3

Skin reactions to Human Papillomavirus 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia

Muriel van den Hende, Mariëtte I.E. van Poelgeest, Jeanette M. van der Hulst, Joan de Jong, Jan W. Drijfhout, Gert Jan Fleuren, A. Rob P.M. Valentijn, Amon R. Wafelman, Gijs M. Slappendel, Cornelis J.M. Melief, Rienk Offringa, Sjoerd H. van der Burg and Gemma G. Kenter

Adapted from Int J Cancer 2008; 123:146-152

Abstract

We have tested the safety and feasibility of a synthetic long peptide-based HPV16-specific skin test to detect cellular immune responses to HPV16 E2, E6 and E7 *in vivo*. Women with cervical neoplasia (n = 11) and healthy individuals (n = 19) were intradermally challenged with 8 different pools of HPV16 E2, E6 and E7 peptides. The skin test was safe as the injections were perceived as mildly painful and no adverse events were observed. The majority of skin reactions appeared significantly earlier in HPV16+ patients (<8 days) than in healthy subjects (8-25 days). The development of late skin reactions in healthy subjects was associated with the appearance of circulating HPV16-specific T cells and the infiltration of both HPV16-specific CD4+ Th1/Th2 cells and CD8+ T cells into the skin. These data show that the intradermal injection of pools of HPV16 synthetic long peptides is safe and results in the migration of HPV16-specific T cells into the skin as well as in an increase in the number of circulating HPV16-specific T cells. The use of this test to measure HPV16-specific immunity is currently tested in a low resource setting for the measurement of spontaneously induced T-cell responses as well as in our HPV16 vaccination trials for the detection of vaccine-induced immunity.

Introduction

The main cause of cervical carcinoma and cervical intraepithelial neoplasia (CIN) is a persistent infection with one of the high-risk, oncogenic human papilloma viruses [Bosch 2002; Munoz 2003]. Anogenital infection with a high-risk HPV type is very common [Burk 1996; Karlsson 1995; Koutsky 1997]. The rate of incidence of infection is estimated to be 80 to 85% worldwide [Jenkins 1996]. Fortunately, the majority of infected subjects will clear the virus infection within a year [Evander 1995; Ho 1998] and only a small proportion of women will become persistently infected and are at risk to develop HPV related malignancies [Kjaer 2002; Remmink 1995]. Cell mediated immune responses play an important role in controlling HPV infections [Ozsaran 1999; Sun 1997].

Delayed type IV hypersensitivity (DTH) reactions are used as a general measure of cell-mediated immunity *in vivo* [Turk 1975]. A local inflammatory reaction (induration and erythema), orchestrated by activated cytokine releasing CD4+ memory T cells, usually appears within 24 to 72 hours after intradermal injection of antigen [Black 1999; Poulter 1982; Vukmanovic-Stejic 2006]. Delayed type hypersensitivity reactions have been used to demonstrate an encounter with pathogens (*e.g.* Mantoux Test), vaccine-induced immune responses [Huebner 1993; Jaeger 1996; Rieser 1999; Thomas-Kaskel 2006], and in particular to show HPV-specific immune responses in various animal models [Chambers 1994; Höpfl 1993; Vambutas 2005]. Höpfl et al. were the first to study HPV16-specific cellular immunity by skin test in humans [Höpfl 1991; Höpfl 2000] and showed that a skin reaction, appearing within 2 to 6 days after intradermal injection with HPV16 E7, was correlated with regression of HPV induced CIN lesions.

We have previously studied the HPV16-specific T-cell responses in great detail *in vitro*, and the results of these studies suggested that the HPV16 E2, E6 and E7 specific type 1 and type 2 T-cell response was associated with the control of HPV16 induced disease [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. Based on these results we designed a HPV16-specific skin test, consisting of the most immunogenic regions of the early proteins E2, E6 and E7 [de Jong 2002a; Welters 2003], that might be used to screen spontaneously- and vaccine-induced immune responses to HPV in large groups of individuals and in areas where the access to specialized laboratories is limited. In this study, we have tested the safety and feasibility of a synthetic long peptide-based HPV16-specific skin test to detect cellular immune responses to HPV16 E2, E6 and E7 *in vivo.*

Materials and methods

Study design

A cross-sectional study to analyse HPV16 E2-, E6-, and E7-specific T-cell responses as measured by intradermal injection of pools of clinical grade HPV16 peptides in the upper arm was performed in patients with HPV16-related disorders of the cervix

and in healthy individuals. Since a delayed type hypersensitivity reaction represents a memory T-cell response, there was no prerequisite for HPV16-positivity at the time of analysis. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center.

