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Original Research Report

Correlates of cervical mucosal antibodies to human papillomavirus 16: Results from a case control study ☆

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Abstract

Background. While the cervical mucosal immune response to human papillomavirus (HPV) infection is believed to be central to viral clearance, it is not well characterized. We performed this analysis to determine correlates of HPV-16-specific mucosal antibody response in women at high risk for infection with HPV.

Methods. Cervical mucosal and serum samples were obtained from participants in a case control study that measured demographic risk factors of cervical disease and HPV infection. An HPV-16 L1-virus-like particle ELISA was used to detect HPV-16-specific IgA and IgG. Antibody results were correlated to demographic characteristics, sexual history, cervical disease, and HPV detection.

Results. Cervical anti-HPV-16 IgA and IgG inversely correlated with HPV DNA, HPV-16 DNA, and cervical disease.

Conclusions. These findings suggest that mucosal antibodies may protect against HPV infection and cervical disease. However, additional longitudinal studies evaluating serum and mucosal antibody correlates of incident, persistent, and clearing HPV infection are needed. In addition, standardization of mucosal sample collection and testing methods are required.
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Background

Human papillomavirus (HPV) infection is the most important risk factor for cervical cancer; yet, most infections are transient and asymptomatic. The immune response is one of several host factors that contribute to determining the outcome of infection. Because HPV is an epitheliotropic virus, it is largely shielded from the immune system. Studies have consistently demonstrated that a significant number of infected individuals never have detectable antibodies and that seroconversion is delayed, occurring 3 to 14 months after infection [1].

Studies have reported that humoral immunity is not necessary for viral clearance [2]. Yet, a recent vaccine trial yielded promising results on the effectiveness of systemic immunization to prevent persistent infection [4]. Finally, a

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recent study reported HPV-specific serum IgA and IgG to be protective against subsequent HPV infection [3].

For infections such as HPV, the mucosal immune response may be more important than the systemic response in determining the outcome of infection. However, few studies have examined local humoral immunity in relationship to infection and disease and to epidemiologic and behavioral characteristics. In this case control study, we measured cervical mucosal HPV-16-specific IgG and IgA antibodies and correlated the results with demographic and behavioral history, HPV DNA, cervical disease, and systemic antibodies.

Methods

This study adhered to human experimentation guidelines of the U.S. Department of Health and Human Services. All participants were volunteers who gave informed consent, and their consent forms are filed in their patient charts (cases) and research charts (controls). The study was reviewed and approved by the institutional review boards at the Centers for Disease Control and Prevention, M.D. Anderson Cancer Center, and the National Cancer Institute.

Study design

Details of the case control study design, eligibility requirements, and epidemiology have been published elsewhere [5,6]. In brief, the study was conducted in Harris County, Texas, between September 1991 and August 1994 at M.D. Anderson Cancer Center and the referral county public health department clinics. Cases of newly diagnosed cervical intraepithelial neoplasia (CIN) were identified among women (18 to 60 years old) referred to the M.D. Anderson Cancer Center Colposcopy Clinic by the county health departments. Each patient had an abnormal Papanicolaou smear and was found to have CIN I–III on cervical biopsy and endocervical curettage. Controls were recruited from the two health department clinics that referred the cases. Control volunteers had to have a history of no warts, no other evidence of HPV infection, and a history of normal Papanicolaou smears. Women with a history of cervical neoplasia, hysterectomy, or current pregnancy were excluded. Detailed medical history and demographic information was obtained via questionnaire which was obtained by research assistants [5,6]. Cases and controls had a complete history, physical examination, pan-colposcopy (vulva, vagina, cervix), a Papanicolaou smear, a wet drop if vaginal discharge was present, gonorrhea and chlamydial testing, and a swab of mucous that was frozen for later HPV analysis.

Blood samples were obtained by venipuncture, and serum was separated by centrifugation. Endocervical swab and endocervical brush samples were obtained during a pelvic examination. The swab was placed in Digene transport media for later analysis using the Hybrid Capture system (Digene Corporation). The endocervical brush was placed in an empty microfuge tube, immediately frozen on dry ice, and stored at −70°C until testing. The frozen cytobrushes were thawed in a 1 ml mixture of 0.01 M PBS and 5 mM EDTA, pH 7.4, and vortexed to dislodge cells. Following centrifugation, the supernatant containing solubilized endocervical secretions was collected and stored at −70°C for use in antibody assays.

