Occasional Memory Cytotoxic T-Cell Responses of Patients with Human Papillomavirus Type 16-positive Cervical Lesions against a Human Leukocyte Antigen-A*0201-restricted E7-encoded Epitope


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ABSTRACT

Most cervical carcinoma (Cxca) cells constitutively express human papillomavirus type 16 (HPV16) E6 and E7 oncoproteins. These proteins are, therefore, attractive targets for T cell-based immunotherapy. Previously, we identified HPV16 E7-encoded CTL epitopes. In patients with cervical intraepithelial neoplasia or Cxca, little is known concerning T-cell activity against viruses in general and against HPV16 in particular. Here, we have screened the blood of 10 healthy donor controls and of 22 patients with HPV16+ cervical lesions for the presence of CTLs directed against HPV16 E7- and control influenza virus matrix-derived epitopes presented by HLA-A*0201. We detected influenza virus-specific CTLs in all donors and in the majority of patients, indicating that most patients have functioning T-cell responses despite their lesions or therapeutic interventions. Moreover, we show that patients with HPV16+ lesions occasionally have memory CTLs against a HPV16 E7-encoded epitope (sequence YMLDLQPETT), providing evidence for natural CTL immunity against HPV16 in patients with cervical lesions.

Combined, these findings raise possibilities for vaccination with HPV16 E7-encoded peptides to induce or augment CTL responses for treatment or prevention of Cxca.

INTRODUCTION

Cxca is the second most common cancer in women worldwide (1). In more than 90% of these tumors, HPV DNA can be detected, predominantly of the HPV16 and HPV18 genotypes. These types of HPV, as well as other high-risk HPVs, have been implicated in the etiology of cervical cancer (2, 3). In the majority of cervical tumor cells, early regions 6 and 7 (E6 and E7) are constitutively expressed and required for maintenance of the transformed state (4, 5). For these reasons, the HPV16 E6 and E7 proteins are attractive tumor-specific targets for T cell-based immunotherapy of Cxca.

Cellular immune responses, particularly those involving CTLs, are important in the defense against many viral infections and (virus-induced) tumors (6, 7). CD8+ CTLs recognize intracellularly processed peptide epitopes that are presented at the cell surface in the context of MHC class I molecules (8–10).

In the control of HPV-associated tumors, there are several indications for involvement of T cell-mediated immunity: (a) there is an increased prevalence of HPV-related diseases in immunosuppressed patients (11); (b) loss of expression of certain HLA genotypes has been associated with poor clinical stage in patients with cervical lesions (12); and (c) in rodents, rejection of tumors expressing HPV-encoded tumor antigens mediated by tumor-specific T-cell responses has been reported (13–16).

We set out to identify human CTL epitopes derived from HPV16 E6 and E7 following a multistep approach. HPV16 E6- and E7-encoded peptides have been tested with respect to binding affinity to the most common HLA-A molecules (17, 18). Nine peptides capable of binding to HLA-A*0201 have subsequently been used in immunogenicity studies. Some of these peptides were immunogenic both in vitro to PBMCs of HLA-A*0201+ healthy donors and in vivo in HLA-A2 transgenic mice (19). These immunogenic peptides induced CTL responses that recognized HLA-A*0201+ and HPV16+ Casca cells, suggesting that these peptides represent endogenously processed HLA-A*0201-restricted CTL epitopes of HPV16 (19) that can be used as active components in vaccines for the prevention or treatment of human Cxca.

The role of HPV16-specific CTLs in preventing the onset of CIN and the progression to Cxca is not well defined. To probe the role of these CTLs, estimation of the presence of HPV16-specific antitumor CTLs (precursors) in the blood of patients with either premalignant or malignant cervical lesions is desirable. Moreover, knowledge of the strength of the natural CTL response against HPV16 allows evaluation of the potential clinical utility of immunotherapeutic protocols to stimulate anti-HPV16 CTL responses.

