LANGERHANS´ CELLS

AND

LOCAL CELLULAR IMMUNITY

IN THE CERVIX UTERI

SUBMITTED BY

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Dedicated to my father

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DECLARATION

I declare that the preparation and writing of this Thesis has been carried out by myself.

The research described in this thesis was performed by myself except where the help of others has been acknowledged.

Robert J S Hawthorn

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Summary

Dermatological research has shown there is a likely role for Langerhans' cells in initiating the local immune response. They are known to act as antigen presenting cells. Antigen presentation is essential for the generation of the T-cell response. The process involves helper T-cell interaction in the afferent limb of the immune response, with cytotoxic T-cells involved in efferent response. The resultant immune response has been shown to be dependent on the numbers of Langerhans' cells in the epithelium. T-cell mechanisms are primarily involved in immunity to tumours and to viral infections. The study was designed to investigate the possible role of the Langerhans' cell and local immune system in the cervix in relation to neoplastic changes especially since a virus - human papillomavirus (HPV) - is currently thought to be implicated in the aetiology of cervical neoplasia.

The investigation of the local immune system with special regard to tumour immunosurveillance is relevant and well suited to studies in the cervix, although access to tissue is limited without specialised techniques such as colposcopy and biopsy. The well documented and easily identifiable preinvasive phase associated with carcinoma of cervix makes it possible to study the whole spectrum of neoplastic change in the cervix. The study therefore involved examination of normal patients, those with cervical carcinoma, patients with cervical intraepithelial neoplasia (CIN), patients at high risk of such changes and other groups of patients in whom T-cell function is known to be compromised.

Several methods of identifying Langerhans' cells in the cervix were evaluated. However, the indirect immunoperoxidase technique on frozen sections proved optimal since with the range of specific monoclonal available, Langerhans' cells and T-cell subsets could be easily identified in-situ. The simplest method of quantification used a standard length of basement membrane as the denominator (100 basal cells) but counts with computerised image analyser techniques were also used. Good correlation was shown between the Langerhans' cell count per 100 basal cells and the other methods of enumeration investigated.

Studies in normal patients showed that Langerhans' cells were a consistent finding in the cervical epithelium of the transformation zone and ectocervix, being present in both areas in similar numbers. Examination showed the majority of the cellular infiltrate in the epithelium and stroma was of T-cell origin. Helper T-cells were shown to predominate in the stroma and cytotoxic T-cells in the epithelium. The image analyser was most useful in quantifying this lymphocytic infiltrate. The T-cell infiltrate was shown to be similar in the ectocervix and transformation zone.

A series of 142 patients with CIN were investigated using the same techniques. Internal control biopsies of ectocervix were available for comparison in most instances. Analysis of results showed that two groups were distinguishable, one with concomitant histological features of human papillomavirus infection and the other without. The Langerhans' cells were significantly reduced in the first group and significantly increased in the second compared with the numbers in internal control biopsies.

In the lesions showing histological evidence of HPV infection

the lymphocytic infiltrate appeared to be influenced by the presence of class II Major Histocompatibility (MHC) antigens on the epithelial cells. The number of helper and cytotoxic T-cells were significantly greater in the epithelium and stroma of MHC class II positive lesions. The percentage composition of the infiltrate was not different comparing the MHC class II positive with the class II negative lesions.

In CIN with low numbers of Langerhans' cells (< 7 per 100 basal cells) and lacking MHC class II antigens on the epithelial cells, a significant positive correlation was noted between the numbers of Langerhans' cells and number of helper and cytotoxic T-cells in the epithelium. There was no such correlation seen in the stroma.

Investigation of the relationship between Langerhans' cell numbers and specific HPV subtypes, as demonstrated by slot blot DNA hybridisation, showed reductions associated with HPV 18 and a suggestion that the reduction seen in the presence of HPV 16 may be related to viral copy numbers per cell. Increased numbers of Langerhans' cells were confirmed in the absence of HPV genomes.

Female renal transplant recipients on systemic immunosuppressant therapy represent a group at high risk of cervical neoplasia. However, there is little published data on the results of screening this high risk group. Screening by colposcopy and cytology was therefore performed in 38 patients. The local immunocompetent cells were also studied in these patients to determine whether reductions in Langerhans' cells or alterations in other constituents of the local immune system could be implicated in the development of cervical neoplasia. Results of screening confirmed a high prevalence of CIN (6/38) and of HPV

infection (12/38), with cytology alone producing false negative results in three of the six patients with CIN. Immunohistochemical studies showed significantly reduced numbers of Langerhans' cells and helper T-cells in the typical transformation zone of this group compared with the normal transformation zone. There was no difference in the Langerhans' cell or helper T-cell numbers comparing the patients on azathioprine and prednisolone with those on cyclosporin and prednisolone.

Patients in renal failure are also reported to be immunocompromised. Since it was difficult to be certain that the changes seen in the cervix of the transplant patients were not secondary to uraemia, 15 women in chronic renal failure were also studied. The results of colposcopic and cytologic screening suggest that the high prevalence of CIN and HPV infection seen in the transplant patients represents an increased incidence following commencement of systemic immunosuppressant therapy. Compared with the post transplant patients, the patients in renal failure have significantly greater numbers of Langerhans' cells and helper T-cells in the cervix. There were no significant differences in the T-cell subsets in the cervix comparing the patients in renal failure with the normal patients. However, the Langerhans' cells were reduced in seven uraemic patients who were not being dialysed.

Pregnancy is another situation in which T-cell function is impaired. Examination of 20 patients in early pregnancy showed no significant differences in the numbers of Langerhans' cells or in the composition of the T-cell infiltrate of the cervix when compared with normal patients.

The diversity of the local immune response was emphasised

when specimens from 10 patients with stage Ib or IIa carcinoma of cervix were examined. The predominant cells identified stained with T-cell markers although B-cells, macrophages and natural killer cells were also noted. The number of Langerhans' cells in the malignant epithelium varied greatly. Variation was also noted in the pattern of MHC class II antigen expression on the epithelial cells. Loss of MHC class I antigens from the malignant cells was found in one tumour which had metastasised to pelvic lymph nodes.

Chapter 1

Background and historical review of Langerhans' cells and cervical intraepithelial neoplasia

Chapter 1

Introduction

The recent increase in patients with abnormal cervical smears has reached almost epidemic proportions and has been highlighted in the popular press. Deaths from cervical carcinoma remain a significant problem, especially in young women. These deaths are potentially preventable by the diagnosis and appropriate treatment of detectable epithelial changes which preceed the development of cervical carcinoma. Such changes are encompassed by the term cervical intraepithelial neoplasia (CIN).

This thesis explores the role of the immune system in the cervix in relation to CIN. In the first chapter the nomenclature, natural history and aetiology of CIN together with specialised techniques for its detection are introduced. The history, staining characteristics and evidence supporting a central role for a specialised cell - the Langerhans' cell, in coordinating the local immune response to such epithelial changes, are discussed. A historical review of the pertinent literature is included.

Nomenclature used in CIN

The term CIN was introduced by Richart in 1966 (1966a,b) because he believed that the nomenclature used up to that time to describe the histological changes seen in the cervix was inappropriate. For instance, cervical epithelial dysplasia was thought, by some, to be a benign condition whilst carcinoma-in-situ (CIS) was considered pre-malignant. Richart, on the other hand thought that they were both part of the same spectrum of disease which could preceed squamous carcinoma of the cervix. It

took some years before Richart's concept of CIN as a single entity became accepted, but it has now been universally adopted.

Diagnostic criteria

CIN is a histological diagnosis based on the arrangement and nuclear morphology of the epithelial cells. It is characterised by an increased nucleocytoplasmic ratio, variation in nuclear size, abnormal mitoses and the absence of normal cellular maturation and differentiation. It is graded according to the extent of the epithelial changes. If they are confined to the basal third of the epithelium this constitutes CIN I; if the changes extend to occupy two-thirds then CIN II exists. CIN III is present if over two-thirds of the epithelium are involved (Buckley, Butler & Fox 1982). When correlated with the previously used terms, CIN I is synonymous with mild dysplasia, CIN II with moderate dysplasia and CIN III encompasses both severe dysplasia and carcinoma-in-situ.

Natural history

Richart's belief that all neoplastic cellular changes limited to cervical epithelium were part of the same disease process was followed by a study in which he and Barron (Richart & Barron 1969) showed that all grades of dysplasia could advance to higher grades and to carcinoma in-situ. Other studies support these findings by confirming the progression from dysplasia to carcinoma-in-situ (Stern & Neely 1963; Fox 1967).

There is considerable evidence establishing the malignant potential of CIN, although this has not been a uniformly held opinion (Green & Donovan 1970). The first of two studies from Copenhagen reported that the progression rate for intraepithelial

disease to cancer was 11% at three years and 33% at nine years (Petersen 1956). In the second study, Lange (1960), found that in a group of women with lesions bordering on early invasion one third developed carcinoma after 32 months compared to a group with intraepithelial disease alone of whom one fifth progressed to carcinoma after an average of five years. Another long-term prospective study in women with carcinoma-in-situ confirmed the progression to invasive carcinoma (Koss et al. 1963; Jordan, Bader & Day 1964). McIndoe et al. (1984) showed the importance of long-term surveillance and treatment of residual abnormality in patients with CIS when they reported that progression to invasion occurred in 22% of patients with persistent abnormal cytology and in 1.5% of patients with normal cytology after a minimum follow-up of five years.

There is some evidence that the change from intraepithelial disease to invasive carcinoma may occur, in some cases, via an intermediate stage - microinvasive carcinoma (Way, Hennigan & Wright 1968). This stage can be further divided histologically into a group with minimal stromal invasion and another with microcarcinoma (Burghardt 1976).

The malignant potential of CIN is no longer questioned, but its natural history is better illustrated by examining data from large populations. Using the incidence of carcinoma-in-situ and carcinoma Fidler, Boyes & Worth (1968) calculated that 43% of CIS progressed to carcinoma over an average duration of 12 years. A statistical model for the natural history of the condition was constructed by Barron, Cahill & Richart (1978) using figures obtained from population screeening in British Columbia and Barbados. They concluded that the duration of CIS was between 3

and 10 years. Way et al. (1968) agreed that pre-invasive lesions may exist for 10 years, but added that a further 10 years may lapse before symptoms or clinical signs develop.

While CIN may progress to cervical carcinoma in some instances, it is recognised that many more lesions regress. Mild (CIN I) and moderate (CIN II) dysplasias regress much more frequently than severe dysplasia and CIS (CIN III) (Koss 1963; Richart 1966b; Fox 1967; Hall & Walton 1968).

What factors might influence the natural history of CIN?. Several studies indicate that the biopsy from which the diagnosis is made influences the regression of CIN (Rawson & Knoblich 1957; Koss et al. 1963; Richart 1966b). Regression followed biopsy in over 80% of lesions sudied by Rawson & Knoblich (1957). Where cytology and colposcopy, without biopsy, have been used to follow the natural history, progression has been recorded in up to 60% of patients with abnormal smears (Fox 1967). A study that compared biopsied with non-biopsied CIN confirmed that regression was more common and progression less common after the biopsy (Nasiell, Nasiell & Vaclavinkova 1983). There is not uniform agreement on the effect of biopsy (Stern & Neely 1964) but it is agreed that CIN should be a histological diagnosis (Buckley et al. 1982).

The presence of human papillomavirus (HPV-one of the proposed aetiological factors in cervical neoplasia) with CIN may also influence the progression or regression of the condition. In a recent study Campion et al. (1986) described the progression from mildly abnormal smears to CIN III, within 24 months, in 26% of lesions which contained HPV 16 genome sequences. A defect in this study is that the initial diagnosis was made from colposcopy and cytology alone, whereas the final diagnosis was on histological

criteria. More detailed inspection of the data implicating HPV indicates that patient age and diagnostic criteria are confusing variables. The cytological evidence of viral infection in CIN decreases with increasing age (Syrjanen, Heinonen & Kauraniemi 1981) while conversely Meanwell et al. (1987) showed that viral detection by (HPV) genome sequences increases with age.

The immune response to CIN is likely to be a factor which will influence the natural history as indicated by the high prevalence of the condition in immunosuppressed patients (Schneider, Kay & Lee 1983). The difficulty in any serial study examining local immunity is the requirement of tissue biopsies which, as mentioned above, may themselves contribute to the regression rate.

Colposcopy in the diagnosis of CIN

Cervical neoplasia most frequently develops in the area of cervix known as the transformation zone, which lies between the 'original' and the 'new' squamocolumnar junctions i.e. in the area where columnar epithelium has been transformed to squamous epithelium (Richart 1967; Johnson 1969). The junction between the transformation zone and original ectocervix is marked by the so called histological 'last gland' (Burghardt 1976). The transformation zone may be identified in-vivo using a colposcope which is a modified binocular microscope that allows the cervix to be viewed between 6 and 40 times magnification. Following exposure of the cervix using a speculum, cotton wool swabs are used to remove cervical mucus. The application of saline on cotton wool swabs permits detailed examination of the subepithelial capillary network of the cervix. The vascular pattern is more clearly seen

with the interposition of a green filter which causes the vessels to appear black. Frankly abnormal irregular and cork-screw superficial vessels are suspicious of invasive disease. The vascular features are generally best seen before the application of acetic acid which is used to define epithelial abnormalities.

Acetic acid (5%) causes cell swelling and transiently denatures the nuclear proteins. Areas of abnormal epithelium whose cells contain increased amounts of nuclear protein become opaque and appear 'aceto-white'. The 'punctate' and 'mosaic' appearances that often appear in the aceto-white epithelium occur where the epithelium is thinnest allowing the pink of the capillaries or stromal ridges to show through. Several colposcopic features assist in the differential diagnosis of aceto-white epithelium. These include the presence of sharp borders to the lesion, the response to acetic acid, the surface contour of the lesion, the vascular pattern, the site and extent of the lesion, the response to Schiller's iodine, the presence of multiple abnormalities and the presence of keratinisation (Burghart 1984). Reid et al. (1984) have formed a 'colposcopic index' based on what they considered to be the most important features in distinguishing the lesser degrees epithelial abnormality from the major abnormalities.

Despite the well described colposcopic appearances, biopsy of areas of aceto-white epithelium or areas with abnormal vasculature is required for definitive diagnosis. Biopsies can be taken with punch biopsy forceps such as Leech-Wilkinson forceps. This is an outpatient procedure which causes little discomfort and requires no analgesia. Colposcopy therefore allows accurate identification of the biopsy site (Navratil et al. 1958) and with experience high correlation between the histology obtained from

colposcopically directed punch biopsies and the definitive histology at cone biopsy or hysterectomy can be obtained (Donohue & Meriwether 1972 ; Stafl & Mattingly 1973; Reid et al. 1984).

The technique of colposcopy was first introduced in Europe by Hinselmann (1925) and was initially used, like cytology, as a screening technique. In this country, however, colposcopy is most widely practised following abnormal cervical cytology and is recommended prior to definitive treatment (Jordan, Sharp & Singer 1982). Recent reports have highlighted the problems of relying on cytology alone as a screening test. Walker, Dodgson & Duncan (1986) have shown a poor correlation between mildly atypical cytology and histology. Campion et al. (1986) re-emphasised this when they showed that 26% of young women with mildly abnormal smears had CIN III on biopsy. The need for reintroducing primary screening by colposcopy in identifiable patients at particular high risk of cervical neoplasia has been proposed (Cordiner, Sharp & Briggs 1980).

In addition to the diagnostic benefits afforded by colposcopy and biopsy, they have permitted a trend towards more conservative treatment of CIN which is especially appropriate for younger women who have not completed their families. Multiple punch biopsies, cold coagulation, electrodiathermy, electrocautery, cryosurgery and carbon dioxide laser ablation have all been used as alternatives to formal cone biopsy or hysterectomy in the management of CIN.

Aetiology of cervical neoplasia

Inherent in Richart's concept of CIN as a single entity preceding cervical carcinoma is that the same aetiological factors

are implicated in both.

Sexual activity has long been associated with cervical neoplasia (Rigoni-Stern 1842). Epidemiological studies have shown that early age at first intercourse, multiple partners and unstable marital relationships are commonly found behavioural characteristics in women who develop cervical neoplasia (Wynder et al. 1954; Rotkin & King 1962; Meisels, Begin & Schneider 1977; Harris et al. 1980). Perhaps linked to these observations is the fact that CIN is more common in imprisoned women prostitutes than in other women (Keighley 1968).

Associations with CIN have been shown for socio-enconomic status and sexually transmitted disease (Beral 1974). A male factor has also been implicated (Kessler 1976) and Singer, Reid & Coppleson (1976) proposed the concept of the high risk male. Other associations include the presence of genital warts (Walker et al. 1983), smoking (Trevathan et al. 1983), immunosuppression (Schneider et al. 1983) and perhaps the oral contraceptive which seems to predispose to early cervical neoplasia when compared to use of the intrauterine contraceptive device (IUCD) (Vessey et al. 1983), although this effect may be related to duration of pill use (WHO Collabrative Study 1985)

From this background, the search for the aetiological agent causing cervical neoplasia has investigated several sexually related factors. Smegma was implicated following observations of low incidences of cervical neoplasia amongst Jewish and Fijian populations where the practice of circumcision is common (Handley 1936; Kennaway 1948; Weiner, Burke & Goldberger 1951). However studies from USA, Scotland and Lebanon have cast serious doubt on this theory (Rotkin & King 1962; Aitken-Swan & Baird 1965;

Abou-Daoud 1967).

Research from Sydney (Coppleson & Reid 1968; Coppleson 1969) demonstrated that metaplastic cervical epithelial cells possessed the ability to phagocytose sperm. They postulated that the ingested sperm protein or nucleic acids had transforming properties and that mutation resulted in neoplastic cell lines (Reid & Coppleson 1975). Furthermore males from different socio-economic groups were shown to have different amounts of arginine-containing protamine in the sperm head but the signifiance of these findings remains unclear.

Herpes Simplex Virus type 2 (HSV 2) was thought a likely candidate after several large seroepidemiological studies showed a link between the presence of antibodies to HSV 2 and cervical neoplasia (Skinner, Thouless & Jordan 1971; Nahmias, Naib & Josey 1974; Thomas & Rawls 1978). These studies were questioned in the light of cross reactivity between HSV 1 and HSV 2 (Lonsdale 1979). Large variations in the presence of antibodies in control groups made the evidence even less convincing (Melnick & Adam 1978).

A group working in Glasgow (Eglin et al. 1981; Sharp et al. 1981) were able to demonstrate HSV 2 ribonucleic acid (RNA) in biopsies of CIN and invasive squamous cell carcinoma. However, other groups were unable to consistently demonstrate HSV specific deoxyribonucleic acid (DNA) in cervical cancer (zur Hausen et al. 1974; McDougall, Galloway & Fenoglio 1980). The inability to find HSV DNA in neoplastic cervical tissue using the sensitive diagnostic techniques of DNA hybridisation has cast serious doubt on HSV 2 as the causative agent.

The consistent presence of human papillomavirus (HPV) genome sequences in CIN and in invasive carcinoma has led to some

authorities claiming that HPV is the causal agent (Crum et al. 1984; zur Hausen, Gissman & Schlehofer 1984). The venereal transmission of HPV is well documented (Oriel 1983) and Campion, Singer & Clarkson (1985) have shown a relationship between CIN and penile condylomata in the consort.

There are many HPV subtypes and the list is ever expanding. Types 6 and 11 have been shown to be present in benign papillomas of the female genital tract and low grade CIN (Gissman et al. 1983) whereas types 18 and 16 have been more commonly found in CIN III and frequently in invasive cancers (Crum et al. 1983; Durst et al. 1983 ; McCance et al. 1985; Millan et al. 1986). HPV 31, 33 and 35 have also been sequenced from invasive cancers (Editorial 1987b). Integration of the viral genome may be a risk factor in malignant transformation by HPV infection (Di Luca et al. 1986) but its prognostic significance is uncertain since integrated HPV genome sequences have been observed in the chronically inflamed cervix (Millan et al. 1986) and in the normal cervix (Cox et al. 1986; Murdoch et al. 1988).

The carcinogenic potential of the papillomavirus is supported by the presence of HPV DNA in cervical cancer-cell lines (Schwartz et al. 1985) and its role in the development of various cancers in animals. For instance the Shope papillomavirus induces malignant transformation of papillomas in domestic rabbits (Rous & Friedwald 1944) while bovine papillomavirus is the causal agent of oesophageal and intestinal carcinoma in cattle (Jarrett 1981). Papillomaviruses have also been implicated in the development of ocular tumours in cattle (Ford et al. 1982) and skin cancer in sheep (Vaneslow, Spradbrow & Jackson 1982). In these situations the papillomavirus is acting with other cofactors such as chemical

carcinogens in rabbits, and bracken in cattle. Ultraviolet light appears to be neccessary for the generation of ocular and skin cancer in these animals.

In man there is also evidence that papillomaviruses require cofactors to enhance their malignant potential. Epidermodysplasia verruciformis (EV) is a rare, possibly autosomally inherited cutaneous disorder in which papillomas undergo malignant transformation when exposed to ultraviolet light (Orth et al. 1979; Kremsdorf et al. 1982). HPV 5 is the subtype implicated with neoplastic change in the warts. In addition to ultraviolet light, impaired T-cell immunity is also a feature of EV and could be another factor involved in the malignant transformation (Glinski et al. 1976). HPV 5 has also been found in benign and malignant lesions in a renal transplant recipient, on azathioprine, in sun exposed sites (Lutzner et al. 1983). Here again exogenous immunosuppression, especially of T-cell function, and exposure to ultraviolet light are possible cofactors.

In cervical neoplasia zur Hausen (1982) has postulated that HSV 2 may act synergistically with the papillomavirus, with HPV initiating cell proliferation and HSV acting as a potent mutagen on the proliferating cells. Smoking may also act as a cofactor via a possible toxic effect on the epithelium (Singer, Walker & McCance 1984) or via an immunosuppressant effect (Miller et al. 1982).

The weight of research opinion behind HPV as the causative agent of cervical neoplasia has diminished from its peak when some authorities were suggesting that routine screening for HPV (type 16) by DNA hybridisation should be introduced (Editorial 1987a). On current evidence Murdoch, Cordiner & Macnab 1987; Murdoch et

al. 1988) have concluded that such screening is not justified.

Immunological considerations

In animals and man there appears to be a link between malignant transformation of HPV induced lesions and immunosuppressive insults such as exposure to ultraviolet light and drugs. The relationship between CIN and immunosuppresion is a theme explored further in this thesis which includes an assessment of the local immune response in cervix in a variety of immunosuppressive conditions. Consideration is also given to the effects of specific HPV subtypes in an attempt to explore the contribution which local immunity plays in the genesis of cervical neoplasia.

The immune system has the capacity to identify and react specifically against foreign antigens, even without having had prior exposure to the antigen and without acting against autologous (self) antigens. The idea that self and foreign antigens would result in tolerance or rejection of transplanted tissue lead to the discovery of a group of antigens in mice which, when matched between donor and recipient, greatly improved the survival of the grafted tissue. These antigens became known as histocompatibility antigens. It was also noted that the gene products of a localised chromosomal area were able to control the process of graft rejection and this area was labelled the Major Histocompatibility Complex (MHC). Although initial experiments were performed using in-bred strains of mice, similar major histocompatibility systems have been shown in all mammals. In man the system was first defined on the human leukocyte system A (HLA). In mice the MHC is present on chromosome 17, where it is

refered to as the H-2 region, but in humans the MHC is located on chromosome 6.

In man the MHC consists of at least four regions named HLA - A,B,C and D. The D region contains several loci DR, DC, DQ and DP. Although first identified by its role in graft rejection the glycoprotein products of the MHC are now known to be involved in many aspects of the regulation of the immune response (Benacerraf 1981). Regions A, B and C (HLA-A,B and C) produce class I major histocompatibility antigens, while region D codes for class II major histocompatibility antigens. Class I antigens are expressed on all nucleated cells while class II antigens are found on certain immunologically competent cells (Roitt, Brostoff & Male 1985).

The immunologically active cells were identified as lymphocytes and shown to differentiate into two groups, T-cells (thymus-derived) and B-cells each with distinct functions. Cell mediated immunity is dictated by T-cells and antibody production from B-cell derivatives, plasma cells.

The complexity of the immune system was emphasised by the demonstration of sub-groups of T-cells with regulatory and effector functions which may be distinguished by cell surface antigens. For instance T4 (CD 4) positive cells have helper - inducer functions in vitro (Thomas et al. 1980; Reinherz et al. 1979, 1981), while in vitro cytotoxic suppressor activity is performed by T8 (CD 8) positive T-cells (Friedman et al. 1981; Reinherz & Schlossman 1981; Thomas et al. 1981). The value of separating the T-cell subsets on the basis of these antigens has been emphasised by Van Waue & Goossens (1981), but the functional separation may not be as exact as once believed (Robins & Baldwin

1985)

The MHC and its class I and II products are the means by which immune recognition between immunologically active cells takes place. In the case of cytotoxic T-cells they recognise foreign antigen such as viral antigens in association with the class I antigens present on the cell surface of the target cell. Helper/inducer cells only recognise foreign antigen in association with class II products. The resultant T-cell function is wholly dependent on this MHC recognition. The importance of this feature will be discussed in relation to the findings in CIN.

In addition to the B-cells and T-cells described above there are other cells with important immunological functions. Effector cells include killer (K) cells and natural-killer (NK) cells and activated macrophages. Macrophages also have the capacity to present antigen to the immune system, a role dependent on the possession of surface class II antigens. These antigens are also found on Langerhans' cells present in squamous epithelium. The main immune response to tumours and to viral infections is thought to be T-cell mediated (Lee & Eisinger 1976; Robins & Baldwin 1985). The evidence suggesting a central role for the Langerhans' cell in the implementation of T-cell immunity will now be discussed following review of the history of the Langerhans' cell and its staining characteristics since these are essential to the work carried out in this thesis.

History of the Langerhans' cell

An epithelial dendritic cell was first described by and named eponymously after Paul Langerhans (Langerhans 1868). He utilised a gold chloride staining method on human skin. This method also

stained neural crest elements and led to the Langerhans' cell initially being labelled as of neuroectodermal origin. Other early opinions considered them to be lymphocytes, worn out melanocytes or artefacts.

The electron microscopic demonstration of a characteristic rod shaped granule in the cytoplasm of these cells (Birbeck, Aodan & Everall 1961) stimulated an upsurge in interest as these dendritic cells of unknown origin and function could now be identified specifically. In the light of electron microscope studies the neuroectodermal and melanocyte theories were challenged when Birbeck granule containing cells were demonstrated outside skin in Histiocytosis X (Bassett & Turiaff 1965).

A possible mesenchymal origin was postulated (Breathnach et al. 1968; Wolff 1972). Experiments to determine such an origin were performed by Katz, Tamaki & Sachs (1979) using skin transplantation and chimera studies in mice. First, parental skin was transplanted to F1 hybrids. By three weeks later the majority of Langerhans' cells in the transplanted skin were shown to be of recipient origin, whereas the keratinocytes were of donor origin. In the second part of the same study irradiated mice, which had subsequently undergone marrow transplantation, were shown to have a Langerhans' cell population derived from the donor marrow. Combining these observations they proposed that the Langerhans' cells were derived from a pool of precursor cells located in the bone marrow and the mesenchymal bone marrow origin of Langerhans' cells was thus accepted.

Identification of Langerhans' cells

Special staining techniques are required to identify Langerhans' cells as they are not recognised in normal haematoxylin and eosin sections. These have been extensively reviewed by Wolff (1972) and Rowden (1981) and are summarised below.

Histochemical markers

1) Gold Methods. A gold chloride method was the first used (Langerhans 1868) and although other gold methods have been utilised none have been widely adopted due to their poor reproducibility.

2) Osmium and osmium-zinc-iodide. These method gives good results in some laboratories. A variation described by Niebauer et al. (1969) showed the Birbeck granule staining positively. Rodriguez & Caorsi (1978) published a method allowing progression from light to electron microscopy. However other studies have indicated that melanocytes and keratinocytes may also be stained with osmium-zinc-iodide (Breathnach & Goodwin 1965).

3) Adenosine Triphosphatase (ATP-ase). ATP-ase is a cell membrane enzyme which is present in Langerhans' cells and allows their demonstration. There are many minor variations of the technique, some apparently being specific for Langerhans' cells while others show ATP-ase actvity in melanocytes and keratinocytes. Wolff & Winklemann (1967) showed that ultrastructurally ATP-ase was present in cells containing Birbeck granules, ATP-ase activity being observed in the cytomembrane and dendrites with none in the cytoplasm or cellular organelles. This study also showed ATP-ase activity was almost totally confined to Langerhans' cells, with virtually none found in melanocytes thus

confirming the apparent 'specificity' of this particular technique. Thomas et al. (1984) showed, by using a double labelling method, that ATP-ase marked the same epidermal cells as a Langerhans' cell specific monoclonal antibody NA 1/34 (see T6 antigen).

One of the disadvantages of using ATP-ase in orthagonally sectioned specimens is that these sections require to be cut several times thicker than routine sections for haematoxylin and eosin staining; this significantly impairs the histological interpretation. Thick sections are required in order to expose more of the cell membrane containing the enzyme to the substrate.

The use of ATP-ase in epidermal sheets does not avoid the problem of histological interpretation, but does permit full visualisation of the Langerhans' cell and its dendrites thus facilitating easy identification and quantification.

The inconsistency of ATP-ase when compared to monoclonal antibodies is a limitation confirmed by Nordlund & Ackles (1981a) but is to some extent offset by the low cost, simplicity and results obtained especially in epithelial sheets.

4) Langerhans' cells contain other enzymes which have been used to demonstrate their presence. These include aminopeptidase, non-specific esterase, alkaline phosphatase, acid phosphatase, peroxidase, cholinesterase and mannosidase. Some, such as acid phosphatase are associated with phagocytic activity but the low levels of such enzymes suggest that Langerhans' cells are poorly phagocytic although metabolically very active. None of these methods have become popular principally because of their lack of specificity.

5) Light microscopic observation of Langerhans' cells is also

possible by using L-dopa or other catecholamines, especially in epithelial sheets. Supravital stains have also been used.

Antigenic markers

In addition to the histochemical markers already described there exists a group of antibodies to antigens present on the Langerhans' cell which can also act as markers for their identification. Also, while there is no known function for cell surface ATP-ase, study of some of the cell surface antigens reveal a little of the likely immunological cellular interactions of the Langerhans' cell.

1) T6 antigen. T6 is an antigen originally detected on immature thymocytes and is found on 70% of T-cells maturing in the thymic cortex. It is a glycoprotein with a molecular weight of 49000 daltons (Van Agthoven & Terhorst 1982). It is not present on circulating T-cells being replaced by antigens associated with the T-cell subsets T4 and T8; other antigens such as T3 (CD 3) and T1 (CD 5) are common to virtually all T-cells.

The same antigen was unexpectedly detected on epidermal cells (Fithian 1981; Fithian et al. 1981; Murphy et al. 1981b). These cells were suprabasal dendritic cells and were thought to be Langerhans' cells. Immuno-transmission electron microscopy confirmed the presence of Birbeck granules in the positively stained cells (Murphy et al. 1981a).

The presence of T-cells in a keratinising environment of ectodermal origin is the current theory behind T6 staining of epithelial but not circulating Langerhans' cells (Patterson & Edelson 1982) although the exact reason for this is unclear.

T6 staining is currently taken as the 'gold standard' for Langerhans'cell enumeration (Harrist et al. 1983; MacKie & Turbitt

1983).

Commercial preparations of anti-T6 antibody are available as OKT 6 (Fithian 1981; Fithian et al. 1981), NA 1/34 (McMichael et al. 1979) and as Leu 6. The preparation used in this study is NA 1/34 (MacKie 1982). Frozen sections are used most widely but epithelial sheets and single cell suspensions may also be stained and immuno-electron microscopy is also possible (Fithian et al. 1981; Murphy et al. 1981a).

2) HLA-DR. Langerhans' cells express class II histocompatibility antigens which are thought to be necessary in generation of the immune response. Ia antigens in mice are the equivalent of the human DR antigens (Rowden, Lewis & Sullivan 1977). As mentioned before (p. 34) MHC class II antigens and their related genes are particularly involved in the interactions between antigen presenting cells and T-lymphocytes (Rosenthal 1978; Braathen & Thorsby 1982). The presence of DR antigens on Langerhans' cells and on macrophages is further evidence that these cells are components of the mononuclear phagocyte system.

Langerhans' cell staining with DR antigens have been shown in-situ and in cell suspensions (Rowden 1980; Tjernlund 1981). Rowden (1977) confirmed the presence of Birbeck granules in positively stained cells.

The problems with anti-DR antibodies in demonstrating Langerhans' cells include the inconvenience of requiring frozen section specimens, neoplastic epithelial cell expression of these antibodies and presence of infiltrating activated lymphocytes with the same antigens (Fu et al. 1978); Ceuppens, Goodwin & Searles 1981). In addition, it has been shown that anti DR antibodies identify consistently fewer Langerhans' cells than other more

specific monoclonal antibodies. Harrist et al. (1983) showed between 18-63% of T6 positive Langerhans' cells were identified by anti-Ia antibodies. MacKie & Turbitt (1983) demonstrated that up to 75% of dendritic cells stained with both anti-DR and anti-T6 antibodies while a further 25% stained with anti-T6 alone. The significance of DR negative/T6 positive and DR positive/T6 positive cells is not known (Murphy et al. 1982). Despite agreement between the above authors Liu, Schroeter & Muller (1986) have claimed that anti-T6 is no better a marker than anti-DR with joint reactivity in 97-100% of dendritic cells in both epithelial sheets and in frozen sections using a double labelling technique.

3) S-100. Staining of epidermal Langerhans' cells and melanocytes with a protein originally identified as a 'brain specific' protein was shown by Cocchia, Michetti & Donato (1981) and Nakajima et al. (1982). It was called S-100 because of its solubility in 100% ammonium sulphate solution at neutral pH (Moore 1965). The protein is present in the central, peripheral and autonomic nervous system mainly in astrocytes, oligodendrocytes, Schwann cells and in some neurones. It has been identifed in tumours of nervous and non-nervous tissue origin (Nakajima et al. 1982a; Nakajima et al. 1985).

The biochemical characteristics of S-100 have been extensively reviewed (Moore 1972 ; Isobe & Okuyama 1978) but as yet no specific function for the protein has been identified. However, it does have similar structure to the calcium binding proteins calmodulin, light chain myosin and troponin C (Isobe & Okuyama 1978) suggesting that conformational changes on binding calcium may be the clue to its function.

Electron microscopic localisation of S-100 staining reveals

that the protein is present in the cytoplasm of the cells and absent from the endoplasmic reticulum, Golgi apparatus, mitochondria and Birbeck granule (Cocchia et al. 1981; Nakajima et al. 1982b). Close relations of the Langerhans' cell ie. interdigitating cells and veiled cells (Derxhage et al. 1979; Thorbecke, Silberberg-Sinakin & Flotte 1980) also exhibit positive staining (Nakajima et al. 1982c) as do the Birbeck granule containing cells of Histiocytosis X (Nakajima et al. 1982c).

S-100 is absent from macrophages and this may indicate some divergence in differentiation between Langerhans' cells and macrophages.

The main advantage of utilising S-100 staining for Langerhans cells is that the protein appears to resist the effects of denaturation by fixatives thus rendering possible Langerhans' cell identification in conventionally fixed and stored tissue which makes retrospective review possible. The disadvantages include the concomitant staining of melanocytes and the great variability in results obtained probably due to variations in fixation medium, time or concentration or temperature of processing. Lastly the numbers of Langerhans' cells identified are substantially less than with anti T-6 or anti HLA-DR antibodies (see chapter 2).

Cytoplasmic and other markers

In addition to the monoclonal antibodies previously described to cell surface antigens, Majdic et al. (1981) showed that antibodies to some B-cell lines reacted with the cytoplasm of Langerhans' cells. Cloning produced a cell line which reacted to antigenic determinants of both Langerhans' cells and B-cells. This anti CY-1 antibody also marks other members of the mononuclear phagocyte system such as the Kupffer cells of the liver and

monocytes of peripheral blood. The antigen is not related to cytoplasmic equivalents of DR antigens nor to S-100 protein.

A further recent addition to the antigenic cytoplasmic markers of Langerhans' cells has been the use of antisera against cellular enzymes eg. beta-glucuronidase (Mackenzie, Bickenbach & Rittman 1982) but whether this proves to have any advantages over the methods previously described remains uncertain.

Mention should be made of other 'immunological' cell surface receptors which may be used as markers of Langerhans' cells. Stingl et al. (1977) demonstrated the presence of receptors for the Fc portion of IgG and for the C3 component of complement on the Langerhans' cell. These observations strengthened the theory of the Langerhans' cell as a member of the mononuclear phagocyte system, but the presence of these receptors does not help in localisation of Langerhans'cells in tissue sections or epithelial sheets. They are however of considerable value in the isolation of Langerhans' cells from epidermal cell suspensions. The Fc and C3 receptors are thus exposed and able to become involved in rosette formation (Berman & Gigli 1980). This rosetting enables the Langerhans' cells to be isolated following density gradient centrifugation. The cells identified by this method are ATP-ase and Ia positive and usually contain Birbeck granules at electron microscopy.

Kashihara et al. (1986) recently described an antibody which specifically stains the Langerhans' cell granule and can be used for light microscopic identification. This antibody is discussed further in the following section.

Electon microscopy (EM)

The Birbeck granule is the definitive marker of the Langerhans' cell. It is only seen at the ultrastructural level. Quantitation studies of Langerhans' cells using electron microscopy are, however, severely limited by the small size of specimen which may be examined.

The major ultrastructural features of the Langerhans' cell were established by Birbeck et al. (1961). They have no desmosomal attachment to surrounding keratinocytes and no tonofilaments, the nucleus is lobulated or indented and the cytoplasm is clear, containing the characteristic organelle. Melanosomes are not found except in association with phagocytic activity and lysosomes are relatively uncommon. The cells rarely appear as dendritic at the ultrastructural level due to the very slim chance of observing the cell in the correct profile. However, the dendrites are commonly observed between the other cells of the epidermis.

Since their original description as disc shaped granules, 0.3 microns in diameter no definite function has been ascribed to Birbeck granules. They are not always granules and often appear as rods with an expanded ends similar to a tennis racket. They tend to be associated with the Golgi apparatus but are seldom uniformly distributed. They have also been observed in the dendrites, nucleus and associated with the cell membrane but their affinity with the Golgi and smooth endoplasmic reticulum has led some to consider them as secretory granules (Niebauer et al. 1969). The similar staining of the Golgi and Birbeck granule with zinc-iodide-osmium has been used to support this idea. However, a more likely explanation is the affinity of the stain for lipid which is present in both organelles. Further support for an origin

from the Golgi apparatus comes from ultrastructural evidence that a Langerhans' cell granule (LAG) antibody stains the Birbeck granule and the associated Golgi apparatus but not the cell membrane (Kashihara et al. (1986).

The associaton of the Birbeck granule with the cell membrane led others to consider a phagocytic role more appropriate (Hashimoto 1971; Takahashi & Hashimoto 1985) but Langerhans' cells are poorly phagocytic. Phagocytosis is performed by small vesicles and not by Birbeck granules.

Electron microscopy has used been used to confirm the specificity of many of the methods described above including iodide-zinc-osmium (Rodriguez & Caorsi 1987), ATP-ase (Hanau et al. 1986) and HLA-DR (Rowden 1977) in addition to S-100 and T-6 already described.

The Langerhans' cell in skin

Potten & Allen (1976) suggested that Langerhans' cells could influence the differentiation and maturation of keratinocytes. However, the only roles supported by experimental evidence involve immunologic functions. Possible secretory and phagocytic functions mentioned above require clarification.

The common origin and relationship of the Langerhans' cell to other cells of the mononuclear phagocyte system, especially macrophages have been discussed. The Langerhans' cell is not, however, a typical macrophage, its phagocytic potential is low, whereas, the antigen presenting capacity appears highly developed.