Detection of HPV DNA

The results of the PCR analysis from the cervical brush, including DNA copy number and type, have been published [6]. Briefly, DNA was extracted from cell pellets by standard phenol-chloroform extraction and resuspended in 200 μl of Tris EDTA [6]. A contamination control, consisting of 1 ml of water, was inserted after every tenth sample and carried through all extraction and polymerase chain reaction (PCR) procedures.

HPV DNA was detected by using ethidium-bromide-stained agarose gel electrophoresis of L1 consensus PCR products as described [6]. Extracts with a visible HPV band and the contamination controls were diluted and tested for HPV-16 DNA, using a quantitative multiplex fluorescent PCR assay for HPV-16 and globin [6]. The threshold for the HPV-16 assay was set at 40 copies.

ELISA for IgG and IgA

Virus-like particles (VLPs) were produced by expression of an HPV-16 L1 recombinant baculovirus in insect cells as described elsewhere [7]. Serum samples were tested in an ELISA assay for HPV-16 VLP-specific IgG [7]. In brief, plates were coated with 0.1 μg of VLPs/well, blocked, washed, and then incubated for 1 h with 50 μl of sera (1:20 in diluent plus 10% insect cell lysate) per well at 37°C. IgG was detected by using alkaline-phosphatase-conjugated goat anti-human IgG (Roche Molecular Biochemicals), and alkaline phosphatase substrate (Sigma-Aldrich) was used for assay development. Mucosal HPV-16-specific IgG and IgA antibodies were also detected by using an ELISA in a manner identical to that used for the serologic assay except samples were tested undiluted and different conjugate antibodies were used. Mucosal IgG was detected by using horseradish-peroxidase (HRP)-conjugated goat anti-human IgG (Kirkegaard & Perry Laboratories) at 1:1000 (2.0 μg/ml) in diluent. For the mucosal IgA assay, goat anti-human IgA HRP conjugate antibody (Sigma-Aldrich) was used for detection. Mucosal assays were developed using ABTS peroxidase substrate (Kirkegaard & Perry Laboratories) for 45 min with gentle rocking.

For serum IgG assay quality control, we used known positive and negative human sera as previously described [8]. The serologic IgG cut-off absorbance value was determined to be 0.450, the point at which (using control

sera) the lowest number of false positive and the highest number of true positive results were observed. Because of the lack of known positive and negative mucosal samples, a valid cut-off could not be established for virus-specific mucosal IgG and IgA. Therefore, results are reported in terms of log-transformed raw absorbencies.

Raw absorbance values were detected at 405 nm in an automated plate reader (MRX II Revelation; Dynex). Each sample was tested in duplicate wells. Quality control for any given day of ELISA testing was evaluated by comparing the daily control absorbance with the mean control absorbances observed throughout the study, as described previously [7]. Repeat tests were performed if ELISA testing failed control measures and if adequate sample was available.

Statistical analysis

Univariate and classical non-parametric methods, using STATA 7.0 (STATA Corporation) were used for data analysis. Subjects with no mucosal sample were excluded from analysis. Correlation coefficients were derived using the Kruskal–Wallis test, a non-parametric measure for one-way analysis of variance. Correlations between continuous variables were tested using Spearman's rank correlation. By nature of the reactivity of an enzyme-based assay, ELISA results can only be positive. Thus, absorbency data were skewed to the right, and data were log-transformed for analysis. Reflecting this log transformation, means reported are geometric.

Results and discussion

Three hundred twenty five case patients and 279 controls met eligibility requirements and were included in the original case control study. Because of limited volume, IgA and IgG antibody determinations could not be made for 8 and 15 of the samples from case patients and controls, respectively. Mucosal samples were available for antibody testing for 291 of the case patients and 252 of the controls.

