Resolution of cervical dysplasia is associated with T-cell proliferative responses to human papillomavirus type 16 E2

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The 'high-risk' human papillomaviruses (HPVs) cause persistent infections of the anogenital region that may resolve spontaneously following activation of a protective immune response. The aim of this study was to determine whether cell-mediated immunity (CMI) to the early protein E2 was associated with disease regression and to establish whether E2 CMI and antibodies to L1 virus-like particles (VLPs) were associated markers of immunity to HPV. Lymphoproliferative responses to histidine-tagged E2 and antibody responses to VLPs were measured in patients with persistent cervical dysplasia, those whose disease had recently resolved and normal controls. Resolvers had significantly higher E2-specific lymphoproliferative responses when compared with normal controls or persisters, whereas there was no significant difference between the persisters and the normal controls. The T cells stimulated by E2 secreted high levels of gamma interferon (IFN-γ), consistent with a type 1 helper (Th1) phenotype. VLP IgG responses were associated with current or previous HPV infection, but not with disease regression or a lymphoproliferative response to E2. Major histocompatibility complex class I-restricted T cells secreted IFN-γ following stimulation with E1, and E2 peptides were detected more frequently in the persister group. The data showed that lymphoproliferative responses to E2 with a cytokine profile indicative of Th1 are associated with disease resolution, supporting the development of a therapeutic vaccine that activates this type of response for the treatment of individuals with pre-existing disease.

INTRODUCTION

The oncogenic ‘high-risk’ types of human papillomavirus (HPV) are the causal agents for cervical cancer (Walboomers et al., 1999), a disease that causes mortality of around 230 000 women worldwide each year (Pisani et al., 1993). Cervical cancer is most frequently attributable to infection with HPV type 16 (HPV-16) (Clifford et al., 2003; Muñoz et al., 2003), with types 18, 45 and 31 being the next most frequently associated (Muñoz et al., 2003). HPV causes persistent infections that may last for around 3–5 years before resolving spontaneously (Schlecht et al., 2003) and the host immune response is believed to play a major role in disease regression, which is associated with an infiltration of immune cells to the site of infection (Tagami et al., 1980). The requirement for an immune response, with cell-mediated immunity (CMI) as the major effector component, is supported by a significantly higher frequency of progression of cervical pre-malignant lesions (Petry et al., 1994) associated with cellular [e.g. human immunodeficiency virus (HIV) infection, Nezelof’s syndrome, transplant recipients] and not humoral (e.g. common variable immunodeficiency) immunosuppression (Conley et al., 2002; Heard et al., 2000; Rudlinger et al., 1986). Although two virus-like particle (VLP) vaccines have been licensed recently, these vaccines are prophylactic and will not contribute to the treatment of those who are already infected (reviewed by Frazer, 2004). Therefore, there is a need to identify the components of the immune response that are associated with disease regression, thereby providing targets for the development of therapeutic vaccines that will complement the prophylactic vaccines now available.

HPV infections are localized to the epidermal layer of mucosal or cutaneous skin. The viral genome is maintained episomally at a low copy number in the keratinocytes in the basal layer of the epidermis and is amplified and packaged as infected cells differentiate and move to the skin surface. This process is regulated by differential viral protein expression, with early protein expression (E1, E2, E5, E6...
and E7) in the lower layers, capsid protein expression (L1 and L2) in the upper layers and E4 expression being more widely distributed throughout the epidermis (Middleton et al., 2003). Whereas the early proteins such as E2 are expressed in cells that are normally in close contact with the Langerhans cells resident throughout the epidermis (Vayrynen et al., 1984), the capsid proteins of intact virus particles may be more readily accessible to the immune system via the mucosa-associated lymphoid tissue (Vayrynen et al., 1985). It is feasible that an immune response may be activated to early viral proteins independent of a late protein response and vice versa.

Expression of both E1 and E2 is necessary for viral episome replication in basal stem cells, which can occur independently of productive viral infection (Amella et al., 1994; Chiang et al., 1992; Ferencyz et al., 1985; Nicholls et al., 2001). Although HPV latency is poorly understood, reactivation of productive infection from cells that maintain viral episomes has the potential to occur, as does integration of the viral genome into chromosomal DNA, an event that is the first step in malignant progression (Jeon et al., 1995; Schifman et al., 2005). Destruction of episome-containing basal stem cells is therefore necessary to obtain sterilizing immunity, and E1 and E2 are potential target antigens for such a response.