Initial evidence for a role as an antigen presenting cell came from experiments conducted in contact sensitivity reactions. Shelley & Juhlin (1976) showed in epithelial sheets exposed to

various contact allergens that they were selectively taken up by high level ATP-ase positive dendritic cells. They concluded that Langerhans' cells formed a reticulo-epithelial network or trap for contact allergens.

Silberberg-Sinakin et al. (1976) found, following intradermal challenge with ferritin, that Langerhans' cells were detectable by electron microscopy in the marginal sinus and cortex of draining lymph nodes as early as four hours. They were seen to migrate via afferent lymphatics. The reaction was more marked when previously sensitised with ferritin. The postulate made was that the Langerhans' cells capture antigenic material in skin, circulate to lymph nodes and cause activation of specific T-cells.

A late effect after antigenic challenge was reduction in number and damage to Langerhans' cells with lymphocytes in apposition. From these observations the authors concluded that the Langerhans' cell could also act as the target for the efferent arm of the immune response in cutaneous delayed-type hypersensitivity reactions (Silberberg-Sinakin & Thorbecke 1980).

In earlier experiments the same group found that Langerhans' cells could also act as the target for the immune response in immune complex reactions. The presence of complement caused cell damage, decreased phagocytosis and impaired their passage to lymph nodes. It was concluded that such immune complex reactions could affect subsequent cell mediated reactions to foreign material (Silberberg-Sinakin et al. 1977).

Experiments by Streilein, Toews & Bergstresser (1980) suggested that the actual numbers of Langerhans' cells could influence the resultant immune response. Sensitising the mouse in areas of low Langerhans' cell density produced less than one-third

of the response to re-challenge compared to initial sensitisation in areas with normal numbers. This held true in areas with normally low numbers and in those where the Langerhans' cell population had been artificially depleted by ultraviolet light exposure. Contradictory evidence has been presented by Cooper et al. (1986) who found decreased antigen presentation was a function of ultraviolet light induced Langerhans' cell damage and bore no relation to numbers.

Some investigators have shown that immune tolerance can result if the allergens are presented in areas of low Langerhans' cell density (Toews, Bergstresser & Streilein 1980a). Ptak et al. (1980) noted a marked impairment in response to further exposure to exogenous antigen if the normal process of antigen presentation to helper T-cells was by-passed. They proposed that the resulant tolerance was induced by 'suppressor' T-cells activated by exogenous antigen. Immunity was able to induced if the suppressor cells were inhibited by cyclophosphamide. Rheins & Nordlund (1986) also demonstrated that decreased numbers of Langerhans' cells suppressed both the afferent and efferent response in contact sensitivity reactions while increased numbers were noted to augment both limbs of the immune response.

Stingl et al. (1978) showed Langerhans' cells isolated from epidermal suspensions from mice and guinea-pigs could substitute for macrophages in an in-vitro test of antigen presenting function. Purified T-cells sensitised to simple chemical haptens or to soluble protein antigens were incubated with a variety of primed stimulator cells and T-cell proliferation was assessed after three days. Langerhans' cells, but not other epidermal cells, proved as efficient as macrophages in stimulating T-cell

proliferation. Similar results have been obtained using human epidermal Langerhans' cells (Braathen, Bjercke & Thorsby 1984). It has also been shown that Langerhans' cells are more potent than blood monocytes in inducing some antigen specific T-cell responses including to viral infection (Braathen et al. 1984).

Modification of the antigen is also part of the presentation process and is necessary before the interaction between the antigen presenting cell and responder lymphocytes can take place. The final result is T-cell proliferation. The basis of the interaction is histocompatibility between the Langerhans' cell and the responder group of T-cells in the gene products coded for in the D-region of the the major histocompatbility complex (Unanue 1981; Braathen & Thorsby 1982).

The in-vitro model for this interaction between antigen presenting cells and T-lymphocytes is the mixed leukocyte reaction (MLR). It has been shown that the majority of the responder cells are T-4 positive - mainly helper T-cells (Biddison et al. 1982). The helper T-cells recognise the processed foreign antigen in association with class II antigens. The interaction is abolished when antibodies to the class II antigens and complement are added to the MLR (Stingl et al. 1978). Aberer et al. (1986), however, suggest that in-vivo effects of anti-Ia on Langerhans' cell function are less than on other antigen presenting cells.

The data presented above suggests that the Langerhans' cell is ideally placed and able to act as the most peripheral lookout in the the afferent limb of the local immune system. If the conclusions of Pehamberger et al. (1983) are confirmed it may be that they are also necessary for full development of the efferent limb - cytotoxic T-cells.

The relationship between Langerhans' cells and skin cancer is complex. Ultraviolet radiation is strongly linked with development of malignant proliferation of melanocytes and keratinocytes (Kripke & Fisher 1976). It also produces various effects on Langerhans' cells depending on the wavelength of incident light (Aberer et al. 1981; Nordlund & Ackles 1981b). Kripke & Fisher (1976) suggested that mice irradiated with ultraviolet light were unable to reject strongly antigenic tumours which were easily rejected by non-irradiated recipients. This may be secondary to a reduction in Langerhans' cell numbers or to impaired function (Sauder et al. 1983) allowing neoantigens to escape immunosurveillance. Suppressive mechanisms may ultimately be induced following this, similar to the situation occuring in contact sensitivity reactions. Thus it may be possible that cells transformed by an oncogenic stimulus may escape detection and subsequently proliferate undetected by the immune system if the change is occurring in an area deficient in Langerhans' cells. These changes could explain the link between exposure to ultraviolet light and the malignant transformation of skin lesions containing HPV 5 mentioned earlier (p. 32).

Rowden (1981) pointed out that in guinea-pigs experimentally infected with cytomegalovirus the viral particles are almost exclusively taken up by Langerhans' cells. It has been postulated that viral infection of Langerhans' cells may alter their function. MacKie (1981) suggested that persistent infection of Langerhans' cells could impair their interaction with helper T-cells allowing the helper T-cells to accumulate in skin in mycosis fungoides.

A possible relationship exists between Langerhans' cells in

skin, human papillomavirus (HPV) and squamous cell neoplasia. In epidermodysplasia verruciformis the possible role of impaired T-cell immunity and ultraviolet light exposure have been discussed (p. 32). However, Haftek et al. (1987) have also shown that the Langerhans' cells are reduced in number in the flat wart lesions compared with non-involved skin. Since these lesions are at high risk of malignant transformation especially in the presence of HPV 5 it is possible that this effect is promoted by impaired local immunosurveillance. Studies on the human cervix will be discussed later but they suggest that Langerhans' cells are decreased in association with HPV infection.

In summary, evidence from skin research shows the Langerhans' cell has antigen presenting properties in the afferent immune response which are dependent on helper T-cell recognition of genetically defined surface determinants. Their circulation locally enables them to intercept antigens at the most peripheral outpost probably engaging in similar cell interactions to the well documented effects following contact hypersensitivity reactions. Escape from the normal immune surveillance may be one trigger to neo-antigen and tumour tolerance in the host.

The Langerhans' cell is assumed to have a similar role in the cervix as has been documented in skin (Toews, Bergstresser & Streilein 1980b). In the context of this thesis it is their immunosurveillance role in relation the effects of virally transformed and neoplastic cells that is investigated further.

Other interactions and cells involved in local immunity

Before discussing the Langerhans' cell in the cervix it is necessary to consider other cells which may be implicated in local

immunosurveillance. Streilein (1978) proposed the concept of skin associated lymphoid tissue (SALT) that involved Langerhans' cells, keratinocytes and T-cells which specifically recirculate through skin and draining peripheral lymph nodes. However, evidence suggesting such a specific T-cell recirculation is, to a large extent, circumstantial. The presence of a malignant epithelial T-cell infiltrate in the Sezary syndrome (Patterson & Edelson 1982) and the discovery of a THY-1 antigen bearing bone marrow derived epidermal cell in murine epithelium have been used as supportive evidence.

Recently, more interest has concentrated on the role of the keratinocyte than on the epidermotrophic T-cell. Antigen presentation to specific T-cells requires initial processing and presentation in association with histocompatible class II antigens. Following this initial interaction soluble factors are released which influence the subsequent response. Interleukin 1 (IL-1) is produced by the Langerhans' cells (Sauder, Dinarello & Morhenn 1984) and interleukin 2 (IL-2) by the helper T-cells. Epidermal T-cell activating factor (ETAF) is a product of keratinocytes (Luger et al. 1981,1983) and is indistinguishable from IL-1, a powerful chemotactic agent for mononuclear, polymorphonuclear cells and T-cells (Luger et al. 1983). It promotes T-cell proliferation and differentiation by stimulating the helper T-cell production of interleukin 2 (T-cell growth factor) and/or increasing the expression of receptors for IL-2. The recruited T-cells may then help other T-cells to become cytotoxic T-cells. Following initiation it is not clear whether subsequent events occur in the epithelium or in the regional lymph nodes. However, T-cell division takes place in the draining lymph

node and effector cytotoxic T-cells exit the lymph node into the blood stream. They are principally active through recognition of MHC class I antigens (HLA A,B,C) in association with the allogenic stimulus.

Keratinocytes have also been shown to secrete other soluble factors including interleukin 3 (Luger et al. 1985) which promotes growth of bone marrow derived cells.

MHC class II antigen expression on keratinocytes has also been reported in various pathologic conditions involving a T-cell response, eg graft versus host disease, allograft rejection and allergic contact dermatitis. In-vitro experiments have shown that gamma interferon (Nickoloff et al. 1986), a product from activated T-cells, can induce synthesis by keratinocytes of class II antigens. The function of these class II antigens is unknown but it has been postulated that they may be the stimulus to Langerhans' cell migration into the epithelium (Daynes et al. 1985). The cytotoxic T-cell response to allogenic targets is greatly enhanced when a class II stimulus is also provided (Sollinger & Bach 1976), so the presence of class II antigens on keratinocytes may be acting as both stimulators and as targets of the immune response.

The work described in this thesis concentrates on the cervical Langerhans' cells as they appear to have a central role in the local immune response but, given the above discussion, it is likely that the epithelial cells have more than just a passive role in the process and their possible contribution especially the MHC class II positive cells, will be considered together with the local lymphocytic infiltrate.

Langerhans' cells in cervix

Zwillenberg (1958) was the first to describe Langerhans' cells in the cervix. He noted the presence of dendritic epithelial cells in separated sheets of cervical (and vaginal) epithelium and reported their presence in both normal and dysplastic cervical tissue.

Younes, Robertson & Bencosme (1968) noted cells with the ultrastructural features of Langerhans' cells in normal cervix. Increased numbers were found in carcinoma-in-situ and were postulated to be secondary to 'abnormal epithelial turnover'. They made no mention of any apparent change in Langerhans' cell morphology in the dysplastic cervix. This publication was followed by another (Younes 1969) repeating these observations. The author suggested that exfoliated Langerhans' cells could result in confusing cytology, but, no confirmatory evidence has since been presented.

Hackemann, Grubb & Hill (1968) compared the ultrastructural detail of normal skin with normal cervix and found that, apart from lack of keratin and absence of melanocytes, both were essentially similar. Once again the presence of Langerhans' cells containing Birbeck granules was confirmed in the cervix. The authors incorrectly believed they were the first to record these features.

Figueroa & Caorsi (1980) were the first to provide any detail on the distribution and morphology of Langerhans' cells in the cervix. In their initial paper they utilised modifications of a method described by Rodriguez & Caorsi (1978) which allowed identification of Langerhans' cells using a zinc-iodide-osmium technique. The method they used stained both the Langerhans' cell

membrane and Birbeck granules when examined ultrastructurally. Light microscopy was used for quantitative and morphologic assessment. They reported their findings from only 3 cervices. The mean number of Langerhans' cells detected in epithelial sheets by this method was 8.34 (SE 0.92) per square millimetre. They reported that distribution was variable with an apparent increase in the vicinity of the external os. The gross morphology of the Langerhans' cells was classified according to the number of dendrites they possessed and the authors postulated that increased surface area in association with increased dendrites represented increased antigen presenting capacity. This theory has not been substantiated.

Armed with their previous observations of the Langerhans' cell in normal cervix they proceeded to investigate their presence in CIN overlying early invasive carcinoma using the techniques they had established (Caorsi & Figueroa 1984). Three cases were examined, with apparently minimal amounts of tissue being submitted for routine histological diagnosis. Quantitative analysis was again performed on epithelial sheets. The average Langerhans' cell density was 30.07 cells/mm^2 (SE 3.62), an increase of more than three fold compared with their previous study. Once again they found highest numbers of Langerhans' cells close to the external os and commented that the degree of intraepithelial neoplasia followed a similar trend, being most severe near the external os and diminishing gradually in severity towards the ectocervix. The more branched variety of Langerhans' cells predominated in the epithelial sheets examined. Electron microscopy confirmed Langerhans'cell migration into the underlying stroma. Hypertrophy of the rough and smooth endoplasmic reticulum

suggested an increase in their metabolic activity. Characteristic surface contact was seen between Langerhans' cells and lymphocytes analogous to the situation in skin. They concluded that Langerhans' cells increased according to the degree of CIN and suggested that this was an immune response to the neoplastic process.

They supported their conclusions in their most recent publication (Caorsi & Figueroa 1986) in which four further cases, with neoplastic epithelium overlying early invasive carcinoma, were examined. Strong correlation was again noted between the degree of CIN and an increase in Langerhans' cells numbers. There was no mention of any histological evidence of wart virus infection in any of the cases presented. The authors proposed three possible mechanisms for the increased numbers of Langerhans' cells found, namely increased bone marrow migration, intraepithelial division or retention in the neoplastic epithelium.

Increased numbers of Langerhans' cells were also observed in CIN by MacLean (1984b) before treatment with laser. His studies concentrated predominantly on the epithelial changes occurring in the post laser cervix. The earliest Langerhans cells were observed 14 days after treatment. These reports were made from sections stained for ATP-ase activity. Morris et al. (1983a) made similar observations regarding maturing metaplastic cervical epithelium stained with an anti T-6 monoclonal antibody (NA1/34).

In addition to the above findings, Morris et al. (1983a) reported that Langerhans' cells were a consistent component of cervical epithelium in subjects ranging from 30 week fetuses to 62 year old adults. They reported little difference between

ectocervix and the transformation zone with the Langerhans' cell density in post-natal life ranging between 74 and 145 cells/mm^2.

Bjercke et al. (1983) also noted similar numbers of Langerhans' cells in pre and post menopausal women, with counts between 1% and 3% of cervical epithelium stained with anti-HLA DR antigens, but they did not directly compare ectocervix with transformation zone.

It is of note that only one 'normal' adult premenopausal patient is included in the study by Morris et al. (1983a). A single pregnant patient also had Langerhans' cell numbers within the range of values obtained in non-pregnant cervical tissue. No statistics from the results were presented.

Morris et al, (1983a) also examined the lymphocytic infiltrate in the cervix with special regard to the type and distribution of lymphocytes in the epithelium and stroma. The vast majority of epithelial lymphocytes in these normal cervices were of the cytotoxic/suppressor type, (T8 positive), whereas in the stroma, helper T-cells predominated. Contacts between the Langerhans' cells and lymphocytes, Langerhans' cells and capillary endothelium and between Langerhans' cells and stromal 'hugging cells' suggested to the authors a central role in the processing and presentation of exogenous antigen to stromal lymphocytes or other immunocompetent cells. B-cells were noted in the stroma occasionally but were absent from the epithelium.

Studies followed on wart virus infection and intraepithelial neoplasia (Morris et al. 1983b). The findings in four patients with 'wart only' features on histology were described. The Langerhans' cells in these were consistently reduced, restricted to the basal zone and had stunted dendrites. The lymphocytic

infiltrate was also markedly reduced in the epithelium corresponding to areas of reduced Langerhans' cell activity. The cytotoxic/suppressor sub-group of lymphocytes predominated in the epithelium. Another finding was patchy epithelial cell membrane labelling to the mid-zones with anti-HLA class II antibodies.

Three patients were studied with CIN III only. The Langerhans' cells were increased and again showed altered dendrite morphology. Class II antigen staining of epithelial cells showed marked perinuclear and cytoplasmic staining extending diffusely throughout the epithelium. In the lymphocytic infiltrate, cytotoxic/suppressor cells predominated with helper cells apparently reduced. The infiltrate appeared increased and was confined to the area of CIN. B-cells were again an uncommon finding. Five patients in the second study (Morris et al. 1983b) had biopsies taken from epithelium adjacent to lesions and in the non-affected epithelium the Langerhans' cell distribution was normal.

The authors speculated that wart virus could be exerting a cytopathic effect on the Langerhans' cells or that it could be causing increased migration from infected epithelium. They postulated that depletion could allow persistence of wart virus infection and possibly promote malignant transformation while the increased numbers in CIN were interpreted as a specific immune response recruting T-cells to the lesion. Once again no statistical analysis was presented from the very small numbers of cases studied.

The finding of class II epithelial cell staining was confirmed in another study by the same group (Morris et al. 1983c) in wart virus infection, CIN, combined HPV / CIN and in carcinoma.

Edwards & Morris (1985) used the same monoclonal antibody (NA 1/34) as used in the studies described here to demonstrate Langerhans' cells. They examined the distribution of Langerhans' cells in cervix, vagina and vulva. Fifteen biopsies were studied from each area. The counts were taken per 100 basal cells and statistical analysis performed by Kruskal-Wallace one way analysis of variance. In the cervix, Langerhans' cells were equally distributed between ectocervix and transformation zone. The count ranged from 4.7 to 22.7 with a median of 12.9 per 100 basal cells. This was significantly greater than counts from vagina (median 5.5) and less than counts from vulva (median 18.7).

Langerhans' cells were observed in proximity to vascular endothelium which also expressed class II antigens. The possibility that antigen primed endothelium could act as the recognition site for returning stimulated lymphocytes was raised.

Examination of the lymphocyte subsets in cervical epithelium showed that cytotoxic/suppressor T-cells exceeded helper T-cells by 8-9:1 in both ectocervix and transformation zone. The stroma of the two areas differed markedly however with the infiltrate being markedly reduced in the ectocervix. This difference between ectocervix and transformation zone prompted Edwards and Morris to propose that the transformation zone was an area of 'enhanced immunological activity' similar to that previously proposed for skin (Streilein 1978). They proposed that 'cervical lymphoid tissue' (CLT) should include the Langerhans' cells, lymphocytes and metaplastic epithelial cells of the transformation zone. They thought the increased lymphocyte population of the transformation zone could be due to more active Langerhans' cells in the transformation zone recruiting lymphocytes or, more likely, that

the metaplasic epithelial cells themselves were contributing to the lymphocyte recruitment and differentiation by production of soluble factors as had previously been shown in skin. This has been discussed more fully in an earlier section (p. 52).

Di Girolamo, Goni & Laguens (1985) compared the Langerhans' cell distribution in the ectocervix of 4 normal patients with biopsies of colposcopically abnormal epithelium in 9 patients which showed immature and mature metaplastic epithelium on histology. They found similar numbers in the mature transformation zone and ectocervix but found no Langerhans' cells in incomplete metaplastic epithelium. This is in contrast to findings by MacLean (1984a) and Morris et al. (1983a) who both noted increased numbers in maturing cervical epithelium.

Vayrynen et al. (1984) studied Langerhans' cells in combined HPV/CIN lesions followed for an average interval of 16 months. They used a monoclonal anti-T6 antibody to identify them in the inflammatory infiltrate of the lesions. No relation was noted between the number of Langerhans' cells and the infiltrate, between the grade of CIN and Langerhans' cells, or between the type of HPV lesion and Langerhans' cells. No relationship was shown between the number of Langerhans' cells in HPV lesions which persisted, regressed or progressed. No age dependency was demonstrated. The authors highlighted the fact that no internal control biopsies were examined. When they examined specimens obtained from lesions that regressed spontaneously they found that the now normal epithelium contained lesser numbers of Langerhans' cells than previously. This study quantified the T-6 population as a proportion of the total inflammatory infiltrate but made no reference to the actual number of epithelial Langerhans' cells.

They concluded that other factors were likely to be implicated and emphasised the role of the Langerhans' cell as only a part of the local immune system in the cervix.

The only other study presenting information on Langerhans' cells in the cervix which was published before commencing the studies reported in this thesis was a comparative study between the immunocompetent cells in oral mucosa, normal cervix and oral papillomas and leukoplakias (Becker et al. 1985). Seven biopsies of cervix were examined. The mean count from 10 randomly selected high power fields of epithelium was used in the analysis. Fewer Langerhans' cells were noted in cervix compared with oral mucosa. However, epithelial cytotoxic/suppressor T-cells were increased. It is not clear whether transformation zone or ectocervix or both were included.

Between the start of this project in August 1986, and the end of 1987 there have been a further four publications on Langerhans' cells and immunocompetent cells in the cervix. M^{C}Ardle & Muller (1986) examined their numbers in CIN and HPV infection using S-100 polyclonal antibody on 79 biopsies stored in paraffin blocks for up to 6 years. Forty-six biopsies contained normal ectocervix (20 were adjacent to abnormal epithelium). No specimens were examined from normal patients, the 'normals' used for comparison being taken from cone biopsies in which ectocervix was fortuitously present. Wart virus alone was found in 14, CIN alone in 21 (14 being CIN III) and combined CIN/HPV in 20 specimens. Langerhans' cell counts were made per square millimetre of epithelium using a projected imaging technique. Statistical analysis between groups was performed by multiple Wilcoxon rank sum tests.

Results from this analysis again showed no difference between Langerhans' cell numbers in ectocervix and transformation zone. The Langerhans' cell population was significantly reduced, ($p=0.01$), in biopsies showing histologic evidence of wart virus infection only, when compared to the biopsies showing normal epithelium. In lesions showing CIN only the numbers were significantly increased ($p=0.01$) and showed the same significance when CIN III lesions were examined. No results were presented comparing normal epithelium with combined CIN and HPV lesions, though inspection of the results suggests that there was no difference (mean densities /mm^2 12.6 and 12.5 for normal ectocervix and lesional epithelium respectively). The authors do not appear to have included any positive or negative control sections in their study and in addition make no comment on the duration of storage affecting antigen positivity. They do not mention the possibility of other epithelial cells staining with S-100 (Takahashi et al. 1985). Perhaps some evidence of the disadvantages of the methodology used is gained by examining the variation in Langerhans' cell numbers within separate groups. The range is up to 100 fold in both the normal and CIN group with the other groups showing at least 10 fold variations. Some of these criticisms are the subject of a letter to the editors of the journal (Hawthorn & MacLean 1987a).

The other three recent papers concerning Langerhans' cells and the local immune response in the cervix come from Singer and his group at the Whittington Hospital, London. In the first paper Tay et al. (1987a) compared the Langerhans' cell populations using T-6, ATP-ase, S-100 and class II antigens in six normal cervices, five with HPV alone and 14 with CIN (all of whom had concomitant

evidence of HPV infection). T-6 antigen and ATP-ase showed the greatest numbers of Langerhans' cells, class II antigens demonstrated 60% of these and S-100 only 35% in the normal cervix. Analysis, using multiple Wilcoxon rank sum tests, comparing the normal with the two abnormal histological types showed that Langerhans' cells were significantly reduced by all methods in HPV infection alone ($p<0.05$). Similar results were obtained for the combined HPV/CIN group. The depletion was most marked in both groups for Langerhans' cells stained with T-6 and S-100.

In a separate study of S-100 positive Langerhans' cells reported in the same paper the numbers in a group of 20 normal patients were compared with 30 patients who had combined CIN/HPV lesions; 63.3% of the abnormal biopsies showed no Langerhans' cells. The was no relation between the numbers of Langerhans' cells and the degree of CIN. The numbers of Langerhans' cells in any normal adjacent epithelium were stated to be normal but these were not directly compared with the numbers in the lesions. The authors noted that the distribution of S-100 positive cells was not uniform, as they appeared with other methods, but were predominantly located around capillaries and associated with lymphocytic infiltrates. This uneven distribution was reflected in poor correlation between S-100 positive Langerhans' cells per unit area and per unit length. The first study illustrated a good correlation in the numbers, identified by T-6 antigen, ATP-ase and class II antigens, between the measurements per unit length and per unit area of epithelium.

From these results the authors concluded that the S-100 population of Langerhans' cells might have a functional significance. Once again the population stained by S-100 showed

the greatest variation in range (8 to 10 fold in normal cervix) which questions the validity of using S-100 to quantify Langerhans' cells in the cervix.

In their second paper (Tay et al. 1987b), the lymphocyte subsets in five normal patients, five with HPV alone and ten with HPV and CIN were examined. The lymphocytic infiltrate was located predominantly in the upper stroma and lower quarter of the epithelium. In the normal epithelium cytotoxic / suppressor cells exceded the helper T-cells but in the stroma the converse was true. In the abnormal epithelium there was a marked reduction in both T-cell subset numbers. The reduction was reported in the same areas as decreased Langerhans' cells and it was considered that the reduction in helper cells was of the magnitude to effectively reverse the mean ratio of helper cells to cytotoxic cells. Activated T-cells were not found and no significant reduction was reported in the stromal infiltrate. This group agreed with the earlier findings of Morris et al. (1983b) that B-cells were uncommon in the normal and abnormal cervix. They reported a larger reduction in the helper subgroup and they speculated this may be related to the reduced Langerhans' cells creating a localised area of intraepithelial immune deficiency.

Tay, Jenkins & Singer (1987) also presented data relating to the presence of natural killer (NK) cells, identified by Leu 7 and Leu 11, in CIN and papillomavirus infection. The numbers were very small with five normals, six HPV infection only and 12 with HPV/CIN. Analysis was performed by subjective semi-quantitative methods. Natural killer cells were rarely present in normal cervix. In the abnormal cervix only one of the 12 patients with CIN had a marked infiltrate of NK cells, with two of the six

patients with HPV infection alone showing a marked infiltrate. It is difficult to comment regarding the role of NK cells in such lesions from such a small, semi-quantitative study with no data analysis.

In a preliminary study Tay et al. (1987c) also examined the macrophage infiltrate in similar lesions, though again in only a small number of specimens. Decreasing stromal and epithelial macrophages were found from HPV infection alone through CIN I to CIN III. The signifcance of these observations is unclear.

In addition to the papers discussed above the inflammatory infiltrate in the cervix had been the subject of other studies. However, in most no attempt was made to distinguish between the epithelial and the stromal infiltrate. In Finland, Syrjanen (1983) used acid alpha-napthyl acetate esterase (ANAE) to distinguish between B-cells, T-cells and mononuclear phagocytes on morphological staining characteristics. This method lacks the specificity of monoclonal antibodies, but differences were shown between CIN with and without the presence of HPV, as identified by HPV antigens. The B-cells declined in relation to increasing degrees of CIN in the lesions without HPV. T-cells and macrophages increased in accordance with the degree of CIN in the HPV positive lesions. Syrjanen suggested that the increase in the T-cells could be due to the presence of increased numbers of cytotoxic T-cells and that the increased inflammatory infiltrate in the HPV lesions may indicate that concomitant HPV infection renders the intraepithelial change more antigenic.

In a subsequent paper Syrjanen et al. (1984) used a similar method to identify the three groups of immunocompetent cells but added monoclonal antibodies to identify the helper and cytotoxic/

suppressor T-cell subsets. They examined patients with HPV or HPV/CIN lesions in 65 women followed for over 12 months. The percentages of T cells, B cells and macrophages were similar in both groups. There was a significant reduction ($p<0.025$) in the ratio of helper T cells to cytotoxic/suppressor cells between the HPV/CIN (1.44) lesions and the pure viral lesions (0.81) which was due to a higher percentage of cyotoxic/suppressor cells in the HPV only lesions. After the period of follow-up, the infiltrates in the lesions that had progressed, regressed or persisted were compared. No significant cell type differences were found in any of the three groups of HPV lesions. The ratio of helper to cytotoxic cells was least in the lesions that progressed but not significantly less than in the other two groups. Only three lesions progressed. The percentage of helper cells was similar in all three groups, the lesser ratio in the progressive lesions being due to increased cytotoxic/suppressor cells. Again Syrjanen et al. speculate on the role of suppressor cells acknowledging that the currently available monoclonal antibodies are unable to separate suppressor cells from those with effector functions. More recently Syrjanen et al. (1985) reported an inverse relationship between the infiltrate and progression of HPV associated lesions but no major differences in the infiltrate were seen comparing the HPV/CIN lesions with those showing CIN only.

This group have also examined prospectively the possible contribution of natural killer cells with Leu 7 phenotype in a study with mean follow up of 16 months in virus only and CIN/HPV lesions (Syrjanen et al. 1986). They concluded that there was no relation between the degree of CIN and NK cells in the associated infiltrate and no relation between NK cells in the lesions that

progressed, regressed or remained static.

In these last four papers the authors made no attempt to distinguish the intraepithelial from the stromal infiltrate. The prospective studies too are difficult to interpret in view of the effects of biopsy on the natural history of HPV and intra-epithelial neoplasia.

Summary and interpretation of current literature (Table 1 p. 68)

It appears that Langerhans' cells are a consistent component of the cervical epithelium as identified by light microscopy using various staining techniques, and by transmission electron microscopy. Similar significance has been attached to their presence in cervix as in skin, acting as the peripheral outpost of the immune surveillance system. The Langerhans' cell population appears equally distributed in the ectocervix and normal transformation zone when different methods for their identification are used but quantitative data comparing the Langerhans' cells of normal ectocervix and normal transformation zone from the same patient has not been presented.

In the small number of patients so far examined, Langerhans' cells are reduced where there is histological evidence of HPV infection alone, however, the reduction has not been quantified in all studies.

Where there are no histological features of HPV associated with CIN the Langerhans' cell population appears to be increased. Quantification of the increase has only been attempted in three studies which include a total of 28 cases. Included in this number are seven patients whose lesions were associated with underlying invasive carcinoma.

In combined HPV/CIN lesions reduced or virtually absent S-100 positive Langerhans' cells are reported. Normal controls are absent in one of the two studies which together total 60 patients.

Where comparative analysis has been performed all investigators have used a separate normal control group. None have attempted to use an internal control such as the ectocervix for comparison with the lesional number of Langerhans' cells. An internal control would appear to be desirable since the variation in Langerhans' cells even in the normal population can be as much as 100 fold depending on the method used. Internal control biopsies also eliminate the possible effects cofactors in cervical neoplasia such as smoking, exposure to semen and contraceptive habits.

Available evidence suggests T-cell mechanisms predominate in the cervix and B lymphocytes appear to have little importance in the normal or abnormal cervix. The predominant lymphocyte subtype in cervical epithelium is the cytotoxic / suppressor group of T-cells. There is disagreement on the proportions of helper and cytotoxic / suppressor T cells in the epithelium but quantification studies are limited. It is suggested that HPV infection with or without CIN is associated with decreased intraepithelial lymphocytes, especially the T-helper subgroup. A reduction in epithelial lymphocytes is suggested in HPV infection. and an increased lymphocytic infiltrate in pure CIN. The prognostic significance of the lymphocytic infiltrate has not been established but prospective studies may add little further information since it may be argued that the biopsy required for immunohistochemical studies is likely to influence the natural history of CIN by itself.

Table 1. Langerhans' cells in cervical epithelium - summary

Author	Normal Cx Tz	Normal Cx NS	Normal Cx Ecto	Abnormal Cx V	Abnormal Cx CIN	Abnormal Cx CIN+V	Comments
Figueroa (1980)			3				Epithelial sheets Tz not mentioned increased Lc's near ext os
Caorsi (1984)					3		Increase X3 vs normals overlying Ca Cx
Caorsi (1986)			2		4		patients with Ca Cx increase Lc's with grade of CIN
MacLean (1984b)					Yes		increased no quantation
Morris (1983a)			(10)				2 normal pre & post menopausal patients Tz = ectocervix
Bjercke (1983)			19				1-3% of epith cells Class II
Morris (1983b)			5				adjacent to abnormal
				4			reduced
					3		increased Class II epith + ve
							no quantiation
Morris (1983c)			4				Class II pattern examined-no Lc's
				4			quantitation
					6		
						3	Class II epith +ve
Edwards (1985)	15						Tz = ectocervix
Di Girolamo (1985)			4				ATP-ase
				(9 patients)			colposcopically abnormal epith- mature TZ = ectocx
McArdle (1986)	46		13				no 'normal' patients Tz = ectocx
							S100-100 fold range
				14			decreased vs ectocx
					21		increased vs ectocx
						20	similar to ectocx
Tay Study 1 (1987a)			20				S100-10 fold range
						30	absent Lc's in 63.3% normal Lc's adjacent to lesions
Study 2			6				S100/T6/Class II/ATP Class II-60% T6/ATP S100-35% T6/ATP
				5			decreased vs normals
						14	decreased vs normals Lc's no relation to grade of CIN

Key: Tz=transformation zone NS=origin not stated Ecto=ectocervix
V=virus only CIN=CIN only CIN+V=combined CIN & virus
ALL NUMBERS REPRESENT NUMBER OF PATIENTS/BIOPSIES STUDIED

Aims of this thesis

1) To elaborate on the distribution of Langerhans' cells in the normal cervix by different staining techniques. To assess any differences between techniques and utilise further the method giving the most reliable and consistent results.

2) To examine the distribution of Langerhans' cells in CIN alone and in CIN with associated histological features of virus infection in sufficient numbers of patients to allow meaningful statistical analysis and interpretation of the results. To determine if the distribution varies in the typical and atypical transformation zone, and to compare the atypical transformation zone with internal control biopsies of adjacent normal ectocervix.

3) To compare the distribution of Langerhans' cells in CIN, where the presence or absence of wart virus infection has been proven by the sensitive and specific method of DNA DNA hybridisation, with internal control biopsies to examine any effects of specific HPV subtypes.

4) To assess whether or not immunosuppression alters the Langerhans' cell numbers by examining patients compromised naturally in pregnancy and by systemic immunosuppressants following renal transplantation. In addition, to examine the Langerhans' cells in a group of patients prior to possible renal transplantation, on peritoneal, haemodialysis and in chronic renal failure and to screen colposcopically and cytologically the immunocompromised female transplant recipient

and her pre-transplant counterpart for evidence of CIN and wart virus infection.

5) To examine the Langerhans' cell distribution in early invasive squamous cancer of cervix (stages Ia, Ib & IIa).

6) To examine other aspects of the local immune response in association with Langerhans' cell numbers.

7) Using these observations to examine further the role of the Langerhans' cell in the cervix with special regard to its role in cervical neoplasia.

Chapter 2

Techniques evaluated to demonstrate and count Langerhans' cells

Chapter 2

Introduction

Prior to commencing this study, published work on Langerhans' cells in skin and cervix suggested that the most suitable methods for their demonstation required frozen sections for staining with a monoclonal antibody to T-6 antigen or ATP-ase. Epithelial sheets had been used with a zinc-iodide-osmium method. S-100 had been used on routinely processed skin.

Normal cervix was examined to detect Langerhans' cells using the methods described in this chapter, the details of which are described in Appendix I(p. 243-250). The recruitment of normal patients is described in chapter 3. Initial experiments were designed with the following aims:

1) to find the most appropriate method of preparation of cervix for cutting frozen sections
2) to compare the various methods for Langerhans' cell identification taking into account the effects of storage of tissue, and to evaluate different methods of enumeration
3) to establish the most appropriate methods for examining the Langerhans' cell population in the various study groups to be examined.

Tissue for frozen section staining

i) Collection and freezing of tissue

Following removal of the cervix from the fresh hysterectomy specimen, 5-10 millimetre blocks of cervix were cut from the endocervical canal through the transformation zone to the

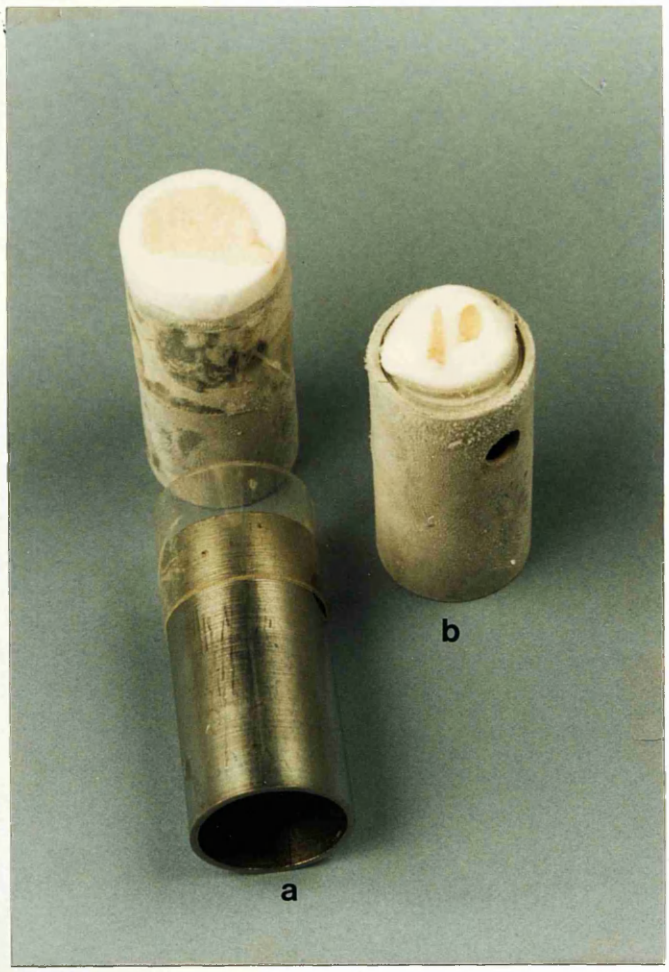

Figure 1.

Sellotape modified microtome chuck (a) with hysterectomy specimen embedded in OCT above; paired cervical biopsies embedded in OCT b).

ectocervix. This was most easily performed on the 'opened' cervix.

Initial specimens were transported to the laboratory for freezing but best results were obtained with snap freezing in the operating theatre followed by transportation to the laboratory in a Dewar Flask. Three methods of freezing were evaluated:

a) specimen placed on microtome chuck in a bath of CO_2 ice and acetone, and embedded in ice

b) specimen embedded in Tissue-Tek II OCT Compound (Lab Tek Products) and snap frozen onto a microtome chuck in liquid nitrogen vapour

c) specimen embedded in OCT and snap frozen onto a microtome chuck held between blocks of CO_2 ice.

The last method produced the most consistent results. This was confirmed for the smaller punch biopsies. The time taken for the OCT to freeze allowed optimal orientation of the tissue and removal of obscuring blood. A similar method has been employed and reported by Fletcher, Smart & Livingstone (1985) in optimally freezing punch biopsies of cervix for rapid diagnosis in a colposcopy clinic. A modification of the microtome chucks with Sellotape overcame the problem of embedding the larger blocks from hysterectomy specimens in OCT (**Figure 1**). 'Small' specimens such as punch biopsies were of dimensions up to 8X8X3 millimeters and large specimens obtained from hysterectomy specimens up to 20X20X8 millimeters.

Freezing in liquid nitrogen gave similarly good results with larger specimens. Cutting of these deeply frozen blocks was, however, often difficult due to chattering of the block on the microtome knife, a problem less frequently encountered following

the slower, less profound freezing with CO_2 ice. Re-orientation of specimens in liquid nitrogen vapour is virtually impossible when compared with CO_2 ice. CO_2 ice is also more easily transported and handled than liquid nitrogen.

Freezing in OCT proved more satisfactory than freezing in distilled water for several reasons. Water produced more ice artefact, distorting and ballooning cells. It did not allow re-orientation of specimens due to rapid freezing and did not permit easy flattening of thin cryostat sections as was the case with OCT. Additionally the rapid freezing of the water made complete embedding of the larger specimens obtained from hysterectomy specimens very difficult.

ii) Cutting frozen sections

All cryostat sections were cut on a Bright 5030 microtome.

Trial and error revealed the optimal temperature for cutting to be $-20^{o}C$.

The thickness of section was varied according to the method of staining used. Seven micron sections were optimal for monoclonal antibody techniques but thicker sections, 20 microns, were required for ATP-ase methods. Initial experimentation with the thickness of sections revealed that thin sections gave the best cellular detail. Seven micron sections represent a compromise between thinner sections which are technically very difficult to cut and thicker sections in which some cellular detail is obscured. Twenty micron sections were used for ATP-ase methods as thin sections did not give such good Langerhans' cell definition due to their lesser content of cell membrane.