Population characteristics

As shown in Table 1, case patients did not differ from controls in the distribution of income, age, current contraceptive use, immunosuppressive disease, or concurrent cervical/vaginal infection. A significantly higher proportion of case patients than controls were HPV DNA-positive

Table 1
Population characteristics

Parameter	Controls		Cases		*P* value[a]
	Normal cytology		CIN I/II/III		
	N	(%)	*N*	(%)	
Population characteristics					
Median age, years	26.9[b]		26.6[c]		0.0626
Income less than $20K	155	(62.0)	247	(85.2)	0.9780
HPV viral risk factors					
HPV DNA-positive	48	(19.1)	211	(72.5)	0.0001
HPV-16 DNA-positive	9	(3.6)	102	(35.2)	0.0001
HPV-16 seropositive	101	(53.2)	123	(50.0)	0.5135
Non-viral risk factors					
Race/ethnicity					0.0001
White	62	(24.6)	134	(46.1)	
African-American	68	(27.0)	66	(22.7)	
Hispanic	122	(48.4)	91	(31.3)	
First sex before age 17	103	(40.9)	163	(63.4)	0.0001
No. of lifetime sex partners					0.0001
1	92	(36.5)	45	(15.6)	
2/3	67	(26.6)	73	(25.3)	
4/5	53	(21.0)	65	(22.5)	
6+	40	(15.9)	106	(36.7)	
History of smoking	66	(26.2)	138	(47.4)	0.0001
Currently use oral contraceptive	153	(60.7)	157	(54.3)	0.1343
Immunosuppressive disease[d]	1	(0.4)	5	(1.7)	0.1411
Concurrent cervical/vaginal infection[e]	23	(9.2)	25	(9.8)	0.8169

[a] Kruskal–Wallis equality of case versus control proportions.
[b] 95% confidence interval (CI), 26.2–27.6.
[c] 95% CI, 25.8–27.4.
[d] Reported health history of cancer, lupus, Crohn's disease, HIV, colitis, or renal transplant.
[e] Reported current gonorrhea, chlamydia, vaginitis, pelvic inflammatory disease, trichomonas, or yeast infection.

(72.5% vs. 19.1%) and HPV-16 DNA-positive (35.2% vs. 3.6%), but HPV-16 seropositivity did not differ between the groups (50.0% vs. 53.2%). A disproportionately higher number of case patients (134; 46.1%) than controls (62; 24.6%) were white, and significantly more case patients than controls reported having sex before age 17 (63.4% vs. 40.9%, respectively). Similarly, case patients reported a significantly higher number of sex partners than did controls, and a significantly higher proportion of case patients than controls had a history of smoking (47.4% vs. 26.6%, respectively).

Antibody correlates

Correlation values of the mean mucosal anti-HPV-16 VLP ELISA absorbance with cervical disease, HPV detection, and HPV seropositivity are presented in Table 2. Geometric mean optical densities (ODs) for HPV-16 IgG and IgA were significantly lower in women with low-grade cervical intraepithelial neoplasia (CIN I) or high-grade CIN (CIN II/III) than in controls. A lower mean OD for mucosal HPV-16 IgG was also found in women with any HPV or HPV-16 DNA compared with those negative for HPV or HPV-16 DNA. A similar relationship was observed for mucosal HPV-16 IgA and HPV or HPV-16 DNA, but the results were not statistically significant. The box plots of normalized mucosal antibody reactivity in relation to cervical disease and HPV-16 DNA detection (Fig. 1) show the same relationship but allow the degree of overlap between categories to be seen.

Higher ELISA levels of mucosal HPV-16 IgG but not mucosal IgA correlated strongly with HPV-16 seropositivity. However, seropositivity showed a significant positive correlation with detection of HPV-16 DNA, in contrast to the negative correlation observed with mucosal IgG and IgA. Mucosal IgG and IgA ELISA OD values were significantly correlated (Spearman's rho = 0.5601; P = 0.000; Fig. 2).