In this study, we have used an in vitro restimulation protocol to screen the PBMCs of 10 HLA-A*0201+ healthy donors, 11 HLA-A*0201+, HPV16+ CIN patients, and 11 HLA-A*0201+, HPV16+ Casca patients for the presence of CTLs directed against two HLA-A*0201-binding peptide epitopes encoded by HPV16 E7 (epitopes 11-20 and 86-93) and, as a reference, against the HLA-A*0201+ restricted influenza virus A matrix epitope 58-66 (20). The relevance of our findings for the use of peptide-based anti-Casca vaccines is discussed.

MATERIALS AND METHODS

Donors and Patients. Donor blood samples were obtained from the Blood Bank, University Hospital Leiden, and patient blood samples were taken in the Departments of Gynaecology, University Hospital Leiden, and Reinier de Graaf Hospital, Delft. Informed consent for blood donations was obtained from all individuals. CIN or Casca status of the patients was diagnosed at the Departments of Gynaecology and Pathology. Additionally, at the Department of Pathology, University Hospital Leiden, and at the Department of Gynaecology, Reinier de Graaf Hospital, Delft, HPV16 typing on samples of the cervical lesions was performed by touchdown PCR analysis using HPV16-specific primers (21).

PBMCs of healthy donors and of patients were purified by centrifugation on...
a Ficoll-Isopaque gradient and were routinely stored in liquid nitrogen in 10% DMSO (Merck, Darmstadt, Germany)/45% FCS.

HLA typing was performed serologically at the tissue typing laboratory, University Hospital Leiden. HLA-A*02-subtyping of the serologically HLA-A2*-typed PBMCs was achieved by the amplification refractory mutation system-PCR method and primer-mixes as described by Krausa et al. (22) in a Gene Amplification PCR System 9600 (Perkin Elmer Cetus), which allows subtyping for HLA-A*0201 to HLA-A*0214.

Table 1 summarizes the characteristics of the healthy donors and of patients with CIN or Cxca included in this study.

**Cell Lines.** Cell lines were cultured in complete culture medium consisting of Iscove's modified Dulbecco's medium with Glutamax I (GIBCO-BRL, Paisley, Scotland) supplemented with 8% FCS, 100 IU/ml penicillin (Brocades Pharma, Leiderdorp, the Netherlands), and 20 μM 2-mercaptoethanol (Merck, Darmstadt, Germany) at 37°C in humidified air containing 5% CO2.

JY is a homozygous HLA-A*0201* EBV-transformed B-cell line. Influenza virus-infected JY cells were prepared by a 1-h incubation at 37°C with a dose of the influenza virus strain A/HK/68 of 150 plaque-forming units/5 × 106 cells, and were subsequently cultured overnight in complete culture medium. CaSkii is a HLA-A*0201* Cxca cell line expressing the HPV16 E6 and E7 proteins (American Type Culture Collection, Rockville, MD; Refs. 4 and 23). CaSkii cells were incubated with 20 units/ml of human IFN-γ (Genzyme Diagnostics, Cambridge, MA) for 48 h before use in 51Cr release cytotoxicity assays. The K562 erythroleukemia cell line is a NK cell-sensitive target cell and was used to block NK-like activity in 51Cr release cytotoxicity assays.

**In Vitro Evaluation of Human CTL Responses.** To determine CTL responses in the subjects, responder PBMCs were cocultured with synthetic peptides according to a recently published protocol (24). In brief, responses in the subjects, responder PBMCs were cocultured with synthetic peptides and antigen-presenting cells. The K562 erythroleukemia cell line is a NK cell-sensitive target cell and was used to block NK-like activity in 51Cr release cytotoxicity assays.

PBMCs were cultured in 24-well tissue culture plates in 1 ml RPMI (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated HPS, 100 IU/ml penicillin, 4 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 μM 2-mercaptoethanol (RPMI-10% HPS medium). At days 3 and 6, wells were fed with 1 ml medium containing human rIL-2-1 (10 IU/ml final concentration; Eurocetus, Amsterdam, the Netherlands). At day 7, the T cells were restimulated as follows. Autologous PBMCs (35 Gray-irradiated; 3—4 × 106 cells/well) were allowed to adhere for 2 h at 37°C in RPMI-5% HPS medium containing 10 μg/ml synthetic peptide and 3 μg/ml human β2-microglobulin (Sigma Chemical Co., St. Louis, MO). After removal of nonadherent PBMCs by gentle washing, 1—2 × 106 responder T cells were added to the peptide-pulsed adherent PBMCs in 1 ml RPMI-10% HPS medium. Two days later, wells received 1 ml rIL-2-containing medium. At days 7 and 14, T cells were assayed for cytotoxic activity.

