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Serological Responses to Human Papillomavirus Type 6 and 16 Virus-Like Particles in Patients with Cervical Neoplastic Lesions

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Serum samples from 36 cervical carcinoma patients, 33 patients with high-grade squamous intraepithelial lesions, and 31 cytologically normal women were tested by enzyme-linked immunosorbent assay (ELISA) using human papilloma virus type 6 (HPV 6) and HPV 16 virus-like particles as antigens. Forty serum specimens from 1-year-old children were used to assign cutoff points. When serum samples from the subjects infected with HPV 16 were tested in an HPV 16 ELISA detecting immunoglobulin A (IgA), IgG, and IgM binding, 61% showed IgA, 44% showed IgG, and 39% showed IgM reactivity. Of HPV 6- or 11- or HPV 18-infected subjects, fewer than 17% showed IgA or IgG responses and 33% showed IgM reactivity. In contrast, 13% showed IgA, 10% showed IgG, and 16% showed IgM reactivity in the HPV DNA-negative controls. The results suggest that the IgA and IgG responses are HPV 16 specific and the IgM response is cross-reactive to different HPV types. On the other hand, the serological responses to HPV 6 did not differ in the patient and control groups. The percentages of patients positive for both IgA and IgG antibodies were significantly higher in the groups with high-grade squamous intraepithelial lesions (12% [4 of 33]; $P = 0.04$) and cancer (17% [6 of 36]; $P = 0.02$) than in the healthy women (0% [0 of 31]), and the percentages for either IgA or IgG were higher for the cancer group (47% [17 of 36]; $P = 0.01$) than in the normal group (19% [6 of 31]). Most sera positive for IgA and IgG in the patient groups showed higher titers than those in the normal group. All these results suggest that high IgA and IgG responses are good indicators for estimating HPV 16 infection.

Human papillomaviruses (HPV) are small DNA viruses isolated from benign and malignant proliferative lesions of squamous epithelium. Advances in molecular biological techniques during recent years have allowed the identification of more than 70 types of HPV DNA in various human tissues. In mucosal epithelium, some of them have been found to be associated with squamous cell carcinomas and their precursor lesions. HPV types 16 (10) and 18 (2) (HPV 16 and HPV 18) are thought to be high-risk types, because they are often found in invasive cervical carcinomas and have transforming ability (34, 36, 38, 39), whereas HPV 30, 31, 33, 35, 45, 51, 52, 56, and 58 are intermediate-risk types (7, 23) because they are found in high-grade intraepithelial lesions but are rare in invasive carcinomas. On the other hand, HPV 6, 11, 44, and 55 are found in benign lesions such as condyloma acuminata, suggesting that they are low-risk types (22, 23).

Despite this evidence, we know little about the life cycle of high-risk HPV types and of immunological responses against the viruses in humans. There is no suitable system for propagation of HPV virions in vitro and no good animal model. However, recently, many groups have produced virus-like particles (VLP) of HPV 1 and HPV 11 in protein expression systems using vaccinia virus (14) or baculovirus (31). VLP of high-risk HPV 16 have also been produced in both baculovirus (18) and *Schizosaccharomyces pombe* (37) systems using the wild-type L1 sequence from newly isolated HPV 16 DNA (18, 30). These in vitro-produced capsids appear to have a structure identical to that of authentic virions as determined by electron microscopic examination.

Immunization with bovine papillomavirus and HPV 11 virions has elicited antibodies in animals which inhibit the infectivity of these viruses for cultured cells (11) and for xenografted tissues (12). These studies suggest that the native capsid structure of HPV may be essential for protective immunization. VLP of HPV 11 are recognized by neutralizing monoclonal antibodies (MAbs) against the native virus and also induce high titers of neutralizing antibodies in both rabbits and mice (5). Immunization of mice with vaccinia virus recombinants and/or VLP of HPV 1 generates serum immunoglobulin G (IgG), IgM, and mucosal IgA antibodies (13). These data suggest that the VLP can substitute for native virions.

A serological assay for genital HPV infection would have several advantages over the other clinical screening tests, since it would detect systemic responses to HPV infection, could be easily carried out in many laboratories, and could evaluate both past and recent exposure to HPV infection. For HPV 16, many studies using serological assays have been reported, but most of them have targeted the viral early proteins (8, 17, 20, 26, 35). Serological responses against HPV 16 L1 and L2 capsid proteins have been detected by Western blotting (immunoblotting) with bacterium-derived fusion proteins (20) or by enzyme-linked immunosorbent assay (ELISA) with synthetic peptides (4, 9, 21). Antibody responses have been detected in patients with cervical neoplasia by these methods; however, it is possible that antibodies recognizing intact viruses have been missed because the peptide antigens and denatured proteins used in Western blotting lack the native conformation. A re-

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cent report shows that more than half of women with cervical HPV 16 infection generate an IgG antibody response to conformationally dependent epitopes of the L1 and L2 proteins in ELISAs using HPV 16 VLP (3, 18, 27).

In this study, we established a system for testing human serum responses to HPV capsids by ELISA using HPV 6 and 16 VLP, produced in *S. pombe*. We observed increases of both IgA and IgG responses against HPV 16 capsids in patients with invasive cervical carcinoma and its precursor lesions compared with those of healthy women with no cervical cytological abnormalities.