Subjects

Eleven women (P) with a history of HPV16 positive cervical carcinoma (n = 8) or CIN (n = 3) and a group of nineteen healthy individuals (HS) participated in this study after providing written informed consent. The clinical characteristics of the patients are summarized in Table 1. The age of the patients ranged from 29-72 years (median age, 46 years). The healthy volunteers displayed a median age of 31 years old (range, 20-51 years) and comprised of 80% women and 20% males. Peripheral blood mononuclear cells (PBMCs) were obtained from all subjects immediately before administration of the skin test. The late appearance of positive skin tests in healthy individuals made us decide to isolate a second blood sample, which could be drawn from 11 of 19 healthy volunteers.

DTH Skin test

Skin tests, based on Delayed Type Hypersensitivity reactions (DTH), can be used as a sensitive and simple method for in vivo measurement of HPV-specific cellular immune responses [Höpfl 1991; Höpfl 2000]. The skin test preparations consisted of 8 pools of long clinical-grade synthetic peptides spanning the whole HPV16 E6 and E7 protein and the most immunogenic regions of HPV16 E2 protein [de Jong 2004]. These clinical

Table 1. Patient characteristics.

Patient	Age (yrs)	Diagnosis	Grade/stage	Treatment	Time (months)[a]	HPV[b]
1	72	CxCa	IB[c]	radical hysterectomy	9	16
2	57	CxCa	IA	radical hysterectomy	18	16
3	50	CxCa	IB	radical hysterectomy	6	16
4	44	CxCa	IA	hysterectomy	36	16
5	34	CxCa	IB	radical hysterectomy	48	16
6	44	CxCa	IB	radical hysterectomy	7	16
7	43	CxCa	IB	radical hysterectomy	39	16
8	44	CxCa	IIA	radical hysterectomy	10	16
9	29	CIN	III	LEEP[d]	2	16
10	42	CIN	II	LEEP	3	16
11	44	CIN	III	LEEP	12	16

[a] Time of treatment before skin tests were performed. [b] HPV detection by polymerase chain reaction in paraffin embedded tissue. [c] Cervical cancer stage according to FIGO. [d] Loop electrosurgical excision procedure.

grade peptides were produced in the interdivisional GMP-Facility of the LUMC. Each pool of the skin test consisted of 2 or 3 synthetic peptides, indicated by the first and last amino acid of the region in the protein covered by the peptides. *Pool 1*: $E2_{31-60}$, $E2_{46-75}$, *Pool 2*: $E2_{301-330}$, $E2_{316-345}$, *Pool 3*: $E6_{1-31}$, $E6_{19-50}$, *Pool 4*: $E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$, *Pool 5*: $E6_{85-109}$, $E6_{91-122}$, *Pool 6*: $E6_{109-140}$, $E6_{127-158}$, *Pool 7*: $E7_{1-35}$, $E7_{22-56}$, *Pool 8*: $E7_{43-77}$, $E7_{64-98}$. Per peptide pool 0.05 ml of 0.2 mg/ml peptides in 16% DMSO in 20 mM isotonic phosphate buffer (10 μg/peptide) was injected intracutaneously. The pools of peptides and a negative control (vehicle of solvent only) were injected separately at individual skin test sites of the upper arm. Skin test sites were inspected at least three times, at 72 hours and 7 days after injection of the peptides [Höpfl 1991; Höpfl 2000] and at 3 weeks following the first report of a very late skin reaction in one of the first healthy subjects. Reactions were considered positive when papules greater than 2 mm in diameter arose no less than 2 days after injection. Healthy individuals with a positive skin reaction were asked to consent to the collection of a punch biopsy (4 mm). When consent was given, the punch biopsies were cut in small pieces and cultured in IMDM (Cambrex Bio Science, Verviers, Belgium) containing 10% human AB serum, 10% TCGF and 5 ng/ml interleukin 7 (IL 7) and IL15 to allow the emigration of lymphocytes out of the skin tissue [Piersma 2008]. After 2 to 4 weeks of culture the expanded T cells were harvested and tested for their HPV-specific reactivity.

Antigen for in vitro immune assays

A set of peptides, similar to the peptides used in the skin test, were used for T-cell stimulation assays and IFNγ ELISPOT assays. The four HPV16 E2 peptides consisted of 30-mer peptides overlapping 15 residues, HPV16 E6 consisted of 32-mers and HPV16 E7 of 35-mers, both overlapping 14 residues. The peptides were synthesized and dissolved as previously described [van der Burg 1999]. Notably, in the IFNγ ELISPOT assays peptide pool 4 and 5 slightly differed from the peptide pools used in the skin test, pool 4 contained peptides $E6_{37-68}$, $E6_{55-86}$, $E6_{73-104}$ and pool 5 comprised peptides $E6_{73-104}$, $E6_{91-122}$.

