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A Prospective Study of Antibody Responses to Defined Epitopes of Human Papillomavirus (HPV) Type 16 in Relationship to Genital and Anorectal Presence of HPV DNA

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The aim of this study was to investigate whether antibody responses against synthetic peptides derived from genital human papillomavirus (HPV) proteins are associated with laboratory-proven genital and anorectal HPV infection. In this study, 158 heterosexual patients (110 women and 48 men) were followed prospectively. At each visit we collected serum samples as well as specimens from several sites in the anogenital area for detection of HPV type 6/11 (HPV-6/11), -16, -18, and -33 DNAs by PCR. Immunoglobulin A (IgA) and IgG responses against disrupted bovine papilloma virions and eight different synthetic peptides derived from HPV-6/11, -16, and -18 were determined for serum samples from the first and the last visits. The subjects attended the Municipal Sexually Transmitted Disease Clinic in Amsterdam, The Netherlands, two to seven times (mean, four times) at approximately 4-month intervals. Women were monitored over a period of 155 person-years, and men were monitored over 65 person-years. The magnitudes of the IgA responses against HPV-16 late protein epitopes L1:13, L1:31, and L2:49 were significantly higher in the sera from the last visit among the currently HPV DNA-positive participants than in HPV DNA-negative persons ($P = 0.02$). When the persons positive for any HPV type at any time during the follow-up period were compared with those who were negative at all times during the follow-up period, we also found a significant elevation of IgA responses against L1:31 and L2:49 ($P = 0.04$ and 0.01, respectively). When the persons who were positive solely for HPV-16 at the last visit were compared with the currently HPV DNA-negative persons, the sera collected at the time of the last visit showed higher IgA titers against the HPV-16 late protein peptides L1:13, L1:31, and L2:49 in the HPV-16-positive group ($P = 0.001$, 0.002, and 0.006, respectively). Comparison of the persons who were solely HPV-16 positive at any time during the follow-up period with the participants who were HPV DNA negative during the study period also showed elevations of IgA reactivity to peptides L1:13 and L2:49 ($P = 0.06$ and 0.02, respectively). Comparison of optical density values for sera collected at the first and the last visits from a given participant revealed an increase in titers of the last sera. Although the results of the cross-sectional analyses for the first and last visits were not consistent, we concluded that among heterosexual men and women at high risk of HPV infection, IgA antibody titers against certain defined HPV-16 late protein epitopes reflect genital and anorectal HPV infection.

More than 70 human papillomavirus (HPV) types are known at present (3, 4, 10, 23). Mucosal HPV types can be differentiated from cutaneous HPV types, and in both groups the oncogenic types can be distinguished from the low-risk HPV types. The HPV types associated with genital disease are 6/11, 16, 17, 31, 33, 35, 39, 42 to 45, 51 to 55, and 59 (17). High-risk types associated with genital neoplasia are HPV type 16 (HPV-16), -18, -45, and -56; some HPVs are considered to be intermediate-risk types, and low-risk types are HPV-6, -11, -42, -43, and -44 (17, 18). Diagnosis of HPV infection is based on clinical examination and HPV DNA detection. For determination of HPV antibody responses, several sources of viral antigens are available. Antigens produced by recombinant techniques or synthetic peptides have been extensively characterized (1, 7, 11, 15, 16, 24). The antibody responses against synthetic peptides derived from HPV proteins have been shown to be preferentially found in sera from patients with cervical intraepithelial neoplasia or cervical carcinoma (5, 7, 9, 20, 21, 25). However, an evaluation of the clinical significance of the HPV antibody responses in relation to laboratory-proven HPV infection has not been performed for groups other than women with cervical cancer. This study reports on the reactivity of sera collected from participants in a follow-up study on the natural history and regional distribution of HPV infections (27, 28). Both immunoglobulin A (IgA) and IgG levels against defined epitopes derived from the early protein E2 of HPV-6/11, -16, and -18, from the late proteins L1 and L2 of HPV-16, and from the E7 transforming protein of HPV-16 were determined.

