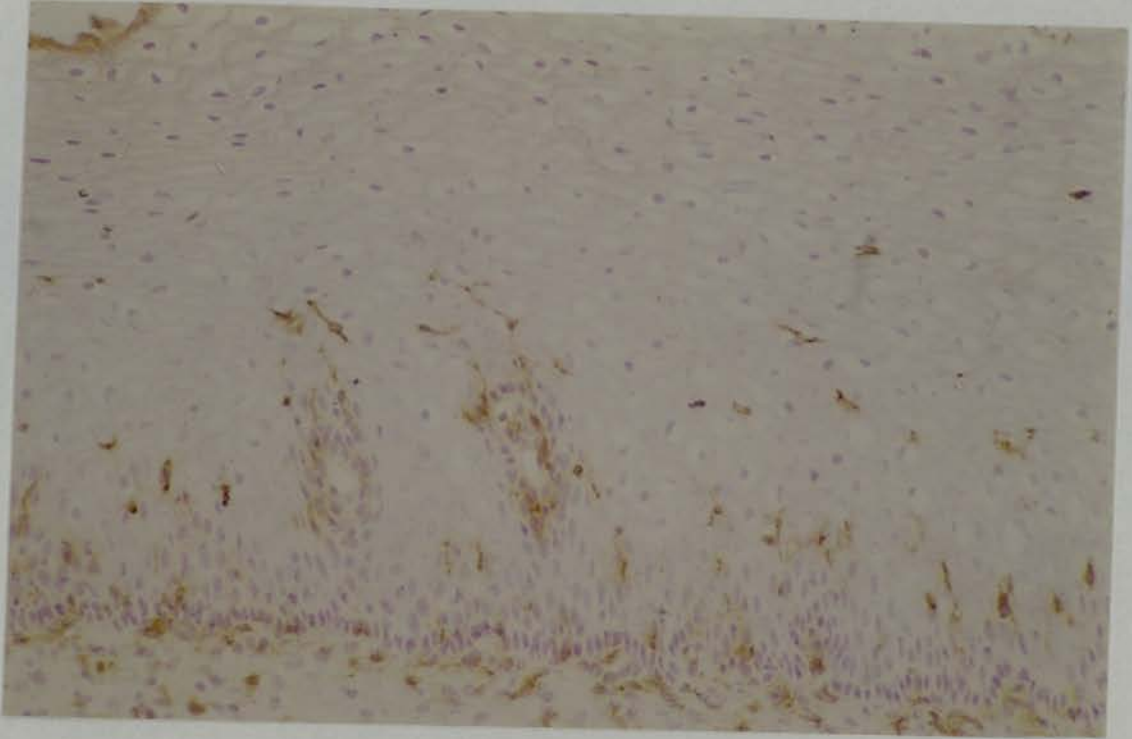
Distribution and number of LC

In specimens taken from normal cervical transformation zones LC were found predominantly in the lower half of the epithelium [figures 16 and 17]. The slender dendritic processes stained strongly with DAKO T6 and appeared to link adjacent LC [see figure 6]. No significant differences in LC morphology, distribution, number or MHC Class II expression were seen if biopsy samples from the two groups ('A' and 'B') of normal controls were compared. In specimens taken from areas of CIN [figures 18 and 19] LC were distributed throughout the entire thickness of the epithelium and their dendrites were much shorter and less numerous. Figure 20 demonstrates that T6 positive LC were significantly reduced in number ($P < 0.01$) in all abnormal groups except CIN I.

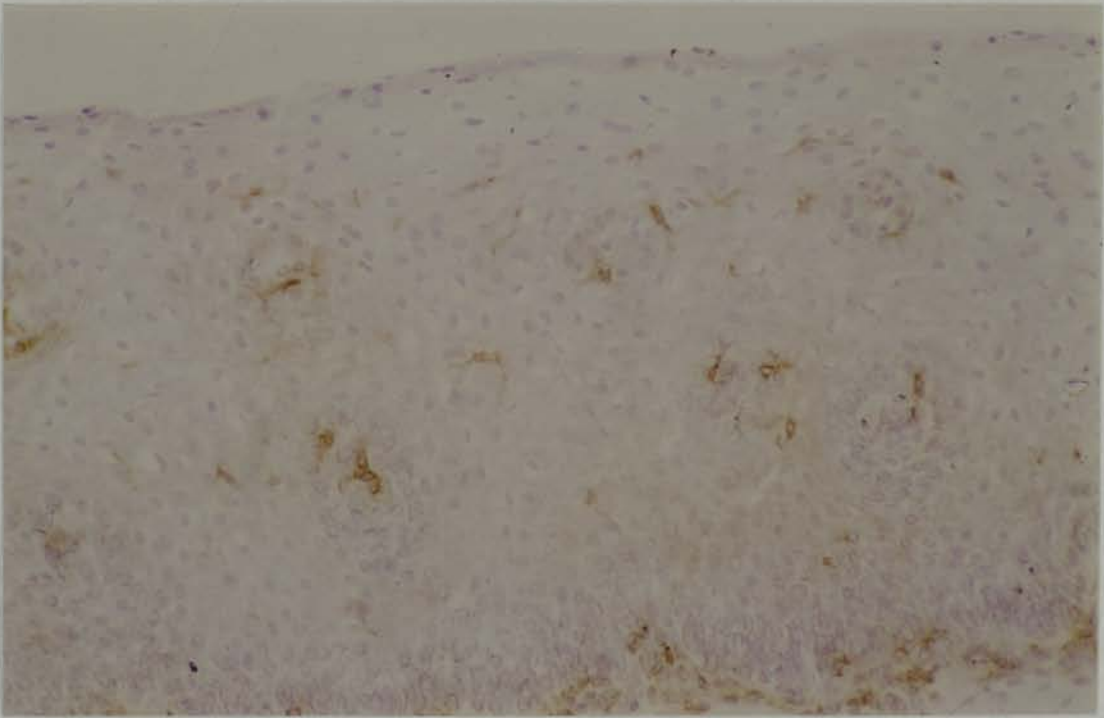
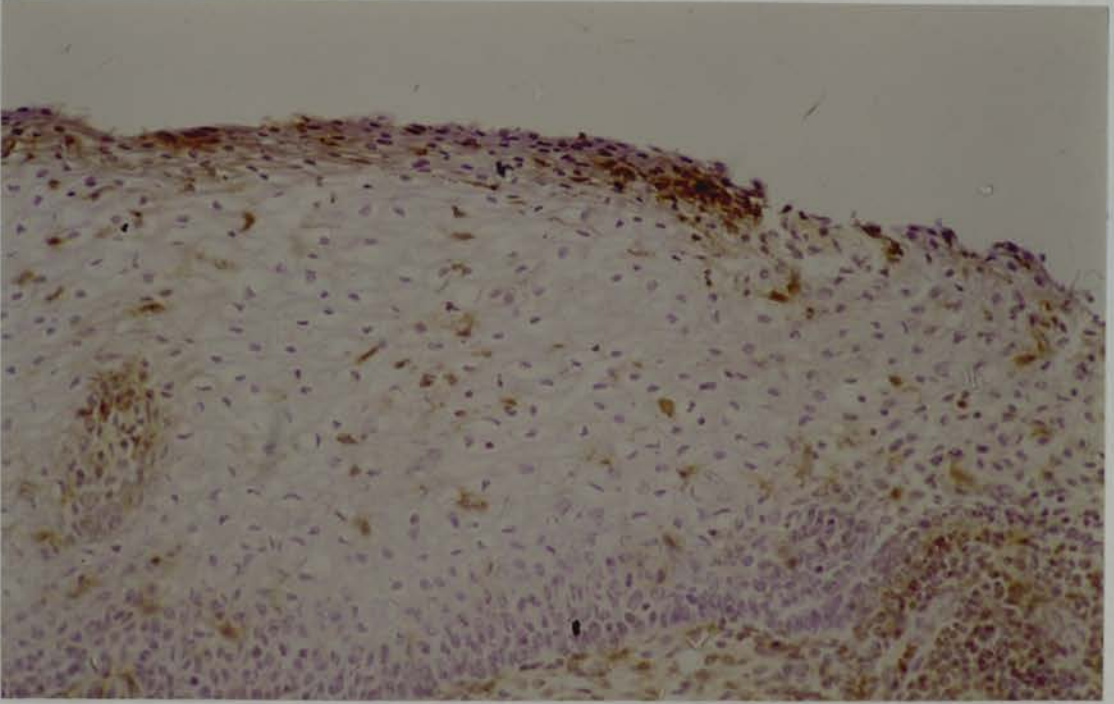
Expression of MHC class II antigens by LC

When the mab DA6.231 was used the number of positively staining dendritic cells closely paralleled the number of T6 positive cells although it can be seen from Figure 20 and Table 8 that T6 positive dendritic cells always exceeded DA6.231 positive dendritic cells in number. Some LC present were therefore not expressing Class II antigens, or were expressing these antigens at undetectable levels. In each specimen there were some DA6.231 positive, T6 negative, non-dendritic cells and these were assumed to be activated T lymphocytes. This was confirmed in some cases (see below) by the use of mabs to T cell markers.

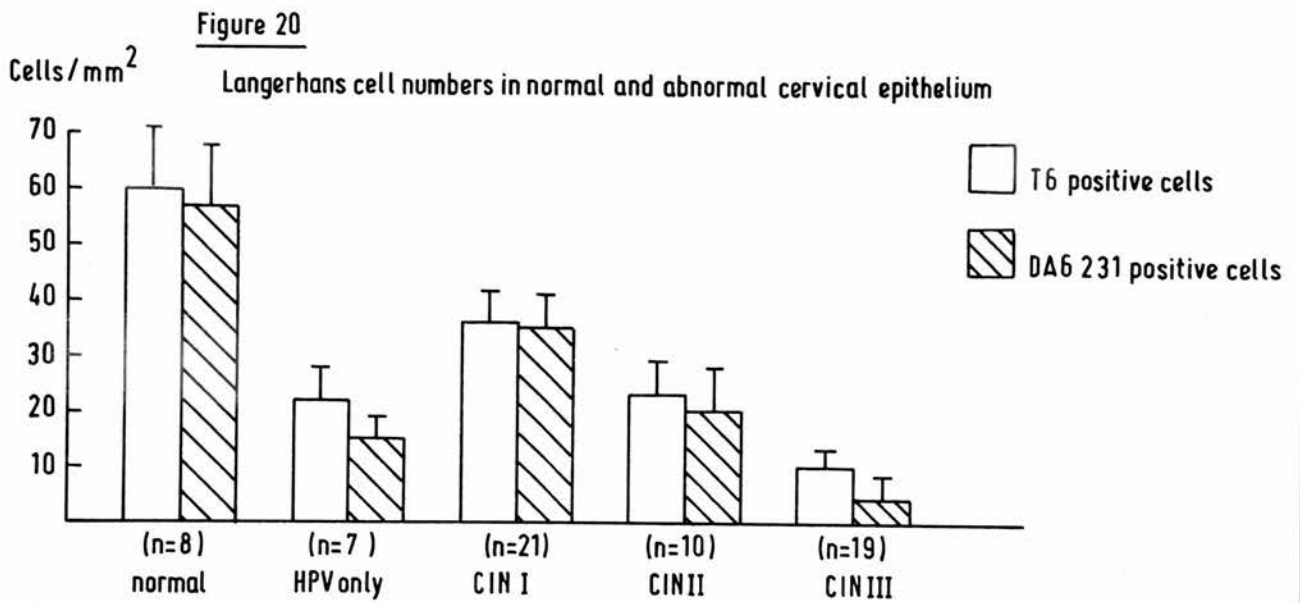
Table 8 shows the pattern of expression of HLA-DP, -DQ and -DR by LC. A significantly higher percentage of LC in HPV infected epithelium and in CIN I and II expressed HLA-DR when a comparison



Figures 16 and 17. DAKO-T6 staining of normal cervical transformation zone showing dendritic Langerhans cells in the lower half of the epithelium (magnification x 125).



Figures 18 and 19. DAKO-T6 staining of CIN I showing Langerhans cells throughout the epithelial thickness (magnification x 125).



Bars represent mean value + S.E.M. in each group. The "normal" group consists of five group 'A' and three group 'B' histologically normal controls. It contains two biopsy specimens which were subsequently analysed by flow cytometry and found to be HPV antigen negative. The remaining six specimens were histologically normal but were not tested for HPV antigen. The "HPV only" group includes six biopsy specimens with histological evidence of HPV infection, without CIN, and one histologically normal biopsy specimen, which was HPV antigen positive. All abnormal groups, except CIN I, contain significantly fewer T6 positive and DA6.231 positive dendritic cells per mm² sectional area than the normal group ($p < 0.01$). Significance calculated by Mann-Whitney U-test.

Table 8

Percentage of LC (T6-positive dendritic cells) expressing MHC class II antigens in normal and abnormal cervical epithelium

	+ DA6.231 mean (range)%	DR mean (range)%	DP mean (range)%	DQ mean (range)%
normal ⁺⁺ (n = 6)	94 (81 - 98)	53 (41 - 73)	42 (13 - 56)	19 (7 - 52)
HPV infection only (n = 5)	76 (63 - 95)	89* (64 - 117)	9* (4 - 26)	23 (9 - 46)
CIN I (n = 14)	93 (61 - 99)	72* (52 - 93)	47 (21 - 82)	65** (10 - 91)
CIN II (n = 8)	88 (65 - 100)	81* (61 - 94)	46 (27 - 69)	59* (8 - 73)
CIN III (n = 11)	72 (53 - 92)	46 (33 - 78)	85* (43 - 98)	57* (4 - 81)

n = number of patients

+ The percentage of LC staining positively with each MHC Class II mab was calculated for individual specimens before obtaining a mean value.

++ The "normal" group consists of three group 'A' and three group 'B' histologically normal controls. It contains two biopsy specimens which were analysed subsequently by flow cytometry and found to be HPV antigen negative. The remaining four specimens were histologically normal but were not tested for HPV antigen. The "HPV infection" group includes four biopsy specimens with histological evidence of HPV infection, without CIN, and one histologically normal biopsy specimen, which was HPV antigen positive.

Significance of differences compared with normal group; * p < 0.05;

** p < 0.01 (Mann-Whitney U-test)

was made with HLA-DR expression by LC in normal cervical epithelium ($p < 0.05$). No increase in HLA-DQ expression by LC was seen in the presence of HPV infection but significantly more LC were HLA-DQ positive in all grades of CIN than in normal epithelium (CIN I $p < 0.01$; CIN II and III $p < 0.05$). Significantly more LC were DP positive in CIN III than in normal epithelium ($p < 0.05$) but the increases seen in CIN I and II were not statistically significant. There was a reduction in HLA-DP expression by LC in HPV infection which reached significance ($p < 0.05$).

Comparison of LC numbers in the cervical transformation zone of combined oral contraceptive pill (OCP) users and non-OCP users

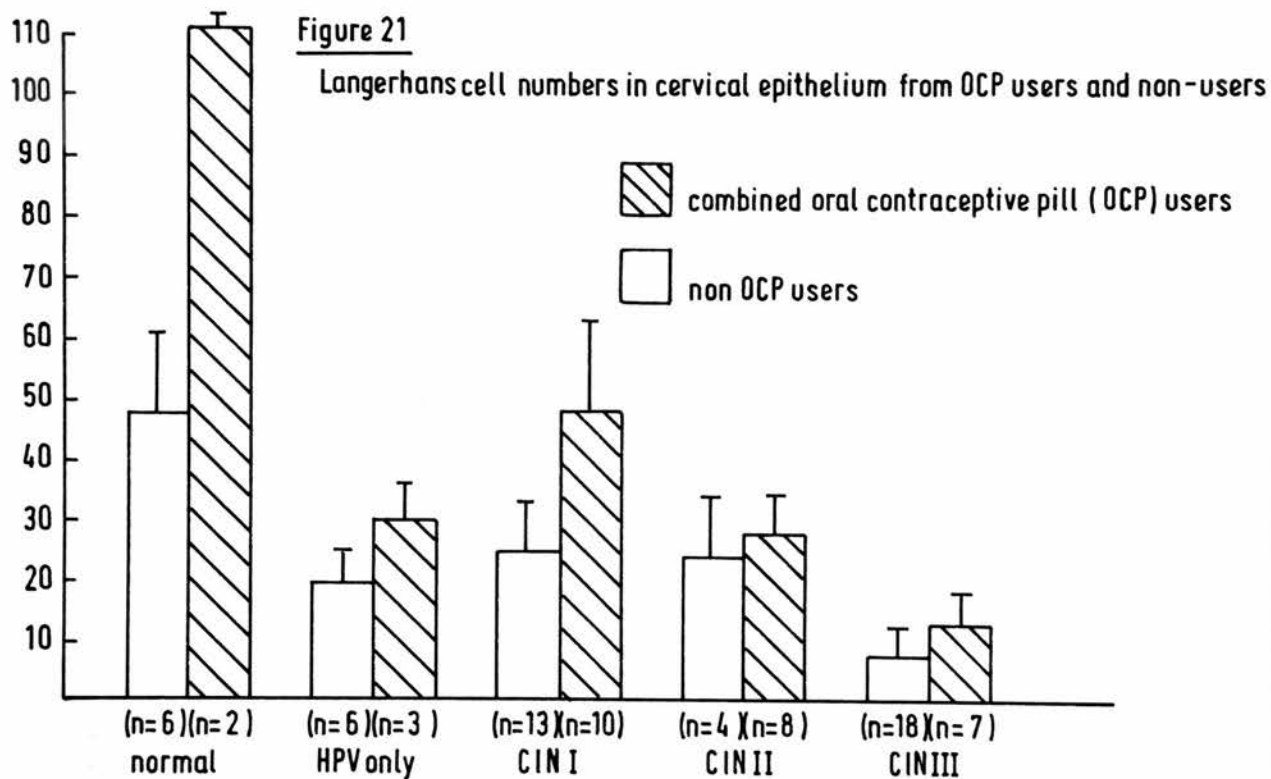
In Figure 21, a comparison of LC numbers in OCP users and non-OCP users can be seen. The number of LC was higher in OCP users than in non-OCP users in the normal and in all the abnormal groups, but the difference was significant only in CIN I ($p < 0.05$).

Comparison of LC numbers in the cervical transformation zone of smokers and non-smokers

In Figure 22, a comparison of LC numbers in current smokers and non-smokers can be seen. There was no significant difference between LC numbers in smokers and non-smokers in any group. When heavy (20 or more cigarettes per day) smokers were compared to non-smokers there was again no significant difference.

Relationship of LC counts to clinical outcome

Of the 21 patients with CIN I studied in this section, 10 were recruited to a study being performed at the Lothian Area Colposcopy Clinic, comparing the outcome in patients who receive laser treatment for CIN I or for cervical HPV infection, with the outcome in those advised to use condoms and those receiving no treatment.

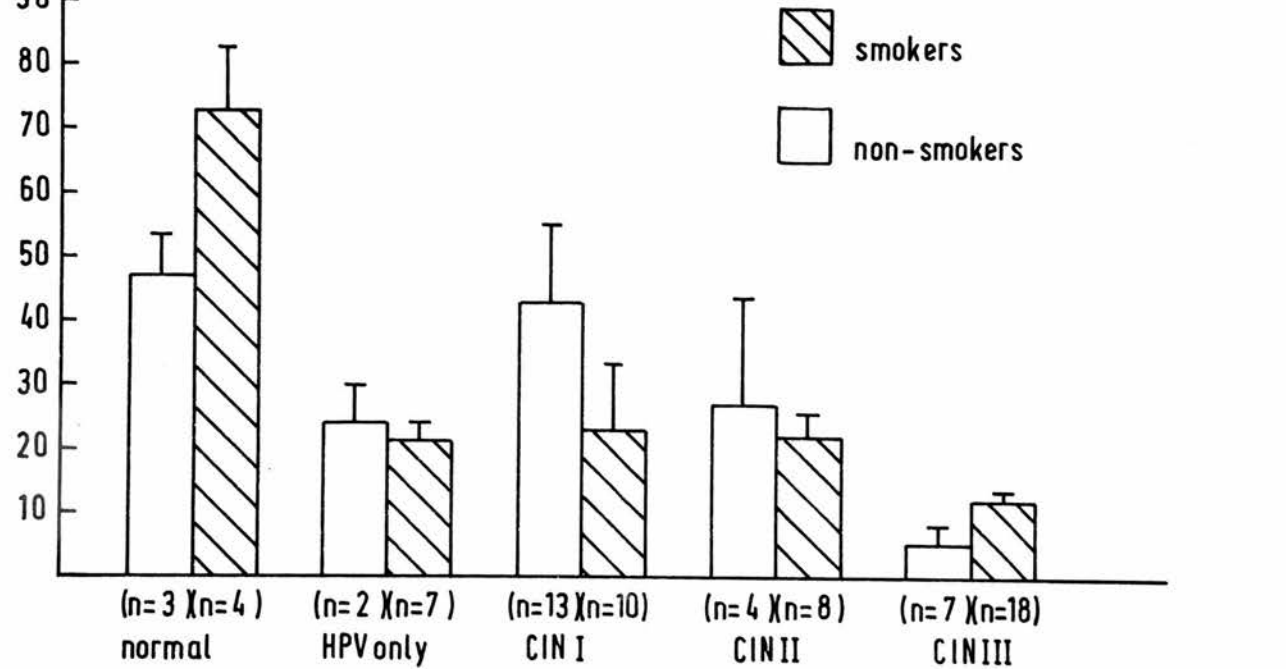
Cells/mm²

The "normal" group consists of four group 'A' and four group 'B' histologically normal controls. It contains two specimens which were analysed subsequently by flow cytometry and found to be HPV antigen negative. The remaining six specimens were not tested for HPV antigen. The "HPV only" group includes seven specimens with histological evidence of HPV infection and two histologically normal specimens which were HPV antigen positive. Bars represent mean value \pm S.E.M. in each group. OCP users did not differ significantly from non-OCP users within any group except in CIN I ($p < 0.05$). Significance calculated by Mann-Whitney U-test.

Cells/mm²

Figure 22

Langerhans cell numbers in cervical epithelium from smokers and non-smokers



The "normal" group consists of three group 'A' and four group 'B' controls histologically normal controls. It contains two specimens which were analysed subsequently for HPV antigen and found to be negative. The remaining five specimens were not tested for HPV antigen. The "HPV only" group includes seven specimens with histological evidence of HPV infection, and two histologically normal specimens which were HPV antigen positive. Bars represent mean value + S.E.M. in each group. Smokers did not differ significantly from non-smokers within any group. Significance calculated by Mann-Whitney U-test.

Six of the 10 were randomised to receive no treatment or to use condoms and of these six, two (who had been advised to use condoms but who had not used them consistently) had CIN III when they were re-examined one year after the biopsy analysed in this study was taken. Four patients were randomised to receive laser treatment and 11 others were excluded from the study and received laser or cold coagulation treatment. One year follow up data is available for two of the other four women who received no treatment. These two women have persistent CIN I. The mean LC count /mm² for the "progressors" (n=2) was 11 (range 6-16), for the patients with persistent CIN I (n=2) 37 (range 24-50) and for the patients (n=8) who had no evidence of residual disease after laser or cold coagulation treatment 39 (range 5-77). These differences are not statistically significant (Mann-Whitney U test). One year follow up information is available for six women with treated CIN II; none of these women had colposcopic evidence of residual or recurrent disease.

Follow up data is available on 18 women with treated CIN III; four (22%) of these patients had residual or recurrent CIN. The mean LC count /mm² (in the original biopsy) for patients with CIN after treatment for CIN III was 8 (range 3-45) and for the patients with no evidence of recurrence 14 (range 2-58). This difference is not statistically significant (Mann-Whitney U test).

T cell numbers

In this section T cell counts in abnormal cervical TZ epithelium were compared with T cell counts in normal cervical epithelium. The abnormal cases were categorised as either HPV infection alone (without associated CIN) (n=4); CIN in which

Table 9

CD3, CD4, CD8 and class II positive T lymphocyte numbers in normal and abnormal cervical epithelium

	CD3 mean (range)	CD4 mean (range)	CD8 mean (range)	CD4:CD8 ratio mean (range)	DA6.231 mean (range)
** normal (n = 4)	65 (20 - 105)	23 (4 - 42)	43 (12 - 103)	0.56 (0.34 - 0.98)	29 (2 - 49)
HPV infection (koilocytosis) (n = 4)	39 (16 - 45)	16 (11 - 30)	22 (15 - 56)	0.73 (0.41 - 1.03)	14 (3 - 41)
CIN (DQ < DR)	52 (4 - 79)	19 (3 - 36)	28 (15 - 78)	0.69 (0.52 - 1.43)	19 (3 - 44)
(n = 5; 2 CIN I 1 CIN II, 2 CIN III)					
CIN (DQ > DR)	57 (20 - 92)	19 (4 - 33)	38 (9 - 82)	0.52 (0.32 - 0.86)	24 (2 - 57)
(n = 3; all CIN I)					

* cells/mm²

** The 'normal' group consists of two group 'A' and two group 'B' histologically normal controls. It contains one specimen which was analysed subsequently for HPV antigen and found to be negative. The remaining three specimens were not tested for HPV antigen.

n = number of patients

No mean value obtained from an abnormal group was significantly different from the corresponding mean in the normal group (Mann-Whitney U test)

the number of HLA-DR positive LC exceeded the number of HLA-DQ positive LC (n=5); and CIN in which the number of HLA-DQ positive LC exceeded the number of HLA-DR positive LC (n=3).