T helper (Th) responses to HPV-16 E2 peptides are detected in normal healthy individuals (de Jong et al., 2002, 2004; van Poelgeest et al., 2006), and interleukin (IL)-2-secreting Th cells specific for the C-terminal domain of HPV-16 E2 have been detected around the time when HPV-16 PCR-positive patients with cervical intraepithelial neoplasia lose PCR reactivity, suggesting that HPV-16 E2-specific T-cell responses may contribute to disease resolution (Bontkes et al., 1999a). However, responses to HPV-16 E2 peptides and protein have also been found in some individuals with high-grade squamous intraepithelial lesions and cervical neoplasia (Lehtinen et al., 1995); therefore, the importance of Th responses to E2 in relation to disease resolution is not entirely clear.

The role of CD8+ cytotoxic T-cell responses to HPV-16 E1 and E2 in natural infection is currently unknown. E1 and E2 MHC class I-restricted epitopes with high binding affinity for HLA-A*0201 have been identified previously, some of which can be presented by HPV-16-infected cells (Konya et al., 1997). Cytotoxic lymphocytes (CTLs) have been stimulated in vitro from the peripheral blood of healthy donors using monocyte-derived dendritic cells pulsed with the C-terminal domain of HPV-16 E2 (Davidson et al., 2001), and E2 chimeric VLPs can stimulate E2-specific CTLs in HLA-A2 transgenic mice (Qian et al., 2006). Whether these cells are stimulated in response to natural infection in humans will be addressed in this study.

A number of studies have shown that antibody responses to HPV VLPs are a type-specific marker of infection (Giroglou et al., 2001; Kirnbauer et al., 1994). VLP-specific IgA is short-lived and considered to be a marker of current infection, whereas the IgG response persists longer after viral clearance (Sasagawa et al., 2003). Although prophylactic vaccination that induces antibody responses to VLPs has a high protective efficacy (Koutsky et al., 2002), antibodies to capsids are not considered to have a role in regression of existing infections (Sun et al., 1999; Viscidi et al., 2004) and little is known about the relationship between humoral responses to late proteins and cellular immunity to early HPV proteins.

In this study, our primary objective was to determine the nature of the cell-mediated immune response to E2 in patients that were resolving symptomatic disease and to establish whether it differed from patients with persistent or progressive disease. To test this, lymphoproliferative responses to E2 and E1 and E2 CD8+ T-cell responses were measured in a cross-sectional analysis of patients who had resolved cervical dysplasia within the last 6 months, in patients with disease that had persisted or progressed over the previous 6 months and in uninfected controls. We found that T cells proliferating in response to E2, secreting high levels of gamma interferon (IFN-γ) consistent with the phenotype of a Th type 1 (Th1) cell, were detected more frequently in patients whose disease had resolved recently. This type of response was observed infrequently in patients with progressive disease and was not detected in the normal control group. The pattern of reactivity was reversed in the HLA-A2-positive patients tested for CD8+ T-cell responses, such that CD8+ T-cell responses specific for E1 or E2 HLA-A2-restricted peptides were detected more frequently in individuals with persistent disease. Antibodies to VLPs were detected in the patient group and not in the normal control group, and did not show any association with disease regression. These data have implications for the design of therapeutic vaccines for the treatment of low-grade cervical dysplasia.

METHODS

Subjects and controls. This study was approved by the Southern Regional Health Authority in accordance with local ethical requirements. Cytological classification of the samples was carried out according to the Bethesda system (ASCUS; atypical cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions), which is the method of reporting used in New Zealand. Individuals attending the Student Health Services (University of Otago) for routine cervical screening were enrolled in the study after providing written informed consent to participate. The normal control group (n=10) consisted of individuals who had no previous history of cervical abnormality and who were confirmed to be cytologically normal at the time of recruitment to the study. The test group included patients with ASCUS, showing changes favouring HPV effect, or LSIL, showing changes consistent with HPV infection, at the time of the first cytological sampling. Individuals with ASCUS showing changes favouring HPV effect were only retained in the study group if their cytological result at the time of the second test had progressed to a higher grade of abnormality (LSIL consistent with HPV infection or HSIL). Cytological and blood samples of the test group were obtained at the 6-month follow-up screening test. The abnormal group was stratified according to the change in cytology between the two smear
samples. Participants who had normal cytology when tested at the 6-month follow-up were classified as ‘resolvers’ (n = 22). Individuals who continued to show evidence of abnormal cytology at the follow-up test were classified as ‘persisters’ (n = 12).

