Frequent Detection of Human Papillomavirus 16 E2-specific T-helper Immunity in Healthy Subjects

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ABSTRACT

The incidence of genital human papillomavirus (HPV) infections is high in young, sexually active individuals. Most infections are cleared within 1 year after infection. The targets for the cellular immune response in this process of viral clearance remain to be identified, but the expression pattern of the E2 protein in early infection and low-grade cervical intraepithelial neoplasia renders this early protein a candidate antigen. Therefore, we studied the HPV16 E2-specific T-cell responses in more detail. Very strong proliferative responses against one or more peptide-epitopes derived from this antigen can be found in peripheral blood mononuclear cell cultures of approximately half of the healthy donors. Additional analysis revealed that at least a majority of these responses represent reactivity by memory CD4 T-helper (Th) 1-type cells capable of secreting IFN-γ on antigenspecific stimulation. Interestingly, all of the E2 peptides against which strong responses were detected are clustered in the key functional domains of the E2 protein, which are conserved to considerable extent between HPV types. This suggests that HPV16 E2-specific Th memory may be installed through encounter with HPV types other than HPV16. Indeed, one HPV16 E2-specific Th clone was found to cross-react against homologous peptides from other HPV types, but three other Th clones failed to show similar cross-reactivity. Therefore, part of the HPV16 E2-specific Th memory may relate to previous encounter of other HPV types, whereas the majority of the immune repertoire concerned is most likely established through infection with HPV16 itself. Our data are the first to reveal that the T-cell repertoire of healthy donors can contain particularly high frequencies of E2-specific memory Th cells and suggest that boosting of this immunity can be used for preventive and therapeutic vaccination against HPV-induced lesions.

INTRODUCTION

Genital infection with HPV is one of the most common sexually transmitted diseases (1–3). So-called high-risk HPV types (e.g., type 16) are causally related to the development of CIN and cervical carcinoma (4). Fortunately, in a majority of immunocompetent individuals the HPV infection is asymptomatic, whereas most premalignant cervical lesions, in analogy to warts, are found to regress spontaneously (5–7). This regression is likely to be mediated by cellular immune responses, because regressing genital warts are predominantly infiltrated with CD4+ T cells and macrophages, consistent with a delayed type hypersensitivity reaction (8, 9). The role of immune surveillance in controlling infection by various HPV types is additionally indicated by the increased incidence of HPV infections, HPV-associated warts, CIN lesions, and cervical carcinoma in immunocompromised subjects (10, 11). Moreover, cervical neoplastic lesions frequently display deficiencies in the expression of HLA class I (12–14).

The investigation of anti-HPV immunity in humans has thus far primarily focused on responses against the E6 and E7 oncoproteins of HPV16/18, because expression of these proteins is tightly associated with HPV-induced dysplasia. Notably, these responses do not necessarily correlate with spontaneous regression (15–19). This suggests that the observed responses are in many cases not capable of eliminating the HPV-infected cells. In fact, although clearance of HPV infections as well as spontaneous regression of especially low-grade CIN lesions is frequently observed, more progressed lesions are rarely cleared. Importantly, the E6 and E7 oncoproteins are primarily expressed at high levels in more advanced lesions. During most stages of the productive infection cycle of HPV as well as low-grade lesions, the expression levels of these proteins are kept in check by the E2 protein and, as a consequence, are modest. The E2 protein is the major regulator of viral DNA replication and gene expression (20), and this antigen is uniformly and abundantly expressed in cells containing episomal HPV genomes. The highest E2 expression is found in koilocytes in low-grade CIN, whereas with progression of the lesions to invasive carcinoma, E2 expression is lost (21, 22). This corresponds with the observation that in high-grade CIN and cervical carcinoma, the E2 gene is frequently disrupted because of linearization of the viral genome, which is required for integration of viral DNA into the cellular genome.