**51Cr Release Cytotoxicity Assay.** CTL activity was measured in a standard chromium release assay as described previously (17). Peptide-pulsed targets were prepared by incubating the cells with synthetic peptide (3 μg/ml) for at least 30 min at 37°C. Target cells were added to various numbers of effectors, which were preincubated with unlabelled K562 cells (1 h at 37°C, in 20-fold excess of the target cells) in a final volume of 150 μl of complete culture medium in 96-well U-bottomed microtiter plates. After 4 h of incubation at 37°C, 100 μl of supernatant was harvested and measured in a counter. The mean percentage specific of triplicate wells was calculated as follows:

\[
\text{% specific lysis} = \left( \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \right) \times 100
\]

To more readily allow comparison of responses, the data are expressed as LUs extrapolated to 106 effector cells; 1 LU is defined as the number of effectors required to induce 30% lysis of 105 target cells during a 4-h assay. Specific CTL activity is obtained by subtracting the LUs obtained in the absence of antigen from the LUs obtained in the presence of antigen. When

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* ND, not determined; NA, not applicable.

* CIN, patient with cervical intraepithelial neoplasia; Cxca, patient with squamous cell carcinoma of the uterine cervix.

* Stage according to Fédération Internationale des Gynécologues et Obstétristes.
In Vitro Evaluation of Human T-Cell Proliferation. To assess proliferative T-cell responses in the subjects, PBMCs (1.5 × 10^6/well) were stimulated in 96-well flat-bottom plates with 4 μg/ml PHA (Wellcome Diagnostics, Dartford, UK) in RPMI-10% HPS medium (experimental wells) or with medium alone (control wells). Routinely, cells were plated in replicate sets of four to six wells per condition. After 7 days, 135 μl of medium was removed from each well and replaced with fresh medium containing IL-2 (10 IU/ml final concentration). 0.5 μCi methyl[^3]H]-thymidine (NEN-Dupont, Boston, MA) was added to each well 48 h later. Cells were harvested on glass fiber mats the next day and quantitated for[^3]H]-thymidine incorporation. The PI was calculated as follows:

$$PI = \frac{\text{mean cpm experimental wells}}{\text{mean cpm control wells}} \times 100$$

Responses were considered positive when the PI exceeded 10.

Statistical Analysis. To determine whether the reference CTL responses against the influenza virus matrix epitope in patients were significantly weaker than those in the healthy donors, a sign test was performed as follows. In each cytotoxicity assay with PBMCs of patients, at least one donor was included as control for test conditions. For each assay, comparison of the LUs of each patient to those of each donor resulted in classification in one of three categories: "<", "=", or ">." Next, from the total in category "<" compared to the total in the combined categories "<" and ">," a 95% CI was deduced.

RESULTS

Evaluation of T-Cell Responses in Healthy Donors and Patients. Because immunosuppressive effects introduced by the malignancy (26) or by radio- and chemotherapy of cancer patients could frustrate specific immunotherapeutic approaches and CTL responses in general, we used the presence of CTLs directed against influenza virus in the blood of HLA-A*0201" subjects (Table 1) as a reference. This widely encountered virus has evoked CTL responses in most, if not all, adult human beings.

As expected, all healthy donors (10 of 10) and the majority of patients (9 of 11 CIN and 8 of 11 Cxca patients) showed evidence of
CTL immunity against the HLA-A*0201-restricted influenza virus matrix epitope (M.58-66; Fig. 1a). CTLs of two representative donors (donors 3 and 4) are capable of lysing influenza virus-infected HLA-A*0201+ target cells, indicating that these CTLs are not only peptide-specific but are also able to recognize endogenously processed antigen (Fig. 2). These results show that most Cxca patients do not suffer from a general inability to exert CTL reactivity against viral CTL epitopes.

