Association Between HLA DQB1 * 03 and Cervical Intra-epithelial Neoplasia

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ABSTRACT

Background: Cervical intraepithelial neoplasia (CIN) and cervical cancer have been shown to be strongly associated with infection by human papillomavirus (HPV). However, other factors may be contributory in the progression from normal epithelium to CIN and cervical cancer, since not all women with HPV infection develop disease. Recently, it was demonstrated that there is a high risk for cervical cancer and CIN in women with HLA DQB1 * 03 (RR = 7.1, $p < 0.0009$) (1). Subsequent reports have been conflicting, due to sample size, genetic heterogeneity and differences in the techniques employed for the detection of HLA DQB1 * 03.

Materials and Methods: DNA from cervical smears of 178 women with CIN and 420 controls with normal cervical cytology was analyzed by polymerase chain reaction (PCR) with type-specific primers for HPV 16, 18, 31, and 33. The DNA from test and control samples were also analyzed by a novel PCR technique, which mutates the first base of codon 40 (DQ alleles) from T to G to create an artificial restriction site for an enzyme *Mlu* I that distinguish DQB1 * 03 from other alleles and are confirmed by digestion of amplified DNA with *Mlu* I. Further analysis of individual DQB1 * 03 alleles was performed using PCR and allele-specific primers.

Results: One hundred forty-four (34%) out of 420 controls (all HPV 16, 18, 31, or 33 negative and normal cytology), 37/66 (56%) of CIN I and 72/112 (64%) of CIN III were positive for DQB1 * 03 (trend test, $p < 0.001$, $\chi^2 = 37.3$). A significant association was observed between DQB1 * 03 and CIN (odds ratio 3.03; 95% CI 2.11–3.45). Of women with CIN, 131/178 (73.5%) had HPV (types 16, 18, 31, or 33) infection. There was a significant association between DQB1 * 03 and presence of HPV (odds ratio 3.43; 95% CI 2.25–5.10). Homozygosity for DQB1 * 03 was more strongly associated with CIN than heterozygosity (odds ratios 4.0 and 2.63, respectively); and for the presence of HPV (odds ratio 4.47; 95% CI 2.58–7.77). HLA DQB1 * 0301 was the most strongly associated allele with CIN and HPV (odds ratios 2.53 and 2.63, respectively).

Conclusions: HLA DQB1 * 03 is associated significantly with CIN and may be permissive for HPV infection. Further analysis of class II HLA typing in CIN is necessary to evaluate this association.

INTRODUCTION

The hypothesis that human papillomavirus (HPV) is causally associated with cervical intra-epithelial neoplasia (CIN) and cervical cancer is well supported by several lines of evidence. First, HPV is detected in the cervical tissue of majority of patients with disease, compared with controls (2,3). Second, the oncogenic types of HPV can transform and immortalize human keratinocytes in vitro (4,5). Third, the HPV16 E_6 and E_7 proteins inactivate endogenous tumour suppressor

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proteins p53 and Rb, thereby abrogating normal cell control mechanisms (6). Despite this, the majority of women infected with HPV do not develop CIN or cancer. Several cofactors have been suggested as being important. A recent analysis of HLA type has shown a significant association between HLA class II, in particular HLA DQB1 * 03, and cervical cancer but not for class I (1). Subsequent reports have not been consistent (Table 1) presumably due to the size and type of population examined and differences in techniques used for the HLA and HPV typing. As cervical intraepithelial neoplasia (CIN) is an early stage prior to the development of cervical cancer, any association with HLA class II that could be demonstrated would be important. This paper reports the results of such a study conducted in a Caucasian population, where both controls and test samples were typed for HPV and HLA DQB1 * 03.

MATERIALS AND METHODS

Sample Collection

Cervical smears were taken using standard procedures from healthy women and those with CIN attending the outpatient clinics at City Hospital, Nottingham; Whittington Hospital, London, and the Margaret Pyke center, London. Further processing of samples for PCR was performed as described (7,8).