It can be seen from table 9 that the total number of T cells (as measured by counting cells expressing the T cell common antigen, CD3) was insignificantly reduced from normal in koilocytic lesions. In CIN the count was intermediate between the counts in normal epithelium and koilocytic lesions. CD8 positive T cell (suppressor/cytotoxic T cell) counts exceeded CD4 positive T cell (helper/inducer) counts within normal and abnormal epithelium and there was no significant difference between the CD4:CD8 ratios in the normal and any of the abnormal groups. There was an insignificant trend for CD8 positive T cell numbers to be reduced to a greater extent than CD4 positive T cell numbers in the koilocytosis only and CIN (HLA-DQ < DR) groups, making the CD4:CD8 ratios higher in these groups. There was no significant difference in T cell counts between the CIN (HLA-DQ < DR) and CIN (HLA-DR < DQ) groups. If the CIN (HLA-DR < DQ) group (which contained only cases of CIN I) was compared with the two cases of CIN I in the CIN (HLA-DQ < DR) group, no significant difference was identified.

Expression of MHC class II antigens by columnar and squamous cervical epithelium

In three of ten specimens from normal transformation zones and 16 of 67 specimens from abnormal transformation zones a portion of columnar epithelium was included in the sections. In all 19 cases this epithelium stained positively with DA6.231 [figure 23] but not with the other mabs.

Normal squamous epithelium from the transformation zone and

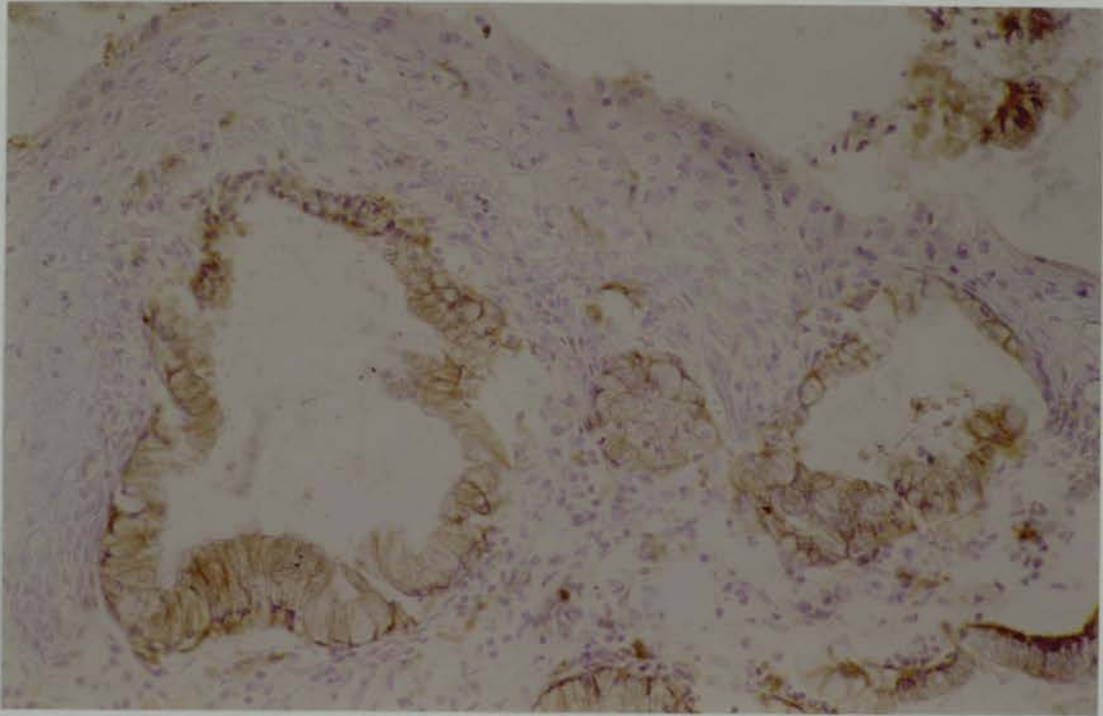


Figure 23. DA6.231 staining of endocervical columnar epithelium; adjacent squamous epithelium is negative (magnification x 125).

specimens of squamous epithelium from areas of koilocytosis only, and of CIN I and II, were consistently negative with all mabs used. In four of 29 (14%) specimens of CIN III positive staining of squamous epithelium was seen using DA6.231, with parabasal cells staining particularly strongly [figure 24]. Staining with other mabs was always negative. These four cases were excluded when DA6.231 positive LC were being counted, as it was not possible to distinguish class II positive LC from positively staining squamous epithelium.

Discussion

The normal controls

The choice of control patients for comparison with patients with HPV infection and with CIN in this and subsequent sections was not ideal. It is recognised that a larger number of normal controls should have been included. In addition, all controls should have undergone colposcopic examination, in view of the well recognised insensitivity of cervical cytology (Coppleson, 1970 and Giles et al 1988). All specimens should have been tested for HPV antigen or viral DNA, as it is now appreciated that a significant proportion of colposcopically, cytologically and histologically normal cervixes contain HPV DNA (Wickenden et al 1985). Ideally the group 'C' controls should have been included in this section. Unfortunately it was not possible to recruit these control patients until after most of the experimental work had been completed.

It is, however, noteworthy that despite these deficiencies it was still possible to demonstrate a significant difference in terms of LC numbers and MHC Class II expression between patients with proven HPV infection and CIN, and "normal" controls. The lack of

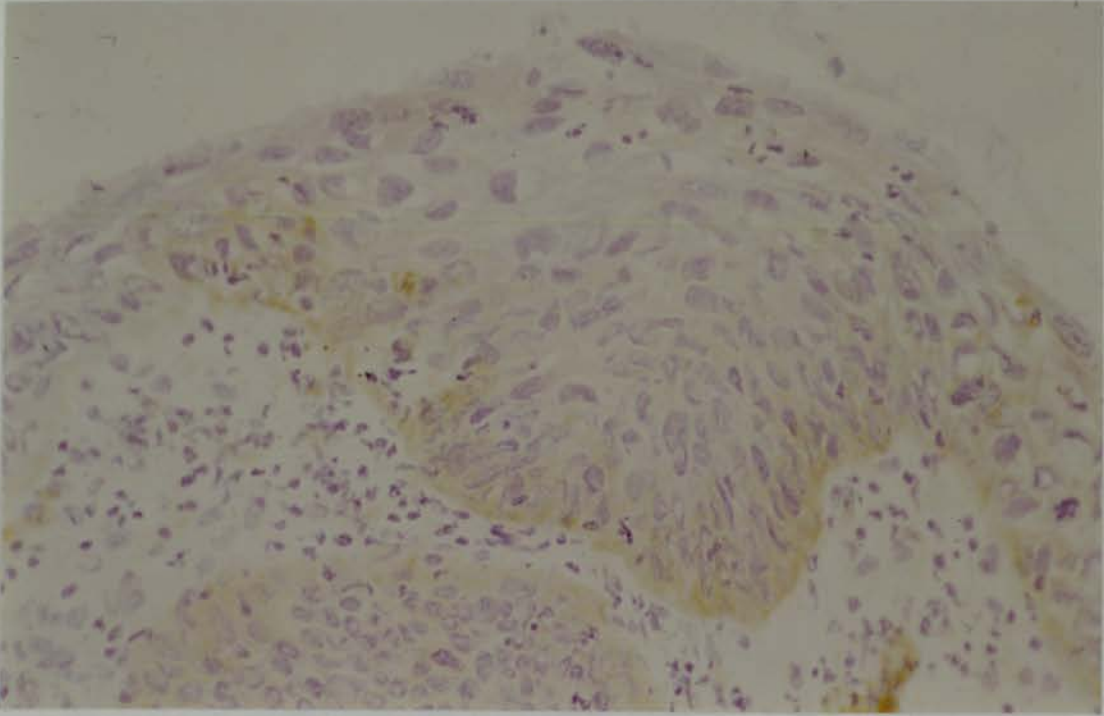


Figure 24. CIN III stained with DA6.231 (magnification x 250).

colposcopic and HPV antigen screening of controls may have led to the inclusion of some patients with HPV infection and CIN in the control group. However it can be predicted that the inclusion of these patients would diminish any differences between controls and patients, thus weakening rather than strengthening the data.

Distribution and numbers of LC and T cells

The work reported here demonstrates that LC are reduced in number in HPV lesions and in CIN II and III, and that their distribution and morphology are altered within abnormal cervical epithelium. This is consistent with a recent report from Tay et al (1987a) although earlier studies reported a decrease in LC numbers in HPV lesions and an increase in numbers in CIN (Morris et al. 1983; McArdle and Muller, 1986).

The reduction in LC numbers and alteration in morphology may be responsible for the reduction in T cell numbers in CIN, and the more marked reduction in T cells in koilocytic lesions relative to normal reported here and elsewhere (Tay et al, 1987b).

It has been postulated (Morris et al, 1983) that the papillomavirus exerts a direct cytotoxic effect upon LC and the loss of dendrites observed here may be a reflection of this effect. It is interesting to note the abnormal distribution of LC throughout dysplastic epithelium and to speculate upon the relationship between this and the pattern of HPV infection of squamous epithelium. HPV DNA can be detected by in situ hybridisation in the parabasal layers but viral antigens are normally detected only in the more superficial layers of the epithelium (Kurman et al, 1983). Perhaps the antigen positive cells attract LCs towards the surface away from their normal

parabasal position. Once at the site of infection LCs may be able to present HPV antigens locally to T lymphocytes or, having been activated by the local viral infection, may leave the site and present HPV antigens in draining lymph nodes. An experiment designed to investigate the latter possibility is described in Chapter 5.

MHC class II expression by LC

In figure 11 it can be seen that not all T6-positive LC were stained with the pan-Class II marker, DA6.231. This has been reported previously (Chardonnet et al, 1986; Tay et al, 1987a; Harrist et al, 1983) using other mabs to Class II antigens, and may reflect the variable expression of these antigens by LC. HLA-DR expression by LC was significantly increased in koilocytic lesions, and in CIN I and II, and HLA-DQ expression was significantly increased in all grades of CIN with the increase being most marked in CIN I (see Table 8). Thus, although LC are reduced in number compared to normal, those present appear more highly "activated".

The parallel increase in HLA-DR and -DQ expression by LC in CIN is consistent with reports from other groups that HLA-DQ expression correlates in general with HLA-DR expression (Sorrentino and Corte, 1986). HLA-DQ is almost always expressed at a much lower level than HLA-DR (Sorrentino and Corte, 1986), so that detection of HLA-DR positive HLA-DQ negative cells may reflect the limit in sensitivity of the technique used.

Most published work suggests that the expression of HLA-DQ and HLA-DP antigens has the same functional significance as the expression of HLA-DR (Gonwa et al, 1983; Nunez et al, 1985; Sanchez-Perez and Shaw 1986). However Navarette et al (1985a and

1985b) have proposed that HLA-DQ has a function distinct from HLA-DR. They report that, while HLA-DR is implicated in the induction of the proliferation of helper/inducer T lymphocytes, HLA-DQ has a predominantly modulatory function involving the generation of suppressor and/or cytotoxic T lymphocytes. This may be of relevance to the present study as a small, insignificant, increase in the relative proportion of CD8 positive T cells was found in the cases where HLA-DQ positive LC exceeded HLA-DR positive LC in number. The number of patients studied was too small for this increase to achieve statistical significance but it is noteworthy that Tay et al (1987b) report an increase in the relative proportion of CD8 positive T cells in CIN. In the present study increased HLA-DQ expression by LC was found to be particularly marked in CIN I. It is possible that the presentation of viral and/or tumour antigens together with HLA-DQ early in the development of CIN (i.e. in CIN I) leads to a predominantly suppressor type response specific for these antigens, thus permitting the disease to progress more rapidly.

HLA-DP specific antibodies have only recently become available so that there is little reliable information on the expression of HLA-DP. The data available suggests that HLA-DP expression is qualitatively indistinguishable from DR (Sanchez-Perez and Shaw, 1986) and the work reported here would support that hypothesis.

Combined oral contraceptive pill (OCP) use and LC numbers

In this section LC were found to be increased in number in the epithelium of the cervical transformation zone of OCP users, when compared to non-OCP users, with the increase reaching significance in patients with CIN I [figure 21]. This has not been previously reported although other workers have demonstrated an impairment of non-specific peripheral blood lymphocyte proliferation in OCP users (Barnes et al, 1974) and an elevation of cervical mucus IgG and IgA in OCP users (Chipperfield and Evans, 1972). Perhaps the increased numbers of LC present are involved in the initiation and amplification of a local antibody response in the cervix. It is, in any case, interesting to note a difference between OCP users and non-OCP users in view of the continuing controversy over the role of the OCP in the aetiology of cervical cancer (Piper, 1985; Francheschi et al, 1986).

Cigarette smoking and LC numbers

No significant difference in LC numbers was found between smokers and non-smokers, in normal or abnormal cervical epithelium. This is in contrast with the recently published work of Barton et al (1988) who found that LC numbers were significantly reduced in the cervixes of smokers. Further work will be necessary to clarify this discrepancy.

Relationship of LC counts to clinical outcome

The results obtained concerning clinical outcome show that there is a trend for women with progressive disease or with recurrence after ablative therapy to have lower LC counts at their first visit. The numbers in each group are too small for this trend to be statistically significant but the finding adds further

support to the hypothesis that LC play an important role in immunosurveillance against neoplastic change within cervical epithelium.

Expression of MHC class II antigens by columnar and squamous cervical epithelium

The expression of class II antigens by columnar epithelium lining endocervical glands was a chance finding as the majority of sections studied contained no columnar epithelium. Expression of HLA-DR by endometrium has been reported to vary with the menstrual cycle (Tabibzadeh et al, 1986a) being highest in the late proliferative phase and thus correlating with oestrogen receptors levels. No apparent correlation between intensity of staining and stage of the menstrual cycle was found in the present study but 11 of 19 subjects in this group were OCP users and therefore not experiencing normal cycles. It would be useful to examine this in more detail.

It was interesting that in some cases (14%) of CIN III the squamous epithelium exhibited positive staining with DA6.231 although no epithelium from normal cervix or from areas of CIN I or II was positive. Morris et al (1983) reported HLA-DR expression by squamous epithelial cells in CIN III and, in patches, in koilocytic lesions with no associated CIN. It is postulated that keratinocytes (Lampert, 1984) and endometrial epithelial cells (Tabibzadeh et al, 1986b) are induced to express class II antigens by lymphokines secreted by infiltrating lymphocytes and thus acquire the ability to act as antigen presenting cells. The detection in this study of class II positive epithelial cells in CIN III may be further evidence to support this hypothesis.

Functional assays should bring further understanding of the interactions taking place between LC, lymphocytes and epithelial cells in the cervical transformation zone and of the significance of class II antigen expression at this site.

CHAPTER 5

CHAPTER 5**FLOW CYTOMETRIC ANALYSIS OF DNA CONTENT C-MYC PROTEIN EXPRESSION AND
PAPILLOMAVIRUS ANTIGEN EXPRESSION IN CERVICAL EPITHELIUM**Introduction

In this section the technique of flow cytometry was employed to analyse patterns of cell division and to look for evidence of DNA aneuploidy in HPV infected and neoplastic cervical epithelium. Aneuploidy is a well recognised characteristic of malignancy (Friedlander et al, 1984a) and it is known that the number of proliferating cells in a tumour increases in parallel with neoplastic change (Laerum and Farsund, 1981).

The techniques of cervical brushing and biopsy to obtain specimens for flow cytometry were compared. The relationship between expression of papillomavirus antigens and the rate of cell division was compared in view of the hypothesis that the papillomavirus acts as a nonspecific mitogen (Reid et al, 1982). C-myc oncogene protein expression was also examined, and expression related to grade of CIN and rate of cell division.

ResultsComparison of cervical brushings and biopsy specimens

10^6 - 5×10^6 (mean 2×10^6) nuclei were obtained from a biopsy specimen and 0.6×10^6 - 6×10^6 (mean 1.6×10^6) from a brush specimen. More cells were obtained by brushing mid cycle than peri-menstrually. Twelve patients (four normal, two with

koilocytosis only, two with CIN I, one with CIN II and three with CIN III) had both brushings and a cervical biopsy specimen taken for flow cytometric analysis, to compare the two methods of obtaining samples. Propidium iodide staining gave very similar results from both brushings and biopsies; if the % S + G₂ + M phase values obtained from a biopsy and brush specimen in each patient were compared the difference was never more than 3%. A DNA aneuploid population was identified in one biopsy specimen; DNA aneuploidy was also detected in the brush specimen from the same patient and no other aneuploid populations were detected in biopsies or brushings from this group.

When c-myc oncogene expression was measured using the antibody MYC-6E10, results from biopsies and brushings from the same patient did not differ by more than 2%.

The percentage of papillomavirus antigen positive nuclei in biopsies and brushings from the same patient varied considerably (see below).

Comparison of different methods of fixation of brush specimens

Two brush specimens were taken from six patients (four normal, two with CIN I) and one specimen fixed in methanol while the other specimen was fixed in 1% formol saline, in order to determine whether these different methods of fixation gave different results with propidium iodide, c-myc or papillomavirus antigen staining. No significant difference was detected when the two methods of fixation were compared, for any of the above stains.

Results

Cell cycle analysis

Table 10 shows that of 89 cervical biopsy samples examined,

Table 10

DNA content of biopsy specimens from normal and abnormal cervical epithelium

Cervical histology	number of samples suitable for parametric analysis	% (SEM) nuclei in S phase in S+G ₂ +M phases	** significance	Number (%) specimens containing aneuploid populations a) compared with CRBC control***	b) separate peak***
Normal* (n = 9)	9/9	10 (1.2)		0 (0)	0 (0)
HPV infection* (n = 12)	11/12	21 (2.8)	p < 0.05	2 (17)	2 (17)
CIN I (n = 14)	14/14	23 (2.5)	p > 0.01	2 (14)	1 (7)
CIN II (n = 21)	20/21	20 (1.7)	p < 0.01	4 (19)	1 (7)
CIN III (n = 33)	31/33	18 (1.0)	p < 0.01	7 (21)	4 (12)

* The "normal" group consists of four group 'A' and five group 'B' histologically normal controls. It contains two specimens subsequently analysed for HPV antigen and found to be negative. The remaining seven specimens were not tested for HPV antigen. The "HPV infection only" group consists of 11 specimens with histological evidence of HPV infection and one histologically normal specimen which was HPV antigen positive.

n = number of patients

** Comparison with normal group by Mann-Whitney U test

*** see text

nine were classified by histopathological criteria as normal squamous epithelium, 12 as containing evidence of HPV infection without CIN, and 68 as showing evidence of CIN; 14 grade I, 21 grade II and 33 grade III. Table 11 shows that brushings were taken from five cytologically, colposcopically and histologically normal cervixes, seven cervixes containing evidence of HPV infection without CIN, six containing CIN I, five containing CIN II and six containing CIN III. Four biopsy specimens and three brush specimens produced histograms on flow cytometry that, because of an abnormal distribution of DNA were unsuitable for parametric analysis and were omitted from this part of the study. Tables 10 and 11 also show the proportion of nuclei in the S phase and in the S + G₂ + M phases of the cell cycle.

Traditionally, studies of cellular proliferation have examined only the fraction of cells in the S phase, as the cells in the G₂ + M phases could not be quantitated by the uptake of tritiated thymidine. In this study it was found that calculating the total proportion of cells in the S + G₂ + M phases gave more consistent results than calculating the proportion of cells in the S phase alone, although as can be seen from tables 10 and 11 the two measurements gave very similar results.

The percentage of nuclei in the S phase increased from 11% (biopsy specimens) and 9% (brush specimens) in normal cervix to 20% (biopsy specimens) and 21% (brush specimens) in epithelium containing evidence of HPV infection ($p < 0.05$). The samples (biopsy and brush) from patients with CIN all contained significantly more cells in the S phase than those from the normal controls. There were no significant differences between the group

Table 11
DNA content of brush specimens from normal and abnormal cervical epithelium

Cervical histology	No. of samples suitable for parametric analysis	% (SEM) of nuclei in S phase in S+G ₂ +M phases	significance**	No (%) of sample containing aneuploid populations (a) compared with CRBC control***	No (%) of sample containing aneuploid populations (b) separate peak***
normal* (n = 5)	5/5	9 (2.6)	19 (2.8)	0 (0)	0 (0)
HPV infection only* (n = 7)	7/7	21 (3.2)	29 (3.9)	p < 0.05	0 (0)
CIN I (n = 6)	5/6	24 (2.6)	30 (2.3)	p < 0.05	1 (13)
CIN II (n = 5)	4/5	22 (2.6)	31 (1.8)	p < 0.05	1 (20)
CIN III (n = 6)	5/6	22 (1.6)	32 (1.2)	p < 0.05	1 (13)

* The "normal" group consists of three group 'A' and two group 'B' controls. Two specimens in the group were tested for HPV antigen and found to be negative. Three specimens were not tested for HPV antigen. The "HPV infection" group contains five brush specimens from cervixes with histological evidence of HPV infection and two HPV antigen positive brush specimens from cervixes which were colposcopically and histologically normal.

n = number of patients

** Comparison with normal group by Mann-Whitney U-test

with evidence of HPV infection only and the group with CIN. When the S and $G_2 + M$ phases were considered together a similar pattern was seen. Biopsy and brush samples from all four groups with abnormalities (HPV infection and CIN I, II and III) contained significantly more cells in the S + $G_2 + M$ phases than the normal control samples but did not differ significantly from each other.

DNA aneuploidy

DNA aneuploidy was classified in two ways: (a) and (b).

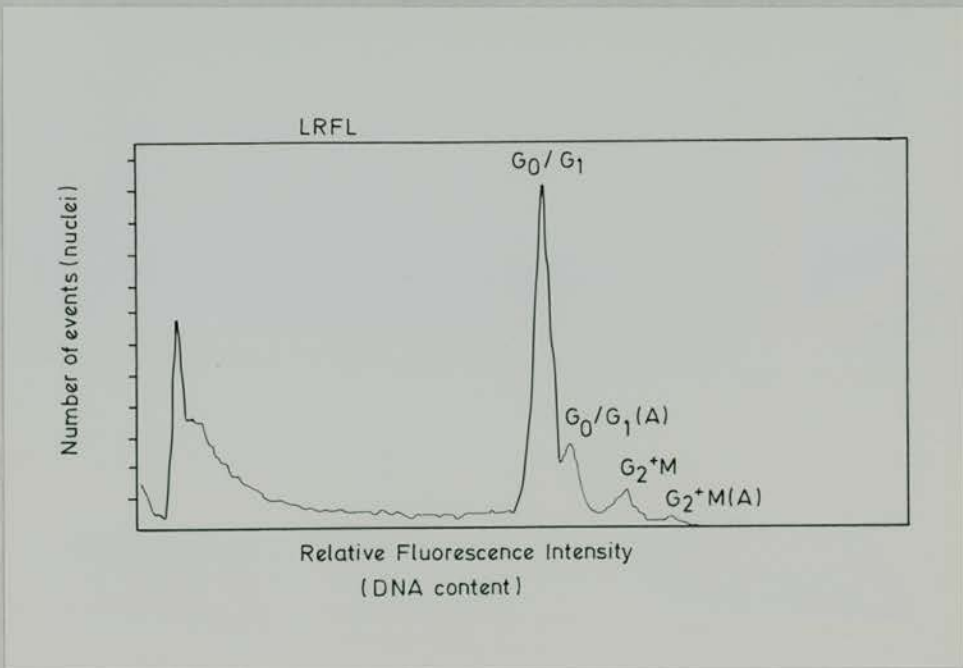
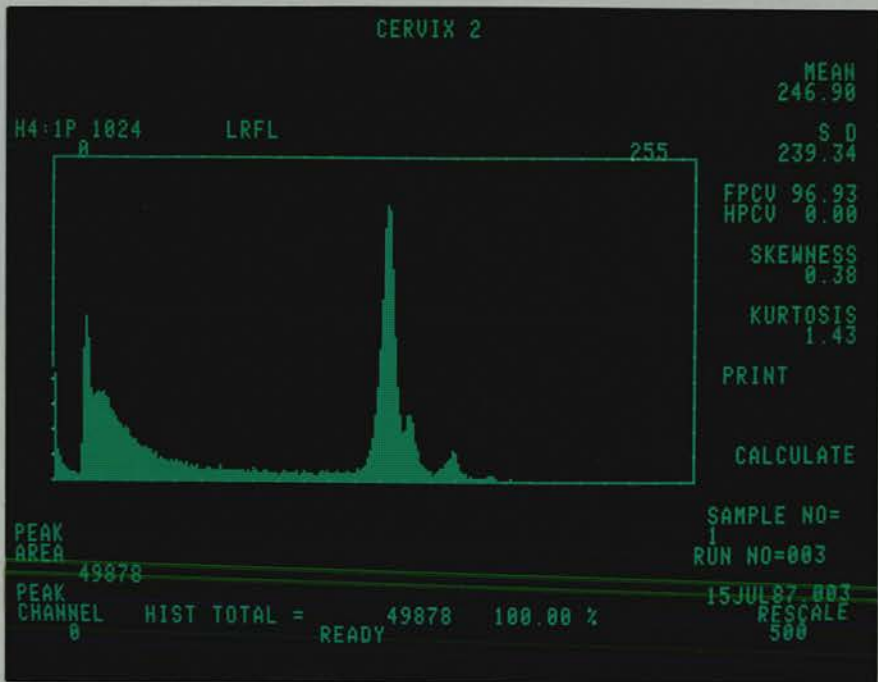
(a) The DNA index was calculated from the ratio between the chicken red blood cell (CRBC) and sample modal channel numbers versus the ratio between the CRBC and lymphocyte modal channel numbers (Vindelov et al, 1983). Thus a DNA index of 1 equalled the DNA index of a diploid cell. A sample was considered to contain a DNA aneuploid component by this classification if the DNA index was less than 0.9 or more than 1.1. Column (a) in tables 10 and 11 contains results obtained according to this classification. All four abnormal groups yielded some aneuploid biopsy samples, the proportion of aneuploid specimens ranging from 14% (CIN I) to 21% (CIN III). Seventeen per cent of "HPV infection only" biopsy samples contained an aneuploid component. Brush specimens gave very similar results; the proportion of aneuploid brush specimens ranging from 13% (CIN I and III) to 20% (CIN II). No DNA aneuploidy was detected in brushings taken from cervixes containing evidence of HPV infection. Of the 17 aneuploid samples identified (15 biopsy samples, two brush samples and one brush and biopsy from the same patient), seven were hypodiploid (DNA index < 0.9) and ten were hyperdiploid (DNA index > 1.1). The DNA index of hyperdiploid cell populations was always less than 1.5.