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MATERIALS AND METHODS

Study population. From May 1989 through December 1990, participants were recruited among patients attending the Sexually Transmitted Disease Clinic of the Municipal Health Service of Amsterdam, The Netherlands. Criteria for enrollment in the study were age of 18 years or older and sexual contact with five or more heterosexual partners within 6 months before entry. This study was originally designed to determine the heterosexual spread of the human immunodeficiency virus. Therefore, hemophiliacs, men with homosexual contacts in the preceding 5 years, and individuals having used drugs intravenously in the preceding 5 years were excluded (14, 26). Written informed consent was provided by all participants.

Participants were asked to return at 4-month intervals. At each visit a physical examination, including colposcopy, was performed, and a standardized questionnaire with questions on medical history, human immunodeficiency virus and HPV risk factors (early age of first sexual intercourse, large number of sexual encounters, use of hormonal contraceptives, history of sexually transmitted diseases, and smoking), sexual behavior, and social-psychological variables was completed. A total of 162 women and 85 men consented to be enrolled in the HPV study (28). Of these participants, 110 women and 48 men could be followed until January 1992. The mean age of the male population was 37 years (standard deviation [SD], 10 years), and that of the female participants was 31 years (SD, 8 years). In this serological study we focus on the cohort that was followed up. The 110 women were monitored over a period of 155.14 person-years (411 visits). For women the mean duration of the follow-up period was 513 days (SD, 256 days; range, 113 to 950 days), and the mean interval between the visits was 188 days (SD, 96 days; range, 72 to 672 days). The 48 men were monitored over a period of 65.4 person-years with a total of 172 visits. The mean duration of the follow-up period was 498 days (SD, 269 days; range, 119 to 902 days), with a mean interval of 193 days (SD, 110 days; range, 75 to 679 days) between the visits. With respect to HPV risk factors, the follow-up group did not differ significantly from the total group that entered the study. Men in the follow-up group had a higher level of education than, and the mean age of women in the follow-up group was higher than, those of the participants who were lost to follow-up.

HPV serology. At each visit blood samples were collected for antibody testing. Detection of antibody to HPV was performed as described previously (7). In short, purified bovine papillomavirus (BPV) or synthetic peptides were applied to microtiter plates (Costar, Cambridge, Mass.). BPV was purified from bovine warts by double CsCl gradient centrifugations, and purity was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Disruption was accomplished by a 2-h incubation at room temperature in carbonate buffer, pH 9.6 (9). The HPV-derived synthetic peptides are presented in Table 1 and Fig. 1. The characteristics of the peptides have been extensively described recently (6, 7). Ten percent horse serum in phosphate-buffered saline (PBS) was added and left for 1 h at 37°C for blocking in the assays with synthetic peptides, and 10% lamb serum in PBS was used for blocking in the assays with disrupted BPV as the antigen. Sera were diluted in 10% horse serum–PBS containing 1 mM EDTA at 1:30 or 1:50 for detection of IgA or IgG against the synthetic peptides, respectively. The dilutions for detection of IgA or IgG against BPV antigens were 1:50 and 1:100, respectively. Incubation for 2 h at 37°C was followed by washing of the plates five times with PBS–0.05% Tween 20. Bound IgA or IgG antibodies were detected with mouse monoclonal antibodies to human IgA or IgG (Eurodiagnostics, Apeldoorn, The Netherlands). Bound monoclonal antibodies were detected with a horseradish peroxidase-conjugated antibody to mouse IgG (Southern BioTechnology, Birmingham, Ala.) by incubation for 1 h at 37°C. After the plates were washed five times, the substrate 2,2′-azino-di(3-ethylbenzthiazolinsulfonate) deammonium salt (Boehringer) diluted 1:50 in 0.1 M citrate buffer, pH 4.0, with 0.9% hydrogen peroxide was added. A_{415}s were recorded. The sera collected at the first and last visits of a given participant were tested in this study and were assayed paired on the same plate. The sera were tested blindly; the technician was not aware of the HPV DNA status. The sera from males were assayed first, and then all specimens from females were tested.