In terms of immune intervention, the E6 and E7 oncoproteins are the major targets in high-grade CIN and cervical carcinoma, because these proteins are constitutively expressed and are required for maintenance of the transformed state (20). However, in the context of productive infection and low-grade CIN, the E2 protein constitutes an attractive target for the immune system, because it is highly expressed in these settings, thereby exhibiting an expression pattern complementary to that of E6 and E7. In the CRPV model, which is the major animal model for cancers associated with papillomavirus infection, proliferative responses against the E2 protein were associated with spontaneously regressing papillomas (23). Moreover, E2-specific immunization was shown to enhance regression of CRPV-induced papillomas (24). Finally, a follow-up study in HPV16-positive women diagnosed with CIN revealed that Th responses against the COOH-terminal domain of the E2 protein were frequently observed at the time of viral clearance, suggesting that these responses may contribute to viral clearance (25).

The proposed role of the E2-specific T-cell response in control of HPV infection and in regression of HPV-induced lesions, as well as the high incidence of genital (subclinical) HPV infection (3, 7), suggests that a pool of E2-specific memory T cells may be present in a sizeable fraction of the healthy population. We performed a detailed analysis of the E2-specific Th response in healthy individuals. Our
data reveal that the E2 protein exhibits several highly immunogenic regions that contain naturally processed Th epitopes. Moreover, the analysis of the CD45RO⁻ memory fraction of PBMC revealed the presence of E2-specific memory Th responses in a major fraction of healthy individuals.

MATERIALS AND METHODS

Lymphocytes. PBMCs and serum for proliferation assays were obtained from HLA-typed anonymous healthy blood donors after informed consent. Furthermore, for enzyme-linked immuno spot (ELISPOT) assays, PBMCs were isolated from buffy coats of anonymous healthy blood donors after informed consent. Because these donors were anonymous, no data on medical history was available. Importantly, donors with a known recent history of infection, including abnormal pap-smear were, as part of normal regulations, discouraged to donate blood.

Antigens. A set of peptides spanning the whole HPV16 E2 protein consisting of 23 overlapping peptides, 22 of which have a length of 30 amino acids and one of which (E2311–360) has a length of 35 amino acids, was used. These peptides share an overlap of 15 amino acids. For epitope fine-mapping of HPV16 E2-specific Th clones, peptides with a length of 15 and 20 amino acids were used. For determining the cross-reactivity of HPV16 E2-specific Th clones, the following peptides derived from different HPV types were used, with a length varying between 25 and 30 amino acids. Amino acid mismatches are italicized; amino acids with similar physico-chemical properties but not identical are indicated in bold: PKFVKQNTLKLAT (HPV16 E2), VKIPKTI (HPV45), QRKQLTVKIPKT (HPV35), YKPQFLV (HPV6B), QRQFLAV (HPV10), VSTGMS (HPV18), VKIPKTI (HPV26). The peptides spanning the HPV16 E2 protein (E2331–360) were used for epitope fine-mapping of HPV16 E2-specific Th clones, peptides with a length of 15 and 20 amino acids were used. For determining the cross-reactivity of HPV16 E2-specific Th clones, the following peptides derived from different HPV types were used, with a length varying between 25 and 30 amino acids. Amino acid mismatches are italicized; amino acids with similar physico-chemical properties but not identical are indicated in bold: PKFVKQNTLKLAT (HPV16 E2), VKIPKTI (HPV45), QRKQLTVKIPKT (HPV35), YKPQFLV (HPV6B), QRQFLAV (HPV10), VSTGMS (HPV18), VKIPKTI (HPV26). The peptides spanning the influenza matrix protein of A/PR/8/34, which were used as control peptides in ELISPOT assays, consisted of 16-30-mer peptides overlapping by 15 amino acids. Peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany) and analyzed by reverse-phase high-performance liquid chromatography. The lyophilized peptides were dissolved in 50 μl of DMSO, diluted in PBS to a final concentration of 2.5 mg/ml. The HPV16 E2 COOH-terminal (E2260–360) protein and HPV16 E7 protein were produced according to procedures described previously (26).