For a more detailed analysis of the detection of aneuploidy in brushings and biopsy specimens see "Comparison of cervical brushings and biopsy specimens" above.

(b) It has been suggested that DNA aneuploidy should be reported only when at least two separate G_0/G_1 peaks are demonstrated (Hiddemann et al, 1984) [see figure 25]. Column (b) in tables 10 and 11 contains the results obtained when DNA aneuploidy was classified in this way. It can be seen from table 10 that this method leads to a smaller percentage of specimens being reported as containing an aneuploid component than method (a), with the fractions ranging from 7% (CIN I and II) to 17% (HPV infection only). When brush specimens were classified by the two different methods the results obtained were identical (table 11).

Relationship of flow cytometric findings to clinical outcome

The majority of patients studied in this section received local ablative therapy to the cervical transformation zone either immediately after the biopsy or brushing was taken or between two and nine weeks later. Four patients with HPV infection only and three patients with CIN I received no treatment or were asked to use condoms as part of the trial described above (see "Relationship of LC count to clinical outcome"). One year follow-up data is available for 78 of the 106 patients. Of these 78, 10 had residual or recurrent disease after treatment, and one had progressed (without treatment) from HPV infection only at her first visit to CIN III one year later. When cell cycle data on the original biopsies or brushings from the patients with disease at one year was compared with data on the disease-free patients at one year no significant difference could be seen. If samples in which



Figures 25a and b DNA histogram of aneuploid sample of CIN III (LRFL = log red fluorescence, $G_0/G_1(A)$ and $G_2 + M(A)$ denote the aneuploid components).

a DNA aneuploid component was detected were compared with samples in which no aneuploid component was detected, again, no significant difference in clinical outcome was detected. Of 67 patients with non-aneuploid specimens on which one year follow up data was available six (9%) had evidence of residual or recurrent disease. Follow up data was available for 11 patients who had had evidence of aneuploidy; of these, one patient had further CIN after treatment (for CIN III) and one patient (who had had HPV infection only originally) now had CIN III. This equals a recurrence or progression rate of 18% (2/11). The other "HPV infection only" patient with evidence of DNA aneuploidy had previously had CIN III treated by laser.

C-myc protein staining

The number of specimens in each histological category staining positively with the anti-c-myc antibody MYC1-6E10 is shown in table 12. It can be seen that the percentage of specimens from normal cervical epithelium which were c-myc antigen positive was higher (63%) than the percentage of specimens from CIN II or III (20%) or invasive carcinomas (0%). These differences were not significant by Fisher's exact probability test at $P < 0.05$ although the difference between normal and invasive carcinoma does reach significance at the $P < 0.2$ level. The range of percentages of nuclei staining positively with MYC1-6E10 (in the positive specimens) is also shown. If c-myc antigen positive specimens in each histological group are compared the percentage of nuclei in each specimen staining positively does not vary by more than 2.3% (table 12).

The percentage of nuclei in the S and $G_2 + M$ phases of the

Table 12

C-myc protein in biopsies and brushings from normal and abnormal cervical epithelium

Cervical histology	Number (%) specimens c-myc antigen positive		Mean (and range) % nuclei expressing c-myc (within c-myc positive specimens)
	brushings	biopsies total*	
** normal (n=5)	2/4 (50)	2/2 (100) 3 (60)	3.2 (2.6 - 4.4)
HPV infection only*** (n=8)	2/4 (50)	1/5 (20) 2 (25)	2.6 (2.0 - 5.4)
CIN I (n=10)	2/5 (40)	3/6 (50) 5 (50)	4.3 (3.0 - 7.4)
CIN II (n=5)	1/3 (33)	0/3 (0) 1 (20)	2.4
CIN III (n=5)	0/2 (0)	1/5 (20) 1 (20)	3.7
invasive carcinoma (n=7)	-	0/7 0 (0)	-

* Seven patients had both brushings and a biopsy processed for flow cytometry

** All five normal control specimens were HPV antigen negative. Three were from group 'A' controls two from group 'B' controls.

*** The "HPV infection only" group contained specimens from five patients with histological evidence of HPV infection and from three patients with no colposcopic or histological evidence of HPV infection, but HPV antigen in a cervical biopsy and/or brush specimen.

n = number of patients

Table 13
Cell cycle analysis and c-myc antigen expression

Cervical histology	% (SEM) nuclei in S + G ₂ + M phases	c-myc negative specimens	c-myc positive specimens
normal*		19 (4.3) (n=2)	20 (3.2) (n=4)
HPV infection only*		29 (3.0) (n=6)	28 (3.2) (n=3)
CIN I		33 (3.7) (n=5)	31 (3.5) (n=5)
CIN II		32 (3.1) (n=5)	32 (n=1)
CIN III		32 (2.7) (n=6)	30 (n=1)

n = number of patients

The biopsy and brush specimens from patients who had both taken are included as separate specimens.

* see footnote to table 12

cell cycle in c-myc antigen negative and c-myc positive specimens is shown in table 13. It can be seen that within each histological group, there was no significant difference in the percentage of cells in the S and G₂ + M phases of the cell cycle when c-myc negative and c-myc positive specimens were compared.

HPV antigen staining

The results obtained from staining cervical nuclei with an antibody to papillomavirus antigens are displayed in table 14. It can be seen that the proportion of specimens exhibiting positive staining with the antibody ranged from 38% (normal cervixes) to 83% (koilocytosis alone). A similar proportion of biopsies and brushings from CIN with (67%) and without (75%) associated koilocytosis stained positively with the antibody. Nine patients had both a biopsy sample and a brush sample tested for the presence of papillomavirus antigen. In seven patients both the brush specimen and the biopsy specimen stained positively; in one patient both were negative, while in one patient with a cytologically, colposcopically and histologically normal cervix the biopsy gave a negative result while the brushing stained positively with antibody to papillomavirus antigens.

When the percentage of nuclei from brush and biopsy samples staining positively with the antibody were compared it can be seen from table 14 that brush samples gave consistently higher percentages than biopsy samples. HPV antigen positive brush samples from the different histological groups did not contain significantly different percentages of positive nuclei; similarly there was no significant difference between the HPV antigen positive biopsy samples from the different histological groups.

Table 14

Papillomavirus antigen expression in biopsies and brushings from normal and abnormal cervical epithelium

Cervical histology	Number (%) specimens papillomavirus antigen positive		Mean (range) % nuclei papillomavirus antigen positive (within antigen positive specimens)	brushings		biopsies
	brushings	biopsies		total*	brushings	
normal† (n=8)	2/4 (50)	2/6 (33)	3/8 (38)	23 (20-26)	4 (3-5)	
Koilocytosis alone (n=6)	3/3 (100)	4/5 (80)	5/6 (83)	24 (8-35)	5 (3-11)	
CIN without koilocytosis (n=8)	4/4 (100)	4/6 (67)	6/8 (75)	18 (4-26)	4 (3-9)	
CIN with koilocytosis (n=9)	4/4 (100)	5/8 (63)	6/9 (67)	15 (6-28)	6 (3-13)	
Benign prostatic hypertrophy (n=6)	-	0/6	0/6	-	-	

* Nine patients had both brushings and a biopsy processed for flow cytometry

** The "normal" group is made up of three group 'A' controls, two group 'B' controls plus the three patients normally excluded from control groups A (n=1) and B (n=2) due to detection of HPV antigen

n = number of patients

The percentage of cells in the S + G₂ + M phases of the cell cycle in HPV negative and positive specimens is shown in table 15. It can be seen that within each group except CIN with koilocytosis, HPV positive specimens contained more dividing cells than HPV negative specimens. The differences were not significant by the Mann-Whitney U test; perhaps because of the small numbers in the HPV negative groups.

The results obtained from staining six specimens of prostatic chippings obtained at transurethral resection of prostate are shown in table 14. No positive staining was obtained with anti-papilloma virus antibody in these specimens.

The results obtained by staining biopsies from cervical carcinomas, from adjacent normal or dysplastic epithelium and from draining internal and external iliac lymph nodes with antibody to papillomaviral antigens are shown in table 16. It can be seen that no carcinoma specimens stained positively with this antibody and that 50% of specimens from adjacent normal cervical epithelium, 67% from adjacent dysplastic epithelium and 73% of draining lymph nodes stained positively. Where both a cervical biopsy and a lymph node specimen were examined the lymph node contained a higher percentage of HPV antigen positive nuclei than the corresponding cervical biopsy, except in one case.

Three of the 11 lymph node specimens tested contained metastatic tumour. Two of these specimens were HPV antigen positive, and one was HPV antigen negative.

Discussion

The normal controls

The same criticisms of the control patients selected can be

Table 15
Cell cycle analysis and papillomavirus antigen expression

Cervical histology	% (SEM) nuclei in S + G ₂ antigen negative specimens	% (SEM) nuclei in S + G ₂ + M phases antigen positive specimens
normal*	21 (3) (n=7)	27 (4) (n=3)
Koilocytosis alone	24 (n=1)	32 (n=7)
CIN without Koilocytosis	21 (4) (n=2)	34 (4) (n=8)
CIN with Koilocytosis	29 (2) (n=3)	30 (4) (n=9)

n = number of specimens

* see footnote to table 14

The biopsy and brush specimens from patients who had both taken are included as separate specimens.

Table 16

**Papillomavirus antigen expression in invasive cervical carcinomas, adjacent epithelium
and draining lymph nodes**

Histology	Number (%) specimens papillomavirus antigen positive	Mean (range) % nuclei papillomaviral antigen positive (within antigen positive specimens)
Stage Ib squamous cervical carcinoma (n = 7)	0 (0)	-
normal epithelium adjacent to carcinoma (n = 4)	2 (50)	5 (3 - 7)
CIN adjacent to carcinoma (n = 3)	2 (67)	6 (4 - 8)
draining lymph nodes (n = 11)	8 (73)	13 (7 - 18)

n = number of patients

made here as were made in chapter 4 (p. 110). However, it should be stressed again that the possible inclusion of patients with HPV infection or CIN in the control group would have the effect of weakening rather than strengthening the reported data, so that the actual differences between controls and patients may be greater than found here.

Comparison of cervical brushings and biopsy specimens

A number of authors have advocated the use of cervical brushes for obtaining specimens for routine cytopathology (Glenthøj et al, 1986), for HPV DNA extraction (McCance et al, 1986b) and for flow cytometric analysis (Elias-Jones et al, 1986). The data obtained in the present study show that cervical brush specimens give very similar results when analysed for DNA content and for expression of the c-myc oncogene product as cervical biopsy specimens, and thus offer a non-invasive alternative to biopsies which causes less discomfort to the patient and is less likely to alter the natural history of cervical lesions.

The results concerning HPV antigen expression obtained from brushings did differ from those obtained from biopsies, in that the percentage of positive nuclei within positive samples was higher in brushings than in biopsies. This is presumably a sampling effect as a brush removes mainly superficial epithelial cells which are more likely to be antigen positive (Kurman et al, 1983) while a biopsy normally removes the full epithelial thickness as well as some underlying stromal cells. Theoretically a brush sample may be preferable as a brush obtains only epithelial cells without the stromal contamination which is inevitable in a biopsy specimen. This contamination might be avoided by employing a technique

similar to that described by Caorsi and Figueroa (1986), in which cervical biopsies are incubated in EDTA, a chelating agent which facilitates the separation of epithelium from stroma. The use of this refinement would help clarify whether the changes described above in biopsy specimens from HPV infected and neoplastic cervixes do relate to the epithelium or are due to changes in the cell population of the underlying stroma. Despite this, when biopsy and brush samples were taken from the same patient the two samples gave the same result (i.e, either HPV positive or negative) as one another in eight of nine patients.

Cell cycle analysis

The analysis of cellular DNA content by flow cytometry shows that normal cervical epithelium can be distinguished from HPV infected epithelium and from epithelium containing CIN. HPV infected or dysplastic epithelium differed from normal in containing a larger proportion of dividing cells (cells in the S and $G_2 + M$ phases) and, in some cases, in possessing a DNA aneuploid component (see below).

It has been postulated that cells infected with HPV initially behave as though stimulated by a non-specific mitogen and that in a semi-permissive epithelium many cells remain in a prolonged S or G_2 phase (Reid et al, 1982). Reid (1983) proposed that cumulative mitotic errors in these stem cells might result in occasional chromosomal mutations spawning clones of aneuploid basal cells. By showing an increased proportion of cells in the S + $G_2 + M$ phases in the koilocytic group and in the HPV antigen positive groups the data presented here support this view. The biopsy samples from CIN II and III contained a smaller proportion of S phase cells than

samples from HPV infected lesions and from CIN I, although this difference was not significant, and was not paralleled by a similar decrease when the S and G₂ + M phases are considered together. The reduction of S phase cells in CIN II and III relative to CIN I and HPV infected lesions is consistent with Reid's hypothesis that the papillomavirus initially stimulates epithelial proliferation followed by S phase arrest and the emergence of aneuploidy.

DNA aneuploidy

The proportion of samples containing DNA aneuploid populations was essentially similar in the four abnormal groups, including the group with HPV infection alone (range 14.4% to 21.2% by the criteria of Vindelov et al, 1983). The prevalence of aneuploidy in CIN III reported here is considerably lower than that reported by Jakobsen et al (1983) and Reid et al (1984). This may be due to differences in the preparation of specimens, in the sensitivity of the various methods or in the definition of aneuploidy. Hendy-Ibbs et al (1987), defining aneuploidy as "the presence of a distinct second peak separate from the diploid distribution" found no aneuploidy in a series of 34 cases of CIN and 64 cases of invasive cervical carcinoma. They noted a positive skew of the diploid peak in 19 of the carcinoma specimens, which they suggest could be due to aneuploidy. In the present study some of the G₀/G₁ peaks were abnormally broad and may have included an aneuploid component merging with the diploid population. The detection of aneuploidy in HPV infected epithelium (without associated CIN) has recently been confirmed by other workers (Watts et al, 1987) and Evans and Monaghan 1985, in related work using

static cytometry, reported aneuploidy in cases of "warty atypia". The finding would support the hypothesis that HPV plays a significant role in the aetiology of cervical carcinoma and has important implications for the management of patients with cervical HPV infection and with CIN I. In many centres these patients are treated conservatively in the hope that the lesion will regress. It has been suggested that the clinical behaviour of these lesions may be predicted on the basis of ploidy (Fu et al, 1983 and Evans and Monaghan, 1985). No evidence was found for this on the basis of results from those patients for whom follow-up data was available (see Results section "Relationship of flow cytometric findings to clinical outcome") but this may be related to the small number of patients in each diagnostic group who had a follow-up colposcopic examination after treatment as well as to the short period of follow-up. The data presented here do, however, show that it was not possible to distinguish koilocytic lesions from CIN III by cell cycle analysis or by ploidy implying that they should perhaps be treated identically.

Oncoprotein staining

It is interesting that the percentage of specimens from normal cervical epithelium which were c-myc antigen positive was higher than the percentage from CIN II or III or invasive carcinomas, although not significantly so. C-myc DNA amplification has been found in more advanced cervical carcinoma (Riou et al, 1984 and Ocadiz et al, 1987) and increased levels of c-myc RNA are associated with early clinical relapse in early (stage I and II) carcinomas (Riou et al, 1987). In contrast Hendy-Ibbs et al (1987) report a higher level of c-myc antigen expression in normal

cervical epithelium than in neoplastic epithelium (in wax embedded tissue) corresponding with the findings reported here. C-myc mRNA and protein have a short half life in stimulated cells (Dani et al, 1985 and Rabbitts et al, 1985) and it has been suggested that they may be particularly susceptible to proteolysis in neoplastic cells during the preparation for the assay, or that MYC 1-6E10 may recognise an epitope of some other nuclear associated proteins. However, another antibody recognising a different region of the protein, gave the same results (Hendy-Ibbs et al, 1987). Thus it seems likely that MYC1-6E10 does recognise the c-myc product.

HPV antigen staining

The finding of HPV antigen expression in 38% of otherwise normal cervixes is consistent with reports published elsewhere that between 11% (Wickenden et al, 1985; Toon et al, 1986; Lorincz et al, 1986) and 50% (Schneider et al, 1987) of otherwise normal cervixes contain HPV DNA. The proportion of dysplastic biopsy specimens containing HPV positive nuclei (71% - see table 14) is higher than is generally reported for immunohistochemical studies of paraffin sections (Kurman et al, 1983). The method of preparation of nuclei for flow cytometric analysis used in this study may expose the viral antigens in a way which makes them more accessible to antibody. The technique may also be more sensitive than standard immunohistochemical techniques as many thousands of nuclei can be examined very rapidly. It is possible that the antibody used cross reacts with an epitope found in cervical epithelium and encoded by cellular DNA, which is exposed when the nuclei are prepared by the method described here and not in paraffin sections. This seems unlikely, as the antibody is prepared

by purifying viral protein from bovine rather than human papillomas. A larger proportion of dysplastic biopsies than normal cervical biopsies stained positively with the antibody, and the antibody did not recognise any epitopes present in prostatic tissue (see table 14).

The relationship between HPV antigen expression and cellular proliferation has been discussed above.

No HPV antigen expression was detected in seven cases of stage 1b cervical carcinoma. This was not unexpected, as antigen expression is known to decrease with increasing severity of neoplastic lesions (Kurman et al, 1983). The identification of papillomaviral antigens in biopsies taken from dysplastic and normal epithelium adjacent to carcinomas was also anticipated, as Kurman et al (1983) report antigen expression in CIN I and II adjacent to antigen negative CIN III, while Ferenczy et al (1985) report the presence of HPV DNA in biopsies taken from normal skin adjacent to treated anogenital warts. The detection of viral antigen in draining lymph nodes was, however, unexpected and has not previously been reported. Two HPV positive nodes contained metastatic tumour, so that the viral antigen may have been present in metastatic tissue. The other six HPV positive nodes were, however, free of metastasis, and it must be postulated that the viral antigen was transported there by circulating LC or T cells. It would be interesting to perform immunohistochemical staining or in situ DNA hybridisation on paraffin sections of antigen positive nodes in order to determine which cells contain viral proteins. This would help further our understanding of the local immune response to epithelial papillomavirus infections. It would also be

useful to examine lymph nodes draining apparently uninvolved epithelium, to discover if they too contain HPV antigen. It was not possible to perform this experiment as part of the present study.

CHAPTER 6

CHAPTER 6**CERVICAL INTRAEPITHELIAL NEOPLASIA IN LYMPHOMA PATIENTS**Introduction

Patients with lymphomas are known to be at increased risk of developing second malignancies, especially acute myeloid leukaemia (Coleman et al, 1982, Tester et al, 1984) and, in the case of patients with Hodgkin's disease (HD), non-Hodgkin's lymphomas (Jacquillat et al, 1984). It has been suggested that the incidence of second malignancies in HD patients correlates with the level of treatment to which patients are exposed (Arseneau et al, 1972) and, for those receiving very intensive chemotherapy and radiotherapy, the risk may increase by as much as 1500 times (Coleman et al, 1982, Tester et al, 1984). There is little evidence of an increased incidence of second malignancies in patients treated with radiotherapy alone leading some authors to postulate that the increase in risk is related to the use of chemotherapeutic agents, especially alkylating agents (Anon, 1985). These drugs are mutagenic and carcinogenic in laboratory systems and are also immunosuppressive (Schilsky and Erlichman, 1982). Procarbazine, an important drug in the treatment of HD, is also highly carcinogenic in experimental systems (Schilsky and Erlichman, 1984).

In this section a defined group of patients suffering from HD or non-Hodgkin's lymphoma were studied in an attempt to discover their relative risk of developing cervical neoplasia. Evidence of

cervical HPV infection was also sought. There is some evidence that immunosuppression may lead to the malignant conversion of papillomavirus induced lesions in animals (Campo and Jarrett, 1987) and in man (Sillman et al, 1984, Rudlinger et al, 1986, Schneider et al, 1983).

Table 17 contains information concerning the lymphoma patients studied. They were compared with the control patients in groups 'A' (n=12) and 'C' (n=67). Those four patients in control group 'A' who were found to have CIN or HPV infection (see p.68) were not excluded from the control group in this section. Biopsies and/or brushings from three group 'A' controls were known to be HPV antigen negative. The HPV antigen status of the remaining five group 'A' controls, and of all group 'C' controls, is unknown.

Results

a) General patient data

It can be seen from table 17 that the lymphoma patients studied were less likely to have been pregnant and reported a later onset of sexual activity and fewer sexual partners than the control patients. In addition fewer of the lymphoma patients smoked. Thirteen (48%) lymphoma patients used no contraception. In nine patients this was because contraception was unnecessary due to post-chemotherapy amenorrhoea which had lasted for at least 18 months. The mean age at onset of amenorrhoea was 34.5 years (range 23-44 years).

b) Haematological data

Details of haematological diagnoses are given in table 18. All HD patients receiving chemotherapy (C/T) were given procarbazine, prednisolone, vinblastine and/or vincristine and

Table 17
Epidemiological data from lymphoma patients and controls

	Mean age (range)	Number parous (%)	Mean age of		Median no. sexual partners (range)	Current smokers (%)	Current combined oral contraceptive pill use (%)
			Coitarche (range)				
All lymphoma patients (n=27)	38.9 (24-59)	18 (67)	21.2 (13-40)	1 (1-30)	6 (22)	6 (22)	
HD (n=19)	36.5 (24-59)	10 (53)	21.1 (16-40)	1 (1-9)	2 (11)	4 (21)	
non-HD (n=8)	44.5 (29-56)	8 (100)	21.5 (13-33)	1 (1-30)	4 (50)	2 (25)	
control patients (n=79)	39 (20-71)	71 (90)	19 (14-27)	2 (1-6)	37 (47)	5 (50)	

n = number of patients

Table 18

Lymphoma patients grouped according to their haematological diagnosis and cervical histology and cytology

Patient	Histology	Stage	Months since diagnosis	Treatment	Cervical cytology class	comments	Cervical Histology
A. HODGKIN'S DISEASE							
<u>i) No significant abnormality</u>							
EH	nodular sclerosing (NS)	1A	34	R/T	1	chronic cervicitis	Squamous metaplasia, active
CMcP	NS	1	26	"	1	Monilia	inflamed endocervical polyp, squamous metaplasia
MY	unclassified	1A	35	"	1	many lymphocytes	uninflamed endocervical polyp
CC	NS	1A	13	C/T	1		no biopsy
JK	NS	2A	52	"	1	atrophic/many lymphocytes	no biopsy
MMcK	NS	3A	38	"	1		squamous metaplasia
DS	NS	2A	54	R/T & C/T	1	atrophic	metaplasia, chronic cervicitis
MG	lymphocyte predominant (LP)	3A	73	"	1		no biopsy
JJ	NS	3	45	"	1	inflammatory, enlarged but bland nuclei	no biopsy

Table 18 (continued)

Patient	Histology	Stage	Months since	Treatment	Cervical cytology class	comments	Cervical Histology
<u>A. HODGKIN'S DISEASE cont'd</u>							
LB	LP	1A	72	R/T	1	Koilocytes	<u>ii Koilocytosis without CIN</u> no biopsy
IF	LP	1A	23	"	1		koilocytosis
IMcK	NS	2A	80	C/T	1+	koilocytes, inflammation	koilocytosis
PP	mixed cellularity (MC)	3B	66	"	1	koilocytes	no biopsy
<u>iii) CIN</u>							
KW	MC	2A	66	R/T	1	koilocytes	CIN II + koilocytosis
NR	NS	3A	46	C/T	1	koilocytes	CIN II + koilocytosis
SLeP	MC	3A	45	"	2		CIN I + koilocytosis
LC	NS	2A	15	"	1+	abnormal endo- cervical cells	CIN II + koilocytosis
MM	MC	4B	24	"	3		CIN III + koilocytosis
EJ	MC	4	38	R/T + C/T	1	koilocytes	CIN II + koilocytosis

Table 18 (continued)

Patient	Histology	Stage	Months since	Treatment	Cervical cytology class	Cervical histology comments	Cervical Histology
B. NON-HODGKIN'S LYMPHOMAS							
<u>i) no significant cervical abnormality</u>							
IP	poorly differentiated lymphocytic	1A	47	R/T	1	inflammation	chronic inflammation
CM	poorly differentiated nodular	3A	18	"	1		no abnormality detected
WH	Lymphocytic	3A	97	R/T + C/T	1		no biopsy
EB	centroblastic centrocytic	3A	8	none	1	atrophic	no biopsy
<u>ii) Koilocytosis only</u>							
JG	poorly differentiated lymphocytic	2A	35	R/T	1	heavy polymorph exudate	koilocytosis
AS	mixed lymphocytic/histocytic	2	66	C/T	1	koilocytes	koilocytosis
WT	poorly differentiated lymphocytic	2	56	R/T + C/T	1+	?chemotherapy effect. Many lymphocytes	koilocytosis
PB	poorly differentiated nodular	2	18	pelvic + C/T	R/T 1	atrophic, inflammatory	koilocytosis

mustine or chlorambucil. Standard dosage regimens were employed. One patient (MM) also received adriamycin. Chemotherapy for non-Hodgkin's lymphomas consisted of cyclophosphamide, adriamycin, vincristine and prednisolone for all patients. One patient (WT) also received bleomycin.