The quality of sections obtained improved with the operator's experience. It was also dependent on the quality of microtome

blade available. Initial sections were cut on 'old' blades but it rapidly became evident that this was unsuitable. The availability of disposable blades led to a marked improvement in quality. Larger blocks had to cut on reground solid blades as they scored and distorted the thinner disposable blades. Cutting of larger blocks was further improved by removing excess fibrous stroma from the specimens.

The best sections were obtained when the specimens were cut with the cervical epithelium at right angles to the knife. This produced less traumatic artefact to the epithelium and gave a larger area for staining and examination.

Up to 20 serial sections were cut from each block at any particular level. They were collected on gelatinised slides to aid adhesion and prevent 'floating' during staining. The slides were numbered appropriately. These were allowed to air dry and stored at -20^{o}C until use. A minimum of three sections at each level were stained with haematoxylin and eosin to allow histological diagnosis and comparison of quality with the routinely processed specimens.

Methods employed for demonstration of Langerhans' cells

1) Indirect immunoperoxidase method (using monoclonal antibody to anti-Human Thymocyte Antigen (NA 1/34), Sera Lab - modified from MacKie (1982); see Appendix Ia (p. 243) for full description of method).

MacKie (1982) described a method for immunoperoxidase staining of frozen sections of skin which, with the following minor modifications, produced consistent results on frozen sections of cervix.

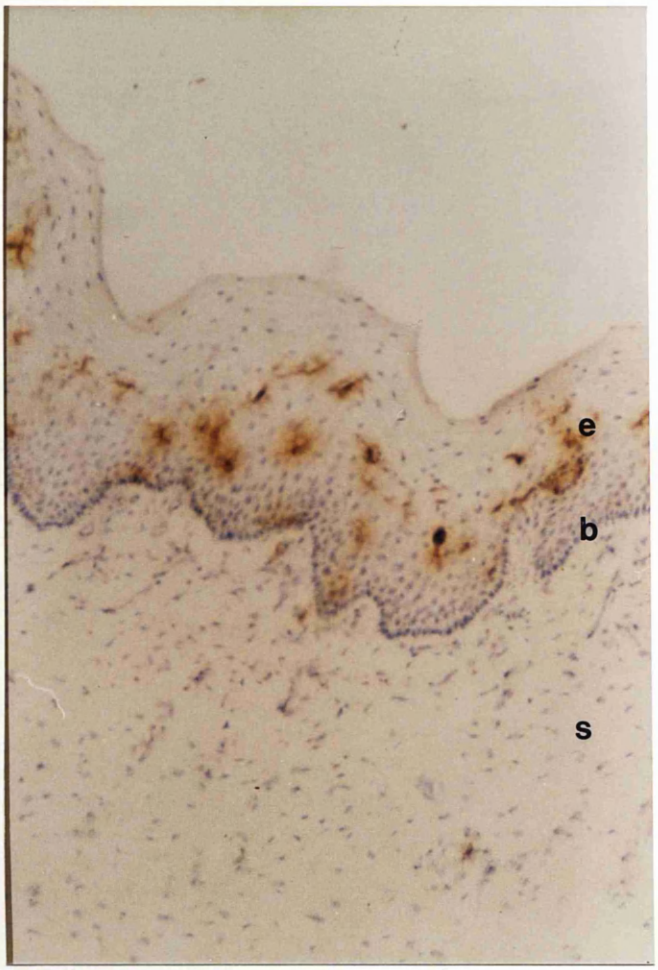

Figure 2.

Langerhans´ cells stained with NAl/34, and diaminobenzidene as the chromogen, in normal cervix.

The brown staining cell bodies are fairly uniformly distributed in the lower third of the epithelium (e). The basement membrane is shown (b) and the stroma (s).

Total magnification X 362.

Hydrogen peroxide (H_2O_2) is used to remove endogenous peroxidase activity. Seven per cent H_2O_2 proved unsatisfactory as cervix contains more peroxidase activity than skin. Its use resulted in sections that were greatly disrupted by oxygen released from the reaction. This effect was most marked in larger sections. Three per cent H_2O_2 was found to be ideal, although longer incubation was required. Fifteen minutes was found to be adequate.

Cervix contains mucus secreting 'glands' not present in skin. This mucus frequently caused non-specific positive staining, this was reduced by prolonging the incubation with normal rabbit serum to 20 minutes.

In practise the method is relatively free of complication but is labour intensive. Forty slides could be comfortably stained in one session. The dilution of the primary and secondary antibodies had been established by Dr M Turbitt. Since the same technique was being used in the laboratory by other workers, cross-checking of staining patterns was easily performed.

Diaminobenzidene (DAB) was the chromogen most frequently used to demonstrate the monoclonal antibody reaction. It is carcinogenic and has to be treated with care. It is made up in a fume cabinet. Pelliniemi, Dym & Karnovsky (1979) described a method that allows batches to be made and stored in aliquots which can be subsequently thawed for use when required.

DAB gives a brown reaction product (**Figure 2**). The main benefit of using DAB is that once stained, dehydrated and mounted the preparation is permanent allowing sections to be stored for examination.

Another commonly used method to demonstrate the antibody

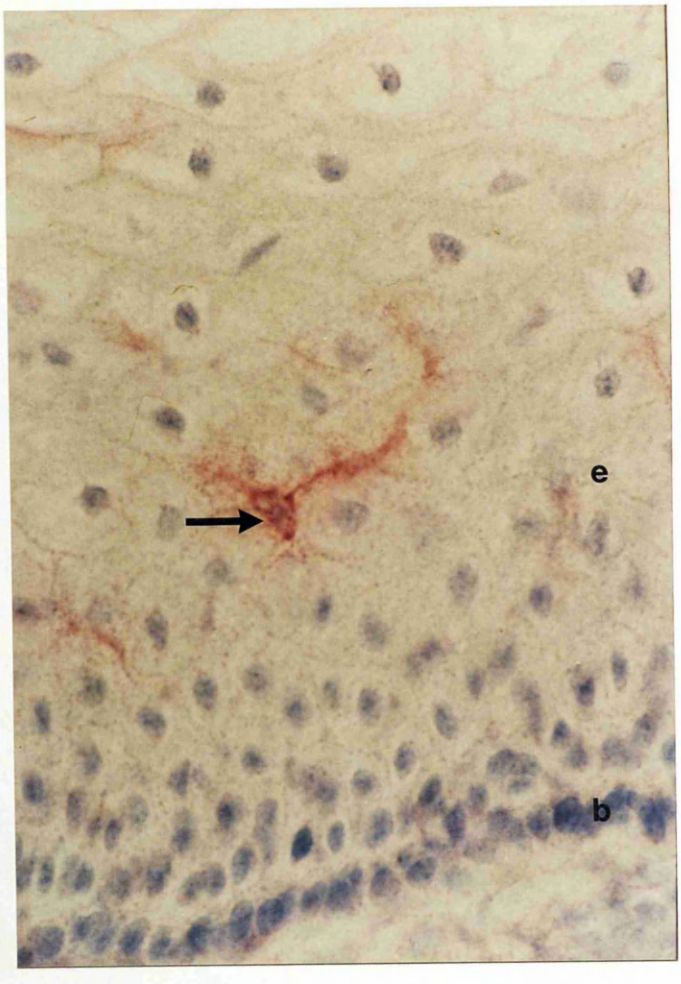

Figure 3.

Langerhans´ cell stained with NA 1/34, and APAAP and Fast Red Salts as the chromogen, in normal cervix.

The cell body is indicated with dendrites extending into the epithelium (e). A granular deposit results with the Fast Red Salts. The basement membrane is indicated (b).

Total magnification X 1450.

antigen reaction is alkaline phosphatase complex (APAAP). This method allows amplification of the process by repeating the steps using the uncongugated rabbit antimouse antibody and APAAP complex (Cordell et al. 1984).

The final reaction product can be red (**Figure 3**) or blue depending whether fast red or blue salts are used. The use of the salts leaves a granular deposit. The sections can only be counterstained lightly with haematoxylin, and slight over staining cannot be removed. Sections have to be water mounted resulting in a preparation which is less permanent than DAB. The technique is also more time consuming, with no better Langerhans' cell staining resulting.

The specificity of the monoclonal antibody NA 1/34 is good with no other epithelial cell staining positively (Figures 2 & 3).

The demonstration of Langerhans'cells with the APAAP method offers no real advantages over DAB after initial labelling with NA 1/34. The methods can be successfully combined in immunoperoxidase doubles eg. to demonstrate Langerhans' cells and helper T-cells in the same section. APAAP was also used to demonstrate Langerhans cells in an ATP-ase, NA 1/34 double method when the brown reaction product of the DAB would have been difficult to distinguish from the black of the ATP-ase.

2) Control Sections

Despite the apparent specificity of NA 1/34, multiple sections were stained together with at least one control known to be positive. Negative controls consisted of substituting other monoclonal antibodies for NA 1/34 in the primary incubation. The primary antibody was also omitted from one section. In practise negative controls were rarely of any practical use such is the

specificity of the Langerhans' cell staining.

Before a new batch of any antibody was used it was compared with the one in use. Details of the Langerhans' cell marker and other antibodies used are shown in Table 2.

Table 2. Monoclonal antibodies utilised in the study of Langerhans' cells and local cellular immunity in the cervix uteri.

Monoclonal antibody	CD Code	Specificity & Comments
NA 1/34	1	Cortical thymocyte marker of T-6 antigen which has a molecular weight of 49,000 Daltons and is found on Langerhans' cells and dendritic cells in thymus and lymph nodes.
UCHT 1	3	Marker of T-3 antigen expressed on the cell membrane of T cells.
UCHT 4	8	Marker of T-8 antigen present on T cells exhibiting cytotoxic/suppressor functions.
OKT4a	4	Marker of T-4 antigen (60,000 Daltons) present on about 2/3 of circulating T cells including most T cells with helper/inducer functions.
OKM1	15	Marker of 170,000 Dalton antigen expressed by monocytes & granulocytes.
Leu 11b		Marker of 50-70,000 Dalton antigen expressed on Natural Killer (NK) cells and neutrophils.
Leu 7		Marker of HNK-1 antigen on cells with Natural Killer/Killer function
Leu 12	22	Marker of 130,000 Dalton antigen on B cells.
DA6 231		Marker of MHC class II antigen HLA-DR.
DA6 147		Marker of MHC class II antigen HLA-D.
W6/32		Marker of MHC class I antigens HLA A,B,C.

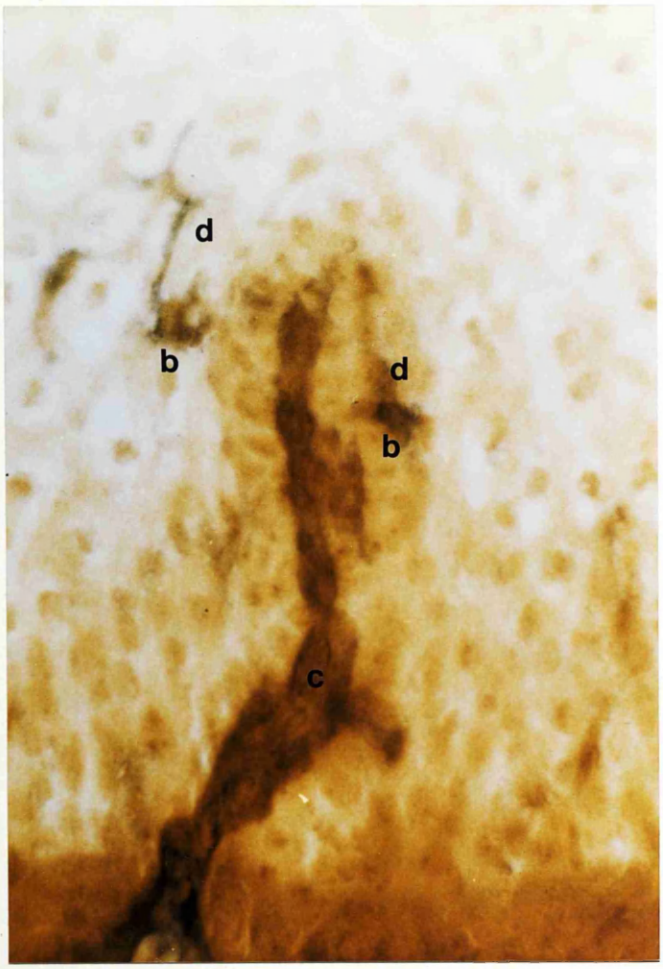

Figure 4.

Langerhans´ cells stained with ATP-ase in a frozen section. Cell bodies are indicated (b) with dendites (d) extending into the epithelium adjacent to a capillary (c).

Total magnification X 1450.

3) Other monoclonal antibodies used

The substitution of other monoclonal antibodies for NA 1/34 in the indirect immunoperoxidase method means that a panel of antibodies can be utilised to stain neighbouring frozen sections. This gives the benefit of being able to identify other cellular components of the immune system. The monoclonal antibodies used and their specificities are shown above (Table 2) and further details in Appendix II (p. 251).

4) Adenosine Triphosphatase (ATP-ase)

Several different methods have been used that depend on ATP-ase activity of the Langerhans' cell for their demonstration. The most useful and reproducible was a modification of the Calcium Method for Adenosine Triphosphatase reported in Pearse (1968).

Twenty micron frozen sections of cervix, as previously described produced the best results. The technique is simply performed (Appendix Ie, p. 247). Forty sections are easily stained in one session.

The effect of incubation time was investigated as initial results showed no Langerhans' cells in cervix despite their demonstration in control sections of skin after 30 minutes incubation. Sections were withdrawn from the incubating medium at 15 minute intervals to a maximum of 180 minutes. Dendritic cells were best demonstrated in cervix after 90 minutes **(Figure 4)**.

The most apparent drawback of a prolonged incubation time is an increase in the ATP-ase activity of vascular endothelium in the sections. This effect may obscure the Langerhans'cell population. In addition at longer incubation times some non-specific epithelial cell nuclear staining becomes evident.

The cost of this method is considerably less than that of the monoclonal antibody technique. However, it is more liable to failure especially towards the end of the shelf life of the stock reagents. Difficulties controlling the pH were also encountered. These problems are not experienced with the monoclonal antibody method.

5) S-100

Reports that S-100 was a useful marker for Langerhans' cells in fixed blocks stored in paraffin appeared attractive as it would have obviated the need for the collection and cutting of frozen sections. In addition it would be possible to examine specimens retrospectively and specimens which were not suitable for frozen sections, such as cone biopsies taken to exclude the possibillity of early stromal invasion.

A technique was evaluated to attempt to demonstrate Langerhans' cells in fixed tissue and is described in Appendix Ig (p. 249) with the APAAP modification in Appendix Id (p. 246).

Initial results were very poor compared with NA1/34 in frozen sections from the same cervix despite the fact the inbuilt positive control (nerve fibres) were staining across a wide variety of incubation times, dilutions and temperatures.

Sections for S-100 staining were cut from routinely fixed blocks of cervix by Mr R Muirhead of the Department of Pathology, Western Infirmary. Best results were obtained with 4 micron sections.

Pre-treatment of sections with trypsin was found to help. However, sections had then to be collected on poly-lysine coated slides to prevent trypsin digestion of gelatin that resulted in

the sections floating from the gelatinised slides.

S-100 staining was attempted on frozen sections in the hope of giving a more direct comparison between anti T-6 staining and S-100 in adjacent serial sections of cervix. No staining, even of nerve fibres, was detected. Brief fixation of frozen sections was attempted with formalin, glutaraldehyde and periodate-lysine-paraformaldehyde. In no instance was any positive staining seen in the fixed frozen sections. The penetration of S-100 into tissue would seem to be dependent on long fixation which totally blocks the sensitive surface T-6 antigens on the Langerhans' cell. The loss of antigenicity is associated fixation and processing of tissue (Hsu, Raine & Fanger 1981).

Other disadvantages of using S-100 to identify Langerhans' cells are that only a small proportion of those stained with anti T-6 are identified by S-100. Additionally even in the same cervix there are only occasional areas that show any S-100 activity. This great variation is probably due to vagaries of fixation and especially of fixation time. The importance of a standardised fixation time for specimens for immunohistochemical studies is highlighted in a thesis being submitted to The University of Glasgow by Dr Iain Brown, Department of Pathology, Western Infirmary. None of the published studies reporting S-100 positive Langerhans' cells have attempted any such standardisation.

6) Other methods of tissue fixation for antibody staining

As a result of the inconsistent findings with S-100 an alternative aproach to studying fixed biopsies was evaluated. McLean & Nakane (1974) reported that periodate-lysine-paraformaldehyde could be used as a tissue fixative for material

subsequently to be stained with monoclonal antibodies. The advantages of a reliable reproducible method would have been considerable as specimens could have been used for routine processing and for immunohistochemistry, thus removing the need for frozen sections. A more extensive range of cervical pathology would also have been amenable to study, especially cone biopsy specimens including microinvasive carcinomas. Long term use of such a fixative would also have allowed retrospective analysis. Follow through to electron microscopy was also reported to be possible.

Traditional fixation causes loss of tissue antigenicity partly because of altered tertiary structure secondary to the formation of cross linkages. The conformational change limits antibody recognition of antigen. Paraformaldehyde, lysine and dichromate were reported to offer a compromise between good fixation and good ultrastructural preservation with retention of tisue antigenicity (McLean & Nakane 1974). No previous study had reported results of staining with an anti-T6 (CD1) antibody. Various T and B cell markers had been successfully employed although none had been used on the cervix.

Solutions were prepared according to the method of McLean & Nakane (1974).

No Langerhans' cell staining was observed despite the good results in paired frozen sections when stained with NA 1/34.

Variations were tried with and without 5% potassium dichromate and using various pH's. Again the Langerhans' cells showed no T-6 activity in the fixed tissue (Appendix Ib, p. 244).

Enzyme digestion was also performed as removal of some of the cross linkages can improve staining eg. S-100 (see above).

Digestion with trypsin and pepsin produced no significant improvement.

Varying the antibody concentration, even the use of undiluted NA 1/34 produced no recognisable Langerhans' cell staining over incubation periods ranging from one hour to overnight.

Demonstration of the antibody antigen reaction was also attempted using the sensitive amplification APAAP method, although again without success.

Hand processing and low temperature wax embedding was also tried with no success before the technique was abandoned.

7) Effects of storage on Langerhans' cell markers

The blocks of tissue for frozen sections, both from hysterectomy specimens and from punch biopsies, were cut within 24 hours of collection. As mentioned above the labelled slides were stored in a refrigerator at $-20^{o}C$. The remainder of the biopsy specimen was removed from the microtome chuck and placed in a box labelled with the number of the specimen and date of collection. In case further sections were required the cut surface of the block was covered with a thin layer of OCT to prevent the specimen drying out.

Staining of sections with monoclonal antibodies was performed within one week of collection. However, the effects of storage on the cut sections was investigated comparing especially NA 1/34 and ATP-ase.

Paired sections from two specimens were cut at 7 and 20 microns. Nine pairs of sections were cut to allow comparison of the staining patterns of NA 1/34 and ATP-ase over an eight week period.

Results of this comparison showed slight loss and dispersal of membrane ATP-ase activity when compared to NA 1/34. The quality of the monoclonal antibody staining remained constant throughout the eight week period and showed little subjective loss in quality when stored for periods of up to 1 year.

8) Other Methods - Langerhans' cells in epithelial sheets

Preparation of epithelial sheets

In addition to orthagonal sections of cervix, epithelial sheets were prepared by tetrasodium ethylenediamine tetracetic acid (EDTA) chelation of calcium in biopsies. The observation of Langerhans' cells in such sheets allows their complete 3-dimensional profile to be observed. The advantages of Langerhans' cell counting in such sheets are discussed later in this chapter.

Once collected the biopsies were placed in a 'transport medium'. Calcium and magnesium free Hanks Buffer was found to produce the best results.

After storage at $4^{o}C$ for at least 1 hour, tetrasodium ethylenediamine tetracetic acid was added (Scaletta & McCallum 1972). Following incubation at $37^{o}C$ for 1 hour the epithelium could be stripped from the underlying stroma using fine forceps under the stereo dissecting microscope.

a) ATP-ase

Using sheets prepared in the above manner ATP-ase staining was performed using the method outlined in Appendix If, p. 248.

This proved a very satisfactory alternative method in large specimens (Figure 5). However, when smaller punch biopsies of

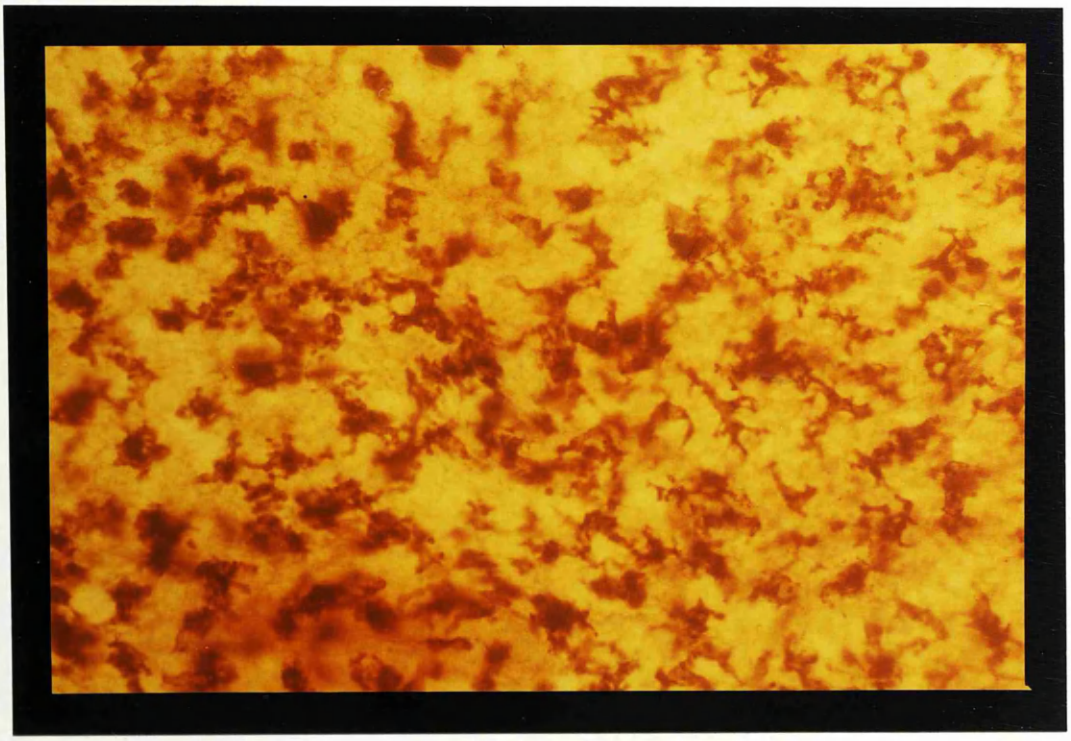

Figure 5.

Langerhans´ cells stained with ATP-ase in an epithelial sheet. The cell bodies with dendrites are best seen on the right of the figure. The full thickness of the epithelial sheet allows the 3-dimensional profile of the Langerhans´ cell to be studied by adjusting the depth of focus.

Total magnification X 725.

cervix were prepared, there was often difficulty separating the epithelium due to presence of epidermidised cervical crypts in the transformation zone. Even when successful separation was acheived the presence of the epidermidised crypts made counting of the Langerhans´ cells in superficial epithelium virtually impossible. In addition it is impossible to establish the exact histological diagnosis in the area where the Langerhans´ cells have been counted, even allowing for examination of strips of epithelium from the edges of the specimen (Hawthorn & MacLean 1987b).

b) Anti T-6 antibody - NA 1/34

Following the separation of the epithelial sheets, monoclonal antibody staining of Langerhans´ cells was attempted using a method modified from the indirect immunoperoxidase method described in Appendix Ia, p.243. This method has been shown to work satisfactorily on suction blisters of skin. After incubation with EDTA, however, no Langerhans´ cell activity was demonstrated in sheets of cervical epithelium.

c) Zinc-iodide-osmium

Caorsi & Figueroa (1984; 1986) reported a method, modified from Niebauer et al. (1969) for Langerhans´ cell identification in separated epithelial sheets, which was suitable in their hands, for examination at light and at transmission electron microscopy. No consistent staining of any kind was seen even with light microscopy and the method was abandoned.

9) Transmission electron microscopy

One millimetre cubes of cervical epithelium were cut from normal cervix and fixed in 2.5% glutaraldehyde. They were post fixed in 2% osmium tetroxide in Sorensen´s phosphate buffer (pH

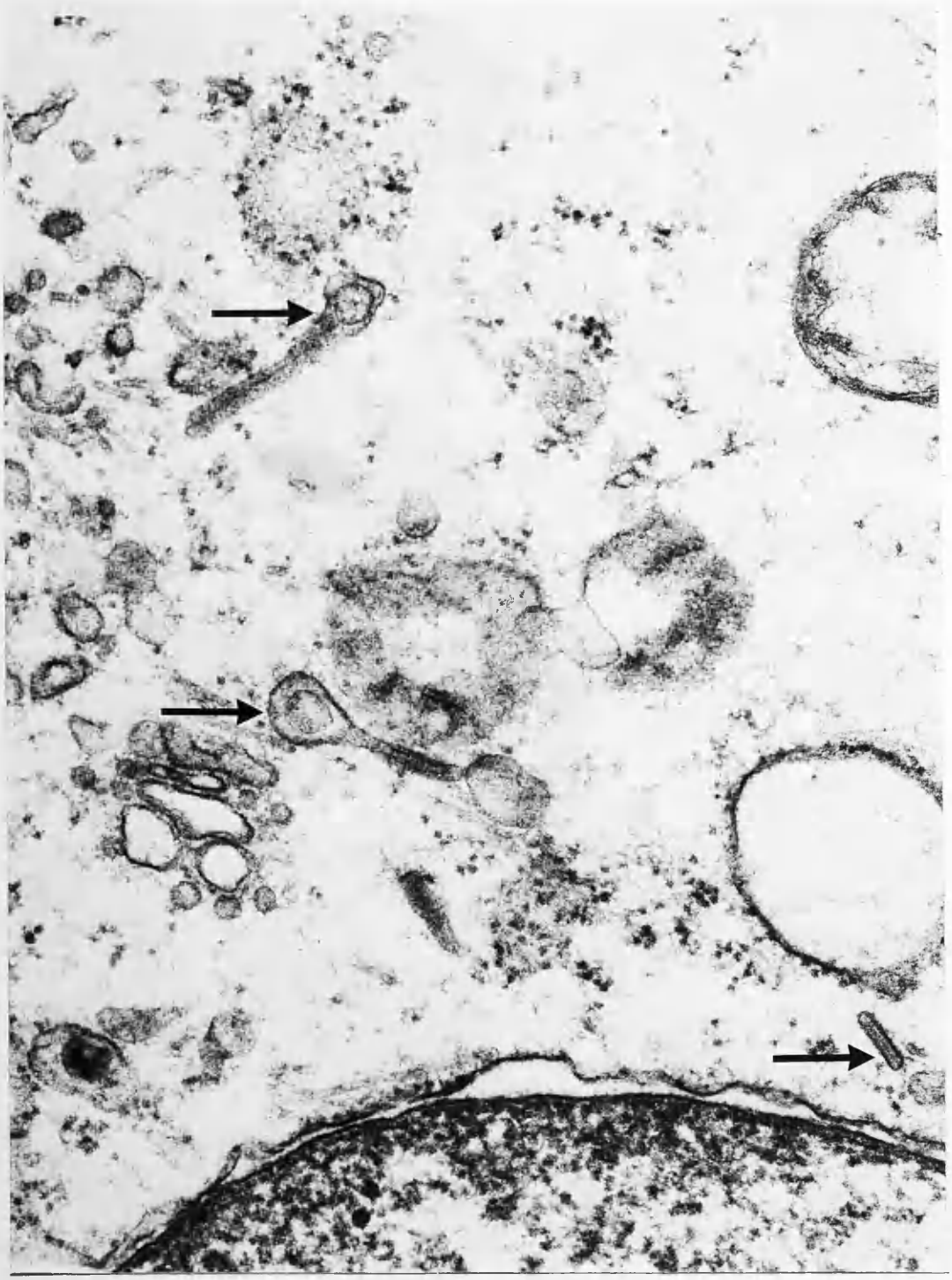

Figure 6.

Birbeck granules in a Langerhans´ cell from cervix.

The Birbeck granules with their characteristic racket-shaped heads are indicated in the electron micrograph.

Total magnification X 63,000.

7.38), before being dehydrated through alcohol to propylene oxide and embedded in araldite. Thick sections were stained with toluidine blue to identify the suprabasal layers where Langerhans' cells were thought most likely to be found. Appropriate thin sections were then cut on an LKB ultratome 1, double stained with uranyl acetate and lead citrate before being viewed in a Phillips EM 301 G microscope. The processing for EM was supervised by Mr T Downie, Department of Pathology, Western Infirmary, Glasgow.

Birbeck granule containing cells were identified in several of the specimens examined (**Figure 6**). The small sample size capable of being examined with EM meant that Langerhans' cells were not always observed. The Birbeck granules were seen mainly in relation to the Golgi apparatus and were not uniformly distributed through the cytoplasm. This too could account for the sampling errors experienced.

10) Electron microscopy following other methods

In attempts to specifically identify Langerhans' cells by methods described above, progression from T-6 antibody and ATP-ase was attempted.

a) Indirect immunoperoxidase

The method for progression from monoclonal antibody staining is shown in Appendix 1h (p. 250). This was adapted from several papers which had used electron microscopy to identify specifically other cells stained with monoclonal antibodies (Foidart et al. 1980; Holden, Morgan & MacDonald 1982; Mutasim et al. 1985).

The method used followed the first steps of the indirect immunoperoxidase technique described in Appendix Ia . Following the reaction with DAB the sections were fixed in glutaraldehyde.

The stroma underlying the epithelium was then removed with a scalpel using a stereo dissecting microscope. This maximised the area of epithelium available for ultrastructural examination. Post fixation in osmium tetroxide served to enhance the DAB reaction.

Langerhans' cells could be identified in the sections following fixation and before sectioning for EM. Unfortunately, the intracellular ultrastructural detail was insufficiently preserved to allow Birbeck granule observation. Even after initial cryoprotection with sucrose impregnation, Birbeck granules were not detected. Holden et al. (1982) were able to demonstrate Birbeck granules in T-6 positive cells employing a similar method.

b) ATP-ase

Hanau et al. (1986) described a method allowing progression from ATP-ase to EM. Separated sheets of epithelium were used. Once again Langerhans' cells could easily be identified before EM processing, but due to poor cellular preservation Birbeck granules could not be demonstrated in positively stained cells.

Quantitation of Langerhans' cells

There have been various methods used to quantitate cells in tissue sections. Where the cells are in the epithelium the simplest method would be to use a standard length of epithelium eg. 1 mm. This would be a useful, reproducible method if the epithelium were perfectly flat or undulated in a regular fashion. When considering the cervix, the ectocervix has a relatively smooth basement membrane and the numbers measured per linear millimetre of epithelium would be expected to correlate more closely with the numbers measured per millimetre length of basement membrane. This relationship would not hold where the

basement membrane was subject to marked undulations. The transformation zone of the cervix lies between the 'last gland' and new squamocolumnar junction and is characterised by the presence of epidermidised cervical 'glands' (Burghardt 1976). The basement membrane elongates greatly round these gland crypts and to make valid measurements the length of the basement membrane would require to be measured.

A more logical approach is to use an easily measured segment of basement membrane. A recognised method is to use the basal cells as the denominator and make the counts relative to a given number of basal cells. In the laboratory where this work was performed the standard length of basement measured is 200 basal cells (MacKie & Turbitt 1983; Ashworth, Turbitt & MacKie 1986). This is suitable for skin but in cervix the areas of abnormality eg showing viral features may be limited to very small areas. In addition the time taken to accurately count 200 basal cells is considerable. At higher power magnifications the slide has to be moved on the microscope stage thus interrupting the count. These are lesser problems when measurements are taken per 100 basal cells. The disadvantage of this method is the time involved in counting the 100 basal cells. The basal cells were counted with a hand counter from a mark on a calibrated eye-piece and the mark of the 100th cell was noted.

An Olympus BHS 312 microscope, with a photomicrography unit attached, was used to record and confirm the findings throughout the studies. The Langerhans' cell bodies were most easily counted over the 100 basal cells at 250 times magnification.

The Langerhans' cells were counted in representative areas of each specimen. The modal count was used where there was less than

two cells variation per 100 basal cells in five fields. Where any marked variation was obvious (2 or greater cells difference per 100 basal cells in 5 fields) the count was made in up to 10 areas and the mean value chosen for statistical analysis. Only definite cell bodies were counted in epithelium which showed a good basal array in areas cut at right angles to the surface.

In the normal cervices the transformation zone and ectocervix were identified as above using the 'last gland' as the boundary. Where abnormal biopsies were being evaluated appropriate areas were identified in adjacent haematoxylin and eosin stained sections.

Observer errors in Langerhans' cell counts per 100 basal cells

The results of inter and intra observer variation are shown in Table 3 & 4. The intra-observer variation was calculated using the same field of transformation zone counted on two occasions one month apart in the 25 normal patients described in the next chapter. An assessment of the inter-observer variation was made by two observers counting Langerhans' cells simultaneously using a 'double headed' microscope (Table 4). The frequency of the difference between the two counts are shown for the specimens with low and high numbers of Langerhans' cells.

Table 3. Intra-observer variation in Langerhans' cell (LC) counts per 100 basal cells.

	Intra-observer differences				
	-2	-1	0	+1	+2
low Lc counts (7/100 or less)			9	1	
high Lc counts (8/100 or greater)		7	7	1	

Table 4. Inter-observer variation in Langerhans' cell (LC) counts per 100 basal cells.

	Inter-observer differences				
	-2	-1	0	+1	+2
low Lc counts (7/100 or less)		1	8	1	
high Lc counts (8/100 or greater)	3	3	4	4	1

It can be seen from the two tables that the variation in Langerhans' cell counts per 100 basal cells is related to whether there are low or high counts. At low numbers there was no difference in the counts by the same observer in 90% of the cases, and between observers there was no difference in 80% of the cases. Where there are higher numbers the correlation is less good

with 7 of 15 cases counted by the same observer showing no difference and only 4 of the cases between observers showing no differrence. Despite the variation dependent on the native population of Langerhans' cells, with the same observer making the counts there was no instance where there was more than one cell difference.

Langerhans' cell counting in epithelial sheets

In epithelial sheets the measurement of the denominator is simpler and the counts are usually recorded per square millimetre of epithelium. Using the above equipment and a calibrated square counting grid in the eye-piece Langerhans' cells were quantitated per square millimetre.

The dendritic processes could be followed through the epithelium and the cell bodies were easily identified. The counts were made in small marked squares, Langerhans' cells lying on or partly on the top and right border of each square were excluded but those lying on or partly on the left and bottom borders of each square were included. The small squares of the graticule represent 1/625 mm^2.

The presence of epidermidised cervical crypts in the separated sheets of transformation zone made counting the Langerhans' cells almost impossible due to the three dimensional distortion caused. The difficulties in achieving adequate separation of the epithelium from the stroma have been previously mentioned. This technique therefore proved inadequate for assessing the Langerhans' cell distribution in biopsies from abnormal transformation zones. Quantitation was possible in normal transformation zones obtained from the larger hysterectomy

specimens (see chapter 3).

Langerhans' cells counts by image analyser

Computer assisted methods for Langerhans' cell enumeration in skin and cervix have been reported by several investigators. The counting involves a method of enhancing the cell staining to enable the computer to identify the cell population under scrutiny.

In the specimens used in this study this involved separating the blue counterstain from the brown of the diaminobenzidene reaction product. This was not easily accomplished as the image analyser available utilised a grey scale. Blue filters were used to absorb the light from the counterstained areas and this served to enhance the contrast between positive and counterstained cells. The filters were not always effective as any densely counterstained areas often registered above the threshold of detection thus artificially increasing the cell counts. The sensitivity of detection is easily varied and a suitable compromise was selected. This invariably involved some cells marking as three or four images while other cell bodies marked as one. A control field was counted at the start and end of each session and every hour to ensure no great changes in the sensitivity of the system.

The great advantage of such a system is that large numbers of fields from many specimens can be rapidly evaluated. In all cases Langerhans' cells in 30 epithelial fields were evaluated. This method, although attractive, does not remove the possibility of observer bias but this is reduced by making multiple meaurements.

The analyser allows variation in the area from which the

counts are made, and this area remained constant throughout the analysis. All results have been standardised to express the Langerhans' cell count per unit area of cervical epithelium (0.15 X 0.15 cm^2). The thickness of the cervical epithelium rarely exceeded 0.15 cm.

In this study an Optomax CPU 1 image analyser was used. This equipment is housed in the Department of Opthalmology, Western Infirmary and is available for use by many collaborating departments. The image analyser was interfaced with a Hewlett Packard 9815A calculator on which the programmes for cell counting were designed. The mean cell count per specimen has been used for statistical analysis and the results from a selection of biopsies are presented in the relevant chapters to provide a comparison with the results of the counts made per 100 basal cells.

Image analyser quantitation of the lymphocytic infiltrate

Previous work from the Department of Dermatology had shown up to 30% inter-observer variation when the lymphocytic infiltrate had been evaluated subjectively (Turbitt, personal communication). Some of the disadvantages of using the image analyser have been discussed above, but this technique was most useful in allowing quantitative assessment of the lymphocytic infiltrate. The major limiting factor in performing this part of the study was the qualitative differences in cell staining patterns found with the T3, T4 and T8 antibodies. The T4 (helper cell) marker produces a less dense, frequent pericellular DAB deposit in comparison with the more uniform dense deposits found with the other two antibodies. T3, T4 and T8 antibodies were used on adjacent frozen sections from each biopsy to limit sampling variation. Only the

specimens showing least variation in positively stained cells were used.

In performing the assessment of the infiltrate the total area of positive staining per unit area was used and not the actual cell (pixel) count as used to assess the Langerhans' cells. The Optomax CPUl is better able to measure actual areas than cell counts where there are confluent areas of positive staining as in the stromal lymphocytic infiltrate.

The stromal infiltrate was most frequently concentrated in the area immediately below the basement membrane and all counts were made in the stroma within 0.15 cm of the basement membrane. The infiltrate was quantified in the epithelium up to 0.15cm above the basement membrane. The staining with the pan T (CD3) monoclonal antibody was used as an indicator of the total lymphocytic infiltrate in both the epithelium and stroma. Comparisons with this value were made to allow calculation of the percentages of the helper and cytotoxcic/suppressor components of the infiltrate in both areas. The percentages of T4 and T8 cells in the epithelium and stroma and the actual 'numbers' of each and the total infiltrate as represented by the areas of positive staining have been used for statistical analysis.

Subjective quantitation of other components of the cellular infiltrate

Macrophages and natural killer cells were rarely encountered and image analysis was not attempted. They were scored on a subjective basis from negative to very heavy. In practice these cells were rarely present in any significant numbers.

Class I and II epithelial antigen staining patterns were

classified according to a basal, two thirds or full thickness and negative or diffuse epithelial staining patterns respectively. See Figures 7 and 8 (p. 125) for examples. Class II positive Langerhans' cell were counted in the normal cervix (chapter 3).

Statistics

Advice on statistics was given by Dr Tom Aitchison, University Department of Statistics, Glasgow University. Analysis was performed using SPSS-X, through the Department of Computing, Glasgow University. Non-parametric tests have been used where there are small numbers of cases, but the majority of analyses have been performed using statistical tests for normally distributed data. Most data analysis has been performed to provide the mean, standard deviation of the mean and the 95% confidence interval of the mean. The comparison of data has been interpreted, in the main, using the confidence intervals of the mean to establish differences between the groups analysed. Where multiple comparisons have been performed, appropriate corrections for such analyses have been employed thus avoiding multiple T-tests.

