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Characterization of IgA response among women with incident HPV 16 infection

Takashi Onda,[a] Joseph J. Carter,[a] Laura A. Koutsky,[b] James P. Hughes,[c] Shu-Kuang Lee,[b] Jane Kuypers,[d] Nancy Kiviat,[d] and Denise A. Galloway[a,d,e,*]

[a] *Program in Cancer Biology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109-1024, USA*
[b] *Department of Epidemiology, University of Washington, Seattle, WA 98195-7236, USA*
[c] *Department of Biostatistics, University of Washington, Seattle, WA 98195-7232, USA*
[d] *Department of Pathology, University of Washington, Seattle, WA 98195-7470, USA*
[e] *Department of Microbiology, University of Washington, Seattle, WA 98195-7242, USA*

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Abstract

Previous studies have characterized the prevalence and duration of serum IgG antibodies to human papillomavirus type 16 (HPV 16) in a well-studied cohort of college women, using viruslike particle- (VLP) based ELISAs. In this study IgA antibodies in cervical secretions and sera were examined using a newly developed capsomer-based ELISA and the patterns observed for serum IgG, serum IgA, and cervical IgA antibodies were compared. The median time to antibody detection from the first detection of HPV 16 DNA was 10.5 months for IgA in cervical secretions and 19.1 months for serum IgA. Serum IgA antibody conversion was observed less frequently and occurred later than IgA conversion in cervical secretions ($P = 0.011$) or serum IgG conversion ($P = 0.051$). The median time to antibody reversion, following seroconversion, was 12.0 months for IgA in cervical secretions and 13.6 months for serum IgA, whereas approximately 20% of women with serum IgG antibodies reverted within 36 months. Thus, the duration of IgA in cervical secretions and sera was shorter than the duration of serum IgG ($P = 0.007$ and 0.001).

Keywords: HPV; Papillomavirus; IgA; Serology; Seroconversion; VLP; Capsomer

Introduction

Several human papillomavirus (HPV) types, including HPV 16 and HPV 18, are causative agents of cervical cancer, which is one of the leading causes of death due to cancer among women worldwide (Working Group on the Evaluation of Carcinogenic Risk to Humans, 1995; Vizcaino et al., 2000). Animal studies have demonstrated that papillomavirus vaccines using viruslike particles (VLPs) composed of the major capsid protein (L1) or L1 and L2 provide protection from primary infection (Breitburd et al., 1995; Suzich et al., 1995; Kimbauer et al., 1996). In the canine and rabbit models, protection was shown to be mediated by neutralizing antibodies (Breitburd et al., 1995; Suzich et al., 1995). Vaccine studies conducted in humans have shown that VLP immunization induces high-titer neutralizing antibodies (Harro et al., 2001; Brown et al., 2001). The effectiveness and longevity of protection provided by these vaccines is being evaluated. Some insight into the potential utility of these vaccines may be gained by an understanding of the natural history of the antibody response following naturally acquired infections.

Previous studies by this laboratory have focused on the natural history of serum IgG responses to HPV types 16, 18, and 6 (Carter et al., 1996, 2000). IgA is the second most abundant isotype in serum and plays an important role for protection against pathologic agents at mucosal sites (Mestecky and Fultz, 1999). A number of cross-sectional

* Corresponding author. Mail Stop C1-015, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., P.O. Box 19024, Seattle, WA 98109-1024, USA. Fax: +1-206-667-5815.
E-mail address: jcarter@fhcrc.org (J.J. Carter).

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studies have demonstrated that IgA responses specific for HPV VLPs correlate with IgG responses or with the detection of HPV DNA of that type (Heim et al., 1995; Wang et al., 1996; Sasagawa et al., 1998; van Doomum et al., 1998; Petter et al., 2000). Longitudinal studies are required to characterize the natural history of immune responses. Only a few such studies have been conducted (Bontkes et al., 1999; Hagensee et al., 2000). Thus, the time between the acquisition of HPV infections and the development of specific IgA antibodies and the duration of these responses remains largely undefined.

Most studies of HPV-specific antibodies have employed VLPs produced in insect cells; however, highly immunogenic and genotype-restricted HPV capsid-neutralizing antigenic domains were shown to be contained entirely within capsomers (Li et al., 1997; Rose et al., 1998). The HPV VLP is composed of 72 capsomers, each of which is a pentamer of 5 L1 proteins (Chen et al., 2000). Assembly of capsids from capsomers was shown to be promoted by intercapsomeric disulfide bonds between certain cysteine residues (Sapp et al., 1995; Li et al., 1998; Modis et al., 2002) and mutation of particular cysteines in the C-terminal region of HPV 33 L1 was shown to assemble only into capsomers (Sapp et al., 1998). Comparison of bacterially produced HPV 11 VLPs with HPV 11 VLPs produced in insect cells found them to be immunologically indistinguishable (Li et al., 1997). These results suggested that HPV capsomers can be generated in a bacterial expression system and that capsomers may serve as effective and economical antigens in seroepidemiologic studies of HPV.