Those patients who received both radiotherapy (R/T) and C/T did so because of residual disease after initial R/T, or because they experienced a relapse following R/T. There were two exceptions; JJ was given R/T when she experienced a relapse following initial C/T, and MMcK experienced profound myelosuppression during C/T, necessitating the completion of her treatment with R/T.

Patients with localised disease (stage 1 and sometimes stage 2) were given R/T, and only one patient (PB) received pelvic R/T. C/T was the treatment of choice for more extensive disease. In non-Hodgkin's lymphomas the degree of differentiation was also considered; thus patients with low grade lymphomas received R/T and those with higher grade tumours C/T. In general, therefore, those patients who received C/T had more extensive and/or less differentiated tumours than those who received R/T.

c) Previous cervical cytology

Twenty-one (78%) lymphoma patients (including four patients found to have CIN) reported having had previously normal cervical cytology. Smears from 16 of these patients could be traced and 15 were confirmed normal. One (from a patient found to have CIN III) was reclassified as class 2. The mean length of time since the last normal smear for the whole group was 67.3 months (range 5-240) and for the patients found to have CIN 16.3 months (6-45

months).

One patient (SLeP) had had a class 1+ smear 4 months before being seen at the Colposcopy Clinic. This smear was confirmed class 1+ on review and a diagnosis of CIN I with koilocytosis was made by histological examination of a colposcopically directed punch biopsy.

d) Cervical histology

It can be seen from table 19 that five (19%) lymphoma patients had CIN II/III, compared with two (3%) of the controls. This difference is statistically significant ($p < 0.01$) by the χ^2 test. The percentages of lymphoma patients with koilocytosis alone, and with CIN I, were not significantly greater than the percentages of control patients with these abnormalities. Significantly more lymphoma patients (14 patients; 52%) had evidence of cervical HPV infection than controls (21 patients; 27%) ($p < 0.02$). Table 19 shows that all six lymphoma patients with CIN were HD patients. The proportion of HD patients with CIN (32%) was not, however, significantly different from the proportion of non-Hodgkin's lymphoma patients with CIN (0%) ($p > 0.05$) by Fisher's exact probability test.

Table 20 shows that the lymphoma patients with cervical koilocytosis without CIN were older, more likely to be parous and became sexually active rather later than the patients in whom no significant abnormality was detected. They had also had their lymphomas diagnosed for longer periods. Lymphoma patients with CIN, on the other hand, were younger, commenced sexual activity earlier and reported more sexual partners than did the patients in whom no abnormality was detected, although these differences were not

Table 19

Cervical histology of patient and control groups

Cervical	CONTROLS		LYMPHOMA (n=27)	PATIENTS (%)
	(n=79)	(%)		
no significant abnormality detected	57	(72)	13	(48)
Koilocytosis only	14	(28)	8	(30) NS
CIN I*	6	(8)	1	(3) NS
CIN II/III*	2	(3)	5	(19)**
Koilocytosis alone or in association with CIN	21	(27)	14	(52)***

* Koilocytes were seen in association with CIN in all cases except one control patient with CIN I

** $\chi^2 = 8.33$ $p < 0.01$ (lymphoma patients versus controls)

*** $\chi^2 = 5.8$ $p < 0.02$ (lymphoma patients versus controls)

NS = not significant

Table 20

Epidemiological characteristics of lymphoma patients
(grouped according to cervical histology and cytology)

	Age (range)	Number parous (%)	Mean age at coitarche (range)	Median no. sexual partners (range)	Smoking current (%)	Ex (%)	Months since diagnosis mean (range)
No significant abnormality detected or inflammation only (n=13)	38.8 (26-56)	7 (54)	20.7 (13-40)	1 (1-30)	4 (31%)	0	41.1 (8-97)
Koilocytosis alone (n=8)	43.5 (32-59)	6 (75)	23 (19-33)	1.5 (1-8)	2 (25%)	3 (38%)	57.8 (18-72)
CIN (n=6)	32.6 (24-47)	3 (50)	18.7 (16-20)	4 (1-9)	0	1 (17%)	39 (15-66)

statistically significant ($0.1 > P > 0.05$ by Mann-whitney U test). Lymphomas had been diagnosed in these patients prior to colposcopic examination for approximately the same length of time as in the lymphoma patients with normal cervixes.

Discussion

A significantly higher prevalence of CIN II and III was demonstrated in lymphoma patients than in a control group of women. Ideally each case should have been compared with two or three controls, matched with the case for known risk factors. However, colposcopic examination and biopsy is an invasive procedure and, when carried out on patients anaesthetised for dilatation and curettage or laparoscopic sterilisation, significantly increases the duration of the general anaesthetic. The recruitment and examination of the much larger number of controls necessary for this approach would have presented considerable practical problems and it was therefore decided that it was justifiable to compare lymphoma patients with available controls, matched only for age and oral contraceptive use. In fact the control patients reported on average more sexual partners and an earlier coitarche and were more likely to smoke than the lymphoma patients (see table 17). A higher prevalence of CIN in the controls than in the lymphoma patients would be predicted on the basis of these known risk factors (La Vecchia, 1985, Winkelstein et al, 1984). However the reverse was found, that is a significantly higher prevalence of CIN II and III in lymphoma patients than in controls. This lends support to the contention that lymphoma patients are at increased risk of developing CIN and that this risk is independent of known risk factors such as sexual behaviour and smoking. It is noteworthy

that the lymphoma patients found to have CIN reported having had more sexual partners than did either the lymphoma patients without CIN or the normal controls (see tables 17 and 20).

The malignant potential of CIN II and III is well defined with few cases regressing. The natural history of CIN I, on the other hand, is still controversial (Campion et al, 1986) as studies relying on cytology alone have tended to underestimate the severity of the initial lesion while those employing colposcopic punch biopsy have found this procedure to be curative in some cases, thus artificially increasing the "regression" rate (Koss, 1978). In view of this uncertainty patients and controls with CIN I were separated from those with CIN II and III for the purpose of statistical analysis.

The increased prevalence of CIN in lymphoma patients is in agreement with the increase in other malignancies seen in these patients (Coleman et al, 1982; Tester et al, 1984) and may be due to immunosuppression. Five of the six lymphoma patients with CIN had received chemotherapy, which contributes to immunosuppression in these patients (Schilsky and Erlichman, 1982). It should, however, be remembered that patients receiving chemotherapy tend to have more extensive disease so that the apparent association between CIN and chemotherapy may be due to the immunosuppressive effects of the disease rather than the effects of chemotherapy. This question will be explored further by studying breast cancer patients treated with alkylating agents in an attempt to separate the effects of these agents from the effects of the lymphoma.

It is interesting that no CIN was detected in non-Hodgkin's lymphoma patients treated with chemotherapy. Less is known about

the risk of second malignancies in non-Hodgkin's lymphomas than in HD but the risk of acute myeloid leukaemia in patients treated with alkylating agents for non-Hodgkin's lymphomas appears to be similar to the risk seen in HD patients treated with these agents (Pedersen-Bjergaard et al, 1985). In this study there were only four patients with non-Hodgkin's lymphomas who received chemotherapy so that no firm conclusions can be drawn.

Of the six lymphoma patients with CIN, five had had cervical smears performed within the preceding four years, but only one of these smears was reported, on routine screening, to be abnormal. When smears were taken under optimal conditions at colposcopy and examined carefully three of six cases of CIN were still undetected. The insensitivity of cervical cytology has been appreciated since 1970 when Coppelson reported colposcopic persistence of dysplasia despite reversion to normal of abnormal cervical smears. This finding has been confirmed recently by Davis et al (1987) and Kitchener et al (1987) while Campion et al (1986) studied women with mild cervical atypia and found that of the women whose disease progressed to CIN III, 15% had had two consecutive false negative smears. Giles et al (1988) surveyed 200 normal women and found that 22 (11%) had CIN. Cervical cytology failed to detect seven (32%) of these cases.

The data reported here is in agreement with these results, lending support to the increasingly popular view that cervical cytology alone is inadequate as a screening technique for women at high risk of CIN.

Fifty-two per cent of lymphoma patients had cytological and/or histological evidence of cervical HPV infection, alone or in

association with CIN. This was significantly higher than the proportion of control patients with cervical HPV infection (see table 19). However, it should be noted that the lymphoma patients with koilocytosis only, in the absence of CIN, are, on average, 11 years older than those with CIN and have been sexually active for 6.6 years longer than CIN patients (see table 20). The "koilocytosis only" patients were also diagnosed as suffering from a lymphoma 18 months earlier than the CIN patients (i.e. 57.8 months before colposcopy versus 39 months). These data tend to argue against HPV infection being a premalignant condition and certainly demonstrate that even in immunosuppressed women, neoplastic change does not inevitably follow. Other factors including, in these patients, the degree of immunosuppression and the carcinogenic potential of drugs given, as well as recognised risk factors such as early onset of sexual activity, multiple partners and smoking, are likely to be implicated.

CHAPTER 7

CHAPTER 7CONCLUSIONS

This thesis would undoubtedly have benefitted from the inclusion of a larger number of colposcopically screened normal controls. The controls studied in Chapters 4 and 5 were patients referred to the Colposcopy Clinic on account of a cervix thought to be clinically suspicious, but with normal cervical cytology, colposcopy and histology, and patients undergoing unrelated gynaecological procedures with normal cervical cytology. They were chosen because they were easily accessible, and because taking cervical brushings or biopsies from them did not cause delay in the clinic or the operating theatre and did not inconvenience other members of staff. It was considered clinically appropriate to take cervical biopsies from the normal controls seen at the Colposcopy clinic to confirm the absence of neoplastic disease. Patients undergoing unrelated procedures gave informed consent for cervical punch biopsy, and did not experience discomfort because they were anaesthetised.

The Group 'C' controls who are described in Chapters 3 and 6 are, perhaps, more acceptable as normal controls. They were patients with normal cervical cytology who underwent colposcopy and cervical punch biopsy at the time of an unrelated minor gynaecological procedure such as dilatation and curettage or laparoscopic sterilisation. The recruitment and examination of these patients involved considerable disruption to operating lists which were already full, and it could be argued that they are not

ideal normal controls either. They were older, on average, than the patients with CIN, reflecting the general tendency for patients undergoing gynaecological operations to be older than those attending colposcopy clinics. It might be predicted that women undergoing laparoscopic sterilisation will be more highly parous than the general population but, in fact, there was no significant difference in parity between group 'C' controls and CIN patients. Patients undergoing dilatation and curettage may be suffering from a hormone imbalance related to dysfunctional uterine bleeding and in view of the controversy surrounding the role of oestrogen and progesterone in the aetiology of CIN (see p.27) are perhaps not ideal controls. Perhaps the best controls would be patients attending a family planning or well woman clinic for routine cervical screening. The recruitment and examination of these women would, however, have involved considerable organisation and additional work for both the author and for other members of staff and was therefore not performed.

The group 'B' normal controls (women undergoing unrelated gynaecological procedures) had normal cervical cytology but did not have colposcopic examination carried out. The relative insensitivity of cervical cytology is well recognised (see p.110) so that CIN cannot reasonably be said to have been excluded unless a colposcopic examination has been carried out. The results of the present study confirm this, in that three of six cases of histologically proven CIN in lymphoma patients, and seven of eight cases in control patients were undetected by cervical cytology. In addition all specimens obtained should have been

analysed for the presence of HPV antigen or viral DNA, as 38% of cervical brushings and biopsies from otherwise normal cervixes contained HPV antigen, and similar percentages (in relation to viral DNA) are reported from other groups (Cox et al, 1986; Schneider et al, 1987).

It should, however, be remembered (as discussed in Chapters 4 and 5) that significant differences were detected between the so-called normal controls and the patients with proven cervical HPV infection or CIN, in terms of LC numbers, MHC class II expression, cell cycle analysis and ploidy. If the control group did contain patients with undetected HPV infection or CIN it can be predicted that the differences between a "pure" control group and HPV and CIN patients would be greater, rather than less.

The conclusions drawn from the work described in Chapters 4, 5 and 6 may therefore be regarded as valid and as under-estimating, rather than over-estimating, the differences between normal and abnormal cervical epithelium.

The results reported in Chapter 4 of this thesis support the contention advanced by other investigators that there is a disturbance of local immune function in papillomavirus infected cervical epithelium, and in CIN. LC and T cells were found to be reduced in number in HPV infected epithelium and in CIN. MHC Class II antigen expression by LC was, in addition, significantly increased in CIN.

It has been suggested that HPV has a cytopathic effect on LC (Morris et al, 1983) or that LC surface antigens may be altered or blocked by the presence of HPV (Hawthorn et al, 1988). One or both of these mechanisms may account for the reduced LC numbers and

altered LC morphology noted in HPV infected epithelium. It is also possible, however, that intraepithelial LC are reduced in number because they migrate out of virally infected epithelium in a manner similar to that seen following ultraviolet irradiation of skin (Gurish et al, 1982). It would be interesting, in this context, to study LC numbers in adjacent "normal" cervical epithelium, to discover whether cell counts are unchanged or reduced, relative to normal, as might be expected if the virus destroys intralesional LCs; or increased as might occur if the virus causes migration out of the lesion. Morris et al (1983) examined normal epithelium adjacent to CIN and found LC counts to be similar to counts in normal epithelium from controls. It is, however, difficult to obtain a truly "normal" biopsy as apparently normal epithelium adjacent to CIN or koilocytic lesions has been shown, repeatedly, to contain HPV DNA (Ferenczy et al, 1985; MacNab et al, 1986).

The demonstration here of HPV antigens in the tumour-free pelvic lymph nodes of patients undergoing hysterectomy for cervical carcinoma (see Chapter 5) would support the theory that infected LC, or LC bearing HPV antigen, do migrate from HPV infected epithelium to draining lymph nodes. It would, however, be necessary to employ a double staining technique to identify the cells within the node containing, or bound to, HPV DNA or antigen to ascertain whether these cells are, in fact, LCs. This would be most helpful in advancing our comprehension of the local immune response to HPV which is so limited at present.

Once HPV infection has caused intraepithelial LC depletion (by causing LC damage or migration) it is postulated that neoplastic change can occur, undetected by the normal immunosurveillance

mechanisms (Morris et al, 1983). HPV may play a dual role in the causation of cervical neoplasia, allowing neoplastic change to occur undetected and, in addition, stimulating cell division.

It would be interesting to look at LC numbers in epithelium infected with other viruses, to discover whether LC depletion is a non-specific viral effect. There is little published work in this area to date. Bhawan et al (1986) report a total absence of LC in molluscum contagiosum, with normal or increased numbers in peri-lesional normal skin and postulate that alterations in the differentiation and maturation of keratinocytes in these lesions may lead to an inhibition of LC migration into the epidermis. Nagao et al (1976) studied LC at the site of vaccinia virus inoculation and comment that intralesional LC contained increased numbers of granules. They do not, however, give details of absolute numbers of LC.

In this study LC were also found to be reduced in number in CIN. Other investigators have reported conflicting results (see Chapter 1 p.50), but in general LC are reported to be reduced in number in and around malignant tumours (Gatter et al, 1984). It is interesting that patients with progressive disease, and patients with residual or recurrent CIN following ablative therapy, were found to have had lower LC counts at their first visit than those who were found to be free of disease at one year follow-up. This corresponds with the findings of Syrjanen et al (1984) who noted that LC counts were lower in patients whose disease progressed than in those whose disease regressed, and is further evidence to support the idea that LC play a role in tumour immunosurveillance at this site.

Increased class II antigen expression by LC was demonstrated in koilocytic lesions and in CIN, and may represent a higher level of "activation" of the cells. The work of Navaretti et al (1985a and b) concerning the role of HLA-DQ in the generation of a suppressor type response is discussed in Chapter 5. At present little is known about the functional significance of the differential expression of HLA-DP, DQ and DR but it is possible that increased expression of HLA-DQ in CIN I (as described in Chapter 5) does induce a predominantly suppressor response thus allowing neoplastic progression.

The expression of MHC Class II antigens by epithelial cells in CIN was described by Morris et al (1983) and is confirmed here. It may be a reflection of the lack of differentiation of the epithelium but may also have important implications concerning the local intraepithelial immune response. Again, little is known about the functional significance of MHC Class II expression by non-lymphoid cells but it would be interesting to compare the clinical outcome of untreated Class II positive and negative lesions.

The work reported in Chapter 6 comparing the prevalence of CIN in lymphoma patients with its prevalence in normal controls provides further evidence to support the theory that an intact immune system is necessary to prevent the development of CIN. The number of patients studied was, of necessity, very small but if the detection of CIN in 38% of women with Hodgkin's disease who had received chemotherapy is confirmed by studying a larger group this will have important implications for the management of these patients, the majority of whom have a life expectancy of at least

15 years. It is not clear from this study whether the increased risk of CIN detected in patients with Hodgkin's disease can be attributed to the lymphoma or to the effects of the chemotherapeutic agents used. A study has begun of patients with breast cancer treated with similar agents in an attempt to separate the effects of the lymphoma from the effects of therapy.

The cervical cytology data acquired in this part of the study is of clinical concern and would support the view that cervical cytology alone, without back-up colposcopy or histology, is an unacceptably insensitive technique when used to screen women with known cervical abnormalities. The implications for screening of the general population and of women at high risk of developing CIN, are less clear. The true false negative rate in the general population will not be known until a study combining cytology, colposcopy and histology in the examination of a large number of normal women is performed, but Giles et al (1988) suggest that the rate may approach 32% (see page 153).

The rate of cell division in koilocytic and papillomavirus antigen positive cervical epithelium was found to be higher than in normal epithelium, and to be similar to the proliferative rate in CIN. This supports the hypothesis of Reid et al (1982) that the virus acts as a non-specific mitogen and causes S-phase arrest. Possibly this is the role of HPV in carcinogenesis; by stimulating cell division and by arresting cells in the S-phase it simply provides a greater number of opportunities for mutation and neoplastic change.

It is easier to reconcile this idea with the detection of HPV DNA and antigens in a significant proportion of normal cervixes

(here, and Meanwell et al, 1987) and in other normal tissues (Maitland et al, 1987; Ferenczy et al, 1985) than it is to postulate that the virus consistently causes genetic aberrations and malignant change. It should be noted that HPV antigens are not detected in invasive carcinomas, although viral DNA is known to be present, and there is evidence that viral DNA is not transcribed in all HPV positive tumours (Lehn et al, 1985; Yee et al, 1985) which argues against an active role for the virus as a source of tumour antigen. HPV type and integration of viral DNA may both be important factors which were not explored at all in this thesis.

More surprising, perhaps, was the detection of DNA aneuploidy in koilocytic lesions, in the absence of any detectable CIN. This suggests that the virus induces potentially malignant changes in cellular DNA which can be detected before any phenotypic change has occurred. This appears to be an important piece of evidence supporting the theory that HPV behaves as a carcinogen in the cervical transformation zone. It is important to remember, however, that aneuploidy has been reported in benign lesions with no known malignant potential (see Chapter 1, p.57) and that some aneuploid cervical lesions have been shown to regress (Nasiell et al, 1979). It would be most informative to look for DNA aneuploidy in lesions induced by HPV types 1 and 2 which have no known malignant potential and in warts from immunosuppressed individuals including those with epidermodysplasia verruciformis. The results obtained from the various experiments described in this thesis demonstrate a consistent difference between HPV infected and normal epithelium, and a consistent similarity (in terms of patterns of cell division, ploidy, oncogene expression and LC and T cell

infiltrates) between HPV lesions and CIN. There was no real evidence of an increasing trend in the detection of any of these features as "progression" occurred from HPV infection to CIN, arguing perhaps, against HPV infection being a precursor of CIN. The techniques employed in this study (in particular the use of monoclonal antibodies, and flow cytometry) were not in use when interest centred on herpes simplex virus type 2 as an aetiological agent in cervical carcinoma. It would be very interesting to use these methods to discover whether the abnormalities found in association with HPV infection are also associated with infection by other microorganisms, including HSV-2.

It is, nevertheless, striking that HPV infected and neoplastic epithelium examined by very different methods and assayed for very different criteria should be so difficult to distinguish from one another. Perhaps papillomavirus infection is a necessary precursor for neoplastic change, but progression only occurs in the presence of a factor or factors not identified by these experiments but which probably include immunosuppression (local or systemic) and hormonal status and may include smoking, infection with other viruses, particular characteristics of the male partner's semen and genetic predisposition. Carcinogenesis is well recognised to be a multifactorial phenomenon (Berenblum, 1941) and it seems unlikely that cervical carcinogenesis will be an exception to this rule.

B I B L I O G R A P H Y

BIBLIOGRAPHY

- Aberer, W., Schuler, G., Stingl, G., Honigsmann, H. and Wolff, K.
Ultraviolet light depletes surface markers of Langerhans cells. *J. Invest. Dermatol.* (1981) 76: 202-210.
- Alexander, S., Hubbard, S.C. and Strominger, J.L. HLA-DR antigens of autologous melanoma and B lymphoblastoid cell lines: differences in glycosylation but not protein structure. *J. Immunol.* (1984) 133: 315-320.
- Alloub, M. Personal communication (1987).
- Alzate, H. Sexual behaviour of Colombian male medical students. *Br. J. Sex. Med.* (1977) 5: 19.
- Alzate, H. Sexual behaviour of Colombian female university students. *Arch. Sex. Behav.* (1978) 7: 43-54.
- Andolsek, L., Kovacic, J., Kozuh, M. and Litt, B. Influence of oral contraceptives on the incidence of premalignant and malignant lesions of the cervix. *Contraception* (1983) 28: 505-519.
- Anon. New studies tighten link between herpes viruses and cervical cancer. *JAMA* (editorial) (1975) 234: 1101-1106.
- Anon. Papillomavirus invades esophagus, incidence seems to be increasing. *JAMA* (editorial) (1984) 251: 2185-2187.
- Anon. Second malignancies in lymphoma patients. *Lancet* (editorial) (1985) 11: 1163-1164.
- Anon. Epstein Barr virus and the uterine cervix. *Lancet* (editorial) (1986) 11: 1134-1135.

- Arseneau, J.C., Sponzo, R.W., Levin, D.L. et al. Nonlymphomatous malignant tumors complicating Hodgkin's disease: possible association with intensive therapy. *N. Engl. J. Med.* (1972) 287: 1119-1122.
- Atkin, N.B. Antioncogenes (letter). *Lancet*, (1985) ii: 1189-1190.
- Aubock, J., Romani, N., Grubauer, G. and Fritsch, P. HLA-DR expression on keratinocytes is a common feature of diseased skin. *Br. J. Dermatol* (1986) 114: 465-472.
- Aurelian, L., Strandber, J.D., Mecendez, L.V. and Johnson, L.A. Herpes simplex virus type 2 isolated from cervical tumour cells grown in tissue culture. *Science* (1971) 174: 704-707.
- Aurelian, L., Schumann, B., Marcus, R.L. and Davis, H.J. Antibody to Herpes simplex virus type 2 induced tumour specific antigens in serum from patients with cervical cancer. *Science* (1973) 181: 161-164.
- Ayre, J.E. Selective cytology smear for diagnosis of cancer. *Am. J. Obstet. Gynecol.* (1947) 53: 609-617.
- Baird, P.J. Serological evidence for the association of papilloma-virus and cervical neoplasia. *Lancet* (1983) ii: 17-18.
- Bangle, R., Berger, M. and Levin, M. Variations in morphogenesis of squamous carcinoma of the cervix. *Cancer* (1963) 16: 1151-1159.
- Banks, L., Spence, B., Androphy, E., et al. Identification of human papillomavirus type 18 E6 polypeptide in cells derived from human cervical carcinomas. *J. Gen. Virol* (1987) 68: 1351-1359.

- Barnes, E.W., MacCuish, A.C., Loudon, N.B., Jordan, J. and Irvine, W.J. Phytohaemagglutinin induced transformation and circulating autoantibodies in women taking oral contraceptive pill. *Lancet* (1974) 1: 898-900.
- Barton, S.E., Maddox, P.H., Jenkins, D., Edwards, R., Guzick, J. and Singer, A. Effect of cigarette smoking on cervical epithelial immunity: a mechanism for neoplastic change? *Lancet* (1988) 11: 652-654.
- Basham, T.Y. and Merigan, T.C. Recombinant interferon increases HLA-DR synthesis and expression. *J. Immunol* (1983) 130: 1492-1494.
- Beckman, A.M., Daling, J.R. and McDougall, J.K. Human papillomavirus DNA in anogenital carcinomas. *J. Cell Biochem. (Suppl)* (1985) 9c: 68.
- Bendich, A., Borenfreund, E., Witkin, S.S., Bejn, D. and Higgins, P.J. Information transfer and sperm uptake by mammalian somatic cells. *Progr. Nucleic Acid Res. Mol. Biol.* (1976) 17: 43-75.
- Berenblum, I. The mechanism of carcinogenesis. *Cancer Res* (1941) 1: 807-815.
- Berman, B., Duncan, M.R., Smith, B., Ziboh, V.A. and Palladino, M. Interferon enhancement of HLA-DR antigen expression on epidermal Langerhans cells. *J. Invest. Dermatol.* (1985) 84: 54-58.
- Bernheim, A., Berger, R. and Lenoir, G. Cytogenetic studies on African Burkitts lymphoma cell lines: t(8;14) t (2;8) and t(8;22) translocations. *Cancer Genet. Cytogenet.* (1981) 3: 307-315.