TABLE 1. Immunoreactive synthetic peptides derived from genital HPV types

Peptide	HPV type and region	Sequence	Serotype(s)[a]	Characteristics[b]
245:6	HPV-6 E2 (internal)	HKHAIVTVTYDSEEQRQQC	6, 11 (16, 31)	No disease association; cross-reactive with 245:16
245:16	HPV-16 E2 (C terminal)	HKSAIVTLTYDSEWQRDQC	16, 31 (6, 11)	Association with CIN and CC; IgA lower after treatment
E2:9	HPV-16 E2 (internal)	GDICNTMHYTNWTHIYICEE		Association with CC and anal cancer
245:18	HPV-18 E2 (C terminal)	EKTGILTVTYHSEETQRTKC	18	No cross-reaction with 245:6/16; association with CC
L1:13	HPV-16 L1 (internal)	LCLIGCKPPIGEHWGKGSPC		Association with CC; broad cross-reactivity[c]
L1:31	HPV-16 L1 (C terminal)	VTSQAIACQKHTPPAPKEDPL	16, 33	Broad cross-reactivity[c]; no disease association
L2:49	HPV-16 L2 (internal)	PMDTFIVSTNPNTVTSSTPI	16, 6, 31, 33 (11, 18)	Cross-reactivity with other HPVs; no disease association
E7:5	HPV-16 E7 (C terminal)	KCDSTLRLCVQSTHVDIRTLE	16, 6, 31, 33 (11, 18)	Cross-reactivity with other genital HPVs; association with CC

[a] Serotypes were determined by using human sera. Parentheses indicate reactivity to a lower extent.
[b] CC, cervical cancer; CIN, cervical intraepithelial neoplasia.
[c] Cross-reactivity detectable with hyperimmune sera from experimental animals.

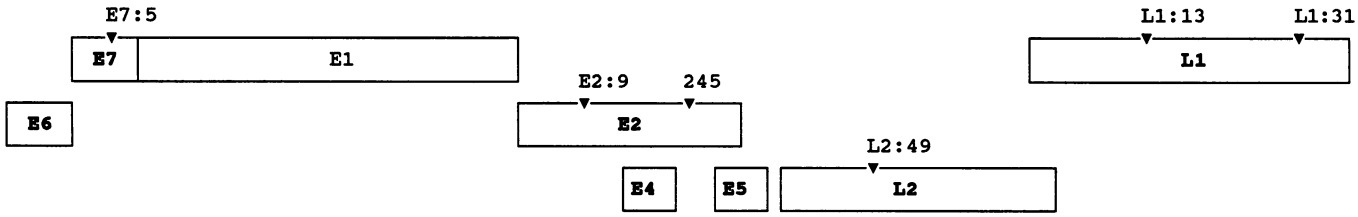

FIG. 1. Schematic presentation of the major defined HPV-16 epitopes. E, early open reading frame; L, late open reading frame.

Calculation and interpretation of the serological results were performed as follows. Absorbances in the linear interval of the optical density (OD) curve, i.e., in the OD interval of 0.2 to 1.0, were used to calculate single-dilution enzyme-linked immunosorbent assay (ELISA) titers (7, 12). In each test run four to six control sera were tested, and the absorbance of the serum to be tested was adjusted according to the absorbances of the control sera, which were between 0.2 and 1.0 OD unit. The absorbances of each plate were corrected by multiplication with a constant derived from the absorbances of the control sera on that plate, i.e., the expected mean of absorbances of positive controls divided by the mean of the actual absorbances of positive controls for each plate. Prior to the adjustment and calculation of OD titers, the absorbance of the sample in the uncoated well was subtracted from the absorbance of the specimen in the corresponding antigen-coated well. Negative values resulting from this subtraction were set to zero. ODs of between 0.100 and 0.200 were considered to be within a grey zone (+/−), ODs of ≥0.200 and <0.500 were considered positive (+), and ODs of ≥0.500 were considered strongly positive (++).

The inter- and intra-assay variations of the tests used are extensively described by Dillner et al. (7). The intra-assay variation was generally low, with a mean coefficient of variation of 4.6%. The interassay variation was more variable; the mean coefficient of variation before correction was 16.8%, and that after correction with the factor based on the absorbances of the positive controls as described above was 7.2%.