**HLA-A*0201 haplotyping.** The presence of the HLA-A*0201 allele was determined by staining peripheral blood mononuclear cells (PBMCs) with the monoclonal antibody BB7.2. Binding of the antibody was detected with biotinylated sheep anti-mouse immunoglobulin (Amersham Biosciences) and phycoerythrin-labelled streptavidin (Zymed Laboratories). The fluorescence intensity of bound antibodies was measured by flow cytometry (FACS Calibur; Becton Dickinson).

**Antigens.** The full-length E2 protein with a C-terminal histidine (His) tag was expressed from *Escherichia coli* BL21 (LysS) cells transformed with pET21d-E2. E2–His protein, purified as described previously (Heinemann et al., 2004), was used for the T-cell proliferation assay. A control protein extract was prepared by carrying out the E2 purification procedure from *E. coli* containing the pET21d plasmid but without an insert. An equivalent volume of this extract was used in control wells in the T-cell proliferation assay. Tetasun toxoid (TT; CSL) was used as a positive control in the T-cell proliferation assay.

The following previously described HLA-A*0201-binding peptides (Konya et al., 1997) were synthesized commercially using 9-fluorenylmethoxycarbonyl chemistry (Commonwealth Biotechnologies): E1 (aa 253–262), E2 I (aa 69–77), E2 II (aa 93–101), E2 III (aa 138–147) and influenza virus matrix K62 (aa 58–66; Flu-MA). The liphilized peptides were dissolved in DMSO at a concentration of 5 mg ml\(^{-1}\) and diluted in PBS to a concentration of 1 mg ml\(^{-1}\).

HPV-16 VLPs, purified from recombinant insect cells (Rose et al., 1994), were a kind gift from Dr R. Rose (University of Rochester Medical Centre, New York, USA).

**T-cell proliferation assay.** T-cell proliferation assays were carried out without prior knowledge of the cytology result of the individuals being tested. PBMCs were isolated from 30 ml heparinized blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences). PBMCs were seeded at a density of 2 × 10^5 cells per well in a 96-well round-bottomed microtitre plate (Nunc) in 200 μl RPMI 1640 supplemented with 2 mM l-glutamine, 100 U penicillin ml\(^{-1}\), 100 μg streptomycin ml\(^{-1}\) (crPMI) and 10 % autologous serum. E2–His protein was added to a final concentration of 1.9, 3.75, 7.5 or 15 μg ml\(^{-1}\) to triplicate samples. Control wells, also in triplicate, contained the equivalent volume of His-only control protein extract. Cells cultured with medium alone were used as a negative control. Cells stimulated with concanavalin A (ConA; 1 μg ml\(^{-1}\)) and TT (15 lethal factors ml\(^{-1}\)) were used as positive controls. Cells were pulsed with \[^{3}H\]thymidine on day 4 and antigen-specific proliferation was measured at day 5. For all samples, the concentration of antigen that gave the maximal difference between E2–His and the His-only control antigen was used. Stimulation indices (SI) are presented as the mean value for ten known HPV-negative women (recruited independently of this study) + 3 SD.

**Data analysis.** Data were analysed on completion of the study, at which time a complete list of cytological results was obtained. Data are presented as means ± SEM unless otherwise stated. Statistical analysis was carried out using non-parametric analyses tested with Kruskal–Wallis ANOVA or Mann–Whitney U-test. Fisher’s exact test was used for some analyses.

**RESULTS**

**Patients with low-grade dysplasia frequently resolve disease without medical intervention**

In this study, the relationship between resolution of cytological abnormality of the cervix and markers of immunological reactivity to HPV was determined. A cytologically
’normal’ control group (n=10) was compared with ‘persister’ patients (n=12) who had previous cytological abnormality 6 months prior to the study (11 LSIL showing changes consistent with HPV infection, one ASCUS showing changes favouring HPV effect) and continued to show abnormal cytology when recruited to the study (four HSIL; seven LSIL showing changes consistent with HPV infection; one ASCUS showing changes favouring HPV effect) and ‘resolver’ patients (n=22) who had cytological abnormality 6 months prior to testing (22 LSIL showing changes consistent with HPV infection) but were cytologically normal when recruited (Table 1). Overall, cytology reverted to normal in 65 % of the participants with abnormalities at the start of the study.