MATERIALS AND METHODS

Patients. Thirty-six patients with invasive squamous carcinoma of the cervix, 33 patients with cervical high-grade squamous intraepithelial lesions (HSIL), and 6 patients with genital condyloma acuminata were studied. All diagnoses were performed histopathologically with tissue specimens obtained at the time of operation or colposcopy according to the Bethesda classification system (24) and the World Health Organization histological typing system (28). These patients ranged in age from 21 to 72 years and were treated between March 1992 and June 1994 at the Department of Obstetrics and Gynecology, School of Medicine, Kanazawa University.

Controls. Thirty-one women were selected as controls, including 18 patients admitted for operations for uterine myoma, ovarian cyst, or uterine prolapse and 13 healthy volunteers; these subjects showed normal cervical cytology. The median age of the controls was 44 years (standard deviation, 10.8; standard error, 1.9). These values did not differ significantly from those for the HSIL group, which had a median age of 43 years (standard deviation, 10.6; standard error, 1.8), and the invasive cancer group, which had a median age of 46.0 years (standard deviation, 9.5; standard error, 1.6).

To assign cutoff points in the ELISA, 40 serum samples were obtained from 1-year-old children in conjunction with a rotavirus vaccination trial at the Department of Pediatrics, School of Medicine, University of Tampere.

Analysis of HPV DNA. DNA samples purified from the cervical scraped cell samples of 15 HSIL patients or 20 healthy women and from the tissue samples of 20 cervical carcinoma or 6 condyloma acuminatum patients were analyzed for HPV typing by dot blot tests using two probe mixtures for HPV 6 or 11 (HPV 6/11) and HPV 16/18 (21). The HPV 16-positive samples were confirmed by Southern blot analysis with an HPV 16 DNA probe under high-stringency conditions (33). The HPV-negative samples in this study, therefore, were negative for HPV 6, 11, 16, and 18 but were not tested for other HPV types. All 20 samples from the healthy women were negative for HPV DNA. The other 11 healthy subjects and 34 patients with the cervical lesions were not examined for HPV DNA.

Purification of VLP from *S. pombe*. HPV 6 and HPV 16 L1 genes had been introduced into yeast expression vector REP, and the recombinants were induced to synthesize the major capsid proteins of each HPV type under the control of the thiamine-repressed promoter. Purification of the VLP was done as previously described (37), with some modifications. Nine liters of *S. pombe* containing an HPV L1 gene was incubated at 30°C for 20 h after induction of expression of the introduced gene and then harvested. The yeast cells were collected by being spun down at 2,000 × *g*, resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl), and mechanically broken with a cell disrupter (Stansted Fluid Power Ltd., Essex, United Kingdom). Total cell lysate was obtained by centrifugation at 10,000 × *g* at 4°C for 10 min using a Sorvall SS 34 rotor. The lysate was centrifuged to equilibrium in 27% (wt/wt) CsCl-HEPES buffer for 20 h at 141,000 × *g* in an SW28 rotor. The appropriate fraction (at a density of 1.29 g/ml) was harvested by puncturing the tube and centrifuged for 2.5 h at 100,000 × *g*. The pellet was resuspended in 27% CsCl-HEPES buffer, and the above process was repeated. The final pellet was resuspended in 25 mM Tris-Cl (pH 7.5)–2 M NaCl–2 mM MgCl and kept at 4°C until used.

Mouse MAbs against HPV capsids. An anti-HPV 6 antibody (H6 10B) and anti-HPV 11 antibodies (H11A3, H11B2, and H11H3) generated by immunization with native virions were kindly provided by N. D. Christensen (6). Anti-HPV 16 antibodies (Camvir 6 and Camvir 7) produced by immunization with HPV 16 VLP produced in the baculovirus system were provided by Margaret Stanley, Department of Pathology, University of Cambridge, Cambridge, United Kingdom. The anti-HPV 16 antibody (Camvir 1) was generated by immunization with HPV 16 L1 protein produced in *Escherichia coli* (25). In the ELISA using mouse MAbs, the MAbs were used at a 1:100 dilution of culture supernatant, and biotinylated sheep anti-mouse immunoglobulin (Amersham) was used at a dilution of 1:1,000. All the other steps were carried out in the same way as for the ELISA using human serum.

Immunoelectron microscopy (IEM). Three microliters of purified VLP was placed on glow-discharged carbon Formvar-coated grids and left for 5 min. After 30 min in blocking solution containing 5% normal goat serum, 5% bovine serum albumin (BSA), and 5% ovalbumin in phosphate-buffered saline (PBS), the grids were transferred onto drops of anti-HPV 6 or HPV 16 capsid mouse MAbs diluted 1:2 in PBS containing 0.5% BSA, 0.5% ovalbumin, and 1% normal goat serum and incubated for 1 h at room temperature. The grids were washed three times in PBS and then incubated with goat anti-mouse immunogold conjugate for 1 h. After three rinses in PBS and in distilled water, the grids were negatively stained with 1% aqueous uranyl acetate and examined with a Zeiss 10C electron microscope. As a negative control, an HPV 16 E2 antibody as the primary antibody or no primary antibody was used in each analysis.