Seroconversion was defined as the occurrence of ODs against at least three different peptides that were higher than 0.200 in the last serum when the ODs of the first serum were 0.100 or less. Seroreversion was considered to have occurred when the first serum showed ODs of greater than 0.200 against three or more antigens and the last serum did not (ODs of less than 0.00).

Collection of specimens for HPV detection. Specimens for HPV DNA detection were taken as described elsewhere (27). Specimens were collected from five standard sites in three different anatomical regions: the genital region (urethra and coronal sulcus in men; cervix and labia minora in women), the anorectal region (anus and rectum in both women and men), and the oral cavity (tongue and buccal epithelium together in both women and men). For women cervical specimens were also obtained from the transformation zone for routine cytological examination.

Detection of HPV DNA. The methods used for HPV DNA detection have been described elsewhere (19). The PCR procedure was performed with four different primer sets as described by Claas et al. (2). Four different reactions were performed, each with HPV-6/11, -16, -18, and -33 primers separately. The amplified products were analyzed by dot blot analysis. The filters were hybridized with a mixture of γ-^{32}P-5′-end-labelled HPV type-specific oligonucleotide probes under conditions of high stringency.

Definition of HPV infection. The following definition of duration of infection in a specific region was applied. The sites of sample collection were divided in three regions: the genital, anorectal, and oral regions. Detection of a specific HPV DNA type in a region at one visit and no detectable HPV DNA within 365 days before and after the HPV DNA-positive visit was regarded as a short, regressing infection. An infection with a medium level of persistency was defined as the detection of HPV DNA of a specific type at two consecutive visits over a period of 365 days in a single region. Long persistency was defined as the detection of the same HPV DNA type in the same region at three or more consecutive visits over a period longer than 365 days without a negative visit in between (28).

Data analysis. Statistical procedures used to compare the cohort that was followed up with the participants who could not be followed up included Student's t test, the chi-square test, and the Mann-Whitney U test. A P value of ≤0.05 (two sided) was considered to be significant. For the comparison of the serological results of the HPV DNA-positive and -negative participants, the Mann-Whitney U test was employed. The Wilcoxon paired signed-ranks test was applied for the analysis of the serological results at the first visit versus those at the last visit. All analyses were performed by using the Statistical Package for Social Sciences (SPSS/PC+) (22).

RESULTS

HPV infections and DNA detection. At entry into the study, 23 of 110 women (21%) in the follow-up group were diagnosed with 26 HPV infections. Seventy-three new infections were observed over the follow-up period of 155.14 person-years, resulting in an incidence in women of 47.1 (95% confidence interval, 33.8 to 60.3) new HPV infections per 100 person-years. In the male follow-up group, 15 of 48 men (31%) had 16 HPV infections at entry into the study. Over a period of 65.41 person-years, 33 newly acquired infections were detected, resulting in 50.5 (95% confidence interval, 29.7 to 71.2) new infections per 100 person-years.

The number of patients with HPV DNA (type 16, 18, and/or 33) positivity at any visit during the study period was 75. At the first visit when serum was collected, a total of 39 subjects were HPV DNA positive; HPV-6/11 was detected in nine cases (6%), HPV-16 was detected in 13 cases (8%), and HPV-18 was detected in 19 cases (12%). The distribution of the HPV types among the 35 HPV DNA-positive cases (22%) detected at the last collection of serum was as follows: HPV-6/11, six cases (4%); HPV-16, 13 cases (8%); HPV-18, 16 cases (10%); and HPV-33, four cases (3%). Because of cases of multiple infections, the total number of HPV-positive subjects differs from the total number of infections observed.

