The Immune Microenvironment in HPV-related Cervical Neoplasia

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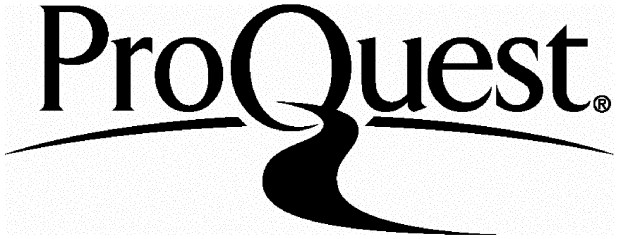

SUMMARY

There is a strong body of evidence implicating human papillomaviruses (HPVs) as aetiological agents in the pathogenesis of cervical cancer and its precursor lesions, i.e. cervical intraepithelial neoplasia (CIN). Host cell-mediated immunosurveillance mechanisms may be of critical importance in the control of HPV-related cervical disease. The main objective of this thesis was to contribute to the understanding of these mechanisms involved in the local immunomodulation of HPV infected and/or transformed cervical epithelium. We have shown that the addition of mussel glycogen during the extraction of DNA for HPV 16 PCR increases the DNA yield and the detection of this oncogenic HPV type. With respect to antigen processing, the aspartic proteinase cathepsin E, which has been reported to play a role in presentation by class II MHC molecules is associated with cellular dedifferentiation in CIN lesions. HLA-DR antigens were expressed constitutively on the majority of Langerhans cells. In contrast, few Langerhans cells express HLA-DQ in normal cervix, but there was a steady upregulation of the proportion expressing HLA-DQ, which paralleled the severity of premalignant disease. In contrast to the epithelium, stromal CD1a+ cells expressed adhesion molecules. CD58 (LFA-3) was expressed constitutively by cervical keratinocytes, but not CD50 (ICAM-3) and CD86 (B7-2). Keratinocyte expression of TNF-α protein was downregulated, and IL-10 protein was detected in some CIN lesions. In high-grade disease there was an overall increase in the number of mucosal infiltrating immune cells, predominantly positive for CD11a (LFA-1). Finally, we have shown different patterns of CD3-induced T cell proliferative responses in the presence of supernatants from cultures of HPV-infected and/or transformed cervical epithelia, but no significant correlations were found between T cell proliferation and the concentrations of IL-1α, IL-10, IL-12 and TNF-α in the culture media. Our results are consistent with a local response to CIN progression characterized by Langerhans cell activation and emigration, alterations in keratinocyte cell surface and cytokine production, and infiltration of immune cells.

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CONTENTS

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

Numerous studies support the view that cervical cancer and its precursor lesions, cervical intraepithelial neoplasia (CIN), are sexually transmitted diseases, and that the major causal agent is human papillomavirus (HPV) (zur Hausen 1989 and 1991, Munoz *et al* 1992, Lorincz *et al* 1992, Schiffman *et al* 1993). This evidence was summarized by Reid and Lorincz in 1991. Cross-sectional epidemiological data show strong, consistent relationships between specific HPV types and both precursor and invasive disease. HPV-immortalized human cells can develop tumorigenic (invasive) properties with long-term culture. Animal papillomaviruses of analogous genetic organization produce invasive cancers in several species. Viral genes (especially E6 and E7) are continuously transcribed within cervical cancer-derived cell lines. E6 and E7 viral proteins bind two cellular "anti-oncogenes" (p53 and pRB) that control cell growth rates. HPV DNA is episomal in benign lesions, but is integrated into the cellular genome of most cancer cells. Integration destroys the viral negative control gene (E2) but preserves the transforming genes (E6 and E7).

Cancer of the cervix is estimated to be the second most common female cancer with an assumed 465,000 new cases per year worldwide (Parkin *et al* 1988). Despite an extensive screening programme, the incidence of this disease in the UK, as well as in other countries is increasing, particularly in young women in the age groups of 25-29 and 30-34 (Beral and Booth 1986). Although the mortality rates from cervical cancer are on decline in most industrialized nations, this cancer remains one of the major causes of cancer deaths in women worldwide (Boffetta and Parkin 1994). In developing countries where disease prevalence is high, it is the most common cause of cancer mortality in women and was responsible for an estimated 340,000 deaths in 1985 (Boffetta and Parkin 1994).

The incidence and prevalence of premalignant cervical disease is very difficult to reliably document. However, accumulated data suggests that there has been a dramatic increase over time in the incidence and prevalence of CIN. The average age of women

with CIN has also been decreasing (Mazur and Cloud 1984, Kock and Johanser 1987). The primary reason for this increase might relate to the efficacy of cervical cytology screening in identifying premalignant lesions. Additionally, there is probably an increased population at risk due to the trend towards early onset of sexual activity among young women, and consequently, earlier age of acquisition of HPV. These observations coincide with the rising incidence of cervical cancer in women under the age of 40 (Beral and Booth 1986, Winkelstein and Selvin 1989).

In this thesis we will be focusing our attention on the study of cervical squamous intraepithelial neoplasia, the precursor of invasive cervical cancer, for several reasons. The majority of cervical malignancies (80 to 90%) are squamous cell carcinomas (Robert and Fu 1990). The prevalence and incidence of both HPV infections and CIN lesions are estimated to be increasing worldwide. The study of these lesions offer an opportunity not only to follow the mechanisms involved in the process of cervical carcinogenesis, but also the host defense mechanisms to suppress the development of neoplasia and retard tumour progression. The understanding of these mechanisms could contribute to the development of new therapeutic strategies and/or assist in the development of original and effective prophylactic modalities to prevent disease and amplify infection/tumour rejection. Research on the prevention of cervical cancer, as well as on detection and control of its premalignant precursors is necessary because, unfortunately, when overt neoplasia becomes apparent the treatment modalities so far available still offer poor results in terms of survival and quality of life.

1.1 The normal cervix

The uterus is divided into the corpus and cervix. The cervix is divided by its vaginal attachments into a supravaginal portion and a vaginal portion, the portio vaginalis, projecting into the vaginal canal. The outer surface of the portio vaginalis is commonly refereed to as the ectocervix and is covered by a stratified squamous epithelium contiguous with the vaginal mucosa. The external os leads to the endocervix, lined by a columnar mucin-secreting epithelium. The zone of transition between the stratified squamous epithelium of the ectocervix and the columnar epithelium of the endocervix, previously known as the squamocolumnar junction, is presently designated as the

transformation zone, and normally coincides with the external os. The transformation zone designates the area of original columnar epithelium which can become squamous epithelium through a physiologic process known as metaplasia, which occurs in pluripotent, reserve cells beneath the columnar epithelium. This area of cellular instability through intermittent metaplastic change is the privileged site of origin for squamous cervical neoplasia (Coppleson and Reid 1968, Abdul-Karim *et al* 1982). Early studies on mapping of CIN and the surrounding epithelia in cone specimens have shown that only 3% of CINs were found in the ectocervix, 10% were surrounded by endocervical epithelium and the remaining 87% of CINs occurred within the transformation zone (Abdul-Karim *et al* 1982). This might be attributed to the more active metabolism and cell-cycle kinetics of the metaplastic epithelium in the transformation zone, whose stem-cells are readily accessible and so more likely to incorporate viral or other carcinogenic material. The most active phases of metaplasia occur during foetal life, but also in the years following puberty and during the first pregnancy (Coppleson and Reid 1968), which generally correspond to the years of most intense sexual activity.

1.2 Histopathology of cervical squamous intraepithelial neoplasia

Cervical cancer precursors can be defined as a reaction to a combination of carcinogenic and noncarcinogenic agents. Excluding changes associated with inflammation, repair and infections, and also some HPV-induced lesions such as condylomata or warts, which are benign conditions with no potential for malignant transformation, a vast spectrum of intraepithelial abnormalities remain. At the lower end, the lesions are minimal or mild and difficult to distinguish from reactive hyperplasia and inflammatory changes. They include manifestations of cervical HPV infection - koilocytic atypia, also designated condylomatous atypia (Brisson *et al* 1988). At the other extreme, the lesions exhibit most or all the morphologic features of malignant cells except for stromal invasion. For decades, these premalignant intraepithelial changes have been known as squamous dysplasia and carcinoma *in situ* (CIS) (Reagan *et al* 1953). This two-tier concept of disease process was replaced by cervical intraepithelial neoplasia (CIN), which encompasses dysplasia and carcinoma *in situ* as a spectrum of the same disease, considered as progressive stages in the development of invasive carcinoma. This was classified into three grades (Richart 1967, Richart and Barron 1969). These grades are

defined depending on the proportion of the thickness of squamous epithelium with abnormal differentiation, as shown by disturbances of cellular maturation and stratification, as well as cytological atypia. In CIN1 (previously known as mild dysplasia) cytoplasmic maturation occurs in the superficial two thirds of the epithelium. In CIN2 (previously known as moderate dysplasia) cytoplasmic maturation starts in the middle third of the epithelium, and in CIN3 (including severe dysplasia and carcinoma *in situ*) cytoplasmic maturation, which is minimal, occurs in only part of the superficial third of the epithelium or is absent (Buckley *et al* 1982).

However, studies on HPV typing (Lorincz *et al* 1992), nuclear DNA ploidy patterns (Fu *et al* 1989), epidemiologic (Narod *et al* 1991), and clinical (Koutsky *et al* 1992) investigations of CIN, favor the adoption of a classification separating CIN lesions into two classes. The Bethesda System for reporting cervicovaginal smears has proposed the terminology low- and high-grade squamous intraepithelial lesions (SILs) (Solomon 1989). Low-grade disease includes koilocytotic atypia and/or CIN1, and high-grade disease includes CIN2 and/or CIN3. The histopathologic criteria characterizing low- and high-grade CINs/SILs (considered synonyms as proposed by (Richart 1990), suggestion that we will be following throughout this thesis) are based on the cellular maturation, cellular organization and polarity, nuclear features and mitotic activity of the lesions. The cellular organization is minimally perturbed in low- and severely altered in high-grade SIL. The nuclei in low-grade SIL are mildly irregular in size and shape and usually round or oval, whereas with increasing severity of the disease they become more irregular reaching the upper half of the epithelium. In low-grade SIL, the mitotic figures are few in number, confined to the lower third and normal in appearance. In high-grade SIL, the number of mitotic figures increase, extend to the upper layers of the epithelium and abnormal forms occur (Fu *et al* 1981).

The adoption of the Bethesda System should not only improve the reproducibility of diagnosis, but also is considered more relevant to the biologic outcome and so, to the clinical management of CIN lesions. It has been shown that CIN 1 is associated with both low- and high-risk HPVs, while higher grade CINs are limited to high-risk HPV types (Lorincz *et al* 1992). In a prospective study, it was found that among women whose HPV DNA status changed from negative to positive, 28% developed CIN 2 or

CIN 3 within 2 years, and in contrast, those whose HPV DNA remained negative, only 3% developed CIN 2 or CIN 3 within the same period of time (Koutsky *et al* 1992). Thus, high-grade CIN may occur through a brief transition time from low-grade CIN or by de novo development. In a study of cervical condylomas and CINs by imaging cytometry (Fu *et al* 1989), all condylomas and 67% of CIN 1 had a predominantly diploid and occasionally polyploid DNA pattern. Aneuploid pattern was detected in 33% of CIN 1, 75% of CIN 2 and 90% of CIN 3. In an earlier study on women with an initial diagnosis of dysplasia and not treated for at least one year, 85% of those whose lesions regressed had diploid or polyploid patterns, and the remaining 15% aneuploid. Among women whose lesions persisted as CIN or progressed to invasive carcinoma 92% had an aneuploid pattern and the remaining 8% diploid/polyploid (Fu *et al* 1981). A further correlation between morphology and DNA ploidy patterns was that lesions with diploid and polyploid patterns retain their normal polarity in the basal layer, where nuclei are relatively uniform in size and lack abnormal mitotic figures. CINs with aneuploid DNA distribution tend to reveal a high cellularity, cellular disorganization and abnormal mitotic figures (Fu *et al* 1979 and 1981).

Therefore, we have adopted a modified Bethesda system to classify the cervical specimens used in this research. A minority of low-grade lesions can also progress to invasive cervical carcinomas. One explanation for this is that these are associated with high-risk HPV types. Furthermore, Anderson *et al* in 1991 emphasized that there was a risk in adopting a two-tier system, as it might encourage the belief that there is a two stage process in the natural history of CIN. Also, there is disagreement over where the division between low- and high-grade lesions should lie, and whether HPV-related lesions should be included with CIN. Finally, diagnostic errors could have a more important impact on patient management than with a three-grade system.

1.2.1 Natural history of cervical intraepithelial neoplasia

The natural history of CIN lesions have been studied for decades using different approaches. However, biases are common and only estimates can be presented. Many prospective studies on untreated patients were biased because patients were not randomly selected. The use of biopsies to monitor disease may completely remove the lesion or alter the natural history of the disease. Lesions could be missed, and both

insufficient length of follow-up and considerable loss of patients to follow-up have occurred. In the few studies in which the initial diagnosis and follow-up were by cytology alone, the underlying histology may not have been reliably established. In studies which analyse patients lost to follow-up, the main criticisms are the small numbers of patients enrolled, length of observation and lack of details on the factors responsible for loss to follow-up that can confound the final analysis. In studies following therapy for carcinoma *in situ* (CIS), the treatment modalities considerably alter the natural history of the disease.

Based on an extensive review of the literature over the past 40 years by (Ostor 1993), 57% of CIN1 regress, 32% persist, 11% progress to CIN3 and 1% progress to invasive disease. The figures for CIN2 are 43%, 35%, 22% and 5%, respectively. Finally, 32% of CIN3 regress, approximately 56% persist and more than 12% progress to invasion. Evaluation of only the most recent studies, which had long follow-up periods, revealed a higher progression rate from CIN3 to invasive cancer varying between 16 and 40%. In a survey of 948 patients followed-up for 5 to 28 years, (McIndoe *et al* 1984) showed that women with untreated CIS have an 18% chance of developing invasive carcinoma of the cervix at 10 years and a 36% chance at 20 years. Data gathered from cervical cancer screening programs subject to statistical analyses estimate transition rates of 19% to 38% for CIN to CIS and 26% to 66% for CIS to invasive cancer. The duration of disease states from these data is estimated to be 3.8 years for CIN to CIS and 8.1 to 12.6 years for CIS to invasive cancer (Boyes *et al* 1982).

Recently, the risk of progression from low- to high-grade CIN was shown to be greater in patients with high-risk HPV infection (Remmink *et al* 1995), persistent HPV infection (Ho *et al* 1995), and in those with high viral load (Ho *et al* 1995) suggesting that viral type, prolonged exposure to viral effects and host factors (e.g. impaired immunosurveillance) are important in the pathogenesis of cervical disease. However, the biological behavior of an individual lesion remains, so far, unpredictable. Although suggestive, it has not been formally established whether most CIN lesions develop progressively through minor stages of abnormality to high-grade CIN, or whether the origin of CIN is multicellular with lesions of CIN1, 2, or 3 developing independently from abnormal cell populations in the transformation zone. A *de novo* development

from benign epithelium to CIN3 is possible, but its frequency remains unknown. Also, a few invasive cancers may arise *ab initio* without a preceding intraepithelial phase.

1.3 Human papillomavirus

1.3.1 Viral structure

Papillomaviruses are icosahedral particles approximately 55 nm in diameter, with an outer capsid coat composed of two structural proteins: L1, which comprises 80% of the total viral protein, and L2, which is a minor component. The protein capsid encloses a 8-Kb double-stranded circular DNA genome (Favre *et al* 1977). All the human papillomaviruses (HPVs) so far studied encode 8 open reading frames (ORFs), which are sequences coding for proteins synthesized "early" (E) or "late" (L) in viral replication, and a noncoding region, referred to as URR (upstream regulatory region) which contains the origin of DNA replication, promoter elements and transcriptional enhancer sequences. These ORFs can be grouped according to their function: E1, E2 - implicated in viral replication and gene transcription control; E3 and E4 - whose function is unknown, but encode proteins which can disrupt the host cytoplasmic keratin network, producing the classical halo effect in infected keratinocytes; E5, E6, E7 - have transforming functions; and finally, L1, L2 - coding for viral capsid proteins (Danos *et al* 1984, Doorbar *et al* 1991, Thierry 1993).

1.3.2 Classification and clinical associations of HPV genotypes

The classification of HPVs was based on differences demonstrated by DNA hybridization: a "new" HPV type was defined if the degree of hybridization between its genomic DNA and that of previously accepted prototype was less than 50% (Coggin and zur Hausen 1979). The biological justification for this approach is the consistent correlation between clinical lesions and presence of specific HPV types. Currently, the criteria for distinguishing "new" HPV types has been modified i.e., when less than 90% homology with the E6/E7 and L1 regions of the prototype is detected, a subtype being 90-98% homologous and a variant more than 98% (de Villiers 1992). The implications of using this system become apparent when, recently, it has been demonstrated not only that HPV16 variants are widely prevalent in cervical cancers, but also that such variants differ in their ability to interact with host cell cycle control mechanisms (Stoppler *et al* 1996). These techniques have so far identified 77 HPV types, 35 of which were isolated

from genital lesions (de Villiers 1994). Specific anatomical tropism of each viral type is related to differences in their nucleotide sequence.

From a clinical perspective, the HPV types can be grouped into two main categories based on frequency of association with invasive cervical cancer. The low risk group (HPVs 6,11,40,42,43 and 44) is never associated with cervical cancer and these viruses are found principally in benign condylomata or low-grade cervical disease. The high risk or "oncogenic" group (HPVs 16,18,31,33,35,39,45,51,52,56,58,66 and 69) are all viral types that have been found in association with cervical cancer and can also be detected in high-grade CIN lesions (Fuchs *et al* 1988, Lorincz *et al* 1992). On the cervix, HPV 6 and 11 are the main types detected in condyloma acuminata. Flat warts may also contain HPV 6/11, but many are associated with high-risk HPV types (Nuovo *et al* 1991). In contrast, HPV 16 is the most frequently detected type in invasive carcinoma (47.1%) and high-grade CIN (also 47.1%), but in only 16.2% of women with low-grade disease and in 1.5% of apparently normal epithelium (Lorincz *et al* 1992). HPV18 despite being the second most common (22-25%) viral type in invasive cancers, is uncommon in any grade of CIN (5%), which suggests that the virus may produce precursor lesions that progress too rapidly to be detected by cytology screening programmes (Kurman *et al* 1988). HPV18 is particularly associated with adenocarcinomas, and its prevalence is equal to or even greater than HPV16 in these cancers (Duggan *et al* 1993). The remaining high-risk HPV types (31,33,35,39,45,51,52,56,58 and 66) exhibit intermediate oncogenicity, and are under-represented in cervical cancer (about 10%) compared with their over-representation in high-grade CIN (30%) (Lorincz *et al* 1992).

Based on a review of recent large studies on HPV prevalence in women with premalignant and invasive cervical disease, using polymerase chain reaction (PCR) or southern blot analysis, (Walboomers *et al* 1994) concluded that HPV 6 is most frequently present in low-grade CIN and almost absent in cancer. HPV 31 is frequently detected in high-grade CIN but in a low percentage of cervical cancer. HPV 16 is almost equally associated with CIN2 and CIN3 and cervical cancer, and HPV 18 is found in a relatively high number of invasive but less in CIN2 and 3 lesions, which suggests that HPV 18 should be considered the most aggressive oncogenic type. Finally, the same analysis showed that in cervical squamous cell carcinoma, HPV prevalence varied

between 84% and 100%, HPV 16/18 being the most prevalent viruses (67% to 72%) found. In a worldwide study of more than 1000 specimens from patients with invasive cervical cancer, HPV DNA was identified in 93% of tumours. HPV16 was present in 50% of the samples, HPV18 in 14%, HPV45 in 8%, HPV31 in 5%. In squamous cell carcinomas HPV16 predominated (51%) but HPV18 was the commonest type found in adenocarcinomas (56%) and adenosquamous tumours (39%) (Bosch *et al* 1995).

In a prospective study of 100 women with recurrent mild dyskariotic smears (Campion *et al* 1986) reported that 56% of those positive for HPV16 progressed to CIN3 within two years, as compared to 4% of those with HPV6. A prospective cohort study of women who were cytologically negative at the beginning of the study but HP16/18 positive revealed CIN2 or CIN3 in 39% within a two year follow-up, but only 3% who were negative for all HPV types. Women positive for HPV31, 33 or 35 had an intermediate progression rate of 22% (Koutsky *et al* 1992).

1.3.3 Carcinogenic (transforming) properties of HPV

The precise role of HPV sequences in the tumorigenesis of cervical epithelium is still not completely understood. However, it is generally agreed that HPV-induced cancers consistently contain the complete E6/E7 gene sequences, often with only some other genomic elements like the upstream regulatory region (URR), all of which are invariably intact and transcriptionally active (zur Hausen 1989). It has also been shown that in human epithelial cells, cooperation between E6 and E7 is necessary for efficient immortalization (Munger *et al* 1989), and their expression is required for maintenance of the transformed phenotype (von Knebel-Doeberitz *et al* 1994). Interestingly, the *in vivo* morphologic features of HPV-infected cervical epithelium can be mimicked *in vitro* using organotypic tissue cultures, where epithelial cells do stratify and differentiate. Under these conditions, human keratinocytes immortalized by E6/E7 genes of HPV 16 and 18 show histological abnormalities similar to high-grade CINs (Rader *et al* 1990). In addition, integration of the viral genome into the host chromosome is often associated with disruption of the viral E1 or E2 genes, which can negatively regulate the transcriptional promoter directing E6 and E7 (Schwarz *et al* 1985), therefore, increasing the expression of these genes. It has been shown that disruption of either E1 or E2 regulatory genes of HPV16 results in an increase in the immortalization capacity of the

viral genome (Romanczuk and Howley 1992). Furthermore, the viral DNA is frequently integrated preferentially into fragile chromosomal sites or in the vicinity of cellular oncogenes (Pfister and Kleiner 1991). HPV16 DNA has been shown in experimental models to cooperate with the mutated *c-Ha-ras* gene in transforming primary cells (Matlashewski *et al* 1987). HPV sequences integrated near the c-myc locus may also be involved in the activation of the proto-oncogene in cervical cell lines (Durst *et al* 1987). Finally, an early event in malignant transformation was suggested to be overexpression of the cell cycle gene Bcl-2, which protects against apoptosis and differentiation, and was more likely to be seen in high-grade rather than low-grade CINs (Saegusa *et al* 1995).

The E6 and E7 proteins encoded by the high-risk HPVs are oncoproteins and contribute to cellular transformation by binding to the cell regulatory proteins p53 and pRB, respectively. The E6 proteins of the high-risk HPVs can form a complex with the tumour suppressor protein, p53, and promote its degradation (Werness *et al* 1990). The subsequent inactivation and enhanced degradation of p53, which occurs after binding to the E6 transforming proteins, appear to have a pivotal role in the mechanism of cervical tumorigenesis, disrupting the normal response of cervical epithelial cells to DNA damage (Kessis *et al* 1993) and creating chromosomal instability. Normally, p53 protein inhibits proliferation by binding to transcriptional regulatory elements in DNA. The protein functions as a surveillance mechanism in which cells that have undergone genetic damage are arrested in the G1 phase of the cell cycle to allow for DNA repair. If this repair is inadequate, p53 can trigger apoptosis (Kastan *et al* 1991). On binding with pRB protein, E7 frees key cell cycle proteins from RB-imposed negative transcriptional regulation, ultimately responsible for chromosomal instability and aneuploidy. In contrast, E6 and E7 proteins from low-risk HPV types 6 and 11, which are expressed at lower levels than those of the high-risk HPVs, only weakly bind p53 and pRB respectively (Lane and Benchimol 1990, Scheffner *et al* 1991) and, thus, these viruses do not have the ability to induce malignant transformation *in vivo* (Schlegel 1990). Also, these low-risk viruses appear to be unable to integrate their DNA into the human genome (Story *et al* 1988). However, infection *in vivo* with the low-risk HPV types results in marked epithelial hyperproliferation and development of condylomatous lesions.

1.3.4 Prevalence of HPV infection

Prevalence estimates of genital HPV infection vary between studies despite the fact that apparently similar populations are examined and the same molecular methods are used. Such differences may be attributable to variations in distribution of age and risk factors for genital infection in the populations examined, the method of sampling (swabs, lavages, wooden spatulas, biopsy or tampons) and inter-laboratory variation in processing, hybridizing and probing techniques. When polymerase chain reaction (PCR) is used, variation in results may be due to differences in the HPV type-specific probes and whether consensus or generic primers are used (Schiffman *et al* 1991).

Genital HPV infection is the most common sexually transmitted virus and may be the most common sexually transmitted disease (Kiviat *et al* 1992). Significantly, in a study on sexually active female adolescents aged 13 to 19 years attending a family planning clinic, HPV was found to be the most prevalent sexually transmitted organism, detectable by dot-blot hybridization in 15% of participants, compared with 10% for *Chlamydia trachomatis*, 4% for *Trichomonas vaginalis* and 3% for *Neisseria gonorrhoeae* (Moscicki *et al* 1990). The prevalence of HPV infection detected cytologically in a private practice varied between 0.7% and 3% (de Brux *et al* 1983). Among women attending sexually transmitted disease clinics, 8-13% of Pap smears (i.e. exfoliative cytology) show signs of HPV infection (Drake *et al* 1987). Based on routine cytological smears analyzed by PCR to identify the HPV types 6,11,16,18,31,33,35,40,45,52, and 58, (Van den Brule *et al* 1991) reported that in cytologically normal smears of asymptomatic women (n= 1243) an overall HPV prevalence of 3.5% was found. The frequencies of HPV types 16, 18, 31 and 33 was 1.5%. In Pap IIIa (CIN1) (n= 435), Pap IIIb (CIN2) (n= 141) and Pap IV (CIN3) (n= 70) smears the overall HPV prevalence was 74%, 80% and 100% respectively. HPV 16, 18, 31 and 33 rates increased from 43% to 57% and 80% in CIN1, CIN2 and CIN3 respectively. In all cervical carcinomas (Pap V) (n= 50) HPV DNA was detected.

Detection of HPV DNA in genital tract specimens is highest among young sexually active women with a prevalence peak between the ages of 20 and 25, in contrast to older women where detection diminishes with age, suggesting that the infection clears in at

least some women and viral production decreases over time, which could be due to a local immune response (de Villiers *et al* 1992b).

1.3.5 Transmission

HPV transmission is predominantly via sexual activity, since 60 to 66% of sexual partners of individuals with genital HPV-induced disease develop detectable HPV-related lesions (Barasso *et al* 1987, Schneider *et al* 1987b). Although genital-oral transmission is possible, the role it plays in the development of cervical disease is not known. Still also unclear is the contribution of vertical or peripartum transmission of HPV. It is possible that HPV-induced extragenital skin lesions may serve as viral reservoirs (Jenison *et al* 1990).

It has been proposed that the virus gain access to the basal cells of the epithelium in sites of microtrauma as a result of intercourse. The viral genome penetrates the cell membrane after shedding its capsid and is transported to the nucleus. Basal cell division may, then, accelerate viral replication (Taichman and La Porta 1987).

1.3.6 HPV-host cell interactions

Each HPV is species specific and has its own degree of oncogenicity, inducing squamous epithelial and fibroepithelial tumours in their natural hosts. These viruses are exclusively epitheliotropic and virus replication is dependent on the terminal differentiation of its host cell, the keratinocyte. The outcome of HPV infection reflects the interaction of the viral type and host cellular determinants. In HPV productive (permissive) infections of squamous epithelia, proliferating basal cells are found to contain low levels of viral DNA and to lack viral capsid antigens, whereas the uppermost levels of the epithelium contain large amounts of viral DNA (as an extrachromosomal episome) and proteins, and many cell nuclei are packed with complete viral particles (Hoepfner and Loning 1986). In these upper epithelial layers, HPV-infected keratinocytes show characteristic cytoplasmic vacuolation (koilocytes) through collapse of the cytokeratins - the koilocytes representing the hallmark of HPV infection. The nucleus of these cells becomes irregular in shape, hyperchromatic, enlarged and often binucleated (Koss and Durfee 1956, Meisels *et al* 1977). Transforming (non-permissive) infection arises when viral replication and vegetative

viral production does not occur and the neoplastic cells no longer show production of virion particles, but retain at least part of the viral genome (usually integrated into the host chromosome) and maintain a consistent but generally low copy number of the HPV DNA (Yee *et al* 1985). It is possible that infection of stem cells (endocervical reserve cells) in the transformation zone, which do not allow permissive HPV infection, results in either aborted or transforming infection. Finally, in latent infection HPV DNA can be found in cytologically, histologically or colposcopically normal epithelium, suggesting viral persistence after regression of lesions or prolonged viral incubation which may be extended indefinitely.

1.4 Cofactors in the development of cervical neoplasia

Although HPV 16 or 18 alone can transform and immortalize primary rodent or human epithelial cells, these cells are not tumorigenic in nude mice (Munger *et al* 1989, Pecoraro *et al* 1991). Also, the high prevalence and incidence of HPV infection among women in comparison with a minority developing cervical cancer, and the time interval (5 to 25 years) between acquisition of infection and development of malignancy (zur Hausen 1986), indicates that factors other than HPV must be involved in the development of this cancer.

Potential cofactors predisposing to the development of cervical cancer and, therefore, promoting progression of preinvasive cervical neoplasia include genetic changes, cigarette smoking, oral contraceptive use, parity, infectious agents and immunosuppression.

Genetic damage with consequent loss of tumour suppressor genes or activation of cellular oncogenes, can lead to cellular immortalization and transformation. These genetic alterations may occur as a result of cell cycle abnormalities induced by HPV gene expression or may be related to environmental factors. Although loss or mutation of conventional oncogenes and tumour suppressor genes is uncommon in cervical neoplasia (Busby-Earle *et al* 1993), cytogenetic studies have demonstrated non-random chromosome abnormalities involving chromosomes 1, 3, 5, 11 and 17 in cervical carcinoma (Atkin 1997).

Epidemiological studies have implicated cigarette smoking as a possible contributing factor in the development of cervical neoplasia. A 2-fold increased risk of CIN and invasive disease has been demonstrated among smokers (Wigle *et al* 1980). Two mechanisms have been postulated to account for this increased risk. Langerhans cell number and function are reduced in both normal cervical epithelium and in CIN lesions (Barton *et al* 1988). Alternatively, the constituents of smoke and their derivatives may interact with HPV. Nicotine and cotinine found at high levels in cervical mucus (Schiffman *et al* 1987) are mutagenic and can also induce proliferation of HPV-transformed cervical cells (Waggoner and Wang 1994).

An increased incidence of cervical cancer and its precursors has been found in women using oral contraceptives (Beral *et al* 1988). Activation of the immature metaplastic process by oral contraceptive-induced eversion of the columnar epithelium could increase the risk of metaplastic transformation. Alternatively, hormones could have a promoting effect in inducing expression of viral oncoproteins. Beta-oestradiol increased by 8-fold the transcription of HPV16 E6 and E7 (Mitriani-Rosenbaum *et al* 1989). Furthermore, glucocorticoids and progesterone have been shown to induce HPV gene expression in cervical keratinocytes (Mittal *et al* 1993), and progesterone has also been shown to increase the efficiency and frequency of transformation of primary rodent cells by HPV16 (Pater *et al* 1990).

The increased incidence of cervical cancer in association with increased parity could be explained by the combination of increased expression of HPV (permissiveness of cell-mediated immunity to the virus during pregnancy) (Purtilo *et al* 1972), and increased vulnerability of the transformation zone (effects of hormones on the cervix or on HPV expression, eversion of columnar epithelium during pregnancy resulting in a new dynamic phase on the immature metaplastic epithelium, trauma at delivery).

Sexually transmitted infectious agents other than herpes simplex virus type 2 (HSV-2) and human immunodeficiency virus (HIV), the later discussed in section 1.6.1, have not shown consistent association with cervical cancer development. However, local chronic inflammation may contribute to promotion of cervical carcinogenesis by inducing

reparative metaplasia in the transformation zone. A synergistic relationship between HSV (acting as an initiator) and HPV (promotor) may enhance malignant transformation (zur Hausen 1982). Accumulation of mutations within specific sites of the host genome and promotion of gene amplification have been documented with HSV-2 infection (Schmitt *et al* 1989). It has been postulated that the putative role of HSV in cervical carcinogenesis could be related to induction of mutations in keratinocytes, thus predisposing to neoplastic transformation (Galloway and McDougall 1983).

It is unclear, however, whether the described factors operate independent of HPV infection or act through mechanisms that enhance HPV oncogenicity. A proposed mechanism of cervical carcinogenesis suggests that oncogenic HPV genome disruption followed by integration of E6/E7 sequences accompanied by inactivation of cellular control elements induced by external factors acting as cocarcinogens in a susceptible individual (immunocompromised or genetically vulnerable), are the key factors. This multifactorial process of cervical carcinogenesis generally occurs over a long period of time, where the evolution from normal precursor cell to a malignant phenotype is the result of successive and/or cumulative genetic damage which provides a selective growth advantage leading to clonal expansion. In the absence of adequate immunosurveillance mechanisms transformed cells are not destroyed and further genomic instability promotes development of cellular clones with invasive potential.

1.5 Cervical-associated lymphoid tissue

Viral and tumour immunity is primarily cell-mediated and, at least in HPV infection, circulating antibodies tend to play a minor role. In some cases, the effector cells are CD8+ cytotoxic T lymphocytes (CTLs), while in other cases CD4+ helper T (Th) cells are the major effectors and work by amplifying CTL responses and acting in consort with macrophages, natural killer (NK), and other activated immune cells (Greenberg 1991, Melief 1992, Biron 1994). The resolution of viral infections may also involve the innate, nonspecific arm of the immune system represented by local antiviral cytokines (e.g. interferon-γ, IFN-γ and tumour necrosis factor-α, TNF-α) (Ramsay *et al* 1993).

Since HPV does not penetrate below the basement membrane, the primary immunological exposure is to epithelial host defense mechanisms. The mucosal immune microenvironment of the cervix include the following: epithelial trophic T lymphocytes, which include mainly CD8+ cells, although CD4+, and CD4- CD8- γδ+ T cells are also present; Langerhans cells, the professional scavenger APCs of the epithelia; macrophages; natural killer (NK) cells; and stromal endothelial cells, which direct cellular traffic in and out of the epithelium. In addition, keratinocytes although not strictly lymphoid themselves, can phagocytize, constitutively produce and release many cytokines, and express not only MHC (major histocompatibility complex) class I molecules but also upregulate class II antigens and accessory molecules, which enables them to act as antigen-presenting cells (APCs). We will be discussing each of these various components of the epithelium and subepithelial stroma necessary to induce an immune response, which represents the primary line of defense against infections in this anatomic site, in more detail.

1.5.1 T Lymphocytes

T lymphocytes are the cells essential for cellular immune responses, and have been shown to play a critical role in antitumour immunosurveillance (Shimizu and Shen 1979, Rosenberg *et al* 1986). T cells are capable of specifically lysing autologous tumour cells *in vitro* (Topalian *et al* 1989). T cells can also specifically secrete cytokines and proliferate in response to stimulation with tumour cells (Barth *et al* 1991). Finally,

tumour antigens recognized by autologous human T cells have been identified by molecular cloning techniques (Boon *et al* 1994).

T cells express molecules on their cell surface called T cell receptors (TCR), which recognize short peptide fragments of antigens (epitopes) generated by proteolytic degradation, which bind to MHC (major histocompatibility complex) molecules displayed by antigen-presenting cells (APCs). MHC molecules are, therefore, the restriction determinants for T cell recognition of foreign peptide antigens.

There are two classes of MHC molecules which interact with two distinct populations of T cells. Class I molecules present antigenic peptides to CD8+ lymphocytes, whereas class II present the peptides to CD4+ lymphocytes. CD8+ T cells on activation are termed cytotoxic T lymphocytes (CTL) because they can lyse target cells through the release of lytic granules and by induction of apoptosis. CD4+ T cells, while also capable of cytotoxic activity, are called helper T cells because they enhance antibody production by activated B cells, promote CTL responses, activate a variety of other immune effector cells (e.g. macrophages), and are responsible for immunological "memory".

The ability of TCRs to signal T cells following contact with antigen depends on the presence of other adjacent proteins. The TCR antigen-binding heterodimers composed of two chains, $\alpha\beta$ or $\gamma\delta$, are closely associated with a complex of additional proteins, referred to collectively as the CD3 complex (Weissman 1994). The intracytoplasmic portions of the CD3 complex are important in mediating signal transduction into the cell following antigen binding. The two TCR/CD3 signaling routes join in a synergistic fashion within the nucleus to initiate gene transcription, for which interleukin-2 (IL-2) is the T cell prototype. T cell activation signals operate largely by triggering genes that contribute to proliferation, differentiation, and effector functions. One example is the induction of IL-2 and its receptor, which are critical elements in promoting proliferation of unstimulated, naive T cells following activation by antigen (Gaulton and Williamson 1994). TCR $\gamma\delta$ cells, a minor T cell subset in comparison with TCR $\alpha\beta$ T cells, are located in the epithelia, and they may contribute to an early line of host defense. The antigen specificity mediated by $\gamma\delta$ T cells is predominantly MHC-unrestricted.

The cell-surface glycoproteins, CD4 and CD8, expressed by mutually exclusive subsets of mature T cells, serve also as coreceptors for delivering the TCR/CD3 signals. Their role as coreceptors is required for most primary responses. Acting as coreceptors during antigen recognition, CD4 and CD8 bind to nonpolymorphic regions of the same MHC molecule that presents antigen to the TCR, thus joining with the complex TCR/CD3. CD4 and CD8 molecules contribute to both the binding avidity of TCR to the MHC-antigen complex and to the generation of cytoplasmic signals (Janeway Jr 1992, Miceli and Parnes 1993).

1.5.1.1 MHC Molecules

Since MHC molecules play a central role in presenting potential immunogenic peptides to T cells, altered expression by virally infected cells and tumour cells may directly influence immunosurveillance mechanisms.

The two major types of MHC molecules, class I and class II are located in chromosome 6, and designated HLA (human leukocyte antigens) in man. They are membrane glycoproteins. The major subregions for class I genes are named A, B and C, while class II molecules originate from three major subregions designated DP, DQ and DR. Other subregions, DM and DO, have recently been described. The presentation of antigens for scrutiny by T lymphocytes is the function of these molecules. The number of MHC molecules that must be occupied by a particular peptide to activate a T cell is thought to be extremely low, on the order of 0.03% of the total MHC or as few as 60 peptide-MHC complexes (Demotz *et al* 1990).

MHC class I and II molecules are among the most highly polymorphic molecules in the genome, and this polymorphism is concentrated in the peptide-binding grooves (Barber and Parham 1993). The polymorphisms form the molecular basis for the preference of different MHC alleles for different peptide sequence motifs, and they enable each MHC molecule to bind a diverse set of peptides. It has been demonstrated that MHC polymorphism causes differences in levels of human resistance to specific infections and also ensures that within a species a broad ability to bind peptides derived from a pathogenic challenge exists (Roy *et al* 1989).