**T-cell proliferation to HPV-16 E2 is associated with disease regression**

Proliferative responses to HPV-16 E2 of the healthy volunteers, patients with persistent disease and the resolvers are shown in Table 2. We found that there was a significant difference between the E2-specific responses (His-tagged E2 stimulated cells/His-only control stimulated cells) of the resolvers, persisters and normal controls (Fig. 1a; Kruskal–Wallis three-way ANOVA, P<0.025). The group of patients where disease resolved had significantly higher T-cell proliferative responses to E2 than the normal control group (Mann–Whitney U-test, P=0.018) and the persisters (P=0.044), consistent with an association between proliferative responses to E2 and disease regression. There was no significant difference between the T-cell proliferative responses to E2 of the persister group and the normal controls (Mann–Whitney U-test, P=0.14). Furthermore, there was no significant difference (Mann–Whitney U-test) in response to ConA or TT between the groups (Table 1), confirming the specificity of the response and showing that there was no global difference in T-cell responsiveness.

**Serum IgG and IgA responses to HPV-16 L1 VLPs are detected more readily in exposed individuals**

Serum IgG and IgA responses are shown in Table 2. There was a significant difference in the levels of L1 VLP-specific serum IgG (Fig. 1b) between the resolver group and the normal controls (Mann–Whitney U-test, P=0.023); however, there was no significant difference in the response of the resolver group compared with the persister group (P=0.982) or between the persisters and the normal controls (P=0.069). These data therefore did not support a correlation between the presence of IgG antibodies to L1 VLPs and resolution of disease. IgA antibody responses to L1 VLPs did not distinguish disease outcome (Fig. 1c), with no significant difference in IgA responses between the resolvers, persisters and the normal control group (Kruskal–Wallis ANOVA, P=0.15).

**IgG antibodies to HPV-16 L1 VLPs and T-cell proliferative responses to E2 are combined indicators of previous or current cervical dysplasia**

The relationship between Th responses to E2 and antibody responses to HPV-16 L1 VLPs was explored. We found that IgG responses to HPV-16 L1 VLPs could be detected in 41.2 % (14/34) of individuals who had previous and/or current evidence of abnormal cytology (Fig. 2), which was similar to the frequency to E2 T-cell proliferative responses (13/34; 38.2 %). The percentage of individuals positive for each parameter differed significantly (Fisher’s exact test:...
Table 2. HPV-16 E2-specific T-cell responses and VLP-specific antibody responses in patients with cervical dysplasia and in healthy volunteers

<table>
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*N, Normal control; P, persister; R, resolver.
†N, Normal; A, atypical squamous cells present showing changes favouring an HPV effect, although of undetermined significance; LG, abnormal squamous cells present showing changes of a low-grade intraepithelial lesion consistent with HPV infection; HG, abnormal squamous cells present showing changes of a high-grade intraepithelial lesion.
‡A positive proliferative response was defined as a stimulation index ≥ 2. Positive responses are shown in bold.
§A positive serological response to HPV-16 VLPs was defined as a mean specific absorbance greater than or equal to the mean of ten known HPV-negative women + 3 SD (0.123 for IgA and 0.124 for IgG). Positive responses are shown in bold.
IgG L1 VLP responders, \( P = 0.018 \); LPA E2 responders, \( P = 0.041 \) between those who had a history of cervical dysplasia and the normal control group.

T-cell proliferative responses to E2 and IgG antibodies to L1 VLPs were independent immune markers. The majority of the participants in the group with a previous history of cervical dysplasia showed either an IgG response to L1 VLPs (10/34) or a T-cell proliferative response to E2 (8/34), with only 4/34 patients having both. The combination of both markers provided a more sensitive measure of previous exposure in this study. Around 65% of patients in the group who had a history of previous or current cervical dysplasia (and 73% of resolvers) had IgG to L1 VLP or proliferative responses to E2 or both responses, whereas none of the normal control group responded (Fig. 2). This result was extremely significant (Fisher’s exact test, \( P = 0.0003 \)) and supports the use of the combination of these immune markers as a more sensitive test for the detection of individuals with previous or current infection with HPV than each of the two markers individually.