MRM, consisting of a mixture of tetanus toxoid (0.75 limus flocculents/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), Mycobacterium tuberculosis sonicate (2.5 μg/ml; generously donated by Dr. P. Katzer, Royal Tropical Institute, Amsterdam, The Netherlands), and Candida albicans (0.005%, HAL Allergenen Lab, Haarlem, The Netherlands), was used to confirm the capacity of PBMC to proliferate and produce cytokines in response to common recall antigens.

Human Leukocyte Antigen-DR (HLA-DR) Peptide Binding Assay. Binding of peptides to HLA-DR was measured as reported previously (27). Briefly, as a source of DR molecules B-LCL homoyzogous for DR were used: LG.1 (DRB*0101, DR1), IWB (DRB*0201, DR2), HAR (DRB*0301, DR3), and BSM (DRB*0401, DR4). DR molecules were purified by affinity chromatography and the purity confirmed by SDS-PAGE. The analysis of peptide binding to purified DR molecules was performed using NH₂-terminally fluorescently-labeled standard peptides. As standard peptide in the binding assays, H₂A₁β₂₅-₃₁₉ (PKKYVKQNTLKLAT, DR1 and DR2), hsp65 3-13 (KTIAYDEEARR, DR3), or H₂A₃β₁₀-₃₁₉ Y 224 F (PKFVKQNTLKLAT) was used.

Short-Term T-Cell Proliferation Assay. Immunogenicity of individual HPV16 E2 peptides was determined by short-term proliferation assays of healthy donor PBMCs with HPV16 E2 peptides, according to procedures described previously (27). Briefly, freshly isolated PBMCs were seeded at a density of 1.5 × 10⁵ cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 200 μl of IMDM (Bio Whittaker, Verviers, Belgium) supplemented with 10% autologous serum. HPV16 E2 peptides were added at a concentration of 10 μg/ml. Medium alone was taken along as negative control; phenylmercuric acetate (0.5 μg/ml) served as positive control, and cells were washed twice with PBS/BSA 0.2%/Saponin 0.1%, and peptides were added with PBS/BSA 0.2%/Saponin 0.1%, and supernatant was removed before 25 μl of PBS/BSA 0.2%/Saponin 0.1% containing 1 μl of FITC-labeled mouse-antihuman IFN-γ (0.5 g/ml; BD Pharmingen), 2 μl of peptides were scored positive, when in both assays the proliferation of >50% of the test wells exceeded the mean proliferation + 3 × SD of the control wells, and the SI of the positive test wells over medium control wells was >3.

Generation and Analysis of Long-Term HPV16 E2-specific Th Cultures. Long-term HPV16 E2-specific T-cell cultures and Th clones were established according to procedures described previously (27). Briefly, PBMC from healthy HLA-typed donors were stimulated in vitro with the following HPV16 E2 peptides: E2271–310 + E2286–315 + E2311–330 + E2316–345, and E2331–360. PBMCs (15 × 10⁵) were seeded in 25-cm² culture flasks (Nalge Nunc) in 6 ml of IMDM supplemented with 10% autologous serum. Peptides were added at a concentration of 5 μg/ml. At day 7, 15 × 10⁵ PBMCs were added, together with fresh medium and peptides. At days 14 and 21 viable T cell numbers were counted, and it was established with an equal amount of autologous irradiated PBMCs and peptide (5 μg/ml). T-cell growth factor (Biotest, Dreieich, Germany) was added 2 days after restimulation at a final concentration of 10%. The T-cell cultures were tested for peptide recognition by proliferation assay at day 28. Peptide-specific T-cell cultures were cloned by limiting dilution, and T-cell clones were subsequently tested for the recognition of E2-peptide-pulsed and -protein-pulsed APCs.