Whereas class I molecules are found on every somatic cell and most tumour cells, class II molecules are detected on antigen-presenting cells (APCs) (dendritic cells, macrophages, B cells), and in some transformed/tumour cells, like epithelial cells. Antigens complexed with MHC class I and class II molecules originate from two different sources. Class I molecules generally present antigens derived from intracellular sources, such as viral proteins in infected cells. Class II molecules usually present endocytosed antigens derived from extracellular sources (peptides derived primarily from membrane glycoproteins or serum proteins) (Chicz and Urban 1994). Although, clear examples exist that contradict this distinction, especially in "professional" APCs.

The ability of a cell to process (proteolytic degrade) antigens via the class II pathway is more specialized that the almost ubiquitous ability of nucleated cells to process antigen via the class I pathway. Most cells in the body express class I peptide complexes, which are the ligands for CD8+ T cells. Both virally infected cells and some tumour cells clearly present antigenic peptides in the context of class I, because specific recognition of these cells by cytolytic CD8+ T cells results in their destruction *in vitro* and *in vivo.* However, tumour cells can escape immune recognition by a number of mechanisms: loss of transporter molecules required for antigen presentation by tumour cells; downregulation of HLA class I expression by these cells; shedding of surface tumour antigens; production by tumour cells of various immunosuppressive molecules (e.g. transforming growth factor-β, TGF-β); and absent, insufficient or inadequate T cell costimulation by tumour infiltrating APCs or by tumour cells themselves. The majority of tumours, like the tissues from which they arise, do not express costimulatory molecules and, therefore, tumour-specific T cells, although present, do not become activated. Viruses can also downregulate T cell costimulatory molecules, which may provide a means to evade the immune defenses.

1.5.1.2 T cell Adhesion and Costimulatory Molecules

T cells cannot in general respond to foreign protein antigens in the absence of additional cells, the APCs. Furthermore, antigen-specific T cell activation requires triggering the TCR/CD3 complex with a specific antigenic ligand - often referred to as the first signal, which consists of an antigenic peptide bound to the specific MHC molecule. Additional

antigen-independent signals that are required for or otherwise enhance T cell activation - signal two (costimulatory or accessory), are provided by selected cell surface ligands on APCs that engage their cognate receptors on T cells (Springer *et al* 1987, Weaver *et al* 1988, June *et al* 1990). With naive CD4+, CD8+ and some memory T cells as well, the first signal alone is commonly not sufficient for their activation, and absence of the second signal can lead to loss of responsiveness of T cells to subsequent antigen-specific stimulation (anergy) or clonal delection (Mueller *et al* 1989).

Some of the molecules that facilitate this type of interaction between a lymphocyte with its target cell are referred to as "adhesion" molecules. However, an increasing number of these molecules also appear to provide signaling activity following engagement with their ligands, either by enhancing the signal generated by the TCR or by contributing independent signals (Schwartz 1992, Fraser *et al* 1993). Hence, they are termed "costimulatory" molecules. Binding of accessory molecules to ligands on APCs, such as LFA-1 (CD11a/18) to ICAMs, CD2 to LFA-3 (CD58), CD5 to CD72, B7 to CD28, enhance conjugation of the two cells and, therefore, raises the likelihood of interactions between the TCR and the MHC-peptide complexes (Shimizu and Shaw 1990, Dustin and Springer 1991). Furthermore, the signals derived from these "accessory" molecules may be crucial when the peptide-MHC complexes are limited in number or avidity. Recently, it has been suggested that the timing and level of costimulation, rather than simply its presence or absence, are critical determinants of the nature and magnitude of T cell responses (Abbas *et al* 1996). However, several examples have shown that for most APC types, two states can be identified: a state in which the APC-derived accessory molecules are expressed at low levels or are absent, is associated with a weak immunostimulatory function; and an activated state in which these molecules are more abundant correlates with increased APC function (Schwartz 1990, Jenkins 1992).

The stimulation requirements of one T cell population can also differ substantially from those of another. For example, CTL precursor cells and unstimulated, naive CD4 cells require both TCR triggering and second signals to proliferate and generate activated effector cells. These activated cells, however, do not require second signals to engage and kill target cells or release cytokines. They do not need to produce IL-2 or proliferate (Chen *et al* 1994). In the context of a viral infection, this allows activated CTLs to

recognize and destroy any remaining infected cells, thus preventing further spread of the infectious agent. Moreover, shortly after antigen stimulation, naive T lymphocytes of both CD4+ and CD8+ subsets acquire increased levels of surface adhesion receptors including CD2 and LFA-1. These adhesion/costimulatory molecules presumably also help to localize activated T cells to the site of antigen accumulation *in vivo* (Butcher 1986). Such increased expression of receptors persists after the stimulated lymphocytes have reverted to the resting state. The changes in surface phenotype of these memory T cells may have important consequences for their localization, because they occupy distinct microenvironments within lymphoid organs and have different recirculation routes (Springer 1990).

In summary, the ultimate T cell response depends on the balance between the state of differentiation of the responding cell, the intensity of its TCR/CD3 signal, and the different modulating signals from its cell surface receptors, which in part reflect the action of cytokines in the environment and the nature of the APC. We will be discussing the adhesion/costimulatory molecules analyzed in this research and their respective receptors on T cells.

a) CD2/LFA-3

LFA-3 (lymphocyte function associated antigen-3, CD58) is widely distributed on leukocytes and non-hematopoietic cells, and binds to CD2 (LFA-2) on T cells (Springer 1990). CD2 was the first molecule for which dual roles in adhesion and costimulation were established (Dustin *et al* 1987), and the interaction of CD2/LFA-3 costimulates T cells in conjunction with TCR/CD3-antigen/MHC, by transducing a signal which augments or synergizes with signals from the TCR. Human LFA-3 and CD2 were transfected into mouse APCs and T cells respectively. In one study (Moingeon *et al* 1989), 2-4 times more IL-2 was produced by a mouse CD4 hybridoma using this technology. In a second study (Koyasu *et al* 1990), transfection of human LFA-3 into mouse APCs enhanced CTL and proliferative responses of cloned human CD4 cells to low doses of peptide.

b) LFA-1/ICAMs

LFA-1 (lymphocyte function associated antigen-1, CD11a/CD18), expressed on T lymphocytes, is a member of the β2 integrin family, which has been primarily recognized as an adhesion molecule (Springer 1990). It promotes adhesion of T cells to a variety of cells that bear its complementary ligands ICAM-1 (intercellular adhesion molecule-1, CD54), ICAM-2 (CD102) and ICAM-3 (CD50). ICAM-3 is constitutively expressed on resting lymphocytes and monocytes, unlike the other two ICAMs (de Fougerolles and Springer 1992). ICAM-1 enhances the efficiency of antigen presentation about 10-fold, that is, the presence of an ICAM-1/LFA-1 interaction allows much lower doses of antigen to induce optimal IL-2 release (Dang *et al* 1990). Studies with ICAM-1 transfectants (Altmann *et al* 1989) or with purified ICAM-1 (Kuhlman *et al* 1991) indicate that LFA-1/ICAM-1 pathway is also involved in costimulation of TCR/CD3-mediated T cell activation.

ICAM-1 expressed on endothelial cells, fibroblasts and keratinocytes promotes the adherence of leukocytes to these cells (Springer *et al* 1987). ICAM-1/LFA-1 interactions are also instrumental in cutaneous leukocyte trafficking, keratinocyte/leukocyte adhesion and antigen presentation by Langerhans cells.

c) CD28/B7

CD28 is a homodimeric transmembrane glycoprotein found on the majority of human peripheral T cells. Activation of T cells enhances the expression of CD28 and also induces the expression of CTLA-4, a structural homologue of CD28 with a lower level of surface expression than CD28 but a far higher avidity for ligands in the B7 family. CD28 and CTLA-4 signals can have opposite effects. Cross-linking CTLA-4 by B7 ligand can inhibit T cell activation, IL-2 production, and subsequent cell proliferation. Therefore, it is suggestive that CTLA-4 may provide a mechanism for downregulating a CD28-mediated T cell response or inducing an antigen-specific apoptotic signal leading to T cell death (Schwartz 1992, Janeway Jr and Bottomly 1994, Robey and Allison 1995).

The B7 family of ligands for CD28 and CTLA-4 has at least two members: B7-1 (CD80), originally called B7 or BB1, and B7-2 (CD86), each of which can trigger second signals. They are both expressed by dendritic cells, and activated macrophages,

B, T, and NK cells. Encoded by separate genes, B7-1 and B7-2 each react with CD28 and CTLA-4 with similar binding affinities, and they may have overlapping and redundant functions. However, there are significant differences in their rates of enhanced expression on activated cells and in their contributions to immune responses (Hatchcock *et al* 1994, Robey and Allison 1995). Given that CD28 and B7-2 exhibit similar temporal patterns of increased expression after activation of naive T and B cells, and that the expression of both CTLA-4 and B7-1 is induced later, it was postulated that B7-2 is the principal ligand for CD28 (initiation of the immune response) and B7-1 for CTLA-4 (regulation of the immune response) (June *et al* 1994). It has also been shown in some *in vitro* systems that B7-1 and B7-2 differentially regulate the development of Th2 and Th1 cells respectively (Kuchroo *et al* 1995).

1.5.1.3 T cell Subsets

The CD4+ and CD8+ subsets of T cells differ from one another in important functional parameters (Sprent and Webb 1987, Fitch *et al* 1993). CD4+ T cells frequently express a helper phenotype as measured by their ability to help B cell responses (antibody secretion) or the responses of other immune cell populations. For example, the generation of CTLs *in situ* generally requires help from other T cells. Helper T cells may produce IL-2 in response to an antigenic challenge, and the cytokine may facilitate the response of CD8+ CTL precursors to their specific antigen.

These diverse helper T cell functions are attributable to different cell subsets. Indeed, CD4+ helper T cells have been subdivided into two groups of cells, Th1 and Th2, on the basis of the patterns of cytokines that they secrete following antigen-specific or polyclonal stimulation (Romagnani 1992, Fitch *et al* 1993, Swain 1995). This distinction was initially made in murine CD4 T cell clones (Mossman *et al* 1986) and, despite a few discrepancies, they are in general agreement with human disease states. These Th1 and Th2 cells seem to represent a later stage of differentiation from Th0 cells, which display an intermediate cytokine profile (Paliard *et al* 1988), although others consider Th0 cells as a third functionally distinct T cell subset (Romagnani 1994).

In contrast to the TCR, which determines the specificity of the T cell, the secreted cytokines determine the function of the T cell. Th1 T cells promote primarily cellular immune responses, and Th2 cells promote humoral immune responses. Hence, Th1 cells secrete primarily IL-2 and INF-γ, but not IL-4, IL-5, IL-6 or IL-10, and they promote delayed type hypersensitivity responses, cytotoxic cell responses, and macrophage activation. Responses by Th1 cells promote cellular immune inflammatory reactions and appear to provide the primary host immune defenses against intracellular pathogens. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 but not IL-2 or IFN-γ, and they promote B lymphocyte responses and the synthesis of antibodies (Romagnani 1997). Responses by Th2 cells promote humoral immunity, allergic reactions, and immediate hypersensitivity reactions, which provide defenses against extracellular pathogens (Mosmann and Coffman 1989, Abbas *et al* 1996). Furthermore, it is suggestive that Th2 activation, inhibiting acute and chronic inflammation, may also have an important physiologic function as regulators of immune responses (Abbas *et al* 1996). However, responses to most pathogens are, in fact, a mixture of different cytokine patterns, with the ultimate effector function being determined by the ratios of the different T cell subsets.

Naive T cells generate primarily IL-2 following stimulation, and the pattern of cytokines or predominance of a given cytokine in the environment where T cell activation occurs, influences whether a Th1 or Th2 type of response develops (Romagnani 1994, Abbas *et al* 1996). Furthermore, the responses of the two classes of helper T cells are influenced differently by many nonspecific factors, including the antigen dose, the type of APC and its membrane-bound costimulatory molecules. The resulting cytokine profile may, in turn, determine resistance or susceptibility to disease depending on the class of effector-specific immune response that is mounted. In most studies, low antigen concentrations and low-dose infections have been shown to favour a Th1 response, whereas higher doses induce Th2 development (Hosken *et al* 1995). Antigen presentation by macrophages and dendritic cells preferentially induces Th1 responses, presumably because of concomitant IL-12 production, whereas antigen presentation by nonprofessional APCs (such as keratinocytes) may stimulate Th2 development (Fitch *et al* 1993, Goodman *et al* 1994). Finally, another factor known to influence T cell development is the nature of costimulation provided by APCs. In fact, high levels of

costimulation promote Th2 responses, probably by increasing IL-4 production by the activated T cells (Lenschow *et al* 1996).

The responses of the two T helper groups are often regulated in a reciprocal fashion, where the influence of cytokines is critical. For example, in mouse models, the presence of IFN-γ and IL-12 and the absence of IL-4 tend to favor the activation of Th1 cells, whereas IL-4 and IL-10 tend to promote the activation of Th2 cells (Gross *et al* 1993, Murphy *et al* 1994). Moreover, IL-10 can inhibit cytokine production by Th1 cells indirectly by inhibiting macrophage activation of the Th1 cells and, therefore, ultimately suppresses cellular immune reactions. Consequently, the final result of cytokine mediated self-amplification and cross-regulation is that once a T cell immune response begins to develop along one pathway, Th1 or Th2, it tends to become progressively polarized in that direction.

Cytokine patterns have also been identified as type 1 or type 2, in regard to those typically produced by Th1 or Th2 cells, to refer to their functions rather than their cellular source. Cells other than CD4+ T cells that also can contribute to the local cytokine pool, include NK cells (e.g. IFN-γ) and macrophages or B cells (e.g. IL-10). Also, CD8+ cytotoxic cells may be subgrouped into those that secrete Th1, the major subgroup, or Th2 patterns of cytokines (Sad *et al* 1995). Similar subpopulations are known to exist among T cells expressing the γδ antigen receptor (Ferrick *et al* 1995).

1.5.1.4 Cytokines

Cytokines are soluble proteins or glycoproteins produced principally by mononuclear cells of the immune system (usually lymphocytes and monocytes) that have regulatory actions (activation, differentiation and proliferation) on other cells of this system, as well as, on non-immune cells. Thus, cytokines are true hormones acting on other cells at a distance from the secreting ones. It should also be remembered that the *in vivo* effects of cytokines, in general, can be very complex, because often they can have one effect on a target cell but different actions on other cells, induce or inhibit the production of other cytokines, and antagonize or enhance the actions of other cytokines (Nathan 1987). Since we have analyzed the expression of the proinflammatory cytokines (IL-1α, IL-12

and TNF-α) and one anti-inflammatory cytokine (IL-10) in the local cervical microenvironment, they will be discussed.

Two different forms of IL-1, IL-1α and IL-1β, each encoded by distinct genes have been identified (Dinarello 1989). IL-1α is the major type released by keratinocytes (Luger and Schwarz 1990). IL-1 is a multifunctional cytokine: it costimulates T cells; enhances the production of other cytokines, such as IL-2, IFN-γ, and colony stimulating factor (CSF) by T cells; induces the expression of IL-2 receptors on activated T cells; is chemotactic for T lymphocytes; enhances Langerhans cell migration; activates macrophages; stimulates fibroblast to proliferate; is one of the mediators of acute phase inflammatory responses, and is the major pyrogen (Dinarello 1989).

Both IL-1 and TNF-α cause strong induction of MHC class I and II molecules as well as ICAM-1 in a wide variety of tissues, namely keratinocytes and endothelial cells. They also greatly increase binding of lymphocytes and monocytes through their cell surface receptor (LFA-1) to these tissues (Springer *et al* 1987).

TNF-α has an array of immunostimulatory properties such as: stimulation of T cell proliferation (T lymphocytes in response to TNF-α express IL-2 receptors and produce cytokines such as IL-2), enhancement of NK cell and macrophage tumoricidal activity, activation and recruitment of macrophages, granulocytes and cytotoxic cells to sites of inflammatory/immune reactions (Beutler and Cerami 1989). TNF-α also plays a role in the host resistance to tumour growth (Sugarman *et al* 1985) and viral infections (Wong and Goeddel 1986, Ramsay *et al* 1993). The cytokine directly suppresses viral replication, exhibits antiviral synergy with INFs (Feduchi and Carrasco 1991), promotes an antiviral state in uninfected neighbouring epithelial cells and, finally, by increasing the expression of MHC molecules on the surface of infected cells, enhances target cell recognition by cytotoxic effector cells.

IL-10 was originally characterized as a factor generated by mouse Th2 cells that inhibits cytokine synthesis by Th1 cells (Fiorentino 1989). It has been shown that IL-10 is also secreted by Th1 cells in humans (Sornasse *et al* 1996). IL-10 has also been found to

synergise with IL-4 in inhibiting Th1 differentiation and effector functions *in vivo* (Powrie *et al* 1993). This inhibitory effect is likely to be mediated by indirect suppression of IL-12 production (D'Andrea *et al* 1993), or by downregulating the expression of B7-1 (CD80) or other costimulatory molecules on the APC (Ding and Shevach 1992, Enk *et al* 1993, Chang *et al* 1995). For example, IL-10 treatment converts Langerhans cells from potent immunostimulatory cells to tolerogenic APCs *in vitro* (Enk *et al* 1993). IL-10 was also shown to downregulate the synthesis of a broad spectrum of proinflammatory cytokines by monocytes/macrophages, CTLs and NK cells, and to promote the release of IL-1 receptor antagonist by macrophages (Mosmann and Moore 1991, Moore *et al* 1993). Such *in vitro* data led to the proposal that IL-10 might inhibit inflammatory processes mediated by Th1 cells *in vivo*. Indeed, systemic administration of IL-10 in rodents suppresses delayed-type hypersensitivity (Li *et al* 1994), and T cell-mediated inflammatory bowel disease (Powrie *et al* 1994). In contrast, IL-10 exerts a wide array of immunostimulatory effects on B cells. Macrophages, monocytes and B cells are thought to be the major sources of IL-10 (Fiorentino 1991).

IL-12 is the principal Th1-inducing cytokine (D'Andrea *et al* 1992, Hsieh *et al* 1993). IL-12 contributes to Th1-mediated effector functions by its ability to increase the cytotoxic activity of T cells and NK cells (Chehimi *et al* 1993) and, therefore, has potent effects against intracellular viruses and tumours in several experimental models (Scott 1993, Brunda *et al* 1993). Others have confirmed that undefined components of viruses can stimulate IL-12 production and induce Th1-dominated immune responses (Trinchieri 1995). Macrophages are very proficient producers of IL-12 (Gazzinelli *et al* 1993), as are dendritic cells (Heufler *et al* 1996). Other cells also capable of producing this cytokine include B cells and monocytes (D'Andrea *et al* 1992).

1.5.1.5 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTLs) show the hallmarks of T cell immune responses. The CTL response is initiated by exposure to antigen; generation and regulation of the response depends on a complex MHC-dependent interaction of APCs and T cells; the TCR provides MHC-dependent antigen specificity for the interaction between CTLs and target cells; several accessory molecules, such as CD2, CD11a/18 and CD44 reacting with their ligands on the target cells also contribute to T cell triggering by the TCRs; and

memory responses generally follow reexposure to the antigen, resulting in faster, longer, and higher-level responses (Shimizu and Shaw 1990, O'Rourke and Mescher 1993, Berke 1994) .

Most CTLs express either CD8 or CD4 molecules (primarily CD8), but rarely both. In mouse models, it has been shown that both CD8+ and CD4+ T cells can mediate tumour rejection (Greenberg 1991). Thus, the distinction between T cells with cytotoxic function and those with helper function is not absolute, since CD4+ Th1 cells may have both functions, as discussed previously (section 1.5.1.3).

1.5.1.6 Peripheral T cell Tolerance

Mature T cells can be silenced in the periphery in an antigen-specific fashion by any of several different mechanisms. These mechanisms are commonly grouped into anergy, delection, or suppression. In addition, clonal ignorance may exist in the periphery.

Antigen-specific T cell silencing involves TCR triggering. Major factors that contribute to whether a T cell is silenced or activated appear to be the presence or absence of additional accessory signals and whether the T cell is in the process of dividing. In a variety of mouse models, naive T cells and some memory T cells become anergic following TCR triggering (signal 1) in the absence of costimulation (signal 2) (Schwartz 1992, Mueller and Jenkins 1995). The anergic cells are not deleted, they produce cytokines other than IL-2, although at subnormal levels, and they generate increased amounts of high-affinity IL-2 receptor, but they do not synthesize IL-2 or proliferate. The anergy results from a block of IL-2 gene transcription, and CD4+ Th1 and many CD8+ naive T cells are susceptible (Fitch *et al* 1993).

Following activation, mature peripheral T cells can be deleted by triggering the TCR. The process referred to as "activation-induced cell death" can affect Th1 and Th2 CD4+, CD8+, and TCR γδ+ T cells (Kabelitz *et al* 1993, Green and Scott 1994). The deletion appears to be caused by apoptosis triggered by Fas, which is upregulated on activated T cells, and it may serve a physiologic function by limiting the height and duration of a T cell response (Russell *et al* 1993).

A high and persistent load of antigen can also induce clonal delection of CTLs and result in a state designated "high-zone tolerance" (Moskophidis *et al* 1993). This form of tolerance may develop through clonal anergy, clonal deletion, or both, and it appears particularly relevant to tumours with high and persistent levels of antigen.

T cell suppression of other T cells can be mediated by a variety of mechanisms. In some instances, suppression appears to be antigen-nonspecific. Possible mechanisms include an effect of cytokines that nonspecifically interfere with proliferation or differentiation of immune cells (Dorf *et al* 1992). Since Th1 cells are preferentially blocked and Th2 responses are often unaffected, this raises the possibility that the state of immunological unresponsiveness may be due to the immunosuppressive cytokines produced by residual Th2 cells (Liblau *et al* 1995). States of increased nonspecific suppression have been observed in several pathologic conditions, including tumour-bearing hosts. In other cases, suppression may be highly antigen-specific (Bloom *et al* 1992, Sambhara and Miller 1994). In one model, suppression was mediated by cytotoxic T cells that recognize unique determinants on the TCR of helper T cells and destroyed these cells. In another model, CD4+ Th1 cytotoxic cells recognize antigen on the APC and destroy it.

1.5.2 Langerhans cells

Based on shared features of phenotype, structure, and function, Langerhans cells are considered as members of the dendritic cell lineage, although some differences exist, such as expression of Birbeck granules, CD1a and FcγR by Langerhans cells (Steinman 1991, Teunissen 1992). In the literature, Langerhans cells refer to cells present in squamous epithelia, whereas dendritic cells refer to cells isolated from other organs.

These cells are bone marrow-derived leukocytes and descend from the same MHC class II negative progenitor cell that gives rise to macrophages and granulocytes (Katz *et al* 1979, Inaba *et al* 1993). Although dendritic cells are of hematopoietic origin, they lack B, T, and NK markers. *In vivo*, these cells are characterized by their peculiar morphology. Their thin axon-like projections are designated dendrites or veils, which increase their surface area.

Dendritic cells are more potent initiators of T cell-dependent immune responses than any other APC (Steinman 1991), and they have a remarkably high density of both class I and class II on their surfaces (Steinman *et al* 1993). The role of epithelial Langerhans cells is to serve as the local APCs (Streilein 1985). Furthermore, whereas various cell types readily present antigen to activated T cells, Langerhans cells are the only APC type capable of priming unstimulated (naive) T cells in the initiation of a cellular immune response (Inaba *et al* 1987, Steinman 1991).

In situ intraepithelial Langerhans cells are very efficient in capturing and processing native protein antigens and generating immunogenic MHC class II-peptide complexes (Romani *et al* 1989). However, they are poor stimulators of resting T cells, hence, are called immature, "tissue dendritic cells" (Schuler and Steinman 1985). Langerhans cells then migrate to the regional draining lymph nodes, whereby they lose the ability to process protein antigens but acquire the necessary costimulatory molecules to stimulate resting antigen-specific T lymphocytes (mature, "lymphoid dendritic cells") (Silberberg-Sinakin *et al* 1976, Romani and Schuler 1992). During this maturation process, Langerhans cells synthesize large amounts of MHC class I and II molecules and begin to express several accessory molecules for T cell stimulation, such as ICAM-1 (CD54), ICAM-3 (CD50), LFA-3 (CD58), B7-1 (CD80) and B7-2 (CD86) (Teunissen *et al* 1990, Larsen *et al* 1990, Steinman *et al* 1995). Local inflammation, keratinocyte cytokines IL-1, GM-CSF (granulocyte macrophage-colony stimulating factor) and TNF-α, and protein antigens, all appear to stimulate Langerhans cell maturation and migration from the epidermis to T cell areas of draining lymph nodes (Larsen *et al* 1990, Cumberbatch *et al* 1994). Langerhans cells can also bind to local intraepithelial and stromal memory T cells, and thus can induce secondary immune responses. They are likely to play an important role in the activation of specific antitumour T cells *in vivo* (Grabbe *et al* 1995).

In summary, compelling evidence exists that Langerhans cells function as sentinels of the immune system, capturing exogenous antigens in their local environment, carrying it via the afferent lymphatics to T cell dependent areas of the regional lymph nodes and, finally, presenting it at this site in an MHC-restricted fashion to naive T cells.

1.5.3 Macrophages

In contrast to CTLs or NK cells, macrophages can be phagocytic, express CD14, CD36, or CD68, and although they may express CD4, they do not express the TCR or CD2, 3, or 8 (Knapp *et al* 1989).

Macrophages can act as APCs, therefore, they express high quantities of adhesion molecules and, importantly, they express the costimulatory molecule B7 on their surfaces. They can also participate as major effector cells in antitumour responses, and in resistance against infectious agents. Finally, macrophages can serve to dampen the immune response by secreting inhibitory cytokines such as IL-10, TGF β-1, -2, and -3, and IL-1 receptor antagonist. Moreover, factors such as IL-4 and IL-10 can inactivate the function of these cells.

Activated macrophages, like NK cells and CTLs, can be nonspecifically cytotoxic for tumour cells by causing either cell lysis or inhibition of division (Drysdale *et al* 1988, Greenberg 1991). Correlations have been established in several animal models between progressive tumour growth *in vivo* and resistance of tumour cells to macrophage cytotoxicity *in vitro*. However, macrophages can also promote growth of some tumours by effects on the tissue stoma, blood supply, or the tumour cells themselves (Mantovani 1994).

1.5.4 Natural Killer cells

Natural killer cells (NKs) are a relatively small population of lymphocytes distinct from T and B lymphocytes. They generally are large granular lymphocytes that originate in the bone marrow, and they do not require the thymus for maturation. These cells are either in a cell lineage separate from that of CTLs or they diverge from the T cell lineage at an early stage of T cell differentiation (Trinchieri 1989, Lanier and Phillips 1992).

The fundamental phenotypic difference between NK cells and CTLs is that NKs do not express the TCR or CD3 complex at the cell surface. Human NK cells, unlike CTL that are cytotoxic only in an MHC-restricted fashion, express CD56 molecules that may promote intercellular adhesion. In summary, human NK cells are primarily TCR/CD3-,

CD5-, CD56+, and these cell surface features have been used both for physically separating the cells and for functionally distinguishing between NK cell and CTL activity (Lanier and Phillips 1992, Moretta *et al* 1994).

The basic cytotoxic mechanisms of NK cells and CTLs appear to be similar (Lanier and Phillips 1992, Yagita *et al* 1992, Shi *et al* 1992). Granule exocytosis, cytolysin (perforin), and granzymes have well established roles in cytotoxicity mediated by NK cells. Like CTLs, NK cells depend on surface accessory molecules for the binding to target cells. However, NK cells do not require specific antigen presentation by accessory cells to become activated. NK cells also appear to express the Fas ligand and to use the Fas-mediated pathway to destroy target cells (Arase *et al* 1995). Like T cells, Fas expression is markedly increased on the surface of activated NK cells, which may provide a pathway for downregulating activated cells through apoptosis. Furthermore, if tumour cells lose MHC class I expression, they escape CTL but they become more susceptible to a subset of NK cells. In addition, NK cells have the ability to spontaneously kill neoplastic and virus-infected target cells (Handa *et al* 1983). Finally, NK cells are not limited functionally to cytotoxic activity. They can also produce an array of cytokines that affect immune responses, such as IFN-γ and TNF-α.

1.5.5 Lymphokine-activated killer cells

Both CTLs and NK cells cultured with relatively high doses of IL-2 show enhanced nonspecific cytotoxic activity, as revealed by their ability to selectively lyse fresh autologous, syngeneic, or allogeneic tumour cells that are relatively insensitive to normal NK-mediated cytotoxicity (Ortaldo *et al* 1986). They are referred to as lymphokine-activated killer (LAK) cells. The greater cytotoxic activity of LAK cells seems to result, in part, from their increased expression of surface molecules that contribute to target-cell binding and to triggering cytotoxic activity.

1.5.6 Keratinocytes

The non-keratinizing and stratified cervical squamous epithelium can be divided into several zones: the basal layer or stratum basale, small cylindrical cells with large nuclei; the prickle cell or stratum spinosum, several layers of polyhedral cells; the granular layer or stratum granulosum, polyhedral cells characterized by the accumulation of intra-

cytoplasmic glycogen; and finally, the stratum corneum where cells flatten as they migrate towards the surface and the nucleus becomes smaller and pycnotic.

Keratinocytes, characterized by an intermediate filament network of keratin polypeptides, comprise more than 95% of the resident squamous epithelial population, the remaining 5% including melanocytes, Merkel cells and Langerhans cells. In addition, there are intraepithelial populations of lymphocytes and macrophages. Keratinocytes constitute a self-renewing population with the basal ones forming the proliferative compartment, since they are mitotically active (Potten 1974). Keratinocytes migrate from this basal layer, differentiating as they progress upwards to be exfoliated from the surface and replaced by new cells from below. The evolution of the basal keratinocyte to a terminally differentiated cornified cell is associated with the sequential induction of a family of differentiation specific proteins, both structural and regulatory, which are specific to keratinocytes (Moll *et al* 1982), and are the result of induction of specific genes tightly coupled to the repression of others (Stoler *et al* 1988). Furthermore, self-renewal in epidermis is also dependent upon the connective tissue matrix supporting the keratinocytes (Leary *et al* 1992). It is estimated that in cervix 7 to 10 days is the minimum turnover time for epithelial renewal.

There is increasing evidence that epidermal keratinocytes may participate in inflammatory processes and immune responses. They have a so-called environmental "sentinel" function represented by nonspecific response to barrier perturbation. Epidermal keratinocytes, which display high-affinity cytokine receptors, have been reported to secrete and release interleukins (IL-1 - especially IL-1α, 3, 6, 8 and 10), colony-stimulating factors (GM-, G-, and M-CSF), TNF-α, TGF-α and -β, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (Kupper 1988, Shimada and Katz 1988, Kolde *et al* 1992, Enk and Katz 1992). Normally, these cytokines are not actively secreted by keratinocytes, but diverse stimuli can induce their production. It is suggestive that the release of cytokines in response to infection is probably an early mobilization signal to the host, initiating the recruitment of inflammatory cells which attempt to destroy the invading pathogen. For example, IL-1 and TNF-α which are constitutively produced at low levels by epidermal keratinocytes,

are rapidly and significantly upregulated, in terms of synthesis and release by environmental perturbation, such as injury and tumour promoters (Kock *et al* 1990).

Keratinocytes also have a specific, accessory/presenting cell function, since antigen presentation is not an exclusive property of APCs from lymphoid origin, the so-called "professional" APCs. Cells that do not normally express class II MHC molecules will present antigen if expression of these molecules is induced on their surfaces. Keratinocytes can be induced to express functional molecules such as ICAM-1 and MHC class II antigens (HLA-DR and -DQ), (e.g. upon stimulation with TNF-α and IFN-γ), which allow them to bind and activate lymphocytes (Nickoloff 1988). This T cell activation and proliferation occurs after selective recruitment of resting T cells within the mucosa, rather than by nonspecific infiltration of T cells activated elsewhere (Nickoloff and Griffiths 1990). Therefore, keratinocytes can function as "nonprofessional" APCs when activated. However, keratinocytes function differently from professional APCs in their interactions with T cells (Nickoloff and Turka 1994), in terms of the costimulation pathways and cytokine environment they provide to T cell proliferation, which will determine T cell effector function and, ultimately, disease outcome.

Finally, keratinocytes infected with viruses or transformed have a target cell role mainly for mucosa-specific T cells. Possible consequences of these keratinocyte/T cell interactions include: further keratinocyte activation, polyclonal or antigen-specific T cell proliferation and antigen-specific T cell anergy (Nickoloff and Turka 1993).

1.6 Immunology of human papillomavirus infection

The interaction between HPV and the immune system is complex and still not fully understood. However, host cell-mediated immunosurveillance mechanisms may be of critical importance in the control of HPV-related cervical disease, as suggested by the following examples.

1.6.1 Immunosuppression and cervical neoplasia

Immunodeficiency, whether congenital, iatrogenic or acquired, predisposes to the development of neoplasia and increased persistence, as well as enhanced neoplastic progression of HPV-related lesions. This phenomenon has been demonstrated in patients with rare congenital immunodeficiency disorders, organ transplant on immunosuppressive drugs, and receiving cytotoxic chemotherapy. Chronically immunosuppressed individuals demonstrate an increased susceptibility to HPV infection, which increases as the immune system becomes more compromised and the duration of the immune dysfunction is prolonged. As T cell responses in these patients are impaired, this suggests that progression of HPV infection is affected by the cellular arm of the immune response. Furthermore, patients with altered cellular immunity have higher incidence of malignancy (lower genital tract cancers) and HPV infection compared to those with immunodeficiency predominantly due to humoral antibody malfunction. This is also supported by the observation that the presence of circulating anti-HPV antibodies, including neutralizing ones, does not induce lesional regression, and that impairment of humoral immune functions in humans does not affect susceptibility to HPV infection (Lutzner 1985). There may be species differences, however, others have shown that anti-viral immunity in the cottontail rabbit model resulted from the presence of neutralizing antibodies directed against the virus (Christensen and Kreider 1990).

With respect to neoplasia, the overall frequency of malignancy in patients with primary cell-mediated immunodeficiencies is approximately 10,000 times greater than in an age-matched population (Gatti and Good 1971). For example, idiopathic CD4+ T-lymphocytopenia has been associated with anogenital neoplasia (Park *et al* 1994). In renal transplant patients on immunosuppressive drugs, reported rates of cervical neoplasia range from 5% to 40%, with the risk of anogenital neoplasia being 9 to 14

times that of matched control subjects (Penn 1986). There is a 14-fold increase in the risk of developing cervical carcinoma *in situ* in transplant recipients (Porreco *et al* 1975). Neoplasia in these patients may also occur at a young age, persist or recur after treatment, and progress (Sillman *et al* 1985).

During pregnancy, immunotolerance seems to be the factor responsible for the exuberant warty lesions which often regress spontaneously a few weeks after delivery. Detection of HPV has also been demonstrated to increase during pregnancy compared with non-pregnant cohorts (Schneider *et al* 1987a, Rando *et al* 1989), possibly as a result of permissiveness of the cell-mediated immunity to the virus (Purtilo *et al* 1972).

HIV infected patients represent a subgroup of immunosuppressed individuals at a great risk of acquiring HPV infection and contracting CIN lesions. HIV infected women have as much as an 18-fold risk for the development of genital warts which are more resistant to therapy (Matorras *et al* 1991). Cervical HPV infection is also especially prevalent in the HIV positive population, with the detection of HPV DNA 1.4 to 7.8 times higher in the HIV-positive group than in HIV-negative women (Tweddel *et al* 1994). In cervical neoplastic tissue of HIV-positive patients, high rates of multiple-type and unspecified-type HPV infection have been detected (Vernon *et al* 1994). Moreover, in this study HPV persistence was associated with HIV infection, as well as with the degree of immunosuppression, as confirmed by others (Sun *et al* 1997). More importantly, HIV infected women may be particularly susceptible to the more oncogenic HPV types. In one study of patients with CIN and CD4 counts less than 200/mm^3, HPV18 was detected in 50% of the samples (Johnson *et al* 1992). Others have confirmed the higher prevalence of oncogenic HPV16 and 18 in CIN patients and shown a correlation of these viral infections with immunosuppression. In a population of women referred for abnormal Pap smears, it has been reported that 51% of CIN lesions were high-grade in the HIV infected group compared to 15% in controls (Conti *et al* 1993). The same author (Conti 1991) has found a 4-fold higher progression and 3-fold lower regression rate of untreated HPV-related lesions in HIV infected women compared to HIV-negative controls. Furthermore, it has been reported that the likelihood of recurrence among patients treated for CIN 3 was highly associated with CD4 count (Maiman *et al* 1993b). A HIV direct effect on cells participating in the local cervical immune response and/or

HIV-induced alterations on paracrine or autocrine factors that influence HPV gene expression, could help to explain these observations and account for the more aggressive cervical pathology that develops in HIV infected women. As an example, it has been shown that tat (soluble HIV protein), activates HPV protein expression *in vitro* (Vernon *et al* 1993). Finally, various studies have demonstrated a relationship between HIV-associated immunosuppression and development of CIN, and the presence and severity of cervical neoplasia correlated with both quantitative and qualitative T cell function (Schafer *et al* 1991, Petry *et al* 1994). Moreover, invasive cervical carcinomas are biologically more aggressive in HIV infected women, which present in a more advanced stage and with higher rates of metastatic disease, in comparison with seronegative controls (Maiman *et al* 1993a).

1.6.2 Immune responses in HPV-related cervical neoplasia

Successful immune responses to viral infection and neoplasia involves a complex cascade of events that include processing and presentation of viral/tumour antigens to T cells, proliferation of antigen-specific lymphocytes and activation of a diverse array of cytokines. Thus, the literature on immune responses particularly against HPV infection of cervical squamous epithelium and in CIN lesions has been reviewed, in order to try to evaluate which of these events might be implicated.

1.6.2.1 Immunogenetics of HPV-related cervical neoplasia

Since different MHC alleles present different peptides for T cell recognition, both quantitative and qualitative variations in particular alleles may contribute to the development of cervical tumours. Certain HLA alleles, particularly HLA-DQ B1*03, are found more frequently in patients with CIN lesions (David *et al* 1992, van den Velde *et al* 1993, Odunsi *et al* 1995) and cervical squamous carcinomas (Wank and Thomssen 1991, Gregoire *et al* 1994) than in the normal population. This suggests that susceptibility to cervical neoplasia may in part be determined by inherent genetic factors governing the immune response to HPV, and implying a T helper-dependent mechanism for host protection. However, others have not found significant association between disease susceptibility and MHC class II loci (Glew *et al* 1992a, Mehal *et al* 1994).

Keratinocyte downregulation of HLA class I antigens is frequent in invasive disease, where 40% to 80% of carcinomas exhibit downregulation of one or more allelic products (Connor and Stern 1990, Cromme *et al* 1993, Glew *et al* 1993, Hilders *et al* 1994). The same authors, however, did not find a direct correlation of class I downregulation and HPV infection. There was downregulation not only of MHC class I molecules, but also of TAP proteins (peptide transporter proteins associated with antigen processing) in the majority of cervical cancers (n= 100) analyzed (Keating *et al* 1995). Follow-up data has also shown that patients with early stage cervical cancers that exhibit HLA class I downregulation have a worse prognosis (Connor *et al* 1993). There is also evidence of an increased incidence of HLA class I downregulation in cervical carcinoma lymph node metastases in comparison with the primary tumours (Cromme *et al* 1994, Hilders *et al* 1995). These findings are consistent with selection of HLA class I negative cells with immunological advantage during tumour progression. By contrast, the basal cells of normal squamous cervical epithelium show uniform staining of HLA class I antigens, and in CIN lesions loss of HLA class I expression by keratinocytes occurs rarely (Glew *et al* 1993, Hilders *et al* 1994). It is possible that the lack of class I downregulation in CIN lesions is due to the fact that the majority of these lesions will not progress.