ELISA for human serum samples. HPV 6 or HPV 16 VLP were resuspended in carbonate buffer (0.1 M, pH 9.6) and incubated at a concentration of 100 ng of VLP per well in ELISA plates at 4°C overnight. To reduce nonspecific reactivity, all serum samples which had been stored at −20°C until the time of assay were preabsorbed with 1% acetone powder of *S. pombe* overnight at 4°C. The VLP-coated plates were washed in PBS twice and blocked with PBS-BSA-fetal calf serum (FCS) (PBS containing 3% BSA and 0.5% FCS) at room temperature for 30 min. A 100-μl volume of preabsorbed human sera diluted 1:20 in the blocking buffer was added to the plates, which were then incubated at room temperature for 3 h. After three washes with PBS, 100 μl of biotinylated sheep anti-human immunoglobulin or biotinylated goat anti-human IgA, IgG, or IgM antibodies (Amersham) diluted 1:1,000 in PBS-BSA-FCS was added to each well, and the plates were incubated for 30 min at 37°C. After the plates were washed, 100 μl of streptavidin-peroxidase diluted 1:2,000 was added to each well. For development of the peroxidase reaction, ABT [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] was dissolved at 100 μg/ml in 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.0075% hydrogen peroxide (Sigma); 100 μl was added to each well, and the plates were incubated for 45 min at room temperature. The A_{405} was read with an automated plate reader (MR 5000; Dynatech).

The optical density (OD) for each serum sample was calculated as the readings for the samples minus the average reading for wells without capsids. Two serum specimens positive for HPV 6 and HPV 16 VLP were used as the internal standards for each VLP ELISA. To assign a cutoff point between positive and negative in each ELISA, we tested 40 serum samples from 1-year-old children who were presumed not to have been exposed to these genital HPV types. The final absorbances were adjusted relative to the internal standard to compensate for interassay variation, and the final OD of each sample was determined by taking the mean value from three different analyses. The cutoff point for the assay was taken as the average absorbance for the children's serum samples plus 2 standard deviations.

Data analysis. The Mann-Whitney U test was used to compare absorbance values. Fisher's exact probability test and the chi-square test were used to compare percent positive results.

RESULTS

Antibody responses to HPV 6 and HPV 16 VLP as determined by ELISA and IEM. We produced preparative amounts of HPV 6 and 16 L1 VLP from *S. pombe* (37) (Fig. 1). Before analyzing human sera, we tested the VLP ELISAs for sensitivity and specificity using MAbs against the HPV 6, HPV 11, and HPV 16 capsids (Table 1). MAbs H11A3, H11B2, and H11H3 and MAb H6 10B are neutralizing MAbs against HPV 11 and HPV 6, respectively. Camvir 6 and Camvir 7 are MAbs generated against HPV 16 VLP synthesized in the baculovirus system, and Camvir 1 is a MAb against L1 protein produced in *E. coli*. The reactivity of these antibodies to yeast-derived HPV 16 VLP was exactly the same as their reactivity to insect cell-derived VLP (data not shown). H6 10B, H11A3, and H11B2 reacted to HPV 6 VLP but not to HPV 16, while Camvir 6 and Camvir 7 reacted to HPV 16 VLP but not to HPV 6 VLP in the ELISA. These HPV type-specific responses were also observed by immunolabelling in IEM (Table 1 and Fig. 1). In contrast, Camvir 1 and H11H3 reacted to both HPV 6 and HPV 16 VLP in the ELISA but reacted to neither HPV 6 nor HPV 16 VLP in IEM. H11H3 and Camvir 1 may react with unassembled L1 proteins of both HPV types contaminating the purified VLP samples or generated by binding to the plate surface.

Optimization of ELISA conditions for human sera. To investigate the reactivity of our ELISA, 11 serum specimens from four patients with HPV 16 DNA-positive cervical lesions, two patients with HPV 6/11-positive condyloma acuminata, two patients with HPV 18 DNA-positive cervical lesions, and three patients with HPV DNA-negative cervical lesions were tested for immunoglobulin class-specific responses by HPV 16