**T cells proliferating in response to E2 predominantly secrete IFN-γ**

Levels of IFN-γ and IL-4 or -5 were measured in the supernatants of T-cell proliferation assays from patients who responded to E2. Cytokine levels were strongly skewed towards the production of IFN-γ over IL-4 or -5 (Table 3). In contrast to IL-4 and -5, where the maximal level detected was 70.8 pg ml\(^{-1}\), IFN-γ levels were notably higher and ranged from 470 to 8850 pg ml\(^{-1}\). IFN-γ was detected in all samples tested, whereas IL-5 was not detected in four out of eight supernatants tested. Although the majority of patients with T-cell proliferative responses to E2 were in the resolver group, cytokine levels in the supernatants of two patients in the group who had persistent disease were also tested. In both cases, the pattern of cytokine secretion was characterized by high IFN-γ and low-to-undetectable levels of IL-4 and -5, and therefore did not differ from the cytokine profiles of the resolver group. Therefore, in every case, proliferative responses to E2 were associated with a cytokine profile indicative of a Th1 response.

**MHC class I-restricted T-cell responses to E2 are detected in HPV-16-negative individuals with cervical dysplasia**

We measured IFN-γ secretion by ELISPOT in response to stimulation with an E1 and three E2 MHC class I-restricted epitopes in all patients who tested positive for HLA-A*0201.
(n=8: three persisters, five resolvers). IFN-γ-secreting cells could be detected in ConA-stimulated cultures from all patients (1269 ± 356 spots per 10^6 PBMCs; range 215–2710 spots per 10^6 PBMCs), confirming that the assayed PBMCs were functionally competent to secrete IFN-γ in response to stimulation. Two of the eight patients (110 and 141) had a T-cell response against Flu-MA that was above the baseline and two other patients (119 and 136) had a response that was above the background level (Fig. 3). Responses to one or more of the E1 or E2 peptides, of a similar magnitude to the Flu-MA response, were detected in four of the eight patients tested. All three of the patients with persistent dysplasia (104, 110 and 141) showed evidence of IFN-γ secretion in response to two or more E1 or E2 peptides (Fig. 3). In the resolver group, one of the patients (094) had a weak response to the E1 peptide, whereas the remaining four patients showed no evidence of reactivity to any of the peptides.

**DISCUSSION**

Although several studies have attempted to address the role of CMI to E2 in response to HPV infection, there are conflicting data surrounding its importance in disease resolution. Here, we showed that stronger E2-specific lymphoproliferative responses were found in patients who had recently resolved low-grade cervical dysplasia, and that they were significantly less likely to be detected in patients with persistent or progressive disease and were not detected in normal individuals.

Several groups have carried out studies in humans attempting to identify immune markers associated with disease resolution, which has been established as either reversion of PCR reactivity for HPV-16 from positive to negative or loss of symptomatic disease (abnormal cytology reversion to normal cytology). In cross-sectional studies of disease regression where PCR has been used as an indicator of HPV disease, no association between E2 lymphoproliferative responses and loss of PCR reactivity for HPV-16 could be established (Kadish et al., 2002; Sarkar et al., 2005). The use of PCR as an indicator of disease regression is limited, because the presence of HPV DNA detected by PCR is in many cases an asymptomatic, transient presence of HPV (Milde-Langosch et al., 2000).

We attribute the proliferative responses measured here primarily to stimulation of an MHC class II-restricted CD4+ T-cell response, as cross-presentation to CD8+ T cells is inefficient following stimulation with soluble antigen (Wick & Pfeifer, 1996). Although the patients tested here may have been infected with HPV types other than HPV-16, others have shown that there is broad cross-reactivity in CMI responses to HPV (de Jong et al., 2002). The correlation between proliferative responses and disease regression shown here is supported by the results of Bontkes et al. (1999a), who showed that Th responses to the HPV-16 E2 C-terminal domain frequently occurred around the time when patients cleared viral infection, and by studies in both rabbit and dog models that have shown that immunization with E2 by itself or with other viral proteins suppresses viral infection (Johnston et al., 2005; Selvakumar et al., 1995a, b).