Normal cervical keratinocytes do not express HLA class II antigens but HLA-DR is upregulated in both CIN lesions, and particularly cervical squamous carcinomas, where HLA-DR expression can be detected in approximately 80% of these cancers (Glew *et al* 1992, Cromme *et al* 1993, Coleman and Stanley 1994). In one of these studies (Glew *et al* 1992), HLA-DQ expression by tumour cells was also present in 54% of cervical cancers. Levels of MHC class II expression in CIN lesions and cervical cancers appear to relate to the degree of keratinocyte differentiation than to HPV infection (Connor and Stern 1990, Glew *et al* 1992, Cromme *et al* 1993).

Keratinocyte expression of the accessory molecule ICAM-1 (CD54) is also upregulated in CIN lesions, especially in high-grade disease, with concomitant increased intraepithelial trafficking of T cells (Coleman *et al* 1993). However, such ICAM-1 upregulation has been found not to be correlated with the presence of HPV DNA or viral antigen (Viac *et al* 1992). The functional consequences of these phenomena, in terms of

effective immunity and disease outcome, are not clear. A significantly enhanced expression of vascular adhesion molecules (ICAM-1, VCAM-1 and E-selectin) has been reported in high-grade lesions in comparison with normal squamous epithelium and low-grade disease (Coleman and Stanley 1994a). They all may contribute to local recruitment of immune cells which supports the view of a local immune response in high-grade lesions.

1.6.2.2 Local cellular immune responses in HPV-related cervical neoplasia

Immunohistochemical studies have demonstrated a significant reduction of Langerhans cells (professional APCs of the epithelia) in HPV infected cervical epithelium and CIN lesions (Tay *et al* 1987c, Hawthorn *et al* 1988, Viac *et al* 1990, Moreli *et al* 1993). Moreover, it has been documented that Langerhans cell morphology is altered in HPV-related cervical disease with reduction in cell size and stunted dendritic arborizations (Barton *et al* 1988, Hawthorn *et al* 1988, Hughes *et al* 1988). However, a direct link between Langerhans cell numbers and outcome of infection is not supported by two studies which compared persistent versus regressive CIN lesions (Fukuda *et al* 1993) and genital warts (Coleman *et al* 1994b), and found no differences in Langerhans cell counts. Although, a direct correlation was reported between the presence of HPV16 and 18 and decreased Langerhans cells in cervical HPV infection and CIN lesions (Hawthorn *et al* 1988, Lehtimen *et al* 1993), others did not find correlation between number of Langerhans cells and HPV6,11,16,18,31,33 and 35 DNA status in CIN lesions (al Saleh *et al* 1995).

In both HPV infected cervical epithelium and CIN lesions, a decrease of total intraepithelial lymphocytes has been found, especially CD4+ T cells with a reversed CD4/CD8 ratio. In contrast, no significant reduction in the number of lymphocytes in the subepithelial stroma was detected (Tay *et al* 1987b). However, the intraepithelial lymphocyte population in high-grade disease has been shown to differ significantly from both normal and low-grade lesions, with an increase in a CD8, CD56 (NK cells) and CD16 (macrophages) population (McKenzie *et al* 1991). Other studies have shown a steady increase in intraepithelial and stromal T lymphocytes (Viac *et al* 1990) and HLA class II positive immune cells (Hilders *et al* 1994) according to the severity of the CIN lesions. Macrophages, found to be scarce in normal squamous epithelium, significantly

increased in number within both epithelium and stroma in HPV infection and CIN lesions (Tay *et al* 1987d). Another study has shown that this increase, which was related to the severity of premalignant disease, was significantly more pronounced in high-grade lesions (al Saleh *et al* 1995).

Natural killer (NK) cells also appear to play a role in the host immune response to HPV. NK cells found predominantly in the subepithelial stroma were generally detected in both HPV infected epithelium and CIN lesions (Tay *et al* 1987a). Decreased production of interferon gamma and interleukin-2 by these cells, as well as depressed natural killer activity, has been detected in condylomata acuminata (Cauda *et al* 1987). In another study, NK cell mediated cytotoxicity was found to be significantly reduced in anogenital carcinomas associated with HPV16 or 33, but not in patients with condylomata or with HPV6 associated verrucous carcinomas (Malejczyk *et al* 1989). In contrast, increased NK cell activity was found to correlate with regression of HPV-associated low-grade lesions, suggesting that antigen non-specific mechanisms also help to control HPV infection (Garzetti *et al* 1995).

A series of immunological events that accompany regression of genital warts have been described (Coleman *et al* 1994b), providing evidence that clearance of HPV from the genital tract is characterized by an active cell-mediated immune response. Non-regressing warts were characterized by a relative lack of immune activity. Mononuclear cells were present predominantly in the stroma with few intraepithelial lymphocytes, the majority of which were CD8+ cells. In contrast, regressing warts were characterized by a mononuclear infiltrate comprised of both T cells and macrophages, both in the epithelium and stroma. CD4+ T cells dominated this infiltrate, expressed activation markers (CD25, IL-2 receptor) and the RO isoform of CD45, indicating that many were "antigen experienced" or memory cells. Significantly, there was no change in Langerhans cell numbers between regressors and non-regressors, and there was a significant induction of HLA-DR and ICAM-1 on the infected keratinocytes in the regressing group. Another study comparing persistent versus regressive CIN lesions has confirmed that, in contrast to Langerhans cells whose numbers were unaltered, subepithelial CD4+ T cells were significantly reduced in the persistent group (Fukuda *et al* 1993).

Finally, several observations support the view that a host type-specific resistance against HPV infection may develop. Wart regression seems to be a consequence of type-specific immune response, since plantar warts induced by HPV1 do not regress in patients with regressing flat warts usually caused by HPV3 (Tagami *et al* 1974). Furthermore, in the immunocompetent host, spontaneous or treatment-aided regression is unlikely to be followed by recurrence secondary to reinfection with the same HPV type, in contrast to same type recurrence in immunocompromised individuals (Nuovo *et al* 1991b).

1.6.2.3 T cell responses against HPV16

In an murine model, the immune response induced in naive mice exposed to grafts of HPV16 E7 transfected keratinocytes was dose dependent: lower doses induced E7-specific tolerance and higher doses an E7-specific delayed-type hypersensitivity (DTH) response (Chambers *et al* 1994). This response is consistent with observations in regressing human genital warts which reflect an effective host immune response to HPV infection. Furthermore, these results suggest that a key determinant of outcome of HPV infection, and of antigen presentation by keratinocytes, may be the quantity of antigen expressed and/or presented to the immune system.

In vitro experiments using murine models, have shown that cytotoxic T lymphocytes (CTLs) could be generated against a tumour antigen encoded by both the E6 and E7 gene from HPV16 in an antigen-specific and MHC class I-restricted manner (Chen *et al* 1991, Chen *et al* 1992a). In another study, restricted CD4+ cytotoxic T cells were found to be the major effectors against HPV16 E7 antigen (Altmann *et al* 1992). Apart from protection via T cell-mediated immunity induced through immunization procedures, recent data also indicate that existing HPV16-expressing tumours in mice can be eradicated via T cell mediated immunity. This was demonstrated either with transfection of B7 molecules into the tumour cells (Chen *et al* 1992) or by adoptive transfer of a CTL clone obtained through peptide vaccination (Feltkamp *et al* 1995). Finally, it has been shown that dendritic cells loaded with an HPV16-derived peptide could effectively treat mice with established HPV16-induced tumours (Mayordomo *et al* 1995).

In humans, T cell proliferative responses to HPV16 E7 peptides have been observed in about 50% of patients with abnormal cervical cytology and concomitant HPV infection (Kadish *et al* 1994). Cutaneous administration of recombinant HPV16 L1 protein in women that have CIN lesions can elicit DTH reactions (Hopfl *et al* 1991). Helper T cell proliferative responses to HPV16 and 18 E6 fusion proteins, but not to E4 have been observed in patients with CIN lesions (Cubie *et al* 1989). All these results indicate that activated CD4+ helper T cells are present in patients with CIN lesions. Furthermore, not only several potential T helper cell epitopes have been identified, but also one study has demonstrated an HLA-DR restriction (Strang *et al* 1990). Occasional CTL clones specific for E7 have also been identified in patients with HPV16 infection and associated cervical cancer (Ressing *et al* 1996). It was demonstrated that a mutant sequence in HPV16 E6 protein from cervical cancers is only found amongst those derived from patients who are HLA-B7+, and that the mutation removes a B7 restricted CTL epitope from the E6 protein (Ellis *et al* 1995). This suggests that the host immune system must be capable of recognizing HPV16 E6 at least in the context of HLA-B7 on the surface of epithelial tumours. However, recently it has been reported that in most CIN lesions only a minority of CTLs are activated and that the percentage of these activated CTLs was not related to expression of MHC class I on epithelial cells (Bontkes *et al* 1997). Overall, these studies have shown directly that human T cell responses, both helper and cytotoxic, against HPV-derived peptides can be demonstrated in CIN and cervical cancer patients.

The summarized data show that, at least in some HPV-related lesions, antigens are effectively presented to the immune system, and that both MHC class I and II restricted expression is important in disease control. However, although the bulk of evidence above described supports an important role to be played by the immune system in determining the course and outcome of HPV-related cervical disease, it should be noted that persistent HPV infection is common, and that a proportion of premalignant lesions will progress to invasive disease if left untreated. This suggests absence or inadequacy of immune response in these cases. As outlined before, HPV replication is totally dependent upon the keratinocyte differentiation programme, which may represent an evolved viral mechanism to evade host immune recognition. Furthermore, the viruses

are not cytolytic and few viral antigens are released to be taken up by Langerhans cells. As far as is known, only keratinocytes are permissive to HPV infection and, therefore, viral proteins are presented to the immune system by these non-professional antigen presenting cells, which may induce peripheral tolerance at least in some circumstances. Finally, HPV is not a passive passenger in the keratinocyte but reprogrammes the cell to allow viral replication. This may result in the establishment of a local microenvironment with disruption of immune surveillance mechanisms. Hence, the main objective of this thesis was to contribute to the understanding of the mechanisms involved in the local immunomodulation of HPV infected and/or transformed cervical epithelium.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical Specimens

Cervical samples were obtained from patients attending the Colposcopy Outpatient Clinic at the Whittington Hospital in London, for diagnostic and therapeutic procedures, after recent evidence of abnormal cervical cytology. Clinical material ranged from colposcopically directed punch biopsies to resection specimens (loop excision). To analyze cytokine levels and the phenotype of emigrated immune cells from cervical mucosa (Chapter 7), clinical material was obtained from colposcopically directed punch biopsies of areas with colposcopic features of cervical intraepithelial neoplasia (CIN). Two biopsies from invasive squamous cell carcinoma were also analyzed. Tissues from patients undergoing total abdominal hysterectomy for benign conditions, with normal cervical cytology and no evidence of previous cervical disease, were also examined.

Human palatine tonsils, obtained from patients aged 1-20 years on whom routine tonsillectomy was performed at the Royal National Ear Nose and Throat Hospital in London, were used as positive controls for immunohistochemistry and *in situ* hybridization.

2.1.1 Preparation of samples

Specimens were immediately snap frozen on dry ice before being stored in liquid nitrogen until processed further. The samples were mounted in OCT compound (BDH, Poole, UK) and serial 6μm cryostat sections were taken for immunohistochemistry, *in situ* hybridization or polymerase chain reaction (PCR) analysis. Intermediate cervical frozen sections were stained with haematoxylin-eosin (H&E) and the biopsies were classified as normal or as representing low-grade (koilocytosis and/or CIN1) and high-grade (CIN2 and/or CIN3) intraepithelial squamous lesions (Solomon 1989).

In the study on explant cultures from cervical mucosa (Chapter 7), cervical biopsies were floated in RPMI 1640 (Gibco, Paisley, UK) supplemented with 100 U/ml

penicillin, 100 µg/ml streptomycin, 250 µg/ml fungizone, and kept on ice prior to being cultured.

2.2 Immunohistochemistry

Cryostat cervical and tonsillar sections mounted on vectabond-coated slides (Vector Laboratories, Peterborough, UK), air dried for 30 minutes at room temperature (RT) followed by acetone fixation, were stained using an indirect immunoperoxidase technique (Isaacson and Wright 1986). Previously, we found that paraformaldehyde fixation should be avoided due to a complete destruction of signal. After rinsing in Tris-buffered saline (TBS) pH 7.6, sections were preincubated with normal rabbit (1:20) serum (Gibco BRL, Paisley, UK) for 15 minutes and incubated for 60 minutes at RT with specific antibodies. After washing, the sections were incubated for a further 60 minutes in a secondary antibody, horseradish-peroxidase-conjugated rabbit anti-mouse (Dako, High Wycombe, UK) at a dilution of 1:50. Antibody binding was visualized using 3,3-diaminobenzidine (Sigma Chemical Co., Poole, UK) at 5mg/ml and hydrogen peroxide (6µl of 60% w/v H_2O_2) for 10 minutes. Slides were counterstained with Mayers Haematoxylin (BDH, Poole, UK), dehydrated and mounted in a resinous mountant (Eukitt, Kindler GmbH & Co., Frisburg, Germany).

2.2.1 Study on cathepsin E expression by cervical epithelium (Chapter 4)

Cryostat cervical sections (n=43) encompassing normal squamous epithelium (n=8), low-grade (n=21) and high-grade intraepithelial squamous lesions (n=14) were fixed in acetone for 4 minutes at RT. The primary antibodies used were: a monoclonal antibody (MAb) directed against recombinant cathepsin E (mouse IgM, CE1.1) (Sealey *et al* 1996), a MAb against non-polymorphic determinants of HLA-DR (mouse IgG_{2a}, L243) (Lampson and Levy 1980), and a MAb against CD1a (mouse IgG_{2a}, NA1/34) (McMichael *et al* 1979). All these MAbs were used as unpurified culture supernatants at a dilution of 1:5.

Tonsillar sections were used as positive controls for cathepsin E and HLA-DR expression (Figure 2.1). As negative controls, we have used a MAb directed against type II collagen (IgG), anti-CD20 (IgM) or no primary antibody (Figure 2.2). Immunostained

endothelial and infiltrating immune cells from the subepithelial stroma, served as a positive internal control for both cathepsin E and HLA-DR expression. The specimens were classified according to the thickness of the cervical epithelium expressing cathepsin E molecules: no staining, basal, basal and parabasal staining, and full-thickness staining. HLA-DR expression by keratinocytes was evaluated as: absent, patchy basal, diffuse basal and full-thickness staining. Langerhans cells were identified as cells possessing at least two dendrites attached to a cell body (De Jong *et al* 1986) and staining positively with the CD1a and HLA-DR antibodies.

2.2.2 Study on HLA-DQ expression by cervical epithelium (Chapter 5)

Cryostat cervical sections (n=67) from normal cervical squamous epithelium (n=12), low-grade (n=30) and high-grade (n=25) intraepithelial neoplastic lesions were fixed in acetone for 4 minutes at RT. The primary antibodies used were: a monoclonal antibody (MAb) directed against non-polymorphic determinants of HLA-DR (mouse IgG_{2a}, L243) (Lampson and Levy 1980) at a dilution of 1:5, two MAbs against monomorphic determinants of HLA-DQ [mouse IgG_{2a}, Ia3 (Shookster *et al* 1987) at a dilution of 1:100 and L2 - gift from Dr. J Bodmer, London, UK used as neat culture supernatant] and finally, a MAb against CD1a which recognizes human Langerhans cells (mouse IgG_{2a}, NA1/34) (McMichael *et al* 1979) used at a supernatant dilution of 1:5.

Tonsillar sections were used as positive controls for HLA-DR (staining pattern similar to that shown in Figure 2.1c) and HLA-DQ expression (Figure 2.3). As negative controls, a MAb directed against type II collagen (IgG) and no primary antibody, were also included, whose staining patterns were identical to those presented in Figure 2.2. Immunostained endothelial and mucosa infiltrating immune cells, served as a positive internal control for both HLA-DR and HLA-DQ expression. The expression of these molecules by keratinocytes was evaluated both in terms of positivity versus absence of staining, and using a semi-quantitative immunohistologic grading system as described previously (Coleman and Stanley 1994). A total score was awarded which represented the sum of the intensity and the extent of keratinocyte staining for each biopsy. The two parameters were scored as follows. Intensity: 0, no staining; 1, weak staining; 2, moderate and 3, intense staining. Distribution: 0, patchy basal positivity; 1, diffuse basal positivity and 2, full-thickness positivity. Langerhans cells were identified as cells

possessing at least two dendrites attached to a cell body (De Jong *et al* 1986). They were counted within the squamous epithelium under light microscopy with an eyepiece graticule used in conjunction with a high-power (x40) objective lens. The cell counts were expressed as numbers of cells/mm^2 sectional area of the epithelium. Both intraepithelial and stroma subepithelial immune cells were identified by their morphology and expression of either HLA-DR or DQ antigens.

2.2.3 Study on T cell costimulatory molecules and cytokine expression in cervical epithelium (Chapter 6)

Cryostat cervical sections (n=53) from normal cervical squamous epithelium (n=12), low-grade (n=23) and high-grade (n=18) intraepithelial neoplastic lesions were fixed in acetone for 10 minutes at -20°C. A signal could be detected for the costimulatory molecules and cytokines under study only using these fixation conditions. The primary antibodies used are presented in Table 2.1. Where more than one MAb was available for a specific antigen, they consistently gave similar staining patterns. Normal human (1:25) serum was added to the secondary antibody to reduce background staining experienced with anti-IL-10 primary MAb and to a lesser extent with anti-TNF-α.

Tonsillar sections were used as positive controls for HLA-DR and HLA-DQ expression, as well as for ICAM-1 (CD54) (Figure 2.4a), ICAM-3 (CD50) (Figure 2.4b), LFA-1 (CD11a/18) (Figure 2.4c), LFA-3 (CD58) (Figure 2.4d), B7-2 (CD86) (Figure 2.4e), TNF-α (Figure 2.4f) and IL-10 (Figure 2.4g). As negative controls, a MAb directed against type II collagen (IgG) and no primary antibody, were also included. The expression of HLA-DR, HLA-DQ and ICAM-1 by keratinocytes was evaluated using a grading system described by (Coleman and Stanley 1994) and presented in the previous section (2.2.2). The specimens were also classified according to the thickness of the cervical epithelium expressing IL-10 and TNF-α: no staining, basal, basal and parabasal staining, and full-thickness staining. HLA-DR, HLA-DQ, ICAM-1, ICAM-3, LFA-1 and LFA-3 immunostained infiltrating cells were counted within the 200 μm of stroma immediately adjacent to the basement membrane of the epithelium. They were counted under light microscopy with an eyepiece graticule used in conjunction with a high-power (x40) objective lens. The median number of these stroma subepithelial immune cells was

determined for each biopsy and all cell counts were expressed as the number of cells/mm^2, as described previously (Coleman and Stanley 1994). Intraepithelial Langerhans cells were identified as cells possessing at least two dendrites attached to a cell body (De Jong *et al* 1986).

2.2.4 Immunocytochemistry of cell smears (Chapter 7)

This technique was used to analyze the phenotype of the emigrated cell population from cultured premalignant cervical mucosa. Cells spun down at 4°C for 5 minutes were resuspended in Tris-buffered saline (TBS) pH 7.6 and 10 µl were spotted onto a vectabond-coated slide (Vector Laboratories, Peterborough, UK), allowed to air dry at RT and fixed in acetone for 4 minutes. After rinsing in TBS, smears were preincubated with normal rabbit serum (NRS) (1:20) for 15 minutes and incubated for 60 minutes with the primary MAbs shown in Table 2.2. The subsequent procedures were identical to those described in section 2.2. Control samples were incubated in TBS/NRS without primary antibody. Blocking endogenous peroxidase activity and counterstaining the slides with Mayers Haematoxylin did not improve the specificity or resolution of the immunocytochemical signal.

Table 2.1 Primary monoclonal antibodies used for immunohistochemistry

Specificity	Clone	Isotype	Dilution	Source
CD1a	NA1/34	IgG2a	1/5	Hybridoma supernatant
HLA-DR	L243	IgG2a	1/5	Hybridoma supernatant
HLA-DQ	Ia3	IgG2a	1/100	Winchester
ICAM-1	6.5B5	IgG1k	1/10	Dako
ICAM-1	B-H19	IgG1	1/100	Vermot, Desroches C [1]
ICAM-1	B-H22	IgG1	1/100	Vermot, Desroches C [1]
ICAM-1	MEM-111	IgG2a	1/100	Horejsi, Vaclav [1]
ICAM-3	KS128	IgG1k	1/10	Dako
CD11a	AZN-L20	IgG1	1/100	Van Kooyk Yvette [1]
CD11a	AZN-L21	IgG2b	1/100	Van Kooyk Yvette [1]
CD18	7E4	IgG1k	1/100	Van Agthoven, Andre J [1]
CD18	MEM-148	IgG1	1/100	Horejsi, Vaclav [1]
LFA-3	AICD58.9	IgG1	1/25	Boehringer Mannheim
LFA-3	MEM-63	IgG1	1/100	Horejsi, Vaclav [1]
B7-2	BU63	IgG1k	1/1	Hybridoma supernatant
TNF-α	4H31	IgG1	1/5	Harlem Sera Lab
IL-10	23738.11	IgG2b	1/5	R&D Systems

[1] Obtained from 6th International Workshop on Human Leucocyte Differentiation Antigens (Kobe, Japan, November 1996).

Table 2.2 Primary monoclonal antibodies used for immunocytochemistry and FACScan analysis

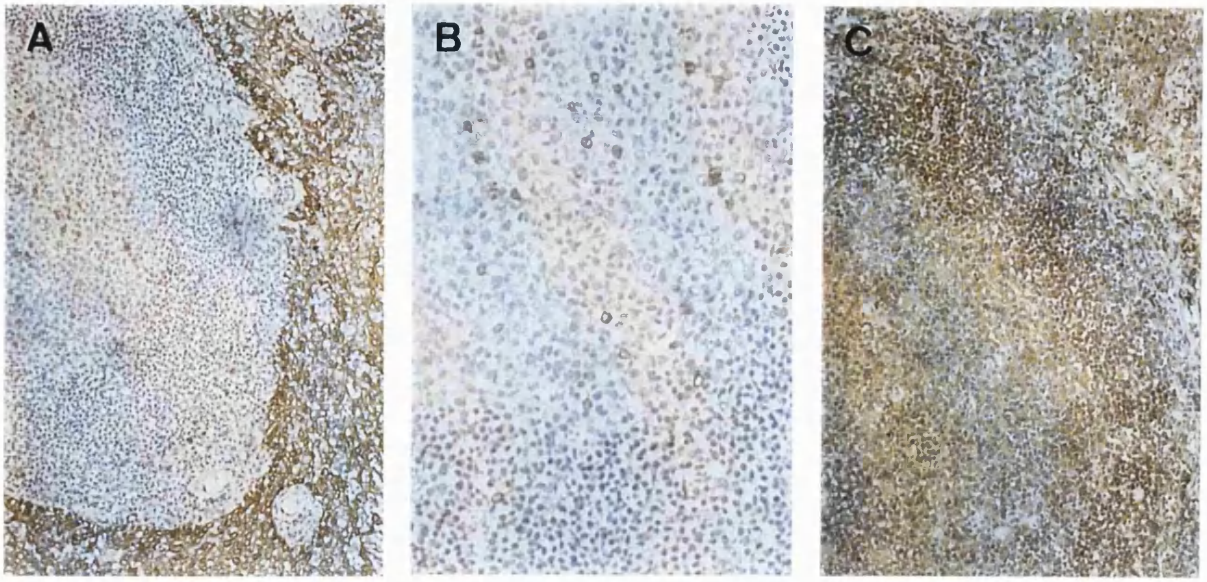
Specificity	Clone	Isotype	Source
CD1a	NA1/34	IgG2a	A. McMichael
CD3	UCH-T1	IgG1	P. Beverley
CD14	HB246	IgG2b	ATCC*
CD19	BU12	IgG1	D. Hardie
CD56	MEM-188	IgG2a	V. Horejsi

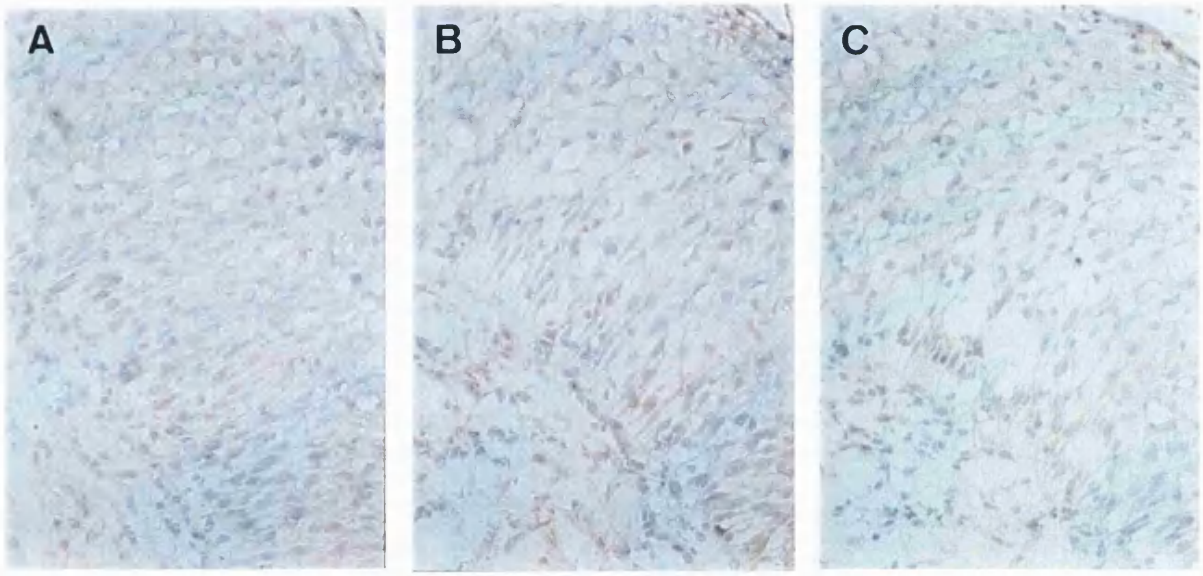
* American Type Culture Collection (cell lines and hybridomas)

Figure 2.1 Lymphoid follicles of a palatine tonsil immunostained with the MAb CE1.1 (cathepsin E) (a,b) and with the MAb L243 (HLA-DR) (c). Antigen presenting cells particularly in the germinal centre are positively stained for cathepsin E (a), which are better illustrated with a higher power, x40 (b). Lymphoepithelial cells also express the molecule (a). Magnification x25. HLA-DR is expressed by cells in all the tonsillar compartments previously referred to (c). Magnification, x25. Samples were counterstained with Mayer's hematoxylin.

Figure 2.2 No staining observed using the primary antibodies IgG (type II collagen) (a) and IgM (anti-CD20) (b) or omitting the primary antibody (c) in a low-grade squamous intraepithelial lesion of the cervix. Samples were counterstained with Mayer's hematoxylin. Magnification, x40.

Figure 2.3 HLA-DQ expression detected using both MAbs (Ia3) (a) and L2 (b) predominates in the mantle zone of tonsilar lymphoid follicles. Samples were counterstained with Mayer's hematoxylin. Magnification, x40.


A
B
C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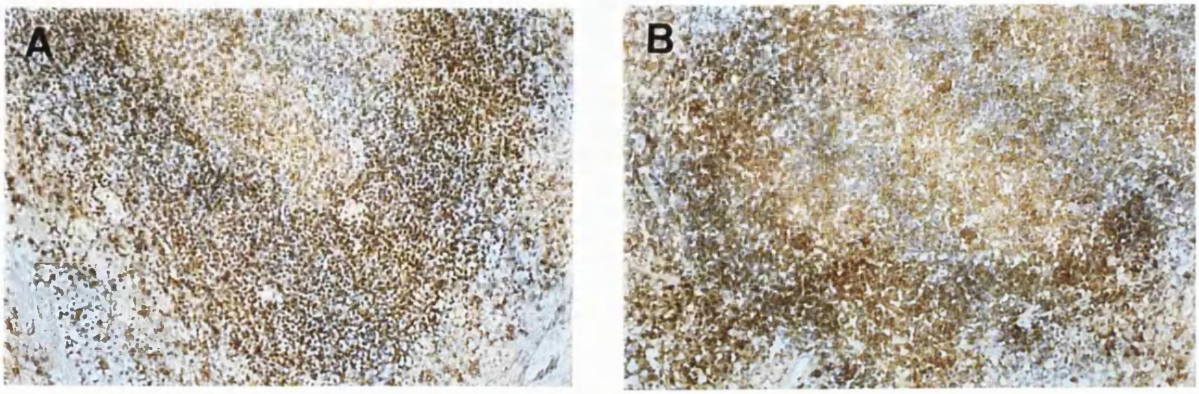
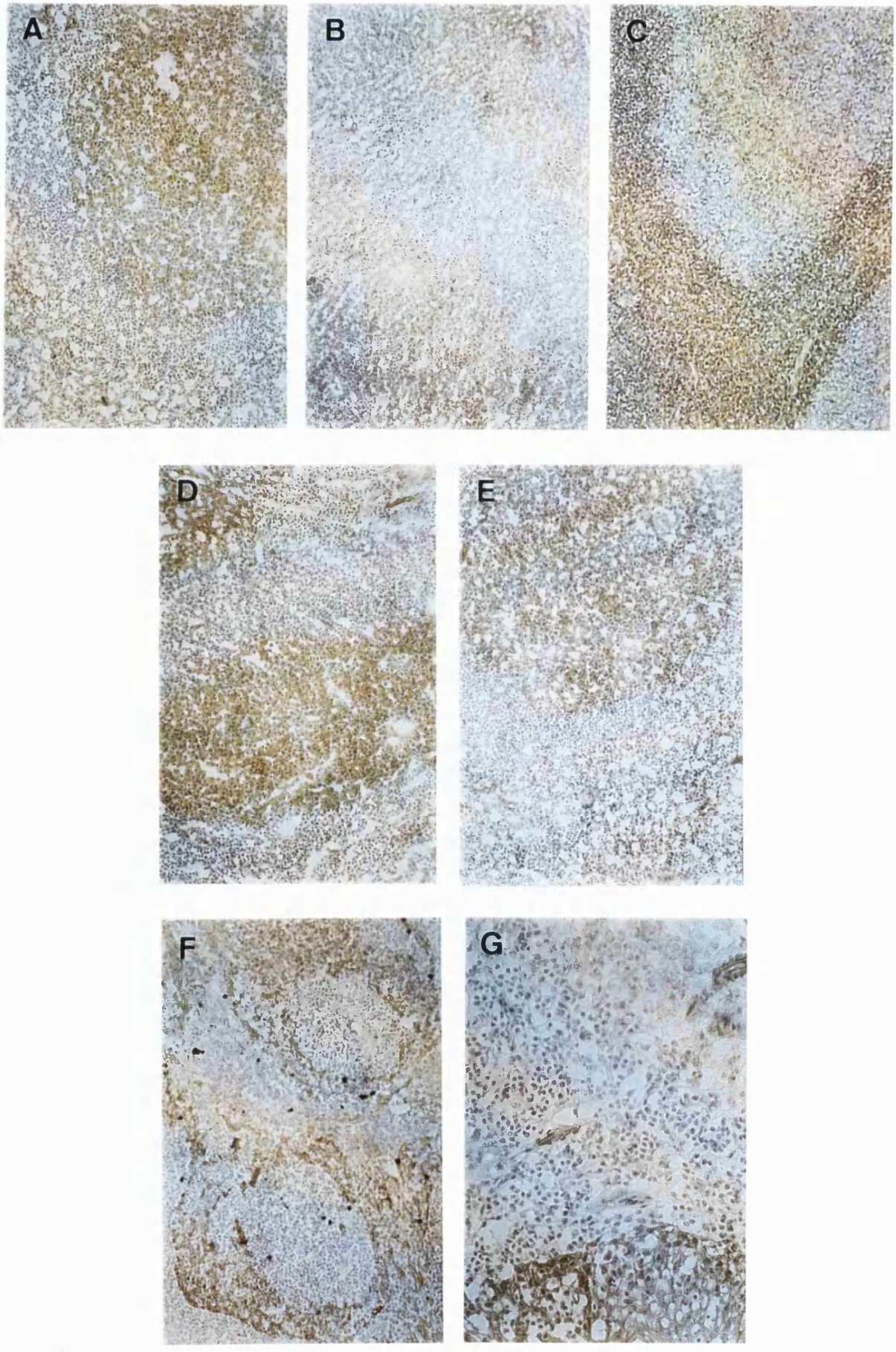

A
B

Figure 2.4 Lymphoid follicles of a palatine tonsil immunostained with the monoclonal antibodies 6.5B5 (ICAM-1) (a), KS128 (ICAM-3) (b), AZN-L20 (α-chain of LFA-1, CD11a) (c), AICD58.9 (LFA-3) (d), BU63 (B7-2) (e), 4H31 (TNF-α) (f) and 23738.11 (IL-10) (g). Similar staining patterns (predominance of positive cells in germinal centres) are observed for the MAbs recognizing ICAM-1, LFA-3 and B7-2. Whereas LFA-1 positive cells prevail in the mantle zone, ICAM-3 positive cells are recognized in both the mantle zone and germinal centre. TNF-α expression was detected in the lymphoepithelial area, as was IL-10, and also in the mantle zone of the follicle as previously reported (Hoefakker *et al* 1993, Harabuchi *et al* 1996). Samples were counterstained with Mayer's hematoxylin. Magnification, x40; except (f), x25.


A
B
C
D
E
F
G

2.3 *In situ* Hybridization

This technique was performed to analyze the expression of cathepsin E, TNF-α and IL-10 mRNA in cervical epithelium.

All solutions used in the pretreatment and hybridization procedures were autoclaved prior to use or made up in diethylpyrocarbonate (DEPC)-treated water. Also, all glassware and slide racks were baked at 180°C overnight to inactivate contaminating RNases.

2.3.1 Tissue preparation

Frozen sections of cervical and tonsillar tissues mounted on vectabond-coated slides (Vector Laboratories, Peterborough, UK), after air drying for 30 minutes, were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4. To improve tissue penetration of cathepsin E mRNA probes, tissue sections were digested with proteinase K (Boehringer Mannheim, Mannheim, Germany) 6μg/ml in 100mM Tris/HCl pH 8.0, 1mM $CaCl_2$ for 15 minutes at 37°C, washed for 2 minutes at room temperature (RT) in 2mg/ml glycine in PBS to inactivate the enzyme, rinsed twice for 1 minute in PBS and postfixed in 4% paraformaldehyde for 20 minutes. This enzymatic procedure was not carried out when TNF-α and IL-10 mRNA probes were used. Before hybridization, the sections were rewashed twice for 2 minutes in PBS, dehydrated through an ascending series of ethanol (30%, 60%, 80%, 95% and absolute ethanol for 2 minutes each) and allowed to air dry for at least 1 hour.

2.3.2 Probe preparation

A 721-bp Hind III fragment of human cathepsin E cDNA had previously been subcloned into the Hind III site of a 2.96-Kb pBluescript KS+ plasmid (Stratagene, San Diego, USA).

A 738-bp EcoRI fragment of human TNF-α cDNA clone 142-4, corresponding to nucleotides 337-1070 of TNF-α mRNA, was subcloned into the EcoRI site of pBluescript SK+ plasmid (Stratagene).

A 410-bp Xho I fragment of human IL-10 cDNA clone H15C, corresponding to nucleotides 0-410 of IL-10 mRNA, was subcloned into the Xho I site of pBluescript II KS+ plasmid (Stratagene).

2.3.2.1 Plasmid digest

The DNA templates were prepared by linearising the plasmids with the appropriate restriction enzymes overnight at 37°C. To confirm that the DNA had been properly cut, an agarose gel electrophoresis of the restriction digest was run (Figure 2.5). Then, the DNA was purified using 3M NaAc, precipitated with 2 volumes of absolute ethanol and incubated at -70°C for 2 hours. After precipitation, the DNA was spun down, washed with 70% followed by absolute ethanol, vacuum dried and resuspended in DEPC-treated water.

2.3.2.2 Transcription and labeling reaction

Transcription reactions were carried out using 1μg of purified DNA template, NTPs (Boehringer Mannheim, Mannheim, Germany), DTT and RNA polymerases (Promega, Madison, USA) and incubated at 37°C for 2 hours. An antisense strand RNA probe for cathepsin E transcripts was synthesized from Not I-linearised plasmid, using T3 RNA polymerase. A control sense strand RNA probe was synthesized from Xho I-linearised plasmid, using T7 RNA polymerase. The antisense probe was used to detect cathepsin E mRNA, whilst the sense probe was used as a negative control on adjacent sections. An antisense strand RNA probe for TNF-α transcripts was synthesized from Not I-linearized plasmid, using T7 RNA polymerase. A control sense strand RNA probe was synthesized from Xho I-linearized plasmid, using T3 RNA polymerase. An antisense strand RNA probe for IL-10 transcripts was synthesized from Xho I-linearized plasmid, using T7 RNA polymerase. A control sense strand RNA probe was synthesized from Hind III-linearized plasmid, using T3 RNA polymerase. All probes were labeled with the non-isotopic hapten, digoxigenin which was attached to the UTPs in the NTP mixture. Approximately 10μg of digoxigenin-labeled RNA were synthesized per 1μg of plasmid DNA template. Transcription reactions were stopped with 0.2M EDTA at 65°C for 2 minutes and a RNA gel electrophoresis with ethidium bromide staining was carried out to assess that the RNA probes had been successfully synthesized (Figure 2.6). The labeled transcripts were then purified by ethanol precipitation to remove unincorporated nucleotides and thus, preventing nonspecific background staining. Three volumes of prechilled absolute ethanol and 0.1 volume of 4M LiCl were mixed to the labelling

reactions and incubated at -70°C for 30 minutes. After centrifugation at 4°C, the RNA pellets were washed with 100µl of cold 70% ethanol, centrifuged, vacuum dried and finally, resuspended in 50µl of DEPC-treated water containing 1µl RNAse inhibitor (500µg/ml) (Promega, Madison, USA) and stored at -20°C.

2.3.2.3 Detection of digoxigenin incorporation into transcripts

To confirm digoxigenin incorporation into the RNA probes and assess the labeling efficiency, 1µl of each probe was spotted onto a nitrocellulose filter. After air drying, filters were incubated in a blocking solution consisting of milk fat for 1 hour at RT and then washed 3 times for 10 minutes in Tris-buffered saline (TBS) pH 7.4. Subsequently, filters were incubated for 1 hour at RT in anti-digoxigenin antibody/alkaline phosphate conjugate, 1:500 (Boeringer Mannhein, Mannhein, Germany) in TBS, washed 3 times for 10 minutes in TBS and incubated in freshly prepared development solution. This solution contained 0.33mg/ml nitroblue tetrazoleum chloride and 0.165mg/ml 5-bromo-4-chloro-3-indoylphosphate (X-phosphate) (Boeringer Mannhein, Mannhein, Germany). Filters placed in this solution were left for 3 hours to overnight until a blue colour had developed (Figure 2.7).