In chapters 4 and 5 where internal control data has been used to provide comparative analyses, the same principal of employing confidence intervals has been utilised for presentation of data. In these paired analyses the confidence intervals of the mean differences in Langerhans' cell counts between normal and abnormal biopsies are presented. If the confidence intervals of the mean differences do not cross '0' this implies a significant difference between the Langerhans' cells in the two areas.

Chapter 3

Langerhans´ cells and local immunity in the normal cervix

Chapter 3

Introduction

The normal cervix was examined by the methods previously described to detect Langerhans' cells. This allowed comparison of methods quantitatively and qualitatively, and gave data for comparison with the abnormal cervix. Some of the results and conclusions from the methods evaluated are presented in the preceeding chapter. This chapter describes the morphological features and distribution of Langerhans' cells in two areas of the cervix, namely the ectocervix and transformation zone. The transformation zone of cervix is the area in which neoplastic change occurs. One of the aims of this chapter was to establish any differences in the Langerhans' cell population between the ectocervix and the transformation zone, comparing various methods, in the hope of being able to use the Langerhans' cell count in the ectocervix as an internal control in later parts of the study. The published data suggest that there are no differences between the two areas but no author has directly compared the results from the ectocervix and the transformation zone of the same patients.

Materials

Specimens were collected initially from patients undergoing hysterectomy for reasons other than disease of cervix; none had any evidence of malignancy. All had had prior smears within 6 months which were reported as normal, and subsequent normal histology on routine examination and on histology from frozen sections (see Appendix IIIa, p. 253).

Punch biopsies of cervix were later used from patients undergoing laparoscopic sterilisation. All had normal cervical

cytology within six months and normal histology on frozen section examination. Hospital 'Ethical Committee' approval had been granted and informed, written consent was obtained from all patients having cervical biopsies taken. Twenty-five cases had specimens taken from the transfomation zone; of these a paired sample of ectocervix was available in 20 cases. Five patients were postmenopausal. Biopsies of vagina and vulva were also obtained with written consent from nine patients undergoing surgery.

Methods

The methods for mounting the blocks from the hysterectomy specimens and punch biopsies have been discussed in chapter 2.

The main staining methods employed were the monoclonal antibody technique (using NA 1/34 and Class II antibodies) and ATP-ase. ATP-ase staining was performed on 20 micron frozen sections and on separated sheets of epithelium from ectocervix and transformation zone. S-100 staining was also attempted.

The Langerhans' cell counts were made as described for each method in Chapter 2.

Results

Transformation zone and ectocervix

a)NA 1/34

No significant differences were found using the paired T-Test to directly compare the Langerhans'cell numbers in the ectocervix and transformation zone of each patient for the three denominators shown in Table 5.

Table 5. Paired T-Test comparison of NA1/34 positive Langerhans' cell numbers in normal ectocervix (ecto) & transformation zone (Tz) counted per 100 basal cells (/100), linear millimetre (/mm) & per unit area by image analyser (/area).

Count	Area	Cases	Mean	SD	SE Diff	Corr	Sig
	ecto		8.2	2.9			
/100		20			0.46	0.80	0.66
	Tz		8.0	3.4			
	ecto		17.9	7.6			
/mm		17			1.18	0.77	0.11
	Tz		15.9	5.9			
	ecto		13.32	3.11			
/area		20			0.61	0.65	0.43
	Tz		12.83	3.35			

NOTE. Mean & SD of mean are of the individual group cell counts. SE Diff (standard error of the mean difference), Corr (correlation) and Sig (significance) relate to the mean difference between the numbers in each paired analysis. A positive correlation indicates that pairing decreases the mean difference. The larger the correlation coefficient the greater the benefit of pairing.

When the individual biopsies from typical transformation zone and ectocervix were taken as two separate groups, no significant differences were found with measurements were made per 100 basal cells (Table 6) or per unit area (Table 7). The mean number of Langerhans' cells and 95% confidence intervals are shown. Results of Langerhans' cell counts per 100 basal cells taken from vagina

and vulva are also included in Table 6. One-way analysis of variance shows significant differences between the numbers in cervix and the numbers in vagina and vulva (F.Prob<0.0001). The cervix contains intermediate numbers. Image analyser counts per unit area of epithelium are shown in Table 7 and confirm the similarities between the normal ectocervix and transformation zone.

Table 6. Analysis of variance of NA1/34 positive Langerhans' cell counts per 100 basal cells in normal ectocervix (ecto), transformation zone (Tz), vulva and vagina.

Area	Cases	Mean	SD	Min	Max	95% Confidence Intervals
Tz	25	8.0	3.1	3	16	6.7 to 9.2
Ecto	20	8.2	2.9	3	13	6.8 to 9.6
Vagina	9	4.7	1.6	3	8	3.5 to 5.9
Vulva	9	12.6	4.5	6	23	9.1 to 16.0

Table 7. Analysis of variance of NA1/34 positive Langerhans' cell counts by image analyser in normal ectocervical (ecto) epithelium and transformation zone (Tz).

Area	Cases	Mean	SD	Min	Max	95% Confidence Intervals
Tz	25	12.43	3.30	7.2	18.8	11.07 to 13.80
Ecto	20	13.32	3.11	7.9	19.2	11.86 to 14.78

NOTE Tables 6 & 7. Mean & SD are of the cell counts in each group. Min and Max are the minimal and maximal counts respectively. The 95% confidence intervals of the mean cell counts are shown.

b)ATP-ase

Paired T-Test comparisons of the number of ATP-ase positive

Langerhans' cells per 100 basal cells and per square millimetre separated epithelium of normal ectocervix with those of the transformation zone (Table 8) show no significant difference in numbers between the two areas. This concurs with the results of paired analyses using the monoclonal antibody NA 1/34 to demonstrate Langerhans' cells (Table 5).

A beneficial effect of pairing the data is again shown with positive high correlations decreasing the variability of the mean difference.

Table 8. Paired T-Test comparison of ATP-ase positive Langerhans' cells in normal ectocervix & transformation zone counted per 100 basal cells(/100) & per square millimetre ($/mm^2$)

Count	Area	Cases	Mean	SD	SE Diff	Corr	Sig
	ecto		8.4	3.3			
/100		8			0.74	0.78	0.28
	Tz		9.2	2.4			
	ecto		595.0	42.8			
$/mm^2$		5			28.07	0.42	0.29
	Tz		628.6	67.2			

See Note Table 5 p. 99.

Pre and postmenopause

The Langerhans' cell population in the ectocervix and transformation zone were compared in the pre and postmenopausal patients. No significant differences were detected between the two areas within each group of patients pairing the data for analysis (Table 9). Comparing the results from transformation

zone and ectocervix of the premenopausal with the post menopausal patients there are no significant differences in either area of cervix (p=1.00, p=0.66 respectively) between the two groups.

Table 9. Paired T-Test comparison of NA1/34 positive Langerhans' cells in ectocervix & transformation zone of pre & postmenopausal patients counted per 100 basal cells (/100).

Group	Area	Cases	Mean	SD	SE Diff	Corr	Sig
	ecto		8.2	3.2			
pre		15			0.53	0.82	1.00
	Tz		8.2	3.4			
	ecto		8.2	2.2			
post		5			0.97	0.83	0.46
	Tz		7.4	3.6			

See Note Table 5 p. 99.

Correlation between methods used

There is good correlation between the numbers of Langerhans' cells in the transformation zone stained by the monoclonal antibody NA 1/34 and counted per 100 basal cells and with the numbers per unit area, the numbers per millimetre of epithelium and the numbers of ATP-ase positive Langerhans' cells per 100 basal cells (r=0.79, 0.82, 0.61 respectively). Similar results are obtained when the ectocervix is examined.

Langerhans' cells and other monoclonal antibodies

The numbers of Langerhans' cells identified with MHC class II antigens (DA6 147, DA6 231), in sections adjacent to those stained with NA 1/34 ranged between 30% and 88% of the counts with NA

1/34. Lower numbers were recorded with DA6 147 and subjectively the staining was less good. The results of MHC class II antigen staining in other groups studied are confined to comments relating to DA6 231. There were no apparent numerical differences between the MHC class II positive Langerhans' cells in the ectocervix and transformation zone.

Langerhans' cell morphology

Anti T-6 antigen (NA 1/34) and ATP-ase consistently identified dendritic epithelial cells in the basal and lower third of normal cervical epithelium. This was not the case for S-100 (see Chapter 2). The Langerhans' cell bodies were usually clearly seen with dendrites extending between adjacent epithelial cells. In some areas, dendrites of neighbouring Langerhans' cells appeared to meet and apparently form a network that stretched between the basal epithelial cells and more superficial layers. The dendrites often reached the basement membrane. These features were seen best following staining with NA 1/34. The problems encountered with other methods have been previously discussed. In many sections dendrites were seen with no apparent cell body. Only cells with identifiable cell bodies were counted for statistical analysis. ATP-ase and MHC class II antigen staining revealed that the dendrites occasionally reached capillaries.

The distribution of Langerhans' cells appeared fairly uniform between ectocervix and transformation zone, occasionally several Langerhans cells were closely related to capillaries of the transformation zone. Very few T-6 positive cells were present in the stromal mononuclear infiltrate frequently observed in the transformation zone.

Lymphocytic infiltrate

An obvious difference in the lymphocytic infiltrate was apparent between the stroma and the epithelium. Cytotoxic T-cells were seen to predominate in the epithelium. In the stroma the helper subgroup was in the majority. Quantitatively the stromal infiltrate is vastly greater. Within the transformation zone the cellular infiltrate directly in the vicinity of the squamo-columnar junction contains larger numbers of polymorphs and occcasionally B-cells. In the stroma of the normal cervix the lymphocytic infiltrate is found mainly in a relatively narrow band immediately below the epithelium of the transformation zone and ectocervix (**Figure 14b** p.161). The infiltrate in the epithelium is predominantly in the basal layers. The infiltrate was graded semi-quantitatively in all of the cases but apart from the clustering around the squamo-columnar junction no difference was apparent between ectocervix and transformation zone.

In order to quantify the infiltrate the image analyser was used, as previously described, in 20 of the cases with normal transformation zone and 15 of those with normal ectocervix. The majority of the epithelial infiltrate was of the cytotoxic/ suppressor (CD8/T8) variety, both in percentage terms and numerically in ratio approximately 4:1 when compared with the helper (CD4/T4) cells.

In the stromal infiltrate T4 positive cells predominated in a ratio of 2:1. Details of the infiltrate are shown in Table 10. Using Scheffe's test for multiple comparisons there are no significant differences between the ectocervix and transformation zone in the infiltrate of CD3 cells in the epithelium and stroma, in the percentage and numbers of helper cells in the epithelium

and stroma or in the percentage and numbers of cytotoxic cells in the epithelium and stroma. No correlation was found between the number of Langerhans' cells and any component of the stromal or epithelial infiltrate in either area of the normal cervix.

Table 10. Analysis of variance of T-cell counts per unit area in epithelium and stroma of normal transformation zone and ectocervix

A) Image analyser counts of T cells in normal transformation zone.

Marker/Area	Cases	Mean	SD	95% Confidence Intervals
CD3 epith (N)	20	161.9	68.0	130.2 to 193.8
CD8 epith (N)	20	165.9	75.1	130.7 to 201.0
CD8 epith (%)	20	104.2	26.7	91.7 to 116.8
CD4 epith (N)	20	41.8	22.3	31.4 to 52.3
CD4 epith (%)	20	28.6	16.5	20.9 to 36.6
CD3 stroma (N)	20	343.7	131.4	282.2 to 405.2
CD8 stroma (N)	20	143.6	78.5	106.8 to 180.3
CD8 stroma (%)	20	42.4	15.9	35.9 to 50.0
CD4 stroma (N)	20	255.5	107.3	205.3 to 305.7
CD4 stroma (%)	20	75.4	23.0	64.7 to 86.2

Note. All cell counts are pixel counts of positively stained cells

CD3 epith=Pan T (T1) cells in the epithelium

CD8 epith=Cytotoxic T (T8) cells in the epithelium

CD4 epith=Helper T (T4) cells in the epithelium

CD3 stroma=Pan T (T1) cells in the stroma

CD8 stroma=Cytotoxic T (T8) cells in the stroma

CD4 stroma=Helper T (T4) cells in the stroma

%= (Number (N) CD4 or CD8 cells X 100)/ (N) CD3 cells)

The SD & 95% confidence intervals of the mean cell counts are shown.

B) Image analyser counts of T cells in normal ectocervix.

Marker/Area	Cases	Mean	SD	95% Confidence Intervals
CD 3 epith (N)	15	190.9	99.1	136.0 to 245.7
CD 8 epith (N)	15	187.7	84.8	140.7 to 234.7
CD 8 epith (%)	15	103.1	28.5	87.3 to 118.9
CD 4 epith (N)	15	57.4	44.9	32.5 to 82.3
CD 4 epith (%)	15	29.9	15.4	21.3 to 38.4
CD 3 stroma (N)	15	350.6	137.1	274.7 to 426.5
CD 8 stroma (N)	15	133.4	71.9	93.5 to 173.2
CD 8 stroma (%)	15	41.0	19.3	30.3 to 51.7
CD 4 stroma (N)	15	262.9	106.1	204.1 to 321.6
CD 4 stroma (%)	15	79.3	22.8	66.6 to 91.9

Other components of the infiltrate

B-cells (Leu 12) and macrophages (OKM1) were rarely encountered outside the region of the squamocolumnar junction. Natural killer cells were an infrequent observation. Image analyser quantiation of these components was not performed.

The pattern of MHC class I antigen activity was consistent, with staining being maximal in the basal one third to half of the epithelium.

The MHC class II antigen activity of Langerhans' cells has been previously described. No class II staining of the epithelial cells was seen although the relation of the Langerhans' cells with capillaries was noted.

Comments

Langerhans' cells have previously been described as a consistent finding in normal fetal, premenarchial and adult

cervical epithelium (Morris et al. 1983a). This study shows that the numbers of Langerhans' cells are similar in pre and post menopausal patients. No significant differences are found between the ectocervix and transformation zone when different methods of identification and different methods of enumeration are used. The previously reported data have suggested that this was the case, but the numbers of patients studied were very small and contained no comparison of staining methods or methods of enumeration. No study has presented internal comparisons between ectocervix and transformation zone.

The results obtained from this analysis confirm that there is large individual variation in the numbers of Langerhans' cells in the normal cervix, a five fold range being noted for both NA 1/34 and ATP-ase. This is considerably less than the variation recorded with other methods, especially S-100 (McArdle & Muller 1986; Tay et al. 1987a). The inconsistency of S-100 staining has been previously mentioned (see Chapter 2, p. 81).

The results presented in this chapter agree with others that Langerhans'cells are identified in approximately equal numbers in cervix using ATP-ase and anti T-6 antigens with around 50-60% of these expressing MHC class II antigens (Tay et al. 1987a).

The Langerhans' cell density of cervix is approximately half that of normal skin. Using a similar monoclonal antibody technique other investigators, working in the same laboratory, report the normal mean Langerhans' cell density as between 11 and 31 cells per 200 basal cells depending on the body site (Ashworth et al. 1986). Rowden (1981) reported that the mean density of ATP-ase positive Langerhans' cells per square millimetre of skin was 460 to 1000 depending on the site of the biopsy, roughly 1.5 times the

number recorded in epithelial sheets in this study. The observation that the Langerhans' cell density of cervix is around 50-70% of that of skin is a useful comparison when other Langerhans' cell markers are compared. The cervix does not show the age dependent decrease in Langerhans' cell numbers seen in skin, presumably because of the lack of effects of ultraviolet radiation on the cervix.

Although neither the T-6 antigen nor membrane ATP-ase activity of Langerhans' cells have any functional significance, there is reasonably good evidence that actual numbers of Langerhans' cells are important when immunological responses are considered. This evidence has been presented in the introduction and relates mainly to the Langerhans' cells required to produce an adequate response to contact allergens.

The good correlation between the numbers of Langerhans' cells per 100 basal cells and other methods of quantitation is a reassuring finding, as is the demonstration that cervix contains 50-70% of the numbers of Langerhans' cells found in skin using different methods for their identification. The good correlation between the number of Langerhans' cells per 100 basal cells and the number per linear millimetre in the transformation zone was not unexpected, despite the reservations expressed in the previous chapter, since the counts made per linear millimetre were performed in areas where the basement membrane was least undulating, usually between cervical crypts. The large hysterectomy specimens permitted easy measurement of the linear millimetre which would not have been consistently possible on the smaller punch biopsies. One hundred basal cells was taken as the denominator for quantification in the later parts of the study.

This measurement was the denominator used by Edwards & Morris (1985) although they reported higher numbers than found in this study in the normal cervix. Additional support for the observations made in this study was obtained by image analyser quantification.

The comparable distribution of Langerhans' cells between ectocervix and transformation zone in individual patients, as shown by paired data analysis, is particularly important since it allows the inclusion of internal controls when the abnormal cervix is examined. This reduces the effects of the large range in Langerhans' cell numbers detected by all methods, with the nett effect of providing a larger control population than would otherwise have been possible. Further support for the use of internal controls is given in the following chapter.

It is difficult to compare the results of the lymphocytic infiltrate reported in different studies, largely because of the many different methods employed and lack of quantitative statistical analysis. Most authors seem to agree that in the epithelium cytotoxic cells predominate with helpers predominant in the stroma. There is no evidence from this study to suggest that the transformation zone is an area of enhanced immunological activity as proposed by Edwards & Morris (1985).

Summary

Langerhans' cells are distributed fairly regularly in the cervical epithelium in similar numbers in the normal ectocervix and transformation zone. Substantial differences in numbers are obvious between patients but the number in the ectocervix is a useful internal control for the transformation zone. Anti T-6

monoclonal antibody demonstrates the largest numbers of Langerhans' cells, although their relation with other cells in the epithelium and with capillaries is better seen with anti-MHC class II antibodies. The numbers of Langerhans' cells in cervix are approximately half that found in skin when several methods are compared. Quantitation per 100 basal cells is a reliable method that correlates well with other methods.

The lymphocytic infiltrate is also similar in the ectocervix and the transformation zone, with cytotoxic T-cells predominating in the epithelium and helper cells in the stroma. B-cells, natural killer cells and macrophages are rarely present in significant numbers. There is no evidence to support a previous comment that the normal transformation zone is a region of enhanced immunological activity.

Chapter 4

Langerhans' cells and the local immune response to cervical intraepithelial neoplasia

Chapter 4

Introduction

This chapter examines the relationship between Langerhans' cell numbers and CIN. Internal control biopsies of ectocervix have been used, where possible, to allow comparison of Langerhans' cell numbers in normal and abnormal cervical tissue from the same patient. The methods used are those established as most appropriate from studies performed in Chapter 2.

Patients

One hundred and seventy-two patients attending the Western Infirmary for laser ablation as treatment of CIN were studied. All were under the care of Dr A B MacLean or Dr J W Cordiner.

The Western Infirmary serves as a referral centre for patients with CIN, amenable to local ablative therapy, from large areas of the west of Scotland. Not all the routine histology from these 172 patients was reported in the Western Infirmary, but the histological diagnosis was available in all instances. The histology taken from frozen sections was reviewed by Dr R A Burnett, Department of Pathology, Western Infirmary and his interpretation was used if there was any discrepancy with the referral histology.

Colposcopically guided laser ablation was performed on all patients. Before the procedure, biopsies were taken from abnormal aceto-white areas of the transformation zone and from normal adjacent ectocervix.

The names of the patients were entered chronologically in the 'Dermatology Research Day Book' and given an identifying number.

Patient details and results were entered using this number on record sheets, later sent to Glasgow University Department of Computing for processing into files which were used for statistical analysis. Patient details including the histological diagnosis are shown in Appendix IIIb (p. 254).

Biopsies were also taken from the previously lasered area in 23 of the patients at routine follow-up. These were taken from the treated area identified colposcopically by changes in the vascular patterns described by MacLean (1984a).

Methods

The paired biopsies were mounted in OCT and orientated optimally as previously described, on a microtome chuck cooled in carbon dioxide ice. Both biopsies were mounted together on one chuck to allow simultaneous cutting (Figure 1 p. 73) and staining. Initial sections from each block were stained immediately with methylene blue to confirm the presence of orthagonally sectioned epithelium in both biopsies. Twenty serial sections were then cut where possible. Every fifth section was stained with haematoxylin and eosin for comparison with the previously reported routine histology. The sections not stained with haematoxylin and eosin were stored at $-20^{O}C$ for immunohistochemical staining. This was performed within one week of collection of the biopsies. The slides not stained initially were kept at $-20^{O}C$ as 'extras'. The biopsies were also stored in labelled numbered boxes at $-20^{O}C$ after they had been cut from the microtome chucks.

The antibodies, methods of staining used and methods of assessment have been discussed previously in Chapter 1. Statistical analysis was performed using packages available with

SPSS-X.

Results

Results of biopsies from 142 patients with histological evidence of CIN on frozen section were available for statistical analysis. Biopsies from another 30 patients were collected but the frozen section histology from these showed no apparent abnormal epithelium, poor preservation or fragments too small for interpretation. Eighty-seven patients had evidence of co-existant HPV infection on well established histological criteria (Meisels et al. 1981; Fletcher 1983). Fifty-five patients had CIN with no apparent HPV infection. The mean ages of these two groups were 28.0 and 29.9 respectively, the viral group being significantly younger ($p<0.05$).

The majority of patients had CIN III (90 patients), being equally split between pure CIN and CIN with viral infection. Thirty-four patients were treated for CIN II with virus and 10 for CIN II only. Eight cases had CIN I, all showed histological features of HPV infection. A paired normal internal control biopsy of ectocervix was available for comparison in 136 of the 142 patients.

a)Paired data

Analysis of the data on Langerhans' cell numbers from the patients in whom paired biopsies were taken was performed in several ways.

Firstly, the difference (normal - abnormal) and the percentage difference ((normal - abnormal X 100)/ normal) in Langerhans' cell numbers between the biopsies were calculated. One-way analysis of variance showed no statistically significant

difference with respect to the histological grade of CIN in either those patients with or those without evidence of HPV infection (Tables 11 to 14).

Table 11. Analysis of variance of the difference in Langerhans' cell counts per 100 basal cells between paired biopsies of normal ectocervix and CIN with HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Interval
CIN I+HPV	7	2.3	3.7	-4	6	-1.2 to 5.7
CIN II+HPV	33	3.4	4.1	-10	10	2.0 to 4.9
CIN III+HPV	44	4.0	4.0	-6	16	2.8 to 5.2
Total	84	3.7	4.0	-10	16	2.8 to 4.5
F.Prob=0.53						

Note. Mean=mean difference (normal-abnormal) in Langerhans' cell count between the paired biopsies. The minimal and maximal differences are indicated with the 95% confidence intervals for the mean difference. F.Prob=probability of a difference between groups, p >0.05 implies no significant difference between groups.

Table 12. Analysis of variance of the difference in Langerhans' cell counts per 100 basal cells between paired biopsies of normal ectocervix and CIN without HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Intervals
CIN I	0					
CIN II	10	-2.1	3.9	-10	3	-4.9 to 0.7
CIN III	42	-1.2	5.9	-11	11	-3.1 to 0.5
Total	52	-1.4	5.5	-11	11	-2.9 to 0.1
F.Prob=0.68						

See Note Table 11.

Table 13. Analysis of variance of percentage difference (((normal-abnormal)X100)/normal) in Langerhans' cells per 100 basal cells between paired biopsies of ectocervix and CIN with HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Interval
CIN I+HPV	7	29.0	66.4	-100	83.3	-32.4 to 90.4
CIN II+HPV	33	36.7	47.9	-125	87.5	19.7 to 53.7
CIN III+HPV	44	45.8	37.5	-60	90.9	34.4 to 57.2
Total	84	40.8	44.3	-125	90.9	31.2 to 50.4
F.Prob=0.52						
See Note Table 11 p.115.						

Table 14. Analysis of variance of percentage difference (((normal-abnormal)X100)/normal)in Langerhans' cells per 100 basal cells between paired biopsies of ectocervix and CIN without HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Intervals
CIN I	0					
CIN II	10	-31.4	56.0	-120	50	-71.4 to 8.6
CIN III	42	-27.4	85.4	-275	88.9	-54.0 to -0.8
Total	52	-28.2	80.1	-275	88.9	-50.5 to -5.9
F.Prob=0.88						
See Note Table 11 p.115.						

In the above tables if the 95% confidence intervals of the mean differences do not cross "0" this implies a significant difference between the Langerhans' cell counts in the normal and abnormal biopsies. Positive values imply larger numbers in ectocervix. Negative values indicate larger numbers in the biopsies of abnormal cervix. Tables 11 to 14 thus show that:

1) there is no variation in the difference in Langerhans' cells

between control and CIN tissue (irrespective of the presence of HPV) dependent on the grade of CIN (F.Prob >0.05),

2) the numbers of Langerhans' cells are significantly greater in CIN without HPV (negative difference best seen in percentage data, Table 14, see below) and significantly less in CIN with virus (positive difference) than in internal control biopsies (95% CI of 'totals' do not cross '0', see p. 95),

3) there are highly significant differences, ($p<0.001$), comparing the two calculated variables in the group with histological evidence of viral infection with the group showing CIN only (comparing Table 11 with 12 and 13 with 14).

As the number of Langerhans' cells in control cervix are small, additional use of the percentage differences (Tables 13 & 14) for analysis increases the sensitivity that small changes in Langerhans' cell numbers between control and abnormal tissue will detected at significant levels (see Tables 12 & 14).

Since the grade of CIN does not influence the numerical or percentage differences in Langerhans' cells, the paired T-test was used on the pooled data from each group to establish the significance of the difference in Langerhans' cell numbers in the paired biopsies (Tables 15 & 16). Results from lesional versus post-laser areas are also included.

The significant increase in Langerhans' cells in the CIN only lesions was confirmed using counts per 100 basal cells ($p=0.033$) and image analyser counts ($p=0.019$). Similar analysis of paired data in the group with histological evidence of viral infection showed a highly significant reduction in Langerhans' cells in lesional epithelium per 100 basal cells ($p<0.001$) and per unit area ($p<0.001$).

Table 15. Paired T-Test comparison of Langerhans' cells in CIN & HPV with ectocervix and post laser epithelium counted per 100 basal cells (/100) and with image analyser (mm^2).

Count	Area	Cases	Mean	SD	Sig
	ecto		8.4	3.1	
/100		84			CIN+HPV<ecto p<0.001
	CIN+HPV		4.7	3.6	
	ecto		11.2	4.2	
$/mm^2$		34			CIN+HPV<ecto p<0.001
	CIN+HPV		5.4	4.6	
	post laser		8.1	2.5	
/100		13			CIN+HPV<post laser p=0.03
	CIN+HPV		4.7	4.8	

Table 16. Paired T-Test comparison of Langerhans' cells in CIN without HPV with ectocervix and with post laser epithelium counted per 100 basal cells and with image analyser ($/mm^2$).

Count	Area	Cases	Mean	SD	Sig
	ecto		7.6	2.5	
/100		52			CIN>ecto p=0.033
	CIN		9.0	5.4	
	ecto		10.7	4.1	
$/mm^2$		24			CIN>ecto p=0.019
	CIN		13.9	6.4	
	post laser		8.1	2.7	
/100		10			CIN>post laser p=0.07
	CIN		10.5	5.2	

Note. Tables 15 & 16 Mean and SD (of mean) refer to cell counts in the individual groups.

Direct comparisons of the Langerhans' cell numbers in abnormal epithelium with those of the post laser areas in the two histological groups again showed significantly lesser numbers are present in lesions with CIN and virus ($p<0.03$). There was no significant difference in Langerhans' cell numbers seen in the biopsies with CIN only using the treated area as a control.

Comparison of the numbers in ectocervix with post laser epithelium showed no significant difference in either the patients with or without histological evidence of HPV ($p=0.084$ and $p=0.228$ respectively).

b) Individual groups

In addition to the paired results presented above, Langerhans' cell counts in the control ectocervix, abnormal and post laser cervix were compared with external control groups. Once again one-way analysis of variance no difference in Langerhans' cells counted per 100 basal cells between the various grades of CIN within the two histological groups (Tables 17 & 19). The total number of cases with CIN only and with CIN plus virus were therefore pooled to give 87 and 55 cases respectively. This was true for the control biopsies of ectocervix (Tables 18 & 20) obtained within each group (and also for the small number of post laser biopsies). Similar results were obtained for Langerhans' cell counts made per unit area of cervical epithelium. The cumulative totals of the cases examined using the image analyser and the results of analysis of variance are shown at the bottom of the relevant table. (See **Figures 10 & 11, p. 149,** respectively for examples of Langerhans' cells in CIN with and without HPV.)

Table 17. Analysis of variance of Langerhans' cell counts per 100 basal cells and by image analyser (/mm^2) in CIN with HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Interval
CIN I+HPV	8	3.7	3.1	1	9	1.2 to 6.3
CIN II+HPV	34	5.3	3.9	1	18	3.9 to 6.6
CIN III+HPV	45	4.2	3.3	1	16	3.2 to 5.2
Total	87	4.6	3.5	1	18	3.9 to 5.4
			F.prob=0.33			
Total(/mm^2)	35	5.3	4.5	0.9	22.1	3.7 to 6.8
			F.prob=0.16			

Table 18. Analysis of variance of Langerhans' cell counts per 100 basal cells and by image analyser (/mm^2) in the ectocervix of patients with CIN and HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Interval
CIN I+HPV	7	6.3	1.9	4	9	4.5 to 8.0
CIN II+HPV	33	8.8	2.8	4	15	7.8 to 9.8
CIN III+HPV	44	8.4	3.3	4	21	7.4 to 9.4
Total	84	8.4	3.1	4	21	7.7 to 9.0
			F.prob=0.14			
Total/mm^2	34	11.2	4.3	4.4	20.3	9.7 to 12.7
			F.prob=0.06			

Note Tables 17 & 18. Mean, SD (of mean) & 95% confidence intervals of the mean refer to cell counts in each group and the maximal and minimal values in each are shown. F.prob indicates the probability of a difference between groups. equal. Only the totals of the data are shown for image analyser counts (Total/mm^2).

Table 19. Langerhans' cells per 100 basal cells and per unit area ($/mm^2$) in CIN without HPV.

Grade	Cases	Mean	SD	Min	Max	95% Confidence Intervals
CIN I	0					
CIN II	10	8.6	4.4	3	19	5.4 to 11.7
CIN III	45	9.1	5.6	1	22	7.4 to 10.8
Total	55	9.0	5.4	1	22	7.6 to 10.5
			F.prob=0.78			
Total/mm^2	26	14.0	6.1	2.5	29.4	11.5 to 16.5
			F.prob=0.19			

See Note Tables 17 & 18 p. 120.

Table 20. Langerhans' cells per 100 basal cells and per unit area ($/mm^2$) in the ectocervix of patients with CIN & no HPV.

	Cases	Mean	SD	Min	Max	95% Confidence Intervals
CIN I	0					
CIN II	10	6.5	1.2	5	9	5.7 to 7.3
CIN III	42	7.8	2.7	4	15	7.0 to 8.6
Total	52	7.6	2.5	4	15	6.9 to 8.2
			F.prob=0.14			
Total/mm^2	24	10.7	4.1	3.9	22.6	9.0 to 12.4
			F.prob=0.53			

See Notes Table 17 & 18 p. 120.

The results from the ectocervix and transformation zone of the normal cervices examined have been presented in Chapter 3.

Analysis of variance on the numbers of Langerhans' cells

between normal transformation zone (mean 8.0; 95% CI 6.7 to 9.2)and abnormal transformation zone from both types of CIN showed significant differences between the sample means (F.prob<0.0001). Using Scheffe's correction for multiple comparisons at the 5% level, the CIN only lesions showed no significant difference in the Langerhans' cell count when compared with normal transformation zone. Those with histological evidence of HPV infection show a highly significant reduction in numbers when compared with normal transformation zone and with the lesions showing no apparent viral features.

No significant differences in Langerhans' cell numbers in ectocervix were detected comparing the patients with pure CIN, CIN plus virus, post laser cervix or normal ectocervix.

The Langerhans' cell count in the biopsies of histologically mixed lesions was significantly less when compared with the numbers in biopsies of ectocervix (normal ectocervix, post laser cervix and control ectocervix).

Direct comparison of CIN with and without virus, as expected from the above results, demonstrated a highly significant reduction in numbers ($p<0.001$) of Langerhans' cells in the lesions of the group with viral features.

The findings were similar using the Langerhans' cell counts per unit area of cervical epithelium.

c)Langerhans' cell morphology in CIN

The morphology of the Langerhans' cells appeared altered in the abnormal epithelium irrespective of the type of lesion, the cell bodies remained obvious but the dendrites were stunted and generally reduced in number. Their distribution in the abnormal

epithelium was little altered compared with normal epithelium, being predominantly situated in the basal / middle layers with the shortened dendrites extending from the cell body to the more superficical and basal layers (see **Figure 11** p. 149). They tended to be related to capillaries extending into the epithelium as in the normal cervix but did not appear to be particularly related to any mononuclear infiltrate that was present. Very few T-6 positive cells were located in the sub-epithelial stroma but they were occasionally seen in the mononuclear infiltrates.

d) Lymphocytic infiltrate

On semi-quantitative asssessment of the epithelial and stromal infiltrate with the Pan T-cell marker, UCHT1, no particular relationship between the infiltrate and degree of CIN was obvious for either the group with evidence of viral infection or the group without. There did, however, appear to be a fairly consistent reduction in the epithelial infiltrate where there was concomitant evidence of HPV infection. Image analysis methods were pursued to investigate this further. Comparisons have been drawn from the results obtained from examination of the typical transformation zone (see Chapter 3) and abnormal transformation zone in both histological types of CIN. The numerical and percentage assessment of the infiltrate and its components have been described in Chapter 2. The results of image analyser quantification of the infltrate in CIN are shown in Table 21.

Table 21. Analysis of variance of T cell counts per unit area in the epithelium and stroma in CIN with (a) and without (b) histological evidence of HPV infection.

a) Lymhocytic infiltrate by image analyser in CIN with HPV.

Marker/Area	Cases	Mean	SD	95% Confidence Interval
CD 3 epith (N)	34	189.0	172.5	128.8 to 249.2
CD 8 epith (N)	34	158.0	122.7	115.2 to 200.8
CD 8 epith (%)	34	86.5	16.5	80.8 to 92.3
CD 4 epith (N)	34	40.1	59.3	19.4 to 60.8
CD 4 epith (%)	34	19.1	4.4	15.2 to 23.1
CD 3 stroma (N)	34	660.6	425.6	512.1 to 809.1
CD 8 stroma (N)	34	257.1	185.5	192.3 to 321.8
CD 8 stroma (%)	34	38.1	4.2	34.2 to 42.0
CD 4 stroma (N)	34	462.4	291.7	360.7 to 564.2
CD 4 stroma (%)	34	70.2	12.2	66.0 to 74.5

b) Lymphocytic infiltrate by image analyser in CIN only.

Marker/Area	Cases	Mean	SD	95% Confidence Interval
CD 3 epith (N)	29	227.6	152.1	169.7 to 285.4
CD 8 epith (N)	29	195.1	130.7	145.4 to 244.8
CD 8 epith (%)	29	86.2	12.7	81.4 to 91.1
CD 4 epith (N)	29	38.3	34.3	25.3 to 51.3
CD 4 epith (%)	29	19.3	12.7	14.5 to 24.1
CD 3 stroma (N)	29	638.2	279.8	531.8 to 744.6
CD 8 stroma (N)	29	234.5	138.7	181.7 to 287.2
CD 8 stroma (%)	29	37.5	14.4	32.1 to 43.0
CD 4 stroma (N)	29	404.6	230.7	316.8 to 492.3
CD 4 stroma (%)	29	63.2	25.7	53.4 to 73.0

See Note Table 10 p. 105.

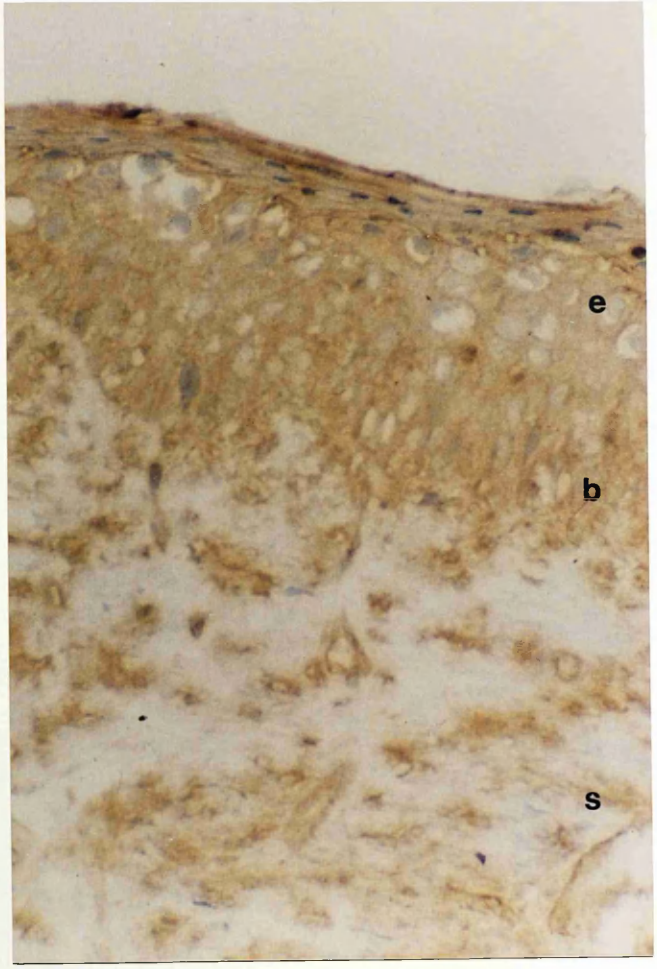

Figure 7.

Uniform epithelial cell staining with MHC class II antibody.

The cells of the epithelium (e) are fairly uniformly stained with DA6 231 in a lesion showing CIN with features of HPV infection (b = basement membrane; s = stroma).

Total magnification X 725.

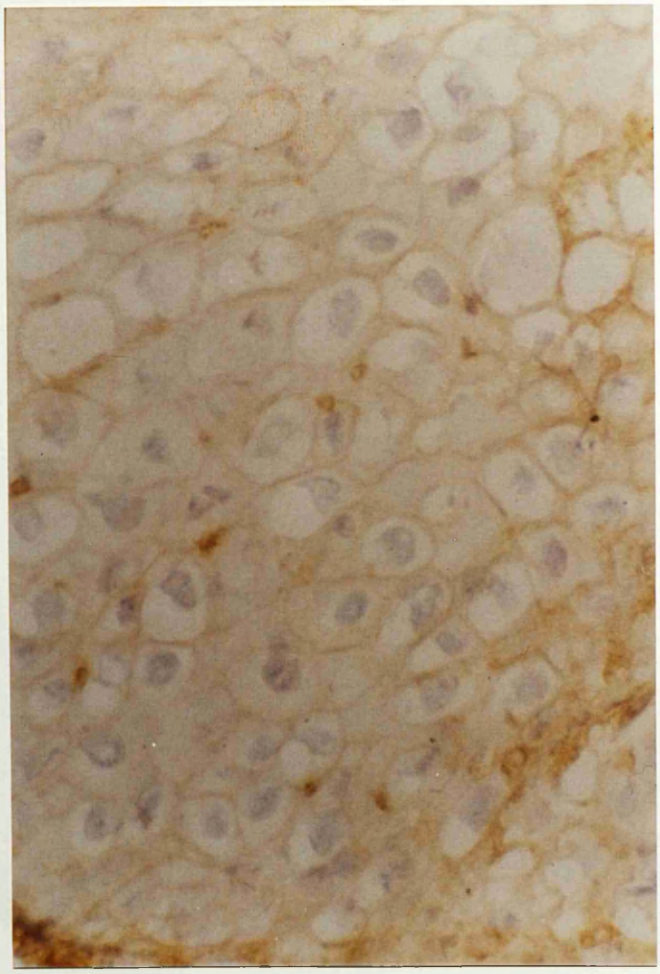

Figure 8.