- Bhan, A.K., Reinherz, E.Z., Poppema, S., McCluskey, R.T. and Schlossman, S.F. Location of T cell and major histocompatibility antigens in the human thymus. *J. Exp. Med.* (1980) 152: 771-782.
- Bhawan, J., Dayal, Y. and Bhan, A.K. Langerhans cells in molluscum contagiosum, verucca vulgaris, plantar warts and condyloma acuminatum. *J. Am. Acad. Dermatol.* (1986) 15: 645-649.
- Birbeck, M.S., Breathnach, A.A. and Everall, J.D. An electron microscopic study of basal melanocytes and high level clear cells (Langerhans cells) in vitiligo. *J. Invest. Dermatol.* (1961) 37: 51-64.
- Bishop, J.M. and Varmus, H.E. Functions and origins of retroviral transforming genes. In *Molecular Biology of Tumour Viruses, Part III RNA Tumor Viruses*, R. Weiss, N. Teich, H. Varmus and J. Coffin, eds. (N.Y. Cold Spring Harbor Press) (1982) p 999-1108.
- Bishop, J.M. Cellular oncogenes and retroviruses. *Ann. Rev. Biochem* (1983) 52: 301-354.
- Bishop, J.M. Viral oncogenes. *Cell* (1985) 42: 23-38.
- Bjercke, S., Braathen, L., Gaudernach, G. and Thorsby, E. Relative efficiency of human Langerhans cells and blood derived dendritic cells as antigen presenting cells. *Acta. Derm. Venereol.* (Stockh). (1985) 65: 374-378.
- Bos, J.D., Zonnenveld, F., Das, P.K., Krieg, S.R., Van der Loos, C.M. and Kapsenberg, M.L. The skin immune system: distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J. Invest. Dermatol.* (1987) 88: 569-578.

- Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, H. Scheurlen, and Zur Hausen, H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.* (1984) 3: 1151-1157.
- Boyle, J., Mackie, R.M., Briggs, J.D., Junor, B.J.R. and Aitchison, T.C. Cancer, warts and sunshine in renal transplant patients. *Lancet* (1984) 1: 702-705.
- Braathen, L.R. and Thorsby, E. Studies on human epidermal Langerhans cells. I. Allo-activating and antigen presenting capacity. *Scand. J. Immunol.* (1980) 11: 401-408.
- Braathen, L.R. and Thorsby, E. Studies on human epidermal Langerhans cells. IV. HLA-D restriction of Langerhans cells dependent antigen activation of T lymphocytes. *Scand. J. Immunol.* (1982) 15: 55-61.
- Braylan, R.C., Diamond, L.W., Powell, M.L. and Harty-Golder, B. Percentage of cells in the S phase of the cell cycle in human lymphomas determined by flow cytometry. Correlation with labelling index and patient survival. *Cytometry* (1980) 1: 171-174.
- Brescia, R.J., Jenson, A.B., Lancaster, W.D. and Kurman, R.J. The role of human papillomaviruses in the pathogenesis and histologic classification of precancerous lesions of the cervix. *Hum. Pathol.* (1986) 17 (6): 552-559.
- Broders, A.C. Carcinoma-in-situ contrasted with benign penetrating epithelium. (1932) *JAMA* 99: 1670-1674.

- Brodersen, I. and Genner, J. Histological and immunological observations on common warts in regression. *Acta. Derm. Venereol. (Stockh.)* (1973) 53: 461-464.
- Brodersen, I., Genner, J. and Brodthagen, H. Tuberculin sensitivity in BCG vaccinated children with common warts. *Acta. Derma. Venereol. (Stockh.)* (1974) 54: 292-292.
- Buckley, J.D., Harris, R.W.C., Doll, R., Vessey, M.P. and William, P.T. Case control study of the husbands of women with cancer of the cervix uteri. *Lancet* (1981) ii: 1010-1015.
- Buckley, C.H., Butler, E.B. and Fox, H. Cervical intraepithelial neoplasia. *J. Clin. Pathol.* (1982) 35: 1-13.
- Burnett, F.M. *Immunologic Surveillance.* (1970) Oxford, Pergamon Press.
- Bussy-Fernandez, A., Chambazaro, F. and Maudnit, G. T cell subsets and Langerhans cells in skin tumours. *Eur. J. Cancer Clin. Oncol.* (1983) 19: 907-913.
- Campion, M.J., McCance, D.J., Cuzick, J. and Singer, A. Progressive potential of mild cervical atypia: prospective cytological, colposcopic and virological study. *Lancet* (1986) ii: 237-240.
- Campo, M.S. and Jarrett, W.F.H. Papillomaviruses and disease. In: *Molecular Basis of Virus Disease* (1987) SGM symposium 40. W.C. Russell and J.W. Almond (eds). pp 215-243.
- Caorsi, I. and Figueroa, C.D. Langerhans cell density in the normal exocervical epithelium and in the cervical intraepithelial neoplasia. *Br. J. Obstet. Gynaecol.* (1986) 93: 993-998.

- Carr, M.M., McVittie, E., Guy, L., Gawkrödger, D.J. and Hunter, J.A.A.
Major histocompatibility class II antigen expression in
normal human epidermis. *Immunology* (1986) 59: 223-227.
- Carson, L.F., Twigg, L.B., Fukushima, M., Ostrow, R.S., Faras, A.J.
and Okagaki, T. Human genital papilloma infections: an
evaluation of immunologic competence in the genital
neoplasia - papilloma syndrome. *Am. J. Obstet. Gynecol.*
(1986) 155: 784-789.
- Cartier, Rene. *Practical Colposcopy*. (1977) Basel, London, New York.
- Caspersson, T.O. Quantitative tumour cytochemistry. In : Clowes
G.H.S. ed. Memorial Lecture. *Cancer Res.* (1979) 39:
2341-2355.
- Cassai, E., Rotola, A., Meneguzzi, G. et al. Herpes simplex virus
and Human Cancer I: Relationship between human cervical
tumours and Herpes simplex virus type 2. *Eur. J. Cancer
Clin. Oncol.* (1981) 17: 685-693.
- Chardonnet, Y., Viac, J. and Thivolet, J. Langerhans cells in human
warts. *Br. J. Dermatol.* (1986) 115: 669-675.
- Chin, J.L., Huben, R.P., Nava, E. et al. Flow cytometric analysis
of DNA content in human bladder tumors and irrigation
fluids. *Cancer* (1985) 56: 1677-1681.

- Chipperfield, E.J. and Evans, B.A. The influence of local infection on immunoglobulin formation in the endocervix. *Clin. Exp. Immunol.* (1972) 11: 219-223.
- Chretien, J.H., Esswein, J.G. and Garagusi, V.F. Decreased T cell levels in patients with warts. *Arch. Dermatol.* (1978) 114: 213-215.
- Christopherson, W.M. Concept of genesis and development in early cervical neoplasia. *Obstet. Gynecol. Surv.* (1969) 24: 842-850.
- Christopherson, W.M., Nealson, N. and Gray, L.A. Noninvasive precursor lesions of adenocarcinoma and mixed adenosquamous carcinoma of the cervix uteri. *Cancer* (1979) 44: 975-983.
- Clarke, E.A., Morgan, R.W. and Newman, A.M. Smoking as a risk factor in cancer of the cervix: additional evidence from a case control study. *Am. J. Epidemiol* (1982) 115: 59-66.
- Clarke, E.A., Hatcher, J., McKeown,, Eyssen, G.F. and Lickrish, G.M. Cervical dysplasia: association with sexual behaviour, smoking and oral contraceptive use? *Am. J. Obstet. Gynecol.* (1985) 151: 612-616.
- Coleman, C.N., Kaplan, H.S., Cox, R., Varghese, A., Butterfield, P. and Rosenberg, S.A. Leukaemias, non-Hodgkin's lymphomas and solid tumors in patients treated for Hodgkin's disease. *Cancer Surveys* (1982) 1: 733-744.
- Coleman, D.V., Wickenden, C. and Malcolm, A.D.B. Association of human papillomavirus with squamous carcinoma of the uterine cervix. *Papillomaviruses: Ciba Found. Symp.* (1986) 120: 175-189.

- Collard, J.G., de Boer, P.A.J., Janssen, J.W.G., Schlijven, J.F. and de Jong, B. Gene mapping by chromosome spot hybridization. *Cytometry* (1985) 6: 179-185.
- Collins, T., Korman, A.J., Wake, C.T. et al. Immune interferon activates multiple class II major histocompatibility genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. *Proc. Natl. Acad. Sci. USA* (1984) 81: 4917-4921.
- Coppleson, M. and Reid, B.L. The etiology of squamous carcinoma of the cervix. *Obstet. Gynecol.* (1968) 32: 432-436.
- Coppleson, M. Cancer of the cervix - epidemiology and aetiology. *Br. J. Hosp. Med.* (1969) 2: 960-980.
- Coppleson, M. The origin and nature of premalignant lesions of the cervix uteri. *Int. J. Obstet. Gynecol.* (1970) 8: 539-550.
- Covone, A.E., Johnson, P.M., Mutton, D. and Adinolf, M. Trophoblast cells in peripheral blood from pregnant women. *Lancet* (1984) ii: 841-843.
- Cox, M.F., Meanwell, C.A., Maitland, N.J., Blackledge, E.G., Scully, C. and Jordan, J.A. Human papillomavirus type 16 homologous DNA in normal human ectocervix. *Lancet* (1986) ii: 157-158.
- Crum, C.P., Mitao, M., Levine, R. and Silverstein, S. Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. *J. Virol.* (1985) 54: 675-681.
- Cubie, H.A. Serological studies in a student population prone to infection with human papillomavirus. *J. Hyg. (Camb.)* (1972) 70: 677-690.

- Cubie, H.A. and Norval, M. Humoral and cellular immunity to papillomavirus in patients with cervical dysplasia. *J. Med. Virol.* (1988) 24: 85-95.
- Czerniak, B., Papenhausen, P.R., Herz, F. and Koss, L.G. Flow cytometric identification of cancer cells in effusions with Cal monoclonal antibody. *Cancer* (1985) 55: 2783-2788.
- Daar, A.S., Fuggle, S.V., Fabre, J.W., Ting, A. and Morris, P.J. The detailed distribution of major histocompatibility class II antigens in normal human organs. *Transplantation* (1984) 38: 293-298.
- Dani, C., Blanchard, J.M., Piechaczyk, M., El Sabouty, S., Marty, L. and Jeanteur, P. Extreme instability of myc mRNA in normal and transformed human cells. *Proc. Natl. Acad. Sci. USA* (1984) 81: 7046-7050.
- Danova, M., Riccardi, A., Mazzini, G. et al. Ploidy and proliferative activity of human brain tumours. *Oncology* (1987) 44: 102-107.
- Davies, K.E., Young, B.D., Elles, R.G., Hill, M.E. and Williamson, R. Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry. *Nature* (1981) 293: 374-376.
- Davis, G.L., Hernandez, E., David, J. and Kunio, M. Atypical squamous cells in Papanicolaou smears. *Obstet. Gynecol.* (1987) 69: 43-46.
- Dawson, D.F., Duckworth, J.K., Bernhardt, H. and Young, J.M. Giant condyloma and verrucous carcinoma of the genital area. *Arch. Pathol.* (1965) 79: 225-231.

- Day, N.E. and Brown, C.C. Multistage models and primary prevention of cancer. *J.N.C.I.* (1980) 64: 977-989.
- Deitch, A.D., Law, H. and Devere White, R. A stable propidium iodide staining procedure for flow cytometry. *J. Histochem. Cytochem.* (1982) 30(9): 967-972.
- De Jong, M.C.J.M., Blanken, R., Nanninga, J., Vanvoorst vader, P.C. and Poppema, S. Defined in situ enumeration of T6 and HLA DR expressing epidermal Langerhans cells: morphological and methodological aspects. *J. Invest. Dermatol.* (1986) 87: 698-702.
- De Villiers, E.M., Schneider, A., Gross, G. & Zur Hausen, H. Analysis of benign and malignant urogenital tumors for human papillomavirus infection by labelling cellular DNA. *Med. Microbiol. Immunol. (Berl.)* (1986) 174(6), 281-286.
- De Villiers, E.M. Compilation of human papillomavirus hybridisation for all sites. In : 6th International Papillomavirus Workshop. Short Review of Current Literature (1987) p 52-63.
- Dezutter-Dambuyant, C., Schmitt, D., Faure, M., Horisberger, M. and Thivolet, J. Immunogold technique applied to simultaneous identification of T6 and HLA-DR antigens on Langerhans cells by electron microscopy. *J. Invest. Dermatol.* (1985) 84: 465-468.
- Diamond, L.W., Nathwani, B.N. and Rappaport, H. Flow cytometry in the diagnosis and classification of malignant lymphoma and leukemia. *Cancer* (1982) 50: 1122-1135.

- Doll, R. Invasive cervical cancer and combined oral contraceptives. (Letter) *Br. Med. J.* (1985) 290: 1210.
- Dunn, A.E.G. and Ogilvie, M.M. Intranuclear virus particles in human genital wart tissue. Observations on the ultrastructure of the epidermal layer. *J. Ultrastruct. Res.* (1968) 23: 282-295.
- Durst, M., Gissmann, K., Ikenberg, H. and Zur Hausen, H. A papilloma-virus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA* (1983) 80: 3812-3815.
- Durst, M., Kleinheinz, A., Hotz, M. and Gissmann, L. The physical state of human papillomavirus 16 DNA in benign and malignant genital tumors. *J. Gen. Virol.* (1985) 66: 1515-1522.
- Edwards, J.N.T. and Morris, H.B. Langerhans cells and lymphocyte subsets in the female genital tract. *Br. J. Obstet. Gynaecol.* (1985) 92: 974-982.
- Eldemiry, M.I.M., Hargreaves, T.B., Bussuttil, A., James, K, Ritchie, A.W.S. and Chisholm, G.D. Lymphocyte subpopulations in the female genital tract. *Br. J. Urol.* (1985) 57: 769-775.
- Elias-Jones, J., Hendy-Ibbs, P., Cox, H., Evan, G.I. and Watson, J.V. Cervical brush specimens suitable for DNA and oncoprotein analysis using flow cytometry. *J. Clin. Pathol.* (1986) 39: 577-580.
- Eriksen, K. Treatment of the common wart by induced allergic inflammation. *Dermatologica* (1980) 160: 161-166.

- Evan, G., Lewis, G.K., Ramsay, G. and Bishop, J.M. Isolation of monoclonal antibodies specific for human and mouse proto-oncogene products. *Mol. Cell Biol.* (1985) 5: 3610-3616.
- Evans, A.S. and Monaghan, J.M. Spontaneous resolution of cervical warty atypia: the relevance of clinical and nuclear DNA features: a prospective study. *Br. J. Obstet. Gynaecol.* (1985) 92: 165-169.
- Evenson, D.P., Darzynkiewicz, A. and Melamed, M.R. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. *J. Histochem. Cytochem.* (1982) 30: 279-280.
- Ewing, J. *Neoplasia diseases* (1940) 4th edition, Saunders, Philadelphia, London.
- Ferenczy, A., Braun, L. and Shah, K.V. Human papillomavirus (HPV) in condylomatous lesions of the cervix. A comparative ultrastructural and immunohistochemical study. *Am. J. Surg. Pathol.* (1981) 5: 661-670.
- Ferenczy, A., Mitao, M., Nagai, N., Silverstein, S.J. and Crum, C.P. Latent papillomavirus and recurring genital warts. *N. Engl. J. Med.* (1985) 313: 784-788.
- Ferguson, A., Moore, M. and Fox, H. Expression of major histocompatibility products and leucocyte differentiation antigens in gynecological neoplasms: a immunohistochemical analysis of the tumour cells and infiltrating leucocytes. *Br. J. Cancer* (1985) 52: 551-563.

- Person, M., Edwards, A., Lind, A., Milton, G.W. and Hersey, P. Low natural killer cell activity and immunoglobulin levels associated with smoking in human subjects. *Int. J. Cancer* (1979) 23: 603-609.
- Fisher, M.S. and Kripke, M.L. Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. *Science* (1982) 216: 1133-1134.
- Fletcher, S. Histopathology of papillomavirus infection of the cervix uteri: the history, taxonomy, nomenclature and reporting of koilocytic dysplasias. *J. Clin. Pathol.* (1983) 36: 616-624.
- Forsum, V., Claesson, K., Hjelm, E. et al. Class II transplantation antigens: distribution in tissues and involvement in disease. *Scand. J. Immunol.* (1985) 21: 389-396.
- Fox, H. and Buckley, C.H. *Pathology for Gynaecologists* (1982). Edward Arnold, London p. 137.
- Francheschi, S., Lavecchia, C. and Talamini, R. Oral contraceptives and cervical neoplasia: pooled information from retrospective and prospective epidemiologic studies. *Tumori* (1986) 72: 21-30.
- French, P.W., Coppleson, M. and Reid, B.L. Effects of serum protamine on human cervical epithelial cells and BHK21 cells in vitro. *J. R. Soc. Med.* (1987) 80: 434.
- Frenkel, N., Roizman, B., Cassai, E. and Nahmias, A. A DNA fragment of herpes simplex type 2 and its transcription in human cervical cancer tissue. *Proc. Natl. Acad. Sci. (USA)* (1972) 69: 3784-3789.

- Friedlander, M.L., Hedley, D.W. and Taylor, I.W. Clinical and biological significance of aneuploidy in human tumours. *J. Clin. Pathol.* (1984a) 37: 961-974.
- Friedlander, M.L., Russell, P., Taylor, I.W., Hedley, D.W. and Tattersall, M.H.N. Flow cytometric analysis of cellular DNA content as an adjunct to the diagnosis of ovarian tumours of borderline malignancy. *Pathology* (1984b) 16: 301-306.
- Fu, Y.S., Reagan, J.W. and Richart, R.M. Precursors of cervical cancer. *Cancer Surveys* (1983) 2: 359-382.
- Furukawa, T., Watanabe, S., Sato, Y., Kodama, T., Nakajama, T. and Shimosato, Y. Heterogeneity of histiocytes in primary lung cancer stained with anti S100 protein, lysozyme and OKT6 antibodies. *Jpn. J. Clin. Oncol.* (1984) 14: 647-658.
- Gagnon, F. Contribution to the study of the etiology and prevention of cancer of the cervix of the uterus. *Am. J. Obstet. Gynecol.* (1950) 60: 516-522.
- Galloway, D.A. and McDougall, J.K. The oncogenic potential of herpes simplex viruses: Evidence for a 'hit and run' mechanism. *Nature* (1983) 302: 21-24.
- Gardner, H.L. and Kaufman, R.H. Condylomata acuminata. *Clin. Obstet. Gynecol.* (1965) 8: 938-945.
- Gatter, K.C., Morris, H.B., Roach, B., Mortimer, P., Fleming, G.K. and Mason, D.Y. Langerhans cells and T cells in human skin tumours: an immunohistological study. *Histopathology* (1984) 8: 229-244.

- Genner, J. Verruca vulgaris II. Demonstration of a complement fixation reaction. Acta. Derm. Venereol (Stockh) (1971) 51: 365-373.
- Giles, J.A., Hudson, E., Crow, J., Williams, D. and Walker, P. Colposcopic assessment of the accuracy of cervical cytology screening. Brit. Med. J. (1988) 296: 1099-1102.
- Gissman, L and zur Hausen, H. Partial characterisation of viral DNA from human genital warts (condylomata acuminata). Int. J. Cancer. (1980) 25: 605-609.
- Gissmann, L., Wolnik, L., Ikenberg, G.H., Koldovsky, U., Schnurch, H. and zur Hausen, H. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Natl. Acad. Sci. USA. (1983) 80: 560-563.
- Gissmann, L., Boshart, M., Durst, M., Ikenberg, H., Wagner, D. and zur Hausen, H. Presence of human papillomavirus in genital tumours. J. Invest. Dermatol. (1984) 83: 265-285.
- Gissmann, L. and Gross, G. Association of human papillomaviruses with human genital tumours. Clinics in Dermatol. (1985) 3 (4) 124-129.
- Glenthoj, J.A., Bostoffe, E. and Rank, F. Brush cytology from the uterine cervix. Acta. Obstet. Gynecol. Scand. (1986) 65: 689-691.
- Gonwa, T.A., Picker, L.J., Raff, H.V., Goyert, S.M., Silver, T. and Stobo, J.D. Antigen presenting capabilities of human monocytes correlates with their expression of HLA-DS, an Ia determinant distinct from HLA-DR. J. Immunol. (1983) 130: 706-711.

- Gonwa, T.A. and Stobo, J.D. Differential expression of Ia molecules by human monocytes. *J. Clin. Invest.* (1984) 74: 859-866.
- Gonzalez-Falco, J., Jurado, M., Madamba, A.M. and Oriol, A. *Gardnerella vaginalis* and mosaic colposcopic pattern of the cervix. *Gynecol. Obstet. Invest.* (1986) 21: 108-110.
- Granberg, I. Chromosomes in preinvasive, microinvasive and invasive cervical carcinoma. *Hereditas* (1971) 68: 165-218.
- Green, G.H. Cervical cancer and population screening in New Zealand. *Br. J. Obstet. Gynaecol.* (1978) 85: 881-886.
- Greenberg, E.R., Vessey, M., McPherson, K. and Yeates, D. Cigarette smoking and cancer of the uterine cervix. *Br. J. Cancer* (1985) 51: 139-141.
- Gross, G., Hagedorn, M., Ikenberg, H. et al. Presence of human papillomavirus structural antigens and of human papillomavirus 16-related DNA sequences. *Arch. Dermatol.* (1985) 121: 858-863.
- Guerry, D., Alexander, M.A., Herlyn, M.F. et al. HLA-DR histocompatibility leukocyte antigens permit cultured human melanoma cells from early but not advanced disease to stimulate autologous lymphocytes. *J. Clin. Invest.* (1984) 73: 267-271.
- Gurish, M.F., Lynch, D.H. and Daynes, R.A. Changes in antigen presenting cells function in the spleen and lymph nodes of ultraviolet-irradiated mice. *Transplantation* (1982) 33: 280-284.

- Gustafson, H., Tribukait, B. and Esposti, P.L. DNA pattern, histological grade and multiplicity related to recurrence rate in superficial bladder tumors. *Scand. J. Urol. Nephrol.* (1982) 16: 135-139.
- Guy, K., Heyningen, V., Cohen, B.B., Deane, D.L. and Steel, C.M. Differential expression and serologically distinct subpopulations of human Ia antigens detected with monoclonal antibodies to α and β chains. *Eur. J. Immunol.* (1982) 12: 942-948.
- Hackermann, M., Grupp, C. and Hill, K.R. The ultrastructure of normal squamous epithelial cells of the human cervix uteri. *J. Ultrastruct. Res.* (1968) 22: 443-453.
- Halliday, G.M., McCardle, J.P., Knight, B.A. and Miller, H.K. New methodology for assessment of the Langerhans cell network. *J. Pathol.* (1986) 148: 127-134.
- Hann, S.R., Thompson, C.B. and Eisenman, R.N. C-myc oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature* (1985) 314: 366-369.
- Hardie, I.R., Strong, R.A., Hartley, L.C.J., Woodruff, P.W.H. and Clunie, G.J.A. Skin cancer in Caucasian renal allograft recipients living in a subtropical climate. *Surgery* (1980) 87: 177-183.
- Harris, R.W., Brinton, L.A., Cowdell, R.H. et al. Characteristics of women with dysplasia or carcinoma in situ of the cervix uteri. *Br. J. Cancer* (1980) 42: 359-369.

- Hawthorn, R.J.S., Murdoch, J.B., MacLean, A.B. and Mackie, R.M.
Langerhans' cells and subtypes of human papillomavirus in
cervical intraepithelial neoplasia. *Br. Med. J.* (1988) 297:
643-646.
- Harrist T.J., Muhlbauer, J.E., Murphy, G.F., Mihm, M.C. Jr and Bhan,
A.K. T6 is superior to Ia (HLA-DR) as a marker for
Langerhans cells and indeterminate cells in normal
epidermis: a monoclonal antibody study. *J. Invest.*
Dermatol. (1983) 80: 100-103.
- Hedley, D.W., Friedlander, M.I., Taylor, I.W., Rugg, C.A. and
Musgrove, E.A. Method for analysis of cellular DNA content
of paraffin-embedded pathological material using flow
cytometry. *J. Histochem. Cytochem.* (1983) 31: 1333-1335.
- Hedley, D.W., Friedlander, M.L. and Taylor, I.W. Application of DNA
flow cytometry to paraffin embedded archival material for
the study of aneuploidy and its clinical significance.
Cytometry (1985) 6: 327-333.
- Heim, S., Mandahl, N. and Kristoffersson, U. et al. Reciprocal
translocation t(3;12) (g27;q13) in lipoma. *Cancer Genet.*
Cytogenet. (1986) 23: 301-304.
- Hellberg, D., Valentin, J. and Nilsson, S. Smoking and cervical
intraepithelial neoplasia. An association independent of
sexual and other risk factors? *Acta. Obstet. Gynecol.*
Scand. (1986) 65: 625-631.
- Hendy-Ibbs, P., Cox, H., Evan, G.I. and Watson, J.V. Flow cytometric
quantitation of DNA and c-myc oncoprotein in archival
biopsies of uterine cervical neoplasia. *Br. J. Cancer*
(1987) 55 (3): 275-282.