2.3.3 *In situ* Hybridization procedure

2.3.3.1 Pretreatment and hybridization

Sections were prehybridised for 1 hour at 50°C with 150 µl of hybridisation solution [4x SSC, 50% formamide, 1x Denhardt's solution, 5% dextran sulphate, 0.5 mg/ml salmon sperm DNA and 0.25 mg/ml yeast tRNA (Sigma, Poole, UK)]. This was drained and replaced with an equal volume of hybridization solution containing 100ng of digoxigenin labeled probes (antisense or sense). The sections were covered with siliconised coverslips (Sigmacote, Sigma, Poole, UK) avoiding trapping any air bubbles and incubated at 50°C for cathepsin E, and at 55°C for TNF-α and IL-10, overnight in a sealed humid chamber lined with paper soaked in 2x SSC/50% (w/v) formamide.

2.3.3.2 Post-hybridization and development

After hybridization the coverslips were removed by immersion in 4x SSC at 42°C. The slides were then washed twice in 4x SSC, once in 2x SSC, 0.2x SSC and finally 0.1x

SSC each for 15 minutes at 42°C. Sections were washed for 5 minutes in buffer A (100mM Tris/HCl pH 7.5, 150 mM NaCl) and blocked for 30 minutes in buffer A containing 0.3% w/v Triton X-100, 2% w/v normal sheep serum. The sections were then incubated for 2 hours at RT with anti-digoxigenin antibody/alkaline phosphate conjugate, 1:500 (Boeringer Mannhein, Mannhein, Germany) in buffer A containing 0.3% (w/v) Triton X-100, 1% (w/v) normal sheep serum, washed twice for 15 minutes in buffer A and once in buffer B (100mM Tris/HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$) at RT, and placed in freshly prepared development solution. This solution contained 0.33mg/ml nitroblue tetrazoleum chloride, 0.165mg/ml 5-bromo-4-chloro-3-indoylphosphate (X-phosphate) and 1mM levamisole. Slides were left in this solution, in the dark, and checked for the development of a blue colour reaction by light microscopy. After approximately 20 minutes the reaction was terminated by immersing the slides in a solution containing 100mM Tris/HCl pH 8.0, 2mM EDTA. Some sections were then counterstained with 2% nuclear fast red and all mounted in a resinous mountant (Eukitt, Kindler GmbH & Co., Frisburg, Germany).

As positive controls for every batch of cathepsin E hybridization, we have used tonsillar sections (Figure 2.8) and EBV transformed B cells (FC7) shown to express cathepsin E by *in situ* hybridization (Figure 2.9) and reverse-transcriptase PCR (Sealey *et al* 1996). Tonsillar sections were also used as positive controls for TNF-α (Figure 2.10) and IL-10 (Figure 2.11) message.

2.4 Polymerase Chain Reaction (PCR)

This technique was used to detect the presence of HPV16 in tissue sections from normal cervices and CIN lesions. The procedures for DNA extraction and HPV DNA PCR are described in Chapter 3 (section 3.2), which analyses the yield of cellular DNA with and without mussel glycogen for subsequent HPV16 DNA PCR. In later studies on which the HPV16 status was investigated (Chapters 4 and 5) the DNA extraction was conducted in the absence of mussel glycogen.

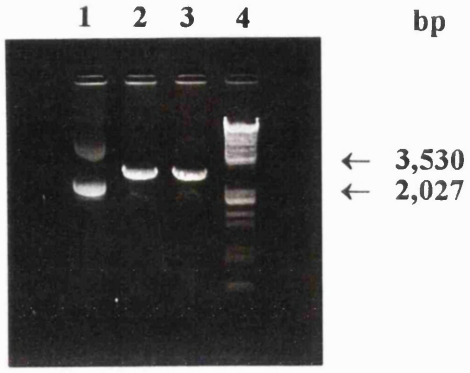


Figure 2.5 Agarose gel electrophoresis of cathepsin E DNA restriction digest. Lane 1: uncut plasmid (control); Lane 2: plasmid cutted with Xho I; Lane 3: plasmid digested with Not I; Lane 4: λ EcoRI + Hind III - DNA marker. Restriction digests run between 2,027 and 3,530 bp, as assessed by the DNA marker. Circular single strand DNAs (lanes 2 and 3) run in parallel with supercoiled uncut DNA (lane 1).

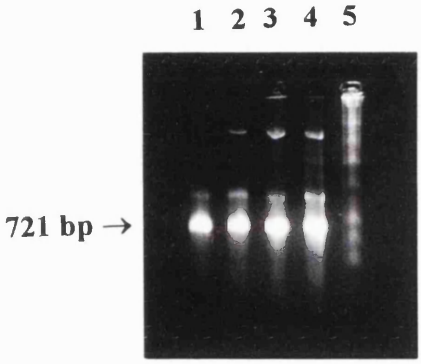


Figure 2.6 RNA agarose gel electrophoresis of cathepsin E transcription reactions. Lanes 1 and 2: old probes; Lanes 3 and 4: newly synthesized probes; Lane 5: Promega 0.28-6.58 Kb - RNA marker. On lanes 1 and 3 control sense strand RNA probes were synthesized from Xho I-linearised plasmid. On lanes 2 and 4 antisense RNAs synthesized from Not I are shown. 721 bp transcripts run between 623 and 955 bp of the RNA marker.

Figure 2.7 Nitrocellulose filters confirming successful digoxigenin incorporation into two cathepsin E RNA probes. Both, Not I (1 and 3) and Xho I transcripts (2 and 4) have been used. On the left, filters were incubated in development solution for 3 hours and on the right, overnight.

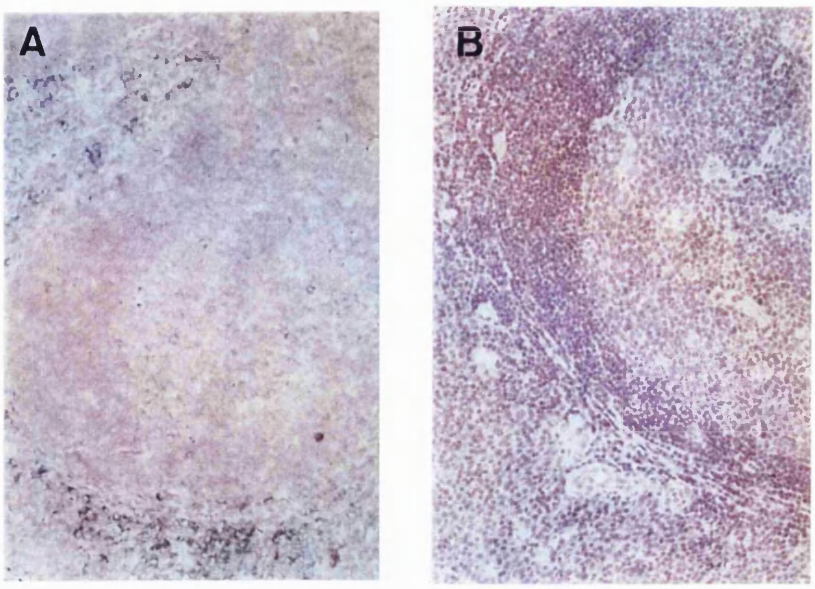


Figure 2.8 Cathepsin E transcripts observed in the germinal centre and especially lymphoepithelial area of a palatine tonsil (a), which correlates to protein expression (Figure 2.1). No signal is detected with the sense control probe (b). Samples were counterstained with nuclear fast red. Magnification, x40.

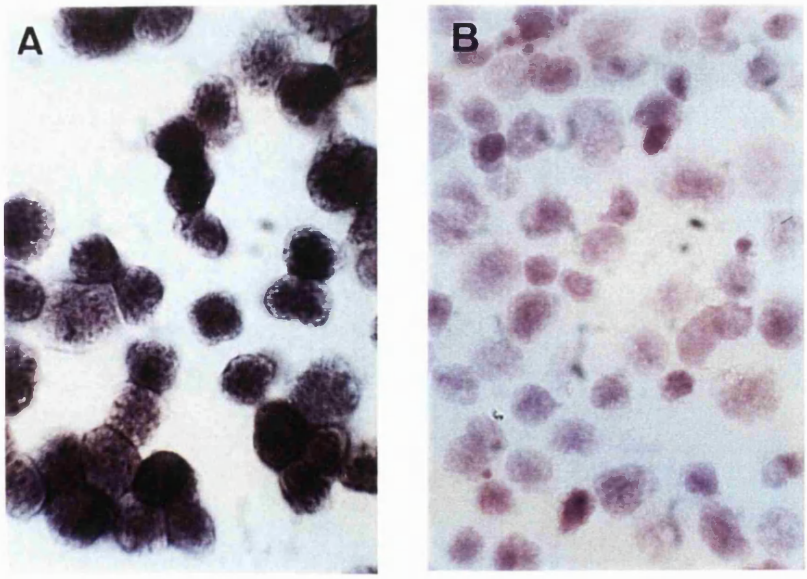


Figure 2.9 *In situ* hybridization of cathepsin E message in a FC7 cell line. Cathepsin E mRNA is detected with the antisense probe (a), whereas the sense control probe shows no signal (b). Cells were counterstained with nuclear fast red. Magnification, x100.

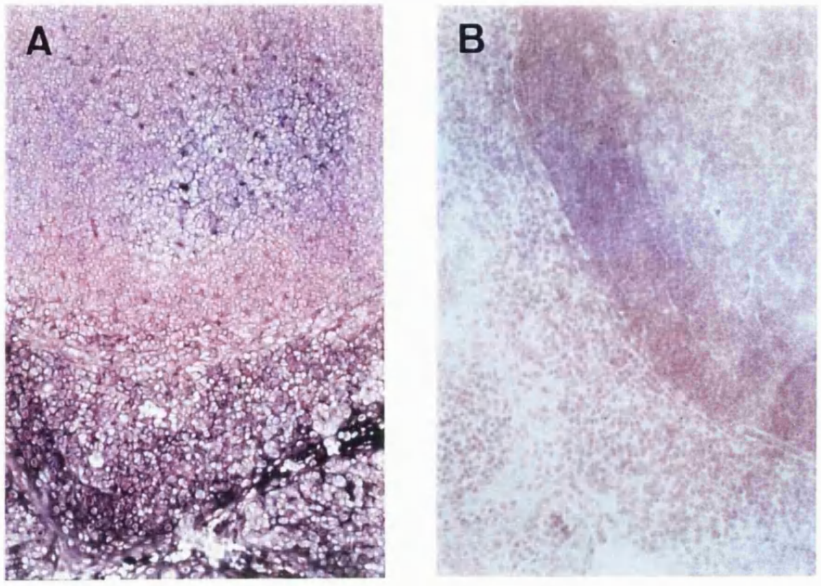


Figure 2.10 TNF-α transcripts predominate in the lymphoepithelial area and to a lesser extent in the germinal centre of a palatine tonsil (a), which correlates to protein expression (Figure 2.4f). No signal is observed with the sense control probe (b). Samples were counterstained with nuclear fast red. Magnification, x40.

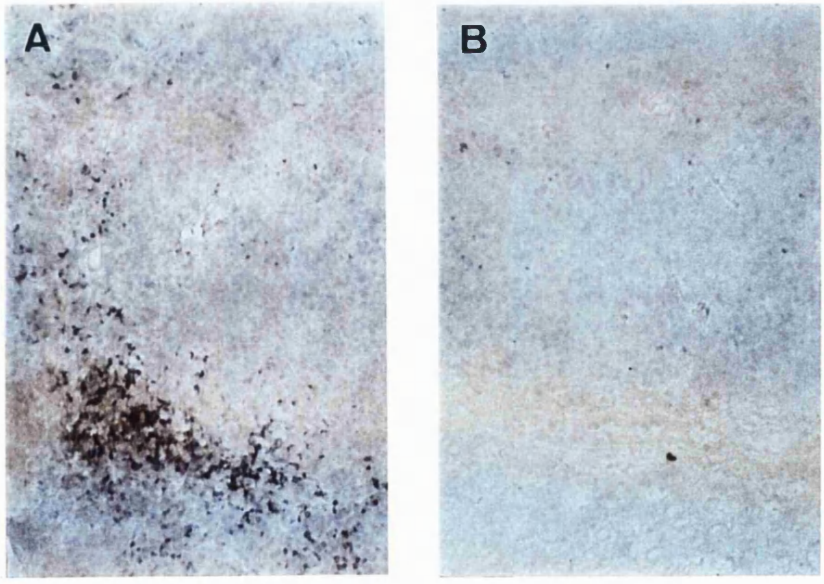


Figure 2.11 IL-10 transcripts detected in the lymphoepithelial area of a palatine tonsil (a), which correlates to protein expression (Figure 2.4g). Minimal background levels of signal observed with the sense probe (b). Magnification, x40.

2.5 Culture of Cervical Mucosa

Explant cultures of cervical mucosa were used to analyze the phenotype of emigrated cells into the medium, quantify the levels of cytokines in the cultured media, and to assess the effects of these media on *in vitro* T cell proliferation assays.

Cervical specimens were washed twice in RPMI 1640 (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml fungizone to remove blood, mucus or cellular debris. If necessary, an area of the stroma was cut off, so that every sample (~3 mm in extension by ~2 mm in depth) would encompass the epithelium and the immediate adjacent subepithelial stroma, areas where the mucosal immune cells predominate. Then, samples were placed stromal side down on top of a grid, each in 400 µl of either complete culture medium (RPMI 1640 supplemented with 10% foetal calf serum, 50 µM 2-mercaptoethanol [Gibco, Paisley, UK], 100 U/ml penicillin and 100 µg/ml streptomycin) for functional assays or in RPMI 1640 for phenotypic analysis, in 35x10 mm dishes (Falcon, Becton and Dickinson, USA) for 24 hours. Figure 2.12 shows a diagram of this culture system.

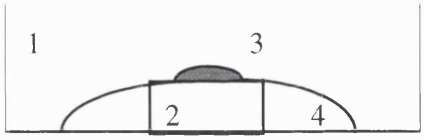


Figure 2.12 Diagrammatic representation of culture system showing petri dish (1) with grid (2), cervical sample (3), and culture medium (4).

2.6 Immunofluorescent Flow Cytometry

The cells which emigrated from cervical explants cultured for 24 hours were analyzed using a FACScan flow cytometer (Becton and Dickinson, USA) and WinMDI software. Cells at a concentration of $2x10^5$/ml, spun down to remove the RPMI, were preincubated on ice in Hanks' balanced salt solution (HBSS) with 10% normal rabbit serum (NRS) (Gibco, Paisley, UK) for 15 minutes to prevent non-specific binding of the secondary antibody. Cells were then incubated for 45 minutes on ice with the primary MAbs at a dilution of 1:500 (Table 2.2). After washing cells twice in HBSS/10% NRS, they were incubated for 45 minutes on ice with FITC rabbit anti-mouse IgG (Dako, Glostrup,

Denmark) diluted 1:20 in HBSS/10% NRS. Cells were washed twice in HBSS/10% NRS, fixed in 3.7% formaldehyde in PBS, and no less than 5000 events were acquired on a FACScan. Data was examined relative to a negative control sample where no primary antibody had been added.

2.7 Enzyme-Linked Immunoabsorbent Assays (ELISA)

Presence of cytokines (IL-1α, IL-10, IL-12 and TNF-α) was detected in cultures of cervical mucosa using commercially available sandwich kits (Biotrak, Amersham, UK). Media were spun down at 4°C for 5 minutes to remove cells, and samples were split into two (for ELISA and proliferation assays), and stored at -84°C.

U-bottom 96 well plates precoated with goat anti-human cytokine were washed twice with PBS/Tween and blocked with 1% bovine serum albumin in PBS for 1 hour at RT. After a further wash, 50-100µl of the supernatants and cytokine standards were added and the plates were incubated for 1 hour at RT. Following 3 washes, 100µl of biotynylated anti-cytokine MAb at a concentration of 1µg/ml was added to each well and plates incubated for 1 hour at RT. The plates were then washed 3 times and 100µl of 1/1000 dilution of streptavidin conjugated horseradish phosphatase added to each well and incubated for 1 hour at RT. After 3 final washes, the colour of the reaction was developed using 100µl of 3,3',5,5'- tetramethylbenzidine (TMB) substrate for 45 minutes and the reaction was stopped with 0.18M H_2SO_4. The absorbance was read at OD_{450nm}. Samples were assayed in duplicate wells, using the standards supplied for each kit to obtain a standard curve from which the concentration of the cytokines in the test samples were calculated. After subtracting background readings, the minimum and maximum levels of detection for each cytokine were as follows: IL-1α, 10-400 pg/ml; IL-10, 15-600 pg/ml; IL-12, 25-1000 pg/ml; TNF-α, 0.31-10 pg/ml. Some samples required diluting and the final cytokine concentrations were calculated as means of duplicate readings and adjusted for the dilution factor.

2.8 Accessory and T cell Preparation and Purification

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers. Sixty milliliters of blood were taken into a heparinised syringe (100 U/ml monoparin

sodium heparin, CP Pharmaceuticals Ltd., Wrexham, UK). This was diluted 1:2 in phosphate-buffered saline (PBS), layered over Histopaque 1077 (Sigma, Poole, UK) and centrifuged for 30 minutes at 1600 rpm at RT. The interface was recovered and washed three times in HBSS. PBMCs were incubated in 6 well tissue culture plates for 2 hours at 37°C, 5% CO_2 in complete culture medium. Non-adherent cells were removed and the adherent cells cultured in fresh medium with 100 ng/ml human recombinant GM-CSF (gift from Dr. S. Devereux, UCL, London, UK) and 50 ng/ml human recombinant IL-4 (Peprotech, London, UK). After 7 days incubation loosely adherent cells were collected and any remaining lymphocytes removed by incubation with anti-CD3 and anti-CD19 mAbs, followed by anti-mouse Ig coated immunomagnetic beads (Dynal, Merseyside, UK). These cells were washed three times in HBSS and used as dendritic cells (DCs) in proliferation assays.

Monocytes were obtained from an identical 2 hour incubation of PBMCs. However, after removal of non-adherent cells (used for preparation of T cells), instead of addition of cytokines as above, adherent cells were incubated at 37°C, 5% CO_2 for 1 hour with 3 mM ethylene-diamine tetra-acetic acid (EDTA) in HBSS, harvested and washed three times in HBSS. Then, both monocytes and T cells were purified by negative immunodepletion using anti-CD2 (XIX.8, Harlan-Sera Lab, Loughborough, UK), anti-CD3 and anti-CD19 MAbs for purification of monocytes and anti-CD14, anti-CD19 and anti-HLA-DR (L243, P. Beverley, UCL, UK) MAbs to purify T cells.

2.9 T cell Proliferation Assays

Responder T cells (10^5 cells/well) were incubated at 37°C, 5% CO_2, with either autologous DCs or autologous monocytes ($3x10^4$ cells/well), both previously irradiated (3000 rads), for 2 hours in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark). We have just used one T cell concentration and one APC concentration based on previous data from our laboratory (Woodhead *et al* 1998). Test supernatants (50µl) from cultures of cervical mucosa were added to this coculture. Anti-CD3 MAb (Harlan-Sera Lab, Loughborough, UK) was added at 0.1 µg/ml, to give a final volume of 200 µl. The cells were incubated for 48 hours and pulsed with 1 µCi of ^3H thymidine (ICN Biomedical, High Wycombe, UK) for 18 hours. Cells were harvested on filters and

proliferation measured by liquid scintillation counting. Assays were expressed as mean counts per minute (c.p.m.) of triplicate cultures.

Allogeneic assays were performed with T cells from a different donor to that of DCs. The same procedures were carried out as described above, with the exceptions that no anti-CD3 mAb was added to the coculture and the cells were incubated for 5 days.

Blocking experiments were undertaken in parallel cocultures in the presence or absence of purified and neutralizing anti-human IL-10 mAb (50 ng/ml) (JES3-19F1, Pharmingen, San Diego, USA).

As positive controls we have used T cells and accessory cells alone, or in the presence of supernatants derived from cultures of normal cervical squamous epithelium, and culture medium where no sample had been incubated with. Finally, T cells alone and accessory cells alone were also always examined, as negative controls.

2.10 Statistical Analysis

Differences on HPV16 status between samples were calculated with the Fisher's exact test (chapters 4 and 5).

Differences between groups of biopsies for HLA-DR and DQ expression by keratinocytes were evaluated using the nonparametric Kruskal-Wallis test, Wilcoxon rank-sum test for trend and the Chi-square test for independence. To compare the numbers of Langerhans cells positive for CD1a, HLA-DR and DQ, and the proportion of CD1a cells which were HLA-DR or DQ positive the first two tests were used. The Kruskal-Wallis test was designed to reject the hypothesis that the three histological groups under study (normal, low- and high-grade) have the same median, the second test investigates if there is a linear trend across these three groups and the Chi-square to compare positive versus absent keratinocyte staining among the three groups. The number of Langerhans cells expressing MHC class II molecules in relation to the HPV16 was analyzed with the nonparametric Wilcoxon rank-sum test. All p values

shown are two-sided. There was no significant difference in patient age, smoking history or contraceptive use among the three groups studied (chapter 5).

Differences between the three histological groups of biopsies (normal, low- and high-grade) for HLA-DR, HLA-DQ and ICAM-1 expression by keratinocytes were analyzed with the nonparametric Wilcoxon rank-sum test. To compare the number of stromal immune cells in relation to keratinocyte expression of HLA-DR and ICAM-1, the same test was used. The expression of HLA-DR, ICAM-1 and the cytokines (IL-10 and TNF-α) by keratinocytes in CIN lesions was evaluated with the Fisher's exact test. The correlation between the number of subepithelial immune cells expressing both MHC class II and T cell costimulatory molecules was investigated with the nonparametric Spearman rank correlation. In all these statistical tests p values are two-sided (chapter 6).

All data of proliferative T cell responses were expressed as mean values of triplicate cultures ± SD (error bar). They were statistically evaluated using the unpaired Welch alternate t test (assume populations with different SDs), considering $p< 0.05$ (two-sided) as the level of significance. To correlate the levels of cytokines to T cell proliferation, the Spearman nonparametric correlation was used (chapter 7).

CHAPTER 3

PCR detection of the human papillomavirus: improved DNA recovery from frozen biopsies

3.1 Introduction

Human papillomavirus (HPV) is a DNA virus known to be associated with cervical intraepithelial neoplasia (CIN) and cancer, and HPV16 is the most prevalent type found in these lesions (Bergeron *et al* 1992). In order to detect the presence of HPV in infected tissue a number of methods, including *in situ* hybridization and Southern blotting, have been used (Walboomers *et al* 1994). However, due to its specificity and sensitivity the amplification of viral DNA by the polymerase chain reaction (PCR) is probably the preferred method.

Several techniques have been developed for the extraction of DNA from fixed or frozen tissue (Sepp *et al* 1994, Luqmani *et al* 1992). They involve lysis of cells by procedures such as boiling in distilled water, detergent, proteinase treatment or sonication. In all these methods DNA can be further purified with a phenol-chloroform extraction step and ethanol precipitation.

In order to achieve increased DNA yields we have examined the effect of the addition of glycogen in the ethanol precipitation step, since it has been shown previously that glycogen is an effective carrier in the precipitation of DNA present in very low concentrations (Tracy 1981), will redissolve readily in Tris-based buffers and does not seem to affect PCR efficiency. The use of glycogen in the extraction of DNA from frozen tissue, formalin fixed tissue or cervicovaginal smears for HPV DNA PCR, has not been reported before. Our results, using frozen tissue, suggest that this approach may have advantages over previously reported methods.

3.2 Materials and Methods

3.2.1 Clinical Specimens

Cervical samples were obtained from 55 patients attending the Colposcopy Outpatient Clinic at the Whittington Hospital in London, for diagnostic and therapeutic procedures after recent evidence of abnormal cervical cytology. Clinical material ranged from colposcopically directed punch biopsies to resection specimens (loop excision). Patients undergoing total abdominal hysterectomy for benign conditions (n=8), with normal cervical cytology and no evidence of previous cervical disease, were also examined. The age of the population under study was 19 to 51 years (29.5±8).

All biopsies were snap frozen immediately on dry ice and stored in liquid nitrogen. Batches of biopsies were retrieved and the samples were mounted in OCT compound (BDH, Poole, UK). Serial 6μm cryostat sections were taken for PCR analysis. Intermediate sections were stained with haematoxylin-eosin (H&E) and the biopsies were classified as normal, or as representing low-grade (koilocytosis and/or CIN1) or high-grade (CIN2 and/or CIN3) intraepithelial squamous lesions (Solomon 1989).

3.2.2 DNA extraction

Presence of HPV16 was analysed in cryostat tissue sections from normal cervices and CIN lesions. Five 6μm sections of a frozen biopsy (~3mm^3 in volume) were mixed with 500μl lysis solution [50mM Tris-HCl (pH 8.5), 1mM EDTA, 500μg/ml proteinase K, 5mg/ml SDS] for 3 hours at 50°C. The sample was then extracted with 1 volume of phenol-chloroform (1:1 mixture) and the aqueous phase split into two 1.5ml microcentrifuge tubes. 10μl of mussel glycogen (1mg/ml stock solution) was added to one tube only, followed by 2 volumes of absolute ethanol to both tubes. Samples were kept at least 1 hour at -20°C and DNA pelleted for 20 minutes at 13,000rpm in a microcentrifuge. Supernatant was discarded, the pellet washed once with 70% ethanol and air-dried. Finally, the material was resuspended in 25μl TE [10mM Tris-HCl (pH 8.0), 1mM EDTA].

3.2.3 Polymerase Chain Reaction (PCR)

PCR reactions were conducted in a total volume of 25 μl containing 1 μl of the extracted specimen DNA, either with or without the presence of glycogen, and used as template for amplification. The additional components of the PCR reaction were: 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride,

100μg/ml gelatin, 5 mM deoxynucleotide (dATP, dCTP, dGTP and dTTP; Promega, Madison, USA) and 2.5 μM of each primer. The reaction mixture was overlayed with an equal volume of mineral oil. The synthetic oligonucleotide primers used in the amplification reaction were H4 (5'-AAGGCCAACTAAATGTCAC-3') and H5 (5'-GCGGATCCTGTCTGCTTTTATAC-TAA-3') and produced a 228-bp fragment. Primers H4 recognise a regulatory region of HPV16 which correspond to sequence position 7763-7781, and H5 correspond to position 78-61. Taq polymerase - 1.25 U (Cenbiot, S.Paulo, Brazil) was added at 70°C after an initial DNA denaturation at 99°C for 5 minutes. The subsequent cycle parameters were as follows: a second denaturation at 94°C 90 seconds, annealing at 51°C for 1 minute and extension at 72°C for 1 minute, 35 cycles, and a final extension at 72°C for 8 minutes. Amplification products stained with ethidium bromide were analysed by 2% agarose gel electrophoresis. An HPV16 plasmid standard served as positive control for the HPV PCR and to assess the quality of target DNA a concomitant PCR using human β-globin gene specific primers was carried out. Those primers were B1 (5'-GAAGAGCCAAGGACAGGTAC-3') and B2 (5'-CAACTTCATCCACGTTCACC-3') and a 268-bp fragment was amplified (Figure 3.1). The cycle parameters were: 99°C for 5 minutes, 1 cycle; 94°C 1 minute, 60°C 1 minute, 72°C for 1 minute, 40 cycles, and finally, 72°C for 8 minutes. An irrelevant DNA was used as a negative control.

3.3 Results

A summary of the data is presented in Table 3.1. HPV16 was not detected in normal epithelium from 8 biopsies, either with or without the use of mussel glycogen for DNA extraction. In 30 low-grade lesions oncogenic HPV16 was present in 10 samples extracted with glycogen in comparison with 7 in its absence. Finally in 25 high-grade lesions, HPV was found in 16 specimens on which glycogen had been used, versus 14 without the addition of this reagent. Thus, in high-grade disease, the sensitivity of PCR for HPV16 DNA detection increased from 56% to 64% with the use of glycogen. Overall, in our study of 63 specimens analysed by PCR, 26 proved to be positive for HPV16 using the glycogen protocol whereas only 21 were detected in its absence.

A representative example of the results obtained in the extraction of DNA for HPV PCR is shown in Figure 3.2. On average, 15 times more DNA is observed with the addition of glycogen than without, as detected by densitometry (Biomed Instruments, Advanced American Biotechnology, CA, USA). With the use of glycogen DNA yield varied from 306 to 1454 units of density with a median of 915 units, and in its absence it ranged from 0 to 602 with a median of 63 units.

The agarose gel electrophoretic analysis of PCR products showed three distinct staining patterns amongst those 26 samples positive for HPV DNA. Examples of these findings are illustrated in Figure 3.3. The first pattern observed was that similar amounts of HPV DNA were detected with or without the addition of mussel glycogen during the cellular DNA extraction for PCR (n=12; 4 low- and 8 high-grade lesions). In the second, higher levels of HPV DNA were observed with the use of glycogen than without it (n=9; 3 low- and 6 high-grade lesions). Finally, 5 samples were positive for HPV16 DNA when the glycogen was added, and in contrast, they were negative in its absence (3 low- and 2 high-grade lesions). These 5 samples did not correspond to those which had a small DNA yield (the median amount of recovered DNA was 120 units in the absence of glycogen). A possible explanation for this finding is that these samples had low levels of HPV16 DNA and, therefore, the virus was only detectable when a higher yield of DNA was obtained with the use of glycogen.

Table 3.1 Summary of cellular DNA yields for HPV16 PCR in the presence or absence of mussel glycogen, as assessed by densitometry (expressed as units of density) and the corresponding HPV16 detection in normal squamous epithelium and CIN lesions.

			Cellular DNA yield		HPV16	
			Glycogen		Glycogen	
Case	Age	Hist	Present	Absent	Present	Absent
1	39	N	nt	nt	-	-
2	39	N	nt	nt	-	-
3	42	N	nt	nt	-	-
4	46	N	823	60	-	-
5	51	N	928	132	-	-
6	47	N	642	0	-	-
7	44	N	1120	320	-	-
8	48	N	918	36	-	-
9	28	L	nt	nt	-	-
10	20	L	nt	nt	-	-
11	32	L	1014	69	+	+
12	24	L	688	0	-	-
13	34	L	nt	nt	-	-
14	26	L	nt	nt	✚	+
15	26	L	828	120	+	+
16	23	L	530	0	-	-
17	30	L	598	134	+	-
18	29	L	1148	112	-	-
19	32	L	nt	nt	-	-
20	26	L	602	0	-	-
21	51	L	1320	280	-	-
22	27	L	728	0	-	-
23	23	L	470	97	✚	+
24	28	L	nt	nt	-	-
25	21	L	nt	nt	-	-
26	21	L	423	28	+	+
27	24	L	1220	112	+	-
28	31	L	1040	20	-	-
29	22	L	746	0	-	-
30	27	L	836	62	+	-
31	32	L	1212	48	-	-
32	26	L	1380	28	-	-
33	23	L	418	0	-	-
34	20	L	1264	50	-	-

35	35	L	306	27	-	-
36	34	L	737	206	-	-
37	24	L	928	68	+	+
38	23	L	1248	78	✚	+
39	27	H	nt	nt	-	-
40	32	H	nt	nt	+	+
41	23	H	nt	nt	✚	+
42	28	H	915	434	+	+
43	24	H	586	42	✚	+
44	33	H	1269	602	-	-
45	24	H	819	197	-	-
46	19	H	460	32	+	+
47	22	H	1246	69	✚	+
48	36	H	1422	246	✚	+
49	33	H	820	68	-	-
50	27	H	nt	nt	-	-
51	20	H	nt	nt	✚	+
52	28	H	476	80	+	+
53	32	H	826	120	+	-
54	24	H	980	0	-	-
55	29	H	786	0	-	-
56	27	H	1218	228	+	-
57	31	H	1454	320	+	+
58	22	H	928	64	+	+
59	28	H	536	28	✚	+
60	21	H	946	116	+	+
61	30	H	1120	62	+	+
62	23	H	1160	36	-	-
63	36	H	915	34	-	-

Hist, histology; N, normal histology; L, low-grade (koilocytosis and/or CIN1) and H, high-grade intraepithelial squamous lesion (CIN2 and/or CIN3); Present/Absent, indicates the use/omission of glycogen during the cellular DNA extraction for HPV PCR; nt, not tested; -, HPV16 absent; +, HPV16 present; ✚, higher levels of HPV16 DNA were observed with the addition of glycogen.

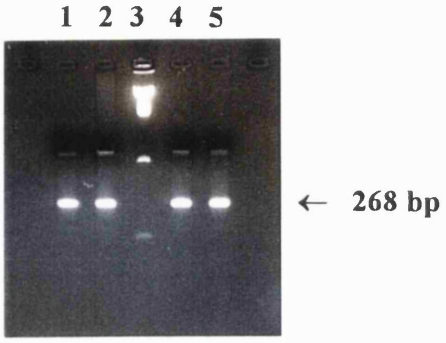


Figure 3.1 Agarose gel electrophoresis showing the 268 bp amplified products of the β-globin gene from four cervical samples. β-globin DNA PCR products run between 125 and 664 bp of the λ Hind III mW marker (lane 3).

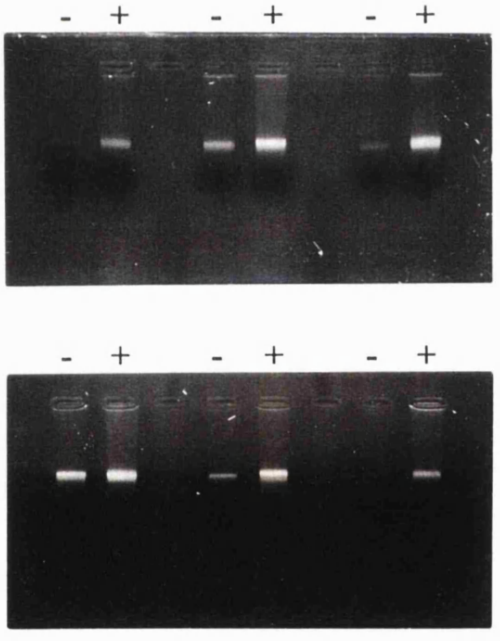


Figure 3.2 Agarose gel showing the results obtained in the extraction of DNA from six independent specimens with an histological diagnosis of CIN. DNA from each sample was ethanol precipitated in the presence (+) or absence (-) of mussel glycogen and analysed by electrophoresis. 10μl of the extracted DNA (out of a total volume of 25μl) were used per well. Subsequent densitometry quantified the DNA present in each lane.

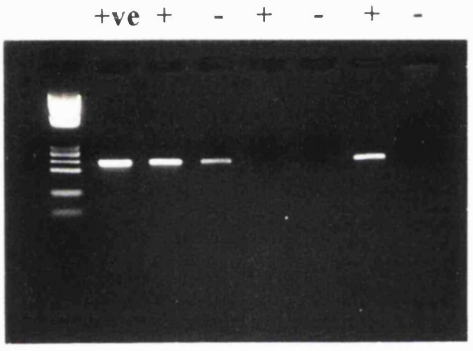


Figure 3.3 Agarose gel electrophoresis showing the products of HPV16 DNA PCR with the use (+) or in the absence (-) of mussel glycogen for cellular DNA extraction. Lane 1: pBR328 - DNA marker; Lane 2: HPV16 plasmid standard (100 fg per PCR reaction) - positive control (+ve); Lanes 3 and 4: high-grade lesion where increased HPV DNA levels are found using glycogen; Lanes 5 and 6: no HPV DNA was detected in this low-grade lesion with or without the use of glycogen; Lanes 7 and 8: low-grade lesion, the glycogen allowed the identification of HPV DNA positivity.

HPV DNA PCR products run between 220 and 234 bp, as assessed by pBR328. Half the total volume of the PCR reaction (12.5µl) was used per well.

3.4 Discussion

We have demonstrated that the addition of mussel glycogen to a frequently used DNA purification protocol can improve the detection of oncogenic types of HPV. In a recent editorial (Walboomers and Meijer 1997) argue that it appears that an almost 100% HPV association exist for cervical cancer. Factors which may explain the rare cases in which no HPV DNA is detectable include not only disruption of HPV by integration events, existence of still unidentified HPVs, and mechanism of transformation, but also improper sampling and sensitivity of the method, and thus false negative results can be obtained especially in cases with low HPV copy numbers per cell. In our study, the samples which proved to be HPV positive with the use of glycogen do not necessarily imply low levels of infection in the corresponding cervices, since quantitative differences are likely to reflect HPV heterogeneity in terms of viral load within the same biopsied specimen, and/or the samples ultimately analysed may not have been representative of the major lesion.

Since the Pap smear (i.e. exfoliative cytology) has a high false negative rate, HPV DNA PCR in combination with cytology has been advocated as an improved method particularly in secondary screening for cervical neoplasia (Cox *et al* 1992, Meijer *et al* 1992, Hatch *et al* 1995). It is known that women with normal cytology but harbouring high risk HPV types have a higher risk of progressing to high-grade squamous intraepithelial lesions (Brinton 1992). However, the minute quantities of target DNA available for examination in cervical smears might result in a high percentage of high-grade lesions proving negative for HPV as detected by PCR. For example, 25% of CIN 2/3 lesions have been shown to be negative for the oncogenic HPV types 16, 18, 31 and 33 (Cuzick *et al* 1995). Therefore, optimising DNA extraction from a small sample, such as that provided by a cervical smear, will increase the likelihood of detecting oncogenic HPV types by PCR. It is particularly difficult to achieve optimum results in cervical smears, where HPV detection can be hampered by admixture of non-neoplastic cells. Samples with a very low HPV copy number per cell may be scored negative, and the use of high sensitivity HPV DNA detection methods is thus essential to ensure reliable HPV analysis (Walboomers and Meijer 1997). Our proposed protocol is applicable to these problems and of wide potential usefulness since the DNA extraction

step only involves washing off the cell smear from the slide, pelleting and proceeding to the standard DNA purification by adding the lysis solution.

This protocol will be clinically significant, influencing the management of equivocal cytology/histology or low-grade cervical disease by clarifying uncertain morphological data and helping to ascertain the neoplastic potential of a particular lesion. It is now so well established that the presence of an oncogenic HPV DNA is the common denominator separating premalignant lesions from a variety of microscopically confusing but biologically harmless mimics (Reid *et al* 1995), that any approach which improves detection would be helpful. In addition, if economic pressures are a concern, and further studies and/or treatments cannot be offered to all the patients tested positive for oncogenic HPV types, at least women requiring a closer follow-up would be identified. Furthermore, rescreening intervals could be extended for those who have tested negative for oncogenic HPV types.

The sensitive HPV DNA test now available will improve the accuracy of diagnosis of HPV-related neoplastic lesions of the genital tract. The clinical merits of the approach are emphasised further by the observation that the combination of an essentially normal colposcopic examination and a negative virus test confers a negative predictive value greater than 98%. Conversely, a positive HPV test will identify a subset of women in whom there is either an occult squamous intraepithelial lesion or an emerging one will manifest within one year (Nuovo *et al* 1992).