Histological Diagnosis

Histological classification into the three groups of normal, CIN I, and CIN III were carried out according to established criteria (9,10).

HPV Typing

HPV typing by PCR was performed using type specific oligonucleotide primers (HPV 16, 18, 31, 33) as previously described. (7,8)

HLA Typing

A rapid system to type the HLA DQB1 * 03 was developed using primer directed mutagenesis by PCR and restriction enzyme digestion (artificial restriction fragment lenth polymorphism [ARFLP]-PCR) (11). All DQB1 * 03 alleles possess an A as the last base of codon 38 followed by CGC (codon 39) and TTC (codon 40) (Fig. 1). By mutating the first base of codon 40, from "T" to "G", a *Mlu* I site (ACGCGT) is created in the DQB1 * 03 allele. Non DQB1 * 03 alleles possess a "G" as the last base of codon 38, and a *Mlu* I site cannot be created this way. The forward primer A is used in conjunction with the reverse mutagenesis primer B:

A: 5′ AGGGATCCCCGCAGAGGATTTCGTGTACC 3′

(forward)

B: 5′ CCGGTACACCCCCACGTCGCTGTCGA<u>C</u>GCG 3′

(reverse)

(The mutated base is underlined.) PCR was carried out as described in 50 νl volume using a Techne-PC 3 machine, according to manufacturer's instructions. The initial denaturation was at 94°C for 8 min followed by 30 cycles each at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. There was a final extension step at 72°C for 15 min.

Following amplification 10 μl of the PCR product was restricted with 50 units of *Mlu* I (Boehringer Mannheim) in a volume of 20 μl at 37°C overnight using manufacturer's buffer. The products were analyzed by electrophoresis on 4% agarose gels (Metaphor, Flowgen) (Fig. 2).

Further typing for HLA DQB1 * 03 for 0301, 0302, 0303, and 0304 in samples that were positive for the HLA DQB1 * 03 phenotype was performed by PCR using a combination of sequence specific primers (12) (Table 2). The annealing temperature for all the primer pairs was 60°C, and other PCR conditions were as published (12). All PCR reactions were performed with both positive and negative controls. The amplified products were separated by agarose gel electrophoresis and visualized by ultraviolet (UV) light.

Statistical Analysis

Odds ratios and their approximate 95% confidence intervals were calculated for all variables by a χ^2 test for 2 x 2 tables without a continuity correction (13). For 2 x k tables the χ^2 test for trend was calculated (14). The unit of sampling was the individual in all analysis except when studying specific alleles. In that case, each allele was taken as an independent observation so that the sample size was twice as large for allele specific comparisons.

TABLE 1. Summary of studies on CIN, cervical cancer, and HLA

Study	Patients	Controls	Population	HLA Typing Method	HPV Typing	Results
Wank and Thomssen (1991) (1)	66 (SCC)	109 (local panel)	German	Serology	Not done	1. DQW3: RR = 7.1 2. DR5: Increased risk 3. DR6: Decreased RR 12.7
Wank et al. (1992) (32)	66 (SCC)	109 (local panel)	German	PCR-SSO	Not done	Increased risk of SCC for DQB1 * 0301 & 0303
Helland et al. (1992) (26)	213 (SCC)	181 (local panel)	Norwegian	PCR-SSO	Not done	DQW3: RR = 2.0
Vandenvelde et al. (1993) (32)	71 (CIN)	323 (CIN0, not typed for HPV)	Belgian	PCR-ASO	Method and HPV types not stated	DQB1 * 03: RR = 2.647 for HPV-associated CIN
David et al. (1993) (29)	50 (CIN)	99 (CIN0, blood donors)	British	PCR-SSO	Not done	DQB1 * 03: RR = 2.5 for CIN III
Glew et al. (1993) (30)	65 (SCC)	857 (organ donors)	British	PCR-SSO & Serology	Southern blotting, HPV 16 only	No HLA association
Apple et al. (1994) (27)	98 (SCC)	220 (CIN0, includes HPV-positive women)	Hispanic	PCR-SSO	PCR consensus primers and oligonucleotide probing	1. DRB1 * 1501-DQB1 * 0602 OR = 4.78 for HPV 16 +ve cases 2. DRB1 * 0407-DQB1 * 0302 OR = 2.19 3. DR13: OR = 0.29 (−ve) 4. DQB1 * 03: No association
Gregoire et al. (1994) (31)	66 (SCC)	214 (local panel, not typed for HPV)	African-American	PCR-SSO	PCR consensus primers and oligonucleotide probing	1. DQB1 * 03: RR = 2.3 2. DQB1 * 0303: RR = 5.2 3. DQB1 * 0604: RR = 5.2