This study was designed to characterize the natural course of IgA responses in cervical secretions and sera against HPV 16 capsid proteins in comparison with IgG responses in sera. The women in this study had few previous sex partners and were HPV 16 DNA and serum antibody negative upon entry into the study. It is, therefore, likely that the antibody responses characterized here resulted from an initial exposure to the virus. The detection of HPV DNA in women who had entered the study HPV DNA and serum negative was considered to be evidence of incident infection. These studies employed an ELISA using bacterially expressed HPV 16 capsomers as antigen in order to develop a lower cost, and potentially more sensitive, antibody detection assay.

Results

Expression and purification of 16L1 capsomers

To produce HPV 16 L1 capsomers in bacteria, mutations were made in the HPV 16 L1 sequence such that cysteines 175 and 427 were converted to serines. HPV 16 L1 protein expression, under control of the *Lac* promoter, was induced and the cells harvested as described under Materials and Methods. Purification consisted of centrifugation, ammonium sulfate precipitation, and two rounds of ion-exchange chromatography as described under Materials and Methods and as shown in Fig. 1. L1 was eluted from the column in the final two washes (lanes 4 and 5). Only the L1 in the final wash (lane 5) was used for antibody testing.

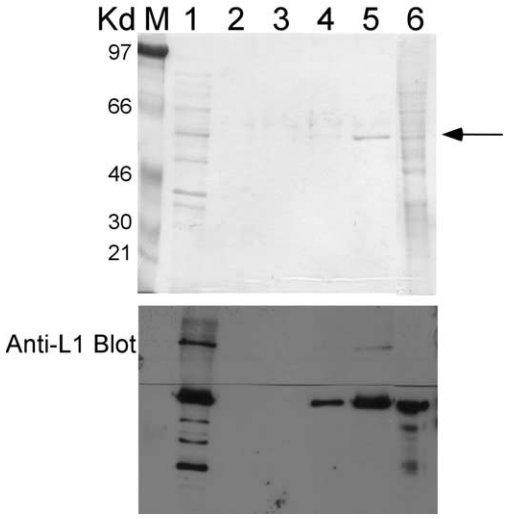

Fig. 1. Purification of 16L1 capsomers. Fractions were saved during the purification of 16L1 capsomers from bacteria and run a 10% SDS–PAGE. The top panel shows the Coomassie blue-stained gel. The lanes are marked as follows: (M) molecular weight size markers, (1) crude lysate applied to the column, (2) flow-through from the P11 column, (3) elution using 250 mM NaCl buffer, (4) elution using 500 mM NaCl buffer, (5) elution using 1 M NaCl buffer, HPV 16 L1 from vaccinia virus. 16L1 was detected using Camvir-1 in the immunoblot shown in the lower panel.

Reactivity of HPV 16 capsomers in capture assay and direct assay

Our previous serological studies have used a capture ELISA format in which monoclonal antibodies that recognize conformational epitopes on the VLP are used to tether the VLPs to the plate (Carter et al., 2000). However, several other investigators bind VLPs directly to the plate (Wang et al., 1996; Wideroff et al., 1995). We investigated how the capsomers would perform in both capture and direct ELISAs. The ELISA values of 48 serum samples from HPV 16 DNA-positive women were measured in a direct capsomer assay for IgG and compared with capture VLP IgG ELISA values (Fig. 2). The sera were selected from a cohort of women who were monitored for the presence of HPV DNA. All of the sera shown here were from women who tested HPV 16 DNA positive prior to or at the time sera were drawn. The ELISA values for the direct capsomer assay correlated well with those in the capture-VLP assay. The R values (Pearson's coefficient of correlation) and P values were $R = 0.846$ and $P < 0.0001$. Capsomers were also used in a capture assay and a somewhat better correlation with the capture-VLP ELISA values ($R = 0.935$) was observed. However, from this and other experiments (not