3.5 Abstract

One of the main obstacles to the reliable detection of human papillomavirus in cervical intraepithelial neoplasia is that a minute quantity of infected tissue is often all that is available. In this work, proteinase K-phenol-chloroform treated sections of frozen cervical biopsies were split in two. Half of the material was ethanol precipitated in the presence of glycogen, and in the remaining half glycogen was absent. On average a 15-fold increase in total DNA yield was obtained with glycogen. As a result, in 63 cases analysed by PCR for HPV16, we were able to detect nearly 20% more positive samples when glycogen was used. The implications of the improved accuracy of diagnosis are that women requiring a closer follow-up can be identified, and conversely rescreening intervals can be extended for those who have tested negative for oncogenic HPV types with more confidence than in the past.

CHAPTER 4

Cathepsin E expression by normal and premalignant cervical epithelium

4.1 Introduction

Cathepsin E is an intracellular aspartic proteinase which has been implicated in proteolytic degradation (processing) of antigen for presentation by class II MHC (major histocompatibility complex) molecules. Unlike its counterpart cathepsin D, which is ubiquitous, cathepsin E has a relatively limited distribution in tissues where its localisation varies and appears to be associated with the specialised function of each cell type (Sakai *et al* 1989). Recent studies have shown that throughout the human gastrointestinal epithelium - stomach (Samloff *et al* 1987, Fiocca *et al* 1990), ileum and colon (Samloff *et al* 1985, Finzi *et al* 1993), jejunum, rectum and appendix (Finzi *et al* 1993), the levels of expression of cathepsin E are greater than those of other processing enzymes. Expression by M cells (a specialised epithelial cell covering gut lymphoid follicles) in rat and human ileum, and M-like cells in human palatine, lingual and pharyngeal tonsil (Finzi *et al* 1993) suggests that cathepsin E may be important in the processing of macromolecules and micro-organisms transported by these cells. The same hypothesis would be consistent with the observation that high levels of cathepsin E are also detectable in lymphomedullary tissues, such as bone marrow (rabbit/rat), spleen (bovine/rat) and cervical lymph node (rat) (Sakai *et al* 1989) as well as in epidermal keratinocytes (human/rat) (Bennet *et al* 1992, Solcia *et al* 1993, Hara *et al* 1993) and in human epidermal Langerhans cells (Solcia *et al* 1993). This functional view is supported by the demonstration that cathepsin E, localized to a non-lysosomal compartment of the endoplasmic reticulum, is essential for antigen processing and presentation by class II MHC molecules by a murine B cell lymphoma cell line (Bennet *et al* 1992). In disease, the enzyme has also been demonstrated in several types of adenocarcinomas, including cervical (Tenti *et al* 1994) and gastric (Matsuo *et al* 1996), but in normal cervix and in squamous epithelium from CIN lesions, i.e. premalignant cervical disease, cathepsin E expression has not been documented.

In cervical cancer and CIN lesions, there is a strong body of evidence which implicates the human papillomavirus (HPV) as a major causal agent (Munger *et al* 1989, Lorincz *et al* 1992, Vousden 1993) and HPV16 is the most prevalent type found in these lesions (Bergeron *et al* 1992). Several studies have demonstrated intracellular co-localisation of MHC class II HLA-DR and cathepsin E (Guagliardi *et al* 1990, Finzi *et al* 1993). Cervical keratinocytes upregulate the expression of class II molecules, especially HLA-DR, during the development of neoplasia (Glew *et al* 1993, Coleman and Stanley 1994), which led some authors to suggest that they can act as antigen-presenting cells (Nickoloff *et al* 1993, Mutis *et al* 1993). Our laboratory has shown that cathepsin E expression is under tight transcriptional control (Sealy *et al* 1996), and is co-ordinately regulated with class II MHC in some cell types (unpublished observation). Therefore, in this study we have examined whether cathepsin E expression is upregulated during CIN development, and whether this upregulation is correlated with the presence of HPV16 and/or the expression of HLA-DR in the local cervical micro-environment. In addition, the existence of a clearly defined "premalignant" phase in the development of cervical neoplasia offers a unique opportunity to follow the molecular changes which accompany the transformation process, and thus to examine the link between cathepsin E overexpression and malignancy.

In normal squamous epithelium (n=8), low-grade (n=21) and high-grade intraepithelial squamous lesions (n=14) of the uterine cervix, the expression of cathepsin E and HLA-DR was investigated by immunohistochemistry, cathepsin E mRNA expression examined by *in situ* hydridization, and finally the presence of HPV16 detected by polymerase chain reaction.

4.2 Results

Table 4.1 summarises the expression of cathepsin E and HLA-DR in the 43 samples studied, and relates them to epithelial localization, CIN and HPV16 status.

4.2.1 Cathepsin E expression by cervical epithelium

Figure 4.1 shows representative examples of expression of cathepsin E protein by normal, low-grade and high-grade intraepithelial squamous lesions. Cathepsin E is distributed diffusely throughout the cytoplasm of the epithelial cells. Such distribution appears to be restricted to undifferentiated cells, namely stem-cells of the basal layer in normal epithelium and the neoplastic/dyskariotic cells of CIN lesions. Protein expression was paralleled by message distribution, as demonstrated by *in situ* hybridization (Figure 4.2). Cathepsin E protein expression was localized to the basal layer of 7 out of 8 normal squamous epithelia. In low-grade disease (21 patients), the enzyme was present in basal (12 cases) or in basal and parabasal cells (7 cases). Finally in 14 high-grade lesions, expression of the molecule was restricted to basal and parabasal cells in 6 samples compared to 8 samples with full-thickness staining of the epithelium (Table 4.2). In 3 samples there was no expression of the molecule at all. The majority of keratinocytes infected with HPV by morphologic criteria (koilocytes) did not express cathepsin E - in 3 cases out of 16 occasional koilocytes were positive.

Cathepsin E was not expressed by Langerhans cells, although they were present in the squamous epithelium of all 43 specimens analysed in this study, as detected by antibodies against CD1a and HLA-DR (Figure 4.3).

We also documented the expression of cathepsin E by endocervical epithelium in 14 biopsies. Cathepsin E was detected in 4 of the 11 biopsies with normal endocervical epithelium. All 3 patients who had endocervical hyperplasia showed cathepsin E expression in the glandular epithelium.

4.2.2 HLA-DR expression by cervical epithelium and its relation to cathepsin E

In normal squamous cervical epithelium (n=8) HLA-DR molecules were never expressed. In low-grade lesions (n=21) only 5 cases showed patchy or diffuse staining of basal keratinocytes for HLA-DR. Finally, 9 out of 14 high-grade lesions (64.3%) expressed HLA-DR, often with staining of full-thickness of epithelium (Figure 4.3). No significant correlation between the distribution of HLA-DR and cathepsin E expression by cervical keratinocytes was found. In low-grade disease HLA-DR was focal, whereas cathepsin E was demonstrated in the whole basal or parabasal stratum of 19 specimens and in high-grade disease, although 64.3% of the lesions were HLA-DR positive

(compared to 100% cathepsin E positive), there was no correspondence to the epithelial level involved.

4.2.3 HPV16 in relation to cathepsin E and HLA-DR expression

HPV16 was not detected in normal epithelium. Its prevalence ranged from 24% (5 out of 21 cases) in low-grade lesions to 57% (8 out of 14) in high-grade disease, which is in accordance with previous studies in Western women (Bergeron *et al* 1992).

There was no simple relationship between cathepsin E expression and the detection of the HPV16. Increased numbers of cells expressing cathepsin E were found in 11 HPV16 negative lesions compared to 10 positive ones. Likewise, there was no correlation between HLA-DR expression in CIN lesions and HPV16 (Fisher's exact test, p= 1.0), (Table 4.3).

Table 4.1 Summary of expression levels for cathepsin E, HLA-DR and HPV16 status in normal squamous epithelium and CIN lesions

case	Hist	Cath E	HLA-DR	HPV16
1	N	b	0	-
2	N	b	0	-
3	N	b	0	-
4	N	b	0	-
5	N	0	0	-
6	N	b	0	-
7	N	b	0	-
8	N	b	0	-
9	L	b	0	+
10	L	b	pb	+
11	L	b	0	-
12	L	b+p	0	+
13	L	b	pb	-
14	L	b+p	0	-
15	L	b	0	-
16	L	b	0	-
17	L	b+p	pb	-
18	L	b+p	db	-
19	L	0	0	-
20	L	b	0	-
21	L	b	0	-
22	L	b	0	-

case	Hist	Cath E	HLA-DR	HPV16
23	L	b+p	db	+
24	L	0	0	-
25	L	b	0	+
26	L	b	0	-
27	L	b+p	0	-
28	L	b+p	0	-
29	L	b	0	-
30	H	b+p	0	+
31	H	b+p	pb	-
32	H	b+p	f	+
33	H	f	f	-
34	H	f	0	+
35	H	b+p	0	+
36	H	f	f	-
37	H	f	pb	-
38	H	b+p	db	+
39	H	f	0	+
40	H	f	0	+
41	H	f	db	+
42	H	f	f	-
43	H	b+p	pb	-

N, normal histology; L, low-grade (koilocytosis and/or CIN1) and H, high-grade intraepithelial squamous lesion (CIN2 and/or CIN3); 0, no staining; b, basal and b+p, basal and parabasal staining; pb, patchy basal and db, diffuse basal positivity; f, full-thickness of the epithelium stained; +, HPV16 present and -, HPV16 absent.

Table 4.2 Immunohistochemical analysis of cathepsin E expression by cervical squamous epithelium in normal and premalignant lesions according to the epithelial level involved

Histology	Epithelial level	n+	n
Normal	basal	7	8
Low-grade	basal	12	21
	basal + parab	7	
High-grade	basal + parab	6	14
	full-thickness	8	

Normal, normal histology; Low-grade, koilocytosis and/or CIN1; High-grade, CIN2 and/or CIN3; n, number of cases; n+, number of positive cases.

Table 4.3 HLA-DR expression patterns in CIN lesions positive or negative for HPV16

	HPV16+	HPV16-
HLA-DR+	5	9
HLA-DR-	8	13

HLA-DR+, keratinocytes positive for HLA-DR and HLA-DR-, negative for HLA-DR as determined by immunohistochemistry. (Fisher's exact test, p= 1.0).

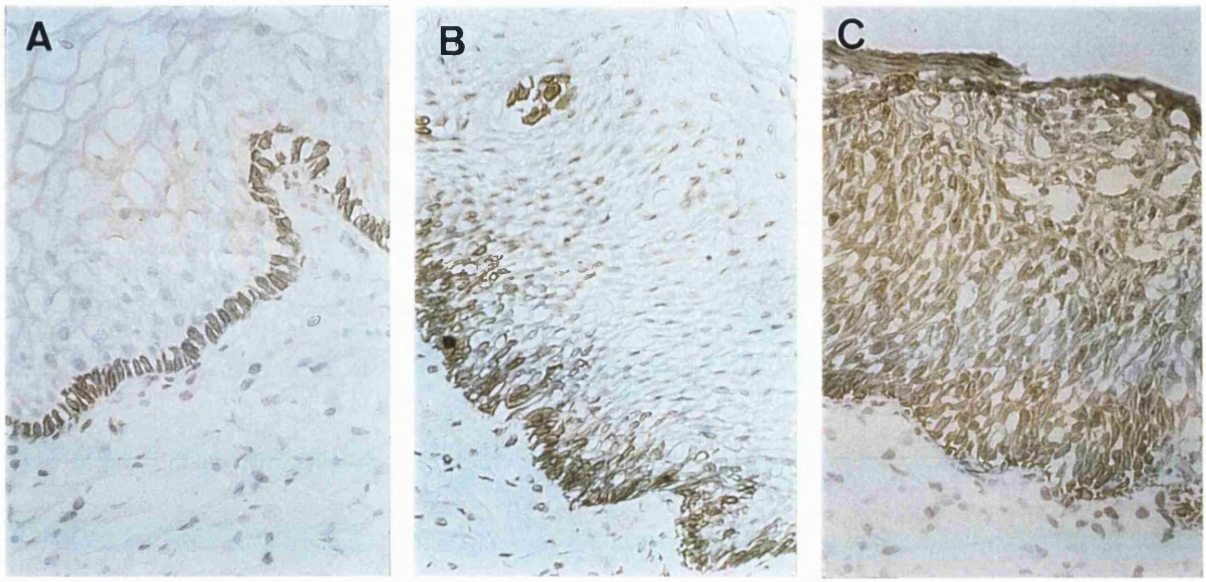


Figure 4.1 Immunohistochemical analysis of cathepsin E expression in cervical squamous epithelium. (a) Normal ectocervix. Only basal epithelial cells are positively stained. (b) Low-grade lesion. Intense staining of cells from the basal and parabasal layers. (c) High-grade lesion. The full-thickness of the neoplastic epithelium is stained for cathepsin E. All samples were counterstained with Mayer's Haematoxylin. Magnification, x40.

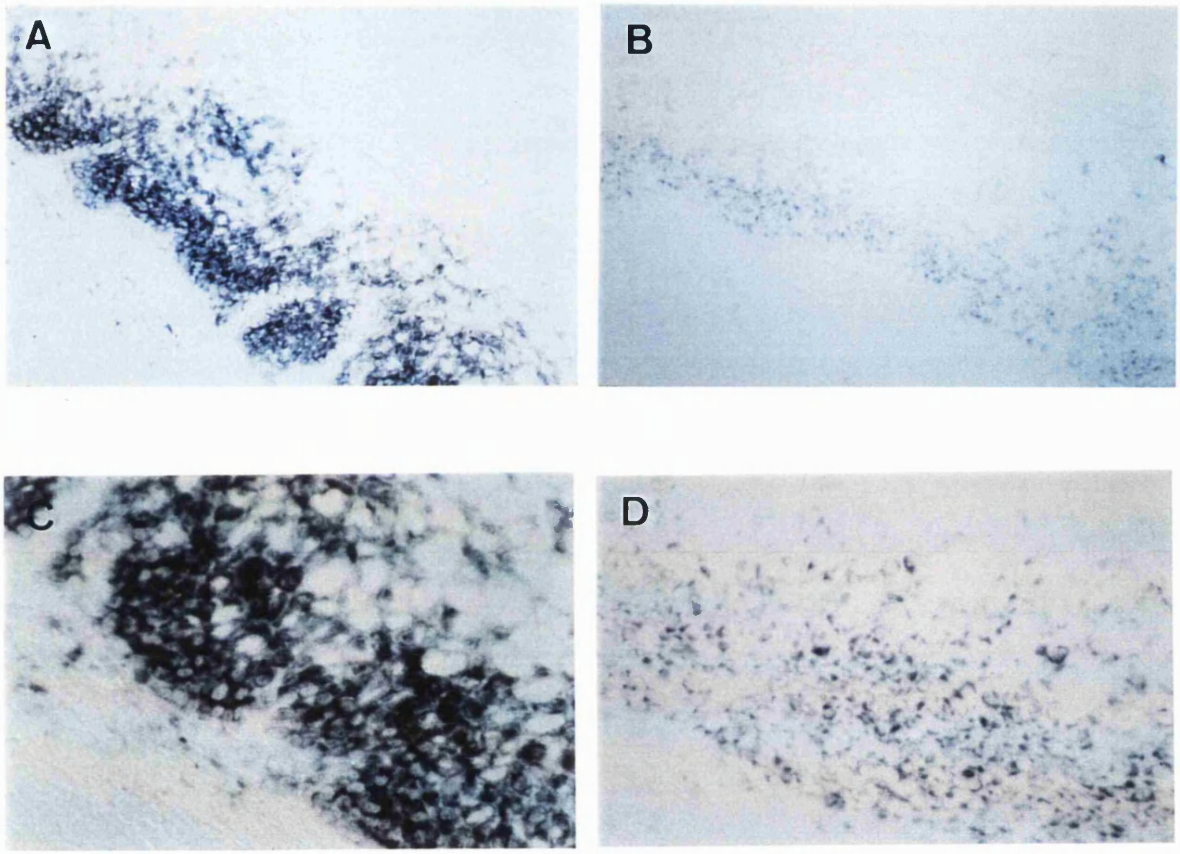


Figure 4.2 Cathepsin E mRNA expression detected by *in situ* hybridization in a low-grade (a,b) and high-grade (c,d) intraepithelial squamous lesions of the uterine cervix. (a) Antisense probe recognising cathepsin E transcripts in basal and parabasal dysplastic cells; Magnification, x25. (c) The lower two-thirds of the epithelium are displaying cathepsin E mRNA; Magnification, x40. (b,d) Sense, control probes showing weak background levels of signal.

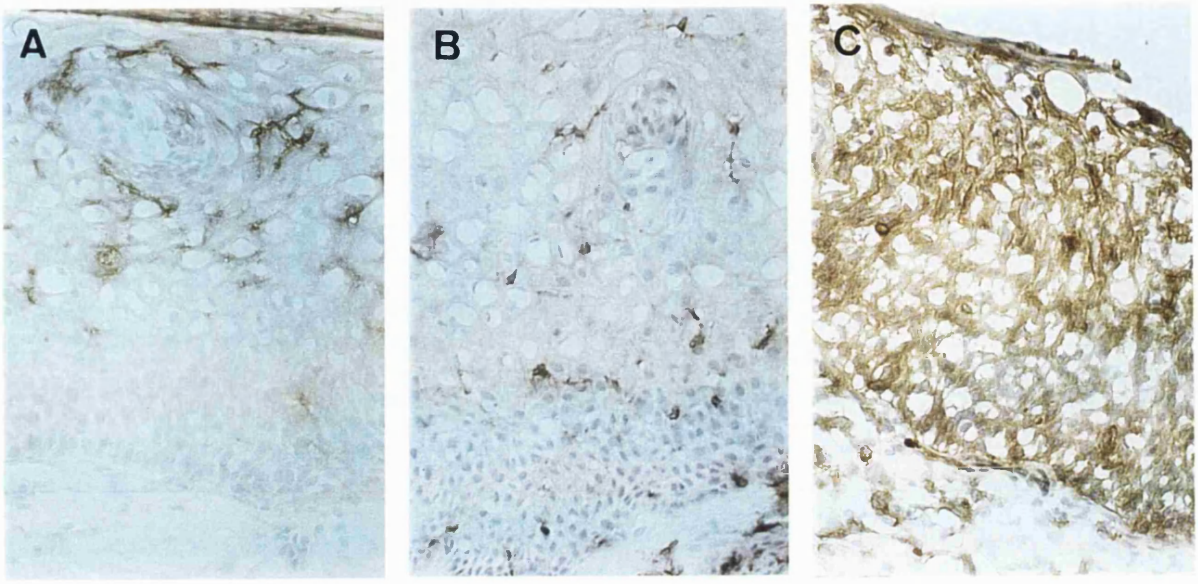


Figure 4.3 Two low-grade (a and b) and one high-grade (c) intraepithelial squamous lesions of the cervix showing: (a) Langerhans cells identified by a specific marker - CD1a, (b) Langerhans cells expressing HLA-DR and (c) keratinocytes stained positively with HLA-DR. Samples were counterstained with Mayer's Haematoxylin. Magnification, x40.

4.3 Discussion

Several lines of research suggest that cathepsin E is involved in the processing of antigens to be presented by class II MHC molecules in order to initiate a T cell-dependent immune response (Sakai *et al* 1989, Bennet *et al* 1992). Keratinocytes from cervical intraepithelial neoplasia (CIN) have been shown to upregulate expression of class II MHC molecules (Glew *et al* 1993, Coleman and Stanley 1994) and, at least theoretically, to act as antigen-presenting cells (Nickoloff *et al* 1993, Mutis *et al* 1993). This keratinocyte-mediated presentation could lead to elimination of infecting human papillomaviruses (HPVs) and/or destruction of HPV transformed cells. Therefore, the regulation of expression of processing enzymes, such as cathepsin E, which prepare the antigen for presentation, might play a role in the natural history of HPV associated disease. Thus, the immediate objectives of the present study were to immunolocalize cathepsin E in these cervical epithelia (keratinocytes and Langerhans cells) and correlate its expression with neoplastic transformation, class II MHC expression (HLA-DR) and HPV16 status.

The results of the immunohistochemistry and *in situ* hybridization show that in normal cervices only the stem-cells of the basal layer of the epithelium contain cathepsin E. There is parallelism between the expression of cathepsin E by the squamous epithelium and the degree of cervical dysplasia: increasing severity of the CIN lesion is related to upregulation of cathepsin E expression. Expression was found to be distributed diffusely in the cytoplasm of the keratinocytes involved. In contrast, koilocytes - keratinocytes harbouring a productive HPV infection - rarely express cathepsin E. Thus, only the basal precursor immature cells (i.e. less differentiated along the keratin formation pathway) or transformed cells, can synthesise this molecule, while "moribund" HPV infected cells and differentiated cervical keratinocytes lose that capacity.

Our observations about specific pattern of cathepsin E staining are in disagreement with previous studies which looked at the expression of cathepsin E in rat and human skin. Using a polyclonal antiserum (Hara *et al* 1993) localized cathepsin E to the cytoplasm of almost all epidermal cells, with the concentration increasing above suprabasal cells. Similarly, we did not find expression of cathepsin E by Langerhans cells, potent

professional antigen-presenting cells, in the squamous epithelium of either normal cervices or CIN lesions, although Langerhans cells were always present and distributed evenly throughout the epithelium, as detected by two monoclonal antibodies. In contrast, (Solcia *et al* 1993) using a specific rabbit anti-human cathepsin E serum detected the proteinase in Langerhans cells of the skin. It is unclear whether these discrepancies reflect real differences in enzyme distribution between skin and cervix, or differences in methodology or reagents. However, it may be relevant that in other keratinized squamous epithelium, such as on the surface of the vulva, Langerhans cells are present in a suprabasal position (Edwards *et al* 1985), which is different from the distribution usually described. This argues in favour of tissue differences, namely in distribution of antigen presenting cells between different epithelia, such as non-keratinizing (cervix) and keratinizing epithelia, and hence of the molecules associated with antigen processing and presentation.

HLA-DR expression by cervical epithelium in CIN lesions was consistent with previous reports (Glew *et al* 1993, Coleman and Stanley 1994), showing that there is an upregulation of the expression of the antigen in relation to the degree of the dysplasia. However, such HLA-DR upregulation was independent of cathepsin E expression in CIN lesions. This is also different from observations in other tissues, such as intestine, lung and gastric epithelia (Finzi *et al* 1993). In addition, both upregulation of cathepsin E and HLA-DR expression by squamous epithelium of CIN lesions was independent of the presence or absence of the HPV16 in those lesions, although we cannot rule out the presence of other HPV types in the HPV16 negative samples.

In conclusion, we have found that cathepsin E expression in cervical epithelium is tightly regulated, and different from other sites. In view of the correlation between immunohistology and *in situ* hybridization, we suggest that this may be regulated at transcriptional level, although additional post-transcriptional regulation cannot be excluded. Cathepsin E in HLA-DR expressing keratinocytes may play a role in antigen processing as has been suggested in other cell types. However, it is clear that cathepsin E expression and HLA-DR expression are regulated independently. The close relationship between cathepsin E levels and keratinocyte differentiation and transformation in the cervix, a key "transition" zone where both epithelial cycling and

maturation are ongoing, suggests that the enzyme may play a role in epithelial proliferation and differentiation as well as in antigen processing. The precise nature of this role remains speculative, but could involve the processing of growth or differentiation factors, which are synthesised within the keratinocytes themselves, as well as being taken up from the surrounding extracellular milieu. Several examples of such enzyme processing are quoted in the literature: transforming growth factor-beta (TGF-β), a potent growth regulatory protein secreted by virtually all cells in a latent form, is processed by human furin convertase into a biologically active peptide (Dubois *et al* 1995). If this mechanism is operative, the regulation of cathepsin E expression in keratinocytes of high-grade CIN lesions is another indication of the defect in the normal differentiation pathway of the epithelial cells in this condition.

4.4 Abstract

We have investigated the expression of the aspartic proteinase cathepsin E, HLA-DR and the presence of HPV16 in normal squamous epithelium (n=8), low-grade (n=21) and high-grade intraepithelial squamous lesions (n=14) of the uterine cervix. Immunohistochemistry of cervical biopsies revealed that upregulation of cathepsin E expression was related to increasing severity of the cervical intraepithelial neoplasia (CIN). Upregulation of protein was associated with increased message as assessed by *in situ* hybridization. Langerhans cells and the majority of koilocytes did not express detectable cathepsin E levels. Although there was also an upregulation of HLA-DR expression by cervical keratinocytes in CIN lesions, as determined by immunohistochemistry, no significant correlation was found between HLA-DR and cathepsin E expression in these lesions, neither was expression of cathepsin E correlated to the presence of HPV16, detected by polymerase chain reaction. The expression of cathepsin E, an aspartic proteinase which is reported to play a role in antigen processing for presentation by class II MHC molecules, is associated with cellular dedifferentiation in CIN lesions.

CHAPTER 5

Differential regulation of HLA-DQ expression by keratinocytes and Langerhans cells in normal and premalignant cervical epithelium

5.1 Introduction

There is a strong body of evidence implicating the human papillomaviruses (HPVs) as etiological agents in the pathogenesis of squamous cell carcinoma of the uterine cervix and its precursor lesions, i.e. cervical intraepithelial neoplasia (CIN), with HPV16 as the most prevalent type found in these lesions (zur Hausen 1991, Bergeron *et al* 1992). This is supported by several lines of evidence. HPV is found in the cervical tissue of a greater proportion of women with disease compared with controls (Lorincz *et al* 1992). There is an increasing prevalence of the oncogenic HPV types 16 and 18 with increasing severity of dysplasia to cervical carcinoma (Lungu *et al* 1992). The HPV16 E6 and E7 proteins inactivate endogenous tumour suppressor proteins p53 and Rb, abolishing normal cell control mechanisms (Vousden 1993). However, although the oncogenic types of HPV can transform and immortalise human keratinocytes *in vitro* (Munger *et al* 1989, Pecoraro *et al* 1991), these cells are initially non-tumorogenic in animals. Furthermore, 87 to 92% of CIN3 lesions harbour an oncogenic HPV type as detected by polymerase chain reaction (PCR) (Lungu *et al* 1992), only 16 to 40% of these lesions will progress to invasive cancer if left untreated (Ostor 1993). This indicates that additional factors other than infection with high-risk HPVs are involved in determining the clinical outcome following HPV infection of the cervix.

The cofactors which have been implicated in the development of cervical neoplasia include environmental (sexual activity, cigarette smoking, oral contraceptives, pregnancy, dietary deficiencies and local chronic inflammation), immunological and genetic, reviewed by (Cox 1995). Host cell-mediated immunosurveillance mechanisms may be a cofactor of critical importance in the control of HPV-related cervical disease. It has been shown that immunosuppression, as a result of therapy or as a consequence of

HIV infection, is associated with a very significant increased risk of cervical neoplasia and enhanced neoplastic progression to malignancy (Rudlinger *et al* 1986, Petry *et al* 1994). Generalised warts have been documented in patients with inherited immune deficiencies (Ormerod *et al* 1983). Changes in local populations of immune cells have been reported in HPV-related disease. For instance, there is a reduction in the number of Langerhans cells (professional antigen-presenting cells - APCs of the epithelia) and MHC (major histocompatibility complex) class II restricted CD4+ helper-inducer T cells in CIN lesions and genital warts. However, in both conditions no significant differences in the number of Langerhans cells were found comparing persistent versus regressive lesions, in contrast to CD4+ T cells which were significantly reduced in the persistent group (Fukuda *et al* 1993, Coleman *et al* 1994). Finally, several recent studies have demonstrated an association between CIN progression and specific HLA haplotypes , with attention focused on HLA-DQ (David *et al* 1992, Van den Velde *et al* 1993, Odunsi *et al* 1995). Furthermore, although the mechanisms of this association remain unknown, the association between specific DQ alleles and immunological non-responsiveness has been documented previously, for example in immunological responses to allergens and in autoimmune disease (Matsushita *et al* 1987; Sasazuki *et al* 1989).

In order to gain further insight into the role of HLA-DQ molecules in the context of intraepithelial neoplasia, we have investigated the expression of HLA-DQ and HLA-DR in a series of cervical samples with varying degrees of neoplastic transformation. Although there have been reports of increased HLA-DR expression during the development of neoplasia (Glew *et al* 1993, Coleman and Stanley 1994), literature review shows only one published study on the expression of HLA-DQ molecules in CIN lesions (Hughes *et al* 1988). The relationship between MHC class II expression and the HPV16 status was also documented, because a significant positive association between HLA-DRB1 and -DQB1 haplotypes and HPV16-mediated CIN lesions, in contrast to HPV16 negative ones, has been demonstrated previously (Apple *et al* 1995). Since the level of MHC expression and the nature of the cell on which the molecule is expressed, can profoundly influence the type of the response, leading either to anergy or activation of responding T cells, the cellular distribution of the MHC molecules was also recorded. The implications of our findings to the understanding of the role of class II HLA-DQ

molecules in the pre-invasive stage of HPV-related cervical neoplasia development are discussed.

5.2 Results

Table 5.1 summarizes the expression of MHC class II molecules by keratinocytes and Langerhans cells, and relates them to HPV16 status in 67 samples studied.

5.2.1 HLA-DR and HLA-DQ expression in cervical epithelium

MHC class II expression by cervical keratinocytes was determined using three monoclonal antibodies (L243, HLA-DR; L2 and Ia3, HLA-DQ) which recognise monomorphic determinants. In 12 biopsies of normal ectocervix there was no keratinocyte expression of either HLA-DR or DQ molecules. In 30 low-grade intraepithelial squamous lesions, whereas HLA-DR expression by epithelial cells was detected in 8 specimens, HLA-DQ expression by epithelial cells was never found. In 25 biopsies with high-grade disease, only 4 samples showed expression of HLA-DQ molecules by keratinocytes, while 60% (15 cases) showed positive expression of HLA-DR. Figure 5.1 shows representative examples of HLA-DR and HLA-DQ expression by cervical keratinocytes in adjacent sections from the same biopsies. It was noticed that in low-grade lesions the positive staining of the epithelial cells for HLA-DR was moderate in intensity, generally patchy and basal, koilocytes or superficial keratinocytes were rarely positive. In high-grade disease HLA-DR staining was often full thickness, with variable but frequently strong intensity. Of the 4 lesions positive for HLA-DQ antigens, 3 showed patchy, moderate basal staining and in one full thickness positive staining was present. In the positive specimens cell surface expression was associated with diffuse cytoplasmic staining. The differences in the expression of HLA-DQ by epithelial cells in the three groups under study (normal, low- and high-grade) were not significant, when analysed using a semi-quantitative grading system previously described by (Coleman and Stanley 1994). However, for HLA-DR expression there was a statistically significant difference between the three groups (Table 5.2). To confirm the conclusions of the semi-quantitative analysis, the number of samples with positive keratinocyte staining were compared to the negative ones, irrespective of the extent or intensity of

such staining. Using the Chi-square test, the same statistically significant differences for HLA-DR were observed (Table 5.2).

In normal epithelium and CIN lesions, subepithelial immune cells from the stroma positive for HLA-DR antigens were always observed. Particularly in high-grade lesions (5 out of 19) an intense infiltration of cells expressing HLA-DR molecules was noticed. In normal squamous epithelium, 5 out of 11 specimens (45%) showed positive HLA-DQ staining of the stromal immune cells and 18 out of 26 (69%) were positive in low-grade disease in comparison with 15 out of 19 (79%) in the high-grade disease group. Although the number of HLA-DQ expressing cells was always less than those expressing HLA-DR antigens, there was a small but steady increase of HLA-DQ positive subepithelial immune cells in parallel with the severity of disease. The same was observed for the number of HLA-DQ positive infiltrating immune cells within the epithelium. In normal ectocervix no DQ positive cells were identified, whereas in low-grade lesions they were detected in 20 out of 29 samples (69%) and in high-grade disease in 19 out of 23 biopsies (83%).

The expression of MHC class II molecules by endocervical columnar epithelium was analysed in 17 biopsies. While all samples were positive for HLA-DR, HLA-DQ antigens were present in 7 of the 14 biopsies with normal endocervical epithelium. All 3 specimens with endocervical hyperplasia were also positive for HLA-DQ.

5.2.2 MHC class II expression by Langerhans cells

In the normal ectocervix, Langerhans cells were found predominantly in the lower half of the epithelium, whereas in CIN lesions they were evenly distributed throughout the whole thickness of the epithelium. Table 5.3 shows that CD1a positive cells, i.e. Langerhans cells identified by this specific marker, were significantly reduced in number from normal epithelium to low and high-grade disease. The same observation was verified for Langerhans cells expressing HLA-DR. An opposite trend was found for HLA-DQ expressing cells. Although the number of cells expressing HLA-DQ was significantly smaller than the number of HLA-DR positive Langerhans cells in all groups, there was an increase in the proportion of Langerhans cells expressing HLA-DQ molecules which was related to the degree of dysplasia. The proportion of HLA-DQ

positive cells increased from 6% in normal epithelium to 13% in low-grade lesions and further to 31% in high-grade disease. This was highly statistically significant (Wilcoxon trend test, p= 0.001). It can be seen from Table 5.3 that most Langerhans cells expressed HLA-DR, and the proportion of CD1a cells which were DR positive did not vary according to disease status. In (Figure 5.2) typical examples of MHC class II expression by Langerhans cells in CIN lesions are depicted from serial frozen sections. No correlation was found between MHC class II expression by keratinocytes and the number of Langerhans cells expressing the same antigens.

5.2.3 HPV16 in relation to MHC class II expression by keratinocytes and Langerhans cells

Using the polymerase chain reaction (PCR), HPV16 was not detected in normal epithelium from 12 biopsies. Its prevalence ranged from 23% (7 of 30 cases) in low-grade lesions to 56% (14 of 25) in high-grade disease, results which concur with previous studies in Western women (Bergeron *et al* 1992).

In the CIN group, although the epithelial cells from 23 specimens out of a total of 55 were negative for both HLA-DR expression and presence of HPV16, HLA-DR upregulation was found in 11 HPV negative samples compared to 12 positive ones, which is not statistically significant (Fisher's exact test, p= 0.09). In high-grade neoplasia 4 specimens have shown upregulated expression of HLA-DQ molecules by keratinocytes, 2 belonging to the HPV16 negative group and the others to the positive one.

Once again, within the CIN group, there was no statistically significant difference between the number of Langerhans cells expressing HLA-DR (p= 0.8) and DQ molecules (p= 0.13) in relation to the HPV16 status, as assessed by the Wilcoxon rank-sum test. For HLA-DR, the median number of Langerhans cells was 40 in the HPV16 positive group versus 42 in the HPV16 negative one, and for HLA-DQ the figures were 13 versus 5.5 respectively.

Table 5.1 Summary of HLA-DR and HLA-DQ expression by keratinocytes and Langerhans cells in relation to the HPV16 status in normal squamous epithelium and CIN lesions

		Keratinocytes		Langerhans cells			
case	Hist	HLA-DR	HLA-DQ	CD1a	HLA-DR	HLA-DQ	HPV16
1	N	0	0	68	55	6	-
2	N	0	0	114	98	6	-
3	N	0	0	nt	56	10	-
4	N	0	0	124	98	8	-
5	N	0	0	64	28	11	-
6	N	0	0	nt	90	0	-
7	N	0	0	104	88	0	-
8	N	0	0	87	76	8	-
9	N	0	0	71	66	0	-
10	N	0	0	nt	62	8	-
11	N	0	0	86	74	6	-
12	N	0	0	94	82	0	-
13	L	0	0	54	47	*	-
14	L	0	0	62	58	0	-
15	L	0	0	92	78	25	+
16	L	1	0	nt	*	8	-
17	L	0	0	48	*	14	-
18	L	0	0	62	56	8	+
19	L	1	0	36	23	12	+
20	L	0	0	*	*	12	-
21	L	0	0	48	42	4	-
22	L	0	0	nt	70	0	-
23	L	2	0	56	49	4	-
24	L	0	0	nt	30	4	-
25	L	2	0	*	62	0	-
26	L	0	0	72	60	16	-
27	L	0	0	72	63	32	+
28	L	0	0	68	*	38	-
29	L	0	0	51	47	0	-
30	L	0	0	nt	*	0	+
31	L	0	0	64	63	6	-
32	L	0	0	42	38	12	-
33	L	0	0	nt	10	2	-
34	L	0	0	34	27	4	-
35	L	1	0	42	32	6	-
36	L	2	0	48	42	0	-
37	L	0	0	nt	48	3	-

38	L	0	0	54	42	24	-
39	L	2	0	42	32	4	-
40	L	0	0	44	46	0	-
41	L	1	0	nt	*	0	+
42	L	0	0	nt	49	7	+
43	H	5	1	22	*	4	-
44	H	3	2	52	47	16	+
45	H	2	0	*	26	6	+
46	H	0	0	24	18	0	+
47	H	0	0	26	22	0	+
48	H	4	0	nt	*	8	-
49	H	0	0	42	38	19	-
50	H	0	0	48	32	17	+
51	H	0	0	48	40	0	+
52	H	3	0	68	60	25	+
53	H	0	0	nt	31	*	-
54	H	0	0	76	58	23	-
55	H	3	0	*	52	39	+
56	H	3	0	39	*	9	+
57	H	4	1	52	*	24	-
58	H	3	0	26	23	5	-
59	H	3	0	56	*	21	-
60	H	0	0	*	28	0	-
61	H	3	0	42	36	14	+
62	H	4	0	46	*	18	+
63	H	3	0	35	27	16	+
64	H	3	0	nt	*	15	+
65	H	4	4	38	*	*	+
66	H	0	0	nt	30	16	-
67	H	0	0	48	46	6	-

N, normal histology; L, low-grade (koilocytosis and/or CIN1) and H, high-grade intraepithelial squamous lesion (CIN2 and/or CIN3); keratinocyte expression is shown using a scale of 0-5, scores evaluated by a previously reported grading scale to analyse HLA-DR expression by keratinocytes; the number of Langerhans cells expressing CD1a, HLA-DR or DQ are shown per mm^2 sectional area; +, HPV16 and -, HPV16 absent; nt, not tested; *, background staining prevented accurate identification of Langerhans cells.

Table 5.2 Analysis of HLA-DR and HLA-DQ staining of cervical keratinocytes

		HLA-DR		
Histology	n	n+	Mean	Range
Normal	12	0		
Low-grade	30	8	0.4	0-2
High-grade	25	15	2.0	0-5

		HLA-DQ		
Histology	n	n+	Mean	Range
Normal	12	0		
Low-grade	30	0		
High-grade	25	4	0.3	0-4

A previously described semi-quantitative grading scale was employed (Coleman and Stanley 1994). Normal, normal epithelium; Low-grade, koilocytosis and/or CIN1 and High-grade, CIN2 and/or CIN3; n, number of cases and n+, number of positive cases; mean, mean score from all cases; range, range of scores.