RR = relative risk; OR = odds ratio; SCC = squamous cell carinoma.

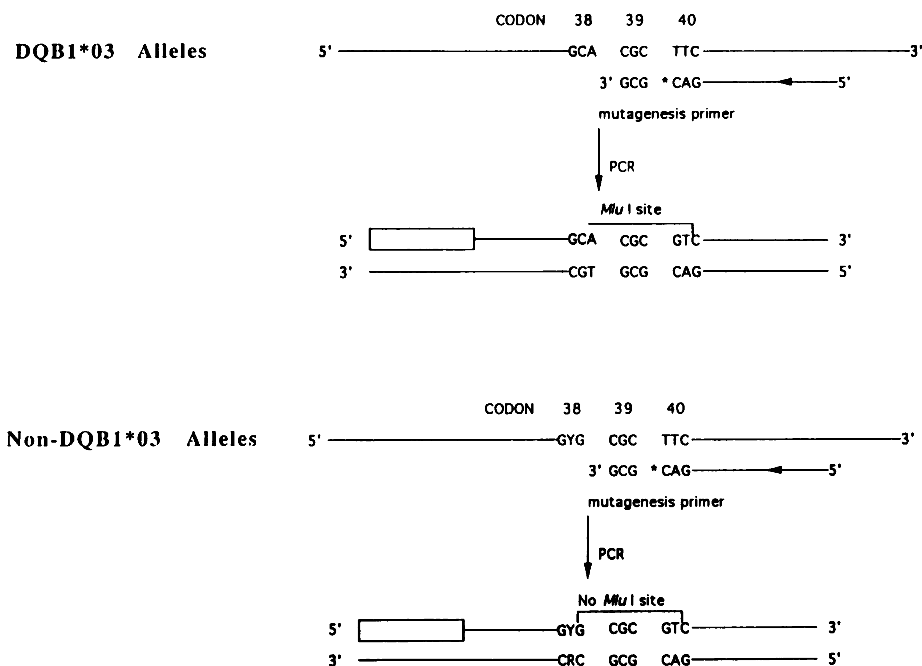

FIG. 1. A schematic diagram showing the principle of ARFLP-PCR

RESULTS

Overall (Table 3)

HLA DQB1 * 03 typing was performed on DNA from cervical smears of 178 women with CIN (CIN I = 66; CIN III = 112) and 420 healthy women who had a normal smear. All samples were successfully amplified for the locus. HPV typing was performed for types 16, 18, 31, and 33 on all the test and control samples.

Of CIN cases, 61% were positive (56% of CIN 1, 64% of CIN III) for the HLA DQB1 * 03 type, compared with 34% of controls. This was significant (χ^2 trend = 37.3, $p < 0.001$), and the odds ratio for CIN overall was 3.03 (95% CI 2.11–4.35). The association was significant for both CIN III and CIN I (odds ratio 3.45 versus 2.45), stronger for CIN III, but not significantly different from CIN I.

One hundred thirty-one patients with CIN (73.5%) were positive for one or more HPV types 16, 18, 31, and 33. Of HPV-positive CIN, 64% were of the type DQB1 * 03. There was a significant association between DQB1 * 03 and HPV (χ^2 trend = 23.2, $p < 0.001$) with an odds ratio of 3.43 (95% CI 2.28–5.15).