HPV serology. The reactivities of the sera collected at the first and last follow-up visits against the different peptides are shown in Table 2. Sera from one woman could not be tested for the presence of HPV antibody because of the lack of sufficient

TABLE 2. ODs and percentages of positive and negative sera for sera collected from 157 heterosexual women and men at entry into study and at the last visit, according to reactivity against the different peptides

Virus and peptide	Ig	First visit					Last visit				
		Median OD (range)	% of sera[a]:				Median OD (range)	% of sera:			
			−	+/−	+	++		−	+/−	+	++
BPV virions	IgG	350 (0–2,295)	11	17	39	32	471 (0–2,080)	4	10	42	45
	IgA	190 (21–677)	19	36	38	7	235 (7–1,826)	11	26	53	10
HPV-6 2:245	IgG	141 (0–2,016)	34	29	29	7	170 (0–2,157)	24	33	31	12
	IgA	159 (0–2,056)	34	23	31	11	208 (0–1,782)	19	31	36	14
HPV-16 2:245	IgG	225 (0–1,319)	22	20	46	11	243 (0–1,870)	15	18	50	16
	IgA	181 (0–1,486)	29	29	35	6	201 (0–1,469)	17	33	42	8
HPV-16 E2:9	IgG	80 (0–1,690)	66	24	8	3	94 (0–1,919)	54	32	10	4
	IgA	93 (0–2,275)	53	31	14	3	113 (0–2,471)	44	29	23	4
HPV-18 2:245	IgG	35 (0–2,072)	80	11	7	2	33 (0–1,848)	73	17	7	3
	IgA	34 (0–588)	88	9	3	1	38 (0–625)	84	10	4	2
HPV-16 L1:13	IgG	33 (0–2,833)	70	7	8	15	49 (0–2,600)	67	10	10	14
	IgA	60 (0–1,902)	65	18	11	6	88 (0–2,221)	55	20	21	4
HPV-16 L1:31	IgG	14 (0–1,946)	68	10	13	10	29 (0–1,931)	64	12	10	14
	IgA	13 (0–1,369)	82	8	6	3	25 (0–1,108)	80	10	6	4
HPV-16 L2:49	IgG	372 (0–2,319)	36	8	9	47	471 (0–2,138)	29	5	17	49
	IgA	99 (0–1,984)	50	16	17	17	98 (0–1,751)	50	14	14	22
HPV-16 E7:5	IgG[b]	43 (0–1,374)	79	12	6	4	46 (0–1,277)	79	12	6	3
	IgA[b]	38 (0–485)	72	17	11		46 (0–1,057)	71	19	6	4

[a] −, OD < 100; +/−, OD = 100 to 199; +, OD = 200 to 499; ++, OD ≥ 500.
[b] Women only.

amounts of serum. For the same reason, for one person the second serum was used instead of the first one, and for four others the next-to-last serum was used instead of the last one. Results for women and men were analyzed together because of the relatively small group of men that could be followed.

The results of the analyses of the ODs in comparison with the current and longitudinal HPV DNA data are shown in Tables 3 and 4, respectively. Sera collected at entry into the HPV study showed a small difference in titer between the 125 participants currently negative for HPV-16, -18, and/or -33 DNAs (mean titer, 117) and the 32 subjects positive for those HPV types (mean titer, 113) in regard to the reactivity against peptide HPV-16 E2:9 ($P = 0.049$). Sera collected at the last visit showed a statistically significant elevation in IgA reactivity against the HPV-16 L1:13, L1:31, and L2:49 peptides for the 30 subjects positive for HPV DNA (types 16, 18, and/or 33) compared with the 127 participants HPV DNA negative at the last visit ($P = 0.02$ for all three peptides). Because subjects currently negative for HPV DNA can have antibody due to infections acquired earlier, the serological HPV status at the last visit was compared between the 75 subjects who were HPV DNA (types 16, 18, and/or 33) positive at one or more visits over the study period and the 82 participants who were negative for HPV DNA at each visit. Compared with those in the HPV DNA-positive group, the titers in the HPV DNA-negative group were significantly lower in IgA reactivity against the HPV-16 L1:31 peptide ($P = 0.04$) and the HPV-16 L2:49 peptide ($P = 0.01$).