Pericellular MHC class II staining.

Pericellular distribution of DA6 231 is seen in the epithelium.

Total magnification X1450.

Multiple comparison of means with Scheffe's correction at the 5% level showed no significant differences in any of the components of the lymphocytic infiltrate comparing the CIN lesions with those showing CIN and HPV. The stromal infiltrate was significantly greater in both types of CIN when compared with the typical transformation zone. In the epithelium the percentage of cytotoxic and helper cells were significantly less in both varieties of CIN lesions compared with the typical transformation zone. Numerically the stromal infiltrate of helper T-cells was greater in both types of CIN than in the typical transformation zone. Cytotoxic T-cells were greater in the stroma comparing normal with virus containing lesions only.

e) Epithelial cell MHC class II expression

Two patterns of epithelial MHC class II antibody staining were obvious in the abnormal transformation zone, uniform cellular staining (**Figure 7**) and pericellular staining (**Figure 8**). MHC class II antibody staining of keratinocytes was found in 78.2% (43/55) of cases with CIN and 60.9% (53/87) of combined lesions. Pericellular staining was most obvious in the lesions showing viral features. As in the biopsies of normal cervix, class II antigens were consistently demonstrated on vascular endothelium.

The components of the lymphocytic infiltrate in the lesions with and without epithelial cell MHC class II antigen expression were compared to highlight whether the epithelial cell expression of these antigens could be influencing the lymphocytic response. There was no significant difference in the numbers of Langerhans' cells in the epithelial cell MHC class II positive and negative lesions in either CIN with or without evidence of CIN.

In the lesions showing CIN only there was no significant difference found in any component of the lymphocytic infiltrate in the stroma or epithelium, comparing the epithelial cell MHC class II positive with the negative biopsies.

In the biopsies with CIN and histological evidence of HPV infection all of the components of the infiltrate measured numerically were significantly reduced in the epithelium and stroma of the lesions lacking epithelial class II antigen positivity ($p<0.05$). These are shown in Table 22. There were no significant alterations in the percentage composition of the infiltrate in either the epithelium or the stroma.

The relationship between class II positivity on the epithelial cells, the number of Langerhans' cells and the lymphocytic infiltrate was further explored by examining the correlation between the number of Langerhans' cells and the infiltrate in the epithelial cell class II positive and negative lesions.

Where CIN only was present there was no significant correlation between the number of Langerhans' cells and any component of the infiltrate in either the epithelial cell class II positve or negative lesions.

In the lesions showing CIN and viral features, significant positive correlations were found between the number of Langerhans' cells and the numbers of epithelial T-cells in the lesions with no concomitant class II positive keratinocytes (Table 23). There was no significant association between the Langerhans' cells and the components of the epithelial infiltrate when calculated as percentages.

Table 22. Analysis of variance of T cell counts per unit area in CIN with HPV, in the presence and absence of MHC class II antigen expression on transformed epithelial cells.

Marker/Area	Class II	Cases	Mean	95% Confidence Interval
CD 3 epith (N)	pos	18	260.1	155.0 to 365.1
	neg	14	112.6	87.0 to 138.3
CD 3 stroma(N)	pos	18	838.9	597.4 to 1080.4
	neg	14	453.7	341.2 to 566.2
CD8 epith (N)	pos	18	220.7	151.6 to 289.7
	neg	14	91.4	70.8 to 112.0
CD 8 stroma(N)	pos	18	325.6	219.0 to 432.2
	neg	14	186.5	125.6 to 247.3
CD 4 epith (N)	pos	18	58.3	20.1 to 96.5
	neg	14	18.0	10.9 to 25.2
CD 4 stroma(N)	pos	18	586.3	426.9 to 745.7
	neg	14	316.3	222.3 to 410.4

See Note Table 10 p. 105.

pos=lesions with MHC class II positive epithelial cells

neg=lesions without MHC class II positive epithelial cells

Table 23. Correlation (Pearson Corr) between Langerhans' cells (/100 basal cells) and epithelial T cell counts per unit area in CIN+HPV in absence of MHC class II expressing epithelial cells.

Marker/Area	Cases	Pearson Corr.	Significance Level
CD 3 epith (N)	14	0.665	p=0.009
CD 8 epith (N)	14	0.559	p=0.038
CD 4 epith (N)	14	0.849	p<0.001

See Note Table 10 p. 105.

In the biopsies showing combined CIN and viral features the significant correlations between the number of Langerhans' cells and the epithelial infiltrate are lost when there is concomitant class II staining of the epithelial cells.

The suggestion of a link between reduced Langerhans' cell numbers, epithelial cell class II antigen expression and the lymphocytic infiltrate was further examined by looking at the latter two variables in all the biopsies showing an atypical transformation zone and less than seven Langerhans' cells per 100 basal cells ie. comparing the lymphocytic infiltrate in the presence and absence of class II antigen expression in all cases (CIN only and CIN+HPV) with low numbers of Langerhans' cells.

No differences were found in the percentage composition of the stromal infiltrate. The numerical composition of the infiltrate in the epithelium and stroma was significantly reduced in the lesions not expressing MHC class II antigens on the epithelial cells (Table 24). The percentage of helper cells was also significantly reduced (p=0.012).

Table 24. Comparison of T cell counts per unit area in lesions with and without epithelial expression of MHC class II antigens in CIN & CIN+HPV with under 7 Langerhans' cells per 100 basal cells.

Marker/area	Class II	Cases	Mean	95% Confidence Interval
	pos	26	247.4	165.4 to 329.5
CD 3 epith(N)				sig p=0.003
	neg	16	120.7	86.4 to 154.9
	pos	26	742.5	654.0 to 920.9
CD 3 stroma(N)				sig p=0.002
	neg	16	424.3	308.1 to 540.5
	pos	26	201.0	143.9 to 258.2
CD 8 epith (N)				sig p=0.002
	neg	16	101.6	71.9 to 133.4
	pos	26	274.0	196.4 to 351.7
CD 8 stroma(N)				sig p=0.025
	neg	16	179.6	121.1 to 238.0
	pos	26	54.9	27.5 to 82.3
CD 4 epith (N)				sig p=0.004
	neg	16	15.1	10.6 to 19.6
	pos	26	504.3	393.7 to 615.0
CD 4 stroma(N)				sig p=0.003
	neg	16	180.3	200.6 to 392.8

See Notes Table 10 p. 105 & Table 22 p. 127.

Significance of the differences in mean T cell counts between MHC class II positive and negative lesions are shown.

f) Epithelial cell MHC class I antigen expression

Lesions with pure CIN more frequently demonstrated increased

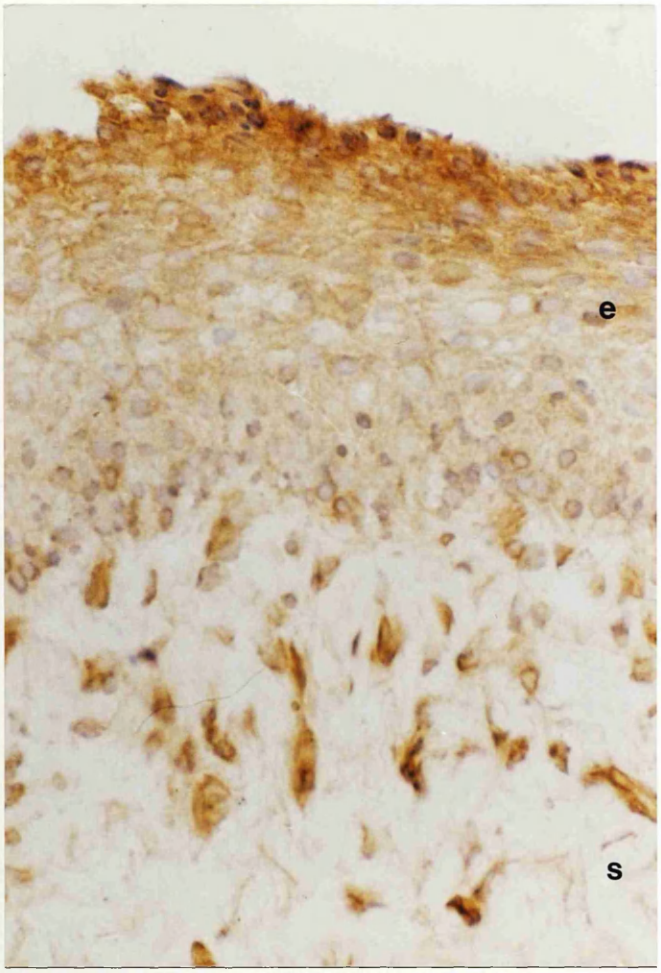

Figure 9.

MHC class I staining in CIN.

W6/32 positive cells occupy the complete epithelium (e). The stroma is shown (s).

Total magnification X 725.

epithelial cell labelling with anti-class I antibody. This increased labelling was apparent throughout most of the epithelial thickness (Figure 9). This staining was predominantly cytoplasmic and pericellular. Where there was histololgical evidence of viral infection the pattern of MHC class I antigen expression was more varied. The staining frequently extended throughout the epithelium but especially in the areas showing marked features of viral infection the pattern was patchy and the staining tended to be more pericellular than cytoplasmic.

g)B-cells, macrophages and natural killer cells

No differences in B-cells, macrophages or natural killer cells were apparent in the abnormal epithelium when compared subjectively with the normal cervix. B-cells were an occasional but inconsistent feature of the infiltrate. Natural killer cells were very rarely found and macrophages were generally present in small numbers. None of these other cells showed any apparent relation to the presence or absence of virus or to the degree of intraepithelial neoplasia.

Comments

The number of Langerhans' cells in CIN appears to be influenced by the presence or absence of viral infection. No other study has presented quantitative data comparing these two histological varieties of CIN. There is no relationship between the numbers of Langerhans' cells and grade of CIN contrary to the claims of Caorsi & Figueroa (1986).

The reduction in the Langerhans' cell population in CIN showing viral features was confirmed in this study using the

sensitive and specific Langerhans' cell monoclonal antibody (NA 1/34) in much larger number of cases than previously reported (Tay et al. 1987a). The reduction in numbers is significant when comparison is made with internal control biopsies and with normal transformation zone.

In CIN with no evidence of viral infection the Langerhans' cells are increased when compared with paired internal controls, although when compared with external control groups this significance is lost. The studies reviewed in Chapter 1 which have suggested similar results in this group of patients have either reported small numbers of cases, included poor control data or had no quantification at all (Morris et al. 1983b; Caorsi & Figueroa 1984, 1986; MacLean 1984b).

The results of this study emphasise the value of internal controls for quantitative analysis especially where CIN without viral infection is being studied. The large quantitative changes in Langerhans' cells are obvious in combined lesions even using external controls.

The use of internal control biopsies adds another dimension to the analysis of Langerhans' cells in cervix. The results from the previous chapter suggest that the ectocervix is an appropriate control for the normal transformation zone. It is possible that colposcopically and histologically normal adjacent transformation zone may have been a valid internal control. However, the number of cases where paired data would have been available would then have been substantially less as in many instances the abnormal acetowhite epithelium involves the complete transformation zone.

The use of internal control tissue also makes allowance for other factors implicated in the natural history of cervical

neoplasia such as smoking, number of sexual partners and contraceptive habits. In the study of the local immune response in the cervix the value of this becomes apparent when the immunosuppressant action of semen (James & Hargreave 1984), smoking (Miller et al. 1982) and the oral contraceptive pill (Hagen & Froland 1972; Barnes et al. 1974) are considered. Although the association of these cofactors with immunosuppression is mainly on in vitro systemic T-cell function, preliminary data from Dr Simon Barton (personal communication), Royal Northern Hospital, London, suggests that smoking per se reduces the Langerhans' cell numbers in the cervix. Hughes, Norval & Howie (1988), however, found no difference in the number of Langerhans' cells in CIN in smokers compared with non-smokers. An increase was noted in pill users versus non-pill users. Although examination of control data was limited in this latter study these findings require clarification, particularly on the effects of smoking and pill use in the normal cervix. They further emphasise the need for internal control biopsies in such studies.

The implication from the other studies reporting reduction in Langerhans' cells in CIN is that the local immune response is impaired where there is evidence of viral infection secondary to the reduction in Langerhans' cell numbers. However, the consistent finding of reduced numbers of Langerhans' cells in CIN with viral infection is suggestive that local "immunological failure" in the cervix does not follow every reduction in Langerhans' cells since only the minority of these lesions actually progress. It is possible there is a critical level of reduction or absolute level at which the antigen presenting capacity of the Langerhans' cell network begins to be compromised. Such a mechanism would then

allow escape of neoantigens from immunological surveillance. They would, in the intact host, be detected and effectively eliminated via the subsequent T-cell response. This could explain why human papillomavirus, even if subsequently shown not to be the actual aetiological agent, may have at least a role as a cofactor in the development of cervical neoplasia. The possibility that low numbers of Langerhans' cells are related to the presence of human papillomavirus DNA sequences is investigated in Chapter 5.

The number of Langerhans' cells present in squamous epithelium has been shown to be a factor in the adequate presentation of antigens to the immune system (Ptak et al. 1980; Toews et al. 1980b; Rheins & Nordlund 1986). The increased number of Langerhans' cells seen in CIN with no apparent viral infection has been interpreted as a specific immunological response to the neoplastic process. However, the results shown in Tables 12 and 14 indicate that reductions in Langerhans' cells also occur in cases of intraepithelial neoplasia with no apparent viral infection. If significant reductions in Langerhans' cells are required, perhaps to a critical threshold, to cause immuno-compromise this would explain why fewer virus negative lesions progress than virus positive lesions. It is not possible from this form of study to determine whether a particular level of reduction or critical threshold number of Langerhans' cells is of importance in the progression of lesions to higher degrees of intraepithelial neplasia and possibly to invasion. As explained in the introduction this short-coming relates to effects of biopsy itself on the natural history of CIN (Rawson & Knoblich 1957; Buckley et al. 1982), biopsy being required for Langerhans' cell enumeration and for initial diagnosis.

The results presented in Tables 22, 23 and 24 suggest that, in the absence of other possible antigen presenting cells, low numbers of Langerhans' cells are positively correlated with reduced epithelial lymphocytes. At higher numbers this association is lost and this perhaps indicates that a threshold number of Langerhans' cells is important for effective immune surveillance.

The assumption has been made above that the decrease in Langerhans' cells in virus containing lesions is real and others have suggested why this may be so. However, the reduction may only be apparent, with the presence of viral infection inducing changes in the surface antigenic markers of the Langerhans' cell. Although surface T-6 has no functional significance it is considered one of the most sensitive markers of Langerhans' cells (Harrist et al. 1983; MacKie & Turbitt 1983).

No consideration was given specifically to the numbers of class II antigen positive Langerhans' cells in the lesions in view of the large percentage that showed class II antigen staining of the epithelial cells. This epithelial cell staining effectively masks the class II activity of the Langerhans' cells.

Although Langerhans' cells act in the afferent antigen presenting limb of the local immune system, the presence of viral and class II antigens on the Langerhans' cells may be one explanation of the reduced Langerhans' cell numbers seen in the virus containing lesions since they may also be the targets for the efferent T-cell response. The electron microscopic studies performed by Silberberg-Sinakin et al. (1977) indicated that Langerhans' could be damaged by immunological reactions and that numbers were reduced following the initial phase of the contact sensitivity reaction (Silberberg-Sinakin & Thorbecke 1980).

The finding that epithelial cells in CIN express class II antigens is an interesting observation that has been noted by others (Morris et al. 1983b, 1983c; Hughes et al. 1988). The results presented in this chapter reveal that the epithelial cells in the majority of CIN lesions exhibit this phenomenon (78.2% of lesions with no apparent viral infection and 60.9% of cases with histological evidence of viral infection). The data from the Edinburgh group (Hughes et al. 1988) indicated this staining occured only in the minority of lesions they examined. Class II antigens are known to be expressed on epithelial cells in other circumstances (Lampert, Suitters & Chisholm 1981; Mason, Dallman & Barclay 1981; Terui et al. 1987). Class II expressing epithelial cells may have an antigen presenting role (Barclay & Mason 1982) but in addition, may also influence the cytotoxic T-cell response (Sollinger & Bach 1976). These new antigens appear to be synthesised by the epithelial cells (Breathnach & Katz 1983) and not to be the result of transfer from Langerhans' cells, lymphocytes or from endothelial cells (Morhenn, Charron & Engleman 1982). The stimulus to their induction in other areas has been shown to be dependent on the production of gamma interferon from T-cells (Pober et al. 1983; Wong et al. 1982). This has been confirmed for keratinocytes in culture (Wikner et al. 1986). The relation between squamous epithelial cells, HLA/DR expression and lymphocytes and interferon production has been discussed by Nickoloff et al. (1986) and Breathnach & Katz (1986) and reviewed in Chapter 1.

The results presented in relation to the lymphocytic infiltrate and the presence or absence of class II antigen expression on the epithelial cells in the two histological

sub-types of CIN studied indicate that at low Langerhans' cell numbers the presence of class II epithelial antigens significantly influences the quantitative infiltrate in both the epithelium and stroma (Tables 22 & 24) without significantly changing its proportions in either area. Combining these observations with the significant correlations between the Langerhans' cell numbers and the epithelial infiltrate at low Langerhans' cell numbers in the absence of class II antigens on the epithelial cells (Table 23) suggests that there is an optimal threshold, or level, of antigen presenting capacity in the squamous epithelium of the cervix, below which the immune response may become defective. Both Langerhans' cells and the class II positive keratinocytes contribute to this threshold. This emphasises that the epithelial cells are likely to have more than merely a passive role in local immunity in the cervix.

It is possible that the epithelial cells with their class II antigens are more important than Langerhans' cells in determining the immune response in abnormal cervical epithelium. They vastly exceed Langerhans' cells in number, and the results presented in Tables 22 and 24 indicate that the Langerhans' cells appear to influence only the epithelial infiltrate while class II epithelial cell positivity is significantly associated with an increase in the epithelial and stromal infiltrate. When the origin of the stimulus to the production of class II antigens on the epithelial cells is considered, this reinforces the notion of 'cervical lymphoid tissue' as a very complex entity dependent on the interaction of many cell types and soluble factors.

In the earlier studies no account has been taken of the influence of epithelial cell class II antigens on the lymphocytic

infiltrate. Tay et al. (1987b) demonstrated significant reductions in the epithelial helper cells of patients with CIN/HPV, and HPV infection compared with normal patients. The findings described in this chapter and in Chapter 3 (Tables 21 & 10) suggest a reduction occurs only in the percentage helper cells in CIN. In addition the percentage of epithelial cytotoxic cells is reduced compared with normal transformation zone while the total stromal infiltrate is increased. In general terms no great differences in the lymphocytic infiltrate are found comparing CIN only with CIN/ HPV. However, the results do indicate that analysis of the infiltrate is not complete without consideration of those lesions with and without class II antigens on the epithelial cells.

The findings in relation to class II antigen expression on epithelial cells may explain the results of others in HPV lesions with no CIN. Several authors have noted significantly reduced Langerhans' cells in such lesions (Morris et al. 1983b; McArdle & Muller 1986; Tay et al. 1987a). Morris et al. (1983c) noted class II antigen expression on the epithelial cells in all of the viral only lesions they examined. Similar results were noted in the earlier paper from the same group (Morris et al. 1983b). It is possible that the antigen presenting capacity is not dramatically impaired in these lesions and that this contributes to the very low malignant potential of pure HPV infection.

This discussion has concentrated on some of the possible factors influencing the afferent immune response. The finding that most lesions demonstrate increased class I antigens suggests that the afferent limb may be the limiting factor in cervical neoplasia. The efferent immune response may have been impaired if there was loss of class I activity on the epithelial cells

preventing recognition by the effector cytotoxic cells. Breathnach & Katz (1986) presented evidence suggesting that although the cytotoxic T-cell response to allogenic cells involved recognition of class I antigens, the response was augmented greatly by the additional presence of class II antigens. Thus in the majority of cases of CIN the epithelial cells may be dictating the afferent and efferent T-cell response. The results presented for both MHC class I and II activity are in-situ observations. The possibility that the actual antigens they define are of no functional or qualitative significance cannot be excluded in studies such as this.

There is no evidence in the large number of cases examined by these in-situ methods that the actual numbers of macrophages or natural killer cells have any role in cervical neoplasia contrary to the findings of Tay et al. (1987; 1987c). However, systemic natural killer cell activity has been reported to be significantly impaired in patients with CIN III. Return to normal activity has been described with alpha-Interferon and used to support possible treatment of CIN by this novel method (Seltzer, Doyle & Kaddish 1983).

Summary

The number of Langerhans' cells in cervical epithelium in CIN is related to the presence or absence of histological features of viral infection. The changes are best seen when internal control biopsies of ectocervix are paired with biopsies from lesions for analysis. In the presence of histological evidence of viral infection the Langerhans' cells are significantly reduced, while in the group without viral features the Langerhans' cells are

significantly increased. The changes are independent of the grade of CIN in each group. Following laser treatment the numbers of Langerhans´ cells return to normal levels.

Quantitative assessment of the lymhocytic infiltrate reveals differences dependent on the number of Langerhans´ cells and on the presence or absence class II antigens on the epithelial cells. It is suggested that Langerhans´ cells and epithelial cells expressing MHC class II antigens are linked together in determining the ´antigen presenting capacity´ of the epithelium in CIN.

The afferent limb of the immune system appears to be the limiting factor in determining the response, with MHC class I antigens generally being increased. Natural killer cells, macrophages and B cells appear unimportant.

Chapter 5

Langerhans' cells and HPV subtypes in CIN

Chapter 5

Introduction

Pure virus infection of the cervix has not been considered in this thesis, as the malignant potential of typical condylomatous change is very low with high rates of spontaneous regression. This is in contrast to the lesions which show atypical mitoses in conjunction with histological features of viral infection (Crum et al. 1984). The previous chapter deals with Langerhans' cells in CIN and in CIN with associated histological features of wart virus infection. These features have been extensively reported (Morin et al. 1981; Fletcher 1983; Dyson, Walker & Singer 1984) but essentially consist of koilocytosis, multinucleation and disordered keratinisation. In addition to histological features of HPV infection, diagnosis may be made from the features in cervical smears (Meisels et al. 1981).

The presence of koilocytosis on histological examination has almost become the pathognomonic feature of HPV infection (Dyson et al. 1984). Alone it is a poor indicator of wart virus infection (Winkler et al. 1984; MacLeod 1986; Kirkup et al. 1982). The picture is further complicated as the individual threshold for reporting the presence of viral infection varies between individual pathologists and cytologists. This has been the subject of a recent investigation, as yet unreported, amongst Scottish pathologists. The histological interpretation becomes more clouded as the frequency of koilocytosis decreases with increasing degrees of CIN (Pilotti et al. 1981). The poor correlation between koilocytes and HPV infection becomes evident when other methods of diagnosing HPV are taken in to account.

Antibodies raised to a group antigen common to all

papillomaviruses, present in the coat protein, allows immunohistochemical detection of papillomavirus infection. This serum, broadly reactive to the common capsid antigen, has been used by many investigators for identification of papillomavirus in 'fixed' tissue sections (Jenson et al. 1980; Woodruff et al. 1980; Morin et al. 1981; Kurman et al. 1981; Ferenczy, Braun & Shah 1981; Dyson et al. 1984). Fletcher (1983) reported koilocytotic atypia in 60% of biopsies showing CIN, whereas Walker et al. (1983) found only 18.3% of biopsies with CIN had demonstrable viral antigen. Others have reported histological evidence of HPV in 44.6% of cases with CIN (Grunebaum et al. 1983) and levels over 90% have been reported (Reid et al. 1982). HPV antigen positivity has been shown in 22% of biopsies in cases studied from the west of Scotland with CIN (Murdoch et al. 1988).

Following the demonstration by Hills & Laverty (1979) of viral inclusion bodies in koilocytes using electron microscopy, Ferenczy et al. (1981) showed the apparent value of immuno-staining for viral antigen. All cases with viral inclusions demonstrated viral antigens. However, a further 23% of cases showed antigen positivity only. Simliar findings were presented by Sillman et al. (1984).

The most sensitive method identifying HPV presence is by using a labelled probe to identify homologous sequences of viral DNA (Coleman & Richman 1985; Jenkins, Tay & Dyson 1986). McCance et al. (1983) presented results comparing histology, antigen staining and DNA/DNA hybridisation studies. Nineteen women had histological features of CIN (15 also with features of virus infection) of these, six demonstrated viral antigens and 12 (63%) contained HPV genome sequences. Not all the lesions expressing

antigen contained viral genomes. DNA hybridisation permits identification of viral subtypes dependent on the homology between known strains. It is reported that HPV 6 and 11 are present in up to 30% of CIN, whereas HPV 16 is reportedly present in 66% of CIN (Singer & McCance 1985). The prevalence of HPV 16 increases with increasing degrees of intraepithelial neoplasia, 77% of CIN III containing HPV 16 (McCance et al. 1985). There is a geographic variation in the prevalence of the HPV and its subtypes. Taking data from the west of Scotland, Millan et al. (1986) found 40% of pure CIN lesions and 73% of those with combined features had demonstrable HPV DNA. HPV 16 was present in 44% of all CIN (II and III). They also reported HPV 18 in 19% of lesions showing CIN (in the United Kingdom HPV 18 had previously only been reported in carcinoma). Murdoch et al. (1988) showed similar results with HPV 16 in 63% and HPV 18 in 15% of biopsies with CIN.

The very high prevalence of HPV 16 (McCance et al. 1985) in squamous cancer of cervix, and its association with abnormal mitoses (Crum et al. 1984), prompted suggestions that HPV 16 and 18 were the types most likely to progress to malignancy. The consistent presence of HPV genomes in some preinvasive lesions and in most squamous cell carcinomas of cervix also lead to some authorities suggesting that HPV causes cervical neoplasia (zur Hausen 1982; Gissman et al. 1983; Crum et al. 1984; Singer et al. 1984).

The findings described in the previous chapter together with the publications noting a link between the histological diagnosis of associated HPV infection in CIN and a reduction in Langerhans' cell numbers prompted the investigation of the possibility of a specific relationship between reduction in Langerhans' cell

numbers and particular HPV subtypes. The distribution of Langerhans' cell in CIN with and without HPV 16 and 18 infection was investigated to test the hypothesis that specific HPV subtypes are associated with reduced Langerhans' cells.

Patients

Tissue was obtained from 23 patients attending for treatment of histologically proven CIN. They form a subgroup of the patients examined in the previous chapter (Appendix IIIb, p. 254).

Methods

Paired biopsies of normal ectocervix and abnormal transformation zone were taken under colposcopic control from each patient. Separate biopsy forceps were used to avoid cross contamination. The two biopsies were halved, part being used for immunohistochemical studies and part for HPV probing. A biopsy taken for histopathology was available for comparison.

1) Immunohistochemical studies

The methods, antibodies and counting of Langerhans' cells were as described in Chapter 2.

2) DNA-DNA hybridisation

The DNA hybridisation was performed by Dr J B Murdoch in the Department of Virology, Western Infirmary under the supervision of Dr Joan Macnab. Dr Murdoch was the recipient of a Wellcome Research Training Fellowship.

HPV 16 deoxyribonucleic acid (DNA) cloned to the BamHl site of pBR322 and HPV 18 cloned to the ECORI site of pBR322 were kindly donated by Professor Harald zur Hausen, Dr Lutz Gissman and

collegues. HPV DNA was released from the vectors by the appropriate restriction endonuclease digestion and purified twice by electroelution after agarose gel electrophoresis.

Biopsies stored at -20^{o} C were chopped finely with a sterile blade and the DNA was extracted in 0.4% sodium dodecyl sulphate, 0.1M sodium chloride, 0.01M edetic acid (disodium salt), 0.05M trometamol (TRIS) hydrochloric acid pH 8.0, 200 mg / l proteinase K (Boehringer). The sample DNA was then extracted with phenol and chloroform before ethanol precipitation. Thereafter the samples were treated with 100 mg / l ribonuclease A. Sample DNA was quantified against a known standard lambda DNA (Gibco, Paisley, Scotland). Three microgrammes of each sample together with appropriate HPV reconstructions containing equivalent amounts of mammalian DNA were transferred through a Minifold 11 slot blot apparatus (Schleicher & Schuell) onto the reprobable nylon membrane Genescreen Plus (New England Nuclear) which had been pre - soaked in 10 X SCC (1 X SCC is 0.15M sodium chloride and 0.015M sodium citrate). The membrane was then treated with a denaturing solution containing 0.4M sodium hydroxide and 0.6M sodium chloride for 20 minutes and transferred to a neutralising solution containing 0.5M TRIS pH7.0 and 1M sodium chloride for 20 minutes before drying at 37^{o}C for 40 minutes. Purified probes were radiolabelled with alpha-phosphorus-32-deoxyadenosine triphosphate and alpha-phosphorus-32-deoxythymidine triphoshate to a specific activity in excess of 1 X 10^{8} counts per minute per microgramme of DNA. The membrane was prehybridised at 42^{o}C in 50% formamide (Fluka), 10% Dextran sulphate , 1% sodium dodecyl sulphate and 1M sodium chloride for 30 minutes. The probes plus 100 mg / l denatured salmon sperm DNA (Sigma) and 0.5 microgrammes cold

denatured pBR322 were treated with 1M sodium hydroxide for 20 minutes at room temperature before neutralising with 1M hydrochloric acid. This solution was then added to the prehybridisation mix and the samples hybridised at 42^{o}C for 18 hours. Cold pBR322 is added to block hybridisation of any residual radiolabelled pBR322 in the purified probe to the sample DNA. Thereafter the membrane was washed in 2 X SCC, 0.1% sodium dodecyl sulphate at 68^{o} C (stringent conditions) and autoradiographed using intensifying screens on X-Omat film (Kodak).

Results

HPV copy numbers per cell and Langerhans' cell numbers from the normal and abnormal biopsies are shown in Table 25. The level of genome expression has been based on densitometry compared with known copy number reconstructions. Under 10 copies per cell of HPV DNA has been taken as a negative result (Murdoch et al. 1987). Low, moderate and high levels of expression have been taken to represent 10-50, 51-100 and over 100 copies per cell respectively.

Table 25. Langerhans´ cells and HPV subtypes in CIN.

Patient	HPV genome (copies/cell) 16	18	Langerhans´ cells (/100 basal cells)	
6007	10-50	<10	7	15
6044	10-50	<10	4	15
6050	10-50	<10	8	4
6054	10-50	<10	11	5
6098	<10	<10	10	13
6121	<10	<10	6	9
6206	<10	<10	6	10
6229	<10	10-50	11	5
6249	10-50	<10	6	6
6267	<10	51-100	14	10
6272	<10	10-50	6	4
6276	<10	>100	12	7
6277	<10	10-50	14	11
6278	<10	51-100	12	6
6281	<10	10-50	7	5
6284	<10	<10	11	21
6285	<10	10-50	8	5
6288	51-100	<10	7	5
6289	<10	<10	11	14
6296	51-100	<10	5	4
6300	<10	<10	9	8
6302	10-50	<10	9	12
6305	51-100	<10	10	6

The vast majority of HPV DNA positive lesions (11/17) showed low levels of genome expression.

Groups were defined according to the presence and level of HPV 16 and 18 genome copies per cell. Statistical analysis was performed on the difference (normal - abnormal) and percentage difference (((normal - abnormal)/ normal) X 100) in Langerhans' cells in the paired biopsies. The median difference and percentage differences are shown with 95% confidence intervals in view of the relatively small numbers involved. Non - parametric testing was also performed using the Wilcoxon matched-pairs signed-ranks test.

HPV 18 > 10 copies per cell is significantly associated with decreased Langerhans' cell numbers (Tables 26 & 27). Inspection of the results in Table 25 suggests that HPV 16 in higher copy numbers per cell (>50) may be producing a similar effect (positive differences in all cases) although the numbers of observations are too small to allow valid analysis.

Any correlation between copies of HPV 18 genome per cell and reduction in Langerhans cells is difficult to evaluate because of the small number of cases with over 50 copies of viral genome per cell.

A significant increase in Langerhans' cell numbers was detected where no virus was present when the normal internal control was compared with the abnormal biopsy (Table 27).

The age range was 19 to 37 in patients with HPV type 16, 23 to 36 with type 18 and 27 to 40 in the absence of viral genomes.

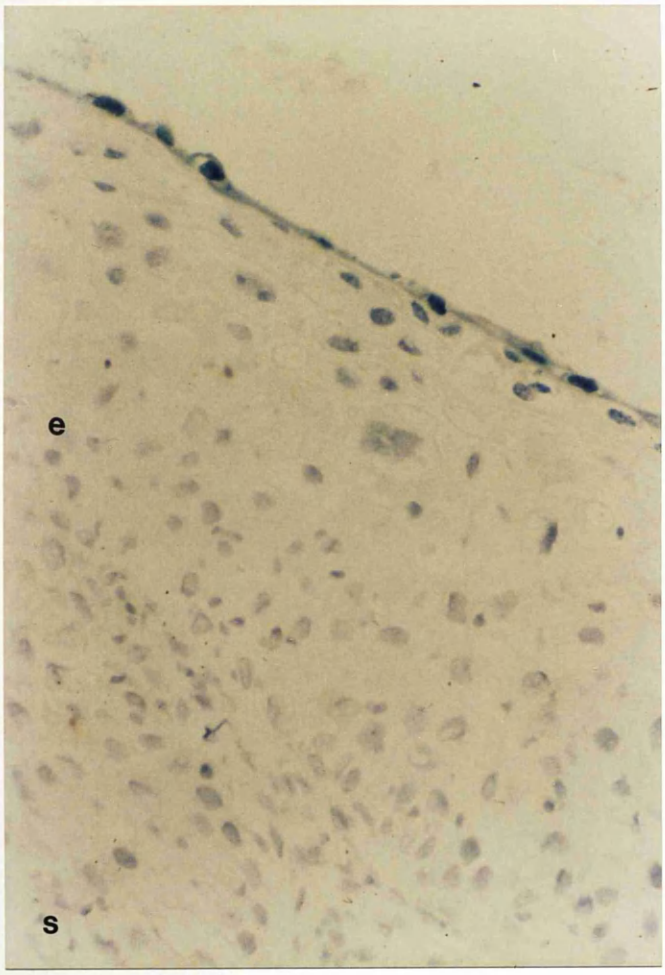

Figure 10.
Langerhans´ cells in CIN with HPV - HPV 18 positive.
No Langerhans´ cells are seen in the epithelium (e) or stroma (s) in this field; a few were seen in other areas of the lesion stained with NA 1/34.
Total magnification X 725.

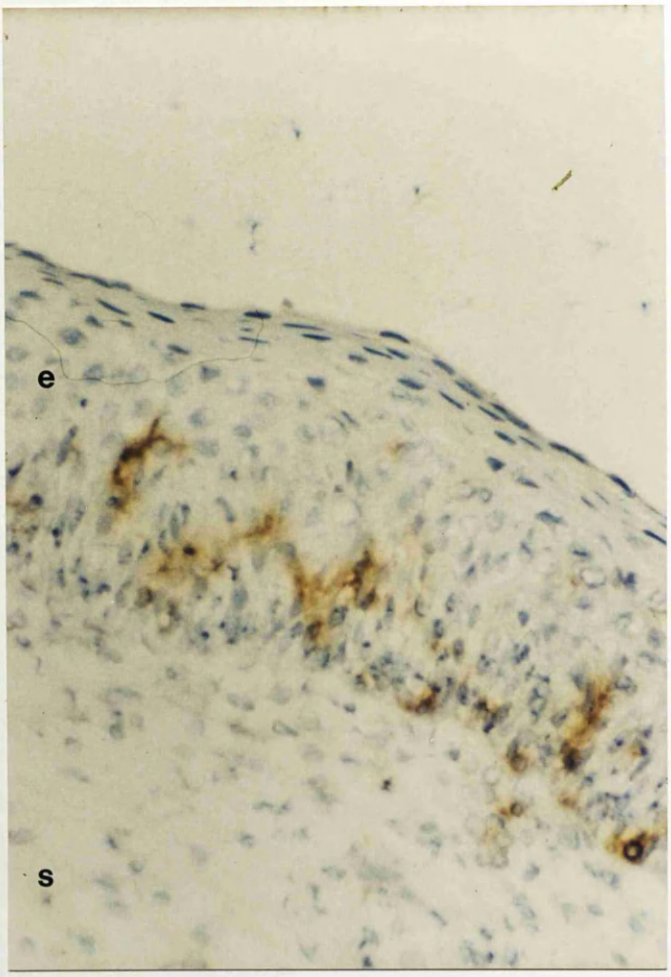

Figure 11.
Langerhans´ cells in CIN - HPV 16 and 18 negative.
Increased numbers of Langerhans´ cells, stained with NA 1/34, are seen in the epithelium (e). Their dendrites are stunted but they are still mainly found in the lower epithelium. (S=stroma)
Total magnification X 725.

Table 26. Relationship between density of HPV 16 and 18 genome expression in CIN and Langerhans' cells per 100 basal cells.

	Copy No /cell	N	Median	Wilcoxon	95% Confidence Intervals % (n-ab/n)	Numbers(n-ab)
HPV 16	10-50	6	-2.0	p > 0.05		-9.5 to 5.0
			-32.1	p > 0.05	-194.6 to 52.3	
HPV 18	10-50	5	3.0	p = 0.031		2.0 to 6.0
			33.3	p = 0.031	21.4 to 54.6	

Note. Median=median difference (normal-abnormal) in Langerhans' cell numbers. 95% CI for median & % difference are shown with Wilcoxon matched-pairs signed-ranks test of significance.

Table 27. HPV 16 and 18 positive and negative biopsies in relation to cervical Langerhans' cells per 100 basal cells.

	N	Median	Wilcoxon	95% Confidence Intervals % (n-ab/n)	Numbers(n-ab)
HPV 16 pos	9	0.5	p > 0.05		-5.5 to 4.0
		3.3	p > 0.05	-123.2 to 41.6	
HPV 18 pos	8	4.0	p = 0.007		2.5 to 5.5
		36.6	p = 0.007	27.4 to 48.1	
No HPV	6	-3.0	p = 0.03		-7.0 to -1.0
		-40.0	p = 0.03	-78.9 to -8.1	

See Note Table 26.

The Langerhans' cell morphology was altered in the biopsies with HPV 16, 18 and in pure CIN. The main changes were, as previously described, to the dendrites with these becoming stunted. Cell bodies appeared unaltered (Figures 10 & 11).

Using Scheffes' correction, at the 5% level, for multiple

comparisons no significant differences were noted in any component of the lymphocytic infiltrate between the HPV 16 and 18 positive lesions and HPV negative lesions.

The small numbers and consequent large confidence intervals precluded any more detailed analysis of the pattern of epithelial cell expression of MHC class II antigens and the lymphocytic infiltrate.

Comments

DNA-DNA hybridisaton is the most sensitive and specific way of detecting the presence of HPV infection. Slot blot detection of HPV genomes does have some limitations when compared with the 'gold standard' Southern blot technique. Murdoch et al. (1987) have shown that slot blot analysis offers low specificity if HPV DNA is present at low copy number but at high copy number (over 10 copies per cell) it is both sensitive and specific. A further limitation of the technique is that it is impossible to differentiate between combined HPV 16 and 18 infection.

The results presented in this chapter show that Langerhans' cells are specifically decreased in the presence of HPV 18 even in low copy numbers and in the presence of HPV 16 at higher copy numbers. The depletion of Langerhans' cells may be a function of both HPV subtype and copy number. The question of whether there is a critical threshold copy number cannot be answered by slot blot hybridisation as this method cannot distinguish between samples with very low copy numbers (<10 copies per cell) and negative samples. It may be that a progressive immunological failure in the afferent limb of the T-cell response results from increased HPV presence. Alternatively, local immunodeficiency may preceed HPV

infection facilitating the infection and subsequent cell transformation by viral gene products (Schneider-Gadicke & Schwartz 1986). No attempt was made to detect possible integration of the viral genome. The presence of preinvasive lesions which do not manifest HPV genomes emphasises that HPV is not likely to be the sole agent and many cofactors are implicated. They may not all involve depletion of Langerhans' cells.