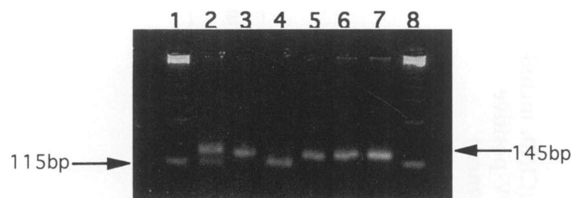

FIG. 2. A 4% agarose gel showing amplified DNA after PCR with primers A and B with and without digestion by *Mlu* I

The size of amplified DNA is 145 bp and on digestion with Mlu I, a 115 and 30 bp product is produced in DQB1 * 03 homozygotes. DNA for all controls were from the British Society for Histocompatibilty and Immunogenetics. Arrows show the 145 and 115 bp products. (Lanes 2 and 3) Heterozygous DQB1 * 03 control with and without digestion by *Mlu* I. (Lanes 4 and 5) Homozygous DQB1 * 03 control with and without digestion with *Mlu* I. (Lanes 6 and 7) Non-DQB1 * 03 control with and without digestion with *Mlu* I. (Lanes 1 and 8) 123 bp markers.

Association Between HLA DQB1 * 03 and CIN (Tables 4 and 5).

The ARFLP-PCR technique used on DNA from cervical smears, following *Mlu* I digestion, identifies women who are negative, heterozygous, or homozygous for the DQB1 * 03 locus (Fig. 2). Of women with CIN, 38% were negative, 37% were heterozygous, and 23% were homozygous for the DQB1 * 03 locus (χ^2 trend = 39.01, $p < 0.001$). Compared with controls, the odds

TABLE 2. Sequence-specific primer pairs for typing the HLA DQB1 * 03 locus

HLA Allele	Primer Sequences FAMP/RAMP	Size/PCR Product (bp)
DQB1 * 0201	5′ GTGCGTCTTGTGAGCAGAAG 3′	205
	5′ GCAAGGTCGTGCCGAGCT 3′	
DQB1 * 0201/0302	5′ GACGGAGCGCGTGCGTCT 3′	129
	5′ CTGTTCCAGTACTCGGCGG 3′	
DQB1 * 0301/0304	5′ GACGGAGCGCGTGCGTTA 3′	122
	5′ AGTACTCGGCGTCAGGCG 3′	
DQB1 * 0302/0303	5′ GACGGAGCGCGTGCGTTA 3′	122
	5′ AGTACTCGGCGTCAGGCG 3′	
DQB1 * 0303	5′ GACGGAGCGCGTGCGTTA 3′	129
	5′ CTGTTCCAGTACTCGCGT 3′	
DQB1 * 0601	5′ GCCATGTGCTACTTCACCAAT 3′	198
	5′ CACCGTGTCCAACTCCGCT 3′	
DQB1 * 0601/0301	5′ GACGGAGCGCGTGCGTTA 3′	129
	5′ CTGTTCCAGTACTCGGCGT 3′	
DQB1 * 0304	5′ GACGGAGCGCGTGCGTTA 3′	129
	5′ CTGTTCCAGTACTCGGCGG 3′	

FAMP Forward amplification primer; RAMP reverse amplification primer.

ratio was greater for homozygosity (4.0, 95% CI 2.43–6.6) than for heterozygosity (2.63, 95% CI 1.75–3.94). Further typing of the DQB1 * 03 locus in positive samples showed that the 0301 allele was present in 40% of CIN as opposed to 9% of controls (odds ratio 2.53, 95% CI 1.79–3.57; χ^2 trend = 28.6, p <0.001). DQB1 * 0302 was present in 32% and 10% of CIN and controls, respectively (odds ratio 1.84, 95% CI 1.29–2.62).