Specificity of the Th clones was analyzed as described previously (27). Notably, in proliferation assays in which Th clones were tested for protein recognition, autologous monocytes were used as APCs. For measurement of proliferation, cultures were pulsed with 0.5 μCi [¹²⁵I]thyminidine (5 μCi/ml; Amer sham, United Kingdom) per well for 18 h. Plates were harvested with a Micro cell Harvester (Skatron, Norway). Filters were packed in plastic covers containing 10 ml of scintillation fluid and subsequently counted on a 1205 Betaplate counter (Wallac, Turku, Finland). HLA class II blocking experiments were performed using murine monoclonal antibodies anti-DQ SPV.L3, anti-DR B8.11.2, and anti-DP B7/21. Supernatants of the proliferation assays were harvested 24 h after incubation and analyzed for the presence of IFN-γ by ELISA (27).

Detection of Memory Th Cells by ELISPOT. Memory cells (CD45RO⁺) were isolated freshly from buffy coats by MACS after incubation with CD45RO microbeads (Miltenyi Biotec, Germany). The purity of the obtained CD45RO⁺ fraction was >95% as determined by flow cytometry after surface staining for CD45RO and CD45RA (Becton Dickinson Biosciences). CD45RO⁺ cells were seeded at a density of 10⁵ cells/well in a 24-well plate (Costar) in 1 ml of IMDM supplemented with 10% FCS. Irradiated autologous cells (10⁶) were added to each well as APCs. The responder cells were incubated with either medium alone, pools of HPV16 E2 peptides at 5 μg/ml/peptide, MRM 1:50 dilution, or pools of influenza matrix peptides (positive controls) and cultured for 11 days to improve the detection of antigen-specific cells (28). The cells were then harvested, washed, and seeded in four replicate wells at a density of 5 × 10⁵ cells/well of a Multiscreen 96-well plate (Millipore, Ettten-Leur, The Netherlands) coated with an IFN-γ catching antibody. Per well, 10⁵ irradiated autologous PBMCs were added as APCs together with 5 μg/ml peptide. ELISPOT analysis was additionally performed according to the instructions of the manufacturer (Mabtech AB, Nutcha, Sweden). Analysis of the number of spots was done with a fully automated computer-assisted-video-imaging analysis system (Carl Zeiss Vision).

ICS of Memory T Cells. Autologous monocytes were isolated from PBMCs by adherence to a flat-bottomed 48-wells plate during 2 h in x-vivo 15 medium (Bio Whittaker, Verviers, Belgium) at 37°C and then used as APCs. CD45RO⁺ cells were stimulated for 11 days with peptide, then harvested, washed, and suspended in IMDM + 0.1% BSA at a concentration of 1.5 × 10⁶ cells/ml. Cell suspension (200 μl) was added to the monocytes + 200 μl of 10 μg/ml HPV16 E2 peptide (stimulated) or 200 μl of medium (nonstimulated control). After 1 h of incubation at 37°C, 800 μl of IMDM + 10% FCS + 12.5 μg/ml Brefeldin A (Sigma Chemical Co.) was added, and cells were incubated for another 5 h. The cells were then harvested, transferred into a V-bottomed 96-well plate, washed twice with ice-cold PBS, and fixed with 50 μl of parformaldehyde 4% for 4 min on ice. After fixation, the cells were washed once with cold PBS and once with PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1%. This was followed by an incubation in 50 μl of PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1% for 10 min on ice. Cells were washed twice with PBS/BSA 0.2%/Saponin 0.1%, and immunofluorescence was performed before 25 μl of PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1% containing 1 μl of FITC-labeled mouse-antihuman IFN-γ (0.5 g/ml; BD PharMingen), 2 μl of
Table 1 Immunogenicity of HPV16 E2-derived peptides

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a PBMC from 8 healthy blood donors were tested against HPV16 E2 peptides in short-term proliferation assays.

b HPV16 E2 peptides are indicated by the first and last amino acid.

c Peptides were scored positive, when the proliferation of ≥50% of the test wells exceeded the mean proliferation + 3 × SD of the control wells, and the mean stimulation index (SI) of all test wells over medium control wells was >3 in two independent assays.