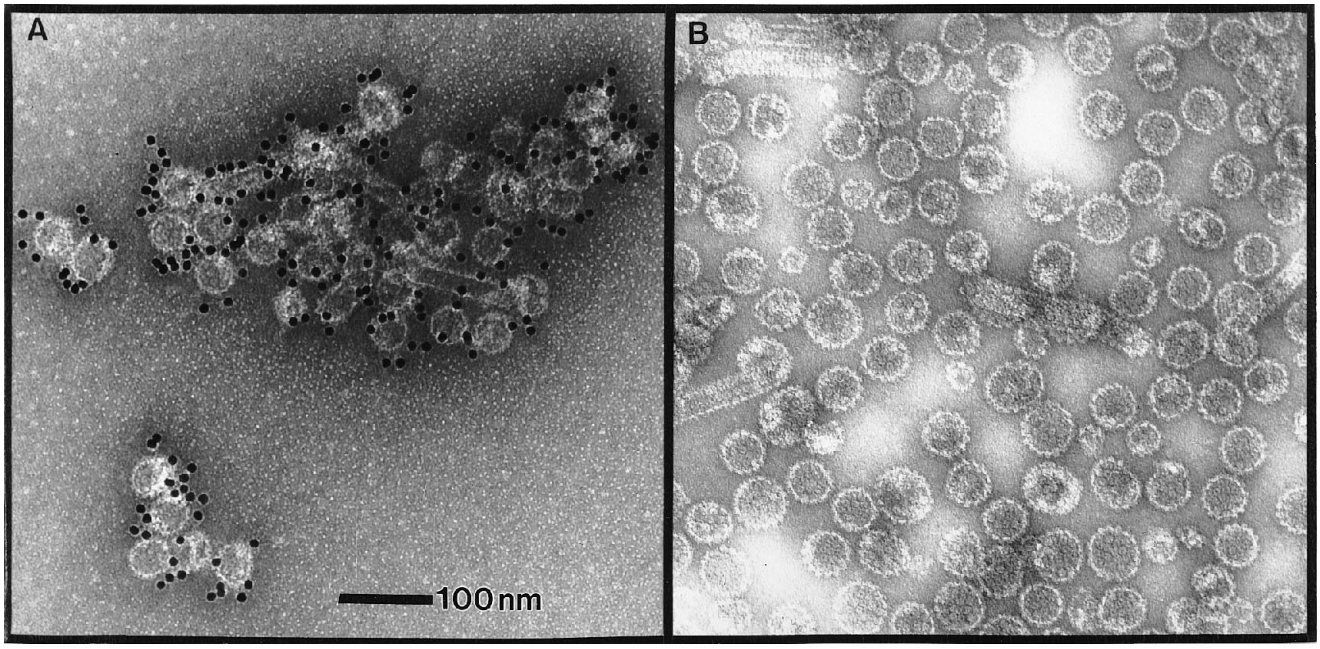

FIG. 1. Immunogold labelling in electron microscopy. HPV 16 VLP are labelled with Camvir 6 (A) but not with control (anti-HPV 16 E2) antibody (B).

VLP ELISA (Fig. 2). Each serum sample was serially diluted in the blocking buffer and tested for reactivity to HPV 16 VLP. Forty serum specimens from 1-year-old children were used as negative controls to assign cutoff points at serum dilutions of 1:10 and 1:20 for all immunoglobulin classes in this ELISA. The cutoff points for IgG and IgA were an OD of 0.2 at a 1:10 serum dilution and an OD of 0.15 at a 1:20 serum dilution, and those for IgM were an OD of 0.4 at a 1:10 dilution and an OD of 0.3 at a 1:20 dilution. For IgA, two of four HPV 16 DNA-positive patients showed positive patterns, whereas all four HPV 6/11-positive and HPV 18-positive patients and three HPV-negative patients were negative (Fig. 2A). For IgG, three of four HPV 16-positive patients and one HPV 6/11-positive patient were positive, whereas the other patients were negative (Fig. 2B). For IgM, one of four HPV 16-positive patients and two of three HPV-negative patients were positive, and two HPV 16-positive patients, two HPV 6/11-positive patients, and one HPV 18-positive patient showed a borderline pattern (Fig. 2C). The absorbance values of the sera showing a borderline pattern were over the cutoff point (OD = 0.4) at a 1:10 serum dilution but under the cutoff point at a 1:20 serum dilution (OD = 0.3). We decided to test all sera at a dilution of 1:20 to minimize the detection of nonspecific reactions in the ELISA.

Correlation between antibody response and HPV infection type. By our HPV test using dot blotting and Southern blotting, 18 of 35 patients (51%) were positive for HPV 16, 6 of 35 (18%) were positive for HPV 18, and the other 11 (31%) were HPV negative. All condyloma acuminatum patients were HPV 6/11 DNA positive. There were no double infections of the different HPV types. All 20 women who were normal in the cytological test were proved to be negative for HPV DNA. Thus, all 11 patients and 20 healthy women who were negative for HPV DNA were categorized as an HPV-negative group (Tables 2 and 3).

We tested the total immunoglobulin response in these subjects by the ELISA using anti-human immunoglobulin as a secondary antibody (Table 2). In the HPV 16 ELISA, the percent positive for the HPV 16-positive group was statistically higher than that for the HPV-negative group, whereas the values for the HPV 6-positive and HPV 18-positive groups were not so high. The median absorbances were also significantly higher for the HPV 16-positive group than for the HPV-

TABLE 1. Reactivity of HPV antibodies to HPV 6 and 16 VLP in ELISA and in IEM

Method and VLP	Reactivity with the indicated MAb[a]							
	Anti-HPV 6, H610B	Anti-HPV 11			Anti-HPV 16			Anti-HPV 16 E2, 261
		H11A3	H11B2	H11H3	Camvir 1	Camvir 6	Camvir 7	
ELISA								
HPV 6	0.473 (0.059)	0.487 (0.092)	0.456 (0.097)	0.408 (0.103)	0.632 (0.068)	0.022 (0.022)	0.025 (0.025)	0.018 (0.018)
HPV 16	0.004 (0.004)	0.012 (0.005)	0.029 (0.016)	0.354 (0.115)	0.562 (0.1)	0.514 (0.077)	0.568 (0.034)	0.022 (0.006)
IEM								
HPV 6	+	+	+	−	−	−	−	−
HPV 16	−	−	−	−	−	+	+	−

[a] ELISA results are expressed as OD (A_{405}); values in parentheses are 2 standard deviations. For IEM results: +, reactive to VLP; −, not reactive to VLP.

