Tonsil T Cell Immunity to Human Papillomavirus in the Absence of Detectable Virus in Healthy Adults

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Background: Human papillomavirus (HPV) is known to infect the epithelium of the upper aerodigestive tract; however, major questions regarding prevalence and persistence of infection, and their relation to local immune response, remain unanswered.

Objectives: To evaluate the tonsil T cell immune response to HPV and compare this to the frequency of detectable virus at this site.

Study Design: A cross-sectional study of cancer-free adults undergoing routine tonsillectomy.

Methods: Mucosal immune responses to recombinant HPV16 L2E6E7 and HPV6 L2E7 antigens were measured by tonsillar T-lymphocyte proliferation assay in 13 subjects. HPV deoxyribonucleic acid (DNA) was assessed by PCR and reverse line-blot hybridization in an expanded population of 44 subjects.

Results: Proliferative T-cell responses to HPV16 and HPV6 were identified in all patients. The presence of a CD45RO/H11001 T cell population responsive to HPV6 L2E7 was confirmed in three of six subjects tested. There were no CD45RO/H11001 responses to HPV16 L2E6E7 and no evidence of current or latent HPV infection of the tonsil.

Conclusions: T cell memory to human papillomavirus can be identified in tonsil tissue from an adult population in the absence of concurrent HPV infection.

INTRODUCTION

Human papillomavirus (HPV) infection is one of the most widespread sexually transmitted diseases worldwide and is the central cause of cervical cancer. While genital HPV infection persisting over 1 to 2 years is relatively common, infection with oncogenic subtypes (HPV16 or 18) leads to the development of intraepithelial lesions or cancer of the cervix in only a small proportion of individuals.1 Serologic studies and virus detection in tumor tissue suggests that HPV also has a causal role in a subset of head and neck squamous cell carcinomas (SCCHN), along with well accepted factors such as smoking and alcohol consumption.2 The strongest link is to oropharyngeal cancer, especially in the tonsil.3 Much less is known about HPV infection in the upper aerodigestive tract compared with in the genital tract. Persistent carriage of high-risk HPV types has been detected in the oral mucosa of 10% of infants during their first 26 months of life.4 Understanding whether these infections persist beyond early childhood and the frequency of new and re-infection later in life will help determine why some individuals are susceptible to this infection-transformation process.

We hypothesize that a memory-type mucosal immune response can be found in individuals who have no active infection in the upper aerodigestive tract. To investigate this and to explore the mechanistic basis for the HPV association with SCCHN, we looked for the immunologic footprint of HPV infection in tonsillar tissue of cancer-free adults and compared this with the frequency of tonsillar HPV.
METHODS

Design

This was a multicenter, hospital-based, cross-sectional biopsy study.

Ethical Approval

The local research ethics committees at North Bristol NHS Trust (LREC Project 139/02) and United Bristol Hospitals NHS Trust (LREC Project E5540) provide ethical permission for the following sampling procedures. The collection and use of clinical material described complies with the relevant guidelines and practices of the aforementioned institutions. All individuals provided informed written consent prior to commencement of the study.

Study Population

Tonsils were obtained from individuals undergoing tonsillectomy for recurrent acute tonsillitis, quinsy, or during surgery for snoring or obstructive sleep apnea. Patients with a past or current history of malignancy were excluded. All subjects completed parts I and II of a health and lifestyle questionnaire based on the National Health and Lifestyle Survey 1991. A sexual attitudes and lifestyle questionnaire was also used with questions taken from the National Survey of Sexual Attitudes and Lifestyles (Natsal 2000) Survey.

Preparation of Mononuclear Cells

Tonsils were stored in decontamination media containing Hanks' balanced salt solution (HBSS) and antibiotics (1,000 U/mL penicillin, 1 mg/mL streptomycin) for transport to the laboratory, prior to isolation of tonsil mononuclear cells (TMNCs) within 12 hours. Tonsil tissue was dissected into 2 mm³ fragments and dispersed into Hanks' balanced salt solution (HBSS) through a steel mesh (Potter & Son, Bristol, U.K.) as described previously. Tonsil mononuclear cells (TMNCs) were obtained by density-gradient centrifugation on Histopaque (Sigma-Aldrich, St. Louis, MO) at 400 g for 30 minutes. Harvested TMNCs were washed three times in HBSS and resuspended in complete RPMI (RPMI 1640 without glutamine supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 4 mmol/L-glutamine and 10 mmol/L HEPES buffer) (Sigma-Aldrich).

Cell Depletions

TMNCs were depleted of CD45RA⁺ populations by magnetic bead-associated cell sorting (MACS) as described. Briefly, 2 × 10⁸ TMNCs were incubated in 1600 μL of cold buffer (calcium magnesium-free PBS supplemented with 2 mmol/liter EDTA and 0.5% human AB serum) and 400 μL of MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) coated with anti-human CD45RA for 15 minutes. Washed cells were separated on an LS⁺ column (Miltenyi Biotec, Bergisch Gladbach, Germany) using a magnetic cell separator. Depleted cells were washed in ice-cold buffer, pelleted, and resuspended in complete RPMI as above. The efficiency of depletion was determined using phycoerythrin-labeled antiCD45RA antibodies (BD Pharmingen, BD, Franklin Lakes, NJ), with at least 95% purity confirmed by subsequent flow cytometry.