Although the magnitude of the E2 proliferative response measured here was modest, it was comparable to responses detected to E2 C-terminal protein in patients with vulval intraepithelial neoplasia (Davidson et al., 2003) but weaker than responses measured following E2 peptide priming of PBMCs in normal individuals (de Jong et al., 2002) and the TT responses measured in this study. The difference between E2 responses measured by de Jong and both Davidson and our study may relate to the relative efficiencies of stimulation with protein antigens, which must be processed by antigen-presenting cells *in vitro*, compared with a higher concentration of peptide presented without natural processing following peptide stimulation. The weak responses relative to TT observed here and by Davidson et al. (2003) suggest a low frequency of HPV E2-specific T cells in peripheral blood. Several studies have shown that there is a large infiltration of T cells at the site of infection in actively regressing lesions (Coleman et al., 1994; Knowles et al., 1996). The low number of T cells in peripheral blood may reflect a loss of circulating T cells due to homing to the site of infection in regressing lesions.

We found that IFN-γ secretion, rather than IL-4 or IL-5, indicative of a Th1 response, predominated from PBMCs stimulated with full-length E2. E2-specific Th1 responses have been detected in normal individuals (de Jong et al., 2002; van Poelgeest et al., 2006), whereas a tendency towards Th2 responses has been reported in patients with high-grade cervical disease (Clerici et al., 1997; de Jong et al., 2004), supporting the hypothesis that E2-specific Th1 cells may have a role in disease regression. However, a Th1 profile predominated in proliferative responses measured in both the persister and resolver groups, which led us to

**Table 3. Cytokine secretion from E2-stimulated cells**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Patient group</th>
<th>IFN-γ (ng ml⁻¹)</th>
<th>IL-4 (pg ml⁻¹)</th>
<th>IL-5 (pg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>012</td>
<td>R</td>
<td>0.98</td>
<td>66.8</td>
<td>NT</td>
</tr>
<tr>
<td>022</td>
<td>R</td>
<td>2.30</td>
<td>13.6</td>
<td>NT</td>
</tr>
<tr>
<td>068</td>
<td>R</td>
<td>0.54</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>084</td>
<td>R</td>
<td>1.63</td>
<td>70.8</td>
<td>1.6</td>
</tr>
<tr>
<td>123</td>
<td>R</td>
<td>0.47</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td>134</td>
<td>R</td>
<td>8.85</td>
<td>NT</td>
<td>49.6</td>
</tr>
<tr>
<td>135</td>
<td>R</td>
<td>8.53</td>
<td>NT</td>
<td>5.9</td>
</tr>
<tr>
<td>136</td>
<td>R</td>
<td>0.63</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>139</td>
<td>R</td>
<td>2.43</td>
<td>NT</td>
<td>9.2</td>
</tr>
<tr>
<td>089</td>
<td>P</td>
<td>1.12</td>
<td>3.4</td>
<td>0</td>
</tr>
<tr>
<td>122</td>
<td>P</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>141</td>
<td>P</td>
<td>1.44</td>
<td>NT</td>
<td>0</td>
</tr>
</tbody>
</table>

R, Resolver; P, persister; NT, not tested.
conclude that the cells stimulated in response to infection were typically Th1 but were more frequently found in the resolvers rather than that there was any polarization of the response towards Th2 in patients with persistent disease.

Not all patients in the resolver group had positive proliferative responses to E2 and a small number of patients in the persister group responded to E2. Cellular immunity to one or more other viral antigens may have caused disease.
regression in some patients in the resolver group. Other
antigenic targets reported to be associated with disease
regression in patients include E6 (de Jong et al., 2004) and
E7 (Kadish et al., 2002), but not L1 (van Poelgeest et al.,
2006), and Th responses to E2 may act in concert with Th
cells to other antigen targets to clear viral infection. The
immune response that we detected in persisters is con-
sistent with a previous report showing that E2 responses
can be detected in some cases in patients with high-grade
disease (Lehtinen et al., 1995).