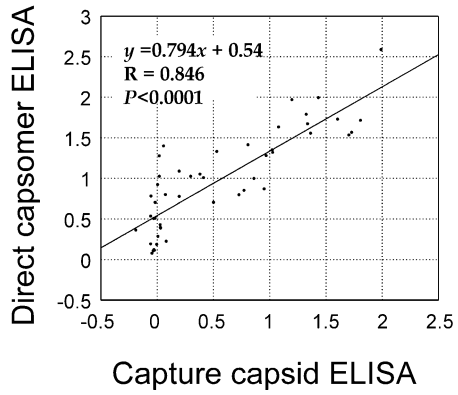

Fig. 2. Reactivity of HPV 16 capsomer with serum samples taken from HPV 16 DNA-positive women was compared with those of an HPV 16 VLP capture ELISA. Figure 1 shows the correlation of ELISA values between a direct capsomer IgG assay and a capture VLP IgG assay.

shown), it was determined that the capture capsomer ELISA was less sensitive than the direct capsomer ELISA. Therefore, capsomer assays for IgA detection in cervical secretions and sera were performed using direct assays.

Correlation of DNA and antibody status

Women with incident HPV 16 infections, that is, women who entered the study HPV 16 DNA negative and HPV 16 seronegative and subsequently had HPV 16 DNA detected at one or more visits during follow-up ($n = 57$), were the focus of this study. In order to be included for analysis, serum or cervical secretions had to be collected within 4 months of the detection of HPV 16 DNA. This resulted in 24 women for the analysis of cervical secretions, 44 women for the analysis of serum IgA, and 52 women for serum IgG.

The presence of IgA in cervical secretions and sera was tested by capsomer ELISA and these results were compared with previous results for serum IgG detection using a VLP-capture ELISA (Carter et al., 1996, 2000). Antibody status was compared with the presence of DNA in these women (Table 1). HPV 16 DNA status was divided into three categories; visits prior to the first HPV 16 DNA detection, HPV 16 DNA positive visits, and visits that were HPV 16 DNA negative from subjects that had previously tested positive. Antibodies were detected more frequently among samples collected at the time of DNA positivity than prior to the detection of HPV 16 DNA for all antibody types.

IgA in cervical secretions was detected in 7 of 66 (10.6%) samples taken prior to HPV 16 DNA detection compared with 23 of 67 (34.3%) samples collected at HPV 16 DNA positive visits. Similarly, serum IgA was detected in only 3 of 172 (1.7%) samples collected prior to the first detection of HPV 16 DNA but in 32 of 135 (23.7%) samples collected coincident with HPV DNA detection; serum IgG was detected in only 8 of 218 (3.7%) samples prior to HPV 16 DNA detection but in 53 of 149 (35.6%) samples collected at the time of HPV 16 DNA positivity.

IgA was detected less frequently in samples collected after HPV 16 DNA was no longer detected compared with samples collected at DNA positive visits, but this pattern was not seen for serum IgG (Table 1). Cervical IgA was detected in 9 of 52 (17.3%) samples and serum IgA was detected in 18 of 115 (15.7%) sera; however, serum IgG was detected in 62 of 120 (51.7%) sera from visits of previously HPV 16 DNA positive women.

The specificity of the IgA assays for HPV 16 was assessed by comparing the antibody status between women who had other types of HPV DNA detected by PCR (other than HPV 16) with women who had had no HPV detected prior to antibody testing. For IgA in sera, there were 3 seropositive women among the 102 (2.9%) specimens (from 38 women) who had tested HPV DNA negative. There were no (0.0%) seropositives among the 70 samples from 34 women in whom other types of HPV DNA had been detected (16 women acquired other types of HPV DNA during the study and thus contributed sera to both categories). Among the cervical specimens there were 4 antibody positives among the 31 (12.9%) samples from 12 women who were HPV DNA negative and 3 antibody positives detected among the 35 (8.6%) samples from 13 women in whom other HPV DNA types were detected. Three women contributed specimens to both categories.

Time to first antibody detection

The median time to first antibody detection was 10.5 months for IgA in cervical secretions, 19.1 months for serum IgA, and 10.8 months for serum IgG (Fig. 3). The cumulative rates of antibody conversion at 18 months for IgA in cervical secretions, serum IgA, and serum IgG were 87.3, 47.7, and 64.7% respectively. Serum IgA conversion was slower than conversion for cervical IgA (log rank, $P = 0.01$, Cox) and serum IgG ($P = 0.05$, Cox). There was no significant difference in the conversion rate for IgA in cervical secretions vs the conversion rate for serum IgG (P

Table 1
Correlation between DNA status and antibody status

	HPV16 DNA status		
	DNA-negative-Pre	DNA-positive visits	DNA-negative-Post
Cervical IgA			
Negative	59	44	43
Positive	7	23	9
Total	66	67	52
Serum IgA			
Negative	169	103	97
Positive	3	32	18
Total	172	135	115
Serum IgG			
Negative	210	95	57
Positive	8	53	62
Total	218	148	119