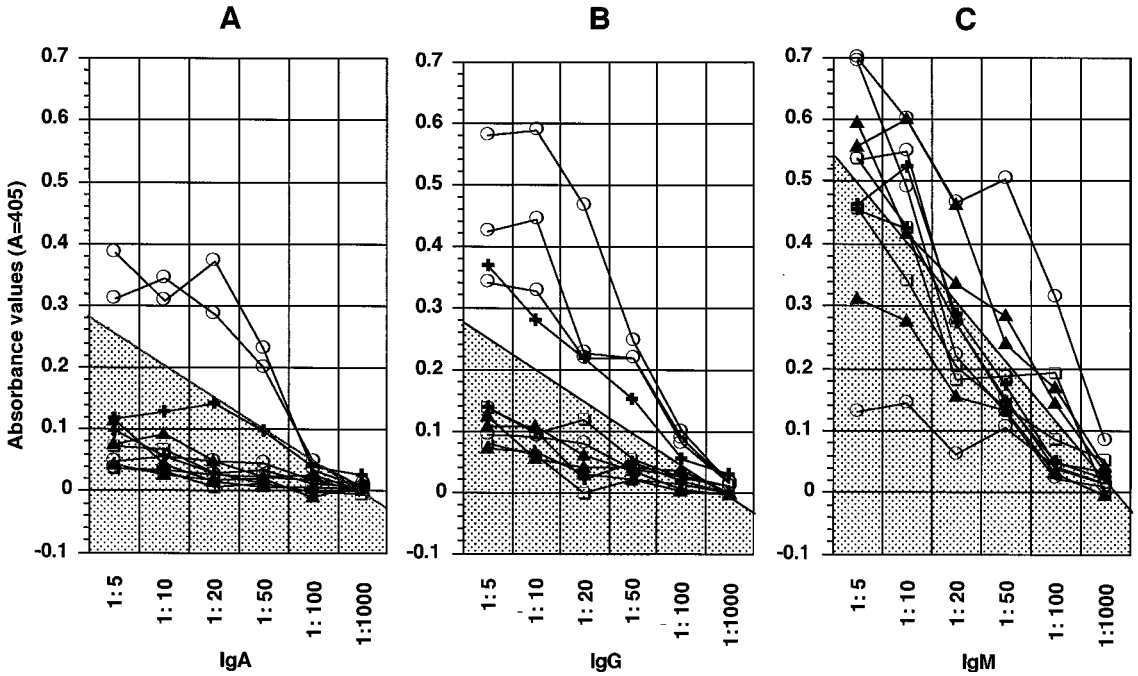

FIG. 2. Human serum responses in HPV 16 VLP ELISA. Nine serum specimens from patients with cervical carcinomas and two serum samples from patients with condyloma acuminata were evaluated in an ELISA using HPV 16 VLP as the antigen. Absorbances are shown for IgA (A), IgG (B), and IgM (C). Results are shown for cervical cancer patients infected with HPV 16 (○) or HPV 18 (□) or negative for HPV DNA (▲) as determined with HPV 6/11 and HPV 16/18 probes and for condyloma patients infected with HPV 6/11 (✚). Cutoff points were calculated by adding 2 standard deviations to mean absorbances for 40 serum specimens from 1-year-old children. The negative zones are shown as a dark areas.

negative group. Among the HPV-positive patients, the median absorbances of the HPV 16-positive group were higher than those of the HPV 18-positive group ($P = 0.044$) but not statistically different from those of the HPV 6/11-positive groups ($P > 0.1$). In contrast, in the HPV 6 VLP ELISA, none of the values differed among the HPV-positive groups and controls, suggesting that the serum responses observed in the HPV 16 ELISA may represent a response to HPV 16 or its related HPV types but not to HPV 6. Therefore, further analyses were carried out mainly by HPV 16 VLP ELISA.

Immunoglobulin class-specific responses were examined in the HPV 16-ELISA. As shown in Table 3, the percentages of subjects positive for IgA and IgG were statistically higher for the HPV 16-positive group than for the HPV-negative group, whereas no differences were observed between the other HPV-positive groups and the HPV-negative group. In comparison of median absorbances, the HPV 16-positive group had higher values than the controls for the IgG response, and the HPV 16-positive and HPV 6/11-positive groups had higher values than the controls for the IgA or IgM response. Among the HPV-positive groups, the IgA response was greater in the HPV 16-positive group (OD = 0.215) than in the HPV 18-positive group (OD = 0.062; $P = 0.005$) but was not different from that of the HPV 6/11-positive groups (OD = 0.120; $P > 0.1$). The IgG response was greater in the HPV 16-positive group (OD = 0.142) than in the HPV 6/11-positive (OD = 0.062; $P = 0.016$) and HPV 18-positive (OD = 0.051; $P = 0.003$) groups. For IgM response, no differences were observed in percent positive and in median absorbances among all HPV-positive groups. All the results suggest that the IgM response in this HPV 16 ELISA seems to be cross-reactive to different HPV types, and IgA and IgG tests are useful for predicting HPV 16 infection.

TABLE 2. Total immunoglobulin response to HPV 16 and HPV 6 VLP in women infected with HPV as determined by ELISA

Group (n)[a]	HPV 16 VLP ELISA result		HPV 6 VLP ELISA result	
	% Positive (P)[b]	Median absorbance (P)[c]	% Positive (P)	Median absorbance (P)
HPV 16+ (18)	50 (0.04)	0.571 (0.03)	22 (NS[d])	0.484 (NS)
HPV 6/11+ (6)	17 (NS)	0.443 (NS)	50 (NS)	0.511 (NS)
HPV 18+ (6)	17 (NS)	0.329 (NS)	17 (NS)	0.452 (NS)
HPV− (31)	13	0.442	18	0.434

[a] Patients with cervical lesions infected with HPV 16 (HPV 16+) or with HPV 18 (HPV 18+), patients with condyloma acuminata with HPV 6/11 (HPV 6/11+), and patients or healthy women in whom HPV was not detected (HPV−).
[b] P values indicate significant differences in HPV groups compared with the HPV-negative controls by Fisher's exact probability test.
[c] P values indicate significant differences in HPV groups compared with the HPV-negative controls by Mann-Whitney U test.
[d] NS, not significant.