Memory response mix (MRM 50x), consisting of a mixture of tetanus toxoid (0.75 *Limus flocculentius*/ml; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (5 μg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands), and *Candida albicans* (0.15 mg/ml, HAL Allergenen Lab., Haarlem, The Netherlands) was used as a positive control. Recombinant HPV16 E2, E6 and E7 proteins were produced in recombinant *Escherichia coli* as described previously [van der Burg 2001].

Analysis of Antigen-specific Th Cells by IFNγ ELISPOT

The presence of HPV16-specific Th cells was analyzed by ELISPOT as described previously [van der Burg 2001]. Briefly, fresh PBMCs were seeded at a density of 2 x 10^6 cells/well of a 24-well plate (Costar, Cambridge, MA) in 1 ml of IMDM (Cambrex Bio Science) enriched with 10% human AB serum, in the presence or absence of the

indicated HPV16 E2, E6 and E7 peptide pools. Peptides were used at a concentration of 5 µg/ml/peptide. After 4 days of incubation at 37°C, PBMCs were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100µl IMDM enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT was performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted-video-imaging analysis system (Bio Sys, Karben, Germany). Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in experimental wells [van der Burg 2001].

T-cell proliferation assay

T-cell cultures of the skin biopsies were tested for recognition of the specific peptides and protein in a 3-day proliferation assay [van der Burg 2001]. Briefly, autologous monocytes were isolated from PBMCs by adherence to a flat-bottom 96-well plate during 2 h in X-vivo 15 medium (Cambrex Bio Science) at 37°C using $2x10^5$ PBMC per well. The monocytes were used as APCs, loaded overnight with 10 µg/ml peptide and 20 µg/ml protein. Skin test infiltrating lymphocytes were seeded at a density of 2-5 x 10^4 cells/well in IMDM supplemented with 10% AB serum. Medium alone was taken along as a negative control, phytohemagglutinine (0.5 µg/ml) served as a positive control. Proliferation was measured by [^{3}H]thymidine (5 µCi/mmol) incorporation. A proliferative response was defined specific as the stimulation index (SI) ≥ 3. Supernatants of the proliferation assays were harvested 48 hours after incubation for the analysis of antigen-specific cytokine production.

Analysis of cytokines associated with HPV16-specific proliferative responses

The simultaneous detection of six different Th1 and Th2 cytokines: IFNγ, tumor necrosis factor α, IL2, IL4, IL5 and IL10 was performed using the cytometric bead array (Becton Dickinson, Erebodegem-Aalst, Belgium) according to the manufacturer's instructions. Cut-off values were based on the standard curves of the different cytokines (100 pg/ml IFNγ and 20 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and >2x the concentration of the medium control [de Jong 2004].

Intracellular Cytokine Staining (ICS)

The specificity and character of the T-cell cultures derived from positive skin reaction sites was tested by ICS as reported previously [de Jong 2005]. Briefly, skin test infiltrating lymphocytes were harvested, washed and suspended in IMDM + 10% AB serum and 2-5 x 10^4 cells were added to autologous monocytes that were pulsed overnight with 50 µl peptide (10 µg/ml) or protein (20 µg/ml) in X-vivo medium. Medium alone was taken along as a negative control, phytohemagglutinine (0.5 µg/ml) served as a positive control. Samples were simultaneously stained with FITC-labelled anti-IFNγ (clone

4S.B3, BD PharMingen, San Diego, CA), PE-labelled anti-IL5 (clone JES1-39D10, BD PharMingen), APC-labelled anti-CD4 (clone SK3, BD PharMingen) and PerCP-labelled anti-CD8 (clone SK1, BD PharMingen). After incubation at 4°C, the cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Statistical Analysis

Fisher's Exact test (2-tailed) was used to analyze the relationship between the detection of IFNγ-producing HPV-specific T cells in PBMC, the presence of a skin test reaction or the presence of HPV-specific T cells in skin biopsies, as well as differences between patients and healthy controls with respect to the size or the number of the skin reactions within these groups. Statistical analyzes were performed using Graphpad Instat Software (version 3.0) and Graphpad Prism 4.

Results

Skin reactions to intracutaneous injection with HPV16 E2, E6 and E7 peptides

We studied skin reactions in both healthy subjects and patients with a history of HPV16 induced disease after intracutaneous injection with HPV16 E2, E6 and E7 peptides. The injections were perceived as mildly painful and no adverse events were observed, indicating that the use of this skin test was safe. Positive skin reactions appeared as flat reddish papules of 2 to 20 mm of diameter, arising within 2 to 25 days after injection. A positive skin reaction was detected in 46 of the 152 test sites in the control group and in 22 out of 88 test sites in the patient group.