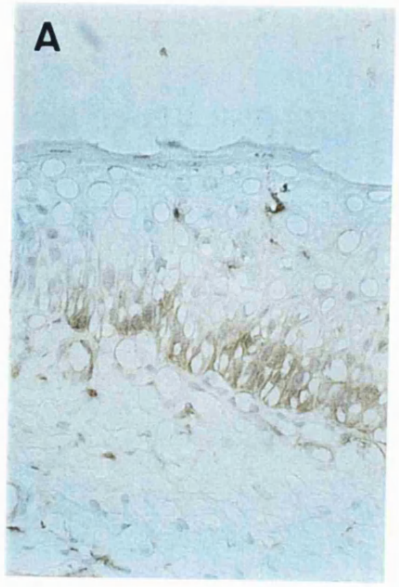
	p values		
	Kruskal-Wallis	Wilcoxon trend	X^2
HLA-DR	0.001	<0.001	<0.001
HLA-DQ	0.55	0.01	-

Table 5.3 Median Langerhans cell counts (per mm^2) positive for CD1a, HLA-DR and HLA-DQ, and ratios in cervical epithelium

				p values	
	Normal	Low-grade	High-grade	Kruskal-Wallis	trend
CD1a	87 (71-104)	52.5 (43-63)	44 (35-52)	<0.001	<0.001
HLA-DR	75 (59-89)	47 (35-59)	32 (27-46)	<0.001	<0.001
HLA-DQ	6 (0-8)	4 (0-12)	15 (5-19)	0.035	0.014
Ratio DR/CD1a	0.86 (0.81-0.87)	0.88 (0.79-0.92)	0.85 (0.77-0.89)	0.58	0.86
Ratio DQ/CD1a	0.06 (0-0.09)	0.13 (0.07-0.29)	0.31 (0.18-0.38)	0.004	0.001

Results are expressed as numbers of cells per mm^2 of sectional area. Median values are given, with the inter-quartile range of scores in parenthesis (interval from 25% percentile data to 75% percentile of data). Medians were calculated using all the data shown in Table 5.1. Normal, normal epithelium; Low-grade, koilocytosis and/or CIN1 and High-grade, CIN2 and/or CIN3.

Figure 5.1 Immunohistochemical analysis of MHC class II expression by keratinocytes in low-grade (a,b) and high-grade (c to f) intraepithelial squamous lesions of the cervix. The left panel shows HLA-DR expression, while on the right a serial section from the same sample is stained for HLA-DQ. Whereas in both grades of CIN lesions keratinocytes show focal or diffuse HLA-DR positivity, HLA-DQ expression is detected in the epithelial cells only in high-grade disease. In (e and f) the entire thickness of the dysplastic epithelium shows cell membrane and cytoplasmic staining for both class II MHC molecules. Stroma subepithelial HLA-DR positive immune cells were always present (a,c,e), but there was an increased number of these cells expressing HLA-DQ antigens in high-grade disease (as exemplified in f). Specimens were counterstained with Mayer's Haematoxylin. Magnification, x40.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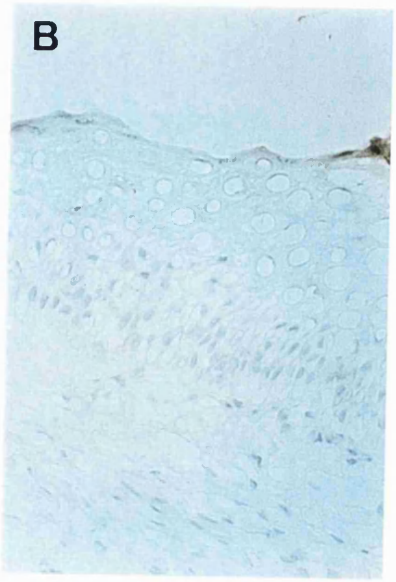
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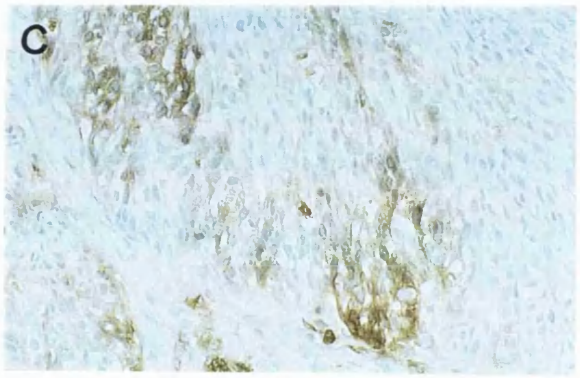
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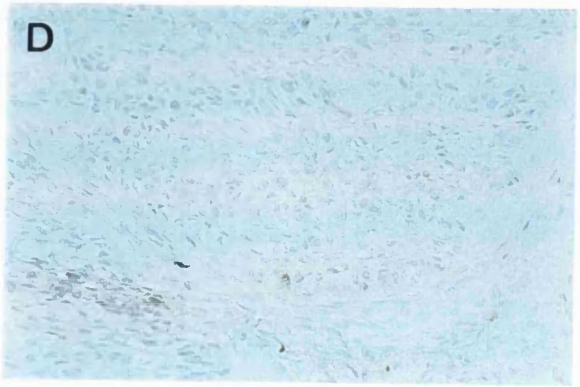
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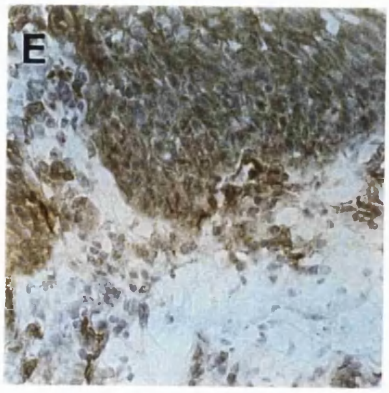
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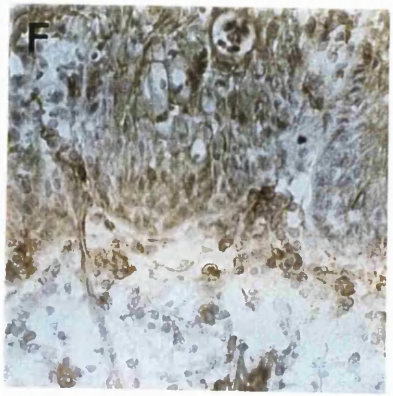
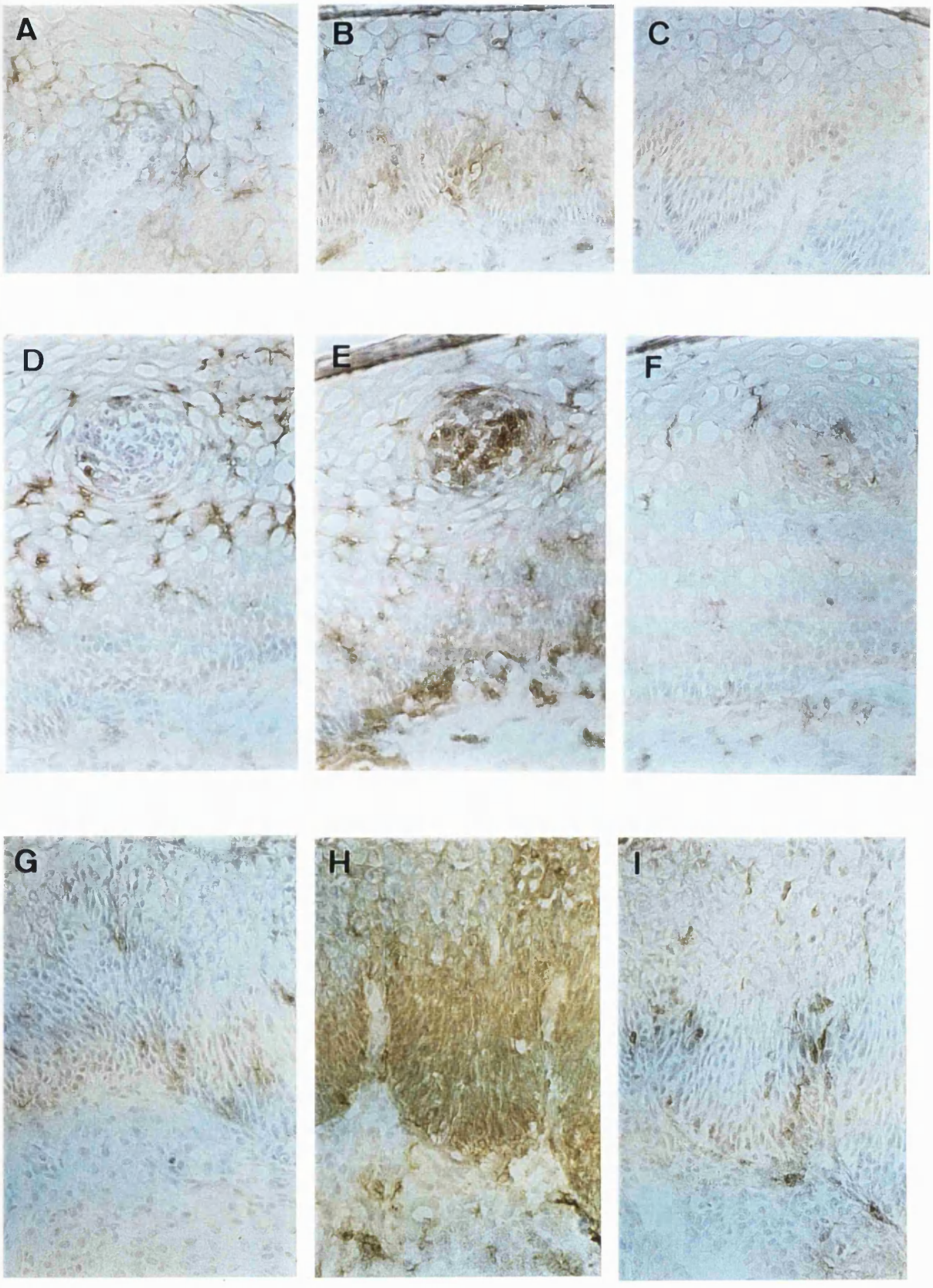

F

Figure 5.2 MHC class II expression by Langerhans cells in two low-grade (a to f), and one high-grade (g,h,i) intraepithelial squamous lesions of the cervix. Langerhans cell staining for a specific marker (CD1a) is shown in the left panel. The number of Langerhans cells expressing HLA-DR antigens (middle panel) was greater than those HLA-DQ positive (right panel), in serial cryostat sections. In (h) keratinocyte staining masks Langerhans cell identification in the consecutive related sectional area under examination. Samples were counterstained with Mayer's Haematoxylin. Magnification, x40.


A
B
C
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I

5.3 Discussion

Changes in HLA class I and II expression can directly affect the immunogenicity of HPV-infected or transformed cervical cells (Smith *et al* 1985), by altering the presentation of viral antigens to specific T cells. One example of such a change is the reduction of class I MHC expression which has been documented in 70 to 80% of cervical carcinomas (Cromme *et al* 1993, Hilders *et al* 1994). In premalignant cervical lesions, however, it has been shown that there are no alterations in HLA class I expression by epithelial cells (Glew *et al* 1993, Hilders *et al* 1994) with the exception of one study in which locus-specific down-regulation was observed (Cromme *et al* 1993). These findings suggest that HLA class I down-regulation occurs after development of malignancy. In contrast, changes in HLA class II molecules may play a role in immunomodulation in pre-invasive cervical lesions and, possibly, in determining the course of such lesions.

We have analysed the expression of MHC class II molecules (HLA-DR and DQ) by keratinocytes and Langerhans cells (epithelial APCs) in 30 low-grade (koilocytosis and/or CIN1) and 25 high-grade (CIN2 and/or CIN3) intraepithelial squamous lesions of the cervix. Normal squamous epithelium (12 specimens) was used as control. Our results show that there is a significant upregulation of HLA-DR expression by cervical keratinocytes, which is related to the severity of the CIN lesion. These data are concordant with previous studies (Cromme *et al* 1993, Coleman and Stanley 1994) although contradictory to others where no expression was reported (Hilders *et al* 1994). HLA-DQ upregulation on keratinocytes was only detected in high-grade disease (4 out of 25 samples - 16%) using two monoclonal antibodies L2 and Ia3, which gave consistently similar staining patterns. In contrast, (Hughes *et al* 1988) did not find such expression using a different anti-DQ reagent, TU22. These differences could be attributed to distinct epitope recognition by the respective antibodies or to differences in staining sensitivity. The predominance of HLA-DR expression over DQ suggests that the two antigens are differentially regulated, a conclusion which is suggested also by the absence of positive correlation between upregulation of HLA-DR and DQ molecules by the epithelial cells.

The mechanisms responsible for epithelial MHC class II upregulation in HPV-related cervical disease are still poorly understood. It is possible that expression could be related to tissue dedifferentiation and clonal expansion during the process of cellular transformation of immature basal cells. This hypothesis is supported by several observations: the relationship between grade of disease and MHC class II expression; the finding that in the same biopsy there are different patterns of MHC expression in contiguous epithelial areas, ruling out the sole role of local cytokines in the induction of MHC class II antigens; and finally, the lack of correlation between the presence of HPV16 DNA and upregulation of class II expression, suggesting that MHC class II expression was not, at least directly, a consequence of HPV16 infection, although we cannot rule out the presence of other HPV types in the HPV16 negative samples.

The decrease in number of Langerhans cells seen both in this and previous studies of HPV-related disease has been interpreted by some authors as a sign of local immunosupression. However, a direct link between Langerhans cell numbers and outcome of infection is not supported by two studies which compared persistent versus regressive lesions, and found no differences in Langerhans cell counts (Fukuda *et al* 1993, Coleman *et al* 1994). In the present study the decrease in number of Langerhans cells is associated with an increase in HLA-DQ expressing cells. Upregulation of HLA-DQ is a characteristic feature of the differentiation of Langerhans cells into fully mature lymphoid dendritic cells (Sallusto *et al* 1994). Decreased numbers of Langerhans cells may, therefore, reflect increased activation and migration from the epithelium to draining lymph nodes, in order to present viral or tumor associated antigens to local lymphocytes. Using an *ex vivo* human skin organ culture, it was shown that maturation of Langerhans cells is not only associated with strong upregulation of HLA-DQ expression, but also marked depletion of these cells due to spontaneous migration out of the epidermis into the dermis and then out of the skin (Rambukkana *et al* 1991). In our study, the increased number of Langerhans cells expressing DQ antigens in high-grade disease correlated with increased number of infiltrating immune cells positive for this antigen within both the epithelium and the subepithelial stroma which, again, suggests immune activation in at least some lesions.

The role of host immune recognition in the natural history of CIN progression remains poorly understood. However, recent genetic studies showing linkage between CIN, cervical carcinoma and particular HLA-DQ alleles (David *et al* 1992, Van den Velde *et al* 1993, Odunsi *et al* 1995; Wank *et al* 1991, Gregoire *et al* 1994) strongly implicate a role for immune recognition. We have found keratinocyte HLA-DQ expression in only a very small number of CIN lesions and expression was low and patchy. It seems likely, therefore, that any association between CIN progression and HLA-DQ is mediated by expression of this molecule on Langerhans cells rather than keratinocytes. The increased level of DQ we report also suggests that CIN lesions are sites of Langerhans cell activation and migration, a process which is usually correlated with immune activation. HLA-DQ has been implicated as a risk factor in several tumors (Han *et al* 1992, Lee *et al* 1994). Although the reason for this negative regulation by HLA-DQ is still unknown, the differences in regulation of expression between HLA-DR and DQ may well be important. This differential regulation is clearly illustrated in this study, both on epithelial cells and Langerhans cells. Since DQ polymorphism's are also associated with differential gene regulation (Andersen *et al* 1991), quantitative differences in expression level of DQ between alleles rather than their differences in specific peptide antigen binding, may play a role in disease modulation. Further studies correlating HLA-DQ expression to particular DQ alleles will be required to answer this question.

5.4 Abstract

HLA-DQ alleles have been associated with progression of cervical intraepithelial neoplasia to malignancy. Expression of HLA-DR and HLA-DQ by keratinocytes and Langerhans cells from normal cervical squamous epithelium (n=12), low-grade (n=30) and high-grade (n=25) intraepithelial neoplastic lesions, was investigated by immunohistochemistry. Expression of these molecules was correlated to the HPV16 detected by polymerase chain reaction. Keratinocytes in histologically normal samples did not express either MHC class II molecules. In low-grade lesions expression was confined to HLA-DR (8 of 30), while in high-grade disease there was expression of HLA-DR (15 of 25) and occasional expression of HLA-DQ (4 of 25). The number of Langerhans cells decreased significantly from normal ectocervix to low- and high-grade disease, as assessed by CD1a. HLA-DR was expressed constitutively on the majority of Langerhans cells. In contrast, few Langerhans cells express HLA-DQ in normal cervix, but there was a steady upregulation of the proportion expressing HLA-DQ which paralleled the severity of disease. There was no direct correlation between HPV16 and the expression of HLA-DR and DQ molecules by keratinocytes and Langerhans cells. Significant upregulation of HLA-DQ molecules by Langerhans cells is observed in high-grade intraepithelial cervical lesions, suggesting antigen presenting cell activation in HPV-related premalignant disease.

CHAPTER 6

Regulated expression of cytokines and T cell costimulatory molecules in normal and premalignant cervical epithelium

6.1 Introduction

Human papillomaviruses (HPVs) play a major role in the etiology of cervical cancer and its precursor lesions, i.e. cervical intraepithelial neoplasia (CIN) (zur Hausen 1991, Bergeron *et al* 1992). HPV16, the most prevalent HPV type in these lesions, has been shown to encode a tumour specific rejection antigen which triggers a cytotoxic T cell response (Chen *et al* 1991). Several other lines of research have also implicated host cell-mediated immunological mechanisms as critical factors in the control of HPV-related cervical disease (Fukuda *et al* 1993, Petry *et al* 1994).

It is generally accepted that resting T cells require at least two signals for induction of cell proliferation and effector functions (Schwartz 1990, Jenkins 1992). Signal 1 is delivered by the interaction of the T cell receptor (TCR)/CD3 complex with the antigenic peptide bound to the specific major histocompatibility complex (MHC) molecule. Signal 2, costimulatory or accessory signal, is antigen independent, utilizes different transduction pathways distinct from those tranduced through the TCR/CD3 complex, and is provided by cell-surface molecules on the antigen presenting cells (APCs) which interact with their cognate receptors on T cells (Weaver *et al* 1988, June *et al* 1990). Antigens presented by MHC molecules to T cells in the absence of this second signal induce anergy or clonal deletion of the antigen specific T cell clone (Mueller *et al* 1989).

MHC class II molecules (HLA-DR and -DQ) are usually expressed primarily on professional APCs, such as Langerhans cells. However, keratinocytes (squamous epithelial cells) which make up about 95% of the cell mass of epidermis, have also been shown to express these molecules following cytokine stimulation *in vitro*, during inflammatory states or upon cellular transformation *in vivo*, allowing them to act as APCs. Furthermore, *in vitro* studies have demonstrated that MHC class II positive

epithelial cells are able to provide accessory costimulating signals to T cells, and thereby, are able to induce T cell proliferation, which can initiate or assist local immune responses (Nickoloff *et al* 1993a). It has previously been shown that cervical keratinocytes can upregulate the expression not only of HLA-DR (Coleman and Stanley 1994) but also the adhesion molecule ICAM-1 (intercellular adhesion molecule-1, CD54) (Coleman *et al* 1993). However, the expression by cervical keratinocytes of other fundamental accessory molecules, such as, ICAM-3 (CD50), LFA-3 (lymphocyte function associated antigen-3, CD58) and B7-2 (CD86) has not been reported. Epidermal (cutaneous) keratinocytes do not express B7-1 (CD80) (Nickoloff *et al* 1993a) and B7-2 (CD86) may be a more relevant stimulatory ligand for the CD28 receptor on T cells than is B7-1 (Hathcock *et al* 1993). It has been shown that efficient T cell activation and clonal expansion is not achieved if inadequate or insufficient costimulation takes place. Moreover, the functional consequences of T cell-non-professional APC interaction may differ depending on the nature of the costimulatory pathways available and/or utilized. Therefore, in this study, we have analyzed the expression of ICAM-1, ICAM-3 and their T cell receptor LFA-1 (both CD11a, α-chain and CD18, β-chain), LFA-3 and B7-2 by keratinocytes and stroma subepithelial immune cells in cervical epithelium and correlated their expression to MHC class II molecules (HLA-DR and HLA-DQ).

The mucosa-associated lymphoid tissue (MALT) comprises not only effector immune cells and keratinocytes which can act as APCs, but also potent professional APCs specialized to initiate primary immune responses, which include epidermal Langerhans cells and dermal dendritic cells. We have identified this cell population with a specific marker - CD1a, and recorded their differential expression of costimulatory molecules in cervical epithelium.

The local cytokine microenvironment in which T cells undergo stimulation is thought to drive their differentiation into CD4+ T helper 1 (Th1) or Th2 cells with diverse cytokine profiles and effector functions. The APC type involved in T cell priming can also influence the cytokine pattern and function of the activated T cells. In contrast to professional APCs, epithelial cells cannot produce interleukin 12 (IL-12), a cytokine that has been shown to be a key factor for differentiation of naive T cells into Th1 T cells (Hsieh *et al* 1993, Seder *et al* 1993), which mediate cellular immunity and, at least in

some cases, are the major effectors in the host defense against viral infections and cellular transformation (Nagarkatty *et al* 1990, Greenberg 1991). Moreover, activated epidermal keratinocytes can secrete IL-10 (Enk and Katz 1992), a Th2 type cytokine which has immunosuppressive functions towards Th1 T cells (Fiorentino *et al* 1991), and inhibits the antigen-presenting cell (APC) function, presumably by downregulation of costimulatory molecules expressed on the surface of these cells (Ding and Shevach 1992, Enk *et al* 1993). It has also been documented that epidermal keratinocytes can produce TNF-α (Kolde *et al* 1992), an inflammatory cytokine which has an array of immunostimulatory properties, such as activation and recruitment of macrophages, granulocytes and cytotoxic cells, induction of adhesion molecules and MHC class I and II antigens by immune and epithelial cells, and thus, plays a role in the host resistance to infection (e.g. direct suppression of viral replication) and tumour growth (Sugarman *et al* 1985, Wong and Goeddel 1986, Beutler and Cerami 1989). Since there is compelling evidence for local antiviral activity entirely mediated by cytokines and since T cell effector functions vary accordingly to the local cytokine milieu with either activation, unresponsiveness/inappropriate response or anergy, we have analyzed the expression of IL-10 and TNF-α in the local microenvironment of cervical epithelium.

In this study all these issues have been addressed by using immunohistochemistry to examine a series of cervical samples with squamous intraepithelial lesions ranging from koilocytosis (i.e. HPV infected keratinocytes without disturbed epithelial maturation) to varying degrees of neoplastic transformation (CIN lesions). Normal squamous epithelium was used as a control. Furthermore, the TNF-α and IL-10 immunohistochemical evidence of protein expression was correlated with mRNA using *in situ* hybridization. The implications of our findings to the understanding of the development of the immune response within CIN lesions is discussed.

6.2 Results

Table 6.1 summarises the expression of MHC class II antigens, T cell costimulatory molecules and cytokines by keratinocytes in 53 samples studied. Table 6.2 summarises the number of immune cells in the subepithelial stroma expressing MHC class II antigens, T cell costimulatory molecules and CD1a in 53 cervical samples.

6.2.1 Expression of MHC class II antigens and costimulatory molecules by cervical keratinocytes

In 12 biopsies of normal ectocervix there was no keratinocyte expression of either HLA-DR, HLA-DQ or ICAM-1 (CD54). In 23 low-grade intraepithelial squamous lesions, whereas HLA-DQ expression by epithelial cells was never found, HLA-DR expressing keratinocytes were detected in 7 specimens and ICAM-1 in 4. Finally, in 18 biopsies with high-grade disease, only 2 samples showed patchy, basal expression of HLA-DQ antigens by keratinocytes, while 61% (11 cases) showed positive expression of HLA-DR and 50% (9 cases) were also positive for ICAM-1 (Table 6.3). The differences in the expression of HLA-DQ by epithelial cells were not significant in the three groups under study (normal, low- and high-grade), when analyzed using a semi-quantitative grading system previously described (Coleman and Stanley 1994). However, for HLA-DR expression there was a statistically significant difference between the normal group versus high-grade disease, and also low-grade versus the high-grade disease group. ICAM-1 expression by cervical keratinocytes was only significantly different comparing the normal and the high-grade disease group (Table 6.3). Furthermore, there was a very strong positive correlation between the expression of HLA-DR and ICAM-1 by keratinocytes in CIN lesions. The epithelial cells from 22 specimens out of a total of 41 were negative for both HLA-DR and ICAM-1 expression, and upregulation of these molecules was detected in the same 13 samples (Fisher's exact test, $p<0.0001$). In addition, in the positive specimens, co-localisation of the expression of these molecules by keratinocytes in premalignant cervical disease was always observed (Figure 6.1). In low-grade lesions the staining of the epithelial cells for HLA-DR and ICAM-1 was moderate in intensity, generally patchy and basal, and koilocytes or superficial keratinocytes were rarely positive. In high-grade disease HLA-DR and ICAM-1 staining was often full thickness, with variable but frequently strong intensity. However, HLA-DR expression was generally more intense and diffuse than that of ICAM-1 in the positive samples.

Expression of both ICAM-3 (CD50) and B7-2 (CD86) by epithelial cells in normal (n=12) or premalignant cervical squamous epithelium (n=41) was never observed, although the antibodies to both these molecules showed strong reactivity in tonsil

(Figure 2.4 b and e). In contrast, LFA-3 (CD58) was constitutively expressed by basal or basal and parabasal epithelial cells in normal epithelium, and this expression, which was detectable in all tissues studied, increased in parallel with the severity of the cervical intraepithelial lesion. LFA-3 protein was located diffusely throughout the cytoplasm of the epithelial cells in normal and premalignant epithelium (Figure 6.3, f).

6.2.2 Expression of TNF-α and IL-10 in cervical epithelium

In normal squamous epithelium (n=12), while TNF-α protein was always identified in the basal or basal and parabasal epithelial cells, IL-10 expression was always absent. In 23 biopsies with low-grade lesions, IL-10 expression by keratinocytes was detected in 12 specimens (52%), and in contrast, TNF-α expression was lost in 3 samples (13%). Finally, in 18 biopsies with high-grade disease, whereas IL-10 continued to be upregulated in 8 cases (44%), TNF-α expression by epithelial cells was further downregulated and the cytokine was undetectable in 6 out of 18 specimens (33%) (Table 6.4). No correlation between the expression of IL-10 and TNF-α by keratinocytes in premalignant cervical disease was found, which suggests that they are independently regulated. Both molecules were found in 17 samples, both were absent in 6, but in 14 TNF-α was present and IL-10 was not, and in 2 the opposite occurred (Fisher's exact test, p=0.2). Once again, within the CIN group, there was no statistically significant difference between the expression of TNF-α and HLA-DR (p=0.1) or ICAM-1 (p=1.0) by the epithelial cells. Likewise, no correlation was observed for the expression of IL-10 and HLA-DR (p=1.0) or ICAM-1 (p=1.0) by cervical keratinocytes.

Figure 6.2 shows characteristic examples of TNF-α and IL-10 protein expression in normal and premalignant cervical squamous epithelium. Both cytokines were distributed diffusely throughout the cytoplasm of the epithelial cells and keratinocyte staining for TNF-α was always more intense than for IL-10. Epithelial infiltrating or stromal immune cells rarely showed detectable expression of either TNF-α or IL-10. These differences might be attributed to the sensitivity of the immunohistochemical method used and/or differences in storage. It is possible that whereas immune cells synthesize and promptly release the cytokines to fulfill their effector functions, keratinocytes are able not only to produce but also to store both cytokines, which can be released when

they are activated. The distribution of mRNA for both cytokines was similar to that seen for protein, as documented by *in situ* hybridization (Figure 6.3), with higher levels found in basal and parabasal cells.

6.2.3 Expression of MHC class II antigens and costimulatory molecules by immune cells in the subepithelial stroma

Table 6.5 summarizes the expression of HLA-DR and -DQ, ICAM-1, ICAM-3 and their T cell receptor LFA-1, and LFA-3 by subepithelial immune cells in normal squamous epithelium and CIN lesions. It is apparent that there is a steady increase in the number of infiltrating cells expressing all the above mentioned molecules in relation to the severity of HPV-related cervical disease. LFA-1 positive cells predominate in the cervical stroma, as well as within the epithelium particularly in high-grade disease. A high number of both ICAM-3 and HLA-DR positive immune cells are also present. Fewer cells express ICAM-1 and HLA-DQ with only a small proportion expressing LFA-3, whereas B7-2 positive cells were undetectable. Figure 6.3 depicts representative examples of the expression of costimulatory molecules by mucosa infiltrating immune cells in premalignant cervical epithelium.

In the CIN group, statistically significant correlations were found between the number of subepithelial immune cells expressing HLA-DR and -DQ molecules (Spearman rank correlation, $p<0.0001$), DR and ICAM-1 ($p=0.02$), DR and ICAM-3 ($p=0.05$), DR and LFA-1 ($p=0.0002$), DR and LFA-3 ($p=0.0009$), DQ and LFA-1 ($p=0.002$), DQ and LFA-3 ($p=0.002$), ICAM-1 and LFA-1 ($p=0.01$), ICAM-3 and LFA-1 ($p=0.0001$), and finally, LFA-1 and LFA-3 ($p=0.04$). In both high-grade disease and CIN lesions as a whole, a greater number of stromal immune cells expressing LFA-1 and HLA-DR antigens were recorded in the specimens showing keratinocyte expression of HLA-DR and ICAM-1, in comparison to those samples where epithelial cells were negative. However, the differences were not statistically significant (Wilcoxon rank-sum test). In the CIN group, using the same test, the number of stromal cells expressing MHC class II or adhesion molecules was not correlated to the expression of TNF-α or IL-10. This result suggests that production of both cytokines by cervical keratinocytes is not induced by local infiltrating immune cells.

6.2.4 CD1a positive cells in the cervical mucosa

Intraepithelial Langerhans cells were identified with a specific marker, CD1a, and they were present in all tissues studied (n=53). This cell population was invariably positive for HLA-DR, occasionally for HLA-DQ and was never found to express adhesion molecules (ICAM-1, ICAM-3, LFA-1, LFA-3 and B7-2) or cytokines (TNF-α and IL-10). Figure 6.4 is a representative example of the staining patterns observed. However, in one sample with normal epithelium (n=12) and in 5 out of 41 CIN lesions, CD1a positive stromal dendritic cells were detected and expressed all the above mentioned adhesion molecules, as observed in serial cryostat sections (Figure 6.5).

Table 6.1 Summary of expression of MHC class II antigens, costimulatory molecules and cytokines by keratinocytes in normal and premalignant cervical squamous epithelium

case	Hist	DR	DQ	ICAM-1	ICAM-3	LFA-3	B7-2	IL-10	TNF-α
1	N	0	0	0	0	b+p	0	0	b+p
2	N	0	0	0	0	b+p	0	0	b+p
3	N	0	0	0	0	b+p	0	0	b
4	N	0	0	0	0	b+p	0	0	b+p
5	N	0	0	0	0	0	0	0	b+p
6	N	0	0	0	0	b	0	0	b+p
7	N	0	0	0	0	b	0	0	b
8	N	0	0	0	0	b	0	0	b
9	N	0	0	0	0	b	0	0	b
10	N	0	0	0	0	b+p	0	0	b+p
11	N	0	0	0	0	b	0	0	b
12	N	0	0	0	0	b	0	0	b
13	L	0	0	0	0	b	0	0	b+p
14	L	4	0	3	0	b+p	0	f	f
15	L	0	0	0	0	f	0	f	f
16	L	3	0	0	0	f	0	0	0
17	L	4	0	0	0	f	0	0	b
18	L	0	0	0	0	b+p	0	b	b+p
19	L	0	0	0	0	b+p	0	0	b
20	L	0	0	0	*	*	0	0	b
21	L	0	0	0	0	f	0	0	b+p

22	L	3	0	3	0	b+p	0	0	f
23	L	4	0	3	0	f	0	b+p	0
24	L	0	0	0	0	f	0	0	b
25	L	3	0	2	0	f	0	0	0
26	L	0	0	0	0	b+p	0	b	b
27	L	0	0	0	0	b+p	0	0	b
28	L	0	0	0	0	b+p	0	b	b+p
29	L	0	0	0	0	b+p	0	0	b
30	L	0	0	0	0	b+p	0	b+p	b+p
31	L	0	0	0	0	b	0	f	b
32	L	0	0	0	0	b+p	0	b	b
33	L	0	0	0	0	0	0	b	b
34	L	2	0	0	0	b+p	0	b+p	f
35	L	0	0	0	0	b+p	0	b	b
36	H	0	0	0	0	f	0	f	0
37	H	5	0	0	0	f	0	0	0
38	H	3	0	2	0	f	0	0	b
39	H	5	0	3	0	b+p	0	0	0
40	H	0	0	0	0	b+p	0	0	b+p
41	H	3	0	2	0	f	0	f	f
42	H	5	0	4	0	f	0	f	f
43	H	4	0	2	0	f	0	f	f
44	H	0	0	0	0	f	0	0	f
45	H	0	0	0	0	f	0	0	0
46	H	5	0	3	0	f	0	f	f
47	H	4	2	3	0	f	0	0	f
48	H	5	0	3	0	f	0	0	f
49	H	0	0	0	0	f	0	b+p	f
50	H	4	0	2	0	f	0	0	b+p
51	H	0	0	0	0	f	0	0	0
52	H	4	3	0	0	f	0	f	0
53	H	0	0	0	0	f	0	f	f

Table 6.2 Summary of immune cell numbers per mm^2 in the subepithelial stroma of normal and premalignant cervical squamous epithelium positive for MHC class II antigens, costimulatory molecules and CD1a

case	Hist	DR	DQ	ICAM-1	ICAM-3	LFA-1	LFA-3	B7-2	CD1a
1	N	393	164	192	137	842	9	0	0
2	N	338	0	98	*	169	0	0	0
3	N	256	16	35	529	2601	36	0	0
4	N	225	0	0	81	961	0	0	0
5	N	92	75	16	169	1242	0	0	0
6	N	0	0	0	196	*	16	*	0
7	N	529	0	121	1225	841	12	0	0

8	N	460	0	225	64	966	16	0	9
9	N	625	0	324	36	420	25	0	0
10	N	169	0	81	158	225	*	0	0
11	N	289	16	121	64	*	0	0	0
12	N	324	0	49	86	361	28	0	0
13	L	2190	0	1532	6370	*	*	0	0
14	L	4110	1187	2958	2814	8750	529	0	81
15	L	1240	0	726	256	1470	124	0	0
16	L	2180	0	246	84	631	*	0	0
17	L	1360	0	*	81	434	0	0	0
18	L	2096	1095	0	2527	2730	169	0	*
19	L	121	0	216	783	434	0	0	*
20	L	1756	400	225	*	6561	*	0	0
21	L	864	0	0	6241	6370	121	0	0
22	L	2932	602	1308	1141	6139	961	0	121
23	L	3136	625	2124	4284	4725	*	0	0
24	L	1260	*	306	2079	3220	64	0	0
25	L	2068	375	181	1369	3584	*	0	16
26	L	4096	1225	*	1849	6084	*	0	0
27	L	2209	81	1369	5184	3136	441	0	0
28	L	961	289	676	256	1681	121	0	0
29	L	2304	16	526	2126	2236	25	0	0
30	L	*	121	788	2621	9604	36	0	0
31	L	*	121	289	6724	7569	169	0	36
32	L	3481	324	721	1849	6369	961	0	0
33	L	3844	576	441	5625	5025	0	0	9
34	L	1024	25	9	324	1849	64	0	0
35	L	2401	1089	1936	6241	9604	784	0	0
36	H	1315	*	196	1160	5677	226	0	0
37	H	4615	498	0	1881	1980	1681	0	0
38	H	2126	*	652	5476	6170	*	0	0
39	H	3751	516	1849	2415	8624	267	0	0
40	H	2450	*	1528	*	*	144	0	0
41	H	961	169	81	1296	2209	121	0	0
42	H	5041	1521	*	*	9801	*	0	0
43	H	3136	256	625	*	5744	*	0	0
44	H	1849	256	169	3136	2601	1220	0	0
45	H	1369	1225	256	676	2209	*	0	0
46	H	4672	1126	961	547	8624	226	0	0
47	H	1681	441	*	*	2601	256	0	0
48	H	4225	660	1644	6780	6220	1826	0	0
49	H	3760	729	144	3249	6328	1367	0	0
50	H	1024	100	961	2116	3476	144	0	25
51	H	1681	121	1225	3776	5404	529	0	0
52	H	2704	484	*	4489	7604	*	0	0
53	H	3592	1296	867	2787	4761	*	0	0

*background staining prevented accurate cell count.

Table 6.3 Semi-quantitative analysis of MHC class II and ICAM-1 expression by cervical keratinocytes

		Histology		
		Normal	**Low-grade**	**High-grade**
Antigens	Values	(n= 12)	(n= 23)	(n= 18)
	n+	0	7	11
HLA-DR	Median	0	0	3.5
	Range		0-4	0-5
	n+	0	0	2
HLA-DQ	Median	0	0	0
	Range			0-3
	n+	0	4	9
ICAM-1	Median	0	0	1.0
	Range		0-3	0-4

A previously described grading scale was employed (Coleman and Stanley 1994). Normal, normal epithelium; Low-grade, koilocytosis and/or CIN1; High-grade, CIN2 and/or CIN3; n, number of cases and n+, number of positive cases; Median, median score from all cases assessed; Range, range of scores.

	p values Wilcoxon rank-sum test		
	normal/low-g	normal/high-g	low/high-g
HLA-DR	0.2	0.008	0.02
HLA-DQ	0.9	0.9	0.7
ICAM-1	0.6	0.03	0.08

Table 6.4 Immunohistochemical analysis of cytokine expression in normal squamous epithelium and premalignant cervical lesions according to the epithelial level involved

		IL-10	**TNF-α**
Histology	Epithelial level	n+	n+
Normal	basal	0	6
(n= 12)	basal+parab	0	6
	basal	6	11
Low-grade	basal+parab	3	5
(n= 23)	full-thickness	3	4
	basal	0	1
High-grade	basal+parab	1	2
(n= 18)	full-thickness	7	9

Normal, normal epithelium; Low-grade, koilocytosis and/or CIN1; High-grade, CIN2 and/or CIN3; n, number of cases and n+, number of positive cases.

Table 6.5 Median number of cells (per mm^2) expressing MHC class II antigens and costimulatory molecules in cervical subepithelial stroma

Histology	**HLA-DR**	**HLA-DQ**	**ICAM-1**	**ICAM-3**	**LFA-1**	**LFA-3**
Normal	306.5 (0-625)	0 (0-164)	89.5 (0-324)	137 (36-1225)	841.5 (169-2601)	12 (0-36)
Low-grade	2180 (121-4110)	205 (0-1225)	526 (0-2958)	2102.5 (81-6724)	4154.5 (434-9604)	121 (0-961)
High-grade	2577 (961-5041)	498 (100-1521)	652 (0-1849)	2601 (547-6780)	5677 (1980-9801)	261.5 (121-1826)

Results are expressed as numbers of cells per mm^2 of sectional area. Median values are given, with the range in parentheses. Medians were calculated using all the data shown in Table 6.2. Normal, normal epithelium; Low-grade, koilocytosis and/or CIN1; High-grade, CIN2 and/or CIN3.