Results

High Reactivity of Healthy Donor PBMCs against HPV16 E2-derived Peptides. We examined the proliferative responses of healthy donor PBMCs against HPV16 E2 protein by using an array of overlapping 30-mer peptides covering the entire E2 sequence. Incubation of freshly isolated PBMCs of eight HLA-typed donors with each of the 23 E2-derived 30-mer peptides showed that four of eight donors reacted to 2 or more of the peptides. The observed E2 peptide-specific proliferative responses were remarkably strong (Table 1). In all of the cases >75% of the parallel microcultures reacted against the stimulating peptide. For instance, in two independent experiments we found the peptide-specific proliferation of donor 8 against peptides E2<sub>231–60</sub>, E2<sub>246–75</sub>, E2<sub>91–120</sub>, E2<sub>151–180</sub>, E2<sub>271–300</sub>, and E2<sub>286–315</sub> to exceed background proliferation in 75–94% of all eight of the parallel microcultures tested (Fig. 1). This points at the presence of a very high frequency of E2-specific T cells in the PBMC isolates. In particular, PBMCs of donors 3, 5, and 8 displayed strong responses with a broad specificity. Please note that the 30-mer peptides have a 15 amino acid overlap with their neighboring peptides. Consequently, responses against adjacent peptides (e.g., donor 3, E2<sub>231–60</sub> and E2<sub>246–75</sub>) most likely involve the same epitope, whereas responses against nonadjacent peptides are directed against distinct epitopes. Not only the frequency of responding cultures but also the magnitude of the proliferative responses were remarkably high. The peptide-specific proliferation of several cultures from donors 3, 5, and 8 exceeded background with mean stimulation indices ranging from 9.2 to 16.5 and, as such, are comparable with responses found against the tetanus toxoid antigen in several of the donors (3, 4, 5, and 7; SI ranging from 13.3–25; see Table 1). These stimulation indices clearly indicate a high immunogenicity of the E2 protein contained in these peptides.
overlapping peptides comprised in this COOH-terminal region revealed to contain several highly immunogenic peptides. The thereby focusing on the COOH-terminal region, which our data (Table detailed analysis of the nature and specificity of such responses, immunogenic Th epitopes within HPV16 E2. We performed a more analysis of the specificity of these Th clones revealed that they and suggest that these responses may reflect T-cell memory.

HPV16 E2-specific Th Cultures Recognize Naturally Processed Epitopes. The proliferation data pointed at the existence of multiple immunogenic Th epitopes within HPV16 E2. We performed a more detailed analysis of the nature and specificity of such responses, thereby focusing on the COOH-terminal region, which our data (Table 1) revealed to contain several highly immunogenic peptides. The overlapping peptides comprised in this COOH-terminal region (E2\textsubscript{271-300}, E2\textsubscript{286-315}, E2\textsubscript{301-330}, E2\textsubscript{316-345}, and E2\textsubscript{331-365}) were tested for their capacity to bind to HLA-DR molecules. Each of the five peptides showed intermediate to strong binding to two or more of the common HLA-DR molecules tested (Table 2), which supports the notion that these peptides can indeed represent class II MHC-restricted Th epitopes. Subsequently, long-term E2-specific Th cultures were generated through stimulation of PBMCs from HLA-typed healthy blood donors at weekly intervals with either peptides E2\textsubscript{271-300} and E2\textsubscript{286-315} or peptide E2\textsubscript{331-365}.