A classical DTH reaction, within 24 to 72 hours after injection, was only observed in 1 patient (P4, Figure 1). The time for the other skin reactions to appear, differed considerably between the groups of patients and healthy volunteers. Significantly more patients (6 out of 11) developed a positive skin reaction within 8 days as compared to healthy subjects (2 out of 19, p = 0,03 two tailed Fisher's exact test; Figure 1). Whereas the chance to detect a later skin reaction was significantly higher in the healthy control group (p = 0,007, two tailed Fisher's exact test; Figure 1). More than 90% of the positive skin reactions (43 out of 46) in this control group were detected at 8 or more days after injection. In 3 of the patients and 1 healthy control (P3, P10, P11 and HS5), both early and late positive skin reactions were observed within the same subject. Over all, each peptide-pool present in the skin test was able to induce a positive skin reaction in one or more subjects. Noteworthy, the majority of early positive skin reactions in the patients was directed against one or more of the E6 peptide pools (10 out of 14 test sites), or to E2 in combination with an E6 response. Only one patient (P7) developed an early positive skin reaction not accompanied by an E6 immune response (Figure 1). In the control group the majority of reactions were against $E2_{31-75}$ (10 out of 19 subjects) and $E6_{41-95}$ (9 of 19). This reaction pattern resembles that of the responses previously found in PBMC [de Jong 2002a; Welters 2003], as well as resembles the skin reaction pattern observed in the patient group (Figure 1). The size of the skin reactions did not differ between the two groups.

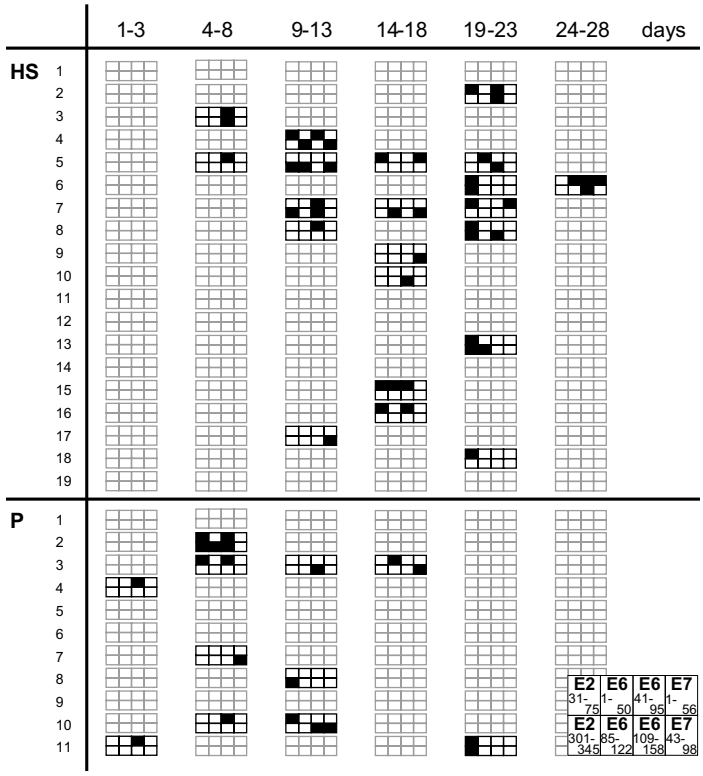

Figure 1. Summary of skin reactions in patients and healthy subjects. An overview of the number, day of appearance and injected antigen that induced a positive skin reaction in the group of 19 healthy subjects (HS) and 11 patients (P) with a history of HPV16 related cervical neoplasia. Skin reactions were considered positive when papules greater then 2 mm in diameter arose no less then 2 days after injection. The indicated layout is used for the 8 peptide pools, the first and last amino acid in the protein of the peptide pool used is indicated. The layout printed in bold indicates at least one positive reaction within this timeframe; a filled square represents a new developed, positive skin reaction to the indicated peptide pool. Significantly more patients developed a positive skin reaction within 8 days as compared to healthy subjects ($p = 0.03$ two tailed Fisher's exact test). Whereas the chance to detect a later skin reaction was significantly higher in the healthy control group ($p = 0.007$, two tailed Fisher's exact test).

Late skin reactions in healthy subjects are associated with an increase of HPV16-specific T cells in the peripheral blood.