- Hezenberg, L.A., Bianchini, D.W., Schroder, J., Can, H.M. and Iverson, G.M. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence activated cell sorting. Proc. Natl. Acad. Sci. USA (1979) 76: 1453-1455.
- Hiddemann, W., Schumann, J., Andreef, M. et al. Convention on nomenclature for DNA cytometry. Cytometry (1984) 5: 445-446.
- Hille, J.J., Margolins, K.A., Markowitz, S. and Isaacson, C. Human papillomavirus infection related to oesophageal carcinoma in black South Africans: a preliminary study. S. Afr. Med. J. (1986) 69: 417-420.
- Hinselmann, H. Einführung in die Kolposkopie Hartung (1933), Hamburg.
- Hoffken, H. and Soost, H.J. in Current Topics in Pathology: cervical cancer. (1981) ed. G. Dallenbach-Hellweg. Cervical cytology as a screening method. pp 21-65 (Springer-Verlag).
- Houghton, A.N., Thomson, T.M., Gross, D., Oettgen, H.F. and Old, L.J. Surface antigens of melanoma and melanocytes. Specificity of induction of Ia antigens by human interferon. J. Exp. Med. (1984) 160: 255-269.
- Howley, P., Yang, Y-C., Spalholz, B.A. and Rabson, M.S. Papillomavirus transforming functions. In Ciba Foundation Symposium (1986) 120: Papillomaviruses pp 39-52.
- Hughes, D.A., Haslam, P.L., Townsend, P.J. and Turner-Warwick, M. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. Clin. Exp. Immunol. (1985) 61: 459-466.
- Hunter, J.A.A. The Langerhans cell: from gold to glitter. Clin. Exp. Dermatol. (1984) 8: 565-592.

- Husby, G., Hoagland, P.M., Strickland, R.G. and Williams, R.C.
Tissue T and B lymphocyte infiltration of primary and metastatic cancer. *J. Clin. Invest.* (1976) 57: 1471-1482.
- Ikenberg, H., Gissmann, L., Gross, G., Grussendorf-Conen, E. and zur Hausen, H. Human papillomavirus DNA in Bowen's disease and in bowenoid papulosis. *Int. J. Cancer* (1983) 32: 563-566.
- Iochim, H.L. The stromal reaction of tumors: An expression of immune surveillance. *J.N.C.I.* (1976) 57: 465-475.
- Isaacs, J.T., Wake, N., Coffey, D.A. and Sandberg, A. Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostatic adenocarcinoma system. *Cancer Res.* (1982) 42: 2353-2361.
- Ivanyi, L. and Morison, W.L. In vitro lymphocyte stimulation by wart antigen in man. *Br. J. Dermatol.* (1976) 94: 523-527.
- Jablonska, S. and Orth, G. Epidermodysplasia verruciformis. *Clinics in Dermatology* (1985) 3: 83-96.
- Jacquillat, T.C., Khayat, D., Desprez-Curely, J.P. et al. Non-Hodgkin's lymphoma occurring after Hodgkin's disease. *Cancer* (1984) 33: 459-462.
- Jakobsen, A., Kristensen, P.B. and Poulsen, H.K. Flow cytometric classification of biopsy specimens from cervical intraepithelial neoplasia. *Cytometry* (1983) 4: 166-169.
- Jakobsen, A. Ploidy level and short-term prognosis of early cervix cancer. *Radiother. Oncol.* (1984a) 1: 271-275.
- Jakobsen, A. Prognostic impact of ploidy level in carcinoma of the cervix. *Am. J. Clin. Oncol.* (1984b) 1: 475-480.

- Jenson, A.B., Kurman, R.J. and Lancaster, W.D. Detection of papillomavirus common antigen in lesions of skin and mucosa. *Clinics in Dermatology* (1985) 3 (4): 56-63.
- Johannessen, J.V., Sobrino-Simoes, L., Lindmo, T. and Tangen, K.O. The diagnostic value of flow cytometric DNA measurements in selected disorders of the human thyroid. *Am. J. Clin. Path.* (1982) 77: 20-25.
- Johansson, E., Niemi, K.M., Siimes, M. and Pyrhonen, S. Fanconi's anaemia, tumor-like warts, hyperpigmentation associated with deranged keratinocytes and depressed cell-mediated immunity. *Arch. Dermatol.* (1982) 118: 249-252.
- Juhlin, L. and Shelley, W.B. New staining techniques for the Langerhans cell. *Acta. Derm. Venereol.* (Stockh.) (1977) 57: 289-296.
- Katz, S.I., Cooper, K.D., Ijima, M. and Tsuchida, T. The role of Langerhans cells in antigen presentation. *J. Invest. Dermatol.* (1985) 85: 96S-98S.
- Kelly, K. and Siebenlist, U. The regulation and expression of c-myc in normal and malignant cells. *Ann. Rev. Immunol.* (1986) 4: 317-330.
- Kessler, I.I. Venereal factors in human cervical cancer. *Cancer* (1977) 39: 1912-1919.
- Kienzler, J-L., Lemoine, M.T. and Orth, G. Humoral and cell-mediated immunity to human papillomavirus type 1 (HPV-1) in human warts. *Br. J. Dermatol.* (1983) 108: 665-672.
- Kienzler, J-L. Humoral immunity to human papillomavirus. *Clinics in Dermatology* (1985) 3 (4): 144-155.

- Kingston, R.E., Baldwin, A.S. and Sharp, P.A. Transcription control by oncogenes. *Cell* (1985) 41: 3-5.
- Kinlen, L.J. and Spriggs, A.I. Women with positive cervical smears but without surgical intervention. *Lancet* (1978) 11: 463-465.
- Kinlen, L.J., Sheil, A.G.R., Peto, J. and Doll, R. Collaborative UK-Australasian study of cancer in patients treated with immunosuppressive drugs. *Br. Med. J.* (1979) 2: 1461-1466.
- Kirchner, H. Immunologic surveillance and human papillomaviruses. *Immunology Today* (1984) 5: 272-276.
- Kitchener, H.C., Burnett, R.A., Wilson, E.S.B. and Cordiner, J.W. Colposcopy in a family planning clinic : A future model? *Br. Med. J.* (1987) 294: 1313-1315.
- Klareskog, L., Sandberg-Tragardh, L., Rask, L., Lindblum, J-B., Curman, B. and Peterson, P.A. Chemical properties of human Ia antigens. *Nature* (1977) 265-51.
- Klareskog, L., Forsum, V. and Peterson, P.A. Hormonal regulation of the expression of Ia antigens on mammary gland epithelium. *Eur. J. Immunol.* (1980) 10: 958-963.
- Klinkert, W.E.F., Labadie, J.H. and Bowers, W.E. Accessory and stimulating properties of dendritic cells and macrophages isolated from various rat tissues. *J. Exp. Med.* (1982) 156: 1-19.
- Kolstad, P. and Stafl, A. Atlas of Colposcopy. 3rd edition (1982). Universitats forlaget, Oslo. Churchill Livingstone, London.
- Koss, L.G. Dysplasia: a real concept or a misnomer? *Obstet. Gynecol.* (1978) 51: 374-379.

- Koss, L.G. Diagnostic cytology and its histopathologic basis.
3rd ed. (1979). Philadelphia, Toronto: Lippincott 278 and
345.
- Koss, L.G. Pathogenesis of cancer of the uterine cervix. In Current
Topics in Pathology (1981) 70: 112-142.
- Kossard, S., Xenias, S.J., Pacestone, R.F., Scheen, I.I.I. Sr. and
Winkelmann, R.K. Inflammatory changes in verruca vulgaris.
J. Cutan. Pathol. (1980) 7: 217-221.
- Kovi, J., Tillman, R.L. and Lee, S.M. Malignant transformation of
condylomata acuminatum. A light microscopic and
ultrastructural study. Am. J. Clin. Pathol. (1974) 61:
702-710.
- Kreider, J.W. and Bartlett, G.L. Shope rabbit papilloma - carcinoma
complex: a model system of human papillomavirus
infections. Clinics in Dermatology (1985). 3 (4): 29-36.
- Kripke, M.L. and Morison, W.L. Modulation of immune function by
ultraviolet radiation. J. Inv. Dermatol. (1985) 85:
62S-66S.
- Kurman, R.J., Shah, K.H., Lancaster, W.D. and Jenson, A.B. Immuno-
peroxidase localisation of papillomavirus antigens in
cervical dysplasia and vulval condylomas. Am. J. Obstet.
Gynecol. (1981) 140: 931-935.
- Kurman, R.J., Jenson, A.B., Perry, S. and Lancaster, W.D. Papilloma-
virus infection of the cervix II. Relationship to intra-
epithelial neoplasia based on the presence of specific viral
structural proteins. Am. J. Surg. Pathol. (1983) 7: 39-52.

- Kurman, R.J., Jenson, A.B., Sinclair, C.F. and Lancaster, W.D.
 Detection of human papillomaviruses by immunocytochemistry.
In Advances in Immunohistochemistry, ed R.A. Delellis, Masson
 Publications USA (1984) pp 201-221.
- Kute, T.E., Linville, C. and Barrows, G. Cytofluorometric analysis
 for estrogen receptors using fluorescent estrogen probes.
Cytometry (1983) 4: 132-140.
- Laara, E., Day, N.E. and Hakama, M. Trends in mortality from cervical
 cancer in the Nordic countries: association with organised
 screening programmes. *Lancet* (1987) i: 1247-1249.
- Laerum, O.D. and Farsund, T. Clinical application of flow cytometry:
 a review. *Cytometry* (1981) 2 (1): 1-13.
- Lampert, I.A. Expression of HLA-DR (Ia-like) antigens on epidermal
 keratinocytes in human dermatoses. *Clin. exp. Immunol.*
 (1984) 57: 93-100.
- Lampson, L.A. and Levy, R. Two populations of Ia-like molecules on
 a human B cell line. *J. Immunol.* (1980) 125: 293-299.
- Langerhans, P. *Über die Nerven der menschlichen Haut.* *Virchows*
Arch (A) (1868) 44: 325-337.
- La Vecchia, C., Franceschi, S., Decarli, A., Fasoli, M., Gentile, A.
 and Tognoni, G. "Pap" smear and the risk of cervical
 neoplasia: quantitative estimates from a case-control
 study. *Lancet* (1984) ii: 779-782.
- La Vecchia, C. The epidemiology of cervical neoplasia. *Biomed.*
Pharmacother. (1985) 39: 426-433.
- La Vecchia, C., Franceschi, S., De Carli A., Fasoli, M., Gentile, A.
 and Tognoni, G. Cigarette smoking and the risk of cervical
 neoplasia. *Am. J. Epidemiol.* (1986) 123: 22-29.

- Leder, P., Baltey, J., Lenoir, G. et al. Translocations among antibody genes in human cancer. *Science* (1983) 222: 765-771.
- Lee, A.K.Y. and Eisinger, M. Cell mediated immunity to human wart virus and wart associated tissue antigens. *Clin. Exp. Immunol.* (1976) 26: 419-424.
- Lehn, H., Krieg, P. and Sauer, G. Papillomavirus genomes in human cervical tumors: Analysis of their transcriptional activity. *Proc. Natl. Acad. Sci. USA* (1985) 82: 5540-5544.
- Linden, W. A., Ochlich, K. Baisch, H. et al. Flow cytometric prescreening of cervical smears. *J. Histochem. Cytochem.* (1979) 27: 529-535.
- Lisi, P. Investigation of Langerhans cells in pathological human epidermis. *Acta. Derm. Venereol. (Stockh.)* (1973) 53: 425.
- Little, C.D., Nau, M.M., Caney, D.N., Gazdar, A.F. and Minna, J.D. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature* (1983) 306: 194-196.
- Londei, M., Lamb, J.R., Bottazzo, G.F. and Feldmann, M. Epithelial cells expressing aberrant major histocompatibility class II determinants can present antigen to cloned human T cells. *Nature* (1984) 312: 639-641.
- Lookingbill, D.P., Kreider, J.W., Howett, M.K., Olmstead, P.M. and Conner, G.H. Human papillomavirus type 11 in bowenoid papulosis, intraoral papillomas, and squamous cell carcinoma of the tongue. *Arch. Dermatol.* (1987) 123: 363-368.
- Lorincz, A.T., Lancaster, W.D. and Temple, G.F. Detection and characterisation of a new type of human papillomavirus. *J. Cell. Biochem.* (1985) (Suppl) 9c: 75.

- Lorincz, A.T., Temple, G.F., Patterson, J.A., Jenson, A.B., Kurman, R.J. and Lancaster, W.D. Correlation of cellular atypia and human papillomavirus deoxyribonucleic acid sequences in exfoliated cells of the uterine cervix. *Obstet. Gynecol.* (1986) 68: 508-512.
- Luckey, E.B. and Nass, G.D. A comparison of sexual behaviour in an international sample. *J. Marriage and the Family* (1969) 31: 364-379.
- Luesley, D.M., Jordan, J.A., Woodman, C.B.J., Watson, N., Williams, D.R. and Waddell, C. A retrospective review of adenocarcinoma in situ and glandular atypia of the uterine cervix. *Br. J. Obstet. Gynaecol.* (1987) 94: 699-703.
- Lutzner, M.A., Orth, G., Dutronquay, V., Du Casse, M-F., Kreish, H. and Crosnier, J. Detection of human papillomavirus type 5 DNA in skin cancers of an immunosuppressed renal allograft recipient. *Lancet* (1983) 2: 422-424.
- McArdle, J.P. and Muller, H.K. Quantitative assessment of Langerhans cells in human cervical intraepithelial neoplasia and wart virus infection. *Am. J. Obstet. Gynecol.* (1986) 154: 509-516.
- McCance, D.J., Walker, P.G., Dyson, J., Coleman, D.V. and Singer, A. Presence of human papillomavirus DNA sequences in cervical intraepithelial neoplasia. *Br. Med. J.* (1983) 287: 784-788.

- McCance, D.J., Chester, P.M., Campion, M.J., Clarkson, P.K., Jenkins, D. and Singer, A. Prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. *Br. J. Obstet. Gynaecol.* (1985a) 92: 1101-1105.
- McCance, D.J., Clarkson, P.K., Dyson, J., Walker, P.G. and Singer, A. Human papillomavirus types 6 and 16 in multifocal intra-epithelial neoplasias of the female lower genital tract. *Br. J. Obstet. Gynaecol.* (1985b) 92: 1093-1100.
- McCance, D.J. Human papillomaviruses and cancer. *Biochim. et Biophys. Acta.* (1986) 823 (3): 195-205.
- McCance, D.J., Kalache, K., Ashdown, K. et al. Increased risk of cervical neoplasia in consorts of men with penile condylomata acuminata. *Int. J. Cancer* (1986a) 37: 55-60.
- McCance, D.J., Campion, M.J. and Singer, A. Noninvasive detection of cervical papillomavirus DNA. *Lancet* (1986b) 1: 558-559.
- McCartney, J.C., Camplejohn, R.S., Alder, J., Stone, M.G. and Powell, G. Prognostic importance of DNA flow cytometry in non-Hodgkin's lymphomas. *J. Clin. Pathol.* (1986) 39: 542-546.
- McDevitt, H.O., Decovitch, T.L., Press, J.L. and Murphy, D.G. Genetic and functional analysis of the Ia antigens. Their possible role in regulating the immune response. *Transplant Rev.* (1976) 30: 197-235.
- MacDonald, H.N. Smoking and oral contraception in cancer of the cervix. *Lancet* (1982) 11: 989.

- McGuire, W.L. and Dressler, L.G. Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. *JNCI* (1985) 75: 405-410.
- McGuire, W.L., Meyer, J.S., Barlogie, B. and Kute, T.E. Impact of flow cytometry on predicting recurrence and survival in breast cancer patients. *Breast Cancer Res. Treat.* (1985) 117-128.
- McIndoe, W.A., McLean, M.R., Jones, R.N. and Mullins, P.R. The invasive potential of carcinoma in situ of the cervix. *Obstet. Gynecol.* (1984) 64: 451-458.
- MacNab, J.C., Walkingshaw, S.A., Cordiner, J.W. and Barklie Clements, J. Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. *N. Eng. J. Med.* (1986) 315: 1052-1058.
- Maitland, N.J., Kinross, J.H., Busuttil, A., Ludgate, S.M., Smart, G.E. and Jones, K.W. The detection of DNA tumour virus-specific RNA sequences in abnormal human cervical biopsies by in situ hybridization. *J. Gen. Virol.* (1981) 55: 123-137.
- Maitland, N.J., Cox, M.F., Lynas, C., Prime, S.S., Meanwell, C.A. and Scully, C. Detection of human papillomavirus DNA in biopsies of human oral tissue. *Br. J. Cancer.* (1987) 56: 245-250.
- Martin, R.G. The transformation of cell growth and transmigration of DNA synthesis by SV40. *Adv. Cancer Res.* (1981) 34: 1-68.

- Meanwell, C.A., Cox, M.F., Blackledge, G. and Maitland, N.J. Human papillomavirus 16 DNA in normal and malignant cervical cancer: implications for the aetiology and behaviour of cervical neoplasia. *Lancet* (1987) 1: 703-707.
- Meisels, A. and Fortin, R. Condylomatous lesions of the cervix and vagina. I. Cytologic patterns. *Acta. Cytol. (Baltimore)* (1976) 20: 505-509.
- Meisels, A., Fortin, R. and Roy, M. Condylomatous lesions of the cervix. II. Cytologic, colposcopic and histopathologic study. *Acta. Cytol. (Baltimore)* (1977) 21: 379-390.
- Melamed, M.R. and Klein, F.A. Flow cytometry of urinary bladder irrigation specimens. *Hum. Pathol.* (1984) 15: 302-305.
- Mellors, R.C. Tumour cell localization of the antigens of the Shope papilloma virus and the Rous sarcoma virus. *Cancer Res.* (1960) 20: 744-746.
- Meyer, R. Carcinoma uteri In Henke F, Lubarscho (Hrsg), Weibliche Geschlechtsorgane. (1930) Springer, Berlin (Handbuch der Speziellen Pathologischen Anatomie und Histologie, Bd VII/1 Kap28).
- Miller, L.G., Goldstein, G., Murphy, M. and Ginns, L.C. Reversible alterations in immunoregulatory T cells in smokers. *Chest* (1982) 82: 526-529.
- Mitchison, N.A. and Kinlen, L.J. Present concepts of immune surveillance. *Progr. Immunology* (1980) 4: 641-645.
- Mogaji, N.A. A case of carcinoma of the cervix in a virgin. *Int. J. Gynaecol. Obstet.* (1973) 11: 122-125.

- Montecucco, C.M., Riccardi, A., Merlin, G.P. et al. Plasma cell DNA content in multiple myeloma and related paraproteinemic disorders. *Eur. J. Cancer Clin. Oncol.* (1984) 20: 81-90.
- Morison, W.L. In vitro assay of cell mediated immunity to human wart antigen. *Br. J. Dermatol.* (1974) 90: 531-534.
- Morison, W.L. Viral warts, herpes simplex and herpes zoster in patients with secondary immune deficiencies and neoplasms. *Br. J. Dermatol.* (1975a) 92: 625-630.
- Morison, W.L. Cell mediated immune responses in patients with warts. *Br. J. Dermatol.* (1975b) 93: 553-556.
- Morris, H.H.B., Gatter, K.C., Sykes, G., Casemore, V. and Mason, D.Y. Langerhans cells in human cervical epithelium: effects of wart virus infection and cervical intraepithelial neoplasia. *Br. J. Obstet. Gynaecol.* (1983) 90: 412-420.
- Morris, H., Edwards, J., Tiltman, A. and Emms, M. Endometrial lymphoid tissue: an immunohistological study. *J. Clin. Pathol.* (1985) 38: 644-652.
- Mounts, P. and Kashima H. Association of human papillomavirus subtype and clinical course in respiratory papillomatosis. *Laryngoscope* (1984) 94: 28-33.
- Mowat, A. McI. The regulation of immune responses to dietary protein antigens. *Immunol. Today* (1987) 8: 93-98.
- Muhlbauer, J.E., Harrist, T.J., Murphy, G.F., Mihm, M.C. and Bhan, A.K. Monoclonal anti T6 antibody labels more epidermal cells than monoclonal anti Ia (HLA-DR) antibodies. *Lab. Invest.* (1982) 46: 59A.
- Munroe, J.S. Progesteroids as immunosuppressive agents. *J. Reticuloendothel. Soc.* (1971) 9: 361-375.

- Murphy, G.F., Bhan, A.K., Sato, S., Harrist, T.J. and Mihm, M.C. Jr. Characterisation of Langerhans cells by the use of monoclonal antibodies. *Lab. Invest.* (1981) 45: 465-468.
- Murphy, G.F., Bhan, A.K., Harrist, T.J. and Mihm, M.C. Jr. Identification of indeterminate cells in normal human dermis by the use of monoclonal anti T6 antibody. *Lab. Invest.* (1982) 46: 60 (A).
- Nagao, S., Inaba, S. and Tijima, S. Langerhans cells at the sites of vaccinia virus inoculation. *Arch. Dermatol. Res.* (1976) 256: 23-31.
- Nahmias, A.J., Naib, Z.M. and Josey, W.E. Herpes virus hominis type 2 infection in association with cervical cancer and perinatal disease. *In Perspectives in Virology* (1971) 7: M. Pollard, New York and London: 73-88.
- Naib, Z.M., Nahmias, A.J. and Josey, W.E. Cytology and histopathology of cervical Herpes simplex infection. *Cancer* (1966) 19: 1026-1031.
- Nasiell, K., Aver, G., Nasiell, M. and Zetterberg, A. Retrospective DNA analyses in cervical dysplasia as related to neoplastic progression or regression. *Anal. Quant. Cytol.* (1979) 1: 103-106.
- Natali, P.G., De Martino, C., Quaranti, V. et al. Expression of Ia-like antigens in normal human nonlymphoid tissue. *Transplantation* (1981) 31: 75-78.
- Navarrette, C., Jaraquemada, D., Fainboim, L. et al. Genetics and functional relationship of the HLA-DR and HLA-DQ antigens. *Immunogenetics* (1985a) 21: 97-101.

- Navarrette, C., Jrraquemada, D., Hui, K., Awad, J., Okoye, R. and Festenstein, H. Differential functions and association of HLA-DR and HLA-DQ (DC) antigens shown by serological cellular and DNA assays. *Tissue Antigens* (1985b) 25: 130-141.
- Neill, W.A. Cell mediated immune responses to cytomegalovirus in patients with abnormalities of the uterine cervix. M. Phil Thesis (1984).
- Nowell, P.C. The clonal evolution of tumor cell populations. *Science* (1976) 194: 23-28.
- Nunez, G., Ball, E.J., Myers, L.K. and Stastny, P. Allostimulating cells in man. Quantitative variation in the expression of HLA-DR and DQ molecules influences T cell activation. *Immunogenetics* (1985) 22: 85-91.
- Obalek, S., Glinski, W., Hatleck, H., Orth, G. and Jablonska, S. Comparative studies of cell mediated immunity in patients with different warts. *Dermatologica* (1980) 161: 73-83.
- Oguchi, M., Komura, J., Tagami, H. and Ofuji, S. Ultrastructural studies of spontaneously regressing plane warts, Langerhans cells showed marked activation. *Arch. Dermatol.Res.* (1981) 271: 55-61.
- Ostrow, R.S., Bender, M., Miimura, L. et al. Human papillomavirus DNA in cutaneous primary and metastasised squamous cell cancers from patients with epidermodysplasia verruciformis. *Proc. Natl. Acad. Sci. USA* (1982) 79: 1634-1638.
- Ostrow, R.S., Manias, D.A., Fong, W.J., Zachow, K.R. and Faras, A.J. A survey of human cancers for human papillomavirus DNA by filter hybridisation. *Cancer* (1987) 59: 429-434.

- Pagano, J.S. Diseases and mechanisms of persistent DNA virus infection: Latency and cellular transformation. *J. Infect. Dis.* (1975) 132: 209-223.
- Papanicolaou, G.N. The sexual cycle in the human female is revealed by vaginal smears. *Am. J. Anat.* (1933) 52: 519-637.
- Papanicolaou, G.N. and Marchetti, A.A. The use of endocervical and endometrial smears in the diagnosis of cancer and of other conditions of the uterus. *Am. J. Obstet. Gynecol.* (1943) 46: 421-422.
- Papanicolaou, G.N. Atlas of exfoliative cytology. Cambridge, Mass: Harvard, 1954, supplement 1960.
- Pater, M.M., Hughes, G.A., Hyslop, D.E., Nakshatri, H. and Pater, A. Glucocorticoid-dependent oncogenic transformation by type 16 but not type 11 human papillomavirus DNA. *Nature* (1988) 335: 832-835.
- Patten, S.F. Diagnostic Cytopathology of the Uterine Cervix. 2nd edition. Basle: Karger (1978).
- Pawelec, G.P., Shaw, C., Ziegler, A., Muller, C. and Wernet, P. Differential inhibition of HLA-D or SB directed secondary lymphoproliferative responses with monoclonal antibodies detecting human Ia-like determinants. *J. Immunol.* (1982) 129: 1070-1075.
- Peden, K., Mounts, P. and Hayward, G.S. Homology between mammalian cell DNA sequences and human herpes virus genomes detected by a hybridization procedure with a high complexity probe. *Cell* (1982) 31: 71-80.

