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Antibodies to Human Papillomavirus 16, 18, 58, and 6b Major Capsid Proteins among Japanese Females

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Among genital human papillomaviruses (HPVs), the so-called high-risk (HPV 16, 18, etc.) and intermediate-risk (HPV 58, etc.) viruses are believed to be etiologically associated with cervical cancer. To estimate the extent of infection with common HPVs among Japanese females, we examined 328 sera from healthy donors (201) and patients with cervical intraepithelial neoplasia (CIN) (22), cervical cancer (67), and condyloma acuminatum (CA) (38) for IgG antibodies against L1 capsid protein by enzyme-linked immunosorbent assay using virus-like particles of HPVs 16, 18, 58 and 6b (low-risk) as antigens. Antibodies recognizing conformational epitopes were found in the sera from both the patients and the healthy donors. The prevalences of anti-HPV 16, 18, and 58 antibodies in the sera from the patients with CIN (45%) and cervical cancer (49%), and that of anti-HPV 6b in the sera from the patients with CA (55%), were significantly higher than those in the sera from the age-matched healthy donors (12%, 14%, and 23%, respectively). Anti-HPV 16 was not found in some of the sera from patients with HPV 16-DNA positive CIN or cervical cancer, suggesting that HPV infection may not always induce production of anti-capsid antibodies or that the level of antibodies may not always be maintained until development of CIN or cancer. Some of the sera contained antibodies against more than one type of HPV, suggesting that the donors had been infected with different HPVs. The type-specific antibodies against capsid L1 protein of one type of HPV may not be able to prevent infections with other types of HPVs.

Key words: HPV — Cervical cancer — Anti-HPV capsid antibodies

Human papillomaviruses (HPVs) are nonenveloped icosahedral particles (with a diameter of 55–60 nm) composed of two capsid proteins [major (L1) and minor (L2)] and double-stranded genomic DNA (8,000 bp). Molecular biological studies of the presence of viral DNA in biopsy specimens revealed that certain types of HPV (referred to as genital HPVs) among more than 70 genotypes recorded so far are associated with the development of a variety of proliferative genital lesions including condyloma acuminatum (low-risk HPVs, 6/11, 42, 43, and 44), cervical intraepithelial neoplasia (CIN), and invasive cervical cancer (high-risk HPVs, 16, 18, 45, and 56; intermediate-risk HPVs, 31, 33, 35, 52, and 58).[1–3] Despite the accumulated epidemiological DNA data, seroepidemiological studies to elucidate the ecology of HPVs have been hampered by the lack of cell culture systems to produce HPV particles.

Recently, the use of HPV virus-like particles (VLPs), which are self-assembled icosahedral particles in cells expressing L1 protein via the baculoviral vector system[4–7] or the vaccinia vector system,[8,9] has made seroepidemiological studies of HPV infection possible. HPV VLPs are excellent antigens to detect antibodies in human sera, whereas bacterially expressed HPV capsid proteins and synthetic peptides are not.[10] The presence of antibody reactive with HPV 16 VLP is related to the presence of HPV 16 DNA in cervical lesions (CIN and cervical cancer).[11–15] The prevalence of antibody against HPV 16 VLP in cervical cancer and cervical intraepithelial neoplasia grade III is significantly higher than that in controls.[14] The antibody reactive with HPV 6/11 VLP is found specifically in the sera from patients with genital warts.[4,8,15]

In this study, we produced VLPs of some of the prevalent genital HPVs (16, 18, 58, and 6b) and examined 338 sera from Japanese females for anti-L1 capsid antibodies (IgG) by enzyme-linked immunosorbent assay (ELISA) using the purified VLPs as antigens. Unlike previous studies, this study involved multiple HPV antigens to test the reaction of single serum samples. The antibodies against these L1 capsid proteins were found in the serum samples from both the patients (CIN, cervical cancer, or condyloma acuminatum) and the healthy donors. Some of the sera contained antibodies to more than one type of HPV.

MATERIALS AND METHODS

Human serum samples Sera were collected from 328 Japanese females: from 201 healthy donors (21 serum

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samples from the age group 0 to 9, 30 samples from each of the age groups 10 to 19, 20 to 29, 30 to 39, 40 to 49, 50 to 59, and over 60 years), 22 patients with CIN, 67 patients with cervical cancer, and 38 patients with condyloma acuminatum. The sera were obtained before treatment, except for three sera obtained during surgery for condyloma acuminatum.