To compare the results of the skin test with the presence of circulating HPV16-specific type 1 T cells, an IFNγ ELISPOT assay was performed with PBMC's collected before the intradermal peptide-challenge was given. In the 2 healthy subjects with an early skin reaction we were able to detect a HPV16-specific immune response to the corresponding peptides by IFNγ ELISPOT. In contrast, HPV16-specific IFNγ producing T cells were not detectable in the pre-challenge blood sample of 5 of the 6 patients displaying an early positive skin reaction, which is consistent with the findings of our

previous studies on HPV16-specific immunity in patients with HPV16+ lesions [de Jong 2004; Welters 2003]. In addition, in the pre-challenge blood sample of 3 patients and 3 healthy controls we detected small numbers of IFNγ producing T cells against peptides that were not positive in the subsequent skin test (not shown). Interestingly, the late positive skin reactions detected in healthy controls were not associated with the detection of HPV16-specific type 1 T cells in the pre-challenge blood sample.

To assess the frequency of HPV-specific T cells at the time that a late skin reaction appeared, additional blood samples from 11 of the 13 healthy volunteers, displaying a late positive skin reaction, were collected. In these individuals 39 out of 88 test sites were positive. In 25 of the 39 positive skin sites these reactions coincided with the detection of an HPV 16-specific T-cell response to the same peptides in the post-challenge blood sample. However, we also observed an HPV16-specific T-cell response in the post-challenge blood sample to peptides injected in 10 of the 49 negative skin test sites (Figure 2A). The detection of circulating HPV-specific IFNγ-producing T cells in the post-challenged blood sample and the presence of a positive skin reaction was significantly correlated ($p < 0.0001$, Fisher's exact test; Figure 2A). This shows that the frequency of HPV16-specific T cells in the blood of healthy volunteers is significantly higher following an intradermal challenge with HPV16 peptide and indicates that intracutaneous injection of peptide antigens enhances the number of HPV16-specific T cells in the blood of healthy volunteers.

Positive skin reaction sites are infiltrated by both HPV16-specific CD4+ Th1/Th2 cells and CD8+ T cells.

Approximately 25% of the late positive skin reactions of healthy volunteers were not associated with the detection of HPV16-specific IFNγ-producing T cells in the blood,

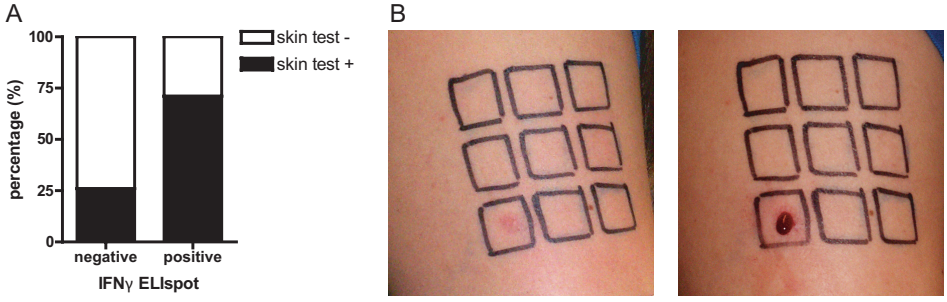

Figure 2. Relation between a positive skin test and the presence of circulating HPV16-specific T cells in the post-challenge blood sample. A. Association between the appearance of a positive skin reaction and the simultaneous detection (IFNγ ELISPOT) of circulating HPV16-specific T cells in the post-challenge blood sample of healthy subjects ($p < 0.0001$, two tailed Fisher's exact test). From a total of 88 skin tests, 39 were positive. Twenty-five of these 39 reactions were associated with a positive reaction in ELISPOT (T-cell frequency ≥ 5 in 100.000 PBMCs). Of the 49 skin test sites that did not show a skin reaction, 10 were associated with a positive ELISPOT. B. Example of a healthy subject (HS10) displaying a positive skin reaction at day 14 to peptide pool 6 ($E6_{109-140}$, $E6_{127-158}$) (left panel). Punch biopsy of the positive skin reaction site (right panel).