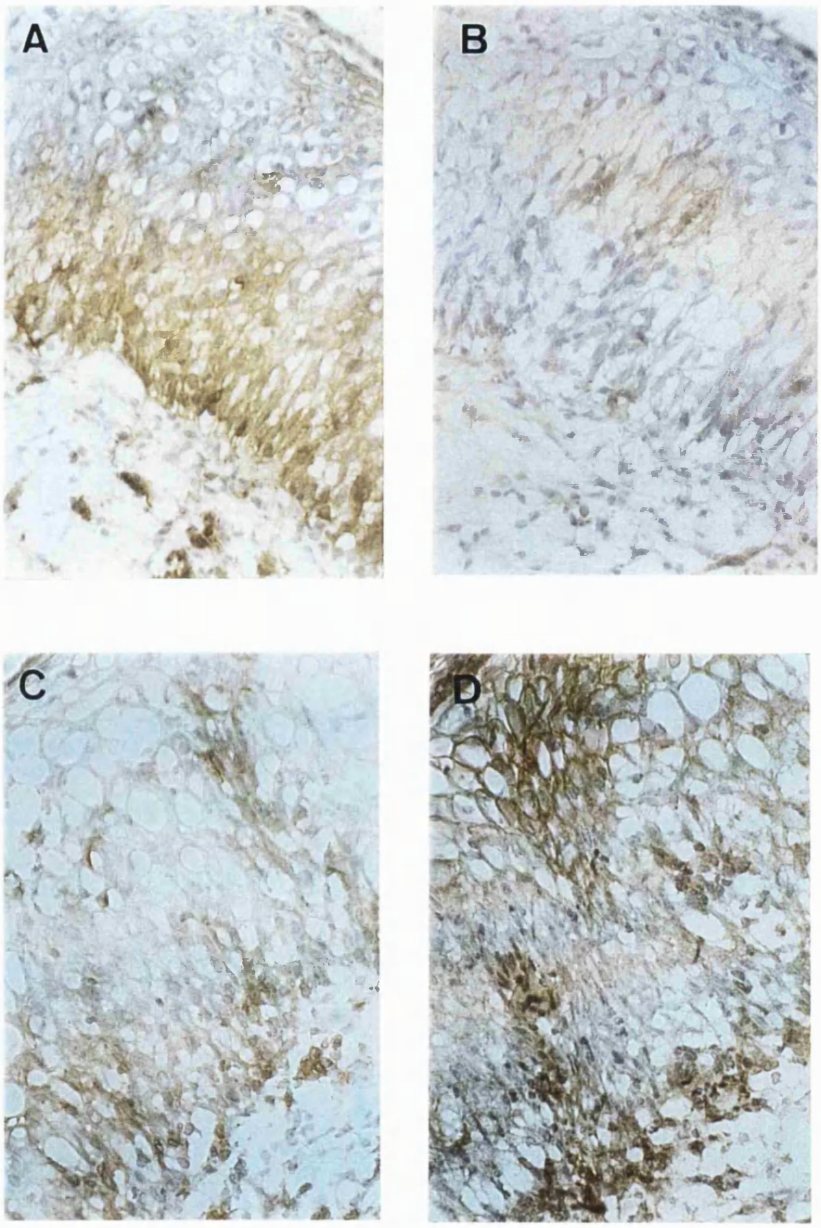
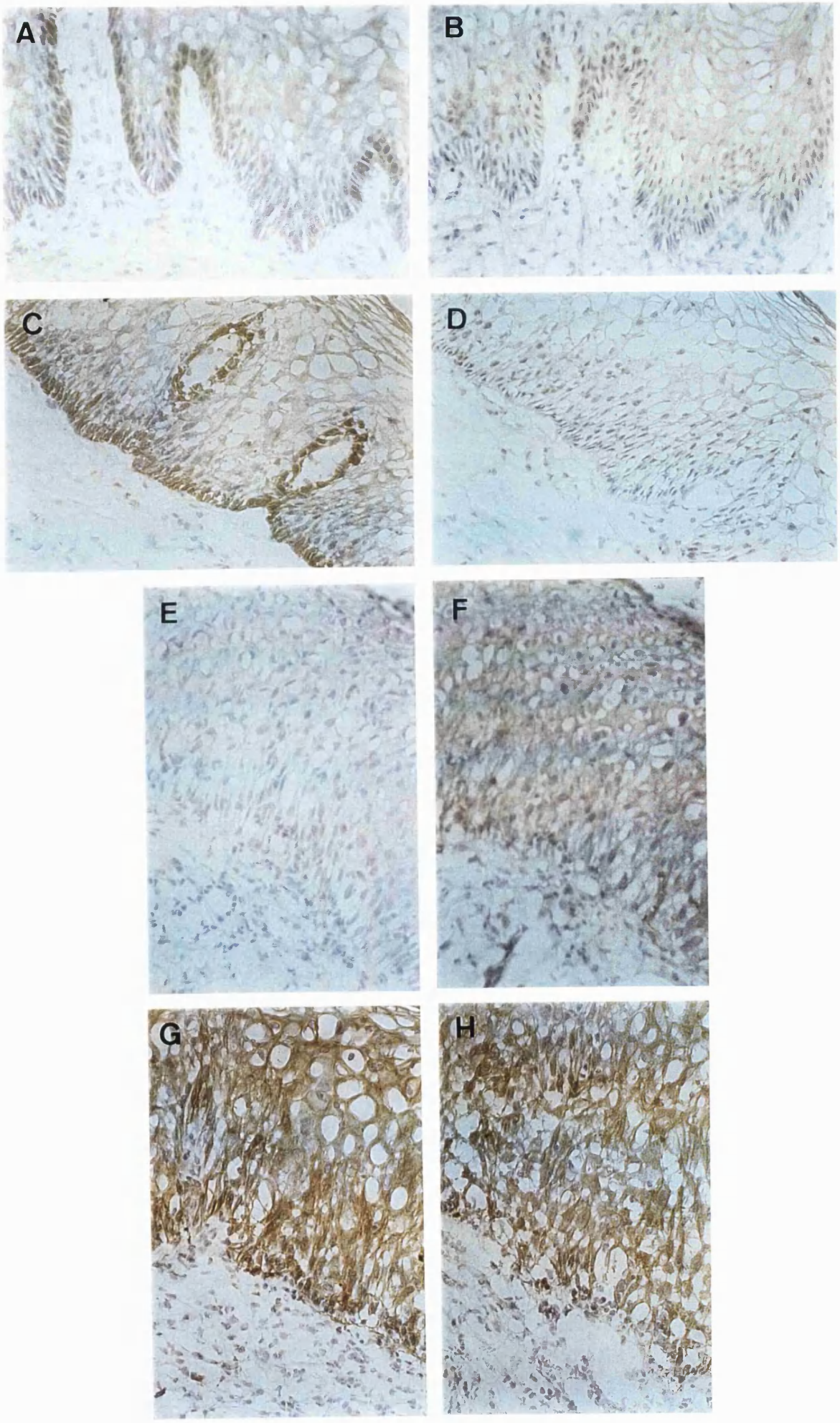


Figure 6.1 Expression of HLA-DR (L243) (a,c) and ICAM-1 (6.5B5) (b,d) by keratinocytes in serial cryostat sections of two low-grade intraepithelial squamous lesions of the cervix. Samples were counterstained with Mayer's Hematoxylin. Magnification, x40.

Figure 6.2 Immunohistochemical analysis of TNF-α (left panel) and IL-10 expression (right panel) in consecutive frozen sections of cervical squamous epithelium. (a,b) Normal ectocervix. Only basal epithelial cells are positively stained for TNF-α, and no IL-10 protein is observed. (c to h) Low-grade lesions. Whereas TNF-α expression was undetectable (as exemplified in e), IL-10 was upregulated in some CIN lesions (f and h). In (g and h) the full-thickness of the dysplastic epithelium shows strong cytoplasmic staining for both cytokines. Samples were counterstained with Mayer's Hematoxylin. Magnification, x40.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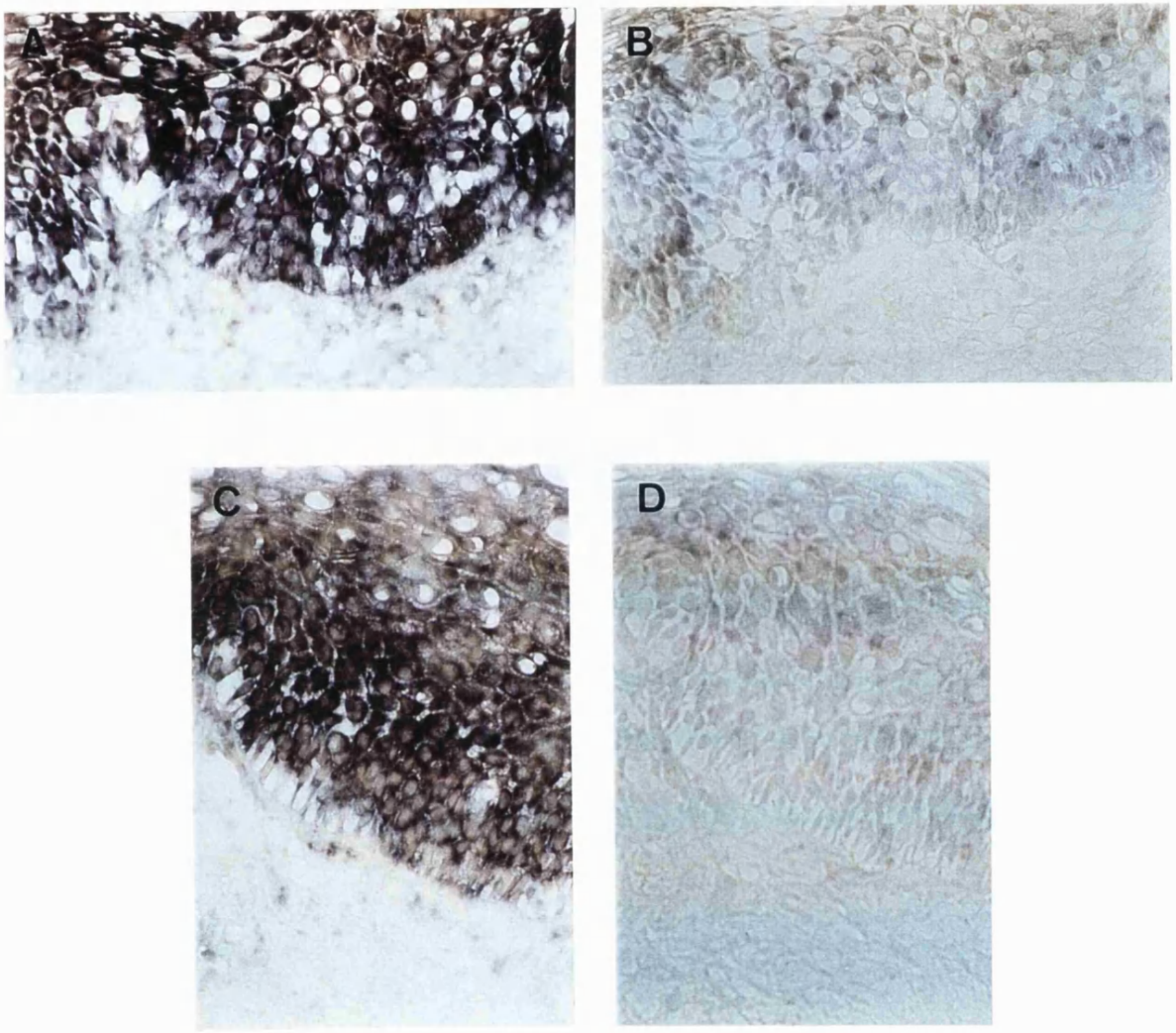
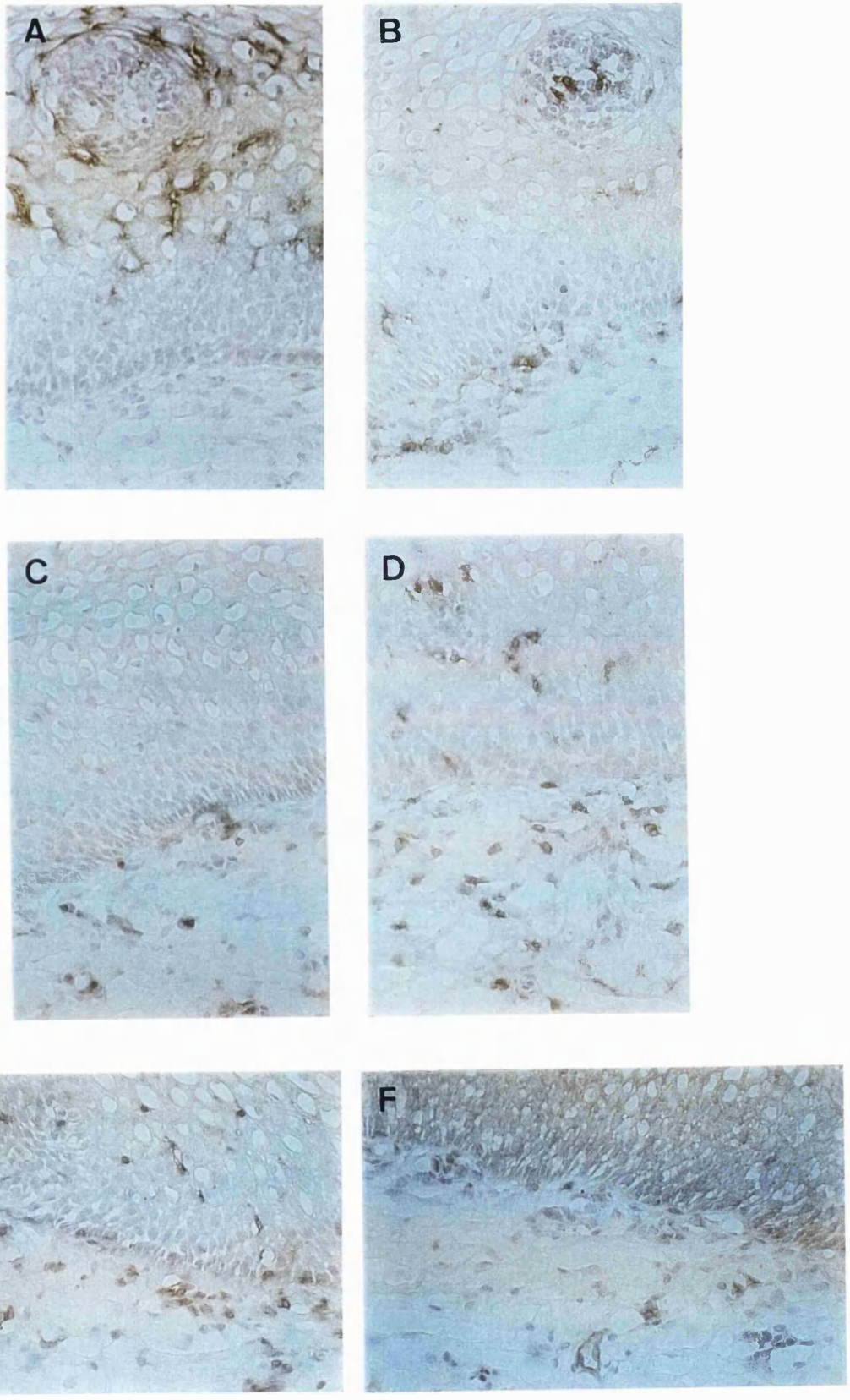


Figure 6.3 Representative examples of IL-10 mRNA expression in a low-grade (a and b), and TNF-α mRNA expression in a high-grade (CIN2) (c and d) intraepithelial squamous lesions of the cervix, detected by *in situ* hybridization. (a) Antisense probe recognizing IL-10 transcripts in the full-thickness of the epithelium. (c) The lower half of the epithelium is displaying TNF-α mRNA. (b and d) Sense, control probes showing almost absent background levels of signal. Magnification, x40.

Figure 6.4 Langerhans cell staining for a specific marker, CD1a (a) and infiltrating immune cells positive for HLA-DQ (Ia3) (b), ICAM-1 (B-H19) (c), LFA-1 (AZN-L20) (d), ICAM-3 (KS128) (e), and LFA-3 (AICD58.9) (f) in serial cryostat sections of a low-grade intraepithelial squamous lesion of the cervix. Note that Langerhans cells do not stain for adhesion molecules. Intra and subepithelial immune cells predominantly express LFA-1 and ICAM-3. In (f) keratinocytes are positively stained for LFA-3. Samples were counterstained with Mayer's Hematoxylin. Magnification, x40.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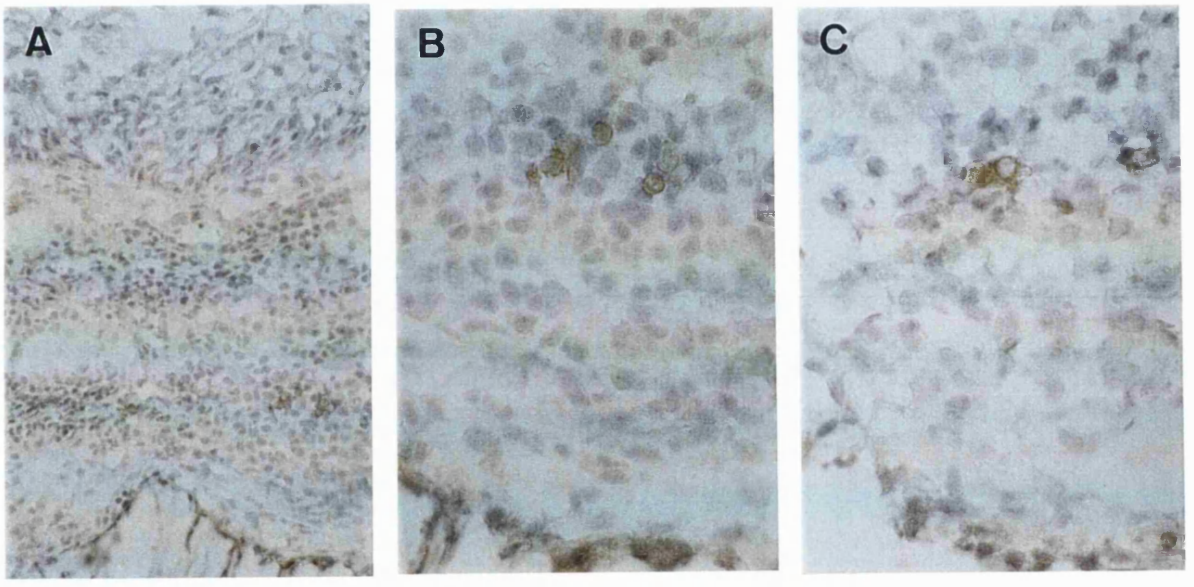


Figure 6.5 Stromal CD1a+ dendritic cells (a and b) expressing ICAM-1 (6.5B5) (c) in a CIN lesion. Samples were counterstained with Mayer's Hematoxylin. Magnification: a, x25; b and c, x100.

6.3 Discussion

It is well established that keratinocytes have the potential to interact with CD4 T cells, since functional MHC class II molecules (HLA-DR and HLA-DQ) can be induced on their surface (Nickoloff *et al* 1993a). However, in order to activate a productive T cell response, keratinocytes must express not only class II but also sufficent cell-membrane adhesion/costimulatory molecules. As in previous studies of CIN lesions, we find upregulation of HLA-DR expression by keratinocytes which parallels the severity of disease (Coleman and Stanley 1994). We also document a similar upregulation in the expression of ICAM-1 (CD54) by the same epithelial cells. Furthermore, there was not only a correlation in the expression of these molecules, but also a co-localisation, as previously reported for genital warts (Viac *et al* 1992). This argues both for co-regulation of expression and for a cooperative functional role for these two molecules in the immune response against viral or tumor-associated antigens in CIN lesions. This would allow keratinocytes to have a limited APC role, as has been demonstrated for human herpetic lesions (Cunningham and Noble 1989). Since expression of HLA-DQ by keratinocytes in CIN lesions was only detected in 2 out of 41 samples, and this expression was patchy and basal, it seems probable that in premalignant cervical epithelium HLA-DR is the principal restriction determinant for T cell activation by keratinocytes.

In addition to disease-related expression of ICAM-1, keratinocytes also expressed a second costimulatory molecule, LFA-3 (CD58). LFA-3 was constitutively expressed by the basal cells in normal squamous epithelium, and in HPV-associated disease its expression was extended to the parabasal layer and/or the full-thickness of the epithelium, and was related to the severity of disease. The expression of accessory molecules by keratinocytes was, however, limited. In contrast to ICAM-1 and LFA-3, the adhesion molecules LFA-1 (CD11a/18), ICAM-3 (CD50), and the costimulatoty ligand B7-2 (CD86) were never expressed by keratinocytes, either in normal cervix or CIN lesions. In these lesions, therefore, there is acquisition of a "partial" or incomplete APC phenotype by cervical keratinocytes. *In vitro* studies have shown that the response induced by keratinocytes acting as APCs can result in antigen-specific activation (Cunningham and Noble 1989) or antigen-specific tolerance (Bal *et al* 1990), and this

could be a consequence of the nature of the costimulatory pathway utilized and/or the cytokines encountered by T cells. The precise functional consequences of the specific phenotype documented in this study in terms of T cell activation and disease outcome remain to be determined. It is possible that the presence of ICAM-1 and LFA-3 may be sufficient for stimulation of memory T cells recruited to the local inflammatory site but not for activation of naive T cells (Bierer *et al* 1988). ICAM-1/LFA-1 interactions contribute to cutaneous leukocyte trafficking and keratinocyte/leukocyte adhesion, and thus could facilitate infiltration by activated T cells and other immune cells leading to lysis of infected/transformed keratinocytes (Kupper 1989). Additionally, a selective stimulation of CD8 rather than CD4 T cells may result.

The presence of increased numbers of both intraepithelial and stroma subepithelial immune cells staining for MHC class II and adhesion molecules in high-grade disease compared to normal squamous epithelium suggests that HPV-related cervical disease, and especially high-grade lesions, are sites of immunologic activation. LFA-1 which has been shown to be present at high levels in activated B and T lymphocytes, monocytes and macrophages (Springer 1990) was the predominant adhesion molecule found on these infiltrating cells. ICAM-3 proposed to be constitutively expressed by resting lymphocytes and monocytes (de Fougerolles and Springer 1992), was present at similar frequency to HLA-DR on stromal immune cells.

Langerhans cells are known to express CD1a (a specific marker commonly used for their identification), HLA-DR and -DQ antigens. Our results show that in normal and premalignant cervical squamous epithelium Langerhans cells *in situ* do not express or express extremely low levels (undetectable by immunohistochemistry) of ICAM-1 and -3, LFA-1 and -3 and B7-2. Viac *et al* 1992, have also not found expression of ICAM-1 by Langerhans cells in genital warts. In contrast, human epidermal (cutaneous) Langerhans cells have been shown to express high levels of ICAM-3 (Griffiths *et al* 1995), but not the other adhesion molecules (Teunissen *et al* 1994). However, subepithelial CD1a positive cells, known as dermal dendritic cells do express all the above mentioned adhesion molecules (Lenz *et al* 1993), as we have observed in 5 out of 41 CIN lesions. This supports the widely accepted view that Langerhans cells *in situ* (immature "tissue dendritic cells") capture antigens and are very efficient in their

processing but relatively poor presenters and only upregulate the expression of adhesion molecules when leaving the epithelia in order to present the peptidic antigens to T cells in the local draining lymph nodes (Lenz *et al* 1993, Steinman *et al* 1995). It has been shown that when Langerhans cells leave the epithelia and become activated (mature "lymphoid dendritic cells") they downregulate the expression of CD1a (Lenz *et al* 1993), which may explain the failure to detect dermal dendritic cells in tissue sections. We have also obtained similar results in cervical epithelia, since in all 53 biopsies studied CD1a positive cells were detected in the epithelium in comparison to only 6 samples where CD1a positive cells were identified in the stroma of the same tissues.

The expression of both TNF-α and IL-10 protein and message in normal and premalignant cervical epithelium has not been reported before. We have shown that TNF-α is constitutively produced by the basal keratinocytes of normal cervical squamous epithelium, in contrast to IL-10 where expression was not observed. Differences in expression of both cytokines by cervical keratinocytes in comparison with epidermal ones are apparent. In normal human epidermis (keratinized squamous epithelium, in contrast to nonkeratinizing cervical squamous epithelium) TNF-α protein is localized to the upper level keratinocytes (Kolde *et al* 1992). Immunohistochemically, IL-10 has also been identified throughout all levels of epidermis but with accentuation in upper level keratinocytes (Nickoloff *et al* 1994a). In CIN lesions, the absence of correlation between the expression of TNF-α and IL-10 observed argues in favor of an independent regulation of these cytokines. In these lesions, whereas the expression of TNF-α by cervical keratinocytes was downregulated especially in high-grade disease, IL-10 was upregulated in some samples. The differential expression between a pro-inflammatory cytokine like TNF-α and an anti-inflammatory one (IL-10) in CIN lesions might have consequences in the modulation of the local immune response and ultimately in the outcome of HPV-related premalignant cervical disease. The biologic properties of TNF-α and IL-10 have been extensively characterized. It can be hypothesized that absent or decreased TNF-α production by cervical keratinocytes could facilitate persistent HPV infection, namely by deficient control of viral replication, and precluding antiviral resistance to uninfected neighboring keratinocytes. In addition or alternatively, the presence/predominance of IL-10 in the local microenvironment during T cell maturation

and differentiation could induce an inadequate effector immune response (i.e. Th2) against viral and/or tumor antigens.

The overall conclusion from this study is that premalignant cervical lesions are sites of partial, and selective immune activation. The upregulation in HLA-DR and ICAM-1, in the presence of constitutive levels of TNF-α and LFA-3 is suggestive of the initiation of both inflammatory and specific immune responses within the cervical microenvironment. However, the subsequent downregulation of TNF-α and/or production of IL-10 by the epithelial cells, and the complete absence of ICAM-3 and B7-2 expression, could contribute to persistence of infection, and hence, cellular transformation in some HPV-related cervical lesions.

6.4 Abstract

It is generally assumed that the immune response within the cervical microenvironment is an important variable in the natural history of cervical intraepithelial neoplasia (CIN) but the pattern of this response, and how it is regulated has not been documented in detail. Therefore, in this study, expression of MHC class II (HLA-DR and -DQ), costimulatory/adhesion molecules (ICAM-1, ICAM-3, LFA-1, LFA-3 and B7-2) and cytokines (TNF-α and IL-10) was investigated by immunohistochemistry in normal squamous epithelium (n=12), low-grade (n=23) and high-grade (n=18) intraepithelial squamous lesions of the cervix. There was a strong correlation between keratinocyte expression of HLA-DR and ICAM-1. LFA-3 and TNF-α were expressed constitutively by normal cervical keratinocytes, but ICAM-3, B7-2 and IL-10 were not detectable. In some CIN lesions, keratinocyte expression of TNF-α was decreased and IL-10 protein was detected. Expression of TNF-α and IL-10 was confirmed by mRNA analysis as assessed by *in situ* hybridization. Increased numbers of mucosal infiltrating immune cells were observed in high-grade disease. *In situ* epithelial CD1a+ cells (Langerhans cells) do not express ICAM-1, ICAM-3, LFA-1, LFA-3 and B7-2, but upregulate the expression of all these molecules when present in the stroma in CIN lesions. The restricted expression of costimulation/adhesion molecules and the nature of the cytokine microenvironment within the epithelium may act to limit effective immune responses in some CIN lesions.

CHAPTER 7

Characterization of soluble factors from cultures of premalignant cervical epithelium

7.1 Introduction

There is a strong body of evidence implicating human papillomaviruses (HPVs) as aetiological agents in the pathogenesis of cervical cancer and its precursor lesions, i.e. cervical intraepithelial neoplasia (CIN) (zur Hausen 1991, Bergeron *et al* 1992). There is also convincing data suggesting that cell-mediated immunosurveillance mechanisms may be of critical importance in the control of HPV-related disease (Fukuda *et al* 1993, Petry *et al* 1994). Thus, local cellular immune responses within the cervix are most likely the major factors in determining the clinical outcome following HPV infection. It has previously been reported that there are changes in the populations of immune cells within the cervix in HPV-related disease. A depletion of Langerhans cells, professional antigen-presenting cells (APCs) of the epithelia, is a well documented feature (Tay *et al* 1987c). T lymphocytes are the predominant immune cells in CIN lesions, with small increases in the number of macrophages and natural killer (NK) cells in these lesions (Tay *et al* 1987d, Viac *et al* 1990).

The local cytokine microenvironment in which T cells undergo stimulation is thought to drive their differentiation and, hence, influence their effector functions with either activation, unresponsiveness or anergy, which ultimately have fundamental consequences in terms of disease outcome. It has been reported that epidermal (cutaneous) keratinocytes can produce interleukin 1α (IL-1α) (Kupper 1988), IL-10 (Enk and Katz 1992), tumour necrosis factor-α (TNF-α) (Kolde *et al* 1992) but not IL-12 (Goodman *et al* 1994). We have also demonstrated that TNF-α is constitutively produced by the basal cells of normal cervical squamous epithelium and its synthesis is lost in some CIN lesions. In contrast, whereas IL-10 protein is not detected in normal squamous epithelium, keratinocytes in many CIN lesions do express IL-10 (chapter 6). Cytokines produced by keratinocytes are critical components of the host response to

infection. IL-1α is a multifunctional cytokine: it enhances the production of other pro-inflammatory cytokines, induces the expression of IL-2 receptors in activated T cells, is chemotatic for lymphocytes, activates macrophages, and induces endothelial cells to express adhesion molecules (Dinarello 1989, Luger and Schwarz 1990). Another pro-inflammatory cytokine, TNF-α activates and recruits immune cells, enhances production of cell-surface adhesion molecules and histocompatibility antigens class I and II, and plays a role in the host resistance to viral infection and tumour growth (Sugarman *et al* 1985, Wong and Goeddel 1986, Beutler and Cerami 1989). IL-12 is considered important in directing CD4+ T cells to a T helper 1 (Th1) phenotype (Hsieh *et al* 1993), which mediate cellular immunity and, at least in some cases, are the major effectors in the host defence against viral infections and cellular transformation (Nagarkatty *et al* 1990). Finally, and in contrast, IL-10 is a cytokine which has immunosuppressive functions towards Th1 T cells (Fiorentino *et al* 1991), inhibits the antigen-presenting cell (APC) function of accessory cells (Ding and Shevach 1992), and may play a role in damping/terminating immune responses (Malefyt *et al* 1991).

Using *in vitro* cultures of premalignant cervical epithelium we have characterized the emigrated cell population from the explants. We have also quantified the concentration of cytokines (IL-1α, IL-10, IL-12 and TNF-α) present in the culture medium. Finally, we have used an *in vitro* proliferation assay (T cells as responding cells and autologous monocytes as stimulators) to analyse the influence of supernatants from cultured cervical epithelium on T cell proliferative responses. Furthermore, we correlated these T cell responses to the levels of cytokines (IL-1α, IL-10, IL-12 and TNF-α) present in the culture supernatants. Normal cervical squamous epithelium was used as a control. Three distinct patterns of T cell proliferative responses were observed in the presence of culture medium from cervical explants, but there did not appear to be any correlation between the concentrations of cytokines in the supernatants and the level of T cell proliferation observed.

7.2 Results

7.2.1 Cell populations which emigrated from the epithelium and subepithelial stroma of CIN lesions

When biopsies of premalignant cervical lesions were cultured for 24 hours, some cells emigrated into the culture medium. However, the FACScan analysis of emigrated immune cells from cervical mucosa was often inconclusive. This resulted from the fact that few immune cells had emigrated to the culture supernatant or contamination with cellular debris and erythrocytes prevented reliable phenotypic analysis. Contaminating keratinocytes were rarely observed under light microscopy in the collected culture supernatants. In a few experiments, 6 to 10% of this cell population stained positive for CD1a (Langerhans cells in the epithelium and dendritic cells in the stroma), while the majority of cells stained positively for CD3 (T lymphocytes), and few (less than 5%) B lymphocytes (CD19), macrophages (CD14), and natural killer (NK) cells (CD56) were present. Figure 7.1 shows a representative example, using FACS analysis for the phenotypic characterization of the emigrated cells. In Figure 7.2 two cell populations are identified in immunostained smear preparations.

7.2.2 Cytokines present in supernatants from cultures of cervical mucosa

Cytokines released from keratinocytes and/or produced *in situ* by the emigrated cell population from cervical explants (n=35) were quantified in culture supernatants (Table 7.1). Table 7.2 summarises the concentrations of IL-1α, IL-10, IL-12 and TNF-α detected by ELISA in cultures of normal, premalignant squamous epithelium, and in two squamous cell carcinomas of the cervix. There was a decrease in the concentration of IL-1α comparing normal squamous epithelium to CIN lesions, but this did not reach statistical significance. The cytokine IL-12 was detectable in 15 out of 35 explant cultures of cervical mucosa. The majority of cultures from cervical mucosa (normal, premalignant epithelium and cervical carcinoma) had detectable levels of IL-1α and TNF-α. Fewer gave positive results for IL-10 (26 out of 35 samples) or IL-12 (15 out of 35). The most striking feature observed was the variability of cytokine concentration amongst samples, as shown in Figure 7.3.

7.2.3 Effects of supernatants from cultures of cervical mucosa on T cell proliferation

We investigated the patterns of CD3-induced T cell proliferation in the presence of supernatants from cervical epithelia cultured for 24 hours. The first proliferative assays

were carried out using dendritic cells (DCs) (purified from peripheral blood mononuclear cells and cultured for 7 days with GM-CSF and IL-4) as accessory cells. Figure 7.4 shows one representative example of the proliferative patterns observed using both allogeneic and autologous DCs. The addition of three different supernatants from cultured CIN lesions resulted in a significant increase in the proliferation of T cells when allogeneic DCs were used. In contrast, no differences in T cell proliferation were observed using the same supernatants in the autologous DC assay, probably because maximal proliferation of T cells was induced under these conditions.

To test whether the effects observed were a characteristic of using DCs as APCs, we tested the same supernatants with purified monocytes (Mo), instead of DCs, as the accessory cells. Figure 7.5 shows the results of one such experiment. As expected, DCs were much more potent stimulators of T cells than Mo, as indicated by the total thymidine incorporation. However, the proliferative patterns seen in the presence of the supernatants were similar to those observed with allogeneic DCs (shown in Figure 7.4a). To avoid the use of cytokines (GM-CSF and IL-4) for DC maturation and maximal stimulation of T cells, autologous Mo were used as accessory cells in all the subsequent proliferation assays. In total, six independent experiments of this type were carried out, testing a total of 38 supernatants from cervical epithelium explants (Table 7.1 and Figure 7.6). Supernatants were tested once only because of insufficient material. In each experiment, supernatants collected from a culture set up in an identical manner, but without any cervical sample, was tested as a control and did not alter the level of T cell proliferation observed. We also assessed the effects of supernatants derived from cultures of normal squamous epithelium which consistently gave T cell proliferative responses not significantly different from the positive control (T cells and Mo in the absence of culture supernatant).

Three different patterns of T cell proliferation were observed in the presence of supernatants from cultured neoplastic cervical epithelium (n=38). Most frequently the T cell proliferation was similar to the positive control (22 out of 38, 57.9%). In many instances there was a statistically significant decrease in T cell proliferation (11 cases, 28.9%). Finally, in a few cases an increased proliferation of T cells was observed (5 cases, 13.2%).

As shown above, many of the explant supernatants were found to contain measurable levels of the cytokines IL-1α, IL-10, IL-12 and TNF-α. However, comparison of the level of each cytokine with the T cell proliferation did not show any significant correlation between the individual cytokine and the functional effects observed (Spearman test). Since IL-10 is recognised as being suppressive of T cell responses, the role of this cytokine in the downregulation of proliferation caused by some supernatants was tested directly by the addition of a neutralising anti-human IL-10 MAb added at the beginning of the cocultures in three independent experiments. Figure 7.7 is a typical example showing that anti-IL-10 MAb has no significant effect on T cell proliferation.

To exclude the hypothesis that the supernatants were cytotoxic, we measured cell viability in the cocultures, both at 24 and 48 hours by trypan blue exclusion. Cell death was less than 5% in each case.

Table 7.1 Cytokine concentration (pg/ml) present in explant cultures of cervical mucosa and T cell proliferation in the corresponding samples

Sample	Hist	IL-1α	IL-10	IL-12	TNF-α	T cell Prolif
1	N	ND	ND	ND	ND	0.77
2	N	112.8	ND	ND	0.4	0.87
3	N	175.8	ND	ND	ND	1.08
4	N	48.2	32.4	72.4	3.6	0.91
5	N	204.0	26.9	ND	3.6	1.1
6	N	2230.8	ND	54.6	3.1	0.8
7	CIN	ND	104.1	55.3	0.8	0.08 *
8	CIN	36.2	ND	534.2	ND	0.9
9	CIN	153.4	47.5	ND	0.4	1.35 *
10	CIN	41.3	32.7	52.7	2.0	1.53 *
11	CIN	28	57.6	ND	26.9	0.94
12	CIN	31.2	25.6	ND	0.4	0.9
13	CIN	72.9	ND	ND	0.7	0.86
14	CIN	34.8	ND	ND	0.4	1.07
15	CIN	178.1	24.8	ND	5.9	0.09 *
16	CIN	93.7	20.8	ND	0.4	0.3 *
17	CIN	33.9	ND	ND	ND	1.07
18	CIN	36.3	ND	ND	0.4	0.16 *
19	CIN	38.5	20.8	ND	0.4	0.27 *
20	CIN	37.4	26.9	ND	2.5	0.81
21	CIN	39.2	30.1	66.6	2.9	0.73
22	CIN	32.7	27.4	ND	1.8	0.76
23	CIN	26.9	40.7	62.5	2.5	0.44 *
24	CIN	29.5	26.6	ND	2.3	1.07
25	CIN	123.9	32.8	70.8	ND	1.16
26	CIN	69.4	27.7	60.4	3.1	0.92
27	CIN	43.2	27.0	56.5	2.8	1.04
28	CIN	34.2	31.0	ND	2.4	0.48
29	CIN	1564.1	27.4	53.7	1.4	0.6
30	CIN	216.8	29.5	57.5	2.4	0.35 *
31	CIN	ND	29.0	56.5	ND	0.94
32	CIN	35.7	30.3	54.9	2.3	1.16
33	CIN	45.5	29.8	56.6	4.3	0.85
34	Cancer	34.3	44.6	ND	1.3	0.09 *
35	Cancer	27.6	27.1	ND	2.4	0.21 *

Hist, histology; Normal, normal squamous epithelium; CIN, cervical intraepithelial neoplasia; Cancer, squamous cell carcinoma of the cervix; ND, not detected; *, significantly different from positive control. T cell proliferation is presented as the ratio of proliferation between the test sample versus the positive control. A further 9 CIN lesions were tested for T cell proliferation only, and gave ratios of 1.92*, 1.60*, 1.36*, 0.80, 1.03, 0.38*, 0.49*, 0.92 and 0.83.