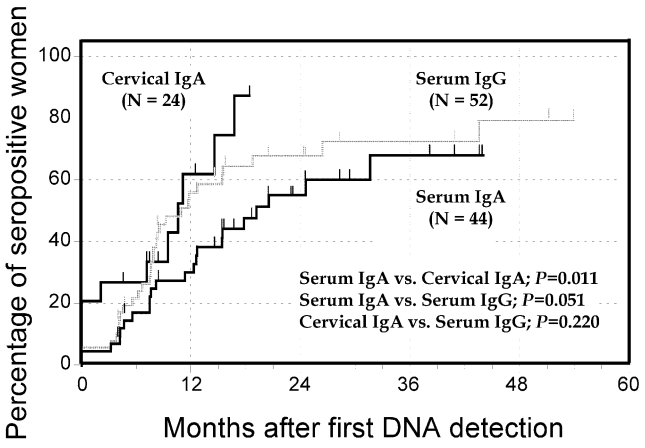

Fig. 3. Time to first antibody detection was analyzed using Kaplan–Meier analysis. Solid black, solid dark gray, and solid light gray lines show the positive rates of IgA in cervical secretions, IgA and IgG in serum samples, respectively.

= 0.22, Cox). Figure 3 shows the results of analysis, including 477 serum samples from 52 women and 124 cervical secretions from 24 women (total of 55 women). Concerning the analysis comparing serum IgG with IgA, similar results were obtained when the analysis was restricted to the 337 samples from 44 women for whom there was data for serum tests for both IgA and IgG (not shown). When the analysis was restricted to 20 women for whom there were cervical IgA as well as serum IgA and serum IgG results, serum IgA conversion was still significantly slower than cervical IgA ($P = 0.04$).

Persistence of antibodies

For the analysis of antibody persistence, the same set of women with incident HPV 16 infection as described above was used with the additional criterion being that they must have converted from an antibody negative to an antibody-positive status. Fourteen women were used for the analysis of IgA antibody persistence in cervical secretions, 25 women for serum IgA, and 34 women for serum IgG (42 different women in total). Figure 4 shows the rates of reversion to a negative antibody test among women who had at least one positive antibody test. The median time to antibody reversion was 12.0 months for IgA in cervical secretions and 13.6 months for serum IgA. More than 50% of women who developed serum IgG remained seropositive throughout follow-up. Serum IgG was more likely to persist than IgA in cervical secretions and sera ($P = 0.007$ and 0.001, Cox). There was not a significant difference between IgA in cervical secretions and serum IgA antibody persistence ($P = 0.73$, Cox).

Figure 4 shows the results of analysis, including all women eligible for the analysis (42 women in total). When analysis was restricted to the 21 women who developed both serum IgA and serum IgG, IgG was more likely to persist than serum IgA ($P = 0.007$). Only 4 women provided persistence information for all three antibody types.

DNA duration and antibody conversion

Production of antibodies and the DNA-positive periods were related (Fig. 5). Women were divided into two categories (i.e., women who were DNA positive at one visit only and women with multiple HPV 16 DNA-positive visits). The rate of antibody conversion was analyzed by Kaplan–Meier survival methods. The percentage of antibody conversion among women with multiple DNA-positive visits and women HPV 16 DNA positive only once was, respectively, 72.5 and 0% at 1 year for IgA in cervical secretions, 59.9 and 25% at 2 years for serum IgA, and 75.2 and 20% at 2 years for serum IgG. Though differences between the serum IgA curves did not reach statistical significance ($P = 0.299$, log rank test), the differences between cervical IgA curves and serum IgG curves were statistically significant ($P = 0.030$ and 0.040, log rank test). The influence of DNA duration on antibody conversion was further analyzed by subdividing the women with multiple DNA-positive results into groups based upon the duration of DNA positivity (e.g., less and not less than 12 months or less and not less than 6 months of DNA duration). These analyses revealed no differences between the groups for the production of three antibodies (data not shown).