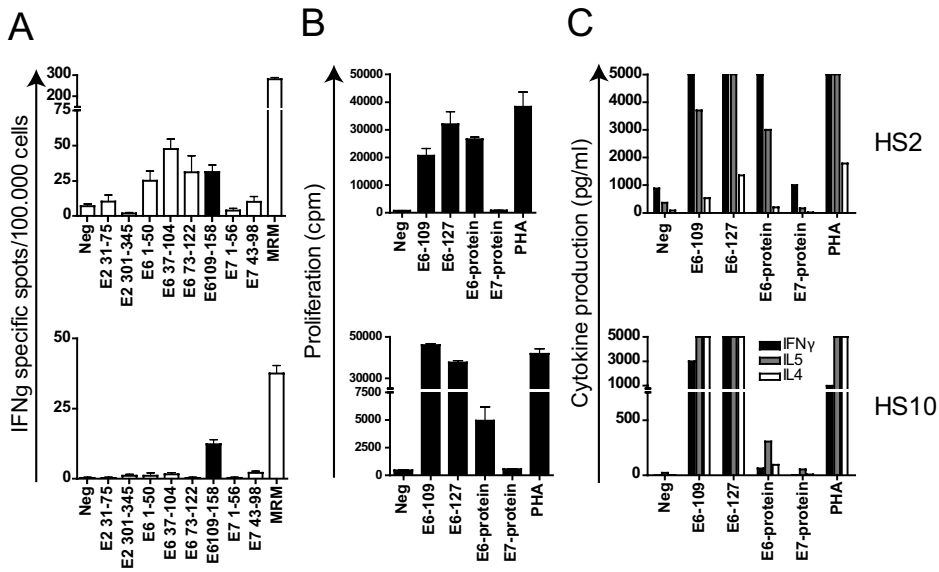

Figure 3. Infiltrating HPV16-specific T cells produce type 1 and type 2 cytokines. A. Two representative examples of HPV16 specific T-cell responses detected by IFNγ ELISPOT in the post-challenge blood sample of healthy subjects displaying a positive skin reaction. The mean number of spots per 100.000 PBMCs are depicted. Memory response mix (MRM) was used as a positive control. The filled bar indicates the positive skin reaction site of which a punch biopsy was taken and put in to culture. B. T lymphocytes ex-filtrating from punch biopsies were, after a 14 to 28 day period of cytokine driven expansion, tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptides (10 μg/ml) – as injected in the skin test – or with protein (20 μg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H]thymidine incorporation and a proliferative response was defined specific as the stimulation index (SI) ≥ 3. Both healthy subjects 2 and 10 (HS2, HS10) are examples for skin reaction sites comprising HPV-specific T cells. C. Supernatants of the proliferative responses in B were analyzed for the presence of IFNγ, interleukin 4 (IL4), IL5 and tumor necrosis factor α, IL2, IL10 (not shown) by cytometric bead array. Cut-off values were based on the standard curves of the different cytokines (100 pg/ml IFNγ and 20 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cut off level and >2x the concentration of the medium control.

suggesting that other, non IFNγ-producing types of T cells may infiltrate the skin after intradermal injection of HPV16 peptides. In order to characterize the cells that infiltrated a positive skin reaction site, punch biopsies were taken from consenting individuals (Figure 2B). In total, 8 biopsies were taken from different positive skin reaction sites of 7 healthy controls (HS2 (2x), 10, 13, 15, 16, 17, 18) and cultured with a cocktail of cytokines that allowed the outgrowth of T cells *in vitro* in the absence of any additional antigenic stimulants [Piersma 2007]. T cells ex-filtrated the tissue, expanded within 3-4 weeks and were tested for their specificity in a short-term proliferation assay. In 4 cases we were able to detect HPV16-specific T cells in the cultures (HS2, 10, 15, 16), in 3 cases the T cells did not respond (HS2, 17,18; not shown) and in

one case no T cells could be cultured from the biopsy (HS13; not shown). Figure 3 shows examples of T-cell cultures that specifically proliferated upon stimulation with autologous monocytes pulsed with the pool of peptides, which was also injected in this site during the skin test (HS2, HS10), as well as with monocytes pulsed with HPV16 E6 protein (Figure 3B). This indicates that these T cells were capable of recognizing their cognate HLA-peptide complexes after the antigen was naturally processed and presented. Analysis of the supernatants of these proliferative T-cell cultures revealed a mixed Th1/Th2 cytokine profile in that the HPV16-specific T cells produced IFNγ, IL-4 and IL-5 (Figure 3C). The detection of HPV 16-specific T cells in the biopsy culture coincided with the detection of HPV16-specific IFNγ-producing T cells the post-challenge blood sample (Figure 3).

Co-staining of the biopsy-derived T cells by CD4 and CD8 cell surface markers showed that not only HPV16-specific CD4+ but also HPV16-specific CD8+ T cells infiltrated the skin site upon intradermal challenge with HPV16 peptide (Figure 4). Overall, in 3 out of 4 biopsies infiltrated by HPV16-specific T cells; we were able to detect HPV16-specific CD8+ T cells. The CD8+ T cells isolated from the biopsy (pool 6) of HS2 responded to both overlapping peptides of the injected skin test: HPV16 $E6_{109-140}$ and $E6_{127-158}$ (not shown). While the CD8+ T cells of both subjects HS15 and HS16 responded to HPV16 $E6_{37-68}$ (see example for HS15 Figure 4).