To evaluate cross-reactivity of the sera positive for HPV 16 with HPV 6 VLP, the same samples were examined for reactivity in an HPV 6 VLP ELISA. Among the specimens seropositive for HPV 16 in the HPV 16-positive group, 3 of 11 samples (27%) positive for IgA, 1 of 8 samples (13%) positive for IgG, and 3 of 7 samples (43%) positive for IgM were also positive for HPV 6. One of two samples positive for IgM in the HPV 18-positive group was also reactive to HPV 6 VLP, whereas none of eight serum samples positive for HPV 16 in the HPV DNA-negative group responded to HPV 6. With regard to IgA and IgG antibodies, the double positives in the HPV 16-positive group may be due to antibodies generated against both HPV 6 and HPV 16 infections. In contrast, IgM

TABLE 3. IgA, IgG, and IgM responses to HPV 16 VLP in women infected with HPV as determined by ELISA

Group (*n*)[a]	IgA		IgG		IgM	
	% Positive (*P*)[b]	Median absorbance (*P*)[c]	% Positive (*P*)	Median absorbance (*P*)	% Positive (*P*)	Median absorbance (*P*)
HPV 16+ (18)	61 (0.001)	0.215 (<0.0001)	44 (0.001)	0.142 (0.01)	39 (NS[d])	0.226 (0.03)
HPV 6/11+ (6)	0 (NS)	0.120 (0.02)	17 (NS)	0.062 (NS)	33 (NS)	0.173 (0.02)
HPV 18+ (6)	17 (NS)	0.062 (NS)	0 (NS)	0.051 (NS)	33 (NS)	0.151 (NS)
HPV− (31)	13	0.069	10	0.101	16	0.160

[a] Patients with cervical lesions infected with HPV 16 (HPV 16+) or with HPV 18 (HPV 18+), patients with condyloma acuminata with HPV 6/11 (HPV 6/11+), and patients or healthy women in whom HPV was not detected (HPV−).
[b] *P* values indicate significant differences in HPV groups compared with the HPV-negative controls by Fisher's exact probability test.
[c] *P* values indicate significant differences in HPV groups compared with the HPV-negative controls by Mann-Whitney U test.
[d] NS, not significant.

double positives are likely due to a cross-reaction because of high percentages of IgM cross-reactivity.

Antibody response to HPV 16 VLP in patients with HSIL or with invasive cervical carcinoma and in healthy women. IgA, IgG, and IgM responses to HPV 16 in 31 cytologically normal women, 33 patients with HSIL, and 36 patients with invasive carcinoma were investigated, irrespective of HPV typing (Table 4 and Fig. 3.) The percentages of subjects positive for IgG were significantly higher and for IgA were slightly higher in the cancer group than in the normal group, but the same values did not differ between the HSIL and normal groups or between the HSIL and cancer groups. In IgM responses, the percentages positive were not significantly high in the patient groups compared with those for the healthy women.

When the absorbance values for IgA and IgG were plotted in a graph in each case (Fig. 3), 4 of 33 members (12%) of the HSIL group and 6 of 36 members (17%) of the cancer group were positive for both IgA and IgG, whereas none of the healthy women were positive for both. The percentages positive for both IgA and IgG were statistically higher for the HSIL ($P = 0.041$, chi-square test) and cancer ($P = 0.017$, chi-square test) groups than for the healthy women. In the responses to either IgA or IgG, 10 of 33 patients (30%) in the HSIL group and 17 of 36 patients (47%) in the cancer group were positive, while 6 of 31 subjects (19%) in the normal group were positive. Many HPV 16-positive patients and some patients with unknown HPV types showed high-level IgA and IgG responses, whereas most of the healthy women and patients negative for HPV DNA showed weaker responses (Fig. 3). Such high IgA and IgG reactivities seem to increase the median absorbances in the HSIL and cancer groups compared with those in the normal group (Table 4). From these results, a high-level IgA or IgG response or both IgA- and IgG-positive response may indicate an infection with HPV 16 or its related type, whereas low-level responses of these antibodies may show a prior HPV 16 infection or a cross-reaction of other HPV types.

DISCUSSION

Recently, we established a system for purification of HPV 6 and HPV 16 VLP of L1 proteins synthesized in *S. pombe* (37). VLP are a candidate for prophylactic vaccination against HPV. In preliminary experiments, there were no antigenic differences between yeast-derived and insect cell-derived VLP. In the ELISA using yeast VLP, the anti-HPV 6 MAb (H6 10B) and anti-HPV 11 antibodies (H11A3 and H11B2) reacted to HPV 6 VLP but not to HPV 16 VLP, while anti-HPV 16 antibodies (Camvir 6 and Camvir 7) reacted to HPV 16 VLP but not to HPV 6. It is reported that HPV 11, HPV 16, and HPV 18 virions are antigenically distinct from one another when tested with anti-HPV 11 antibodies generated in rabbits (32). The neutralizing anti-HPV 11 antibodies, which we used in this study, recognize conformationally dependent epitopes of HPV 11 and show the type-specific reaction (6). Interestingly, the results of the present study showed that HPV type-specific reactivity of MAbs in the ELISA seemed to be correlated with the ability to recognize VLP by IEM (Table 1). On the other hand, MAbs H11H3 and Camvir 1, which could label neither HPV 6 VLP nor HPV 16 VLP in IEM, bound to both HPV 6 and HPV 16 VLP in the ELISA (Table 1). These results suggest that unassembled capsomeres or denatured L1 proteins may be present on our VLP-coated ELISA plates as well as intact VLP, and Camvir 1 seemed to react with the denatured L1 proteins of HPV 6 and HPV 16. Since H11H3 antibody was reported to recognize a conformation-dependent epitope, it may react with a refolded L1 protein rather than a denatured one. A preliminary study showed that the results described above were not changed except for a slight reduction