Although the implication above is that Langerhans' cells are reduced secondary to HPV infection, reduction in Langerhans' cells may be permissive to HPV infection. Similar problems relate to the study of HPV infection, genital tract neoplasia and systemic T-cell function, with some authors suggesting that underlying immunocompromise is a factor (Seski et al. 1977; Seski et al. 1978; Carson et al. 1986). Separating the 'horse from the cart' in longitudinal studies of local immunity would be impossible because of the previously mentioned effects of biopsy.

Another method of pursuing the possible relationship of Langerhans' cells to the development of wart virus infection and cervical neoplasia is to examine populations at high risk of such changes. The following chapter deals with Langerhans' cells and the in-situ local immune response in immunosuppressed renal transplant patients. Examination of immune compromised women in renal failure and in pregnancy follow in Chapters 7 and 8.

Chapter 6

Langerhans' cells and local immunity in the cervix of renal transplant recipients

Chapter 6

Introduction

Immunosuppressed renal transplant recipients are at increased risk of neoplasia. Hoover & Fraumeni (1973) reported this risk to be of the order of 35 times for lymphomata and of the order of 350 times for reticulum cell sarcoma. Skin and lip cancers were also increased by a factor of four. In the female population they studied there were five cases with CIS. Porreco et al. (1975) found gynaecological malignancies in 21 of 224 female patients who developed tumours after transplantation. Cervical neoplasia was present in 18 patients, with two lesions being invasive carcinomata. They calculated the increased risk of cervical intraepithelial 'carcinoma' in this group compared with aged matched controls to be 14 fold. Abnormal cytology on routine smear examination was the commonest presentation. All patients had received azathioprine and prednisolone in their immunosuppressant regimes. It was postulated that 'immunologic surveillance' may be impaired, that oncogenic viruses may be potentiated or that the immunosuppressive regimes themselves may act as cofactors in tumour development.

The high prevalence of cervical disease (39 0f 529 tumours) was emphasised in a later paper (Penn 1978). Penn (1986) also found carcinoma of cervix was present in 1.8% of the females (14 of 777) in a series of 2150 patients with anogenital carcinomas. A field change was reported in some women with lesions in the squamous epithelium of the vulva, vagina and cervix.

In general the incidence of neoplasia appears to increase with length of time since transplantation (Penn 1978). In the

latest paper by Penn (1986) he noted that the genital tumours in females tended to occur later than the other tumours (88 versus 56 months) and at much younger ages than the same tumours in the general population.

The relationship between wart virus infection in the female genital tract and immunosuppression has been examined in two studies (Schneider et al. 1983; Sillman et al. 1984). Schneider et al. (1983) used koilocytosis as being diagnostic of wart virus infection and retrospectively reviewed all cervical material taken from 132 patients identified as at risk of cervical neoplasia, from a total of 472 transplant recipients over a 17 year period at the Medical College of Virginia. Eleven patients had histological evidence of wart virus infection with 5 also having intraepitheial disease (4.5%). One invasive squamous tumour was identified. The average duration between transplant and the diagnosis of neoplasia was 38 months. The 8.8% prevalence of cervical condyloma represents a 5.5 times increase compared with that detected in a routine screening programme (Morin et al. 1981). Schneider et al. (1983) proposed that immunosuppression be added to the list of factors associated with development of cervical neoplasia.

Sillman et al. (1984) studied the problem from another perspective. Twenty women with genital neoplasia on immunosuppressants (predominantly prednisolone for lupus erythematosis) were examined. Colposcopy with directed biopsy was performed in 19 of these patients. Histological examination showed koilocytosis in all the patients. Multifocal intraepithelial disease was also noted in 11 of the 20 patients. Confirmatory evidence of papillomavirus presence was obtained from electron microscopic and immunohistological studies (in 50% and 60% of

cases respectively). T-cell functions were also studied in the peripheral blood of 10 patients using the blastogenic response to phytohaemaglutinin (PHA) and pokeweed mitogen (PWM) as the means of assessment. An impaired T-cell response was found in nine of the patients. In addition examination of the circulating B-cell and T-cell subsets, using rosetting and immunofluorescence techniques, showed a decreased helper/suppressor ratio in nine patients. They also mentioned that those with the most aggressive neoplasia on follow up had the greatest depression of cellular immunity.

Despite the reports noting an increased prevalence of CIN and cervical carcinoma in renal transplant recipients, there are few studies where the at-risk patients have been systematically screened following the advice of Porreco et al. (1975):-

> 'all such patients require gynecologic examination before commencement of treatment and at regular intervals thereafter so that malignancies may be diagnosed at an early stage and treated effectively'

Considering the recommendations of Porreco et al. (1975) it seemed appropriate that this group of patients be screened looking for evidence of papillomavirus infection and cervical neoplasia. A previous study from the Western Infirmary, Glasgow by Cordiner et al. (1986) found five patients, screened colposcopically and cytologically, with CIN. No further screening had been conducted since that study. The local immune system of the cervix was also examined to detect any changes that could predispose to these changes, particulary considering the recent introduction of cyclosporin A to the immunosuppressant regime and its effects on

the T-cell subsets (Hess, Colombami & Esa 1986).

Recruitment of patients

Forty patients attending the renal transplant clinic at the Western Infirmary, Glasgow under the care of DrJ D Briggs and Dr M A Watson were asked for consent to a biopsy of cervix being taken during colposcopic examination. The study had Ethical Committee approval. All patients had had renal transplantation performed a minimum of six months before the examination and were taking systemic immunosuppressant therapy.

Two of the patients approached declined to participate.

Materials and methods

A cervical smear was taken in the usual manner before colposcopic examination. Punch biopsies of cervix were taken from areas identified as normal transformation zone from all patients who showed no evidence of aceto-white epithelium after the application of 5% acetic acid. In those with abnormal aceto-white epithelium two biopsies were taken from the abnormal area and where possible one from adjacent normal ectocervix. One of the biopsies taken from the colposcopically abnormal area was submitted for routine histological examination and used for comparison with frozen section histology. The other was used for immunohistochemical studies and processed and stained with the panel of antibodies described in Chapter 2.

Colposcopic examination of the vulva was also performed. The patients recruited also had a full dermatological examination by Dr D Tillman, Department of Dermatology, Western Infirmary, Glasgow. He examined the group looking especially for any evidence

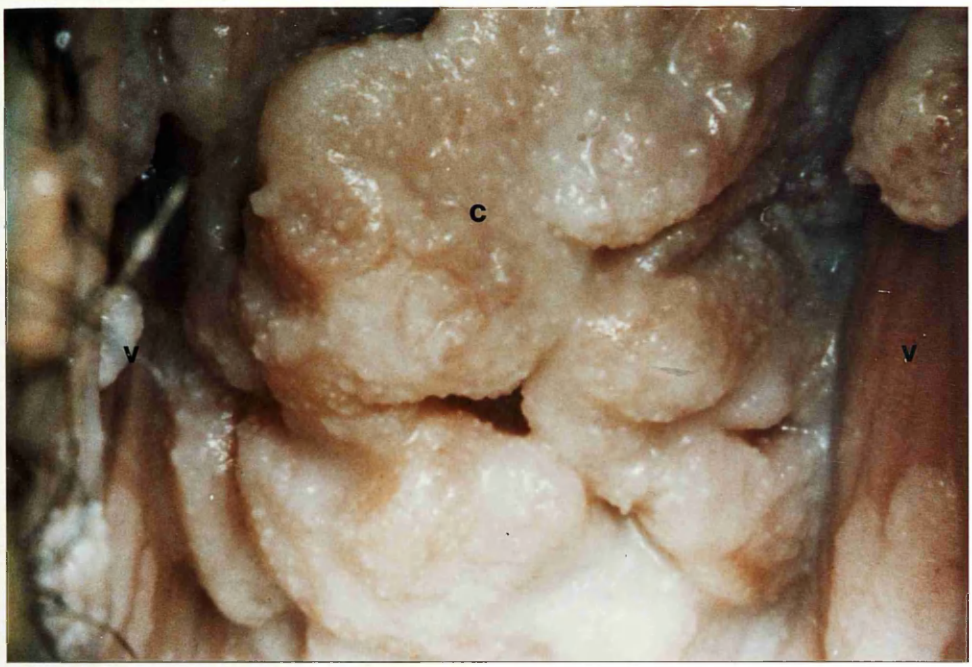

Figure 12.

Colpophotograph of genital warts in a renal transplant recipient. Multiple warts are seen on the cervix (c) and on the vagina (v).

of cutaneous pre-neoplastic, neoplastic and viral lesions.

Results

1) Patients

Patient details are shown in Appendix IIIc (p. 257). Twenty patients were receiving cyclosporin and prednisolone and 14 were receiving azathioprine and prednisolone at the time of the study. Three were on prednisolone only and one patient was on azathioprine and cyclosporin. The mean duration since transplant was 44 months (range 6-200 months). The mean age of the patients was 38.3 years (range 23-62). One patient had been included in the previous study by Cordiner et al. (1980).

2) Cytology

Dyskaryotic smears were reported in three patients. A further four patients had smears with features suggestive of viral infection only. Evidence of monilia was found in eight smears and trichomonas vaginalis infection in two.

Eleven of the patients had never had a previous smear and a further nine patients had not had a smear within five years.

3) Colposcopy

Aceto-white epithelium was identified in 14 patients. A colpo-photograph of multiple warts on the cervix and vagina of one patient is shown in **Figure 12.** Vulval warts were observed in two patients.

4) Histology

The results of histology from the biopsies taken from the aceto-white epithelium showed CIN I in two patients, CIN II in three and CIN III in one patient. All had histological associated features of wart virus infection. A further six patients had

histological evidence of HPV infection only. The remaining two biopsies were negative.

5) Skin survey

Cutaneous warts were found in 65% of the patients examined. No cases with cutaneous neooplasia were detected.

6) Immunohistochemical studies

As mentioned above the transplant population do not receive a uniform immunosuppression regime. There are essentially two groups, one receiving azathioprine and prednisolone and the other receiving cyclosporin A and prednisolone. The mean and 95% confidence intervals for the numbers of Langerhans' cells per 100 basal cells and per unit area of the typical transformation zone are shown in Table 28 in association with the immunosuppressant regime used.

Table 28. Analysis of variance of Langerhans' cells per 100 basal cells and per unit area of cervical epithelium in the typical transformation zone of patients following renal transplantation.

a) per 100 basal cells

Regime	Cases	Mean	SD	95% Confidence Interval
Cyclo	17	4.1	2.7	2.7 to 5.5
Aza	9	3.9	2.4	2.1 to 5.7
Total	28	4.1	2.7	3.1 to 5.2

(inc. 2 Pred only)

b) per unit area

Regime	Cases	Mean	SD	95% Confidence Interval
Cyclo	17	4.36	5.61	1.37 to 7.34
Aza	9	6.50	4.45	2.78 to 10.47
Total	28	4.87	5.12	5.30 to 8.19

(inc. 2 Pred only)

Note. Cyclo=Cyclosporin/Prednisolone

Aza =Azathioprine/Prednisolone

Totals include 2 patients on Prednisolone (Pred) only

Mean, SD & 95% CI of mean for each group are shown.

Using Scheffe's test for multiple comparisons, at the 5% level, the Langerhans' cells in both groups are significantly less than the numbers seen in normal transformation zone (mean 8.0 & 12.43; 95% CI 6.7-9.2 & 11.07-31.80 respectively for counts per 100 basal cells and by image analyser, Tables 6 & 7 p. 100). There is no significant difference in Langerhans' cells comparing the azathioprine with the cyclosporin group.

In addition to the decrease in Langerhans' cells, the other

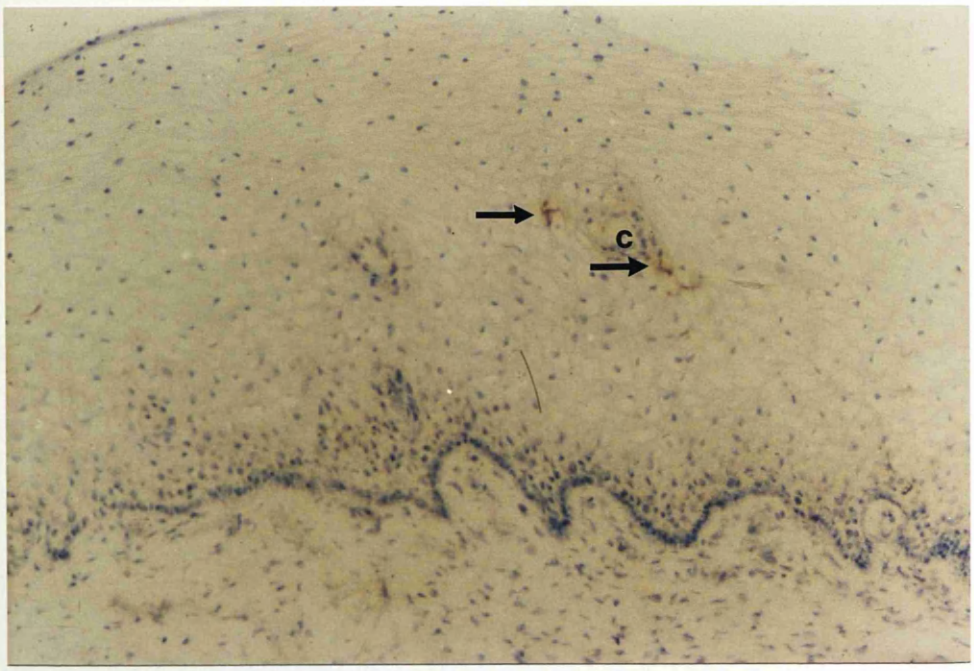

Figure 13.

Langerhans´ cells in the typical transformation zone of a renal transplant recipient.

The Langerhans´ cells in the epithelium (e) are reduced in number; two cell bodies are indicated, stained with NA 1/34, close to a capillary (c).

Total magnification X 362.

main difference was a significant decrease in the helper T-cell infiltrate in percentage and numerical terms in the stroma and epithelium of the transformation zone of both groups of transplant patients compared with the results obtained from the normal patients presented in Chapter 3 (Table 10 p.105). Details are shown, in Table 29, of the mean, SD and 95% confidence intervals for the relevant variables. Scheffe's correction at the 5% level was used to make the multiple comparisons. No difference in percentage or number of helper cells was detected comparing the group on cyclosporin with the group on azathioprine.

Photomicrographs of the Langerhans' cells and helper T-cell infiltrate in the transformation zone in one of these patients are shown in **Figures 13 & 14a** and comparisons with normal cervix in **Figures 2 & 14b.**

Table 29. Comparison of Helper T-cell (CD 4) infiltrate in typical transformation zone of patients on two immunosuppressant regimes folllowing renal transplantation.

Area/Marker	Regime	Cases	Mean	SD	95% Confidence Interval
CD 4 epith (%)	Cyclo	15	12.7	11.2	6.5 to 19.0
CD 4 epith (%)	Aza	8	9.0	9.4	1.1 to 16.7
CD 4 stroma(%)	Cyclo	15	51.3	23.5	38.3 to 64.3
CD 4 stroma(%)	Aza	8	43.7	21.9	25.4 to 62.1
CD 4 epith (N)	Cyclo	15	16.3	11.5	10.0 to 22.7
CD 4 epith (N)	Aza	8	9.9	12.3	-0.4 to 20.2
CD 4 stroma(N)	Cyclo	15	162.4	94.1	110.3 to 214.6
CD 4 stroma(N)	Aza	8	131.6	115.2	35.3 to 227.9

See also Note Table 10 p. 105. %=((No. of positive cells X 100) /total infiltrate (CD 3)). Cyclo=cyclosporin/prednisolone
Aza=azathioprine/prednisolone

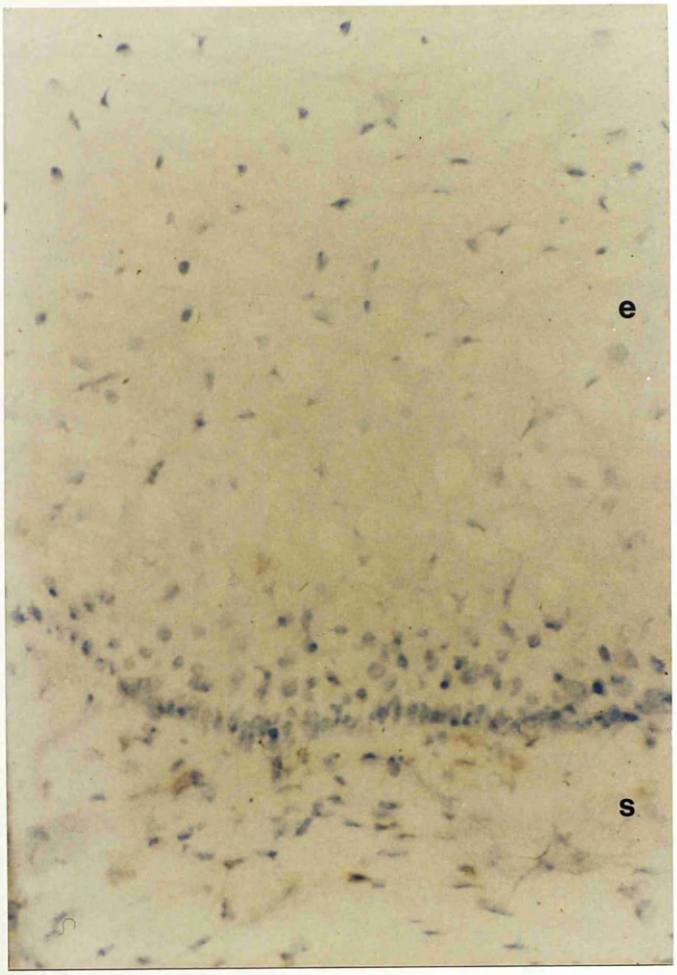

Figure 14a.
Helper T-cells in the typical transformation zone of a renal transplant recipient.
The epithelium (e) and narrow subepithelial band (see Figure 14b) are shown with markedly reduced numbers of Leu 3a positive cells in the stroma (s).
Total magnification X 725.

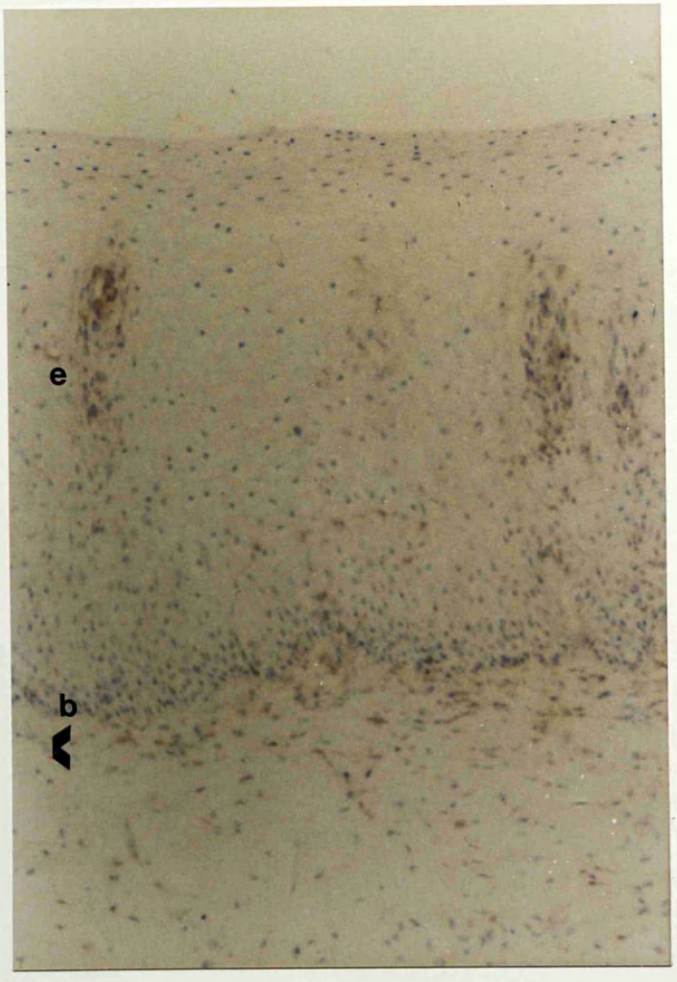

Figure 14b.
Helper T-cell infiltrate in normal transformation zone.
Leu 3a positive cells are seen in the epithelium (e) and especially in the narrow band indicated immediately below the basement memrane (b)(see Figure 14a).
Total magnification X 362.

Despite the changes in the helper T-cell infiltrate there was no significant differences in the percentage or numbers of cytotoxic T-cells comparing the two transplant groups and the non-compromised patients (Chapter 3). There were no differences in any other component of the infiltrate comparing the patients on cyclosporin with those on azathioprine. The quantitative analysis of the infiltrate in the normal transformation zone from the whole group (including two patients on prednisolone alone) are shown in Table 30. Graphical comparison of the results presented in Tables 10 & 30 is shown in Appendix IV.

Table 30. Analysis of variance of T cell counts per unit area in the typical transformation zone of renal transplant recipients.

Marker/Area	Cases	Mean	SD	95% Confidence Intervals
CD 3 epith (N)	25	139.0	76.9	107.2 to 170.7
CD 8 epith (N)	25	142.4	106.9	98.3 to 186.6
CD 8 epith (%)	25	99.6	21.9	90.6 to 108.7
CD 4 epith (N)	25	14.3	11.6	9.6 to 19.1
CD 4 epith (%)	25	11.2	10.2	7.0 to 15.4
CD 3 stroma (N)	25	300.6	135.6	244.6 to 356.5
CD 8 stroma (N)	25	180.2	102.1	138.0 to 222.3
CD 8 stroma (%)	25	60.2	17.7	52.9 to 67.5
CD 4 stroma (N)	25	149.6	100.5	108.1 to 191.1
CD 4 stroma (%)	25	47.8	23.2	38.2 to 57.3

See Note Table 10 p. 105.

The number of renal transplant recipients with CIN were small but represent over 15% of the patients screened. There was no

significant difference in the numbers of Langerhans' cells or in any component of the lymphocytic infiltrate between this group and those with CIN and histological features of HPV infection presented in Chapter 4. Four of the six patients were receiving azathioprine and prednisolone and one cyclosporin and prednisolone. Fisher's exact probability test showed no significant difference (p=0.079) between the azathioprine patients (4/14) and the cyclosporin patients (1/20). The remaining patient was receiving both cyclosporin and azathioprine.

Comments

Prednisolone forms the basis of the immunosuppressant regimes post transplantation being combined with either cyclosporin or azathioprine.

Prednisolone itself has been shown to decrease Langerhans' cell density. This is seen following topical application to the skin (Breathnach & Katz 1986; Halliday, Knight & Muller 1986) rather than after systemic use (Belsito et al. 1982). Prednisolone has also been shown to decrease Langerhans' cell surface markers and impair function by decreasing class II surface antigens (Belsito et al. 1982; Berman et al. 1983; Aberer et al. 1984). The surface T-6 antigens appear not to be affected (Berman et al. 1983). The effects of other immunosuppressants, including azathioprine and cyclosporin, applied topically and administered systemically to mice showed similar effects (Halliday et al. 1986) when ATP-ase is used as the Langerhans' cell marker. The suggestion is that reduction in Langerhans' cells results from the marrow supressant effects of the various agents.

Sontheimer et al. (1984) presented evidence from a small

number of cases that showed the Langerhans' cell population was significantly reduced in skin of patients following renal transplantation and maintained on prednisolone and azathioprine compared with matched controls. They also found the antigen-presenting capacity of the Langerhans' cell was excessively reduced when compared to the mononuclear cells of the peripheral blood. The changes were most marked on the Langerhans' cells from sun exposed areas where pre-malignant and squamous epithelial tumours develop most commonly in this group of patients.

The introduction of cyclosporin to the immunosuppressant regime followed reports that its use resulted in less graft rejection and less myelotoxicity than azathioprine (Hess et al. 1986). Its main disadvantage is its effect on T-cells especially decreasing helper T-cell function (Hess et al. 1986). The dual effect of a cyclosporin and prednisolone regime may theoretically result in a greater propensity to neoplastic transformation or viral infection because both the antigen presenting cell and its receptor T-cell are reduced in number and in function. The possibility of promoting neoplastic change reflects one of the worries in long term use of cyclosporin. No previously published study has reported any cases of CIN in patients receiving cyclosporin. The findings of this study do not substantiate this theory, indeed comparing the observed and expected cases in the azathioprine and cyclosporin groups showed greater numbers of patients in the azathioprine group. This is not wholly unexpected since the number of malignancies increases with time from transplantation and cyclosporin is usually given for six months during the first year. It is reassuring that cyclosporin does not appear to substantially effect the Langerhans' cells or helper

cells when compared with azathioprine, perhaps emphasising that the effects of both these immunosuppressants on local epithelial immunity are secondary to bone marrow suppression.

The effects of immunosuppression on the prevalence of abnormalities in the cervix is not confined to the patients which have had a renal transplant. A high incidence of abnormal cervical cytology has been reported in women treated with azathioprine for systemic lupus erythematosis (Nyberg, Eriksson & Westberg 1981). Azathioprine has also been implicated in the development of CIN in patients treated for chronic active hepatitis (Schramm 1970; Norfleet & Sampson 1978).

Routine cervical screening of the female renal transplant recipient has been recommended for some time (Porreceo et al. 1975). Cytological screening only has been performed by some groups (Kay, Frable & Hume 1970; Ingoldby et al. 1980). Kay et al. (1970) reported two cases of intraepithelial neoplasia developing after transplantation in a group of 28 patients. Ingoldby, et al. (1980) followed a group of patients undergoing renal transplant longitudinally and found pre-existing dysplasia in two of 38 patients prior to transplant and no new cases on follow-up.

There are four reported studies where colposcopy and cytology have been performed. Donohue (1974) reported abnormal cervical cytology or colposcopy in nine of 35 patients in his series although no CIN was found. Husslein, Breitenecker & Tatra (1978) found abnormal cytology in only two of 29 patients although 14 had abnormal colposcopic appearances. Dysplastic features were present in two cases with a case of endometrial carcinoma being found. The photomicrograph presented as Figure 1 in this paper shows changes more in keeping with HPV infection, koilocytosis and multinucleate

cells being present, rather than CIN. Cordiner et al. (1980) found five cases of CIN in 26 patients examined, but, if cytology alone had been used only two of these cases would have been detected. They make no mention of the number of cases where HPV infection only was a finding on cytology or colposcopy. MacLean et al. (1986) found no cases with CIN in 24 transplant recipients but noted genital HPV infection in 21%. They also studied seven patients on haemodialysis and found one patient with vulval intraepithelial neoplasia and another with an atypical transformation zone on colposcopy but negative histology and cytology.

In this current study the high prevalence of CIN has been further substantiated in women following renal transplantation with a prevalence rate of almost 16% being found. In an attempt to examine whether this was likely to reflect a true incidence rate a separate group of patients, prior to possible transplantation, was studied using the same screening techniques (Chapter 7).

The results presented in this chapter confirm those of others (Ingoldby et al. 1980; Schneider et al. 1982; Sillman et al. 1984; MacLean et al. 1986); Gassenmaier et al. 1986; Rudlinger et al. 1986) that there is also a high prevalence of HPV infection in the genital tract and skin of renal transplant recipients. In the cervix evidence of HPV infection, with or without CIN, was found in greater than 30% of cases.

It is also clear from this study that cytology alone does not appear to be an effective screening method in this high risk population with prevalence of CIN >10%, with as many as 60% of cases giving false negative results (Cordiner et al. 1980). This high false negative rate may be due to the high rate of

concomitant infection (protozoal, viral or fungal) as noted in this study and in others (Ingoldby et al. 1980), but it could possibly reflect that exfoliation of cells in this group is reduced. It is also clear that available cytological services are not being applied to an easily identified high-risk group that frequently attend other hospital clinics with over 25% of patients never having had a smear and a further 25% not being screened within five years. An appropriate screening regime would be to recommend that cervical cytology becomes part of the pre-transplant work-up and that following transplantation yearly colposcopy and cytology is performed.

It is possible that the reduced Langerhans' cell numbers are contributing to the high prevalence of CIN and high prevalence of HPV infection with or without CIN in the group of female transplant recipients examined. They also show a high prevalence of cutaneous papillomavirus infection. Others have indicated that as many as 80% will develop cutaneous warts on prolonged follow-up. The work of Sontheimer et al. (1984) and Ten Berge et al. (1981) suggests that functional aberrations in the Langerhans' cell may also be implicated.

Chapter 7

Langerhans´ cells and local immunity in the cervix of women in renal failure

Chapter 7

Introduction

Following the observations made in Chapter 6, this chapter and the following one extends the study to include examination of Langerhans' cells and the lymphocytic infiltrate in the cervix of other naturally immunocompromised patients, some of whom are reported to show increased prevalence rates of cervical epithelial abnormalities.

The group examined in this chapter are patients attending with chronic renal failure (CRF), for haemodialysis (Haemo) or peritoneal (CAPD) dialysis. They were studied in order to quantify the Langerhans' cells and lymphocytic infiltrate in the cervix prior to transplantation, since within the time constraints of the study, longitudinal examination of patients before transplantation through to post-transplant follow-up was not possible. In addition these patients have received little consideration in cervical screening regimes despite the known risks following transplantation and the reported increased risk of neoplasia in patients undergoing long- term intermittent dialysis (Linder, Farewell & Sherrard 1981). They were therefore used as a control group for comparison with the transplant patients, colposcopic and cytological screening being performed to determine the prevalence of abnormal cervical epithelium before transplantation. Another aim was to determine whether the high prevalence of CIN and HPV seen after transplantation reflects the role of renal disease or immunosuppression by drugs, especially since uraemia, itself, is asociated with depressed immune function (Lawrence 1965; Dobbelestein 1976).

Patients

Fifteen patients attending the renal clinic under the care of Dr JD Briggs were recruited. All were part of group of both male and female patients being investigated by Dr K McKerrow, Dept of Dermatology, University of Glasgow. His studies included evaluation of the Langerhans' cells and immunocompetent cells in skin and in peripheral blood. Patients with multisystem autoimmune disease or who had rejected a kidney transplant were excluded. Those treated conservatively had a serum creatinine value of less than 500 micromoles per litre.

Six patients on continuous ambulatory peritoneal dialysis, two patients on haemodialysis and seven patients in chronic renal failure were studied. All patients had been in their respective subgroup for over six months. Ethical committee approval had been given for taking biopsies of skin and of cervix. Informed consent was obtained from all patients. Patient details are shown in Appendix IIId (p. 258).

Materials and methods

Routine cervical cytology was taken from the patient before colposcopic screening. Directed punch biopsies were taken from normal transformation zone for immunohistochemical studies. Any colposcopically abnormal areas were also biopsied and a second specimen sent for routine pathology.

Frozen sections were obtained and the same monoclonal antibodies and immunoperoxidase method used as described previously.

Results

1)Langerhans' cells and lymphocytic infiltrate

Analysis of variance between the individual sub-groups showed no significant differences in the numbers of Langerhans' cells or in the components of the lymphocytic infiltrate. Close inspection of the results did however suggest that the Langerhans' cells may be reduced in the sub-group with untreated renal failure returning towards normal levels following peritoneal dialysis or haemodialysis (Table 31). There are only two patients in the latter sub-group making any comparisons on such small numbers invalid; they are, however, included in the table.

Table 31. Langerhans' cells per 100 basal cells in the typical transformation zone of three groups of uraemic patients.

Group	N	Mean	SD	95% Confidence Interval
CAPD	6	7.0	3.6	3.2 to 10.8
CRF	7	5.3	2.7	2.8 to 7.8
Haemo	2	6.0	2.8	-19.4 to 31.4
Total	15	6.1	3.0	4.4 to 7.7

Note. CAPD =continuous ambulatory peritoneal dialysis
CRF =chronic renal failure
Haemo=haemodialysis

The results from the 15 patients were pooled for comparison with other study groups.

Multiple comparisons of means using Scheffe's correction at the 5% level shows no significant differences between the components of the infiltrate (see Table 10 p.105 & Appendix IV) or Langerhans' cells when compared with the results from the typical

transformation zone (mean 8.0(/100 basal cells); 95% CI 6.7-9.2) presented in Chapter 3. The numbers of Langerhans' cells are greater in the pre-transplantation patients (Table 31) than in the post transplant group (mean 4.1(/100 basal cells); 95%CI 3.1-5.2).

Compared with the results presented in the previous chapter, (Table 30 p.161, Appendix IV) there are no significant differences in the number of cytotoxic T-cells in either the epithelium or stroma, but there is a significant reduction in the cytotoxic T-cell infiltrate in both areas in percentage terms in the uraemic patients (Table 32). This arises due to the reduction in the number of helper T-cells seen in the transplant patients.

Table 32. Analysis of variance of T cell counts per unit area in the typical transformation zone of women in 'untreated' renal failure, on peritoneal or haemodialysis.

Marker/Area	Cases	Mean	SD	95% Confidence Intervals
CD 3 epith (N)	9	160.4	84.3	95.6 to 225.3
CD 8 epith (N)	9	136.7	76.4	77.9 to 195.4
CD 8 epith (%)	9	85.9	8.6	79.3 to 92.5
CD 4 epith (N)	9	35.9	37.7	6.9 to 64.9
CD 4 epith (%)	9	18.8	11.3	10.0 to 27.5
CD 3 stroma (N)	9	419.8	236.9	237.7 to 601.9
CD 8 stroma (N)	9	164.4	104.0	84.4 to 244.3
CD 8 stroma (%)	9	38.6	9.7	31.1 to 46.0
CD 4 stroma (N)	9	303.7	194.2	154.4 to 453.0
CD 4 stroma (%)	9	70.9	11.9	64.7 to 86.0

See Note Table 10 p. 105.

2)Cytology and Colposcopy

Similar percentages of the pre-transplant and transplanted patients had never had a previous smear (4/15 versus 9/38). No evidence of cervical neoplasia was found in the uraemic patients. One patient on CAPD had colposcopic features of wart virus infection and this was confirmed on histology of the punch biopsy.

Comments

It is difficult to make any comment on the apparent finding of reduced numbers of Langerhans' cells in the patients with untreated renal failure in view of the small number of patients involved. Should this result be confirmed in larger numbers of patients it would be tempting to suggest the reduction follows effects on the bone marrow. In chronic renal failure there is marrow suppression but following treatment the suppressive effects are less, with a possible resultant return towards normal numbers of Langerhans' cells in cervix. Following transplantation there is exogenous marrow suppression with the various immunosuppressants and the Langerhans' cell numbers again decrease.

There is no evidence from this part of the study that the pre-transplant patients represent a group at high risk of cervical neoplasia or of HPV infection, although the implication from the earlier work presented is that patients with chronic renal failure could be a further high risk group worthy of more in depth investigation. MacLean et al. (1986) found no cases of cervical neoplasia in seven patients, but vulval neoplasia was found on colposcopic examination of one patient and abnormal findings were noted in another although cytology and histology were negative. The findings of Linder et al. (1981) suggest that a cautious

approach be adopted in interpreting the results from this small number of patients.

Routine cytological screening of the patients showed few inflammatory smears and, although conclusive evidence is lacking, the implication is that cervical cytology alone is probably adequate in these patients. Recruitment of larger numbers of patients to a cytological and colposcopic screening programme could substantiate this.

It is difficult to be certain that the increased prevalence of HPV infection and CIN seen following renal transplantation and commencement of immunosuppressant regimes represent true increased incidence. Screeening all patients with chronic renal failure entering the appropriate clinics could answer the question. Inevitably some of these patients will receive peritoneal dialysis and haemodialysis, and some will ultimately receive a donor kidney. The actual numbers of such patients are relatively small but without long-term follow up the true incidence rates will not become apparent.

It is hoped to develop this plan of approach at the Western Infirmary with all patients entering the transplant programme receiving a cervical smear before transplantation and all those attending the relevant clinics who have never had a smear to be adequately screened in the first instance. In an already busy routine colposcopy service it could be argued that it is a misuse of resources to use the service as a screening tool, however,in women with chronic renal failure, on peritoneal or haemodialysis cytology is likely to be adequate.

Chapter 8

Langerhans' cells and local immunity in the cervix of women in early pregnancy

Chapter 8

Introduction

Pregnancy represents a state of endogenous immunosuppression with the mother exhibiting tolerance to the fetal allograft. The incidence of neoplasia in pregnancy is of the order of one per thousand pregnancies (Potter & Schoeneman 1970). Few tumours are known to be adversely affected by pregnancy eg malignant melanoma and breast carcinoma. However, the changes occuring in the genital tract and in the breast in response to pregnancy may influence subsequent management. The most common tumour of the lower genital tract in pregnancy is carcinoma of cervix with an incidence around 1 in 2200 pregnancies (Hacker et al. 1982). The reported rate for abnormal cervical cytology in pregnancy is about five fold greater than in the non-pregnant population (Shepherd 1984). The opportunity to perform cervical cytology in this 'captive' population probably contributes to the overall detection of carcinoma-in-situ in 1 in 750 pregnancies.

As mentioned previously the main immune response to viral infections and to tumours is thought to be T-cell mediated. This is the same mechanism responsible for graft rejection. An idea of in-vivo impaired T-cell function is obtained when the clinical course of viral infections in pregnancy are considered. Vulval condylomata proliferating throughout pregnancy and regressing spontaneously in the peurperium serve as one example. In-vitro studies of cell mediated immunity in human pregnancy relate to results of the mixed lymphocyte reaction (MLR). This reaction depends on the ability of specifically sensitised (maternal) lymphocytes to undergo cell division and proliferation in response

to foreign (fetal or paternal) antigens. Purtillo, Halgren & Yunis (1972) reported impaired T-cell blast response to phyto-haemaglutinin (PHA) when lymphocytes from peripheral blood of pregnant women were compared with the response of non-pregnant patients. Hsu (1974) performed similar experiments but found that the impaired response to PHA was dependent on the lymphocyte culture being performed in serum from the pregnant patient. When culture was performed in reference serum the impaired response was no longer significant when compared with controls, implying that in pregnancy there were inhibitory circulating factors affecting normal lymphocyte function.

Since pregnancy also produces a state of naturally compromised T-cell function that seems to predispose to cervical epithelial abnormalities, the Langerhans' cell numbers and lymphocyte sub-populations in cervix were studied in a small group of women in early preganncy.

Recruitment of Patients

Pregnant patients undergoing termination of pregnancy gave consent to a punch biopsy of cervix being taken during the procedure. Ethical Committee approval was obtained prior to collection of biopsies. Twenty patients agreed, all were under the care of Dr A B MacLean in the Western Infirmary, Glasgow and all had negative cervical cytology on smears taken in the outpatient department earlier in their pregnancies. Details of their ages, parity and time since last menstrual period are shown in Appendix IIIe (p. 259). It was deemed unjustifiable to take biopsies from patients in later continuing pregnancy because of the risk of bleeding caused by the increased vascularity of the cervix.

Patients presenting with abnormal cytology in pregnancy are not usually biopsied, unless microinvasion or frank carcinoma is suspected on colposcopy, because of the risk of bleeding and are hence not studied in this chapter.

Normal histology was confirmed on haematoxylin and eosin staining of the frozen sections cut from the punch biopsies taken.

Materials and methods

The punch biopsies taken were mounted and frozen as previously described before cryosections were cut. The same indirect immunoperoxidase technique and monoclonal antibodies were used as throughout this study.

Results

There were no differences in the number of Langerhans' cells per 100 basal cells counted in the transformation zone comparing the pregnant patients (mean 7.3; SD 2.7; 95% CI 6.0 to 8.6) with the normal (mean 8.0; 95%CI 6.7-9.2) and pre-transplant patients (mean 6.1; 95%CI 4.4-7.7). Significantly greater numbers were found in the pregnant patients when compared with the renal transplant recipients (mean 4.1; 95%CI 3.1-5.2). Scheffe's correction at the 5% level was used for the multiple comparisons performed.