TABLE 3. Summary of distribution of HLA DQB1 * 03

Patients (No.)	HLA DQB1 * 03 (Positive) (%)	Odds Ratio (95% CI)
CIN (178)	109 (61)	3.03 (2.11–4.35)
CIN 1 (66)	37 (56)	2.45 (1.45–4.12)
CIN 3 (112)	72 (64)	3.45 (2.23–5.33)
HPV negative CIN (47)	25 (53)	2.18 (1.19–3.97)
HPV positive CIN (131)	84 (64)	3.43 (2.28–5.15)
Controls (420) (HPV negative	144 (34)	1*

* Reference category.
χ^2 trend for controls, CIN 1, and CIN 3 = 37.3, $p < 0.001$.
χ^2 trend for controls, HPV negative, and HPV positive = 38.6, $p < 0.001$.

Association Between HPV and HLA DQB1 * 03 (Tables 6, 7, and 8).

HPV typing was performed for the major oncogenic types, HPV 16, 18, 31, and 33. Of CIN cases, 57% were positive for HPV 16, 7% for HPV 18, 12% for HPV 31, and 7% for HPV 33, and 16% were positive for multiple types. All types correlated strongly with DQB1 * 03, but there was insufficient data to find differences in the strength of association between the types. The highest odds ratio was found for women with HPV 18 or multiple types. There was significant correlation with "gene dosage" at the DQB1 * 03 locus, with 39% of HPV-positive CIN being heterozygous and 24% homozygous (χ^2 trend = 37.9, $p < 0.001$). Homozygosity was significantly associated with HPV-positive CIN (odds ratio 4.47, 95% CI 2.58–7.77). Further typing of the HLA DQB1 * 03 locus in positive samples

TABLE 4. Association between HLA DQB1 * 03 and CIN

HLA	Controls (%)	CIN (%)	Odds Ratio (95% CI)
Non-DQB1 * 03	276 (65)	69 (38)	1*
Heterozygous for DQB1 * 03	102 (24)	67 (37)	2.63 (1.75–3.94)
Homozygous for DQB1 * 03	42 (10)	42 (23)	4.0 (2.43–6.60)
Total	420	178	

* Reference category.
χ^2 trend = 39.01, $p < 0.001$.

showed that the 0301 allele was most strongly associated with HPV infection (odds ratio 2.69, 95% CI 1.88–3.94; χ^2 trend = 32.9, $p < 0.001$).

DISCUSSION

Cancer of the uterine cervix is the second most common cancer world-wide and the most common cancer in developing countries (15). The annual incidence in the UK is 4,000 cases per year, with 2,000 dying as a result of the disease. Cervical intraepithelial neoplasia which precedes cervical cancer is even more common. Both cervical cancer and CIN have been shown to be strongly associated with the oncogenic types of the human papillomaviruses (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58) (16,17). An overview of studies show that the incidence of HPV infection detected by PCR based techniques in women with normal cervical cytology varies from 6–53% (18). There also exists a long latency period between HPV infection and cervical cancer (19). Therefore, additional factors are probably involved in the transformation of normal epithelium to CIN and cervical cancer after HPV infection. One such factor is possibly immunological, as in other virus induced cancers, such

TABLE 5. Association between HLA DQB1 * 03 allele and CIN

HLA DQB1 * 03 Allele	CIN 3 (2n = 224)	CIN 1 (2n = 132)	Controls (2n = 840)	Odds Ratio (95% CI)	χ^2 Trend
0301	49 (21)	25 (19)	79 (9)	2.53 (1.79–3.57)	28.6 ($p < 0.001$)
0302	45 (20)	16 (12)	85 (10)	1.84 (1.29–2.62)	15.5 ($p < 0.001$)
0303	5 (2)	7 (5)	21 (2.5)	1.36 (0.67–2.76)	0.05 ($p = 0.82$)
0304	0	0	1 (0.1)	0	
Non-DQB1 * 03	125	84	654	1*	
Total	224	132	840		

* Reference category.