Table 7.2 Concentration of cytokines present in cultures of cervical epithelia harvested after 24 hours

	IL-1α	IL-10	IL-12	TNF-α
Normal	554.3±939.1	29.6±3.9	63.5±12.6	2.7±1.5
n = 6	(48.2-2230.8)	(26.8-32.4)	(54.6-72.4)	(0.4-3.6)
	(n+ = 5)	(n+ = 2)	(n+ = 2)	(n+ = 4)
CIN	123.1±304.6	34.1±17.7	95.2±132.0	3.02±5.4
n = 27	(26.9-1564.1)	(20.8-104.1)	(52.7-534.2)	(0.4-26.9)
	(n+ = 25)	(n+ = 22)	(n+ = 13)	(n+ = 23)
Cancer	30.9±4.8	35.8±12.4		1.8±0.8
n = 2	(27.6-34.3)	(27.1-44.6)		(1.3-2.4)
	(n+ = 2)	(n+ = 2)	(n+ = 0)	(n+ = 2)

Results (pg/ml) are expressed as mean ± SD of the positive samples (n+); n, total number; range is given in parenthesis; Normal, normal squamous epithelium; CIN, cervical intraepithelial neoplasia; Cancer, squamous cell carcinoma of the cervix.

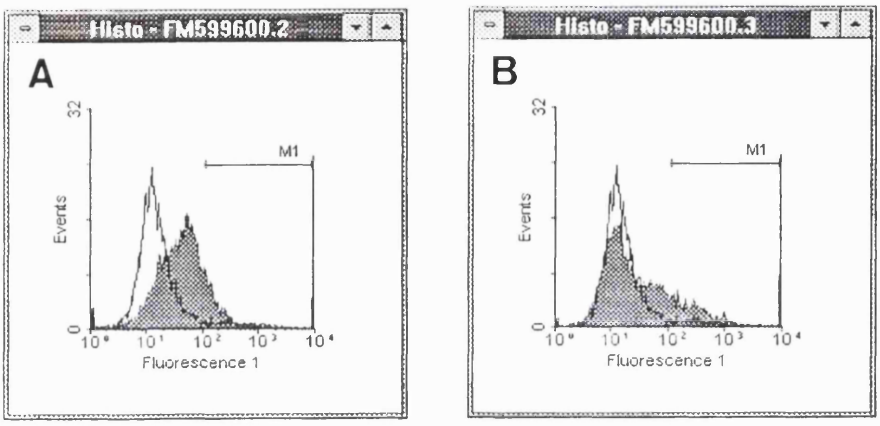


Figure 7.1 FACScan analysis of CD3 (a) and CD1a (b) expression on mucosal migrant cells from explant cultures of CIN lesions. Control (light area).

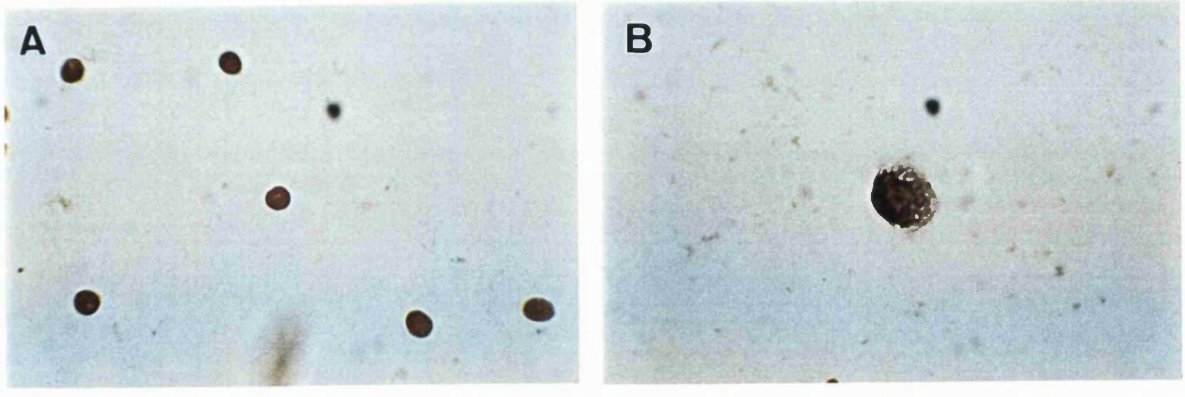


Figure 7.2 Immunocytochemical analysis of smears from explant cultures of CIN lesions showing mucosal CD3+ T cells (a) and CD1a+ dendritic cells (b). Magnification, x100.

Figure 7.3 Cytokine levels in supernatants from explant cultures of premalignant cervical lesions

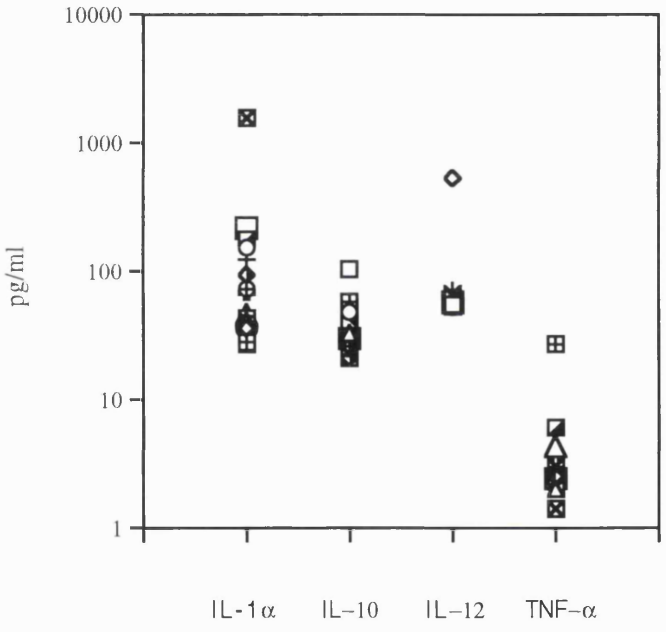
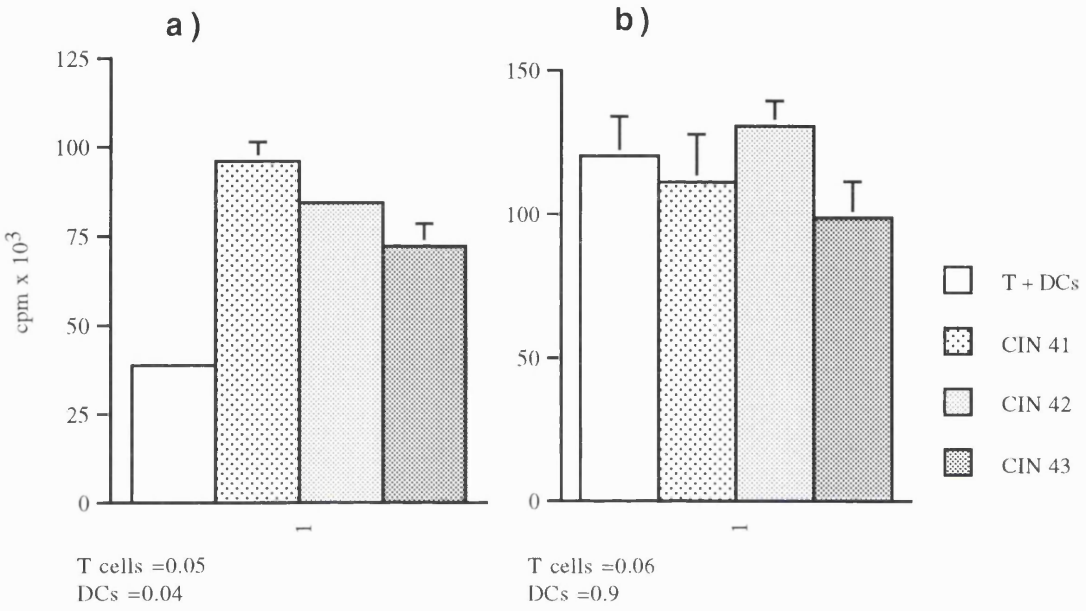
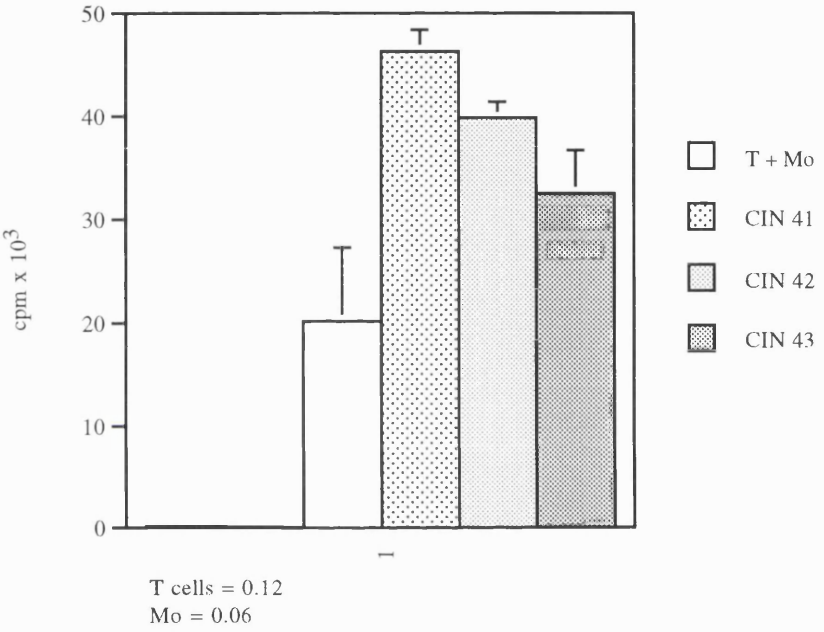


Figure 7.4 Proliferation of T cells in the presence of supernatants from mucosal explant cultures of CIN lesions using allogeneic (a) or autologous (b) dendritic cells as accessory cells



T cells (10^5 cells/well) were cultured with purified DCs ($3x10^4$ cells/well) for 2 days with anti-CD3 MAb (autologous) or 5 days (allogeneic). T cell proliferation was quantified by ^3H thymidine incorporation during the last 18h of culture. Results are shown as the mean cpm ± SD of triplicate cultures. The mean cpm of T cells and DCs alone are given.

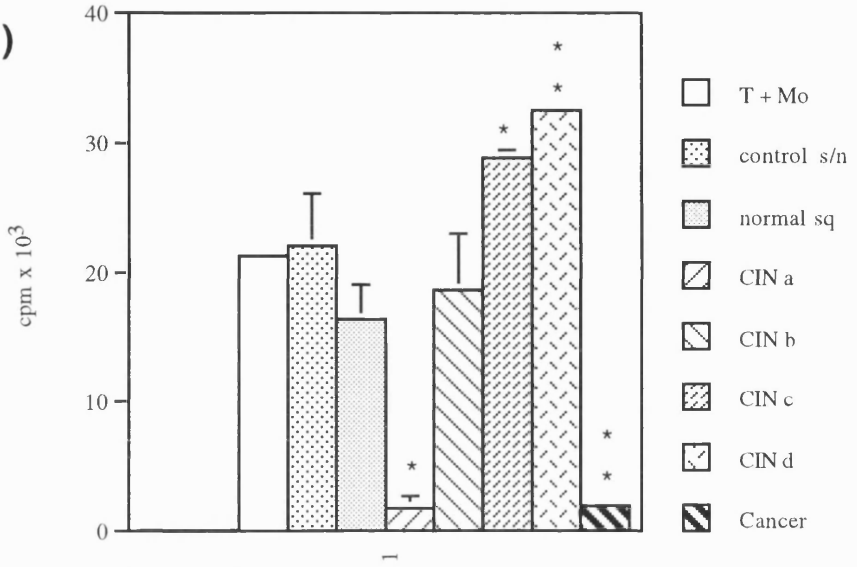
Figure 7.5 Proliferation of T cells in the presence of supernatants from mucosal explant cultures of CIN lesions using autologous monocytes as accessory cells



T cells (10^5 cells/well) were cultured for 48h with purified autologous Mo ($3x10^4$ cells/well) and anti-CD3 MAb. T cell proliferation was quantified by ^3H thymidine incorporation during the last 18h of culture. Results are shown as the mean cpm ± SD of triplicate cultures. The mean cpm of T cells or Mo alone are given.

Figure 7.6 Proliferation of T cells in the presence of supernatants from mucosal explant cultures of cervical epithelia using autologous monocytes as accessory cells (a), and cytokine concentration in the corresponding supernatants (b)

a)



T cells =0.052
Monocytes =0.042

Significant differences from control (T+Mo)
*, p<0.01; **, p<0.001 (Welch t test)

b)

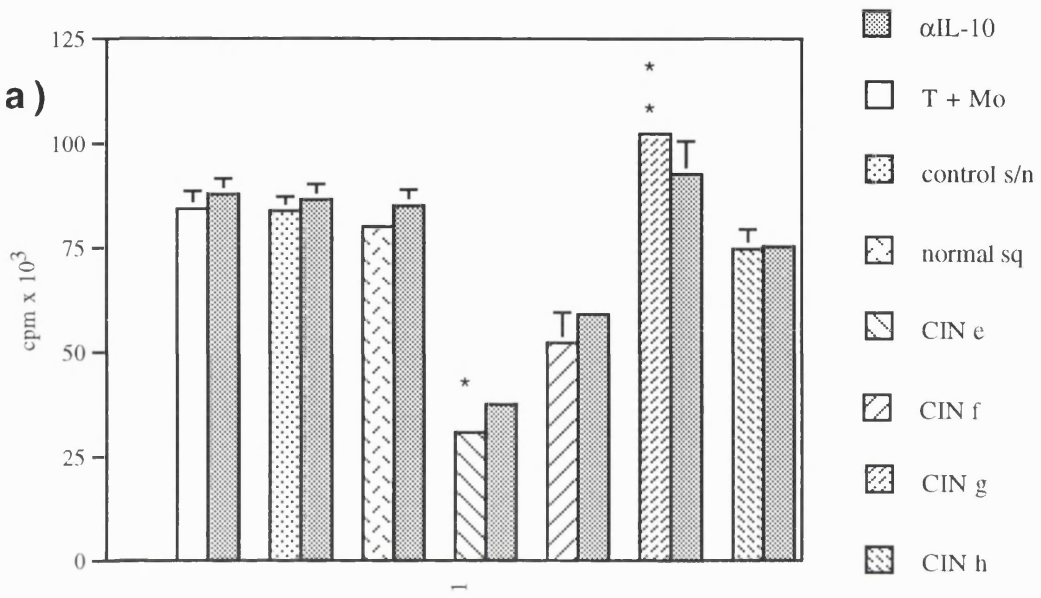
	IL-1α	IL-10	IL-12	TNF-α
Normal sq	ND	ND	ND	ND
CIN a	ND	104.1	55.3	0.8
CIN b	36.2	ND	534.2	ND
CIN c	153.4	47.5	ND	0.4
CIN d	41.3	32.7	52.7	2.0
Cancer	34.3	44.6	ND	1.3

Cytokine concentration (pg/ml); ND, not detected

T cells (10^5 cells/well) were cultured for 48h with purified autologous Mo ($3x10^4$ cells/well) and anti-CD3 MAb. T cell proliferation was quantified by ^3H thymidine incorporation during the last 18h of culture. Results are shown as the mean cpm ± SD of triplicate cultures. The mean cpm of T cells or Mo alone, and the level of significance between test samples and control are given.

Figure 7.7 Effect of anti-IL-10 on the proliferation of T cells in the presence of supernatants from mucosal explant cultures of cervical epithelia using autologous monocytes as accessory cells (a), and cytokine concentration in the corresponding supernatants (b)

a)



T cells =0.18
Monocytes =0.05

*, p =0.006; **, p =0.01 (Wech t test)

b)

	IL-1α	IL-10	IL-12	TNF-α
Normal sq	48.2	ND	72.4	3.6
CIN e	216.8	29.5	57.5	2.4
CIN f	1564.0	27.5	53.7	1.4
CIN g	35.7	30.3	54.9	2.3
CIN h	45.5	29.8	56.6	4.3

Cytokine concentration (pg/ml); ND, not detected

T cells (10^5 cells/well) were cultured for 48h with purified autologous Mo ($3x10^4$ cells/well) and anti-CD3 MAb. T cell proliferation was quantified by ^3H thymidine incorporation during the last 18h of culture. Results are shown as the mean cpm ± SD of triplicate cultures. The mean cpm of T cells or Mo alone, and the level of significance between test samples and control are given.

7.3 Discussion

In the work described in this chapter, using a functional rather than histochemical approach, we have begun to analyse the immune microenvironment in HPV-related cervical lesions. The experimental system used was based on the culture of small biopsy samples on a raised metal "grid", thus replicating as close as possible the *in vivo* situation, where the epithelium lies at the air/liquid interface. There are no previously published studies using this system on cervical epithelium. Although samples were taken on the basis of colposcopic evidence of premalignant cervical disease, a more detailed definition of the grade of the lesion in the biopsy would have required histological examination at the end of the culture. However, when this was performed the histopathology was unreliable mainly due to tissue disruption. Furthermore, although histology was available in each case for adjacent tissue (for clinical purposes) the large amount of micro-heterogeneity within the cervix made this information less relevant.

In a first series of experiments, we attempted to define the population of cells which migrated out of the cultures during the first 24 hours. We have confirmed the results of previous work on skin explants (Pope *et al* 1995), showing that immune cells emigrate from exocervical mucosa into culture medium, both in normal and HPV-related cervical disease. Preliminary phenotyping suggested that the majority of emigrants were indeed haematopoietic, with a predominant T cell and a smaller Langerhans cell component, reflecting the major populations present within the cervical mucosa. However, cell yield was low, and in many cases we observed significant numbers of red blood cells, suggesting contamination from blood vessels within the biopsy. Because of the variability observed and the low cell numbers, which precluded any functional studies of the emigrated populations, this approach was not pursued in detail. Instead, the project focused on the analysis of soluble factors (cytokines) present in the culture medium.

The role of cytokines produced by antigen-presenting cells (APCs), such as Langerhans cells (potent professional APCs of the epithelia) and non-professional APCs (keratinocytes), is well established in the immunomodulation of T cell responses against infectious agents, leading either to T cell activation or induction of tolerance (Luger and Schwarz 1990, Norris 1990). Moreover, the cytokine pattern produced by activated T

cells, either Th1 or Th2, might also determine the course of disease. Finally, local cytokines acting isolated or in synergy (e.g. IFN-γ and TNF-α) are effector molecules in the resolution of viral infections by direct suppression of viral replication and induction of an antiviral state in uninfected neighbouring cells (Ramsay *et al* 1993). We have shown that pro-inflammatory cytokines (IL-1α, IL-12 and TNF-α), as well as the anti-inflammatory cytokine IL-10 are detectable, although generally at low concentrations, in the culture media from explants of CIN lesions and from two squamous cell carcinoma of the cervix. IL-12 is known not to be produced by epidermal keratinocytes (Goodman *et al* 1994), whereas all the other cytokines, IL-1α (Kupper 1988), IL-10 (Enk and Katz 1992) and TNF-α (Kolde *et al* 1992) can be synthesised by these epithelial cells. Since we have previously shown that in CIN lesions when IL-10 and TNF-α are present frequently the full-thickness of the epithelium is positively stained (chapter 6), the consistent very low concentration of these cytokines detected by ELISA, suggests that they are stored within the cervical keratinocytes and are not released (or are released at very low concentrations) into the culture medium. Cytokine release may be actively triggered during local inflammation. Alternatively, the cytokines detected by ELISA could be produced by the immune cells emigrated into the culture medium, which is supported by the detection of IL-12 in 15 out of 35 culture supernatants.

Analysing the effects of culture supernatants derived from explants of exocervical mucosa (36 CIN lesions and 2 carcinomas) on CD3-induced T cell proliferation using autologous monocytes as accessory cells, we have observed three patterns of proliferation. Frequently, the T cell proliferation was similar to the positive control (T cells and monocytes without test supernatant, and cultured medium in the absence of explant) in 22 out of 38, 57.9%, followed by a statistically significant decrease in T cell proliferation in 11 cases (28.9%). Finally, in 5 (13.2%) there was a significant increased proliferation. If these *in vitro* observations could be extrapolated to a clinical perspective they might reflect the natural history of premalignant cervical disease. The majority of HPV-related lesions will not progress to invasive disease, some lesions will persist and if additional carcinogenic cofactors come into play they will progress, and finally, some women, although infected by the virus, will never express clinically evident HPV-related cervical disease and will become HPV DNA negative in follow-up testings.

Furthermore, it is interesting to note that whereas the T cell proliferation was "normal"/unaltered (not significantly different from the positive controls) with supernatants from explants of normal squamous epithelia, there was a significantly reduced T cell proliferation with explants from invasive cancer. However, no statistically significant correlation was found between T cell proliferation and the concentration of cytokines (IL-1α, IL-10, IL-12 and TNF-α) in the culture supernatants. Since prostaglandins are known to inhibit T cell proliferation (Kumar and Das 1994), they could have been produced locally and released into the culture supernatants in those cases where decreased T cell proliferation was observed. Other soluble factors (e.g. TGF-β) present in the supernatants could be responsible for the T cell proliferative patterns observed. It can also be hypothesised that the balance amongst various cytokines (the studied ones and/or others) rather than individual variations in their concentration is important in determining the degree of T cell proliferation. Finally, our results suggest that in the local cervical microenvironment of HPV-related disease diverse immune responses are elicited, which may be capable of determining its course.

7.4 Abstract

We have analysed the phenotype of immune cells which emigrated out of organ cultures of premalignant cervical epithelia, by FACS and immunocytochemistry. T cells and CD1a+ dendritic cells predominated in these cultures. Cytokines IL-1α, IL-10, IL-12 and TNF-α were present in the culture supernatants at low levels as quantified by ELISA. Three patterns of T cell proliferation were observed when culture-derived supernatants were added to a CD3-dependent *in vitro* proliferation assay using autologous monocytes as accessory cells. No statistically significant correlation was found between T cell proliferation and the concentration of cytokines IL-1α, IL-10, IL-12 and TNF-α present in the corresponding culture supernatants. Our results suggest that several distinct immune responses can occur in the local cervical microenvironment in HPV-related disease, which may play a role in determining its clinical outcome.

CHAPTER 8

GENERAL DISCUSSION

We have shown that the use of mussel glycogen can significantly improve cellular DNA recovery from frozen biopsies for subsequent HPV DNA detection by PCR. This improved DNA recovery correlated with higher detection of oncogenic types of HPV, which is in agreement with one study showing that the proportion of HPV positive women increased with increasing amounts of cellular DNA in cytological samples (Guerrero *et al* 1992). However, both studies have shown that this relationship between sample DNA content and HPV positivity was not a straightforward linear correlation. We have also confirmed previous studies on Western women, documenting a very high prevalence of HPV16 infection particularly in high-grade squamous intraepithelial lesions of the cervix.

Since cervical cytology, so far the standard technique for screening cervical cancer precursor lesions, has high false negative rates (low sensitivity) as well as false positive ones (low specificity) of the order of 20% and up to 15% respectively (Koss 1993), HPV DNA testing has been proposed to be used to reduce these rates. Furthermore, combining routine cytology with HPV DNA testing could reduce costly and unnecessary colposcopic examinations, biopsies, and in many instances treatments.

One of the major contentions in cervical pathology relates to the clinical management of patients with "equivocal" or "inconclusive" atypical squamous cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesions (LSILs) on their cervical smears (Richart and Wright 1993). One school recommends colposcopy for all women under this category, the other advocates follow-up. Since some LSILs can progress to invasive disease and the type of HPV infection may strongly influence the clinical outcome of the lesion, HPV testing could identify women as being at high risk for either developing or having high-grade lesions. It has been shown that viral testing identified patients with LSILs who were at risk of progressing to high-grade SIL. In two follow-up studies, 25% of women with morphologically low-grade lesions but with the

oncogenic high-risk HPV16 and 18 progressed to CIN3 (Kataja *et al* 1990, Gaarenstroom *et al* 1994). Similarly, HPV testing would distinguish variants of physiologic epithelium, e.g. immature squamous metaplasia, from the large pool of cytologically minor lesions which will "spontaneously" regress, and finally from those epithelial abnormalities containing oncogenic HPV DNA. Only patients with ASCUS/LSILs smears who test positive for high-risk HPVs would be referred to colposcopy, whereas all others could be followed with cervical smear screenings. This is further supported by the finding that two negative HPV tests for oncogenic HPVs confer a negative predictive value of near 100% (Reid and Lorincz 1991, Cox *et al* 1995). Recently, the diagnostic value of viral testing for detecting high-grade cervical disease has been demonstrated (Wright *et al* 1995, Cox *et al* 1995). In cytological samples with ASCUS or LSILs, the sensitivity of viral testing to detect high-grade lesions was 85%, and when HPV DNA testing was used as an adjunct to cytology, detection rates of histologically high-grade lesions rose from 93% to 100%.

It has also been reported that the presence of a high viral load is indicative of neoplasia. Cytologically negative HPV DNA positive patients who have a low level of infection are at low risk of developing a significant lesion, whereas those with high levels of HPV DNA have a significant risk of developing high-grade lesions (Moscicki *et al* 1993, Cuzick *et al* 1994). Hence, it has been proposed that HPV testing for only high-risk HPV types and using quantitative measurements of HPV DNA levels would be the ideal approach to achieve high specificity and preserving sensitivity for high-grade lesions (Cox *et al* 1995). However, our proposed protocol increasing the detection rate of HPV DNA would improve the accuracy of diagnosis of HPV infections and HPV-related neoplastic lesions, avoiding false negative results especially in cases with low HPV copy numbers per cell. This would be relevant for research purposes answering questions concerning prevalence, incidence, transmission and natural history of HPV infections (e.g. factors associated with latency, persistence and progression of HPV-related CIN lesions). Furthermore, the minute quantities of target DNA in cervical smears may hamper a reliable HPV DNA analysis. The implications of the improved accuracy of diagnosis are that women requiring a closer follow-up can be identified and rescreening intervals could be considerably extended for those testing negative for oncogenic HPV types.

We have demonstrated that cathepsin E, an aspartic proteinase which has been reported to play a role in antigen processing for presentation by MHC class II molecules, is associated with cellular dedifferentiation in CIN lesions. Both protein and mRNA cathepsin E expression by cervical keratinocytes was shown to be correlated to the severity of premalignant cervical disease. Such expression seems to be independent of HPV infection, since no correlation was found between cathepsin E upregulation and the HPV16 status in CIN lesions. However, we cannot rule out that other less prevalent HPV types could regulate the expression of the enzyme. Furthermore, although there was no direct qualitative correlation between cathepsin E and HLA-DR expression by epithelial cells, in view of the role of the enzyme in processing in other sites, it is possible that cathepsin E mediated processing may be taking place in the keratinocyte. This view is consistent with the observation that in normal squamous cervical epithelium the undifferentiated stem-cells of the basal layer expressed the enzyme, whereas the majority of "moribund", HPV infected keratinocytes (koilocytes) did not.

We have confirmed previous work showing that in CIN lesions there is upregulation of HLA-DR expression by keratinocytes which is related to the severity of disease. This expression was significantly correlated to that of the accessory molecule ICAM-1 (CD54), both in terms of upregulation of the molecules in the same samples, as well as co-localization of their distribution. In contrast, whereas LFA-3 (CD58) was constitutively expressed by basal or basal and parabasal epithelial cells in normal squamous epithelium and this expression increased in parallel with the severity of the cervical intraepithelial lesion, LFA-1 (CD11a/18), ICAM-3 (CD50) and B7-2 (CD86) were never expressed by keratinocytes either in normal or premalignant cervical epithelium. Furthermore, we have observed in some CIN lesions, and particularly in high-grade disease, an intense infiltration of immune cells both in the subepithelial stroma, and more importantly within the epithelium. We have shown that these cell populations predominantly expressed LFA-1, ICAM-3 and HLA-DR molecules, which argues in favour of cell activation. These findings suggest that non-specific signals (production and release of cytokines by infected keratinocytes), but also induction of MHC class II molecules on keratinocytes, as well as antigen-specific T cell activation by emigrated Langerhans cells, could all contribute to the local recruitment of immune cells

to the site of infection/cellular transformation. Two previous studies have shown differences in the number of mucosal immune cells comparing persistent versus regressive HPV-related lesions. In regressing genital warts a significant increase in the number of both intra and subepithelial T lymphocytes and macrophages was shown (Coleman *et al* 1994b). In regressing CIN lesions a significant increase in subepithelial CD4+ T cells was documented (Fukuda *et al* 1993). Hence, it is probable that activated intraepithelial immune cells in CIN lesions, the majority of which are T cells (Viac *et al* 1990, McKenzie *et al* 1991), and as confirmed in our phenotypic analysis of cells that have emigrated from cervical mucosa, are destroying infected and/or transformed epithelial cells. With respect to antigen presentation, HLA-DR positive keratinocytes can invoke a secondary T cell response composed of CD4 and CD8 T cells (Nickoloff 1988, Kupper 1989, Nickoloff and Griffiths 1990), and thus cervical keratinocytes expressing MHC class II molecules, as well as the accessory molecules ICAM-1 and LFA-3 may act as proficient APCs for antigen-specific T cell activation, as demonstrated in cutaneous herpetic lesions (Cunningham and Noble 1989). Furthermore, significant upregulation of both HLA-DR and ICAM-1 by epithelial cells correlated with genital wart regression, in comparison to persistent lesions where expression of both molecules was not detected (Coleman *et al* 1994b). However, it is important to remember that antigen presentation by keratinocytes has been shown to differ from that mediated by professional APCs (Nickoloff and Turka 1994). The major differences are related to the nature of the cytokine environment and the costimulatory pathways provided for T cell activation. Thus, antigen presentation by cervical keratinocytes may be occuring, but may induce "unresponsiveness" (i.e. anergy or tolerance) in T lymphocytes, rather than activation.

HLA-DQ expression by keratinocytes was only found in a few (6 out of 43, 14%) high-grade lesions, and its expression was patchy and basal. This suggests that HLA-DR, rather than HLA-DQ, is the principal restriction determinant for T cell activation by keratinocytes in premalignant cervical disease.

Langerhans cells were reduced in number in CIN lesions, which confirms previous reports. However, the proportion remaining in the epithelium was shown not only to

express HLA-DR antigens constitutively, but most important, to upregulate expression of HLA-DQ in parallel with the severity of disease. Since upregulation of HLA-DQ is a characteristic feature of Langerhans cell differentiation, decreased numbers of Langerhans cells may reflect increased activation and migration out of the epithelium to regional draining lymph nodes, in order to present viral and/or tumour antigens to local T lymphocytes, and thereby an antigen-specific T cell response could be initiated. Reduced numbers of intraepithelial Langerhans cells in HPV-related premalignant cervical disease have been generally interpreted as a sign of local immunosuppression. However, a direct link between Langerhans cell numbers and outcome of infection is not supported by two studies which compared persistent versus regressive CIN lesions (Fukuda *et al* 1993) and genital warts (Coleman *et al* 1994b). Moreover, in cutaneous herpes simplex virus (HSV) infection, the decline in epidermal Langerhans cell numbers is accompanied by T cell infiltration of the epidermis that results in viral clearance (Memar *et al* 1995). Recent studies have shown a linkage between CIN progression and particular HLA-DQ alleles. This implies that antigen presentation mediated by the products of these "risk" alleles would induce unresponsiveness on the antigen-specific T cell clone. Our results suggest that such association may be mediated by expression of HLA-DQ on Langerhans cells.

We have also demonstrated that intraepithelial Langerhans cells do not express, or express extremely low levels, of accessory molecules such as ICAM-1 and ICAM-3, LFA-1 and LFA-3 and B7-2. However, in a few CIN lesions (5 out of 41 samples), CD1a positive stromal dendritic cells were expressing all the above mentioned adhesion/costimulatory molecules. This supports the hypothesis that *in situ* Langerhans cells, although proficient in capturing and processing antigens, are likely to be poor presenters to local T cells in most instances, upregulating the expression of accessory molecules when leaving the epithelia to activate resting, naive T lymphocytes in draining lymph nodes. This antigen-presenting function can be modulated by the cytokine milieu where T cell activation occurs which will influence T cell effector function (as outlined below).

In CIN lesions, both Langerhans cell and keratinocyte expression of MHC class II molecules (HLA-DR and -DQ) does not seem to be a direct consequence of HPV16

infection, since no correlation was found between expression of these molecules and HPV16 DNA status.

We have demonstrated that the pro-inflammatory cytokine (TNF-α) and the anti-inflammatory one (IL-10) are differentially synthesised by cervical keratinocytes. Protein expression was correlated directly to mRNA distribution for both cytokines. Whereas TNF-α was constitutively produced by the basal epithelial cells of normal squamous epithelium, IL-10 was never observed. In CIN lesions, we have shown that whilst TNF-α expression was downregulated in some samples (9 out of 41 CIN lesions) and more often in high-grade disease, IL-10 was produced by cervical keratinocytes in both low- and high-grade lesions (20 out of 41 samples). This differential cytokine expression in the local microenvironment of premalignant cervical lesions might have consequences in the modulation of the local immune response. As we have previously discussed (chapter 6), it can be hypothesised that absent or decreased TNF-α production by cervical keratinocytes could facilitate persistent HPV infection by deficient control of viral replication, and precluding antiviral resistance to uninfected neighbouring keratinocytes (Wong and Goeddel 1986, Ramsay *et al* 1993). In addition or alternatively, the presence/predominance of IL-10 in the local milieu during T cell maturation and differentiation could induce an inadequate effector immune response (i.e. Th2) against viral and/or tumour antigens. Furthermore, cytokines such as IL-1 and TNF-α have been shown to contribute to Langerhans cell maturation and migration from the epidermis (Larsen *et al* 1990, Cumberbatch *et al* 1994), and IL-10 to inhibit the antigen-presenting function of these cells (Ding and Shevach 1992, Enk *et al* 1993). It can be envisaged that the local cytokine microenvironment provided by cervical keratinocytes could influence Langerhans cell function, either stimulating or inhibiting its antigen-presenting capacity, which would be relevant in terms of disease outcome.

Distinct immune responses in the local cervical microenvironment of CIN lesions are supported by our observations in the CD3-dependent *in vitro* T cell proliferation assays. For this purpose, we tested the effects of supernatants derived from explant cultures of premalignant cervical mucosa on T cell proliferation and suggested that the differences in T cell proliferation observed (increased, unaltered or inhibited), were attributable to

either a soluble factor (e.g. TGF-β or prostaglandins) or an imbalance amongst various cytokines, since no specific significant correlation was found between T cell proliferative responses and the individual concentrations of IL-1α, IL-10, IL-12 and TNF-α. These cytokines were also produced *in vitro* by the immune cells which emigrated from the cervical mucosa of CIN lesions, supporting the idea that immune cells in the local cervical microenvironment may have diverse patterns of cytokine production, which are reflected in the differences in local signals provided for cell activation. The cytokine pattern of these cells may thus ultimately be decisive in terms of disease outcome, and local immune responses in HPV-related premalignant cervical disease may either contribute to eradication of the infection/epithelial cell transformation or allow its persistence and progression to invasion.

On the basis of the data presented in this thesis and from previous studies, we believe it is possible to formulate a useful model of the role of immune regulation in HPV-related premalignant cervical disease. After HPV infection and initial viral replication early "warning signals" may be delivered by infected keratinocytes to the host innate immune system, but little data is available on these very early events. By analogy to other viral infections, upregulation of interferons and TNF-α might play a key role in eliminating infection in the majority of individuals. Natural killer cells may also be involved in this initial phase, non-specifically destroying HPV infected keratinocytes. In terms of adaptive response, the most important event is probably activation and emigration of local Langerhans cells to present viral or tumour associated antigens to both naive CD4 and CD8 T cells which results in a local mononuclear cell infiltrate. Subsequently, viral and/or tumour antigens displayed on the surface of keratinocytes in association with MHC class I molecules, can be recognised by these infiltrating cytotoxic CD8 T cells which will lyse the infected epithelial cells. In parallel, infected/transformed keratinocytes expressing MHC class II molecules (HLA-DR), accessory molecules (ICAM-1 and LFA-3) and providing an adequate cytokine environment can act as APCs, as well as target cells for CD4 T cells with both helper and cytotoxic activities.

However, in some HPV-related lesions the cellular immune response appears to be blocked at this phase. The reasons for this remain unclear. Instead of an adequate

inflammatory response, in these lesions recruitment and activation of T cells is limited. Molecular mechanisms which contribute to this "sub-optimal" adaptive response may well be varied and, indeed, our *in vitro* culture studies suggest that there is a wide spectrum of immunological microenvironments within different cervical lesions. Most simply, there may be insufficient quantity of antigen for presentation resulting in a low peptide-MHC density at the cell surface and incomplete T cell activation. The requirement for higher antigen levels may be particularly important because of the absence of strong costimulatory signals from the keratinocytes, which do not express B7-2 or ICAM-3. In addition, the local cytokine milieu of some lesions may be inhibitory to a full inflammatory response - TNF-α is downregulated, while IL-10 is upregulated in many lesions, for example. It remains an unknown whether this aberrant regulation is due to the HPV itself, or is an indirect result of the type of T cell response generated. Langerhans cells presenting peptides in the context of a particular HLA-DQ (B1*03), for instance, may induce a Th2-like cytokine response. The consequences of all these mechanisms are that the local immune cell infiltration comprises antigen-specific T cell clones which are "unresponsive", have been anergised or generate the inappropriate type of immunological response. We believe that this immune failure is a major factor in determining persistence of HPV infection, which can lead to cellular transformation, eventually to disease progression, and ultimately to cervical carcinoma.

Appendices

1. List of Abbreviations

APC	antigen presenting cell
bp	base pair
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CIN	cervical intraepithelial neoplasia
CIS	carcinoma *in situ*
cpm	couts per minute
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetraacetate
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
H&E	haematoxylin and eosin
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
HSV-2	human simplex virus 2
ICAM-1	intercellular adhesion molecule one
ICAM-3	intercellular adhesion molecule three
IFN-γ	interferon-γ
Ig	immunoglobulin

IL	interleukin
LFA-1	lymphocyte function associated antigen one
LFA-3	lymphocyte function associated antigen three
LPS	lipopolysaccharide
MAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
Pap	Papanicolaou
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute medium
RT	room temperature
SDS	sodium dodecyl sulfate
SIL	squamous intraepithelial lesion
SSC	sodium saline citrate
TBS	Tris-buffered saline
TCR	T-cell antigen receptor
TGF-β	transforming growth factor-β
TNF-α	tumour necrosis factor-α

2. Solutions

Buffer A

100mM Tris-HCl pH 7.5, 150mM NaCl.

Buffer B

100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$.

100x Denhardt's

10g Ficoll, 10g Polyvinylpyrrolidone, 10g BSA in 500ml ddH_2O.

1x PBS

80 mM $(Na)_2HPO_4$, 20 mM NaH_2PO_4, 100mM NaCl, pH 7.5.

20x SSC

300mM Sodium citrate, 3M NaCl.

1x TBS

20mM Tris base, 137 mM NaCl, 3.8mM HCl, pH 7.6.

TE

10mM Tris-HCl, 1mM EDTA pH 8.0.

3. Publications arising from this thesis

Mota F., Kanan J., Rayment N., Mould T., Singer A. and Chain B. (1997). Cathepsin E expression by normal and premalignant cervical epithelium. *American Journal of Pathology*. **150**:1223-1229.

Mota F. and Kanan J. (1997). PCR detection of the human papillomavirus: improved DNA recovery from frozen biopsies. *Clinical Science*. **93**:599-603.

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