To determine the specificity of the serological reactivity, the ODs for participants positive only for HPV-16 or -18 DNA were compared separately with the ODs for participants negative by PCR for the presence of any HPV type. No significant differences in first-serum ODs were found between the 13 persons positive solely for HPV-16 at the first visit and the 118 persons negative for HPV DNA of any type at the first visit. At the last visit 12 persons were positive only for HPV-16

TABLE 3. Analysis of ODs in comparison with current HPV DNA data[a]

Visit	Peptide	HPV DNA	OD		n (%)[b]		P[c] (HPV positive vs HPV negative)
			Median (range)	Mean (SD)	+	++	
First	HPV-16 E2:9 (IgG)	Negative	81 (0–1,315)	117 (152)	12 (10)	3 (2)	0.049
		Positive	67 (0–1,690)	113 (292)	0 (0)	1 (3)	
Last	HPV-16 L1:13 (IgA)	Negative	74 (0–1,466)	136 (184)	25 (20)	3 (2)	0.02
		Positive	155 (0–2,221)	287 (434)	8 (27)	4 (13)	
	HPV-16 L1:31 (IgA)	Negative	22 (0–1,108)	71 (161)	5 (4)	5 (4)	0.02
		Positive	54 (0–838)	108 (166)	4 (13)	1 (3)	
	HPV-16 L2:49 (IgA)	Negative	85 (0–1,632)	251 (378)	17 (13)	22 (17)	0.02
		Positive	309 (3–1,751)	525 (549)	5 (17)	12 (40)	

[a] Results are for sera collected at the first visit from 125 HPV DNA-negative participants and 32 HPV-16, -18, and/or -33 DNA-positive persons at entry into the study and for sera collected at the last visit from the same group (127 HPV DNA-negative and 30 HPV DNA-positive persons) (Amsterdam, 1989 to 1992).
[b] +, OD = 200 to 499; ++, OD ≥ 500.
[c] Tested by Mann-Whitney U test on complete OD scale.

TABLE 4. Analysis of ODs in comparison with longitudinal HPV DNA data[a]

Peptide	HPV DNA	OD		n (%)[b]		P[c] (HPV positive vs HPV negative)
		Median (range)	Mean (SD)	+	++	
HPV-16 L1:31 (IgA)	Negative	18 (0–875)	64 (147)	2 (2)	4 (5)	0.04
	Positive	35 (0–1,108)	94 (177)	7 (9)	2 (3)	
HPV-16 L2:49 (IgA)	Negative	62 (0–1,593)	222 (343)	10 (12)	12 (15)	0.01
	Positive	151 (0–1,751)	392 (491)	12 (16)	22 (29)	

[a] Results are for 157 sera collected at the last visit from 82 participants not diagnosed with HPV DNA and 75 persons diagnosed with HPV DNA (types 16, 18, and/or 33) at one or more visits during the study period, 1989 to 1992.
[b] See Table 3, footnote *b*.
[c] See Table 3, footnote *c*.

DNA, and 122 persons were negative for any HPV DNA type tested. IgA antibody titers against peptides L1:13, L1:31, and L2:49 derived from HPV-16 were strongly elevated for HPV-16 DNA-positive subjects compared with HPV DNA-negative participants ($P < 0.001$, $P = 0.002$, and $P = 0.006$, respectively) (Table 5). IgG antibody titers against HPV-16 peptides L1:13 and L1:31 were less strongly elevated among HPV-16 DNA-positive persons ($P = 0.03$ and 0.02, respectively). The mean ODs of the HPV-16-negative persons who were positive for other HPV types were intermediate between the ODs of the HPV-negative and HPV-16-positive groups. Seventeen persons were positive for HPV-18 at the first visit, and 13 were positive at the last visit. The ODs of the sera from the HPV-18-positive persons did not differ significantly from those from HPV DNA-negative persons at either the first visit or the last visit.

When the titers of the sera collected at the last visit from the 23 persons positive only for HPV-16 over the total follow-up period were compared with those for the persons negative for any HPV type during the study period, IgA antibodies against HPV-16 peptides L1:13 and L2:49 were higher among the HPV-16-positive persons ($P = 0.06$ and 0.02, respectively), but the differences were less strongly significant than those presented in Table 5.