Table 2 also summarizes geometric mean mucosal HPV-16 IgG and IgA ELISA OD values by descriptive

Table 2
Correlates of HPV-16-specific local antibody

	Mucosal IgG (N = 488)			Mucosal IgA (N = 535)			Serum IgG	
	Mean OD[a]	95% CI	P value[b]	Mean OD[a]	95% CI	P value[b]	% Positive	P value[b]
All data	0.210	0.199–0.223		0.193	0.183–0.204		51.4	
Cervical disease								0.682
Normal	0.250	0.229–0.272	–	0.232	0.214–0.250	–	53.2	
CIN I	0.188	0.170–0.208	0.0001	0.161	0.146–0.177	0.0001	47.9	
CIN II/III	0.183	0.164–0.203	0.0001	0.170	0.152–0.189	0.0001	52.8	
HPV viral risk factors								
HPV DNA								
Positive	0.193	0.179–0.208	0.0028	0.184	0.170–0.198	0.0702	51.6	0.926
Negative	0.229	0.211–0.249		0.202	0.187–0.218		51.2	
HPV-16 DNA								
Positive	0.184	0.164–0.207	0.0087	0.174	0.155–0.196	0.0737	61.5	0.027
Negative	0.219	0.205–0.233		0.198	0.187–0.211		48.6	
HPV-16 serology								
Positive	0.241	0.219–0.266	0.0002	0.198	0.181–0.216	0.2224		
Negative	0.186	0.172–0.202		0.183	0.168–0.198			
Race/ethnicity			0.0001			0.019		0.149
White	0.181	0.166–0.199		0.183	0.168–0.200		51.3	
African-American	0.263	0.233–0.297		0.223	0.198–0.250		58.7	
Hispanic	0.211	0.194–0.230		0.185	0.170–0.201		46.7	
First sex before age 17			0.045			0.277		0.752
No	0.226	0.207–0.247		0.201	0.185–0.218		52.3	
Yes	0.201	0.186–0.217		0.187	0.173–0.202		50.7	
Lifetime sex partners			0.124			0.732		0.016
1	0.218	0.195–0.244		0.195	0.175–0.218		43.1	
2/3	0.229	0.203–0.258		0.198	0.177–0.221		46.9	
4/5	0.196	0.173–0.222		0.201	0.178–0.228		63.9	
6+	0.201	0.181–0.223		0.181	0.164–0.199		53.9	
History of smoking			0.000			0.199		0.721
No	0.230	0.214–0.247		0.200	0.186–0.214		50.7	
Yes	0.182	0.167–0.199		0.182	0.167–0.198		52.5	
Currently use oral contraceptive			0.838			0.817		0.708
No	0.211	0.193–0.229		0.194	0.179–0.211		52.6	
Yes	0.210	0.195–0.227		0.193	0.179–0.207		50.8	

[a] Raw ELISA optical density readings are normalized for analysis.
[b] Chi-square test conducted by use of the Kruskal–Wallis test for equality of populations.