Discussion

In the present study we examined IgA responses against HPV 16 L1 in cervical secretions and sera and compared those responses with serum IgG responses among women who acquired what were likely to be primary HPV 16 infections. The most interesting finding was the obvious

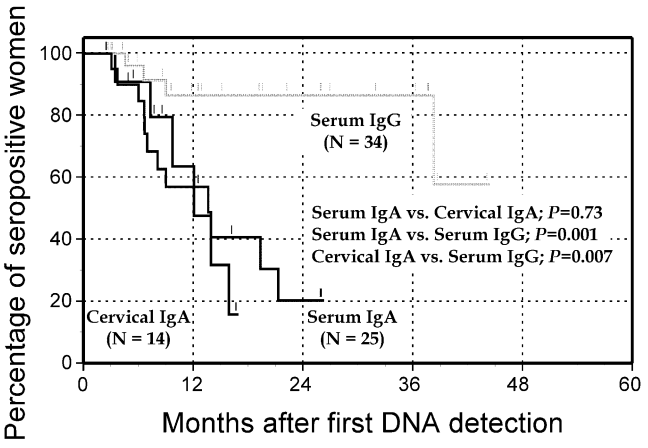

Fig. 4. Durations of antibodies were analyzed using Kaplan–Meier analysis. Solid black, solid dark gray, and solid light gray lines show the positive rates of IgA in cervical secretions and IgA and IgG in serum samples, respectively.

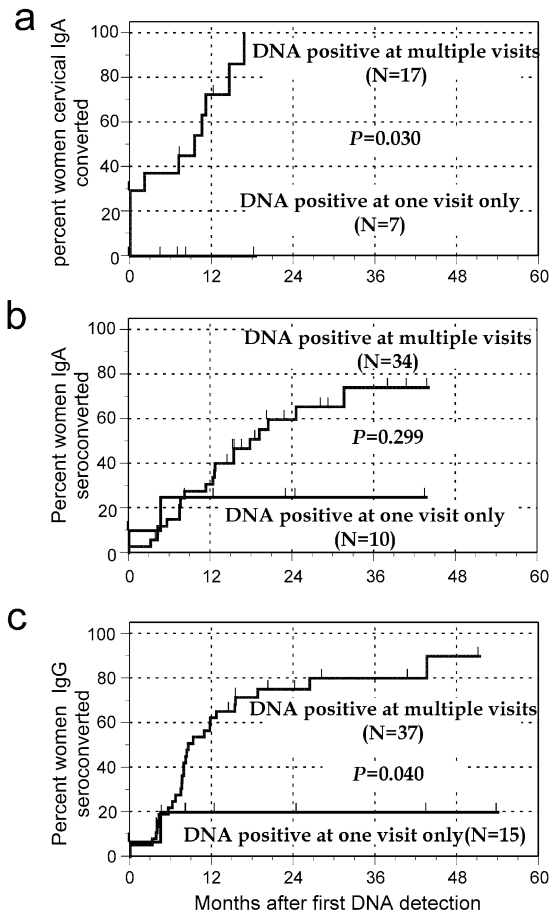

Fig. 5. Association between persistence of HPV 16 DNA detection and antibody detection. Survival curves compare rates of antibody detection between women who were HPV 16 DNA positive only once (solid black lines) and women having several HPV 16 DNA-positive visits (solid dark gray lines) for IgA in cervical secretions (A), IgA in sera (B), and IgG in sera (C).

difference between the persistence of the IgG responses over time and the transient nature of the IgA responses. IgA seroconversion appeared to be delayed relative to the IgG response and, like the IgG response, usually occurred many months after the first detection of HPV DNA.

In line with previous observations using HPV 16 VLPs as antigen (Wang et al., 1996; Sasagawa et al., 1998; Bontkes et al., 1999), the presence of IgA in cervical secretions, IgG in sera, and IgA in sera were associated with the presence of HPV 16 DNA in genital mucosa at the time of collection. IgA in cervical secretions and serum anti-VLP antibodies were detected at much higher frequencies in samples collected coincident with HPV 16 DNA detection compared with samples collected before HPV DNA detection (Table 1).

Concerning the time to antibody detection and duration of serum IgG, we and others reported that IgG seroconversion against HPV 16 generally occurs within 6 (Wikstrom et al., 1995) to 12 (Carter et al., 2000) months after HPV 16 infection and that IgG response remains high over a long period of time (van Doornum et al., 1998; Af Geijersstam et al., 1998; Carter et al., 2000). Limited information is available on the natural history of IgA in cervical secretions and IgA in sera and it is not entirely consistent. Wang et al. (1996) reported that a substantial proportion of women positive for IgA in cervical mucus remained positive at their subsequent visits, although exact intervals between visits were not shown. Sasagawa et al. (1998) demonstrated an early appearance and longer persistence of serum IgA compared to serum IgG, and Elfgren et al. (1996) reported that after conization for cervical intraepithelial neoplasia, serum IgA was more stable than serum IgG and cervical mucosal IgA, though this observation was not based on data from a natural history study.