Analysis of variance and multiple comparisons using Scheffe's correction on the lymphocytic infiltrate showed significantly lower percentages of cytotoxic cells in the epithelium of the pregnant patients compared with normal (Table 10, p. 105) and with the uraemic patients (Table 32, p. 171). The percentage of stromal cytotoxic T-cells was also less compared with the transplant

patients. These features can be explained by the significantly reduced number of helper cells seen in the latter group resulting in greater percentages of cytotoxic cells. The results of quantitative assessment of the infiltrate are shown in Table 33.

Table 33. Analysis of variance of T cell counts per unit area in typical transformation zone of women in early pregnancy.

Marker/Area	Cases	Mean	SD	95% Confidence Intervals
CD 3 epith (N)	20	182.8	84.3	143.4 to 222.2
CD 8 epith (N)	20	140.2	59.5	112.4 to 168.0
CD 8 epith (%)	20	78.6	13.5	72.3 to 85.0
CD 4 epith (N)	20	43.3	39.7	24.7 to 61.9
CD 4 epith (%)	20	22.6	15.4	15.4 to 29.9
CD 3 stroma (N)	20	337.5	128.0	277.6 to 397.5
CD 8 stroma (N)	20	137.3	92.8	93.9 to 180.7
CD 8 stroma (%)	20	40.6	21.2	30.7 to 50.6
CD 4 stroma (N)	20	204.2	111.8	151.8 to 256.5
CD 4 stroma (%)	20	62.6	25.6	50.7 to 74.8

See Note Table 10, p. 105.

No differences were apparent between the pregnant patients and the pre-transplant patients for any variable examined.

There was no significant difference in the numbers of cytotoxic cells in the epithelium or stroma comparing results from the transformation zone of the pregnant patients, with the normal controls (Chapter 3), transplant (Chapter 6) or the uraemic patients (Chapter 7). Graphical comparisons of the lymphocytic infiltrate in the pregnant patients with the normal, uraemic and renal transplant recipients are shown in Appendix IV.

Comments

The results of investigations in pregnancy show no significant changes in the number of Langerhans' cells in the transformation zone of cervix and only minor alterations in the helper/cytotoxic cell ratio when compared with the normal non-pregnant population. The small changes are perhaps an indication that there are greater alterations in later pregnancy when any effect on the marrow would be expected to be more obvious. The drawbacks of pursuing this type of investigation into later pregnancy have been mentioned previously.

The comparison with the transplant group shows no difference in the number of cytotoxic cells present, but the significantly reduced number of helper cells in the exogenously suppressed renal transplant recipients produces alterations in the helper/cytotoxic ratio.

The higher prevalence of abnormal smears and CIN in pregnancy could be explained by opportunistic cervical screening in a fairly captive population, many having their first ever smear. Because of its transient nature it is difficult to imagine that a truly higher incidence of CIN occurs during pregnancy. Although marked immunological changes develop in very early pregnancy, this is not reflected in the cervical epithelium towards the end of the first trimester. The short term immunosuppression of pregnancy could result in some lesions developing or progressing, but with the completion of pregnancy it would be expected that many of these lesions should regress. This is indeed the clinical course of many genital warts in pregnancy.

The renal transplant recipient is not subject to transient

immunosuppression but to long-term immunosuppression and it seems likely that the increased incidence of CIN is secondary to sustained effects on the bone marrow.

Chapter 9

Langerhans' cells and local immunity in carcinoma of cervix

Chapter 9

Introduction

Routine histological methods offer little in the way of predicting the subsequent behaviour of tumours. The marked lymphocytic infiltrate seen in some tumours, eg seminoma of testis and medullary carcinoma of breast, stimulated speculation that the lymphocytic infiltrate may be of some prognositc significance. Underwood (1974) reviewed the published literature and noted that positive prognostic correlation had been reported in carcinoma of cervix for tumour infiltrates of eosinophils (Schoch 1926) and mast cells (Graham & Graham 1966).

The use of monoclonal antibodies permits the examination of various facets of the immune response including the lymphocytic infiltrate in much greater detail than was possible in earlier studies. In-situ studies of Langerhans' cells and the local immune response to tumours derived from squamous epithelium have almost totally concentrated on skin tumours especially basal cell and squamous carcinoma (Synkowski, Schuster & Orlando 1985; Gatter et al. 1984; Murphy et al. 1983; Guillen, Day & Murphy 1985).

Squamous carcinoma of cervix is considered by most authorities to be the result of progression of a minority of pre-invasive lesions (see introduction) and would appear to be a suitable end point of a study which has concentrated on the local immune response to CIN.

Patients

Specimens from 10 patients undergoing radical hysterectomy with node dissection were collected. They were under the care of

Dr J A Davis, Stobhill General Hospital, Glasgow. All had had prior colposcopic assessment and biopsy confirmation of invasive squamous carcinoma of clinical stage Ib or IIa. None had had prior cone biopsy performed. Details of their age and parity, in addition to the findings from the routine histology from the gross specimen including tumour differentiation, extent of local spread and nodal metastases, are shown in Appendix IIIf (p. 260).

Methods

Blocks of cervix were cut from the fresh specimen following amputation of the cervix and removal of the vaginal cuff. The blocks were taken from the margin of tumour, where possible to include the cervical canal and adjacent normal cervix and in such a manner to cause the least disruption to the specimen. Blocks were cut with the pathologist responsible for reporting the routine histology in attendance.

The method of orientating, freezing and cutting the blocks was similar to that described for the study of blocks cut from 'normal' patients undergoing hysterectomy in Chapter 2. Every fifth section was stained with haematoxylin and eosin for comparison with the sections taken from the fixed specimen.

The requirement for a reduced concentration of hydrogen peroxide was most evident in the large sections obtained from these tumours due to the presence of large amounts of endogenous peroxidase activity which caused some sections to 'float'. The same panel of monoclonal antibodies as described in previous chapters was used.

The Langerhans' cells were counted per 100 basal cells in the adjacent normal cervix and per high power field (X400

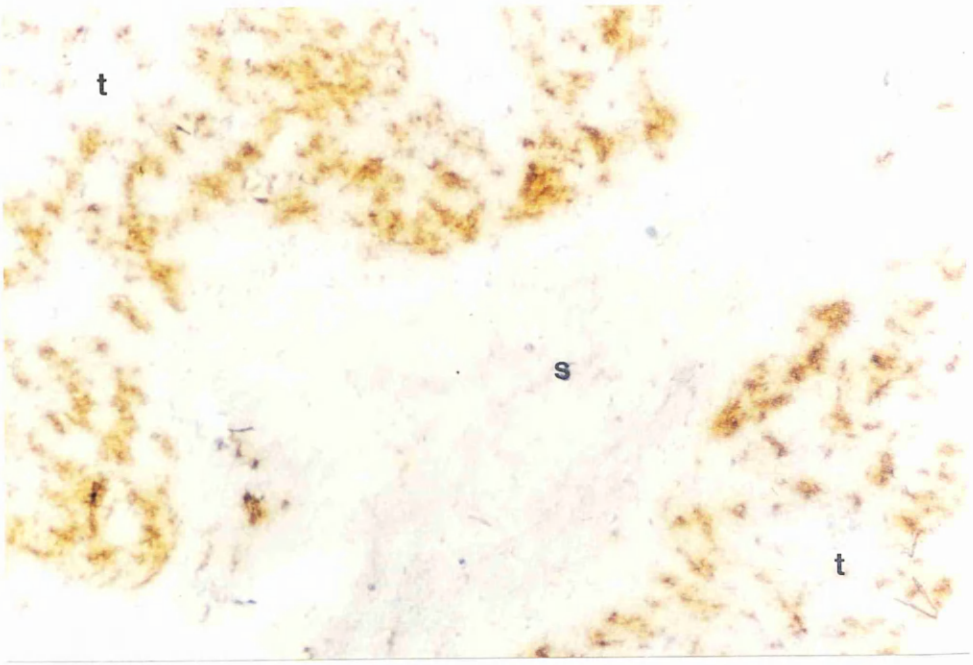

Figure 15.

Langerhans´ cells in cervical carcinoma - abundant cells.

Langerhans´ cells stained with NA 1/34 are seen in large numbers in the tumour (t) being absent from the stroma (s).

Total magnification X 145.

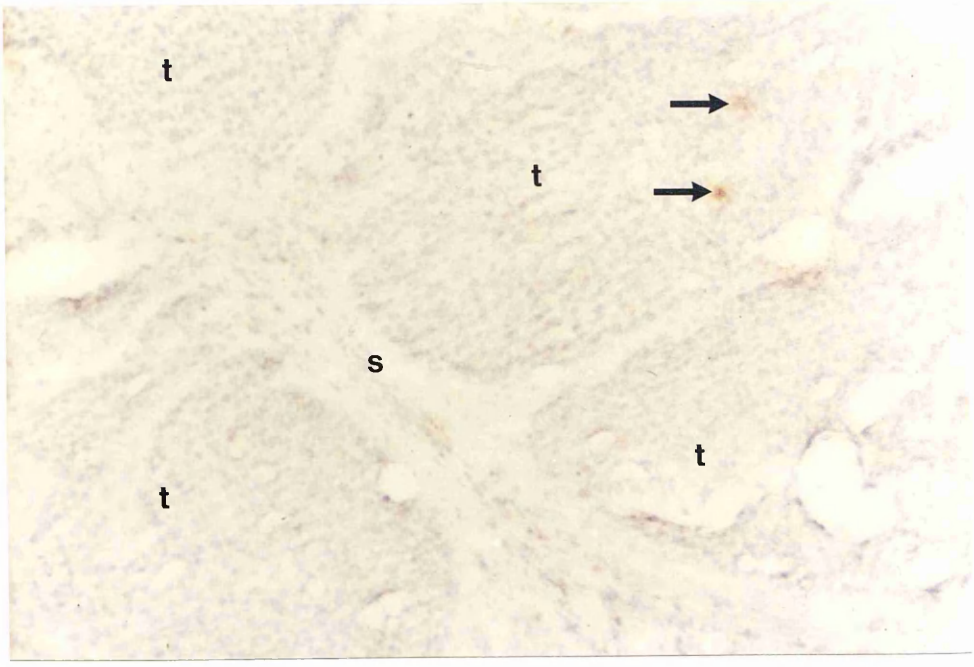

Figure 16.

Langerhans´ cells in cervical carcinoma - reduced cells.

The islets of tumour cells (t), the stroma (s) and two Langerhans´ cells are indicated stained with NA 1/34.

Total magnification X 362.

magnification) in the nests of invasive squamous cells. The lymphocytic infiltrate in the tumour was assessed on a semi-quantitative basis (Whitwell et al. 1984). Image analyser results were also collected for the Langerhans' cell counts and lymphocytic infiltrate.

Results

The numbers of Langerhans' cells in the ectocervix adjacent to the tumours are shown in Table 34. These are significantly less ($p<0.05$) than those counted in normal ectocervix per 100 basal cells (mean 8.2; 95% CI 6.8-9.6) and with the image analyser (mean 13.32; 95% CI 11.86-14.78).

Table 34. Langerhans' cells counted by two methods in ectocervix of patients with stage Ib/IIa squamous carcinoma of cervix.

Measurement	Cases	Mean	SD	95% Confidence Interval
/ 100 basal cells	10	5.8	3.5	3.3 to 8.3
/ unit area	10	7.86	4.49	4.6 to 11.07

Note. Mean, SD & 95% CI (of mean) are of cell counts.

There is no suitable control for the the numbers counted in the malignant epithelial islands, but it is apparent that there are vast differences in Langerhans' cell densities in these squamous tumours (Figures 16 & 15; Appendix IIIf), with some being almost totally devoid of Langerhans' cells while some tumours showed them in large numbers (range 1 to 77 per high power field).

In addition to the vast differences in Langerhans' cell numbers, there were also marked differences seen in the patterns of epithelial class I and class II antigen staining in the islets

Figure 17.

Absent epithelial cell MHC class I expression in cervical carcinoma.

The islets of tumour cells (t) and stroma (s) are indicated.

The malignant epithelial cells lack MHC class I antigen expression when stained with W6/32.

Total magnification X 145.

of malignant cells.

Class II epithelial antigen staining was present in the diffuse patterns seen in the cases with CIN previously described. Four of the tumours demonstrated MHC class II positivity with five being negative. In one patient the normal ectocervix showed diffuse positive staining (Table 35).

Class I epithelial antigen staining was subjectively increased in all but one of the cases, in which the malignant epithelium was virtually devoid of these antigens (Figure 17; Table 35). It is also interesting to note that this was the only tumour in which nodal metastases were found.

Table 35. Epithelial cell expression of MHC class I and class II antigens in stage Ib/IIa squamous carcinoma of cervix.

Patient	MHC class I	MHC class II
5022	+++	neg
5025	++	pos
5031	+++	pos
5046	neg	neg
5058	++	neg
6002	+++	neg
6008	+	neg
6012	++	pos
6014	+++	neg
7041	+++	pos

Note.+=normal expression ++=slight increase +++=moderate increase pos=positive neg=negative

There was no such vast differences noted in the total lymphocytic infiltrate although there were variations in the

proportion of helper and cytotoxic cells ranging from 4:1 to almost 1:1 (Table 36). The bulk of the infiltrate was confined to the stroma with little in the malignant epithelium similar to the situation in normal cervix. In most tumours the infiltrate was maximal at the periphery being lesser towards the centre.

The bulk of the infiltrate was of T-cells, although B-cells were noted in four tumours being marked in one. Macrophages were scant in two tumours but none was totally devoid. Natural killer cells were not recorded in any significant numbers (Table 37).

There was no apparent correlation between the numbers of Langerhans' cells, presence or absence of class II positive epithelial staining and the lymphocytic infiltrate.

Table 36. Image analyser & semi-quantitative analysis of the T cell numbers in patients with stage Ib/IIa cervical carcinoma.

	Image Analyser			Semi-quantitative		
Patient	CD 3(N)	CD 8(%)	CD 4(%)	CD 3	CD 8	CD 4
5022	485	19	81	+++	+	+++
5025	777	46	112	++++	+	+++
5031	638	33	93	+++	+	++
5046	512	48	59	++++	++	++
5058						
6002	649	49	52	+++	++	+++
6008						
6012	618	33	73	++	+	+++
6014	681	50	68	+++	++	+++
7041	533	47	171	+	++	+++

Note. N= number of CD3 cells %=(no. cells/total CD3 cells)X100)
0=neg +/-=occ +=slight ++=mod +++=heavy ++++=v heavy

Table 37. Semi-quantitative assessment of the numbers of B cells, macrophages & NK cells in stage Ib and IIa carcinoma of cervix.

Patient	B-cells CD 22	Macrophages CD 15	Natural Killer (NK) cells
5022	0	++	+
5025	0	+/-	0
5031	+++	+++	+
5046	0	+	+/-
5058			
6002	0	++++	+/-
6008			
6012	++	+++	+/-
6014	++	+/-	0
7041	+	+++	0

Note. 0=neg +/-=occ +=slight ++=mod +++=heavy ++++=v heavy

Comments

The tumours studied represent early localised disease in patients of a generally younger age group than that normally associated with invasive cervical carcinoma. This highlights the fact that the essential treatment for cervical carcinoma is radiotherapy with only a small highly selected group being eligible for surgery. The methods employed in the in-situ study of the local immune response requires that representative samples of tumour are obtained. Superficial biopsies of Stage III or IV tumours, although easily collected in a hospital acting as a referal centre for radiotherapy, would not have been representative of the local immune response because of the

superficial tumour necrosis and infection that occurs, hence specimens were collected only from those with resectable tumours who had no more than small superficial biopsies taken previously for diagnosis. Those who had cone biopsies taken prior to radical hysterectomy were not studied in order to avoid any confusion that may have arisen from the healing change following a large biopsy. Lastly, all tumours had to be of sufficient size to allow blocks to be cut for routine diagnostic histology and for frozen section.

The immune response in squamous cell carcinoma of the cervix has been studied mostly by examining the lymphocyte subsets in the peripheral blood, with additional information on cell mediated immunity being obtained from study of the response to cutaneous challenge with contact sensitising agents. Work by Rand, Jenkins & Bulmer (1977) demonstrated a significantly reduced percentage of T-cells compared with normal controls in advanced carcinoma of cervix (stage 3 and 4), but not in stages 1 and 2. Conversely the percentage of B-cells was significantly increased in the later stages. The significance was lost when absolute numbers of lymphocytes were counted. Ishiguro, Sugitachi & Katoh (1979) also noted decreased numbers of B-cells in advanced carcinoma, however, they noted decreased numbers of T-cells in all stages. They also recorded that the numbers of T-cells binding with sheep rosettes after short incubation periods, so called 'active' T-cells, were also reduced in all stages. In patients with cervical carcinoma, Levy et al. (1978) documented impaired T-cell mechanisms (as assessed by in-vitro lymphocyte stimulation assays and in-vivo delayed hypersenstivity testing to a variety of antigens). Once again patients with advanced disease were found to

have significantly reduced numbers of T-cells and increased B-cells compared with normals but also significantly reduced positive responses to all the contact antigens studied. Lymphocyte stimulation with phytohaemagglutinin was significantly reduced in advanced disease. Impaired cellular immunity was also detected in many of the patients with early disease. When frozen sections of the tumours were examined, using immune adherence to detect T and B cells, there was no obvious pattern linking the local infiltrate in the tumour with the stage of disease and no relation of the individual tumour infiltrate with the in-vitvo response. This prompted the Levy and colleagues to comment that the immune response at the peripheral level is not a good indicator of the response in the tumour. From these studies it is evident that advanced cervical cancer results in depressed T-cells both functionally and quantitatively; however, in early disease there is less agreement although there is the suggestion that a substantial proportion of these tumours are associated with impaired T-cell mechanisims.

The results presented in this chapter agree in some respects with the results from earlier studies, suggesting that there is a diverse immune response in early tumours with no obvious pattern of cellular infiltrate and great variability between different tumours. The predominant infiltrate is of T-cells, with the helper subgroup predominating in the vast majority. Robins & Baldwin (1985) have suggested that the rejection of tumours is essentially a T-cell mediated phenomenon with both main subgroups being important. Antibody dependent responses appear to be of little consequence. This in-situ study shows that B-cells were only present in any quantity in three of the tumours. However, Sano &

Ueki (1987) have claimed that B-cell derivatives (plasma cells) may have a role in limiting the invasive potential of cervical carcinoma.

Despite the anti-tumour role of the natural killer cell there is little evidence that these cells made any significant contribution to the local immune response in the early tumours examined, being only present in the minority of tumours and in very small numbers. Macrophages were a consistent finding.

Once again the main interest remains with the T-cells and their interactions with one another and with the target cell in the efferent response. The great variation in the numbers of Langerhans' cells seen in the tumours could represent large differences in the hosts' ability to present neoantigens to the immune system. It is difficult to be certain of the significance, if any, of the reduced Langerhans' cells adjacent to the lesions which may precede the tumour development or be a consequence of its presence. The presence or absence of class II antigen expression on the tumour cells could also be exerting effects on the afferent and efferent immune response, although evidence of this is not apparent in contrast with the results obtained in CIN. Perhaps the most interesting observation is the loss of class I antigens on the one tumour in which nodal metastases were detected. The dual recognition by the cytotoxic T-cells of the target cell class I antigens and foreign antigen has been mentioned in previous chapters and loss of these antigens in murine virus induced tumours which demonstrate progressive growth has been recorded (Bernards et al. 1983), as have similar findings in some primary breast tumours (Whitwell et al. 1984). By shedding its class I antigens the tumour may effectively be evading the

immune effector cytotoxic T-cells, thus allowing progressive growth and ultimately metastases. Despite this attractive hypothesis, it should be remembered that this class I negative tumour was removed from a patient 20 weeks pregnant at Caesarean hysterectomy and other factors may have been operating to encourage early nodal metastases in this case.

The loss of cell surface antigens and the association with nodal metastases in cervical carcinoma are not totally novel since Davidsohn et al. (1973) reported that A, B and H blood group antigens were lost in 92% of primary tumours and in 91% metastatic tumours in a group of patients who all died of metastatic cervical carcinoma. They interpreted the loss of these antigens as an expression of loss of cellular differentation preceding metastases. These antigens were present on all benign lesions of cervix although their results of findings in preinvasive lesions are not clear. The hypothesis presented above suggests that loss of cell surface antigens may result in local immunocompromise facilitating spread of the tumour. Taking this argument further, prognostic significance has been attached to loss of A,B and H blood group antigens (Lindgren et al. 1986).

The most marked lymphocytic activity was observed in the stroma at the periphery of the tumours with in most instances the reaction sharply localised to this area. There was very little in the islets of malignant epithelium and, although it is difficult to infer T-cell activity merely from their presence, the bulk of the T-cells were class II positive probably supporting this theory. Van Nagel et al. (1978) suggested that a marked lymphocytic infiltrate was significantly associated with decreased nodal metastases and tumour recurrence, but was of no relation to

tumour differentiation in the primary or secondary deposits in patients with stage Ib disease. They found nodal metastases were positively associated with vascular like space involvement.

The results of this chapter are not substantially different from those in the only other study found in which monoclonal antibodies had been used to investigate the local immune response in cervical carcinoma. Ferguson, Moore & Fox (1985) reported a series of 10 patients with adeno- and squamous cervical carcinoma, only five of the latter were definite primary squamous carcinomata. Positive nodal metatases were found in three of these five tumours. MHC class I activity was absent in two and patchy in the other. No obvious relation to tumour differentiation was apparent. The point is well made that the results represent a qualitative assessment of class I activity and that even the negative tumours may be expresssing antigens below levels of detection with the immunoperoxidase technique. MHC class II activity was present in one of the tumours. A heavy leukocytic infiltrate was present in most, the bulk being stromal T-cells of the cytotoxic/suppresssor subtype. They also reported moderate numbers of macrophages and occasional heavy infiltrates of B-cells. Once again natural killer cells were rarely present in the infiltrate. Langerhans' cells were not studied.

From studies containing small numbers of patients and others including various tumour cell types it is impossible to substantiate the claims of Van Nagel et al. (1978), but their conclusions certainly suggest that the local response to the tumour is of importance. Combined with the findings of Davidsohn et al. (1973), Lindgren et al. (1986) and Ferguson et al. (1985) together, with the results of the work presented in this chapter,

it seems apparent that the local immune response in cervical cancer should be studied in larger numbers of patients using monoclonal antibodies to identify the local immunocompetent cells. The results from Langerhans' cell counts and the presence or absence of the major histocompatibility antigens would appear to be greatest interest in the earlier stages of squamous cell cervical carcinoma.

An interesting group to study would have been the very early microinvasive tumours. Most of these, however, are diagnosed on cone biopsies following abnormal cytology and unsatisfactory or suspicious colposcopy. Study of Langerhans' cells in these lesions was attempted using S-100 in specimens recovered from the files of the Pathology Department of the Western Infirmary, Glasgow, which had been diagnosed between 1977 and 1987. Staining with S-100 produced no consistent identification of Langerhans' cells in the areas where the breech in basement membrane had taken place, nor in any adjacent normal or abnormal epithelium, despite the clear staining of nerve fibres present in most blocks. The same problems were encountered in the study of S-100 positive Langerhans' cells in normal cervix (see Chapter 2).

Taking frozen sections from cone biopsies was considered unsuitable since considerable disruption to the specimen could have resulted in less than adequate sampling, with the possibiltiy that prognostically important vascular or lymphatic involvement would have been missed. Study of the local immune response in microinvasive carcinoma was therefore not pursued.

CHAPTER 10

General summary and conclusions

CHAPTER 10

The malignant potential of CIN has been dicussed by many authors. It is now established that CIN represents a continuous spectrum of epithelial abnormality which, in a minority of cases, can progress to carcinoma of the cervix. It remains, however, impossible to predict which lesions will progress and which will not. Factors such as specific subtypes of human papillomavirus eg types 16 and 18 are said to be important in determining the malignant potential of these lesions, but convincing evidence supporting this hypothesis is awaited. Although there is an association between the presence of HPV infection and cervical neoplasia, HPV alone seems unlikely to be the causal agent since viral genomes are now being reported in controlled studies in the normal cervix. Papillomaviruses have been shown to be oncogenic in many species, but in all cases a cofactor has been identified to promote the malignant transformation. Such cofactors, identified in animals, include chemical carcinogens and ultraviolet light.

Ultraviolet light has many effects on squamous epithelium, and amongst these a reduction in the number and antigen presenting capabillities of Langerhans' cells have been identified. Langerhans' cells are a consistent component of normal squamous epithelium, and it has been postulated that they are the sentinels of a local immune system that involves a complex interaction between these cells, squamous epithelial cells and lymphocytes which preferentiallly circulate through the epithelium and local lymph nodes.

Dermatological research has shown that reduction in the number of Langerhans' cells can impair normal T-cell immune

mechanisms and may, in some instances, result in tolerance to the challenging antigens. Moreover, in epidermodysplasia verruciformis there appears to be a link between HPV infection, reduction in Langerhans' cells, exposure to ultraviolet light and malignant transformation of cutaneous lesions.

Impaired T-cell function is also a feature of epidermodysplasia verruciformis, and has been linked to the development of CIN, with systemically immunosuppressed women being recognised as a group at high risk of cervical neoplasia.

Several studies have examined the relationship between Langerhans' cells and local immunity to cervical neoplasia. Most have examined relatively few cases and none have used control tissue in their studies. Difficulties arise in interpreting results from uncontrolled studies since factors associated with cervical neoplasia, such as smoking, oral contraceptive use and exposure to semen, are immunosuppressive in their own right. Moreover, it is only since control tissue has been probed for HPV genomes that the relation between HPV and cervical neoplasia has been doubted.

The work described in this thesis examined the cellular elements involved in local cervical immunity, particularly any changes occurring in these cells in association with cervical neoplasia. Since the main immune response to tumours is thought to involve T-cell mechanisms, the work focussed on the T-cell subsets of helper/inducer and cytotoxic/suppressor cells and on factors stimulating their response. Special interest was therefore directed on antigen presenting Langerhans' cells and MHC class II antigens.

Preliminary work established that the best and most

consistent marker for identifying Langerhans' cells was an anti T-6 monoclonal antibody (NA 1/34) used in an indirect immunoperoxidase method with Diaminobenzidene as the chromogen. Frozen sections cut from biopsies were necessary for application of this method. Substitution of NA 1/34 by alternative monoclonal antibodies allowed other cells of the immune system to be demonstrated.

For satisfactory histological interpretation of the frozen sections, the biopsies from which they were cut had to be carefully prepared and optimally orientated. Tissue-Tek II OCT, frozen in carbon dioxide ice, was the best embedding medium. The most notable conclusion from evaluation of other methods was that S-100 was an unreliable and inconsistent Langerhans' cell marker in routinely fixed and processed biopsies of cervix.

Quantitation of Langerhans' cells was best achieved using a standard length of cervical epithelium basement membrane (100 basal cells) as the denominator. This measurement correlated well with other methods described in this work, but had the benefits of simplicity and reliability. Quantification of the lymphocytic infiltrate was best achieved using a computerised image analyser.

This study confirmed that in the cervix of normal patients the majority of the cellular infiltrate is of T-cells, with helper (T-4) cells predominating in the stroma and cytotoxic (T-8) cells in the epithelium. As no difference was found comparing the infiltrate in the ectocervix with that of the transformation zone, it was concluded that the transformation zone did not represent an area of enhanced immunological activity. More importantly, comparison of the Langerhans' cell numbers in these two areas of cervix, using different methods of enumeration and identification,

indicated that the ectocervix was an appropriate internal control for the transformation zone. For the reasons disdussed previously the ready availability of such a validated control was considered important for the subsequent study of Langerhans' cells in CIN.

In the biopsies examined from 142 patients with CIN, a reduction in the number of Langerhans' cells was found in those lesions where the presence of human papillomavirus infection was shown on histological criteria and by DNA-DNA hybridisation studies. The latter studies showed that the reduced numbers of Langerhans' cells are related to the HPV subtype and amount of virus present.

Impaired local immunity mediated via reduced Langerhans' cells may thus be an important cofactor in HPV related cervical neoplasia, but it is impossible to determine from this type of study whether reduced Langerhans' cell numbers predispose to HPV infection and to cervical neoplasia in otherwise normal women. The increase in Langerhans' cells seen in the absence of HPV in the hybridisation studies suggests that factors other than HPV and impaired antigen presentation are implicated, but when the larger number of biopsies in the main study (see Chapter 4) are examined it is evident that a number of the lesions showing CIN only exhibit reduced Langerhans' cells. Thus in CIN the Langerhans' cell numbers range widely in the lesions showing HPV and in those without, with no significant between the various grades within each subgroup. This effect is not wholly surprising since CIN itself represents a continuous spectrum of disease. One further suggestion from the results, assuming that the immune response is dependent on the number of Langerhans' cells, is that lesions with concomitant evidence of viral infection may be more likely to

progress as a result of local immunocompromise than lesions without HPV.

The results from Chapter 4 also suggest that the neoplastic epithelial cells are implicated in the immune response to cervical neoplasia since the lymphocytic infiltrate was shown to be significantly greater in CIN with virus which expressed MHC class II antigens compared with those lesions which did not. Since the correlation between the number of Langerhans' cells and the lymphocytic infiltrate was only significant where low numbers of Langerhans' cells were present (under 7 per 100 basal cells), and then only in lesions lacking class II antigens, suggests that the cervical epithelium may have a threshold antigen presenting capacity determining local cervical immunocompetence. Compromise appears only to occur in the absence of epithelial cells expressing MHC class II antigens and when low numbers of Langerhans' cells are present. The antigen presentating capacity of abnormal cervical epithelium may thus be influenced by both qualitative and quantitative changes in MHC class II antigen expression, with Langerhans' cells determining the former and the epithelial cells being able to influence the latter. Thus, escape from local immune surveillance in the cervix, secondary to a low antigen presenting capability, could result in tolerance to neoantigens and may ultimately determine the natural history of the lesion. It is possible that the quantitative effect of the epithelial cells in CIN is the most important factor, since their influence was shown to involve the infiltrate in the epithelium and stroma. In addition, the percentage of cases lacking MHC class II antigens almost fits the percentage of cases of CIN that would be expected to progess, some eventually to carcinoma, without

taking account of the number of Langerhans' cells in the lesion.

Although a low antigen capacity may result in immunological compromise, lack of MHC class II antigens and Langerhans' cells are not consistent findings in invasive carcinoma of the cervix. The results emphasise the need to consider other factors, such as loss of MHC class I antigens, in escape from immune surveillance. In the pre-invasive changes of CIN, however, this does not appear to be a feature since the afferent T-cell mechanism seems to be the limiting factor in determining the resultant immune response.

The above conclusions suggest that Langerhans' cells are reduced as a consequence of HPV infection, perhaps due to a cytopathic effect of the virus. However, this hypothesis may be invalid since reduced Langerhans' cells and helper T-cells were shown to be features of the typical transformation zone of 'high risk' renal transplant recipients on immunosuppressive therapy. Thus impairment of antigen presentation by cervical epithelium may permit the development of cervical neoplasia (and HPV infection) in the compromised host. In the non-immune compromised woman, cervical neoplasia may develop as a consequence of a local immune defect in the cervix that is potentiated by cigarette smoking, oral contraceptive use and exposure to semen.

The results from the renal transplant recipients also suggest that, despite the theoretical risk of neoplasia associated with the use of cyclosporin, especially in relation to its effects on helper T-cells, the effects are not seen locally in the cervix nor reflected in a higher prevalence of CIN in women treated with this drug than in those on azathioprine. Indeed, the converse is true.

The increased prevalence of cervical neoplasia in renal transplant recipients seems to result from systemic

immunosuppression, and is not a consequence of long term renal disease as indicated by examination of the patients in Chapter 7. As patients in renal failure are also reported to be immune suppressed, this lends support to the notion that slight reductions in immunocompetence may be not sufficient to place women at risk of cervical neoplasia. However, patients in chronic renal failure who are not being actively dialysed may be a potential high risk category, since they show reduced numbers of Langerhans' cells in the transformation zone. There are reports suggesting that uraemic patients are also at risk of cervical neoplasia but whether or not this relates to the dialysed and/or un-dialysed woman is not known.

The results described in Chapter 7 may be explained on the basis that treatment of renal failure lessens the suppressive effects of uraemia on the bone marrow so that the Langerhans' cells of the transformation zone of cervix return to the normal range, when compared with the reduction seen in the non-dialysed women.

Pregnant patients are another group in which T-cell function is compromised and in which an increased prevalence of CIN is reported. Examination of the cervices from patients in early pregnancy showed no differences in the number of Langerhans' cells or in the lymphocytic infiltrate when compared with normal cervices. This may support the hypothesis that the duration of immune suppression is an important factor in determining the risk of neoplasia. It also implies that factors other than local immune compromise in the cervix contribute to the high prevalence of abnormal smears in pregnancy. Since many women receive their first smear in pregnancy, this may explain most of the reported increase

in abnormal cervical cytology.

It is possible to conclude from the clinical work presented in this thesis that women at high risk of cervical neoplasia represent a screening problem on two counts. Firstly, it has been shown such patients are not being screened effectively by cervical cytology alone; secondly, and of more concern, many are not being screened at all, presumably because they are not thought of as being at high risk. Therefore, further education of patients and practitioners alike seems appropriate since the failure of the current cervical screening programme is mainly due to failure to screen the at risk patient. This situation could be improved if the small number of renal transplant recipients in whom there is poor correlation between histology and cytology had yearly colposcopy as a screening procedure.

Longitudinal screening of patients awaiting renal transplantation should confirm that the high prevalence of CIN, and HPV infection, seen in women after transplantation represents secondary effects of prolonged systemic immunosuppression. It remains to be determined whether or not changes in the surface major histocompatibilty antigens have any prognostic significance in defining the malignant or invasive potential of CIN. This could not be investigated further in the work described in this thesis because it is not considered ethical to leave CIN untreated. Long-term follow-up of patients with cervical carcinoma should determine whether or not there are any features of the local immune response able to predict the behaviour of the tumour. Therefore more detailed examination of the surface antigenic changes on the epithelial cells, throughout the complete spectrum of cervical neoplasia, could form the basis for future studies.

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APPENDICES

Appendix I - Staining techniques

Appendix II - Monoclonal and other antibodies and reagents

Appendix III - Patient details and Langerhans' cell counts

Appendix IV - Graphical comparisons of the lymphocyitc infiltrate (T cell counts) in the epithelium & stroma of typical transformation zone from normal patients, renal transplant recipients, uraemic & pregnant patients

APPENDIX I - STAINING TECHNIQUES

Ia. Indirect immunoperoxidase technique on frozen sections using Monoclonal Antibodies (MacKie 1982).

1)	Frozen sections on gelatinised slides Air dry	3 minutes
2)	Fix in 100% acetone	10 minutes
3)	Wash in tris saline	1 minute
4)	3% H_2O_2 (diluted in tris buffer)	15 minutes
5)	Wash tris saline - 3 changes	5 minutes
6)	Normal rabbit serum (1:5 in tris buffer)	20 minutes
7)	Pour off	
8)	Add monoclonal antibody (1:100 tris buffer)	60 minutes
9)	Wash tris saline - 3 changes	5 minutes
10)	Rabbit anti-mouse antibody/ Peroxidase congugated (DAKO) 1:20 (in 1:1 normal human serum:tris buffer)	45 minutes
11)	Wash tris saline - 3 changes	5 minutes
12)	0.03% Diaminobenzidene (in 100 mls tris buffer + 1 drop 100 vol H_2O_2)	5 minutes
13)	Wash in running tap water	5 minutes
14)	Counterstain with Harris's haematoxylin	2 minutes
15)	Wash in running tap water	1 minute
16)	Differentiate in 1% acid alcohol	30 seconds
17)	Wash in running tap water	1 minute
16)	Blue in Scotts tap water substitute	30 seconds
18)	Wash in running tap water	3 minutes
19)	Dehydrate in methylated spirit	5 minutes
20)	Absolute alcohol - 2 changes	5 minutes
21)	Xylene	5 minutes
22)	Mount in Histomount	

Acetone fixation, all washes, hydrogen peroxide treatment, and diaminobenzidene incubation are performed in COPLN JARS.

Normal rabbit serum blocking, and antibody incubations are performed in moist chambers at room temperature after carefully wiping round sections to ensure a concentrated drop of fluid on each.

Reagents

Tris Buffer

0.01M pH 7.6
6.06g Trizma Hydrochloride (Sigma)
1.39g Trizma Base (SIGMA)
dissolved in 1 litre distilled water

Tris Saline

900mls 0.85% saline
100mls tris buffer

3,3 Diaminobenzidene (Pelliniemi et al. 1980)

600mgs 3,3 Diaminobenzidene Tetrahydrochloride (Sigma)
dissolved in 100mls tris buffer
aliquoted into 5mls vials and frozen at $-20^{o}C$
thawed before use and added to 95mls tris buffer

Ib. Monoclonal antibody technique on fixed tissue sections (including periodate lysine paraformaldehyde fixation).

1)	Take sections to water	
	Xylene	7 minutes
	Absolute Alcohol	5 minutes
	Methylated Spirit	3 minutes
2)	Wash in tris saline	1 minute
3)	Methanol (100 ml) + 0.5% H_2O_2 (0.5 ml) + 0.01% HCl (0.2 ml)	15 minutes
4)	Wash tris saline - 3 changes	5 minutes
5)	Normal rabbit serum (1:5 in tris buffer)	20 minutes
6)	Pour off	
8)	Add monoclonal antibody at varying dilution in tris buffer (room temperature or 37°C)	60 minutes
9)	Wash tris saline - 3 changes	5 minutes
10)	Rabbit anti-mouse antibody/ Peroxidase congugated (DAKO) (1:20 in 1:1 normal human serum:tris buffer)	45 minutes
11)	Wash tris saline - 3 changes	5 minutes
12)	0.03% Diaminobenzidene (in 100 mls tris buffer + 1 drop 100 vol H_2O_2)	5 minutes
13)	Wash in running tap water	5 minutes
14)	Counterstain with Harris's haematoxylin	2 minutes
15)	Wash in running tap water	1 minute
16)	Differentiate in 1% acid alcohol	30 seconds
17)	Wash in running tap water	1 minute
16)	Blue in Scotts tap water substitute	30 seconds
18)	Wash in running tap water	3 minutes
19)	Dehydrate in methylated spirit	5 minutes
20)	Absolute alcohol - 2 changes	5 minutes
21)	Xylene	5 minutes
22)	Mount in Histomount	

Tretment with Trypsin was tried before staining (see S-100 on fixed sections) with no effect. The amplification afforded by the use of the alkaline phosphatase method also showed no significant improvement in staining (see APAAP on fixed and frozen sections).

Ic. Alkaline Phosphatase (APAAP) on frozen sections.

1)	Sections on gelatinised slides. Air dry	5 minutes
2)	Fix in acetone	10 minutes
3)	Allow to dry	
4)	Normal rabbit serum 1:5 tris buffer	10 minutes
5)	Pour off	
6)	Add appropriate monoclonal antibody at correct dilution, at room temperature	60 minutes
7)	Wash tris saline - 3 changes	5 minutes
8)	Uncongugated rabbit-anti-mouse antibody 1:50 (in 1:1 tris buffer and normal human serum) at room temperature	30 minutes
9)	Wash tris saline - 3 changes	5 minutes
10)	Add APAAP complex - 1:100 tris buffer	30 minutes
11)	Wash tris saline - 3 changes	5 minutes
	Repeat steps 8 to 11 to enhance reaction using 10 minute incubations	
12)	Add alkaline phosphatase substrate	15 minutes
13)	Wash tris saline - 3 changes	5 minutes
14)	Wash in tap water	
15)	Counterstain lightly in haematoxylin, only blued in Scotts tap water substitute	30 seconds
16)	Water mount	
17)	Seal with nail varnish	

Reagents.

Alkaline phosphatase substrate.