TABLE 4. Human immunoglobulin responses to HPV 16 VLP in women with normal cervices and malignant cervical lesions as determined by ELISA

Group (*n*)	IgA		IgG		IgM	
	% Positive[a]	Median absorbance[b]	% Positive	Median absorbance	% Positive	Median absorbance
Carcinoma (36)	36 ($0.1 > P > 0.05$)	0.09 ($P = 0.007$)	25 ($P = 0.02$)	0.168 ($P = 0.01$)	39 (P = NS)	0.182 ($0.1 > P > 0.05$)
HSIL (33)	27 (P = NS)	0.115 ($P = 0.009$)	12 (P = NS)	0.15 ($P = 0.005$)	36 (P = NS)	0.173 ($0.1 > P > 0.05$)
Normal (31)	13	0.062	3	0.089	19	0.160

[a] *P* values are given in parentheses, indicating a significant difference of percentages positive in HPV groups compared with that of the normal group by Fisher's exact probability test.
[b] *P* values are given in parentheses, indicating a significant difference of absorbance values in HPV groups compared with that of the normal group by Mann-Whitney U test. NS, not significant.

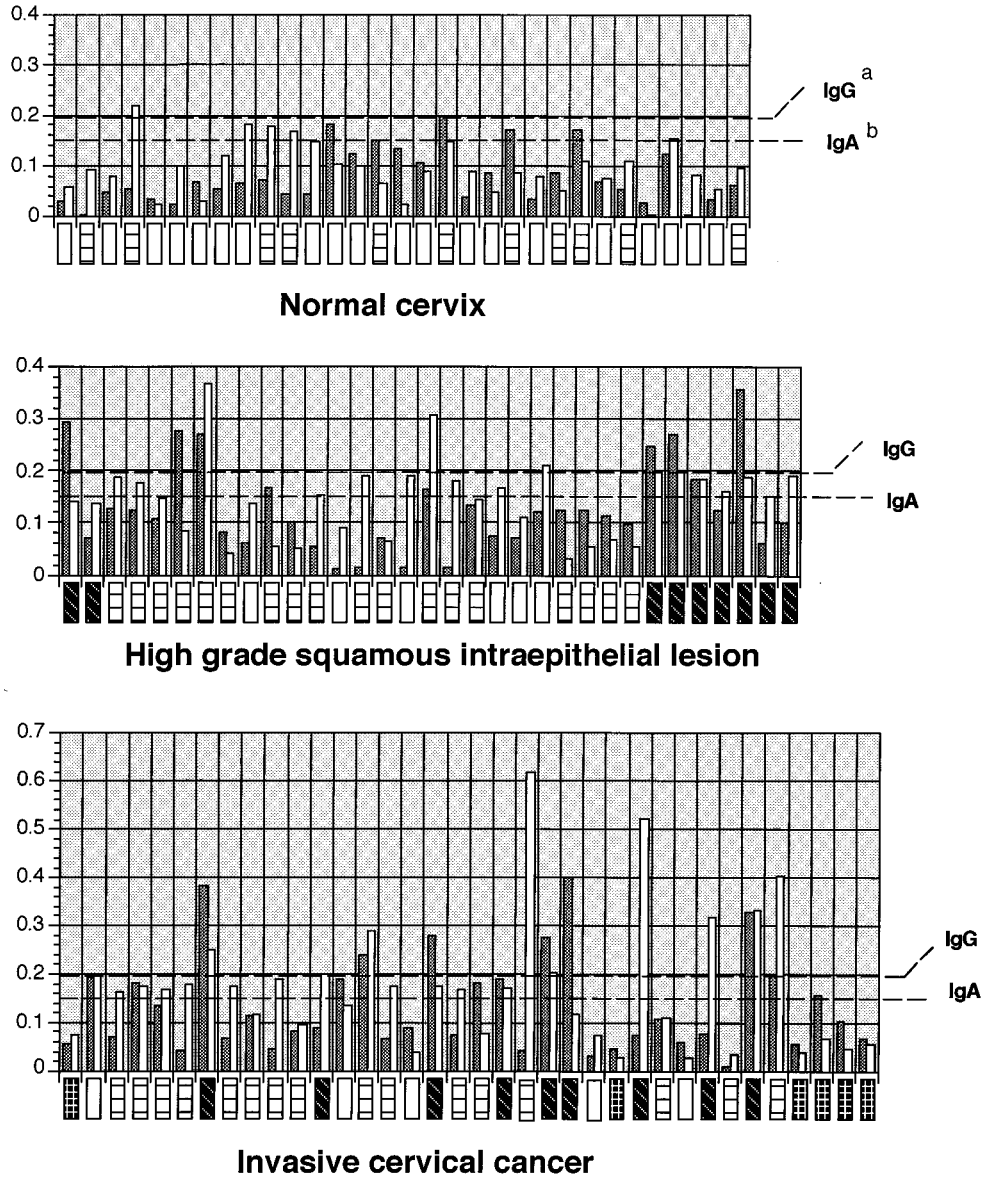

FIG. 3. Human immunoglobulin responses in healthy women and patients with HSIL and cervical carcinomas. Absorbances for IgA (▤) and IgG (□) are plotted for each serum sample. The HPV type in each subject is indicated below the *x* axis as follows: ▮, HPV 16; ▮, HPV 18; ▮, unknown; and ▯, HPV negative. Cutoff points are shown for IgA and IgG at 0.15 (b) and 0.2 (a), respectively.

in absorbances when VLP were incubated in PBS buffer instead of the carbonate buffer we used in the ELISA (data not shown).