Consistent with the results of Bontkes et al. (1999a) who
stimulated Th cells using the C-terminal protein domain of
E2 and contrary to the results of de Jong et al. (2002) who
stimulated cells with E2 peptides, we were unable to detect
lymphoproliferative responses to E2 in normal controls.
The evidence does not support this difference as being
attributed to sample size, as Bontkes et al. (1999a) were
unable to detect any responders to the E2 C-terminal
domain and only one responder to the E2 N-terminal
domain in a larger group of 22 normal controls. The
differences between these studies may be attributed to the
enhanced sensitivity of cell stimulation with peptides
compared with stimulation of cells with whole protein.

Although IgG specific for HPV-16 VLPs was frequently
detected in patients who had symptomatic disease, we, like
Sun et al. (1999), found that disease regression was not
associated with antibodies to HPV-16 L1. In addition, there
was no correlation between T-cell proliferative responses
to E2 or antibodies to VLPs. It has been reported previously
that serum IgA responses to VLPs are associated with
clearance of HPV (Bontkes et al., 1999b). We found that
serum IgA responses to HPV-16 L1 VLP were not specific
markers of HPV-16 infection, as responses were detected in
the normal control group; nor was there any obvious
association with clearance, as this type of response was not
found more frequently in resolvers.

We tested CD8 T-cell responses to four HLA-A2-restricted
E1 and E2 epitopes. These epitopes were originally identi-
fied using an HLA-A2-binding motif and were shown to
bind HLA-A2 and stimulate peptide-specific CTLs in vitro
(Konya et al., 1997). Although 61 E1 and E2 peptides were
identified as containing the HLA-A2-binding motif in that
study, only the four tested here bound strongly to HLA-A2,
and two of those peptides, E1 and E2 I, were shown to
generate peptide-specific CTLs following in vitro priming
of PBMCs from normal individuals (Konya et al., 1997).
We found that E1- and E2-specific CD8 T-cell responses
were detected in all HLA-A2-restricted patients with
persistent disease and in only one of four patients whose
disease had resolved. These data provide the first evidence
that responses to these epitopes can be detected in patients
infected with HPV.

E1- and E2-specific CD8+ T cell responses to one or more of
the peptides tested here were detected more frequently in
persisters than in resolvers, which was surprising as CD8+
T cells are the primary effectors in clearance of a number of
different viruses (Benito et al., 2004; Brown et al., 2004;
Moss & Khan, 2004). There is increasing evidence to
support a direct role for CD4+ rather than CD8+ T cells in
the control and clearance of a number of other viruses
(Miskovsky et al., 1994; Paludan et al., 2002; Zagury, 1991).
In particular, cytotoxic CD4+ T cells are found in chronic
viral infections such as Epstein–Barr virus and HIV
(reviewed by Appay, 2004). The persistent nature of HPV
infection and its ability to block CD8+ T-cell function by a
number of immune evasion mechanisms (Filippova et al.,
2004; Hilders et al., 1994; Thompson et al., 2001; Vambutas
et al., 2001) supports the hypothesis that CD4+ cells are
the primary effector cells involved in viral clearance and
that these cells may have a direct cytotoxic function against
HPV-infected cells, which express increased levels of MHC
class II (Hilders et al., 1995).

The data presented here contribute to the understanding
of the role of CMI to E2 in disease resolution. Disease
regression was associated with IFN-γ-producing T cells
specific for E2, and although CD8+ T cells may yet have
a role in the resolution of HPV infection, there was no
apparent requirement for E2-specific CD8+ T cells against
the peptides tested here. These data support the need for
more extensive analysis of CD8+ T-cell responses to E1
and E2 using a wider array of peptide and MHC back-
grounds and for confirmatory evidence of a direct role for
CD4+ T cells specific for E2 in regressing HPV-infected
lesions to consolidate our findings.

ACKNOWLEDGEMENTS

The authors acknowledge the support of all of the medical and
nursing staff at the University of Otago Student Health Services.
The participation of Southern Health Laboratories in patient sampling
is most gratefully acknowledged. The authors thank Dr Stephen Jones
and CSL Ltd, Australia, for providing the tetanus toxoid and Dr
Robert Rose, Rochester Medical Center, NY, USA, for the VLPs. The
authors thank Alex McLellan for his comments during the pre-
paration of this manuscript. This research was funded primarily by
the Anderson Trust with further support from the Health Research
Council of New Zealand, the Otago Medical Research Foundation
and the Cancer Society of New Zealand.

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