TABLE 6. Association between HLA DQB1 * 03 and HPV type

HPV Type Present	Number of Patients	DQB1 * 03 (Positive) (%)	Odds Ratio (95% CI)
16	75	45 (60)	2.88 (1.74–4.74)
18	9	7 (77)	6.71 (1.56–∞)
31	16	11 (68)	4.22 (1.5–11.84)
33	10	6 (60)	2.88 (0.86–9.64)
Multiple types	21	15 (71)	4.79 (1.88–12.2)
Controls (HPV negative)	420	144 (34)	1*

* Reference category.

as nasopharyngeal carcinoma due to Epstein-Barr virus (20). In immunocompromised women following renal transplantation, the risk for cervical cancer is increased 10 fold (21). HIV infected patients also show an increased frequency of CIN (22). Analysis of HLA class I and II antigens in cervical cancer has shown that loss of class I expression occurs in a third of patients, while the majority demonstrated increased class II expression (23,24).

HLA type has been analyzed by several groups in patients with cervical cancer and CIN with different conclusions (Table 1). The discrepancy in the results between different investigators maybe due to several reasons. Serological typing of DQB1, as in the initial report, is prone to significant error (25). The sample size was adequate in only two studies (26,27). In fact, analysis on a sufficiently large number of cases of CIN has not been performed, though two preliminary studies reported that the DQB1 * 03 locus was associated significantly with CIN (Table 1) (28,29). The controls in several studies were from local donor panels and not necessarily comparable. Typing for HPV was performed in only three studies (27,30,31). To address these issues we have performed a larger study with sufficient number of cases and controls to evaluate the significance of DQBI * O3 association with cervical intraepithelial neoplasia. Further, it is quite important to evaluate the association between CIN, HPV, and DQB1 * 03 using controls that are negative for HPV and have a normal cytology.

The typing for HLA DQB1 * 03 was performed with a rapid technique which was concordant with data based on sequencing (11). This

TABLE 7. Association between HLA DQB1 * 03 and HPV

HLA	Controls (%)	HPV Positive CIN (%)	Odds Ratio (95% CI)
Non-DQB1 * 03	276 (65%)	47 (35%)	1*
Heterozygous for DQB1 * 03	102 (24%)	52 (39%)	2.99 (1.90–4.71)
Homozygous for DQB1 * 03	42 (10%)	32 (24%)	4.47 (2.58–7.77)
Total	420	131	

* Reference category.
χ^2 trend = 37.9, $p < 0.001$.

TABLE 8. Association between HLA DQB1 * 03 allele and HPV

HLA DQB1 * 03 Allele	CIN HPV (Positive) ($2n = 262$) (%)	CIN HPV (Negative) ($2n = 94$) (%)	Controls HPV (Negative) ($2n = 840$) (%)	Odds Ratio (95% CI)	χ^2 Trend
0301	60 (22)	14 (15)	79 (9)	2.69 (1.88–3.94)	32.9 ($p < 0.001$)
0302	45 (17)	16 (17)	85 (10)	1.71 (1.17–2.50)	10.6 ($p < 0.001$)
0303	9 (3)	3 (3)	21 (2.5)	1.35 (0.63–2.89)	0.71 ($p < 0.4$)
0304	0	0	1(0.1)	0	
Non-DQB1 * 03	148 (56)	61 (65)	654 (78)	1*	
Total	262	94	840		

* Reference category.

technique is also informative in assessing whether the individual sample is heterozygous or homozygous for the DQB1 * 03 locus. The results show a significant association between CIN and DQB1 * 03 which is only slightly stronger for CIN III than CIN I. The association between CIN and DQB1 * 03 that we found (odds ratio 3.03) was weaker than that reported by Wank and Thomssen (1). A slightly stronger association in HPV-positive CIN (odds ratio 3.43) was observed than that reported by Van den velde et al. (28). Homozygosity at the DQB1 * 03 locus, was significantly associated (odds ratio 4.0) with CIN and was more strongly related than heterozygosity, a result not reported so far in any of the previous studies. The 0301 allele was the most strongly associated with CIN (odds ratio 2.53, $\chi^2 = 28.6$, $p < 0.001$), but 0302 was also positively related. This agrees with Wank and Thomssen's DNA typing data for 0301 on their original sample of cervical cancer patients (32). A significant association with HPV-positive CIN and DQB1 * 03 was found for all HPV types tested (types 16, 18, 31, and 33). Again, homozygosity at the DQB1 * 03 locus was strongly associated with HPV-positive CIN (odds ratio 4.47) with intermediate risk found for heterozygotes. Typing for HPV has not always been performed in all the previous studies, but in general HPV-positive CIN was significantly associated with the DQB1 * 03 phenotype. In our study, only the most prevalent oncogenic types have been determined, and it is possible that some of the HPV-negative CIN cases are positive for other types. Additional typing for other HPV types would probably increase the strength of the association. These results suggest that the DQB1 * 03 locus is probably an important determinant in allowing HPV infection to be tolerated and increasing the risk for high grade CIN or cancer.