Peptide E2\textsubscript{331-365} peptide in a DR1-restricted manner, both Th clones recognized peptide E2\textsubscript{331-365} in the context of HLA-DQ6(1) (Fig. 2, A and B). Finemapping of the peptides recognized by the two other Th clones revealed a similar relationship, in that one recognized peptide E2\textsubscript{346-355} in the context of DR1(2), whereas the other reacted against the E2\textsubscript{351-365} peptide in a DR1-restricted manner, both present in E2\textsubscript{331-365} (Fig. 2, C and D, and not shown). Notably, these

![Fig. 2.](image-url)
The number of antigen-specific T cells was analyzed by IFN-γ ELISPOT at day 11 (A). The mean number of spots per 50,000 PBMCs are shown; bars, ±SD. A mix of three recall antigens (MRM) was used as a positive control. HPV16 E2-specific T cells are contained in the CD4+ T-cell subset (B). In an independent experiment, CD45RO+ PBMCs of the same subjects were stimulated with indicated peptide pools, and the percentage of IFN-γ-producing cells was analyzed by ICS. The percentage of CD45RO+ PBMCs producing IFN-γ in response to peptide stimulation (right panels) versus medium control (left panels) is indicated.

Additional evidence that the four E2 peptides identified represent physiologically relevant Th epitopes is provided by the fact that the E2-specific Th clones did not only respond against peptide-loaded APCs, but also specifically responded against APCs that were pulsed with the E2 protein (Fig. 2, A–D, top panels). Because in the latter case presentation of the peptide epitopes depends on uptake and processing of the E2 antigen, and not merely on exogenous loading of class II molecules at the APC cell surface, these data provide definite proof that the four E2 peptides recognized by our Th clones correspond to naturally processed epitopes. Finally, all four of the Th clones produced IFN-γ on antigenic stimulation, which is indicative of a Th type 1 cytokine profile. Taken together, our data indicate that the T-cell repertoire of healthy individuals harbors IFN-γ-secreting E2-specific CD4+ Th cells (Fig. 2, A–D, bottom panels).

Detection of HPV16 E2-specific Memory Th Cells in Healthy Individuals. The strikingly frequent detection of HPV16 E2-specific Th immunity in healthy individuals, as described in the first paragraph, prompted us to analyze whether the underlying T-cell repertoire would represent immunological memory as the result of a previous encounter with antigen or whether it would primarily consist of particularly abundant naïve T-cell precursors specific for this antigen. In view of the high incidence of generally transient, genital HPV infections in young, sexually active individuals (2, 3, 6, 32), as well as the prominent expression of E2 during HPV infection (22), it is conceivable that T-cell memory against E2 could be found in healthy subjects. We examined the nature of the HPV16 E2-specific immunity detected by us through analysis of the E2-specific reactivity of the CD45RO+ fraction of healthy donor PBMCs, which contains antigen-experienced T cells but is devoid of their naïve counterparts. Because we found the T-cell repertoire of healthy donors to contain antigen-experienced T cells (Fig. 2), we measured the antigen-specific T-cell responses through IFN-γ ELISPOT. We first analyzed the reactivity of CD45RO+ T cells of two healthy donors while focusing our attention on a selection of E2 peptides that on basis of our previous experiments (Table 1) appeared to be localized in the most immunogenic regions of HPV16 E2. Interestingly, these CD45RO+ PBMCs were found to respond against multiple E2 peptides (Fig. 3A), supporting the notion that healthy subjects can display HPV16E2-specific T-cell memory. We confirmed that the responding IFN-γ-producing cells belonged to the CD4+ Th cell subset by using IFN-γ ICS instead of ELISPOT as a readout (Fig. 3B). A broader survey of E2-specific reactivity against the full array of peptides, using CD45RO+ PBMCs from eight additional healthy donors, revealed that four of these PBMC isolates responded against one or more of the HPV16 E2 peptides (Fig. 4 and not shown). Taken together, our data reveal the presence of CD45RO+ memory-type, IFN-γ-secreting Th cells reactive against HPV16 E2 peptides in approximately half of the healthy donors tested. Notably, the incidence by which these responses are detected is very similar to that of the strong (SI ≥ 3) proliferative responses found in total PBMCs (Table 1), implicating that these responses are also likely to represent reactivity by memory T cells rather than by in vitro primed naïve T cells.