- Pedersen-Bjerjaard, J., Ersboll, J., Sorensen, H.M. et al. Risk of acute non-lymphocytic leukaemia and preleukaemia in patients treated with cyclophosphamide for non-Hodgkin's lymphoma. *Ann. Intern. Med.* (1985) 103: 195-200.
- Pehamberger, H., Stingl, L.A., Pogantsch, S., Steiner, S., Wolff, K. and Stingl, G. Epidermal cell induced generation of cytotoxic T lymphocyte responses against alloantigens or TNP-modified syngeneic cells; requirement for Ia-positive Langerhans cells. *J. Invest. Dermatol.* (1983) 91: 208-211.
- Perry, L.L. and Greene, M.I. Antigen presentation by epidermal Langerhans cells : loss of function following ultraviolet irradiation in vivo. *Clin. Immunol. Immunopathol.* (1982) 24: 204-219.
- Persson, H. and Leder, P. Nuclear localisation and DNA binding properties of a protein expressed by human c-myc oncogene. *Science* (1984) 225: 718-721.
- Peterson, D. Spontaneous course of cervical precancerous conditions. *Am. J. Obstet. Gynecol.* (1956) 72: 1063-1071.
- Pfister, H., Hettich, I., Runne, V., Gissmann, L. and Chliff, G.N. Characterisation of human papillomavirus type 13 from lesions of focal hyperplasia of the cervix. *J. Virol.* (1983) 47: 363-366.
- Piper, J.M. Oral contraceptives and cervical cancer. *Gynecol. Oncol.* (1985) 22: 1-14.

- Pober, J.S., Gimbroni, M.A., Cotran, R.S. et al. Ia expression by vascular endothelium is inducible by activated T cells and by human immune interferon. J. Exp. Med. (1983a) 157: 1339-1353.
- Pober, J.S., Collins, T., Gimbrone, M.A. et al. Lymphocytes recognise human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. Nature (1983b) 305: 726-729.
- Prawer, S.E., Pass, F., Vance, J., Greenberg, L.J., Yunis, E.J. and Elickson, A.S. Depressed immune function in epidermodysplasia verruciformis. Arch. Dermatol. (1977) 113: 495-503
- Pridan, H. and Lilienfeld, A.M. Carcinoma of the cervix in Jewish women in Israel. Isr. J. Med. Sci (1971) 7: 1465-1470.
- Purola, E. and Savia, E. Cytology of gynecologic condylomata acuminata. Acta. Cytol. (Baltimore) (1977) 21: 26-31.
- Pyrhonen, S. and Johansson, E. Regression of warts. An immunological study. Lancet (1975) 1: 592-595.
- Quirke, P. and Dyson, J.E.D. Flow Cytometry. J. Pathol. (1986) 149: 79-87.
- Rabbitts, P.H., Watson, J.V., Lamond, A. et al. Metabolism of c-myc gene products: c-myc mRNA and protein expression in the cell cycle. EMBO J. (1985) 4: 2009-2115.
- Rapp, F. Cytomegalovirus and carcinogenesis. J.N.C.I. (1984) 72: 783-787.
- Rawls, W.E., Adam, E. and Melnick, J.L. An analysis of seroepidemiological studies of herpes virus type 2 and cancer of the cervix. Cancer Res (1973) 33: 1477-1482.

- Reagan, J.W., Seidemand, I.L. and Saracusa, Y. The cellular morphology of carcinoma in situ and dysplasia or atypical hyperplasia of the uterine cervix. *Cancer* (1953) 6: 224-235.
- Reid, B.L., French P.W., Singer, A., Hagan, B.E. and Coppleson, M. Sperm basic proteins in cervical carcinogenesis: correlation with social class. *Lancet* (1978) 11: 60-62.
- Reid, R., Stanhope, C.R., Herschman, B.R., Booth, E., Phibbs, G.D. and Smith, J.P. Genital warts and cervical cancer I. Evidence of an association between clinical papillomavirus infection and cervical malignancy. *Cancer* (1982) 50: 377-387.
- Reid, R. Genital warts and cervical cancer II. Is human papillomavirus infection the trigger to cervical carcinogenesis? *Gynecol. Oncol.* (1983) 15: 239-252.
- Reid R., Fu, Y.S., Herschman, B.R. et al. Genital warts and cervical cancer. VI The relationship between aneuploid and polyploid lesions. *Am. J. Obstet. Gynecol.* (1984) 150: 189-199.
- Reinherz, E.L., Kung, P.C., Goldstein, G., Levey, R.H. and Schlossman, S.F. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T cell lineage. *Proc. Natl. Acad. Sci. USA* (1980) 77: 1588-1592.
- Richardson, A.C. and Lyon, J.B. The effect of condom use on squamous cell cervical intraepithelial neoplasia. *Am. J. Obstet. Gynecol.* (1981) 140: 909-913.

- Richart, R.M. Influence of diagnostic and therapeutic procedures on the distribution of cervical intraepithelial neoplasia. *Cancer* (1966) 19: 1635-1638.
- Richart, R.M. The natural history of cervical intraepithelial neoplasia. *Clin. Obstet. Gynecol.* (1967) 10: 748-784.
- Richart, R.M., Lerch, V. and Barron, B.A. A time lapse cinematograph study in vitro of mitosis in normal human cervical epithelium, dysplasia and cervical intraepithelial neoplasia. *JNCI* (1967) 39: 571-577.
- Richart, R.M. and Barron, B.A. A follow-up study of patients with cervical dysplasia. *Am. J. Obstet. Gynecol.* (1969) 105: 386-393.
- Rigoni-Stern. Fatti statistici relativei alle mallatti ec cancrose che servitono de base alle poche cose dette dal dott. *Giornale per Servivre al Progress e della Patologia e della Terapia* (1842) 2: 507-517.
- Riou, G., Barrois, M., Tordjman, I., Dutronquay, V. and Ortho, G. Presence de genomes de papillomavirus et amplification des oncogenes c-myc et c-Ha-ras dans les cancers. *Comptes Rendus Acad. Sci Paris* (1984) 299: (series III) 575-580.
- Riou, G., Barrois, M., Le, M.G., George, M., Le Doussac, V. and Haie, C. C-myc proto oncogene expression and prognosis in early cancer of the uterine cervix. *Lancet* (1987) 1: 761-763.
- Rodriguez, E.M. and Caorsi, I. A second look at the ultrastructure of the Langerhans cell of the human epidermis. *J. Ultrastruct. Res.* (1978) 65: 279-295.

- Roitt, I.M., Brostoff, J. and Male, D.K. Immunology (1985).
Edinburgh, Churchill Livingstone.
- Ronstrom, L., Tribukait, B. and Esposti, P.L. DNA pattern and
cytological findings in fine needle aspirates of untreated
prostatic tumors. A flow cytofluorometric study.
Prostate (1981) 2: 79-88.
- Rotkin, I.D. A comparison review of key epidemiological studies in
cervical cancer related to current searches for
transmissible agents. Cancer Res. (1973) 33: 1353-1367.
- Rotkin, I.D. Another view of Herpes simplex virus type 2. J.A.M.A.
(1976) 235: 2188-2189.
- Rous, P., Kidd, J.G. and Beard, J.W. Observations of the relation of
the virus causing rabbit papillomas to the cancers deriving
therefrom: I. The influence of the host species and of the
pathogenic activity and concentration of virus. J. Exp.
Med. (1936) 64: 385-400.
- Rowden, G., Lewis, M.F. and Sullivan, L.C. Ia antigen expression on
human epidermal Langerhans cells. Nature (1977) 268:
247-248.
- Rowden, G., Phillips, T.M. and Lewis, M.G. Ia antigens on indeter-
minate cells of the epidermis: immuno-electron microscopic
studies of surface antigens. Br. J. Dermatol. (1979) 100:
531-542.
- Rowden, G. The Langerhans Cell. CRC Critical reviews in Immunology
(1981) 95-180.
- Rubin, I.C. The pathological diagnosis of incipient carcinoma of the
uterus. Am. J. Obstet and Diseases of Women and Children
(1910) 62: 668-676.

- Rudlinger, R., Smith, I.W., Bunney, M.H. and Hunter, J.A.A. Human papillomavirus infections in a group of renal transplant recipients. *Br. J. Dermatol.* (1986) 115: 681-692.
- Sanchez-Perez, M. and Shaw, S. HLA:DP current status. Solheim, B.G., Moller, E., Ferone, S. eds. *HLA Class II antigens A Comprehensive Review of Structure and Function.* Springer-Verlag, (1986): 83-108.
- Sandberg, A.A. and Turc-Carel, C. The cytogenetics of solid tumors. *Cancer* (1987) 59: 387-395.
- Sasson, I.M., Haley, N.J., Hoffman, D., Wynder, E.L., Hellberg, D. and Nilsson, S. Cigarette smoking and neoplasia of the uterine cervix: smoke constituents in cervical mucus. *New Engl. J. Med.* (1985) 312: 315-316.
- Schauenstein, W. Histologische Untersuchungen über atypisches Plattenepithel un der Portio und an der Innenfläche der cervix uteri. *Arch. Gynaek* (1908) 85: 576-616.
- Schiller, W. Untersuchungen zur Entstehung der Geschwulste I. Carcinoma des Uterus. *Virchows Arch (A)* (1927) 263: 279-367.
- Schiller, W. Über Frühstadien des Portiocarcinoms und ihr Diagnose. *Arch. Gynaeck* (1928) 133: 211-283.
- Schilsky, R.L. and Erlichman, C. Late complications of chemotherapy: infertility and carcinogenesis. In : Chabner, B. ed *Pharmacological principles of cancer reflux.* Toronto WB Saunders p 109-131.

- Schneider, A., Sawada, E., Gissmann, L. and Shah, K. Human papillomaviruses in women with a history of abnormal Papanicolaou smears and their male partners. *Obstet. Gynecol.* (1987) 69: 554-562.
- Schneider, V., Kay, S. and Lee, H.M. Immunosuppression as a high risk factor in the development of condyloma acuminatum and squamous neoplasia of the cervix. *Acta. Cytologica.* (1983) 27: 220-224.
- Schottlaender, J and Kermauner, F. Zur Kenntnis des Uterus Karzinoms; monographische Studie über Morphologie, Entwicklung Wachstum, nebst. Beiträgen zur Klinik der Erkrankung Karger, Berlin (1912).
- Schrier, P.I., Bernardis, R., Vaessen, R.T.M.J., Houweling, A. and Van Der Erb, A.J. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus in transformed rat cells. *Nature* (1983) 305: 771-775.
- Schwartz, J., Solt, D.B., Pappo, J. and Weichselbaum, R. Distribution of Langerhans cells in the normal and carcinogen treated mucosa of buccal pouches of hamsters. *J. Dermatol. Surg. Oncol.* (1981) 7: 1005-1010.
- Schwarz, E., Freese, V.K., Gissmann, L. et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* (1985) 314: 111-114.
- Sciplades E J. and Stevenson, K.S. Über die Latenzperiode des beginnenden collum carcinoms und die Dauer seiner symptomfreiheit. *Arch. Gynaek* (1938) 167: 416-464.
- Scottish Health Statistics Advance Tables (1987) : 36.

- Sebastian, J.A., Leeb, B.O. and See, R. Cancer of the cervix - a sexually transmitted disease. Cytological screening in a prostitute population. *Am. J. Obstet. Gynecol.* (1978) 131: 620-623.
- Selby, W.S., Janossy, G., Bofill, M. and Jewell, D.P. Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease.. *Clin. Exp. Immunol.* (1983) 52: 219-228.
- Seski, J.C., Reinhalter, E.R. and Silva, J. Abnormalities of lymphocyte transformation in women with condylomata acuminata. *Obstet. Gynecol.* (1978) 51: 188-192.
- Shelley, W.B. and Juhlin, L. Langerhans cells form a reticulo-endothelial trap for external contact allergens. *Nature* (1976) 261: 46-47.
- Sillman, F.S., Stanek, A., Sedlis, A. et al. The relationship between human papillomavirus and lower genital intraepithelial neoplasia in immunosuppressed women. *Am. J. Obstet. Gynecol.* (1984) 150: 300-308.
- Singer, A., Reid, B.L. and Coppleson, M. A hypothesis: The role of a high risk male in the etiology of cervical carcinoma. *Am. J. Obstet. Gynecol.* (1976) 126: 110-115.
- Smotkin, D., Berek, J.S., Fu, Y.S., Hacker, N.F., Major, F.J. and Wettstein, F.O. Human papillomavirus DNA in adenocarcinoma and adeno-squamous cancer of the uterine cervix. *Obstet. Gynecol.* (1986) 68: 241-244.

- Sorrentino, R., Corte, G. The complexity of HLA-DQ molecules. In: Solheim, B.G., Miller, E., Ferrone, S. eds HLA Class II antigens. A Comprehensive Review of Structure and Function. Springer-Verlag, 1986 p 71-82.
- Spriggs, A.I., Bowey, C.E. and Cowdell, R.H. Chromosomes of pre-cancerous lesions of the uterine cervix uteri. New data and a review. *Cancer* (1971) 27: 1239-1254.
- Spriggs, A.I. and Boddington, M.M. Progression and regression of cervical lesions. Review of smears from women followed without initial biopsy or treatment. *J. Clin. Pathol.* (1980) 33: 517-522.
- Spriggs, A.I. Epidemiology of Cervical Intraepithelial Neoplasia. In Precancerous States, ed Carter R.L. (1984) p 317-355. Oxford University Press, Toronto.
- Steeg, P.S., Moore, R.N., Johnson, H.M. and Oppenheim, J.J. Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. *J. Exp. Med.* (1982) 156: 1780-1793.
- Stern, E. and Neely, P.M. Dysplasia of the uterine cervix. Incidence of regression, recurrence and cancer. *Cancer* (1964) 17: 508-512.
- Stern, E., Forsythe, A.B., Youkeles, L. and Coffelt, C.F. Oral contraceptive pill increases progression from early to more established cervical intraepithelial neoplasia. *Science* (1977) 196: 1460-1463.
- Stevens, J.G. and Wettstein, F.P. Multiple copies of Shope virus DNA are present in cells of benign and malignant non-virus producing neoplasms. *J. Virol.* (1979) 30: 891-898.

- Stingl, G., Wolff-Schreiner, E.C., Pichler, W.J., Gschnait, F., Knapp, W. and Wolff, K. Epidermal Langerhans cells bear Fc and C3 receptors. *Nature* (1977) 268: 245-246.
- Stingl, G., Katz, S.I., Green, I. and Shevach, E.M. The functional role of Langerhans cells. *J. Invest. Dermatol.* (1980a) 74: 315-318.
- Stingl, G., Tamaki, K. and Katz, S.I. Origin and function of epidermal Langerhans cells. *Immunol. Rev.* (1980b) 53: 149-174.
- Stone, K.R., Craig, R.B., Palmer, J.O., Rivkin, S.E. and McDivitt, R.W. Short term cryopreservation of human breast cancer cells for flow cytometry. *Cytometry* (1985) 6: 357-361.
- Streicher, H.Z., Berkower, I.J., Bausch, M., Guro, F.R.C. and Berzofsky, J.A. Antigen conformation determines processing requirements for T cell activation. *Proc. Natl. Acad. Sci. USA* (1984) 81: 6831-6835.
- Strellein, J.W. Lymphocyte traffic, T cell malignancies and the skin. *J. Invest. Dermatol.* (1978) 71: 167-171.
- Strellein, J.W. and Bergstresser, P.R. Ia antigens and epidermal Langerhans cells. *Transplantation* (1980) 30: 319-323.
- Studzinski, G.P., Brelvi, Z.S., Feldman, S.C. and Watt, R.A. Participation of c-myc protein in DNA synthesis of human cells. *Science* (1986) 234: 467-476.
- Stutman, O. Immunologic surveillance and cancer. *In* *Handbook of Cancer Immunology* (1978) ed Waters, H. pp 1-25, Garland STPM Press, New York.

- Svejgaard, H., Hauge, M., Jersild, C. et al. The HLA system. In Monographs in Human genetics 2nd rev. ed. (1979).
- Syrjanen, K., Syrjanen, M., Castren, O., Mantyjarvi, R. and Yliskoski, M. The relation between the type of immunoreactive cells found in human papillomavirus lesions of the uterine cervix and the subsequent behaviour of these lesions. Arch. Gynecol. (1984) 234: 189-196.
- Syrjanen, K., Mantyjarvi, R., Bayrynen, M. et al. Coexistent chlamydial infections related to natural history of human papillomavirus lesions in uterine cervix. Genitourin. Med. (1986) 62: 345-351.
- Tabibzadeh, S.S., Bettica, A., Gerber, M.A. Variable expression of Ia antigens in human endometrium and in chronic endometritis. Am. J. Clin. Pathol. (1986a) 86: 153-160.
- Tabibzadeh, S.S., Gerber, M.A. and Satyaswaroop, P.G. Induction of HLA-DR antigen expression in human endometrial epithelial cells in vitro by recombinant interferon. Am. J. Pathol. (1986b) 125: 90-96.
- Tagami, H., Takigawa, M., Ogino, A. Imamura, S. and Ofuji, S. Spontaneous regression of plane warts after inflammation. Arch. Dermatol. (1977) 113: 1209-1213.
- Tagami, H., Oguchi, M. and Ofuji, S. The phenomenon of spontaneous regression of numerous flat warts: immunohistological studies. Cancer (1980) 45: 2557-2563.
- Tagami, H., Oguchi, M. and Ofuji, S. Immunological aspects of wart regression with special reference to regression phenomenon of numerous flat warts : an experiment on tumor immunity in man by nature. J. Dermatol. Tokyo (1983) 10: 1-12.

- Takahashi, K., Isobe, T., Ohtsuki, Y., Sonobe, H., Yamaguchi, H. and Akagi, T. S100 protein positive human T lymphocytes. *Am. J. Clin. Pathol.* (1985) 83: 69-72.
- Tay, S.K., Jenkins, D., Maddox, P., Campion, M. and Singer, A. Subpopulations of Langerhans cells in cervical neoplasia. *Br. J. Obstet. Gynaecol.* (1987a) 94: 10-15.
- Tay, S.K., Jenkins, D., Maddox, P. and Singer, A. Lymphocyte phenotypes in cervical intraepithelial neoplasia and human papillomavirus infection. *Br. J. Obstet. Gynaecol.* (1987b) 94: 16-21.
- Terhorst, C., Van Acthoven, A., Le Clair, I., Snow, P., Reinherz, E. and Schlossman, S. Biochemical studies of the human thymocyte cell surface antigens T6, T9 and T10. *Cell* (1981) 23: 771-780.
- Terris, M., Wilson, F. and Nelson, J.H. Relation of circumcision to cancer of the cervix. *Am. J. Obstet. Gynecol.* (1973) 117: 1056-1066.
- Tester, W.J., Kinsella, T.J., Waller, B. et al. Second malignant neoplasms complicated Hodgkin's disease: the National Cancer Institute experience. *J. Clin. Pathol.* (1984) 2: 762-769.
- Thivolet, J., Viac, J. and Staquet, M.J. Cell mediated immunity in wart infection. *Int. J. Dermatol.* (1982) 21: 94-98.
- Thomas, D.B. Relationship of oral contraceptives to cervical carcinogenesis. *Obstet. Gynecol.* (1972) 40: 508-58.
- Thomas, D.B. and Anderson, R.I. An epidemiologic and serologic comparison of uterine carcinoma in situ and squamous dysplasia. *Am. J. Epidemiol.* (1974) 100: 113-123.

- Thomas, J.A., Janossy, G., Chilosì, M., Pritchard, J. and Pincott, J.R. Combined immunologic and histochemical analysis of skin and lymph node lesions in histiocytosis. *J. Clin. Pathol.* (1982) 35: 327-337.
- Thomas, L. Reactions to homologous tissue antigens in relation to hypersensitivity. *in* Cellular and Humoral Aspects to the Hypersensitivity State. (1959) ed Lawrence, H.S. p.529.
- Tijo, J.H. and Levan, A. The chromosome number of man. *Hereditas* (1956) 42: 1.
- Todd, I., Pujol-Borrell, R. and Bottazzo, G.F. Epithelial major histocompatibility class II subregion expression in autoimmunity. *Immunology Today* (1986) 7: 6.
- Toon, P.G., Arrand, J.R., Wilson, L.P. and Sharp, D.S. Human papillomavirus infection of the uterine cervix of women without cytological signs of neoplasia. *Br. Med. J.* (1986) 293: 1261-1264.
- Towne, J.E. Cancer of the cervix in multiparous and celibate women. *Am. J. Obstet. Gynecol.* (1955) 69: 606-613.
- Tribukait, B., Gustafson, H., Esposti, P.L. The clinical significance of ploidy and proliferation in the clinical and biological evaluation of bladder tumours: a study of 100 untreated cases. *Br. J. Urol.* (1982) 54: 130-135.
- Van der Leest, R.J., Zachow, K.R., Osjrow, R.S., Bender, M., Pass, F. and Faras, A.J. Human papillomavirus heterogeneity in 36 renal transplant recipients. *Arch. Dermatol.* (1987) 123: 354-357.

- Vayrynen, M., Syrjanen, K., Mantyjarvi, R., Castren, O. and Saarikoski, S. Langerhans cells in human papillomavirus lesions of uterine cervix identified by OKT-6. *Int. J. Gynaecol. Obstet.* (1984) 22: 375-383.
- Verma, R.S. and Dosik, H. Recent advances in detecting human chromosomal abnormalities by various banding techniques. *Pathol. Ann.* (1982) 17: 261-286.
- Vessey, M., Lawless, M., McPherson, K. and Yeates, D. Neoplasia of the cervix uteri and contraception: a possible adverse effect of the pill. *Lancet* (1983) 11: 930-934.
- Viac, J., Bustamante, R. and Thivolet, J. Characterisation of mononuclear cells in the inflammatory infiltrates of cutaneous tumours. *Br. J. Dermatol.* (1977) 197: 1-9.
- Vindelov, L.L., Christensen, I.J. and Nissen, N.T. Standardisation of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. *Cytometry* (1983) 3: 328-331.
- Visser, J.W.M., Jongeung, A.A.M. and Tanke, H.J. Intracellular pH determination by fluorescence measurements. *J. Histochem. Cytochem.* (1979) 27: 32-35.
- Vonka, V., Kanka, J. and Jelinek, J. et al. Prosective study of the relationship between cervical neoplasia and herpes simplex virus type 2. *Int. J. Cancer* (1984) 33: 49-60.
- Walker, P.G., Singer, A., Dyson, J.L., Shah, K.V., To, A. and Coleman, D.V. The prevalence of human papillomavirus antigen in patients with cervical intraepithelial neoplasia. *Br. J. Cancer* (1983) 48: 99-101.

- Waterhouse, J., Muir, C., Correa, P. and Powell, J. eds. Cancer incidence in 5 continents, Vol III. (1976). Lyon : International Agency for Research on Cancer.
- Watson, A.J., Demars, R., Trowbridge, I.S. and Bach, F.H. Detection of a novel human class II HLA antigen. *Nature* (1983) 304: 358-361.
- Watson, J.V., Stewart, J., Evan, G.I., Ritson, A. and Sikora, K. Clinical significance of flow cytometric c-myc oncoprotein quantitation in testicular cancer. *Br. J. Cancer.* (1986) 53: 331-337.
- Watts, K.C., Husain, O.A.N., Campion, M.J. et al. Quantitative DNA analysis of low grade cervical intraepithelial neoplasia and human papillomavirus infection by static and flow cytometry. *Br. Med. J.* (1987) 295: 1090-1092.
- Wickenden, C., Steele, A., Malcolm, A.D.B. and Coleman, D.V. Screening for wart virus infection in normal and abnormal cervixes by DNA hybridisation of cervical scraps. *Lancet* (1985) 1: 65-67.
- Wilbanks, G.D., Richart, R.M. and Turner, J.Y. DNA content of cervical intraepithelial neoplasia studied by two-wavelength Feulgen cytophotometry. *Am. J. Obstet. Gynecol.* (1967) 98: 792-799.
- Wiman, K., Curman, B., Forsum, V. et al. Occurrence of Ia antigens on tissues of non-lymphoid origin. *Nature* (1978) 276: 711-713.
- Winkelstein, W., Shillitoe, E.J., Brand, R. and Johnson, K.K. Further comments on cancer of the uterine cervix, smoking and herpes virus infection. *Am. J. Epidemiol.* (1984) 119: 1-8.

- Wolff, K. and Winkelmann, R.K. Ultrastructural localization of nucleoside triphosphatase in Langerhans cells. *J. Invest. Dermatol.* (1967) 48: 50-54.
- Wolff, K. and Schreiner, E. Uptake, intracellular transport and degradation of exogenous protein by Langerhans cells. An electron microscopic cytochemical study using peroxidase as a tracer substance. *J. Invest. Dermatol.* (1970) 54: 37-47.
- Wolff, K. and Stingl, G. The Langerhans cell. *J. Invest. Dermatol.* (1983) 80: 17-21S.
- Yang, Y-C., Okayama, H. and Howley, P.M. Bovine papillomavirus contains multiple transforming genes. *Proc. Natl. Acad. Sci. USA* (1985) 82: 1030-1034.
- Yee, C., Krishnan-Hewlett, I., Baker, C.C., Schlegel, R. and Howley, P.M. Presence and expression of human papilloma virus sequences in human cervical cancer cell lines. *Am. J. Pathol.* (1985) 119: 361-366.
- Younes, M.S., Robertson, E.M. and Bescosme, S.E. Electron microscope observations of Langerhans cells in the cervix. *Am. J. Obstet. Gynecol.* (1968) 102: 397-403.
- Young, B.D., Ferguson-smith, M.A., Sillar, R. and Boyd, E. High resolution analysis of peripheral lymphocyte chromosomes by flow cytometry. *Proc. Natl. Acad. Sci. USA* (1981) 78: 7727-7731.
- Zang, D.K. Cytological and cytogenetical studies on human meningioma. *Cancer Genet. Cytogenet.* (1982) 6: 249-274.