Cell Culture and Proliferation Assays

Cell proliferation assays were based on methods described by Williams et al. and Plebanski et al. and later modified. TMNCs were cultured in complete RPMI with 1% human AB serum (National Blood Services, UK) at 1.5 × 10⁶, 1.0 × 10⁶, and 0.6 × 10⁶ cells/mL in 24-well flat-bottom plates (Nunc, Roskilde, Denmark) with antigen or medium alone, in volumes of 2 mL per well. The HPV antigens (a kind gift from Xenova, Slough, U.K.), comprising TA-GW (recombinant HPV6 L2E7, 50 μg/mL) and TA-CIN (recombinant HPV16 L2E6E7, 50 μg/mL), were selected for their immunogenicity in human vaccine trials. Influenza vaccine (Fluarix 2003/2004 vaccine, GlaxoSmithKline, Uxbridge, United Kingdom) was used as a positive control antigen (0.2 μg/mL). Cultures were incubated for 10 days at 37°C in 5% CO₂. On days 3 to 9 of culture, 100 μL samples were transferred in triplicate to 96-well round-bottom plates (Nunc), pulsed with 0.4 μCi ³H thymidine (GE Health Care, Chalfont St. Giles, U.K.) and incubated for 20 hours at 37°C in 5% CO₂. Plates were harvested and tritiated thymidine incorporation (³HTdR) was measured using a 1,450 microbeta liquid scintillation counter (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA). Results were expressed as corrected counts per minute (ΔCPM) with background proliferation (derived from pulsed unstimulated cultures) subtracted.

HPV L1 Amplification

Small samples of tonsil epithelium (up to 25 mg) were dissected aseptically, snap-frozen in isopentane, precooled over liquid nitrogen, and stored at −70°C. Deoxyribonucleic acid (DNA) extraction was performed using standard methodology (DNeasy, Qiagen, Venlo, the Netherlands).

Current HPV infection was assessed using a noncommercial PCR HPV detection system, a kind gift from Roche Molecular Systems (Alameda, CA) and a similar commercially available kit (Genome Identification Diagnostics GmbH, Straßberg, Germany). These employ consensus primers to amplify part of the HPV L1-gene. The Roche system amplified each sample with a 5' biotin-labeled PGMY09 & PGMY11 primer set and biotinylated β-globin primers GH20 and PC04 as described. The GenID system employed biotinylated GP5/GP6 primers and GAP-DH amplification control primers. Five μl of purified DNA was used in each PCR mixture. Amplifications were performed in a PerkinElmer 9600 thermal cycler (PerkinElmer). A contamination control and a positive control were included in each test. DNA extract from cultured HeLa cells served as positive control (HPV18 +ve). A tissue positive control derived from a known HPV44 source was also used (a kind gift from Genome Identification Diagnostics GmbH).

Subsequent reverse line-blot hybridization was used to characterize HPV genotypes as described. Following hybridization, each strip was evaluated with the appropriate template. Both procedures employ conjugate and amplification control zones, which had to develop for a valid result.

<table>
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<tr>
<th>TABLE I. Demographic Characteristics of Patients.</th>
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<td>Characteristics</td>
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<tr>
<td>Age range</td>
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<td>History of genital warts</td>
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n = number.
RESULTS

T cell proliferation assays were completed using tonsils from 13 subjects. The demographic characteristics of these patients are shown in Table I. Tonsillar T cell sensitization to TA-GW and TA-CIN was identified in 13 samples (Fig. 1). Proliferation peaked at a median of 5 (range 3–9), 6 (range 4–9), and 6 (range 4–9) days after stimulation with TA-GW, TA-CIN and influenza antigens respectively, suggesting that secondary-type responses were present in some individuals (Fig. 2). The presence of a CD45RA+ depleted T cell population responsive to TA-GW was confirmed in three of six subjects tested (Fig. 3) compared with five of six for influenza. No CD45RA− depleted T cell responses to TA-CIN were identified. No evidence of current HPV infection using PCR was identified in tonsil tissue from 5 of the 13 subjects who had T cell proliferation assays completed (the remaining individuals could not be tested) or from an expanded population of 44 subjects (Fig. 4).

DISCUSSION

Our results indicate that T cell responses to HPV6 and HPV16 antigens are present in tonsillar tissue from a young adult population in the absence of concurrent HPV infection. The kinetics of these responses suggests that some of this immunity represents T cell memory. The demographic characteristics of the two groups were broadly similar—in particular, there was no history of genital warts in the T-cell assay group. Although resting memory T cells may re-express CD45RA in the long-term, depletion experiments showed CD45RO− memory T cell proliferation to the HPV6 but not to the HPV16 antigen. Taken together these findings imply that the tonsil is a frequent site of immune induction in the upper aerodigestive tract; and that infection with the genital wart-associated HPV6 is either more common than the cervical cancer-associated HPV16 or is more immunogenic.

We speculate that although HPV infection may occur in adulthood, acquisition of HPV in infancy may result in long-lasting immune memory. Cross-sectional and longitudinal studies of healthy asymptomatic individuals and women with cervical dysplasia have demonstrated T cell proliferative responses to HPV antigens in peripheral blood.16 It is therefore feasible that this previously undescribed mucosal immunity is due to cross-reactive antigens from other HPV genotypes17 and that priming may be occurring at mucosal sites distant from the tonsil, such as the genital tract.18 Nonetheless, these upper respiratory tract...
mucosal immune responses were seen in both men and women. It is also possible that some of the undepleted T cell proliferation responses may be nonspecific, although they have been specific in other studies using peripheral blood.12 Finally, while persistent HPV infection in the oropharynx may occur below the limit of detection for our assay, our data are consistent with previous PCR-based reports (prevalence range 0%–14%).19,20

**CONCLUSIONS**

Proliferative T cell responses to HPV antigens were consistently identified in tonsil tissue from a cancer-free population in the absence of detectable virus in the epithelium. Although exposure to HPV is common, infection of the upper aerodigestive tract in the healthy adult is likely to be highly transient. Whether parenteral HPV vaccination influences this naturally acquired mucosal T cell immunity remains to be determined.
Acknowledgments

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BIBLIOGRAPHY