Cross-Reactivity of HPV16 E2-specific Th Clones with Peptide Sequences of Other HPV Types. Because of the common nature of HPV infections, a majority of the human population is likely to encounter multiple HPV types (3, 33). Furthermore, the protein sequences of the viral gene products are conserved to considerable extent between HPV types. Therefore, it is possible that at least a fraction of the T-cell repertoire induced by a previous encounter with a given type of HPV could cross-react, and therefore cross-protect, during subsequent infection with other HPV types. Alignment of the sequence of the HPV16 E2 protein with that of various other HPV types revealed that it is most prominently conserved with that of other high risk types (Fig. 5C). Although this conservation is somewhat less conspicuous when the HPV16 E2 sequence is compared with that of low risk or common types (Fig. 5, A and B), it is evident that in all of the cases maximal conservation is confined to certain regions within the E2 sequence. In particular, three areas of HPV16E2 share homology with E2 of other HPV types: the NH2-terminal portions E231–120 and E2151–195 and the COOH-terminal portion E2271–365. These regions colocalize with the major functional domains of E2, in that the NH2-terminal domain harbors the transcriptional activation functions.
of this protein, whereas the COOH-terminal portion mediates its sequence-specific DNA-binding properties. The intervening sequences ranging from residues 210 to 270 constitute the so-called hinge region connecting the two key functional domains, which is poorly conserved between HPV types and also shows considerable intratypic variation for HPV16 (34–36). Interestingly, our analyses of E2-specific responses in short-term proliferation assays have revealed that the most immunogenic peptides are clustered in the two conserved domains of the HPV16 E2 sequence (see Table 1 and summary in Fig. 5D). In view of these considerations, we tested whether our established Th clones, raised against epitopes derived from the COOH-terminal part of the HPV16 E2 sequence (Fig. 2), would be capable of cross-reacting with E2 peptides of a number of other HPV types that shared maximal homology with HPV16 in regard to this particular E2 sequence. Indeed, the DQ6-restricted E2311–325-specific Th clone showed strong recognition of the corresponding peptides of HPV types 26, 31, 33, and 45 (Fig. 6A). The amino acid homology within this epitope varies from 73 to 87% (identical or amino acids with similar physico-chemical properties). Our other Th clones did not reveal considerable cross-reactivity for highly homologous E2 peptides of other HPV types (Fig. 6B and not shown). These data suggest that part of the HPV16 E2-reactive Th memory detected in our assays may relate to encounter of HPV types other than HPV16 but that the majority of this immune repertoire was most likely established through encounter with HPV16 itself.

**DISCUSSION**

Through analysis of the proliferative responses in PBMC cultures from healthy subjects against the HPV16 E2 antigen we have demonstrated that this protein contains highly immunogenic peptide sequences to which strong T-cell reactivity is detected. Subsequent testing of the CD45RO−memory fraction of healthy donor PBMCs of this protein, whereas the COOH-terminal portion mediates its sequence-specific DNA-binding properties. The intervening sequences ranging from residues 210 to 270 constitute the so-called hinge region connecting the two key functional domains, which is poorly conserved between HPV types and also shows considerable intratypic variation for HPV16 (34–36). Interestingly, our analyses of E2-specific responses in short-term proliferation assays have revealed that the most immunogenic peptides are clustered in the two conserved domains of the HPV16 E2 sequence (see Table 1 and summary in Fig. 5D). In view of these considerations, we tested whether our established Th clones, raised against epitopes derived from the COOH-terminal part of the HPV16 E2 sequence (Fig. 2), would be capable of cross-reacting with E2 peptides of a number of other HPV types that shared maximal homology with HPV16 in regard to this particular E2 sequence. Indeed, the DQ6-restricted E2311–325-specific Th clone showed strong recognition of the corresponding peptides of HPV types 26, 31, 33, and 45 (Fig. 6A). The amino acid homology within this epitope varies from 73 to 87% (identical or amino acids with similar physico-chemical properties). Our other Th clones did not reveal considerable cross-reactivity for highly homologous E2 peptides of other HPV types (Fig. 6B and not shown). These data suggest that part of the HPV16 E2-reactive Th memory detected in our assays may relate to encounter of HPV types other than HPV16 but that the majority of this immune repertoire was most likely established through encounter with HPV16 itself.