- Zaninetti, P., Franceschi, S., Baccolo, M., Bonazzi, B., Gottardi, G. and Serraino, D. Characteristics of women under 20 with cervical intraepithelial neoplasia. *Int. J. Epidemiol.* (1986) 15(4): 477-482.
- Zunzunegui, M.V., King, M-C., Coria, C.F. and Charlet, J. Male influences on cervical cancer risk. *Am. J. Epidemiol.* (1986) 123: 302-307.
- zur Hausen, H., Meinhof, W., Schreiber, W. and Burnkamm, G.W. Attempts to detect virus specific DNA in human tumors II. Nucleic acid hybridizations with complementary RNA of human herpes group viruses. *Int. J. Cancer* (1974) 13: 657-664.
- zur Hausen, H. Human genital cancer: synergism between two virus infections or synergism between a virus infection and initiating effects? *Lancet* (1982) ii; 1370-1372.
- zur Hausen, H. Herpes simplex virus in human genital cancer - a review. *Int. Rev. Exp. Pathol.* (1983) 25: 307-326.

A P P E N D I X 1

PUBLISHED PAPERS

- Hughes, R.G., Neill, W.A. and Norval, M. Nuclear DNA analysis of koilocytic and premalignant lesions of the uterine cervix. Br Med J (1987) 294: 267-269.
- Hughes, R.G., Norval, M. and Howie, S.E.M. Expression of major histocompatibility class II antigens by Langerhans cells in cervical intraepithelial neoplasia. J Clin Pathol (1988) 41: 253-259.
- Hughes, R.G., Neill, W.A. and Norval, M. Papillomavirus and c-myc antigen expression in normal and neoplastic cervical epithelium. J Clin Pathol (in press).
- Hughes, R.G., Norval, M. and Howie, S.E.M. Expression of major histocompatibility class II antigens by Langerhans cells in cervical intraepithelial neoplasia (letter). J Clin Pathol (in press).
- Hughes, R.G., Colquhoun, M, Eccles, D.M., Alloub, M., Parker, A.C., Norval, M. and Smart, G.E. Cervical intraepithelial neoplasia in lymphoma patients: a cytological and colposcopic study. Br J Cancer (in press).

CLINICAL RESEARCH

Flow cytometric DNA analysis of koilocytic and premalignant lesions of the uterine cervix

G. HUGHES, WILLIAM A. NEILL, MARY NORVAL

Biopsy samples were taken from 79 patients who had evidence of cervical intraepithelial neoplasia or who had evidence, in the form of koilocytosis, of human papillomavirus infection of the uterine cervix and from 10 women with normal cervixes. The DNA content of the cells in the samples was measured by flow cytometry. Analysis of the data obtained from the biopsy samples from women with cervical intraepithelial neoplasia and human papillomavirus lesions compared with the normal cervixes showed significantly more dividing cells (31.2% of cells from normal cervixes, 29.89%, and 31.76% of cells from cervical intraepithelial neoplasia grades I, II, and III, respectively) than in the normal cervixes (21.6%). The proportion of cells with human papillomavirus infection only (18.2%) did not differ from the group with cervical intraepithelial neoplasia grades I, II, and III (21.2%). The presence of koilocytosis and an increased rate of cellular proliferation are characteristic of malignancy. These results therefore support the view that human papillomavirus plays an important part in the aetiology of cervical carcinoma and are of importance in the clinical management of patients.

There is a close association between infection of the lower genital tract by human papillomavirus and intraepithelial neoplasia. This association has been known for some time, and an increasing body of evidence suggests that this association is causal rather than casual.¹ The

lesions caused by papillomaviruses tend to be persistent and are characterised by increased proliferative activity. These features, as well as the results of animal studies,² make the viruses attractive candidates for playing a part in human carcinogenesis.

Most human cervical carcinomas contain aneuploid cell populations,³ and a variable proportion of cases of cervical intraepithelial neoplasia are also aneuploid.⁴ In addition, the number of proliferating cells in a tumour increases with increasing malignancy.⁵ Since the introduction of flow cytometry it has become possible to identify aneuploid populations with greater accuracy and to quantify the fraction of a cell population in each phase of the cell cycle. In this study we used an EPICS flow cytometer to examine both of these aspects in cervical intraepithelial neoplasia and in infection of the cervix by human papillomavirus.

Patients and methods

Patients—We studied 79 patients aged 22 to 47 referred to the Lothian area colposcopy clinic with abnormal cervical cytology or a clinically suspicious cervix. Ten women aged 25 to 43 who had a history of normal results of smears and who were undergoing minor gynaecological procedures were included as controls.

Biopsies—A cervical biopsy sample was taken from the transformation zone of each patient and control; all the samples from the patients came from areas shown to be abnormal by colposcopy. The specimen was snap frozen on dry ice and stored at -80°C until processed further. A single 8 μm cryostat section was taken from the centre of each specimen and prepared for routine histopathological examination with a conventional haematoxylin and eosin stain. Cervical intraepithelial neoplasia was classified according to recognised criteria.⁶ Koilocytes (balloon shaped cells with a perinuclear halo) were considered to be diagnostic of human papillomavirus infection.⁷ The remainder of the biopsy sample was then thawed, minced, and incubated in 3 ml 0.5% solution of pepsin (Sigma) in 0.01M phosphate buffered saline, with the pH adjusted to 1.5 with hydrochloric acid, at 37°C for one hour. The supernatant was drawn off and centrifuged at 2000 g for 10 minutes, and the resultant pellet was washed in phosphate buffered saline and respun at 2000 g for 10 minutes.

Staining—The nuclear suspension was stained with a modified method of Deitch *et al.*⁸ The pellet was dispersed in 2 ml solution containing 50 μg propidium iodide (Sigma) per ml in 0.01M trometamol (TRIS) (pH 7.0) with 5 mM magnesium chloride, 0.1% Triton X-100, and 15 μg ribonuclease (Sigma) per ml and incubated at 4°C for one to 24 hours. The nuclei

Bacteriology, University of Edinburgh Medical School, 8 9AG

HUGHES, MB, CHB, Melville Trust clinical research fellow
NEILL, MPHIL, research technician
NORVAL, PHD, lecturer

Address: Dr Hughes.

were then centrifuged at 2000 g for 10 minutes and resuspended in phosphate buffered saline.

Controls for flow cytometry—Red blood cells from chickens were run with each sample as an internal control, the DNA content of such cells being 35% of that of a human diploid cell.⁹ They were stained with the propidium iodide solution as described above. Human peripheral blood lymphocytes were stained and run with chicken red blood cells as an external control at the start of each run.

Flow cytometry—The flow cytometer used was an EPICS C (Coulter)

DNA index of 1 equalled the DNA content of a diploid cell. A cell was considered to contain an aneuploid component if the DNA index was less than 0.9 or greater than 1.1 or if it contained a main subsidiary peak distinct from the G₀/G₁ (diploid) and G₂+M peaks. All four groups yielded some aneuploid samples, the proportion of aneuploid cells ranging from 14.3% (cervical intraepithelial neoplasia grade I, grade III). Of the 15 aneuploid samples identified, six were hypodiploid (DNA index <0.9) and nine were hyperdiploid (DNA index >1.1). The DNA index of hyperdiploid cell populations was always less than 1.5.

Proportion of nuclei from cervical biopsy samples in various phases of cell cycle and number of samples containing aneuploid cells

Result of histopathological examination	No of samples suitable for parametric analysis	% (SD) Of nuclei in S phase	% (SD) Of nuclei in S+G ₂ +M phases	Significance*	No (%) of samples containing aneuploid cells
Normal	10/10	11.97 (4.00)	21.60 (4.51)		0 (0)
Koilocytosis alone	10/11	20.64 (8.62)	31.20 (8.53)	p<0.05	2 (18)
Cervical intraepithelial neoplasia:					
Grade I	14/14	23.25 (9.36)	33.06 (8.71)	p<0.01	2 (14)
Grade II	20/21	19.80 (7.74)	29.89 (8.39)	p<0.01	4 (19)
Grade III	31/33	18.27 (5.80)	31.76 (8.38)	p<0.01	7 (21)

*Comparison with normal group by Mann-Whitney U test.

equipped with an argon ion laser that used 200 mW power and emitted light at 488 nm. The nuclei were run at a rate of 150/second until 20 000 stained nuclei had been counted.

Analysis of data—The data obtained were subjected to parametric analysis with the EPICS PARA 1 program, which determined the proportion of nuclei in each phase of the cell cycle. The significance of the differences in each group compared with the control group was calculated with the Mann-Whitney U test.

Results

CELL CYCLE ANALYSIS

The table shows that of 89 cervical biopsy samples examined, 10 were classified by histopathological criteria as normal squamous epithelium, 11 as containing areas of koilocytosis without evidence of cervical intraepithelial neoplasia, and 68 as showing evidence of cervical intraepithelial neoplasia: 14 grade I, 21 grade II, and 33 grade III. Four specimens produced histograms on flow cytometry that, because of an abnormal distribution of DNA, were unsuitable for parametric analysis and were omitted from this part of the study.

The table also shows the proportion of cells in the S phase (the phase of DNA synthesis) and in the G₂ (the phase after DNA synthesis) + M (the phase of mitosis) phases of the cell cycle. The figure shows the data obtained from one biopsy sample.

Traditionally, studies of cellular proliferation have examined only the fraction of cells in the S phase, as the cells in the G₂+M phases could not be quantified by the uptake of tritiated thymidine. Two of us (RH and WN) analysed the histograms independently and found that calculating the total proportion of cells in the S+G₂+M phases was less subject to variation among observers than calculating the proportion of cells in the S phase alone, though as can be seen from the table the two measurements gave very similar results.

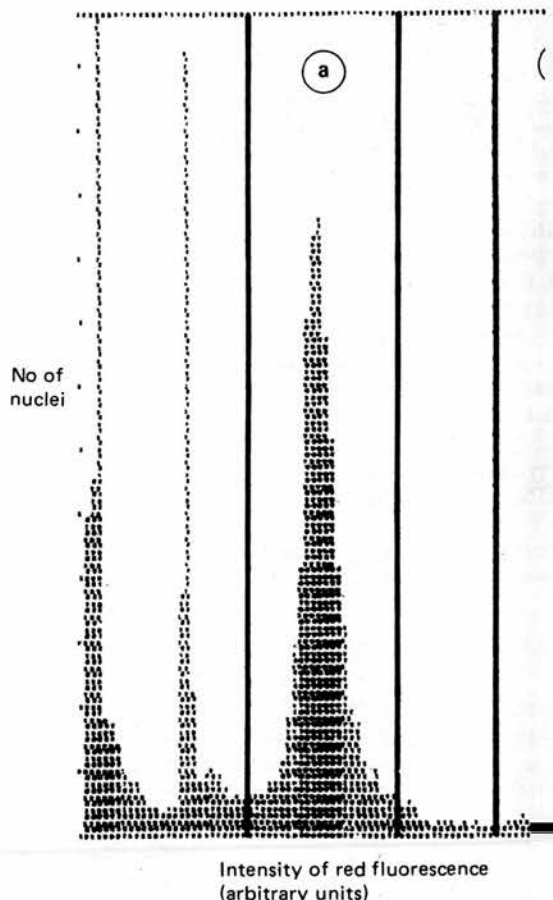
The fraction containing cells in the S phase increased from 11.97% of the total in normal epithelium to 20.64% in epithelium containing koilocytes but no cervical intraepithelial neoplasia (p<0.05). The samples from patients with cervical intraepithelial neoplasia all contained significantly more cells in the S phase than those from the control group. There were no significant differences between the group with koilocytosis and the group with cervical intraepithelial neoplasia. When the S and G₂+M phases were considered together a similar pattern was seen. All four groups with abnormal samples (koilocytosis alone, cervical intraepithelial neoplasia grades I, II, and III) contained significantly more cells in the S+G₂+M phases than the control samples but did not differ significantly from each other.

ANEUPLOIDY

The DNA index was calculated from the ratio between the chicken red blood cell and sample modal channel numbers versus the ratio between chicken red blood cell and lymphocyte modal channel numbers. Thus a

Discussion

Our analysis of cellular DNA content by flow cytometry shows that normal cervical epithelium can be distinguished from epithelium with koilocytosis (evidence of human papillomavirus infection) and with cervical intraepithelial neoplasia. The proportion of cells with koilocytosis or cervical intraepithelial neoplasia did not differ significantly from normal epithelium in containing a larger proportion of



Result of flow cytometry of nuclear DNA from cervical biopsy within set of cursors marked (a) and G₂+M peak within cursor S phase fraction lies between two sets of cursors, and chicken red cell peak to left of (a).

cells in S+G₂+M phases) and, in some cases, possessing a polyploid component. In many systems aneuploidy and an increased rate of cellular proliferation are characteristics of stem cells.

It has been postulated that cells infected with papillomavirus behave as though stimulated by a non-specific mitogen and that in a semipermissive epithelium many cells remain in a prolonged S or G₂ phase.¹⁰ Reid proposed that cumulative mitotic errors in these stem cells might result in occasional chromosomal errors spawning clones of aneuploid basal cells.¹¹ By showing an increased proportion of cells in the S+G₂+M phases in the control group our work supports this view. The samples from the intraepithelial neoplasia grade II and III lesions contained a similar proportion of proliferating cells than samples from the control and cervical intraepithelial neoplasia grade I lesions, and the difference was not significant. This fits Reid's hypothesis that the virus initially stimulates epithelial proliferation and then causes arrest in the S phase and the emergence of aneuploid cells.

In contrast with the work done by other groups^{4,12} the prevalence of aneuploidy did not increase significantly with increasing severity of intraepithelial neoplasia in this study. The proportion of cells containing aneuploid populations was essentially similar in the control and abnormal groups, including the group with koilocytosis (range 14.4% to 21.2%). To our knowledge this is the first published report of aneuploidy in "benign" human papillomavirus infection of the cervix, and it supports the hypothesis that human papillomavirus does have a central role in the aetiology of cervical cancer.

In conclusion, the implications for the management of patients with evidence of cervical human papillomavirus infection or of intraepithelial neoplasia grade I may be far reaching. In these women these women are treated conservatively in the hope that the neoplasia will regress. It has been suggested that the clinical

behaviour of lesions may be predicted on the basis of the degree of ploidy.¹³ On the other hand, our data seem to indicate that so called benign human papillomavirus infections and cervical intraepithelial neoplasia grade III share similar patterns of DNA synthesis and thus should be treated identically.

We are grateful to Drs G E Smart and S Fletcher for enabling us to obtain biopsy material from patients and clinical information and for helpful discussions and to the staff of the Lothian area colposcopy clinic for their help. We thank the Cancer Research Campaign for funding this project and the Melville Trust for a clinical fellowship for RGH.

References

- 1 Anonymous. Genital warts, human papilloma viruses and cervical cancer [Editorial]. *Lancet* 1985;ii:1045-6.
- 2 Pfister H. Biology and biochemistry of papillomaviruses. *Rev Physiol Biochem Pharmacol* 1984;99:111-68.
- 3 Jakobsen A, Bichel P, Sell A. DNA distribution in biopsy specimens from human cervical carcinoma investigated by flow cytometry. *Virchows Arch [Cell Pathol]* 1979;29:337-42.
- 4 Jakobsen A, Kristensen PB, Poulsen HK. Flow cytometric classification of biopsy specimens from cervical intraepithelial neoplasia. *Cytometry* 1983;4:166-9.
- 5 Laerum O, Farsuno T. Clinical application of flow cytometry: a review. *Cytometry* 1981;2:1-13.
- 6 Buckley CH, Butler EB, Fox H. Cervical intraepithelial neoplasia. *J Clin Pathol* 1982;35:1-13.
- 7 Fletcher S. Histopathology of papillomavirus infection of the cervix uteri: the history, taxonomy, nomenclature and reporting of koilocytic dysplasia. *J Clin Pathol* 1983;36:616-24.
- 8 Deitch AD, Law H, White RD. A stable propidium iodide staining procedure for flow cytometry. *J Histochem Cytochem* 1982;30:967-72.
- 9 Vindelov LL, Christensen IJ, Nissen NI. Standardisation of high resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. *Cytometry* 1983;3:328-31.
- 10 Reid R, Stanhope CR, Herschman BR, Booth E, Phibbs GD, Smith JP. Genital warts and cervical cancer. I. Evidence of an association between subclinical papillomaviral infection and cervical malignancy. *Cancer* 1982;50:377-87.
- 11 Reid R. Genital warts and cervical cancer. II. Is human papillomavirus infection the trigger to cervical carcinogenesis? *Gynecol Oncol* 1983;15:239-52.
- 12 Reid R, Crum CP, Herschman BR, et al. Genital warts and cervical cancer. III. Subclinical papillomaviral infection and cervical neoplasia are linked by a spectrum of continuous morphologic and biologic change. *Cancer* 1984;53:943-53.
- 13 Fu YS, Reagan JW, Richart RM. Definition of precursors. *Gynecol Oncol* 1981;12:5220-31.

(Accepted 30 October 1986)

A P P E N D I X 2

LIST OF ABBREVIATIONS USED

APC	antigen presenting cell
BPV	bovine papillomavirus
CIN	cervical intraepithelial neoplasia
CIS	carcinoma <u>in situ</u>
CMI	cell-mediated immunity
CMV	cytomegalovirus
CO ₂	carbon dioxide
CRBC	chicken red blood cells
C/T	chemotherapy
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
EM	electron microscopy
EV	epidermodysplasia verruciformis
FITC	fluorescein isothiocyanate
FLS	forward angle light scatter
HD	Hodgkin's disease
HDF	human dermal fibroblasts
HLA	human leucocyte antigen
HPV	human papillomavirus
HSV	herpes simplex virus
HUVE	human umbilical vein endothelium
IUCD	intrauterine contraceptive device
LC	Langerhans cell
LFLS	log forward angle light scatter
LMI	leucocyte migration inhibition

LIST OF ABBREVIATIONS USED (cont'd)

LRFL	log red fluorescence
LTT	lymphocyte transformation test
Mab	monoclonal antibody
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NRS	normal rabbit serum
NSS	normal sheep serum
OCP	oral contraceptive pill
ORF	open reading frame
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
RNA	ribonucleic acid
R/T	radiotherapy
SCJ	squamocolumnar junction
S.E.M.	standard error of the mean
SPV	Shope papillomavirus
TBS	Tris buffered saline
TZ	cervical transformation zone
UV	ultraviolet

A P P E N D I X 3

LIST OF FIGURES

	Page No.	
Figure 1	Pre-pubertal cervix	5
Figure 2	Post-adolescent cervix	5
Figure 3	The Ayres spatula	7
Figure 4	HPV associated lesions of the uterine cervix	17
Figure 5	Human chromosome 6. Major histocompatibility loci	31
Figure 6	Langerhans cells in normal cervix	35
Figure 7	Antigen presentation - 3 models	39
Figure 8	Antigen presentation by Langerhans cells	41
Figure 9	Schematic representation of flow cytometry	55
Figure 10	The cell cycle	60
Figure 11	The Cytobrush	75
Figure 12	DNA staining method	82
Figure 13	Representative DNA histogram	84
Figure 14	EPICS 'C' flow cytometer	85
Figure 15	Representative DNA histogram (PARA 1 analysis)	87
Figure 16	Langerhans cells in normal cervix	99
Figure 17	Langerhans cells in normal cervix	99
Figure 18	Langerhans cells in CIN I	100
Figure 19	Langerhans cells in CIN I	100
Figure 20	Langerhans cell numbers in normal and abnormal cervical epithelium	101
Figure 21	Langerhans cell numbers in cervical epithelium from OCP users and non-OCP users	104
Figure 22	Langerhans cell numbers in cervical epithelium from smokers and non-smokers	105

LIST OF FIGURES (cont'd)

		Page No.
Figure 23	DA6.231 staining of endocervical columnar epithelium	109
Figure 24	DA6.231 staining of CIN III	111
Figure 25		
(a) & (b)	DNA histogram of aneuploid sample of CIN III	125

A P P E N D I X 4

LIST OF TABLES

		Page No.
Table 1	Human papillomavirus types associated with genital lesions	16
Table 2	Origin and specificity of monoclonal antibodies	78
Table 3	Origin and specificity of antibodies used for flow cytometry	81
Table 4	General clinical data	89
Table 5	A comparison of the results of 5 epidemiological studies of patients with CIN and normal controls a) number of partners	91
Table 6	A comparison of the results of 5 epidemiological studies of patients with CIN and normal controls b) age at coitarche	92
Table 7	A comparison of the results of 4 epidemiological studies of patients with CIN and normal controls c) smoking history	94
Table 8	Percentage of Langerhans cells expressing MHC Class II antigens in normal and abnormal cervical epithelium	102
Table 9	CD3, CD4, CD8 and class II positive T lymphocyte numbers in normal and abnormal cervical epithelium	107
Table 10	DNA content of biopsies from normal and abnormal cervical epithelium	120
Table 11	DNA content of brushings from normal and abnormal cervical epithelium	122

LIST OF TABLES (cont'd)

		Page number
Table 12	C-myc protein in biopsies and brushings from normal and abnormal cervical epithelium	127
Table 13	Cell cycle analysis and c-myc protein expression	128
Table 14	Papillomavirus antigen expression in biopsies and brushings from normal and abnormal cervical epithelium	130
Table 15	Cell cycle analysis and papillomavirus antigen expression	132
Table 16	Papillomavirus antigen expression in invasive cervical carcinomas, adjacent epithelium, and draining lymph nodes	133
Table 17	Epidemiological data from lymphoma patients and controls	143
Table 18	Lymphoma patients grouped according to haematological diagnosis and cervical histology and cytology	144
Table 19	Cervical histology of lymphoma patients and controls	149
Table 20	Epidemiological characteristics of lymphoma patients (grouped according to cervical histology and cytology)	150

RESERVED APPENDIX

1. ANNUAL REPORTS.
Heriot-Watt University Annual Reports from 1969-1970 to 1983-1984.
Edinburgh: Heriot-Watt University.
2. BARKER, A.L. (1979).
'Design and evaluation of an information system for Structured Learning resource materials.
Unpublished Ph.D. Thesis, Heriot-Watt University, Edinburgh.
3. BOLTON, A.
"The Universities and the impact of the Chilver Report". (Personal communication).
Paper 5 - memiographed.
4. BOLTON, A. (1977).
"Problems facing a Head of Department who seeks to rebuild an Engineering Course".
Paper presented to "Education for Engineering Conference".
The British Council Course 638.
Edinburgh - London, Jan-Feb 1977.
5. COWAN, J. (1972).
'Two case histories of curriculum development'.
Lehrsysteme 72, Berlin, April 1972.
6. COWAN, J. (1972).
'Technology in Teaching'.
Paper for a workshop at Callendar Park Conference.
September 1972.
7. COWAN, J. (1974).
"Towards independence in learning in a civil engineering course", in "Independence in Learning selected papers", from "Nuffield Group for Research and Innovation in Higher Education.
London, Nuffield Lodge (1974).
8. COWAN, J. (1974).
'Succour the converted and ignore the unbelievers',
in the Times Higher Education Supplement,
22 November 1974.
9. COWAN, J. (1975).
'Applying educational technology to Scout training'.
Scouting Magazine 2, 69, 87-89.

10. COWAN, J. (1975).
'An experiment involving structured and unstructured tutorial material'.
Paper for Society for Research into Higher Education, Scottish Branch, May 1975.
11. COWAN, J. (1976).
Can tape/overhead compete with tape/slide?
Visual education, May 1976.
12. COWAN, J. (1975).
'A study of the feasibility of resource-based Learning in Civil Engineering Education'.
Unpublished Ph.D. Thesis, Heriot-Watt University, Edinburgh.
13. COWAN, J. (1977).
'Individual Approaches to Problem Solving'.
Paper for "Educational Technology. International Conference", 1977.
14. COWAN, J. (1981).
'Design education based on an expressed statement of the design process'.
Proceedings of the Institution of Civil Engineers, Part 1, 1981, 70, November.
15. COWAN, J. (1982).
Discussion. 'Design education based on an expressed statement of the design process.'
Proc.Instn.Civ.Engrs., Part 1, 1982, 72, November pp. 659-673.
16. COWAN, J. and BROHN, D.M. (1977).
'Teaching towards an improved understanding of structural behaviour', in The Structural Engineer, January 1977.
17. COWAN, J. and McCONNELL, S.G. (1967).
An experiment in the teaching of materials science, in Metals and Material, July 1967.
18. COWAN, J. and McCONNELL, S.G. (1970).
"Project work for undergraduate civil engineers", in University Quarterly, Autumn 1970, 433-439.
19. COWAN, J., McCONNELL, S.G. and BOLTON, A. (1970).
'Learner-directed group work for large classes'.
Paper presented in the Fourth International Conference on Educational Technology, Loughborough, 1970.

20. COWAN, J., MORTON, J. and BOLTON, A. (1973).
'An experimental Learning Unit for structural engineering studies'.
Structural Engineer (1973), 51, 9.
21. COWAN, J., McCONNELL, S.G. and MORTON, J. (1974).
'Two contrasting approaches to curriculum development'. The text read to the audience.
Frontiers in Education Conference, London,
July 1974.
22. PARLETT, M.R. (1971).
'Intensive study in a department of Architecture'.
In Parlett, M.R. and King, J.G.
Concentrated Study, A Pedagogic Innovation
Observed. Research into higher education
monographs, Number 14.
London: Society for Research into Higher
Education.