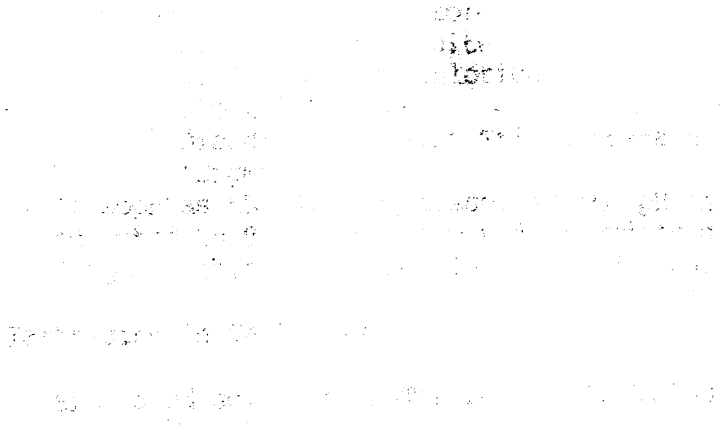
Naphthol AS-MX Phosphate (Sigma) 2 milligrammes (mg)
in 0.2 millilitres (ml) Dimethylformamide
+ 9.8 ml tris buffer pH 8.2
+ 2.4 mg Levamisole (Sigma)

Mix and add Fast Red salts (Sigma) 1 mg per ml
Filter mixture on to slides

Id. Alkaline Phosphatase (APAAP) on fixed tissue sections.

1)	Take sections to water	
	Xylene	5 minutes
	Absolute Alcohol	3 minutes
	Methylated Spirit	1 minute
2)	Wash in tris saline	5 minutes
3)	Normal rabbit serum 1:5 tris buffer	10 minutes
4)	Pour off	
5)	Add appropriate monoclonal antibody at correct dilution, at room temperature	60 minutes
6)	Wash tris saline - 3 changes	5 minutes
7)	Uncongugated rabbit-anti-mouse antibody 1:50 (in 1:1 tris buffer and normal human serum) at room temperature	30 minutes
8)	Wash tris saline, 3 changes	5 minutes
9)	Add APAAP complex, 1:100 tris buffer	30 minutes
10)	Wash Tris saline - 3 changes	5 minutes
	Repeat steps 8 to 11 to enhance reaction using 10 minute incubations	
11)	Add alkaline phosphatase substrate	15 minutes
12)	Wash tris saline - 3 changes	5 minutes
13)	Wash in tap water	
14)	Lightly counterstain in haematoxylin only blued in Scotts tap water subsitute	30 seconds
15)	Water mount	
16)	Seal with nail varnish	

Reagents as for APAAP on frozen sections.



Ie. Adenosine triphosphatase (ATP-ase) on frozen sections.

1)	Twenty micron frozen sections - air dry	3 minutes
2)	Fix in Formol-Cacodylate-Sucrose at 4° C	90 minutes
3)	Wash well in running tap water, finally rinse in distilled water	5 minutes
4)	Place in incubating medium at 37° C	90 minutes
5)	Wash in 1% calcium chloride - 3 changes	5 minutes
6)	Treat in 2% cobalt chloride	3 minutes
7)	Wash in running tap water	5 minutes
8)	Develop in 1% ammonium sulphide	3 minutes
9)	Wash well in running tap water	5 minutes
10)	Dehydrate through methylated spirit, alcohol, Tartrazine in Cellosolve and clear in Xylene - mount in Histomount	

Result ATP-ase activity - Black / Brown
Background - Yellow

Reagents
Formol-cacodylate-sucrose fixative
(Hayashi & Freiman (1966). Journal of Histochemistry and Cytochemistry, 14, 577-581)

Dissolve 40 g paraformaldehyde in 100 ml distilled water at 65° C, add 40% sodium hydroxide dropwise until solution clears to produce methanol-free formaldehyde

0.1 m cacodylate buffer pH 7.2	90 ml
40% methanol-free formaldehyde	10 ml
sucrose	12.5 g
sodium pyrophosphate	125 mg

Adjust pH to 7.4 before use with N sodium hydroxide added dropwise.

Incubating medium
(Padykula and Herman, as in Pearse (1968) Histochemistry, edition III, volume 1,pp 720)

Solution made freshly before use

0.1 m sodium barbiturate	20 ml
0.18 m calcium chloride	10 ml
distilled water	30 ml
Disodium Adenosine Triphosphatase (Sigma)	152 mg

As soon as the ATP has dissolved the pH is adjusted to 9.4 with 0.1 m sodium hydroxide. Slight turbidity may be cleared by filtration.

Tartrazine in Cellosolve

Saturated solution of Tartrazine in Cellosolve

If. Adenosine triphosphatase (ATP-ase) on epithelial sheets.

1)	Flatten sheets in normal saline	
2)	Transfer to Cacodylate Formaldehyde, 4°C	10 minutes
3)	Wash in normal saline, 3 changes	10 minutes
4)	Add 1:10 dilution of 2% Lead Nitrate in Stock ATP and mix well Incubate at 37°C	60 minutes
5)	Wash in normal saline - 3 changes	5 minutes
6)	Develop in 5% ammonium sulphide	90 seconds
7)	Allow to dry	
8)	Mount in glycerin	
9)	Seal with nail varnish	

Reagents.

Cacodylate formaldehyde

Sucrose	6.85 g
40% Formaldehyde	10 ml
0.2M Cacodylic Acid	40 ml
Distilled water	50 ml

Stock adenosine triphosphatase (ATP)

DiSodium ATP (Sigma)	50 mg
Glucose	5 g
tris buffer	40 ml
1.2% Magnesium Sulphate	10 ml

Stock ATP lead nitrate

Stock ATP 5.4 ml + 0.6 ml Lead Nitrate sufficient for 10 sheets.

Ig. S-100 on fixed tissue sections.

1)	Sections on Polylysine slides Take to water as above	
2)	Wash tris saline	1 minute
3)	Wash in pre-warmed tris saline ($37^{O}C$)	10 minutes
4)	Trypsinse. 0.1% CaCl / 0.1% Trypsin (Sigma) in H_2O . Adjust pH to 7.8 with 0.1M NaOH and bring to $37^{O}C$	30 minutes
5)	Wash in cold running water	2 minutes
6)	Methanol (100 ml) + H_2O_2 (0.5 ml) + HCl (0.2 ml)	15 minutes
7)	Wash in tris saline	5 minutes
8)	Normal swine serum : tris buffer (1:5)	15 minutes
9)	S-100 at 1:50, 1:100, 1:200 in tris buffer	60 minutes
10)	Wash tris saline - 3 changes	5 minutes
11)	Swine anti-rabbit immunoglobulins (1:40) (in 1:1 normal human serum:tris buffer)	30 minutes
12)	Wash tris saline - 3 changes	5 minutes
13)	Peroxidase anti-peroxidase 1:100 in tris buffer	30 minutes
14)	Wash tris saline - 3 changes	5 minutes
15)	0.03% Diaminobenzidene + 1 drop H_2O_2	10 minutes
16)	Wash in tap water	1 minute
17)	Dehydrate and counterstain as monoclonal antibodies in frozen sections	

The APAAP method was used as an alternative in order to amplify any reaction but was no better than DAB above.

Ih. Immuno-elctron microscopy method.

1)	Fix in fresh 3% paraformaldehyde with 8.5% sucrose in PBS	4 hours
2)	Wash in 8.5% sucrose	overnight
3)	Embed biopsy in OCT and freeze	
4)	Cut cryosections	
5)	Air dry sections	
6)	3.5% H_2O_2 in tris buffer	10 minutes
7)	Wash tris saline - 3 changes	
8)	Normal rabbit serum 1:5 in tris buffer	10 minutes
9)	Pour off	
10)	NA 1/34 (dilutions 1:5 to 1:100)	1 hour to overnight
11)	Wash tris saline - 3 changes	
12)	Peroxidase congugated rabbit anti-mouse antibody (DAKO) 1:20 in 1:1 NHS tris buffer	45 minutes
13)	Wash tris saline - 3 changes	
14)	Post fix 2% glutaraldehyde	
15)	Wash tris saline - 3 changes	
16)	Excess stroma removed from section with aid of stereo dissecting microscope	
17)	3,3 Diaminobenzidene 5 mls in 95 mls tris buffer + 1 drop H_2O_2	10 minutes
18)	Post fix 2% osmium tetroxide	
19)	Dehyrate through 50%, 70% and 100% alcohol	
20)	Propylene oxide	
21)	Place araldite resin capsules over section allow to harden freeze and snap capsule off slide	

APPENDIX II-Monoclonal & other antibodies & reagents.

Monoclonal Antibodies.

anti-Human Thymocyte Antigen
Sera-Lab
MAS 036C Ascitic fluid
Clone NA 1/34 — Cost £ 45.00

HLA-A.B.C
Sera-Lab
Mouse IgG ascitic fluid
Clone W6/32 — Cost £ 60.00

UCHT 1
Unipath
Code MF 22 — Cost £ 90.00

UCHT 4
Unipath
Code MF 25 — Cost £ 90.00

OK T4a
ORTHO
Code 50140 — Cost £ 207.00

OK M1
ORTHO
Code 50050 — Cost £ 207.00

Leu 7
Becton Dickinson
Code 7390 — Cost £ 205.00

Leu 11b
Becton Dickinson
Code 7530 — Cost £ 205.00

Leu 12
Becton Dickinson
Code 7540 — Cost £ 123.00

Anit-HLA Class II monoclonal antibodies DA6 231 and DA6 147 were available from the MRC Unit, Edinburgh, free of charge.

The Scottish Antiboby Production Unit (SAPU) at Law Hospital is now able to provide some antibodies with the same specificities as those above, free of charge for research purposes.

All antibodies diluted 1:100 in tris buffer for optimal results.

IIb. Other antibodies used.

Peroxidase-congugated Rabbit Immunoglobulins to Mouse Immunoglobulins
DAKO
Code P. 260 Cost £ 165.00

Uncongugated Rabbit Immunoglobulins to Mouse Immunoglobulins
DAKO
Code Z. 259 Cost £ 34.00

Peroxidase Anti-Peroxidase (rabbit)
DAKO
Code Z. 113 Cost £ 59.00

Alkaline Phosphatase Complex (rabbit)
DAKO
Code D. 306 Cost £ 63.00

Alkaline Phosphatase Complex (mouse)
DAKO
Code D. 651 Cost £ 100.00

Swine Anti-Rabbit Serum
DAKO
Code Z. 196 Cost £ 30.00

Other reagents

Normal Swine Serum
DAKO
Code X. 901 Cost £ 17.00

Normal rabbit serum was provided by SAPU free of charge.

IIIa - Normal patients

Name	Age	Unit No	Parity	Spec No	Transformation Zone		Ectocervix		Vagina	Vulva
					/100	/area	/100	/area	/100	/100
JS	65	206776	3+1	5019					3	11
EM	34	294058	1+1	5020	7	12.8	9	15.4		
IH	47	529828	2+0	5021	11	13.9	13	15.8		
ML	37	722109	3+0	5024	8	12.0	7	9.2		
MD	44	635021	2+0	5026	11	13.5	10	15.5		
AC	70	331099	3+0	5027	6	13.3	8	15.3	4	23
MMcK	65	401530	4+0	5032					4	
VR	48	412249	5+0	5034						11
PW	35	628170	1+0	5036	3	8.3	3	10.0		
PD	27	593677	3+0	5037	9	14.1	10	16.3		
DMcN	46	528891	4+1	5038	6	8.3			6	
BC	53	115039	2+0	5039						12
JK	75	808533	1+0	5048	10	15.0	8	12.3	5	6
BM	34	442710	3+1	5049	8	11.0	11	14.6		
JS	44	743088	2+0	5053	12	18.8	10	19.2		
AMcK	42	625832	4+1	5056	9	18.6	11	13.2		
SI	59	757869	4+1	5059	3	8.2	5	10.3	5	12
IL	45	187823	2+0	6017					8	12
JM	82	413022	2+1	6019					4	14
JW	39	756266	4+1	6022	4	10.4	5	10.2	3	12
JR	31	590948	1+0	6033	6	7.2				
SM	23	614755	3+0	6034	9	12.8				
MC	35	623632	1+0	6039	9	12.5				
AH	30	767052	2+0	6041	9	13.4				
JM	38	758873	2+0	6085	8	14.1	7	13.9		
AW	49	670757	2+1	6104	6	9.9	9	14.1		
GMcD	47	637897	4+0	6137	5	8.4	4	7.9		
MG	42	511218	3+0	6147	8	12.7	9	17.3		
KC	48	537848	8+2	6201	12	16.6	11	13.4		
MMcC	35	562335	2+2	6237	16	17.0	11	14.2		
ST	38	822427	0+0	6238	4	8.1	3	8.3		

IIIb - Patients with cervical intraepithelial neoplasia.

Name	Age	Parity	Spec No	Grade	Ecto	Cx	Abn	Tz	Post Rx
MMcM	33	0+1	6018	II	7		10		
AS	30	0+0	6029	II	6		3		5
MP	33	1+0	6069	II	9		19		11
LP	27	0+3	6121	II	6	3.9	9	13.6	12
AA	23	3+0	6146	II	7		10		
CG	29	2+0	6249	II	6	8.3	6	9.4	
JG	24	0+0	6264	II	5		11		
EB	41	1+0	6338	II	5	6.9	7	7.9	
EC	32	1+0	6340	II	7	6.5	5	8.1	
TY	20	0+0	6352	II	7	22.6	6	14.8	
EMcD	31	4+0	6004	III	4		3		
HQ	26	0+0	6007	III	7		15		
EF	30	1+0	6011	III	6		12		6
PT	27	2+0	6013	III	7		4		6
CM	28	3+0	6016	III			3		
MR	31	0+0	6044	III	4	4.7	15	17.3	5
JMcC	31	1+0	6048	III	6		14		10
AP	25	0+0	6054	III	11	13.2	5	5.6	7
MS	27	1+0	6060	III	4		11		8
EF	27	2+1	6098	III	10	9.6	13	15.4	11
GM	32	1+3	6100	III	15		4		
LS	29	1+0	6120	III	7		12		
JG	31	3+0	6143	III	10		7		
EC	27	2+0	6145	III	5		7		
RS	27	0+1	6149	III	5		14		
RD	34	1+0	6150	III	7		3		
MS	29	4+0	6153	III	6		7		
IB	36	2+4	6154	III	7		9		
CB	29	3+0	6160	III	12		4		
MR	29	1+0	6167	III	7		9		
EW	28	1+0	6169	III	7		2		
IM	26	2+0	6183	III	5		14		
CB	31	1+0	6203	III	7	10.1	5	10.3	
MS	40	0+0	6206	III	6	10.9	10	14.9	
JW	32	1+0	6207	III	5	14.1	11	19.5	
AW	29	3+1	6229	III	11	15.4	5	9.6	
WE	25	1+0	6250	III	9		1		
HMcN	28	2+1	6259	III	5		2		
AB	32	1+0	6265	III	5		3		
GC	27	2+0	6266	III	8		6		
LS	26	1+0	6272	III	6	10.6	4	9.4	
LMcK	34	1+0	6280	III	12	10.2	20	24.0	
AB	36	0+0	6281	III	7	9.4	5	6.4	
ST	34	2+0	6284	III	11	10.5	21	21.9	
JL	33	2+0	6289	III	11	15.5	14	17.7	
ID	29	1+0	6290	III	8		9		
AS	30	2+0	6300	III	9	11.6	8	16.6	
RMcG	37	4+1	6305	III	10	15.6	6	8.9	
MMcQ	32	0+3	6309	III	9	6.5	19	18.7	
AK	24	1+0	6329	III	9	7.3	2	2.5	
AP	29	1+1	6335	III	8	9.2	17	17.9	
AS	22	1+0	6350	III			16	14.0	
AM	31	0+0	6351	III			9	16.1	
KD	34	0+0	6355	III	6	11.3	9	14.1	
CH	29	0+1	6359	III	13	13.1	22	29.4	

IIIb (contd) - Patients with cervical intraepithelial neoplasia.

Name	Age	Parity	Spec No	Grade	Ecto Cx		Abn Tz		Post Rx
LB	17	0+1	6005	I+V	4		3		
LT	22	1+0	6124	I+V	4		8		6
AMcL	22	0+1	6126	I+V			2		
JG	23	1+0	6148	I+V	8		9		
MB	40	3+0	6168	I+V	6		1		
ST	46	3+0	6193	I+V	6		1		
AA	37	2+0	6235	I+V	7		3		
EC	25	1+0	6245	I+V	9		3		
CG	26	2+0	6003	II+V	7		4		
EB	30	1+0	6030	II+V	8		18		8
LW	26	2+0	6037	II+V	8	10.2	1	1.3	9
AC	26	1+0	6038	II+V			3	2.5	
JM	33	3+0	6103	II+V	8		1		
AK	25	0+0	6128	II+V	8		14		
SH	34	0+0	6164	II+V	7		1		
AC	28	2+0	6185	II+V	12		2		
JN	29	0+1	6194	II+V	12		9		
JS	35	3+2	6231	II+V	5		5		
LM	28	2+0	6244	II+V	6		1		
JL	23	0+0	6251	II+V	6		6		
EF	23	0+0	6252	II+V	11		7		
SS	20	0+0	6260	II+V	11		5		
MK	28	0+0	6267	II+V	14	15.2	10	9.1	
GG	22	0+0	6274	II+V	9	9.2	2	1.7	
AF	33	0+0	6276	II+V	12	14.1	7	9.0	
PB	23	1+0	6277	II+V	14	20.3	11	12.1	
JS	34	4+0	6279	II+V	7		4		
MC	24	0+0	6285	II+V	8		5		
IB	27	1+0	6288	II+V	7	7.8	5	3.8	
SO	32	2+1	6296	II+V	5	9.1	4	2.0	
SM	29	2+0	6311	II+V	12	12.7	4	1.3	
RM	27	4+0	6314	II+V	4	4.4	2	1.7	
FM	40	2+0	6316	II+V	7	11.4	1	2.8	
FR	33	2+0	6325	II+V	9	12.3	6	11.4	
SC	35	0+0	6330	II+V	11	15.4	5	10.8	
RG	27	0+0	6333	II+V	7	12.1	2	6.2	
MOB	34	2+0	6337	II+V	10	8.5	4	6.5	
MN	19	1+0	6341	II+V	6		8		
YR	22	1+0	6346	II+V	6	19.7	8	22.1	
AM	31	0+0	6354	II+V	9	4.6	5	3.1	
ED	25	1+3	6358	II+V	15	17.6	8	6.2	
BMcP	25	1+0	6360	II+V	10	19.4	2	5.9	
MF	25	0+0	6027	III+V	7		2		8
JR	31	1+0	6032	III+V	6		3		9
EH	25	2+1	6035	III+V	9		4		8
CM	30	2+0	6046	III+V	5		3		4
LI	26	0+0	6050	III+V	8	14.8	4	5.7	8
VL	32	0+0	6051	III+V			1		5
CB	28	2+0	6061	III+V	5		3		12
CP	27	0+0	6067	III+V	7		10		7
CMcD	30	0+1	6068	III+V	5		1		8
CW	29	3+1	6099	III+V	6		3		
JMcD	28	0+1	6125	III+V	21		5		
FC	21	0+0	6129	III+V	10		1		

IIIb (contd) - Patients with cervical intraepithelial neoplasia.

Name	Age	Parity	Spec No	Grade	Ecto Cx		Abn Tz		Post Rx
EH	39	2+0	6130	III+V	4		5		
JMcK	23	0+1	6131	III+V	6		3		13
IK	37	2+0	6134	III+V	4		4		
CC	26	0+0	6138	III+V	9		12		
JB	26	0+1	6139	III+V	8		3		
RA	27	1+1	6151	III+V	10		3		
DM	28	0+0	6161	III+V	11		7		
LN	28	0+0	6163	III+V	7		5		
SG	31	2+0	6166	III+V	5		1		
MC	20	1+0	6177	III+V	7	8.2	2	2.2	
AB	28	3+0	6178	III+V	8	7.9	2	3.0	
CG	29	1+1	6179	III+V	12		8		
PM	28	2+1	6180	III+V	11		5		
EMcK	33	2+0	6184	III+V	8		3		
MH	31	2+0	6202	III+V	6	9.2	3	3.9	
JMcL	22	0+1	6205	III+V	7	7.2	1	2.0	
PP	22	1+0	6230	III+V	9		5		
JS	35	3+2	6234	III+V	8		4		
MF	22	1+0	6248	III+V	5		4		
EMcN	32	2+1	6273	III+V	4		2		
LC	29	2+0	6278	III+V	12	14.2	6	5.8	
JS	34	4+0	6279	III+V	10	15.5	1	0.9	
EC	25	0+0	6297	III+V	10		1		
MMcD	35	2+1	6302	III+V	9	6.5	12	14.2	
LMcD	26	2+0	6306	III+V	17	12.0	4	1.2	
FR	27	0+0	6315	III+V	8	10.3	4	5.0	
IB	35	2+0	6317	III+V	7	5.2	4	1.9	
JW	32	3+0	6319	III+V	12	10.1	10	5.6	
EF	21	2+0	6320	III+V	8	6.2	6	4.8	
JC	26	1+0	6321	III+V	10		16		
CD	24	1+0	6326	III+V	8	9.6	1	1.5	
EI	26	0+0	6331	III+V	8	9.2	3	4.9	
MB	34	4+0	6334	III+V	11	9.4	1	3.3	

Grade = histological grade of CIN
+V = with histological evidence of HPV infection

Ecto Cx = ectocervix
Abn Tz = abnormal transformation zone
Post Rx = post laser cervical epithelium
(first number in each column indicates Langerhans' cell count per 100 basal cells and the second indicates the count with the image analyser)

IIIc - Renal transplant recipients

Name	Age	Parity	Spec No	RX	Norm Tz		Cont Ecto		Abn Tz	
EMcD	48	4+1	6286	CP						
MS	45	2+0	6287	CP	4	1.7				
BM	47	5+0	6291	CP	6	4.6				
MB	44	2+1	6292	CP	4	6.6				
MMcL	36	1+0	6294	P	10	6.2				
CW	41	4+0	6298	CP	2	0.2				
JB	35	1+0	6299	CP	6	1.2				
AC	52	2+0	6304	CP	3	1.7				
EB	37	1+3	6308	CP	4					
CK	23	0+0	6312	CP	6	4.6				
BS	57	2+0	6313	P	1	0.1				
AB	57	0+0	6318	CP	1	0.1				
CC	27	1+0	6322	AC					3	3.7
MMcK	32	1+0	6323	A P						
AM	25	0+1	6324	CP	1	0.2				
FP	41	1+0	6327	A P			5	3.8	2	1.3
LL	27	1+0	6328	A P	6	11.6				
CMcC	37	5+1	6332	CP	10	22.7				
HH	54	4+0	6336	C	1	1.4				
VD	29	1+0	6339	A P	1	1.3				
MG	38	1+0	6345	A P	4	6.8				
CB	20	0+0	6347	CP	3	2.8				
MH	62	1+0	6348	CP	1	3.9				
EG	32	0+0	6353	A P			6	8.0	7	6.5
MMcD	60	6+0	6356	A P	1	3.8				
EMcM	51	1+0	6357	A P	4	6.3				
MH	34	0+1	7002	A P	5	9.8				
JMcG	42	2+0	7003	CP			7	10.3	3	4.5
EMcI	51	3+1	7004	A P	7	12.0				
WG	34	2+1	7005	CP	8	10.7				
MMcD	30	0+0	7006	A P	1	0.4				
JC	52	4+0	7007	CP	3	3.2				
JM	30	1+1	7014	A P			6	9.1	6	8.6
JH	50	5+1	7015	CP	7	4.1				
SB	45	2+0	7019	P						
MH	39	3+0	7020	CP						
AMcK	34	0+0	7040	A P			9		3	
DB	26	1+0	7047	A P	6					

RX = Immunosuppressant regime
I = Azathioprine
C = Cycolsporin A
P = Prednisolone

Norm Tz = normal transformation zone
Cont Ecto = control ectocervix (with Abn Tz)
Abn Tz = abnormal transformation zone
(first number in each column indicates Langerhans' cell count per 100 basal cells and the second indicates the count with the image analyser)

IIId - Women in renal failure

Name	Age	Parity	Spec No	Type	Norm Tz
RC	58	3+0	7023	CAPD	4
WMcM	48	0+0	7024	HAEMO	8
RD	46	2+0	7025	CAPD	6
MS	47	6+0	7027	CRF	8
SC	60	3+0	7043	CRF	8
AF	61	6+0	7044	CRF	
AW	52	5+1	7045	CAPD	11
GMcA	59	0+0	7046	CRF/CA	5
MG	60	3+0	7048	HAEMO	4
MB	55	3+0	7049	CRF	2
HC	45	2+0	7050	CRF	3
AH	54	3+2	7052	CRF	3
ML	44	2+0	7053	CRF	8
SMcL	48	3+1	7060	CAPD	4
MS	48	1+0	7062	CAPD	12
SH	54	3+0	7063	CAPD	5

TYPE = type of regime
CAPD = continuous ambulatory peritoneal dialysis
CRF = end stage renal failure
HAEMO = haemodialysis

Norm Tz = normal transformation zone
(first number in each column indicates Langerhans' cell count per 100 basal cells)

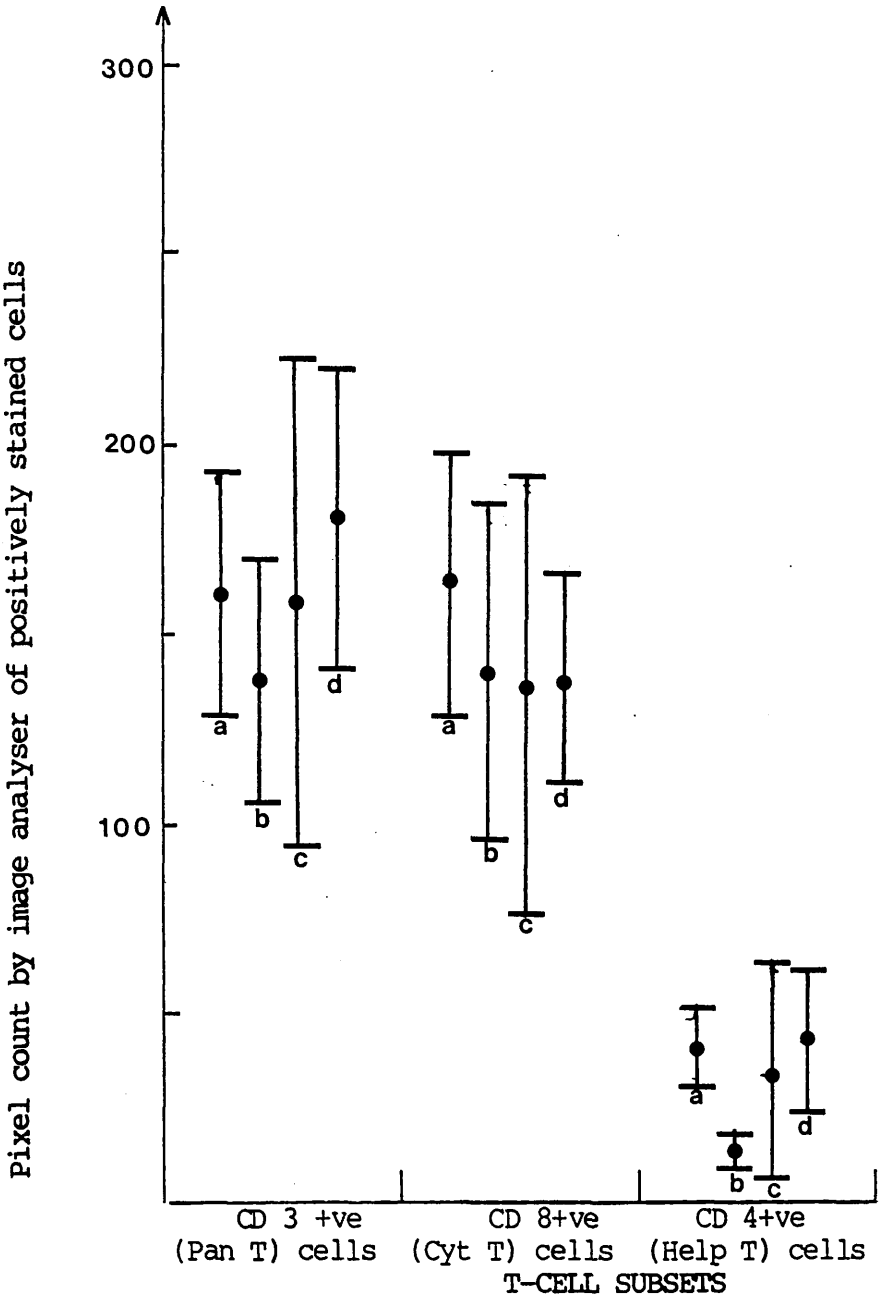
IIIe - Pregnant patients (Normal transformation zone)

Name	Age	Parity	Gest Age	Spec No	Lc's /100	Lc's /area
JH	32	0+0	10	6020	10	14.8
MW	23	0+0	7	6021	2	7.9
MMcK	29	0+0	10	6024	5	7.9
RP	19	0+0	9	6025	9	9.1
SP	19	0+0	8	6036	7	10.0
KC	19	0+0	10	6042	8	12.1
KH	28	0+0	10	6043	10	14.2
CC	19	1+0	8	6097	6	13.3
MMcD	17	0+0	11	6122	5	8.2
KS	27	1+0	9	6127	6	6.2
SH	27	2+0	9	6156	8	8.2
LMcD	25	0+0	11	6165	9	13.3
LN	20	0+0	7	6192	3	4.2
LC	34	1+0	7	6197	9	8.7
HR	26	0+0	10	6208	10	13.1
MMcK	19	1+0	8	6209	6	7.0
VB	23	0+0	8	6253	3	3.6
OM	22	0+0	8	6254	8	6.2
JL	19	0+0	10	6269	11	8.6
RS	32	1+0	7	6270	11	5.7

IIIf - Patients with stage Ib/IIa carcinoma of cervix

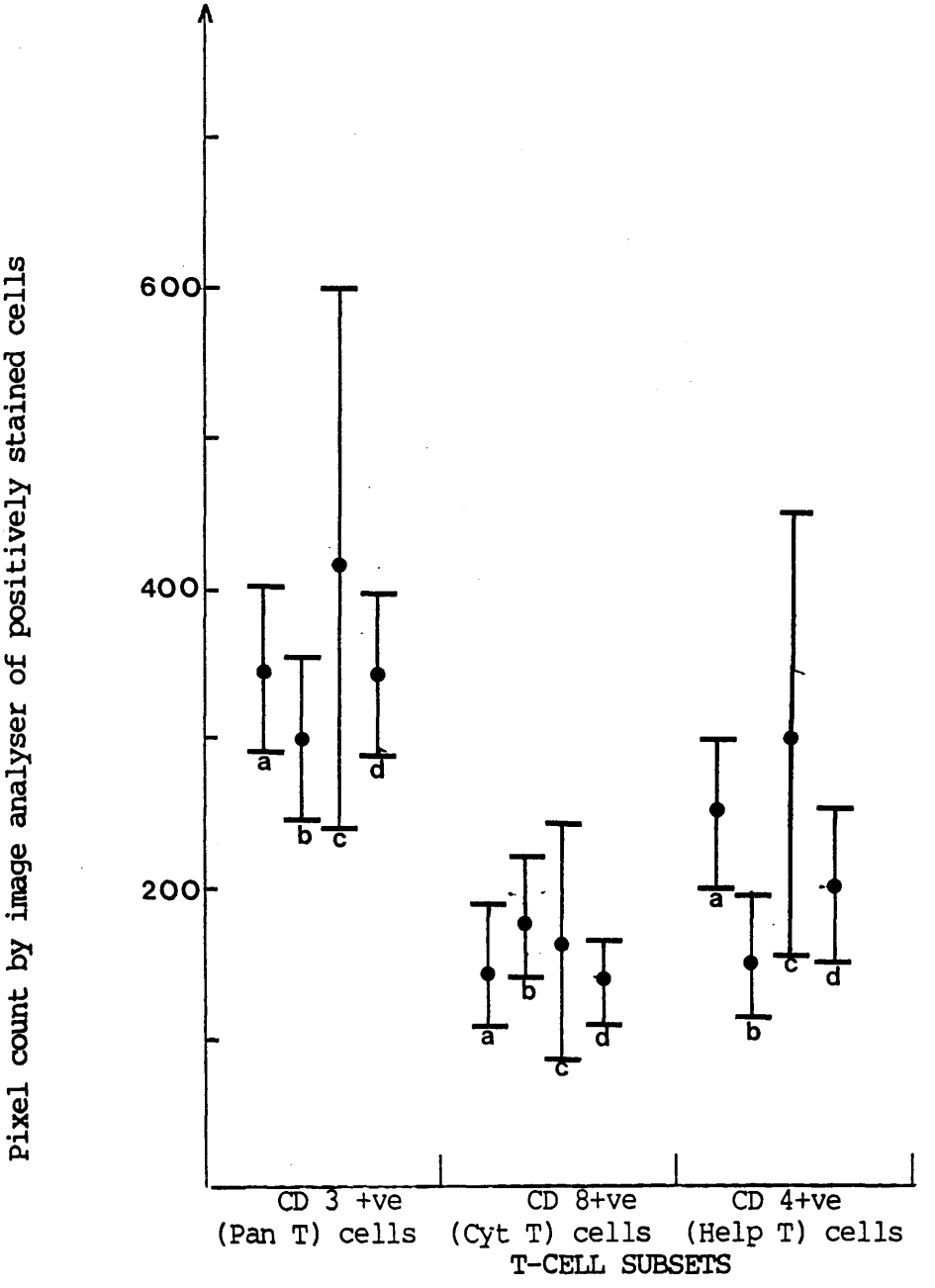
Name	Age	Parity	Spec No	Histology (differentation)	Local spread	Nodes	Langerhans' cells			
							ecto (/100)	tumour (HP)	ecto (unit area)	tumour
EO'N	28	2+2	5022	poor	13mm		13	8	12.7	15.7
CMcN	33	1+0	5025	mod	18mm		8	62	10.0	65.1
JB	27	1+0	5031	poor	13mm		4	77	11.6	48.2
IC	27	19/52 1+0	5046	mod	extensive	+ve	6	5	8.8	13.2
EL	36	1+2	5058	mod	4mm		6	10	6.7	3.3
MY	59	1+0	6002	mod	extensive		9	1	14.9	6.2
DB	49	0+0	6008	mod	5mm		2	20	4.6	23.5
RMcM	50	3+0	6012	poor	5mm		1	7	0.7	15.6
CH	27	2+1	6014	well/poor	10mm		5	3	4.9	4.1
JP	46	1+0	7041	poor	8mm		4	7	3.7	3.2

Appendix IVa.
Graphical comparison of T-cell infiltrate (pixel count) per unit area by image analyser of CD3/T1 positive (Pan T) cells, CD4/T4 positive (Helper) and CD8/T8 (Cytotoxic) T-cell subsets from the epithelium of the transformation zones of normal, renal transplant, uraemic and pregnant patients.



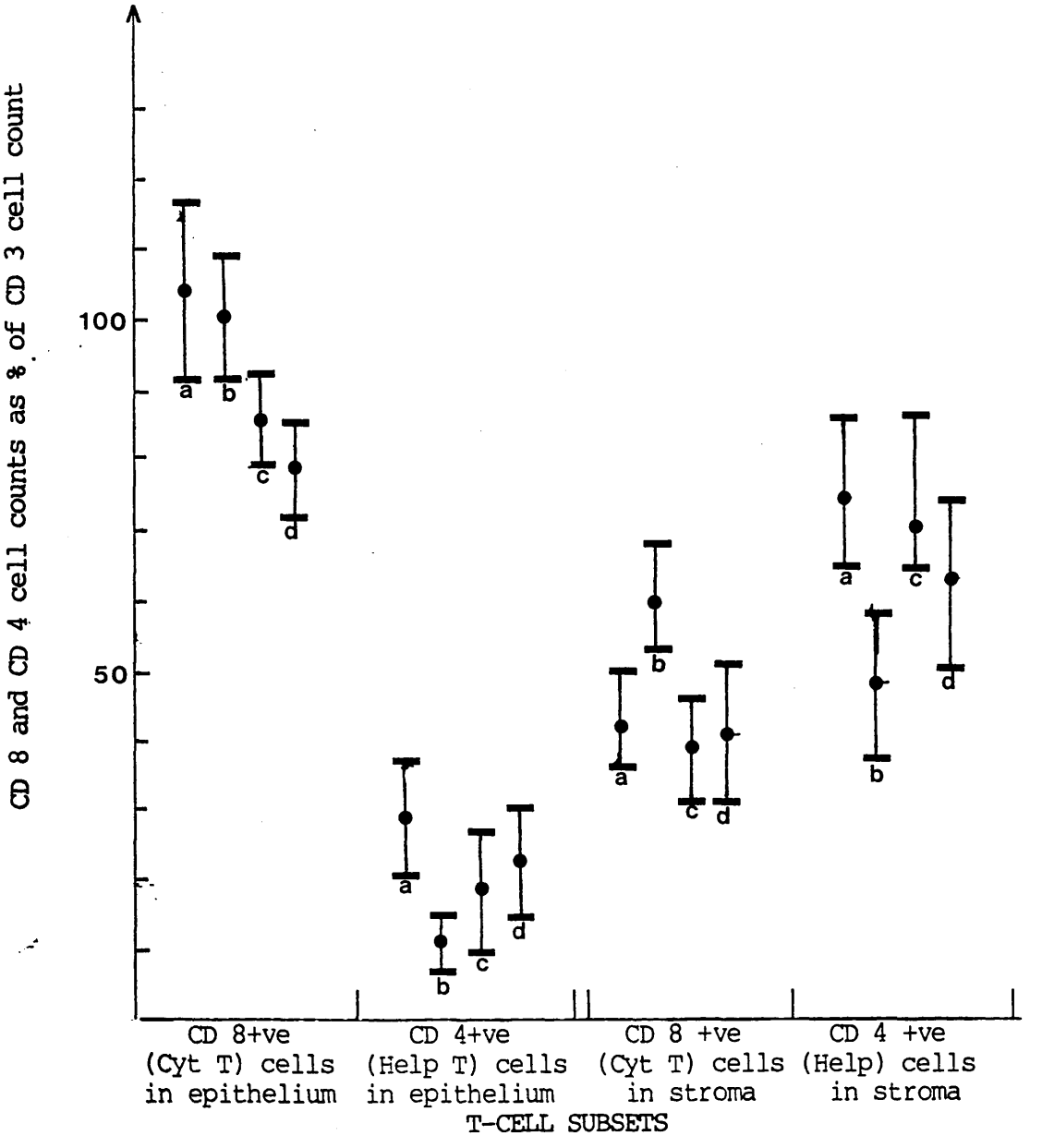
Note. 95% Confidence intervals of means for numbers of cells per unit area of transformation zone are shown ⊢●⊣
a = cell counts from transformation zone of normal patients
b = cell counts from transformation zone of renal transplant patients
c = cell counts from transformation zone of uraemic patients
d = cell counts from transformation zone of pregnant patients

Appendix IVb.
Graphical comparison of T-cell infiltrate (pixel count) per unit area by image analyser of CD3/T1 positive (Pan T) cells, CD4/T4 positive (Helper) and CD8/T8 (Cytotoxic) T-cell subsets from the stromal of the transformation zones of normal, renal transplant, uraemic and pregnant patients.



Note. 95% Confidence intervals of means for numbers of cells per unit area of transformation zone are shown ⊢●⊣
a = cell counts from transformation zone of normal patients
b = cell counts from transformation zone of renal transplant patients
c = cell counts from transformation zone of uraemic patients
d = cell counts from transformation zone of pregnant patients

Appendix IVc.
Graphical comparison of percentages of CD 8/T8 positive (cytotoxic) and CD 4/T4 (helper) T-cells in the epithelium and stroma of the transformation zones of normal, renal transplant, uraemic and pregnant patients.



Note. 95% Confidence intervals of means for % number of cells per unit area of transformation zone are shown ⊢●⊣.
% =((number CD 8 or 4 cells X 100)/ number of CD 3 positive cells)
a = % cell counts from transformation zone of normal patients
b = % cell counts from transformation zone of renal transplant patients
c = % cell counts from transformation zone of uraemic patients
d = % cell counts from transformation zone of pregnant patients