Taken together, the population of immune cells migrating into the skin upon an intradermal challenge with HPV16 peptides comprises HPV16-specific CD4+ Th1 and Th2 cells and CD8+ T cells. This infiltration is paralleled by the appearance of circulating HPV16-specific IFNγ-producing T cells in the blood.

Discussion

Skin tests are commonly used as a simple assay for *in vivo* measurement of cell-mediated immunity. We have tested the safety and feasibility of the skin test assay for the *in vivo* detection of HPV16-specific cellular immune responses directed against the early antigens E2, E6 and E7 and compared the outcome to parallel measurements of T-cell reactivity by *in vitro* assays. Our study reveals that this skin test is safe and that the majority of responding HPV16+ individuals develop a positive skin reaction within 8 days after injection, suggesting that the test can be used to detect HPV-specific immune responses. For instance, as a quick and easy applicable screening method to monitor the induction of specific cellular immunity in vaccination trials.

A classical DTH reaction to the HPV16-specific skin test was only observed in one patient. However, similar to the observations of Höpfl et al. [Höpfl 2000], the majority of patients with HPV16 related neoplasia develop a positive skin reaction within 8 days after intradermal antigen challenge. In fact, this study shows that patients develop significantly more early positive skin reactions than the healthy volunteers ($p = 0.03$, Figure 1) and the majority of the reactions is directed against one or more of the E6 peptide pools. In order

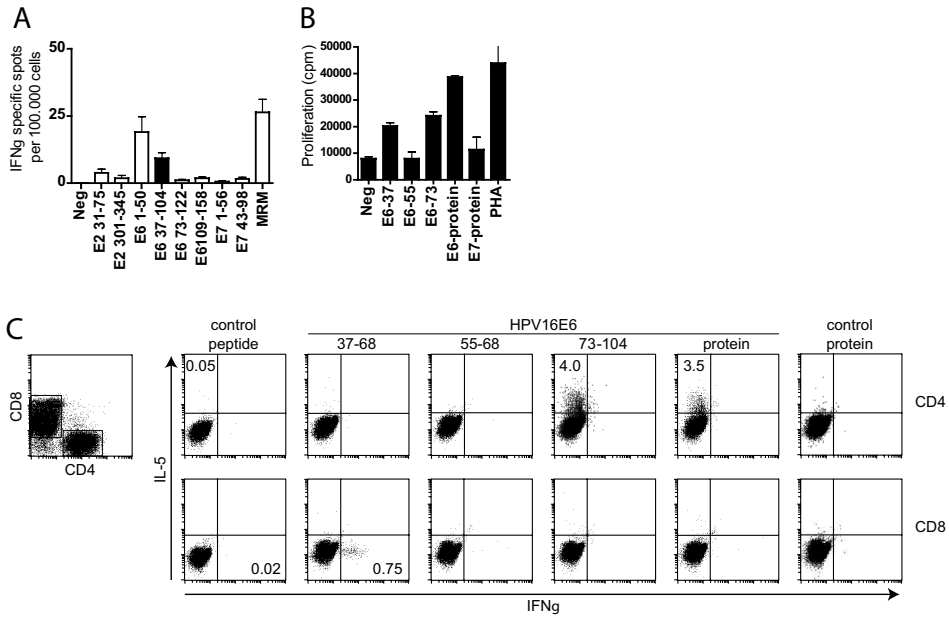

Figure 4. Positive skin test sites are infiltrated by both CD4+ and CD8+ HPV16-specific T cells. A. HPV16-specific T-cell response detected by IFNγ ELISPOT in the post-challenge blood sample of healthy subject 15 (HS15). A punch biopsy was taken from the positive skin reaction site of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$), indicated by the filled bar. B. T lymphocytes ex-filtrating the punch biopsy of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$) of healthy subject 15 (HS15) were tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptides (10 μg/ml) – as injected in the skin test – or with protein (20 μg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H]thymidine incorporation. C. T-cell culture of the skin biopsy of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$) of healthy subject 15 (HS15) consists of both HPV16-specific CD4+ and CD8+ T cells. The specificity of the culture was tested in an intracellular cytokine staining (ICS) against the protein (20 μg/ml) and the peptides (10 μg/ml) corresponding with the injected skin test.