To better characterize the temporal patterns of antibody conversion and duration, we analyzed time to antibody conversion and reversion during longitudinal follow-up. Different patterns of production and reversion for IgA in cervical secretions and IgG and IgA in sera were found (Figs. 3 and 4). After HPV 16 DNA infection, 64.7% of women developed serum IgG antibodies by 18 months (median time to detection; 10.8 M). Duration of antibodies were long (only 1 reverted by year 3). IgA in cervical secretions was detected in 87.3% of women within 18 months of the first detection of HPV 16 DNA and occurred coincident with serum IgG (median time to detection; 10.5 M). Duration of IgA antibodies was short (50% reverted by month 12). IgA in sera developed more slowly than IgA in the cervical mucosa or serum IgG (median time to detection; 19.1 M) and a lower percentage of women developed serum IgA (47.7% within 18 months). Similar to IgA in cervical secretions, the duration of serum IgA antibodies was short (50% seroreverted within 14 months). Several reports have suggested that anti-HPV antibody responses persist (Wang et al., 1996; Elfgren et al., 1996; Sasagawa et al., 1998); however, Wang et al. (2000) reported that serum IgA was associated with recent sexual activity and suggested that this meant serum IgA did not persist. Our results are consistent with Wang et al. and reminiscent of the pattern seen for *Chlamydia trachomatis* serum IgA antibody responses (Osborne et al., 1989) where these responses were a marker for active infections. It must be noted that there were too few women (4) for whom we had samples who converted to all three antibodies (serum IgA and IgG and cervical IgA) to do a comparison.

HPV 16 DNA persistence for longer than one visit has been shown to be associated with IgG seropositivity (Wideroff et al., 1995; de Gruijl et al., 1997; Carter et al., 2000) and IgA seropositivity (Sasagawa et al., 1998; Bontkes et al., 1999) for HPV 16 VLPs. In agreement with these observations, our data showed that the production of serum IgA occurred less frequently among women with only one DNA positive visit. A correlation was also observed between HPV 16-specific IgA responses in cervical secretions and the number of HPV 16 DNA-positive visits. In addition, our data revealed higher rates of both IgA and IgG sero-

conversion among women with versus those without repeated detection of HPV 16 DNA in genital samples. Thus, the duration of HPV 16 DNA in genital mucosa seems to be an important factor for the production of IgA in cervical secretions and IgG and IgA in sera.

The experiments presented here employed a new ELISA system for the detection of IgA antibodies, using bacterially expressed and purified HPV 16 capsomers as antigens instead of HPV 16 VLPs expressed in eukaryotic cells using vaccinia virus or baculovirus vectors (Carter et al., 1996). The new assay was developed as part of a continuing search for improved methods for the detection of antibodies to HPVs. To evaluate the new ELISA, this test was compared to a VLP capture assay using selected human sera (Fig. 2). The results from direct capsomer IgG assays correlated well with those from the capture VLP IgG assay. No samples were identified that reacted only with VLPs. These results suggested that bacterially expressed capsomers possessed conformational epitopes that were similar to those on whole VLPs and that only very weak or no epitopes exist at intercapsomeric regions of VLPs. Therefore, capsomers seemed to serve as useful antigens for detection of anti-HPV 16 L1 antibodies. In a previous study (Hagensee et al., 2000), a luminescence immunoassay was used to improve sensitivity of the detection of IgG and IgA antibodies in cervical secretions. It was not possible to directly compare the results from the capsomer assay with Hagensee's results because the cervical samples previously tested were no longer available. However, the capsomer IgA assay appeared to have a similar or higher sensitivity than the capture-VLP luminescent IgA assay because the proportion of seroconversions observed in women with incident HPV 16 DNA was higher with the capsomer assay than the luminescent assay (data not shown).

If the use of capsomers was less sensitive for the detection of HPV 16 IgA than a VLP based assay it would be anticipated that antibodies would be detected less frequently among HPV 16 DNA-positive women. It was found here that the proportion of women developing antibodies to IgA, as detected by the capsomer ELISA, was not significantly different than the proportion of women seroconverting for IgG, as detected by the capture capsid ELISA (Fig. 3), suggesting that the assays had similar sensitivity.

The specificity of the IgA capsomer ELISA was assessed by examination of the results from women in whom HPV 16 DNA had not been detected at previous or current visits (see Table 1). For cervical specimen 10.6% were IgA antibody positive, whereas only 1.7% of sera were IgA antibody positive. In neither case was antibody positivity associated with the detection of other HPV types in these women (not shown). The results from sera indicated that the use of capsomers produced a highly specific assay. The assay for antibodies in cervical specimen was less specific perhaps due to the use of more concentrated samples.