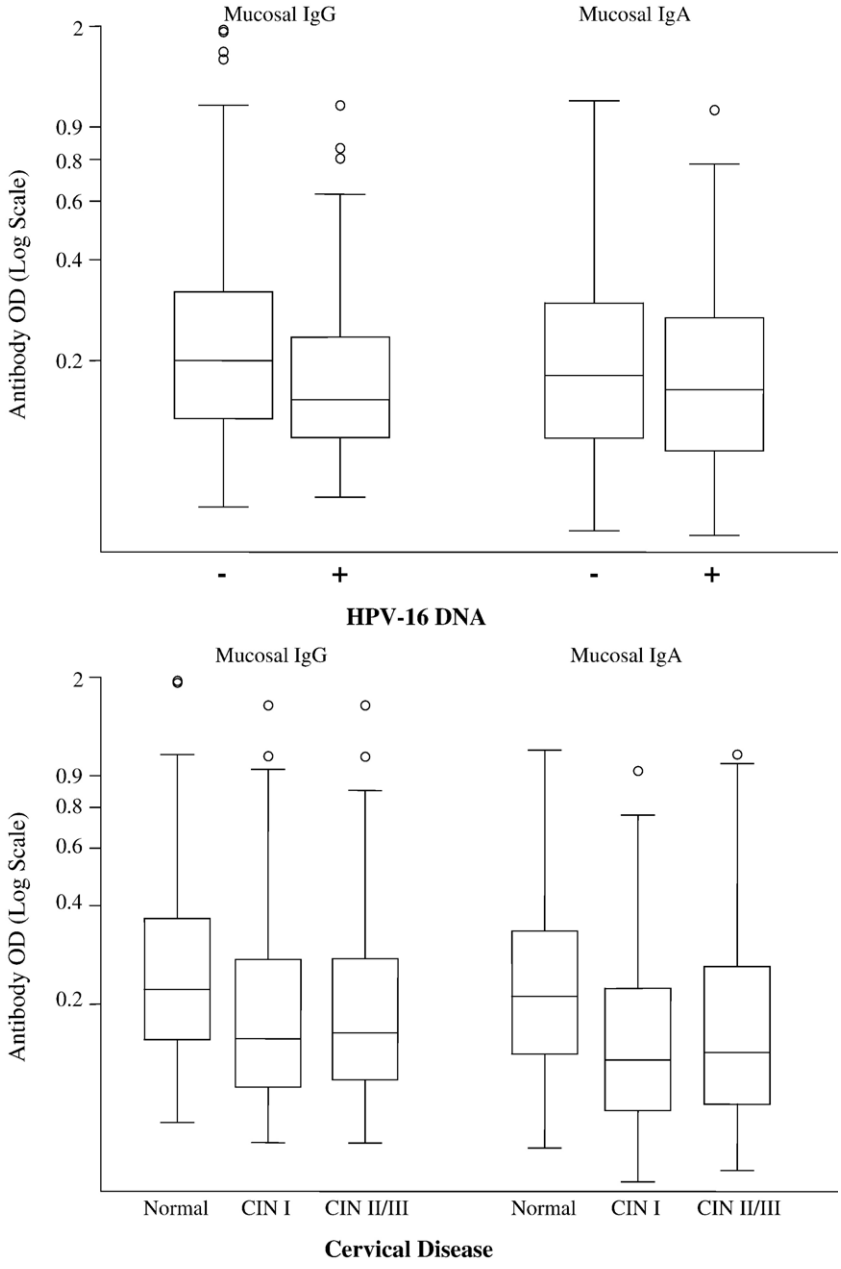

Fig. 1. Box plots of mucosal HPV-16 antibody reactivity versus HPV-16 DNA detection and cervical disease. CIN = cervical intraepithelial neoplasia.

characteristics. Higher mucosal IgG ELISA ODs were found in African-Americans (P = 0.0001) and Hispanics (P = 0.0028) when compared to whites. African-Americans also had higher local IgG reactivity than Hispanics (P = 0.0054). Hispanics and whites had significantly lower mucosal IgA antibody reactivity than African-Americans (P = 0.011 and 0.013, respectively); no differences between Hispanics and whites were evident.

Women who reported first sexual intercourse before age of 17 had higher mucosal HPV-16 IgG but not IgA antibody reactivity. No clear trends between IgG or IgA antibody level and number of lifetime sex partners were evident. However, the distribution of seropositivity varied by the number of lifetime sex partners. The presence of local IgG antibody negatively correlated with a history of smoking, but this correlation was not evident with local IgA. Local antibody absorbance did not correlate with current use of oral contraceptives.

Conclusion

For this study, case patients and controls were recruited from clinics serving minority and economically disadvantaged populations [5,6]. The socio-economic profile of these women combined with reported high numbers of sex