to characterize the immune responses as measured *in vivo*, the results were compared with the detection of HPV-specific T cells by *in vitro* immunological assays. Only a small number of healthy volunteers displayed early positive skin reactions. In this group, known to display HPV16-specific type 1 T-cell responses in vitro [de Jong 2002a; Welters 2003], the appearance of an early skin reaction (within 8 days) was associated with the detection of IFNγ-producing HPV16-specific T cells by ELISPOT, at a frequency of at least 1 per 20.000 PBMC. The same cut-off criteria for a positive reaction in the IFNγ ELISPOT assay are recommended by Jeffries et al [Jeffries 2006], who used mathematical tools to define the appropriate cut-off of the ELISPOT in relation to Mantoux-tests. The low number of circulating memory T cells may explain why the skin reactions appear somewhat delayed compared to classical DTH tests. The T cells need to be boosted or reactivated and start to divide before enough cells are produced to cause a local inflammatory reaction: the positive skin test.

Despite the continuous presence of antigen, 4 out of 11 patients did not respond to the skin test (early or late). This fits with our earlier observation that almost 50% of all cervical cancer patients do not display detectable numbers of proliferating E2, E6, and/or E7-specific T cells in their blood [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. The absence of these circulating HPV-specific T cells may explain the failure to develop a skin reaction. In addition, we recently reported the involvement of regulatory T cells in cervical cancer [Piersma 2007; van der Burg 2007] and one could argue that their presence may prevent the development of early or late positive skin tests. Although we can not formally exclude this it should be noted that also 5 out of 19 healthy controls did not develop a skin reaction suggesting that the development of late skin reactions does not have to occur *per se* following an intradermal peptide challenge. The early positive skin reactions which did appear in the patient population were not associated with circulating HPV16-specific type 1 T cells as measured by IFNγ ELISPOT, suggesting that HPV16-specific T cells producing other cytokines infiltrated the skin test site in these patients. Historically it has been postulated that IFNγ-producing Th1 cells induce DTH responses [Black 1999], however, several studies have now shown that both Th1 and Th2 cells are associated with DTH responses and infiltrate the skin test sites [Wang 1999; Woodfolk 2001]. Also in our study, the skin test sites of the healthy subjects were infiltrated by HPV16-specific CD4+ Th1 and Th2 cells as well as CD8+ T cells (Figure 3 and 4), suggesting that the positive skin reactions in cancer patients are the result of circulating HPV16-specific non-Th1 cells.

Unexpectedly, we observed the majority of skin reactions in healthy individuals to appear 2 to 3 weeks after intradermal injection of the antigen. We were not able to detect HPV-specific CD4+ Th1 cells in the pre-challenge blood sample to the peptides causing these late positive skin reactions. However, in the post-challenge blood sample we detected circulating HPV16-specific IFNγ-producing T cells and their appearance was significantly correlated with the presence of late skin reactions to the corresponding peptides ($p < 0.0001$, Fisher's exact test; Figure 2A). In a number of cases HPV16-specific circulating IFNγ-producing T cells were detected in the post-challenge blood samples but without a concomitant skin reaction (Figure 2A), it is unclear what this means. We hypothesize that the presence of HPV16-specific type 1 T cells in the post-challenge blood sample might reflect a vaccination-induced type of T-cell response. This has also been noted in 29% of patients who underwent a 2-step tuberculin skin testing protocol and who were positive only at the second test round [Akcay 2003]. In general, vaccine-induced T-cell responses peak at 10 to 14 days after vaccination and not at three weeks. However, one should bear in mind that in most vaccine protocols both a higher antigen dose and strong adjuvants are injected. It is therefore reasonable to assume that the T-cell responses induced by intradermal challenge develop slower and peak at a later period. Since the intradermal peptide challenge in healthy volunteers results in the induction of both HPV16-specific CD4+ and CD8+ T cells it could, therefore, be considered as a single, low dose vaccination.

In conclusion, the use of synthetic long peptides in a skin test for the detection of cellular immune response to HPV16 E2, E6 and E7 *in vivo* is safe and feasible. The use of this test to measure spontaneously induced HPV16-specific immune response requires a follow-up of 8 days, because skin reactions to these antigens develop slower (this study and [Höpfl 2000]). Notably, our results indicate that this test does not distinguish between Th1, Th2 or other types of T cells and this should be taken into consideration when the results of this test are interpreted. Currently, this test is used to measure the spontaneously induced HPV16-specific immune response in a large group of HPV-typed patients and healthy individuals in Indonesia. The use of this test to measure vaccine-induced T-cell responses is expected to follow the classical kinetics of a DTH response. In order to study this, we have included the skin test in two of our HPV16 vaccination studies in patients with low and high-grade precancerous lesions of the cervix. Our first preliminary data indicate that skin reactions are strong and appear within 48 hours after application.