The influenza virus M.58-66-specific CTL responses tended to be less vigorous in each of the CIN and Cxca patient groups than in healthy donors (in each group, 67% of the patients showed a reduced immune response, with a 95% CI of 43–87% as deduced from a sign test). Combining the CTL responses observed in both patient groups resulted in weak evidence for significantly reduced influenza virus-directed immunity in the patients (68%, with a 95% CI of 51–81%). This may reflect a reduced CTL immunity due to their cervical lesions and/or therapeutic interventions.

The observation that PBMCs of most patients are not in a functionally inactive state is further supported by the vigorous proliferative responses to the polyclonal mitogen PHA in all subjects tested, with the exception of one Cxca patient (patient 9; Fig. 1b). It is interesting that, of the patients studied, patient 9 had the most severe malignancy (Fédération Internationale des Gynécologues et Obstétristes stage IIIa) and had received both radio- and chemotherapy (Table 1).

Combined, these results show that, in general, PBMCs of both healthy donors and of patients are able to respond to peptide antigens and to mitogens, illustrating that T-cell responses in the carcinoma patients are not fully impaired and may be exploited for antitumor immune intervention.

**In Vitro Detection of HPV16 E7-directed CTL Responses.** The data presented above show that memory CTL activity directed against viral epitopes can be revealed by our in vitro restimulation protocol.

To evaluate the baseline HPV16-specific CTL activity before E7-directed CTL epitope vaccination, it is necessary to assess the presence in PBMCs of CTLs capable of reacting with the E7-encoded peptides to be included in a vaccine. To this end, we tested the CTL-mediated reactivity against two known HLA-A*0201-restricted CTL epitopes (HPV16 E7.11-20 and E7.86-93; Ref. 19; Fig. 3).

None of the donors or patients responded in vitro to restimulatation with HPV16 E7.86-93 (Fig. 3b). Except for a weak and inconsistent response in donor 9, no E7.11-20-directed CTL responses were observed in healthy donors. In contrast, HPV16 E7.11-20-specific CTL responses (>5 LUs) were detected in PBMCs of 2 of 11 CIN patients and 2 of 11 Cxca patients (Fig. 3a). These data indicate that at least some HLA-A*0201+ patients with HPV16 DNA in their cervical lesions have naturally generated a HPV16 E7.11-20-directed CTL response.

**Lysis of Cxca Cells.** In 4 of 22 CIN and Cxca patients tested, CTL responses directed against peptide E7.11-20 were observed. Nonetheless, it is important to know whether these peptide-specific CTLs are capable of lysing HPV16 E7-containing tumor cells.

To this end, we were able to test the lytic activity of CTLs of Cxca patient 2 on HLA-A*0201+, HPV16+ CaSki cells. PBMCs of this patient (Cxca 2), restimulated with peptide E7.11-20, were not only peptide-specific, but also lysed CaSki cells (Fig. 4), indicating that these CTLs recognize the endogenously processed CTL epitope. PBMCs stimulated in vitro with E7.86-93 that were not peptide-specific also did not recognize CaSki cells. These data suggest the existence in at least some Cxca patients of CTLs directed against E7.11-20 that are capable of lysing tumor cells expressing the naturally processed HPV16 E7 antigen.

**DISCUSSION**

Our goal is to develop vaccines to induce or augment CTL responses against the HPV16 E6 and E7 oncoproteins for treatment or prevention of human Cxca. This is based on a C57BL/6 mouse model,
in which vaccination with a HPV16 E7-derived synthetic peptide has been shown to induce protective CTL-mediated immunity against the outgrowth of a lethal dose of HPV16-transformed cells (14). In addition, adoptive transfer of a CTL clone directed against this peptide epitope was capable of eradicating established HPV16-induced tumors (15). It is interesting that this protective immunity was achieved by vaccinating with a subdominant CTL epitope, reactivity to which is not detectable in animals immunized with the tumor cells (14, 15). This indicates that peptide vaccination can have an added value over immunization with tumor cells.