We also analyzed the ODs of the first-visit and last-visit sera from a given participant. The ODs of the last-visit sera were statistically significantly greater in 12 of the 18 various assays, as shown in Table 6. When the 70 participants negative for HPV DNA (type 6/11, 16, 18, or 33) over the study period were analyzed separately, the ODs of the last-visit sera were greater in six different assays.

Seroconversion. Ten women and eight men were found to be seroconverters. IgG, IgA, and IgM antibodies against several epitopes were induced at the same time. IgA and IgM tended to wane after HPV DNA was no longer detectable, whereas IgG tended to persist. Loss of antibody was seen in three women and two men. An extensive analysis of the seroconverters will be described elsewhere (30).

Persistency of HPV infection and abnormal Pap smear. The association between persistency of infection and antibody titer was also analyzed. The ODs for the 13 persons who were diagnosed with a long-lasting, persistent infection with HPV-16, -18, or -33 during the follow-up period were compared with the ODs for the 70 persons who were HPV DNA negative during the follow-up period and, separately, with those for the 144 participants who did not have a persistent infection. No significant differences were found. Only four women were diagnosed with an abnormal Pap smear, and no associated serological responses were found.

DISCUSSION

Our main result is the finding of an association between HPV DNA positivity and IgA (and to a lesser extent IgG) reactivity against peptides derived from the L1 and L2 capsid proteins of HPV-16. This association was highly significant for both IgA and IgG reactivity when the ODs of the last-visit serum samples from the persons currently diagnosed solely with HPV-16 were compared with those for the persons negative for HPV DNA of any tested type. In the longitudinal study, the IgA titers against the HPV-16 L1- and L2-derived peptides were significantly higher among the persons diagnosed with HPV-16, -18, or -33 DNA over the follow-up period than among the participants who were negative for HPV DNA. However, in a cross-sectional comparison of the current HPV DNA status at the first visit and the IgG and IgA antibody

TABLE 5. ODs of 134 sera collected at the last visit from 12 participants diagnosed with HPV-16 DNA and 122 persons not diagnosed with HPV-6/11, -16, -18, or -33 DNA at the last visit

Peptide	HPV DNA	OD		n (%)[a]		P[b] (HPV positive vs HPV negative)
		Median (range)	Mean (SD)	+	++	
HPV-16 L1:13 (IgA)	Negative	74 (0–1,466)	136 (186)	24 (20)	3 (2)	<0.001
	Positive	246 (53–722)	307 (224)	5 (42)	2 (17)	
HPV-16 L1:31 (IgA)	Negative	22 (0–875)	65 (134)	5 (4)	4 (3)	0.002
	Positive	120 (11–379)	139 (111)	4 (33)	0 (0)	
HPV-16 L2:49 (IgA)	Negative	85 (0–1,632)	242 (366)	15 (12)	21 (17)	0.006
	Positive	787 (10–1,751)	790 (642)	0 (0)	8 (67)	

[a] See Table 3, footnote *b*.
[b] See Table 3, footnote *c*.

TABLE 6. Comparison of ODs of sera collected at the first and last visits, according to reactivity against the various antigens

Virus and peptide	Ig	P^a for:	
		Total[b]	HPV DNA negative[c]
BPV virions	IgG	<0.001	NS[d]
	IgA	<0.001	0.003
HPV-6 2:245	IgG	0.001	0.02
	IgA	0.003	NS
HPV-16 2:245	IgG	0.002	0.01
	IgA	<0.001	NS
HPV-16 E2:9	IgG	<0.005	NS
	IgA	<0.001	NS
HPV-18 2:245	IgG	NS	NS
	IgA	0.03	NS
HPV-16 L1:13	IgG	0.03	0.002
	IgA	<0.001	0.003
HPV-16 L1:31	IgG	NS	0.02
	IgA	NS	NS
HPV-16 L2:49	IgG	NS	NS
	IgA	0.01	NS
HPV-16 E7:5	IgG	NS	NS
	IgA	NS	NS

[a] Wilcoxon matched-pairs signed-ranks test, two tailed.
[b] All 157 heterosexual women and men.
[c] 70 participants HPV DNA (type 6/11, 16, 18, and 33)-negative at all visits.
[d] NS, not significant.

titers in sera collected at the same time, we found no significant association. This implies that serology with these epitopes has limited value for determining current infection with HPV but can be an indicator of infection in the past.