Another disease due to HPV infection, recurrent respiratory papillomatosis, has been shown to be associated with the DQB1 * 03 phenotype (33). In a preliminary analysis of 16 patients, 75% were positive for DQB1 * 03. Analysis of HLA class I and II using restriction fragment length polymorphism's in New Zealand rabbits infected with Shopecotton-tail rabbit papillomavirus showed a strong linkage between wart regression and DR locus, and an increased risk of malignant transformation with the DQ locus (34). Thus, based on our study and others, the DQB1 * 03 locus seems to be important for HPV associated disease. The results of the analysis of HLA DR and DQ in squamous cell carcinoma reported by Apple et al. (27) in an Hispanic population are intriguing in that no significant association was found with the DQB1 * 03 locus, although the haplotype DRB1 * 0407-DQB1 * 0302 was associated with increased risk of cervical car-

cinoma. It may be partly related to ethnicity of the population under study.

There are at least three possible ways by which the association between DQB1 * 03 and HPV positive disease can be explained. These women may present peptide antigen to CD4+ T cells ineffectively; clonal deletion of antigen specific T cell during thymic maturation may occur; or there maybe active suppression of immune response to HPV in DQB1 * 03 positive women. Although Mellins et al. (35) using T cell lines and specific HLA DP, DQ, and DR deletion mutants have shown that 20–30% of response to various recall antigens is restricted to DQ, in general, there is a bias against HLA-DQ restriction in human T cell clones reflecting a low level of expression of DQ on antigen presenting cells in the periphery (36). One proposal, in view of the above result, is that DQ is important in thymic selection of T cells. Indeed, there is a high level of expression of HLA DQ in the thymic cortex (37), and a role for negative selection for HPV-specific T cell clones would fit predisposition to HPV-positive CIN by the DQB1 * 03 alleles. The other possible mechanism is based on observations of HLA-associated immunological low responsiveness to antigens such as streptococcal cell wall (38), schistosoma (39), mycobaterium leprae (40), tetanus toxoid (41), and hepatitis surface antigen (42) either after natural exposure or after vaccination. Despite the controversy regarding the function of suppressor T cells, there is evidence to suggest that HLA DQ maybe the preferred restriction element for immunological suppression mediated by CD8-positive T suppressor cells (43,44). It is possible that women who are positive for the DQB1 * 03 phenotype are unable to mount an effective cytotoxic T cell response against HPV infection. This is particularly important as it has been shown that HPV16 E_7 is a target for cytotoxic T cells and can mediate tumor rejection (45).

The present study on CIN lays the framework for a more detailed study of DR and DQ in CIN to identify the significant haplotype. It is obvious that detailed investigation of mechanisms of HLA association with CIN would be important in improving the understanding of the biology of cervical cancer and generally be informative about virus associated cancer. This has obvious importance in the design of vaccines for prevention and treatment of HPV infection and associated disease.

ACKNOWLEDGMENTS

Acknowledgements to Dr. A. Hollingworth, Dr. A. Szarewski, and Prof. A. Singer for samples, and Dr. M. Anderson and Dr. A. M. Hanby for histological diagnosis, and Dr. R. Edwards for statistical analysis. Supported by the Imperial Cancer Research Fund and Wellbeing.

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Contributed by D. J. Weatherall on September 29, 1994.