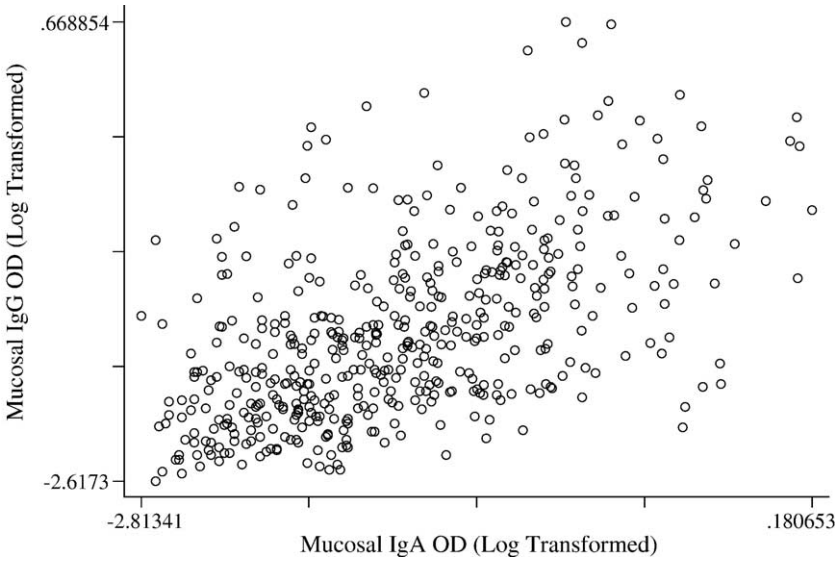

Fig. 2. Scatter plot of local IgA and IgG HPV-16 reactivity. OD, optical density.

partners and first sex at an early age places these women at high risk for HPV infection. Half of the women studied had previous exposure to HPV-16 as indicated by positive serology. This prevalence is more than twice the national seroprevalence of HPV-16 for women aged 12 to 59 years [8].

In this population, which was highly exposed to HPV, we found that local mucosal antibodies to HPV-16 VLPs negatively correlated with cervical disease and HPV-16 infection (that is, higher mean ODs were associated with no disease and no HPV). This finding is in contrast to the positive association that serum IgG seropositivity showed with HPV-16 DNA and suggests that mucosal IgG and IgA reflect a protective response. However, one limitation of our study is that we could not determine an ELISA cut-off value that correlates with a positive antibody response, so the changes in ODs may not reflect meaningful biologic changes. In addition, the measurements presented here are from only one point in time. Little is known of the natural history or immuno-biology of HPV, i.e., how the serological and mucosal antibodies and the T cells react to HPV infection in patients previously HPV-exposed and in those who are HPV-unexposed.

As expected, local HPV-16 IgA and IgG were correlated; however, only IgG showed a significant association with HPV-16 seropositivity. The IgA measurements did not specifically target only secretory IgA, and serum transudates no doubt contribute to the observed mucosal antibodies. This may explain some of the correlation.

Table 3
Summary of prior reports of mucosal antibodies to HPV-16

Author	Reference	Mucosal IgG	Mucosal IgA	Serum IgG	Serum IgA
Rocha-Zavaleta	[14]	19%	15%	n.d.	n.d.
Wang	[15]	n.d.	12%	n.d.	n.d.
Sasagawa	[16]	25%	30%	n.d.	n.d.
Bontkes	[17]	10%	12.5%	20.9%	13.1%

n.d. = not done.

Previous investigations have found positive correlations between disease, infection, and local antibody. However, some of these few investigations examined populations among whom antibody is likely to reflect immune response to primary infection [9–15]. All the studies used different methods to collect the mucosal samples, establish cut-off for positive results, and measure immune response. Table 3 shows the percentage of patients expressing mucosal antibodies against HPV-16. Serological responses are not detailed in most of these studies. Standardization of methods will be required before direct correlation between studies is possible.

Antibody response is not necessary or sufficient for HPV clearance. However, a primary immune response may protect against subsequent infection. A longitudinal study demonstrated that serum IgG and IgA were protective for subsequent infections [4], and a recent vaccine trial demonstrated that immunization prevented persistent infection [3]. The longitudinal study of Sasagawa et al. [16] showed that a mucosal IgA response preceded a mucosal IgG response; once IgG was induced, it persisted long after HPV clearance in the cervix. Thus, in a fully HPV-exposed population, humoral antibody may be indicative of the level of protective humoral immunity. Alternatively, in a fully or partially susceptible population, immune response may reflect differential exposure and thus be a risk factor for infection and disease.

It is difficult to say whether this study is evidence that, in a population with high HPV-16 exposure, a local antibody response may be protective. While vaccine trials provide an opportunity to study natural history in

unexposed populations; more natural history studies of exposed and unexposed patients should be conducted. Further examinations of immune system dynamics, by developing profiles of low- and high-risk individuals, might reveal how this correlation between mucosal antibodies, serological antibodies, and HPV infection and disease is involved with physiological disease progression and immune prevention mechanisms.

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