In CIN and Cxca patients, however, little is known concerning the existence and possible role of T-cell activity against viruses in general and against HPV16 in particular. In this study, we have investigated whether HLA-A*0201-restricted CTLs capable of reacting against the influenza virus M.58-66 epitope and against two HPV16 E7-encoded epitopes (11-20 and 86-93; Ref. 19) are present in the blood of healthy donors (n = 10) and of patients with cervical lesions at various stages of their diseases (n = 22). Our results demonstrate for the first time that both influenza virus-specific and, less frequently, HPV16-specific CTL responses can be detected in the blood of CIN and Cxca patients. In control healthy donors, we only observed immunity against influenza virus.

The detection of CTL immunity in Cxca patients is of particular interest in view of possible immunosuppressive effects induced by their malignancy or by the radio- and/or chemotherapy they underwent. The fact that these patients generally appear to be CTL competent raises possibilities for the priming or boosting of virus-specific CTL responses by immune intervention.

In the present study, we have assessed CTL responses by in vitro restimulation. Therefore, it is likely that the responses observed rep-
resistant memory CTLs induced by viral infection. Indeed, these peptide-restimulated CTLs also recognize target cells expressing the endogenously processed antigens. In the case of HPV16 E7-directed CTL responses, the fact that E7.11-20-specific memory CTLs were not observed among donor PBMCs further supports the notion that CTL responses detected in the patients have been induced in vivo. Alternatively, using a long-term in vitro induction protocol, we demonstrated that CTL precursors against both E7-encoded peptides exist even in the PBMCs of healthy donors, as reported previously (19). Combined, these data indicate that the immune responses detected in this study represent virus-specific memory CTLs.

The HPV16 E7-directed CTL responses, found in 4 of 22 CIN and Cxca patients, are directed against peptide E7.11-20 but not against peptide E7.86-93. Therefore, the response against E7.11-20 appears to be immunodominant over the response against E7.86-93 in all four patients tested. Murine studies demonstrated that tumor-protective immunity can also be established with a subdominant CTL epitope (14, 15). Indeed, in HLA-A2 transgenic mice, CTL specificities against both the E7.11-20 and the E7.86-93 epitopes can be induced by in vivo immunization with a peptide mixture containing both peptides (19). Therefore, both HPV16-encoded peptide epitopes are good vaccine candidates.

One wonders why most HPV16-infected patients have good CTL memory against the influenza virus matrix peptide but not against the E7.86-93 peptide and only occasionally against the E7.11-20 peptide. An important factor may be poor HLA class I expression on HPV-infected cells (27, 28). Alternatively, factors related to immunodominance or to the anatomical site of infection, including poor costimulation and cytokine assistance, may have caused poor natural CTL immunity to the HPV16-derived peptides versus the influenza virus matrix peptide. Also, repeated exposure to influenza viruses may have induced a higher level of CTL memory against influenza virus than against HPV16. We consider this generally much weaker response against two high-affinity HLA-A*0201-binding E7-encoded peptides, however, to be a good starting point for vaccination against these E7 peptides.

Including T-helper epitopes in a CTL epitope-containing vaccine might enhance the generation of a CTL response, as indicated by a recent report on a peptide-based hepatitis B virus trial in healthy volunteers (24). Recently, a suitable pan-HLA-DR-binding T-helper epitope has been designed (29). In immunization protocols in vivo and in vitro, this peptide (965.10), which binds with high affinity to virtually all HLA-DR molecules, induces strong proliferative, primary responses (29).

In conclusion, based on these results added to our previous studies (17—19), the two most immunogenic HPV16 E7-encoded peptides (E7.11-20 and E7.86-93) and a pan-HLA-DR-binding T-helper epitope (965.10; Ref. 29) have been selected as active components in a prototype vaccine for patients with Cxca. Currently, a phase I/II clinical trial is in progress in our institute to assess whether antitumor CTL immunity specific for the E7.11-20 and E7.86-93 peptides can be primed or boosted by peptide immunization of Cxca patients with residual disease after conventional treatment. In this way, we will gain more insight into the role of HPV16-specific CTLs in the prevention and treatment of cervical cancer.

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REFERENCES


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