Cross-reactivity among HPV types has been described for the L1:31 and L2:49 epitopes (29). In the present study, the association between IgA reactivity against these HPV-16 late protein-derived peptides and HPV-16 DNA positivity was stronger than that with positivity for HPV-6/11, -18, or -33 DNA. Analysis of the results for the last-visit samples and current HPV DNA status showed only a comparatively weak association between IgA titers against the L1:13, L1:31, and L2:49 peptides and the current presence of HPV-16, -18, or -33 DNA ($P = 0.02$). This suggests that the antibody responses against these epitopes are primarily induced by HPV-16 itself. The fact that persons positive for HPV types other than 16 showed titers between those for HPV-negative and HPV-16-positive persons suggests that these related types might be able to induce these responses, but at lower titers. A similar association was not observed when the HPV-18-positive persons were compared with the HPV-18-negative participants with regard to titers against the different peptides. An obvious explanation is that only one peptide derived from HPV-18 E2 was tested in the present study, in contrast to six HPV-16-derived peptides.

Further analysis of the inverse relationship between HPV DNA positivity and reactivity against HPV-16 E2:9 revealed that sex was a confounder for this association (data not shown). The mean OD for men was 98, and that for women was 125 ($P = 0.03$). This prompted us to analyze all serological variables for differences between men and women. Surprisingly, ODs of IgG and IgA antibodies against BPV disrupted virions and HPV-16 E2:9 as well as IgG antibodies against peptide 245 (HPV-6) differed significantly between men and women. For the time being we do not have an explanation for this finding, which must be confirmed in other studies especially designed to analyze sex-related differences.

Since both men and women showed an incidence of HPV infections as high as about 50 per 100 person-years, it is conceivable that a large proportion of the HPV DNA-negative persons had been infected in the past. Detection of HPV antibodies in this group can therefore reflect infections occurring before entry into the study. The fact that the IgA response showed a stronger association with current HPV DNA status than the IgG response, which tended to prevail after infection had been cleared, suggests that particularly the IgG response reflected infections acquired some years previous to entry into the study.

In this population of heterosexuals with a large number of sexual partners, waning or loss of HPV antibody could not be observed when we analyzed the first- and last-visit titers for a given participant. On the contrary, even the HPV DNA-negative participants showed an elevation of titers against several antigens. An explanation might be the high incidence of HPV events as detected by PCR. The finding of greater ELISA titers in the last-visit sera among the HPV DNA-negative participants might suggest that even in this subgroup HPV events could have occurred.

The results of this study are discouraging for the understanding of persistency of HPV infection. One can speculate that persistency is associated with an immunodepressed state resulting in the lack of an appropriate serological response. An explanation of the results might be that participants with a persistent infection according to the definition used here in fact have had reinfections with another subtype of HPV. Further analysis of the HPV DNA isolates will be carried out. However, preliminary results of sequence analysis of HPV-16 isolates indicate that in a given participant with a persistent infection, the same isolate is detected at consecutive visits. Attempts to measure local humoral or cellular immune responses were not part of the present study.

The results of the cross-sectional analyses at the first and the last visit with regard to the association between HPV serology and current presence of mucosal HPV infection were not consistent. A methodological bias cannot be an explanation, since both first- and last-visit sera of a given participant were tested on the same microtiter plate. Behavioral changes might explain the inconsistency of both cross-sectional analyses, since we know that unprotected vaginal intercourse decreased over the study period (13). The subpopulation of participants negative for HPV DNA at entry of the study could have previously acquired HPV infection. In any case, it is obvious that the serological assays used in this study should be employed for the time being only in epidemiological studies and not for the diagnosis of individual cases.

In conclusion, it can be stated that among heterosexual men and women at high risk of HPV infection, IgA antibody titers against certain defined HPV-16 late protein epitopes reflect genital and anorectal HPV infection.

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