Among HPV 16-positive patients with HSIL or invasive cervical cancer, 61% showed IgA, 44% showed IgG, and 39% showed IgM responses against HPV 16 capsids in the HPV 16 VLP ELISA. A recent report showed that 51 and 59% of women with HPV 16 DNA-positive invasive cervical cancer and 81 and 73% of women with HPV 16 DNA-positive cervical intraepithelial neoplasia grade III (CIN III) in Colombia and Spain, respectively, were IgG positive in the HPV 16 VLP ELISA (27). The higher percentages positive for IgG observed in that study than in our study are probably caused by differences in determination of cutoff points. The previous investigators assigned cutoff points by a receiver operating characteristic curve, while we did it by adding 2 standard deviations to the mean absorbance value for the serum samples from 1-year-old children. By their method, the sensitivity seems to be higher but the specificity seems to be lower than those of our method. In fact, 43 and 10% of CIN III controls, who were diagnosed as having CIN III but with no HPV 16 DNA, were reactive to HPV 16 VLP in the earlier study (27).

In the present study, percentages positive and median absorbances for IgA and IgG for the HPV 16-positive group were significantly higher than those for the HPV-negative and HPV 18-positive and/or the HPV 6/11-positive groups, but such tendencies were not apparent for the IgM response. Thus, the IgM response seems to be nonspecific to HPV 16 in our VLP ELISA. Strong IgM responses to HPV 16 L1 in patients with cervical neoplasia in an ELISA using synthetic peptides have been reported, and these positive sera were shown to be cross-reactive to an analogous HPV 33 L1 peptide (4). In ELISAs

using synthetic peptides, 17 to 70% of HPV 16-positive women (9) and 31 to 90% of HPV 16-positive women (4) have been reported to be reactive to different parts of the HPV 16 L1 protein. However, 19 to 24% of children are also reactive to these synthetic peptides (4), and no statistical difference between cancer patients and healthy controls was reported in another study (15).

Among the subjects seropositive for HPV 16, 27% also showed IgA reactivity and 13% showed IgG reactivity to HPV 6 VLP. Double infection with HPV 16 and HPV 6/11 has sometimes been reported in epidemiological studies (29), and a high incidence of cervical HPV 16 infection in women with past or present vulvar condyloma acuminata has been reported (16). Therefore, it is not surprising that some sera reacted to both HPV 6 and HPV 16 VLP. In our study, none of the sera positive for HPV 16 in the HPV DNA-negative patient group reacted to HPV 6. These serum responses may be due to prior exposure to HPV 16 or other HPV types which are serologically more cross-reactive to HPV 16 than HPV 6. It has been reported that 38% of serum samples of HPV 31-infected individuals are positive for HPV 16 VLP antibodies, whereas only 9% of the serum samples of HPV 6/11-infected individuals are positive for HPV 16 VLP (18). From this evidence, some high-risk HPV types are likely to be more homologous to HPV 16 in antigenicity than the low-risk types. In the present study, seroreactivity to HPV 6 VLP did not differ in the condyloma acuminatum patients and the other patient and control groups. A possible explanation is that similar populations of women who had been previously exposed to HPV 6/11 were present in all the groups. However, we have to examine larger numbers of serum samples from condyloma acuminatum patients to illuminate this possibility.

When we tested clinical specimens, HPV DNA was detected in 24 of 35 samples (69%) in the HSIL and invasive cervical cancer groups; 18 (51%) were positive for HPV 16, and 6 (17%) were positive for HPV 18. The overall detection rate for HPV in these malignant lesions seems to be lower than those of other studies using PCR; however, the percentages of samples positive for HPV 16 and HPV 18 are almost the same as those reported in a worldwide study using PCR (1). Therefore, our method seems not to be insensitive for HPV 6, 11, 16, and 18 detection, although it could be possible that some other HPV types were missed by our method. In fact, some of the serum samples from the HPV-negative group were positive in the HPV 16 ELISA, and this might be due to a cross-reaction of other HPV related to HPV 16. To clarify this, we should test many serum samples from patients infected with the HPV types other than HPV 16, 18, 6, and HPV 11.

In the present study, about half of the patients with cervical neoplasia infected with HPV 16 had IgA and IgG antibodies against HPV 16. These results are encouraging for the development of a prophylactic vaccine for HPV 16. IgA and IgG antibodies can be produced in naturally infected patients even though very few HPV 16 virions are produced in lesions. Although it is not yet known whether these antibody responses are protective, it should be possible to test this by immunization of healthy individuals and investigation of the correlation between subsequent infection rates and serological responses. This study also indicates that examination of IgG and IgA responses in women may be useful in detecting HPV 16 infection.

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