In conclusion, we developed a new ELISA using HPV 16 capsomers to detect IgA directed against HPV 16 L1 proteins. The ELISA values for the direct capsomer assay correlated well with those in the capture-VLP assay. Compared with IgG serum antibody responses, IgA antibody responses detected in sera and in cervical secretions were more transient.

Materials and methods

Study population

The University of Washington Human Subjects Division approved all protocols and consent forms. The study population was a subset of the 603 female university students enrolled in a study of the natural history of HPV infection described previously (Hagensee et al., 2000; Carter et al., 2000). Clinic visits were scheduled at 4-month intervals. At each clinic visit, cervical and vulvovaginal swabs for the detection of HPV DNA, as well as blood and cervical secretions (from June 1994) for the detection of HPV antibodies, were collected.

Detection of HPV DNA

The presence of HPV DNA was determined by PCR using the MY09 and MY11 consensus primers as described previously (Carter et al., 1996). The PCR products were probed with a biotin-labeled generic HPV probe and type-specific oligonucleotide probes that included a probe for HPV 16. The samples whose PCR products reacted with both generic HPV probe and type-specific HPV 16 probe were determined as HPV 16 positive.

Collection and preparation of cervical secretions

Cervical secretions were collected using two tear flow test strips (Sno-Strips, Abita Springs, LA, USA) placed on the cervical os until they were saturated to the shoulder. After absorption of cervical secretions, strips were removed and placed into a vial containing 500 μl of sterile phosphate-buffered saline (PBS with 0.01% sodium azide), and the samples were kept frozen at -20°C until use. Before use, samples were thawed, mixed with 125 μl of 5$\times$ RIPA buffer [750 mM NaCl, 5.0% Nonidet P-40, 2.5% deoxycolic acid, 0.5% sodium dodecyl sulfate, and 250 mM Tris–HCl (pH 8.0)], and incubated for 1 h at room temperature. Then, the strips were removed by centrifugal filtration (20,000*g* for 15 min.) using a 0.45-μm centrifugal filter (Ultrafree-MC, Millipore, Bedford, MA, USA). In a previous study using cervical secretions (Hagensee et al., 2000), the total amount of protein, the total amount of IgG and IgA, and the presence of blood were measured. None of these variables were found to be useful in the final analysis and the practice was not continued in these studies.

Preparation of HPV 16 capsomers

Primers were designed to make 16 L1 proteins with double Cys to Ser mutations at positions 175 and 428. The primers also contained restriction endonuclease sites for stepwise cloning. [5′-TGACTGAAGCTTGGTCTCACATGTCTCTTTGG (first sense), 5′-CAGTCATCTAGAGTCGACGGGGATCCTTTG (first antisense), 5′-TGACTGTCTAGAGTCGACCAATGTTGCAGTAAATCC (second sense), 5′-CAGTCATCTAGAGCTAGCAATTGCCTGGGATG (second antisense), 5′-TGACTGTCTAGAGCTAGCCAAAAACATACACC (third sense) and 5′-CAGTCATCTAGACTCGAGTTACAGCTTACG (third antisense)]. The 5′ fragment of HPV 16 L1 was generated by PCR using a high-fidelity polymerase (Deep Vent, New England Biolabs, Mississauga, Ontario, Canada). The polymerase chain reaction (PCR) product was digested with restriction enzymes and cloned into pBS- (Stratagene, La Jolla, CA, USA). The other two fragments were generated by PCR and cloned into the same plasmid in a stepwise fashion. The final product was sequenced to verify the changes. The mutated L1 coding region was subcloned into pET19b (Novagen, Madison, WI, USA)(pET19b/HPV 16L1).