**DISCUSSION**

Through analysis of the proliferative responses in PBMC cultures from healthy subjects against the HPV16 E2 antigen we have demonstrated that this protein contains highly immunogenic peptide sequences to which strong T-cell reactivity is detected. Subsequent testing of the CD45RO−memory fraction of healthy donor PBMCs...
revealed overwhelmingly strong responses. Because half of the proliferation assays with HPV16 E2, as described in the present study, identification of memory type T-cell responses (30, 31). In contrast, we choose these nonstringent criteria, because we did not expect to record any HPV16 E7-specific responses in healthy donor PBMCs (19). In this former study we scored all of the responses positive that were above background (mean ± SD).

Fig. 6. Cross-reactivity of HPV16 E2-specific Th clones against corresponding peptide sequences derived from different HPV types. All four of the Th clones were tested for cross-reactivity with peptides derived from other HPV types sharing maximal homology with HPV16 in regard to their cognate epitope. The DQ1-restricted E2311–365-specific Th clone showed strong recognition of the homologous peptides derived from HPV types 26, 31, 33, and 45 (bold), whereas the other three Th clones, including the DR1-restricted E2311–365-specific Th clone (B) recognized only the HPV16 E2-derived peptide. In the CD45RO+ memory-type T cells against HPV16 E2. Although the low numbers of individuals tested precludes definitive conclusions, the T-cell reactivity detected by proliferation assay and ELISPOT revealed a similar peptide-reaction pattern.

Importantly, we have not found any previous report concerning the presence of memory T-cell responses against E2 or any of the other nonstructural HPV16 proteins in healthy individuals. In fact, HPV16-infected individuals diagnosed with CIN were particularly found to display Th responses against the COOH-terminal domain of HPV16 E2 at the time of viral clearance, whereas no E2-specific Th responses were detected in the healthy control group (25). However, the methodology applied for analysis of these responses is essentially different. The interleukin 2 bioassay used in the study by Bonktes et al. (25) measures the total response of all cultured cells and, therefore, lacks the sensitivity of single-cell cytokine analysis applied in the present study. Furthermore, the preselection of the CD45RO+ memory PBMC pool directly ex vivo (Figs. 3 and 4) allows enrichment of HPV16 E2-specific memory T cells, as well as reactivation of so-called central memory T cells by extended culturing of these cells in vitro without the risk of in vitro priming of naïve T cells. By using this highly sensitive method, we now reveal the presence of E2-specific Th cells in healthy individuals. It is interesting to note that HPV16 E2-specific IgG and IgA responses have also been detected in healthy individuals (37, 38). Because antibody isotype switching is Th-dependent, these observations constitute an independent confirmation of the presence of E2-specific Th memory in healthy subjects as reported in the present study.

In preclinical animal models, in particular the CRPV model, it was shown that the E2-specific immune response plays a role in spontaneous papilloma regression (23) and, furthermore, that regression could be enhanced by E2-directed vaccination (24, 39). Also in humans, there are strong indications that the E2-specific immune response is associated with HPV clearance (25). The strong immunogenicity of the E2 protein, together with the presence of E2-specific Th immunity in a large proportion of the human population, could be exploited in immunotherapy and prevention of HPV16-positive CIN lesions. Boosting of preexisting E2-specific Th memory, as established by previous encounters with HPV16 or other HPV types, can provide the powerful, pathogen-specific T-cell help that is required for optimal induction of cytotoxic T lymphocyte responses against various HPV16 antigens including E2, E6, and E7 (40, 41).

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Frequent Detection of Human Papillomavirus 16 E2-specific T-helper Immunity in Healthy Subjects

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