pET19b/HPV 16L1 was introduced into host bacteria (DE3). Bacteria were grown in LB medium with 200 μg/ml of ampicillin, and then L1 proteins were expressed overnight at 30°C after induction with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). L1 proteins were purified through centrifugal fractionation and ion-exchange column procedures generally as previously described (Li et al., 1997) except for omitting the Polymin P step. Bacteria from 2 liters of culture were suspended in 200 ml of buffer A [0.25 M NaCl, 50 mM Tris–HCl (pH 7.9), 5% glycerol, 2 mM EDTA, 15 mM 2-mercaptoethanol (2ME)] and incubated with 200 μg/ml of lysozyme at 4°C for 20 min. Then Triton $\times$ (final concentration of 0.1%) and 4 tablets of Complete (Roche, Indianapolis, IN, USA) were added. The mixture was sonicated with a Branson Sonifier (Model No. 250, VWR Scientific, Willard, OH, USA) for 3 times of 40-s. bursts at 1-min intervals. The sonicated suspension was subjected to 20 rounds of homogenization with Kontes Dounce Tissue Grinder (VWR Scientific). The homogenate was centrifuged at 12,000*g* for 20 min and the pellet extracted with 100 ml of buffer B [1 M NaCl, 10 mM Tris–HCl (pH 7.9), 5% glycerol, 2 mM EDTA, 15 mM 2ME] with homogenization. The suspension was centrifuged at 10,000*g* for 15 min. Supernatants were combined and precipitated with 35% saturation of ammonium sulfate. This precipitate was resuspended in 20 ml of buffer C1 [100 mM NaCl, 10 mM Tris–HCl (pH 7.2), 5% glycerol, 2 mM EDTA, 15 mM 2ME] and dialyzed against the same buffer. Insoluble material was removed by centrifugation at 10,000*g* for 20 min. Supernatant was loaded on DE-52 (Whatman, Clifton, NJ, USA) anion exchange cellulose column with buffer C1. The flow-through was applied to a P11 (Whatman) cation-exchange cellulose column. The column was washed with buffer C2 [250 mM NaCl, 10 mM Tris–HCl (pH 7.2), 5% glycerol, 2 mM EDTA, 15 mM 2ME] and buffer C3 [500 mM NaCl, 10 mM Tris–HCl (pH 7.2), 5% glycerol, 2 mM EDTA, 15 mM 2ME]. Protein (L1) was eluted with buffer C4 [1 M NaCl, 10 mM Tris–HCl (pH 7.2), 5% glycerol, 2 mM EDTA, 15 mM 2ME].

Detection of antibodies against HPV 16 L1 proteins

Detection of serum IgG antibodies was performed using HPV 16 VLPs as previously described (Carter et al., 1996). To detect IgA antibodies in sera and cervical secretions, purified HPV 16 capsomers were used as antigens. Wells of microtiter plates (Immulon 2, Dynatech Laboratories Inc., Chantilly, VA, USA) were coated with or without 200 ng of HPV 16 capsomers in 50 μl of PBS. The plates were incubated at 4°C overnight, washed 3 times with PBS, blocked with 100 μl/well of blocker (5% goat serum and 0.05% Tween 20 in PBS), and incubated at room temperature for 1 h. Fifty microliters per well of sera (1:100 dilution) or cervical secretion (1:10 dilution of sample after extraction from strip as described above) in blocker was added and incubated at 37°C for 1 h. Plates were washed 3 times with PBS and incubated with 1/1000 dilution of rabbit antihuman IgA (Sigma, St. Louis, MO USA) at 37°C for 1 h. After washing 3 times with PBS plates were incubated with 1/1000 dilution of goat antirabbit IgG conjugated with alkaline phosphatase (ALP) (Roche No. 0605415) at 37°C for 1 h. Plates were washed as before and 100 μl of Sigma 104 phosphatase substrate (Sigma) was added [4.33 mg/ml in 0.1 M $NaHCO_3$ and 0.01 M $MgCl_2$ (pH 9.5)], to each well, plates were incubated at room temperature for 30 min. The optical densities were read on an automated plate reader (Elx808, BIO-TEK Instruments Inc., Winooski, VT, USA). Samples were tested in triplicate with and without capsomers. The ELISA values were calculated as the natural log of the median OD measurements with capsomers minus the natural log of the median OD measurements without capsomers as previously described (Carter et al., 1996). The cutoff points were calculated as the mean + 2 SD of the values of samples from 32 study participants who reported no sex partners and who were negative for HPV DNA. To remove the reactivity with bacterial proteins, all samples were preabsorbed with 1% (serum samples) or 0.1% (cervical secretions) acetone powder of DE3 bacterial proteins at 4°C for 1 h. The cross-reactivity of human IgG with rabbit antihuman IgA or goat antirabbit IgG was examined. Human IgG showed no reaction and did not affect the reaction of human IgA (data not shown).

Statistical analysis

Linear regression analyses were performed to compare the reactivity of HPV 16 capsomer and VLP. The association between antibody status and DNA status was analyzed

with Fisher's exact test. For the analysis of time to first antibody detection and duration of antibodies, the Kaplan–Meier product limit method was used (Kaplan and Meier, 1958). Analysis of the differences between the curves was performed using the logrank test or Cox model with adjustment for repeated observations within individual (Lin, 1994).

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