Preparation of VLP L1 genes of HPVs 16,[16] 18,[17] 58,[18] and 6b[19] were expressed in Sf-9 cells using the baculovirus vector. DNA fragments containing the entire L1 open reading frames (nucleotides 5637 to 7154 for HPV 16, 5430 to 7136 for HPV 18, 5565 to 7139 for HPV 58, and 5789 to 7291 for HPV 6b) were prepared by polymerase chain reaction using synthetic primers (5′ ends of sense primers contained *Sma* I recognition sequences and 5′ ends of antisense primers contained *Xba* I [for HPV 16] or *Eco* RI [for HPVs 18, 58, and 6b] recognition sequences) and cloned HPV genome DNA as templates. Because the L1 protein of prototype HPV 16 does not form VLPs,[5] we substituted a histidine residue (amino acid 202) for asparagine by the replacement of C (nucleotide 6240) with G. Each of these DNA fragments was inserted into pVL1393 (the baculovirus-transfer vector, PharMingen, San Diego, CA). DNA (5 μg) of each transfer vector was transfected into Sf-9 cells together with 2.5 μg of baculovirus DNA (Baculo-Gold, PharMingen) to generate recombinant baculoviruses. Sf-9 cells (4×10^9) were infected with recombinant baculoviruses, which had been purified by three single-plaque isolations, at a multiplicity of infection of 3 to 5 plaque-forming units/ml, incubated for 72 h at 27°C, collected by centrifugation, and lysed in phosphate-buffered saline (PBS, pH 7.2) by sonication. Self-assembled VLPs were purified by the method described by Kirnbauer *et al.*[5] The total cell lysate was loaded on a cushion of 40% (w/v) sucrose in PBS and centrifuged in an SW28 rotor (Beckman, Palo Alto, CA) at 25,000 rpm for 2.5 h at 4°C. The resulting pellet was resuspended in 27% (w/v) CsCl in PBS by sonication and centrifuged at 28,000 rpm in the SW28 rotor for 20 h at 20°C. The visible band of VLPs at the density of 1.29 g/ml was harvested. The purification of VLPs by banding in a CsCl density gradient was repeated twice. After extensive dialysis against PBS, the purified VLP preparations were stored at 4°C.

Boiled VLP preparations [for 5 min in PBS containing 1% sodium dodecyl sulfate (SDS)] were used as denatured antigens after having been precipitated with acetone twice to remove SDS.

Characterization of VLPs Purified VLPs were characterized by electron microscopy and electrophoresis. For transmission electron microscopy, purified VLPs were spun onto carbon-coated grids, stained with 1.5% uranyl acetate, and examined with a Hitachi electron microscope (H-7100). Proteins in VLP preparations were electrophoresed in SDS-polyacrylamide gel (8%) and stained with 0.25% Coomassie brilliant blue or analyzed by western blotting with anti-HPV 16 L1 mouse monoclonal antibody (anti-16 L1 MAb) (PharMingen), which cross-reacts with L1 proteins of various HPV types.

Immunization of mice with VLPs Balb/c mice (10 mice for each antigen) were immunized with the VLP preparations of HPVs 16, 18, 58, or 6b. Each mouse was injected intraperitoneally with 20 mg of VLPs of a single HPV type. The immunization was repeated 4 weeks after the initial injection. Sera were collected 2 weeks after the final immunization.

ELISA Purified VLP preparations in PBS or mock samples (50 μl) were added to each well of microtiter plates (Immulon II microtiter plate, Dynatech Laboratories, Inc., Chantilly, VA). The wells were blocked for 16 h with PBS (150 μl) containing 0.2% gelatin at 4°C and then washed three times. Forty-five microliters of serum sample (human serum was diluted 1 : 10 in PBS) was added to the wells immediately after the last washing. The plates were incubated for 1 h at room temperature and washed six times with PBS containing 0.05% Tween-20. Then 50 μl of peroxidase-conjugated goat anti-human IgG or anti-mouse IgG antiserum (Cappel-Organon Teknika Corp., West Chester, PA), diluted 1 to 1000 in PBS containing 1% bovine serum albumin (BSA) prior to use, was added to each well. The plates were incubated for 30 min at room temperature with gentle rocking and washed five times. A mixture of 0.01% H_2O_2 and *o*-phenylenediamine (2 mg/ml) in 0.1 *M* citrate buffer (pH 4.7) was added to the wells. The absorbance (A) at 450 nm was measured after incubation for 15 min at 20°C. Since some of the human serum samples reacted with gelatin used for blocking, specific absorbance was calculated by subtracting the absorbance of mock samples, measured in wells that received gelatin only.[20]

The cut-off values were determined statistically.[20, 21] First, the mean and standard deviation (SD) were calculated for the 328 samples, and those with specific absorbance higher than the mean+2 SD were considered to be seropositive. Second, the mean and SD were calculated again for those with specific absorbance lower than the initial mean+2 SD, and those higher than the second mean+2 SD were considered to be positive. The calculation was repeated until there were none to be excluded for the subsequent calculation, and the last mean+2 SD was chosen as the cut-off value.

RESULTS

VLPs used as antigens for ELISA The electron microscopic observations showed that the purified preparations of HPV 16, 18, 58, and 6b VLPs contained similar spherical particles with a diameter of 50 to 60 nm. These

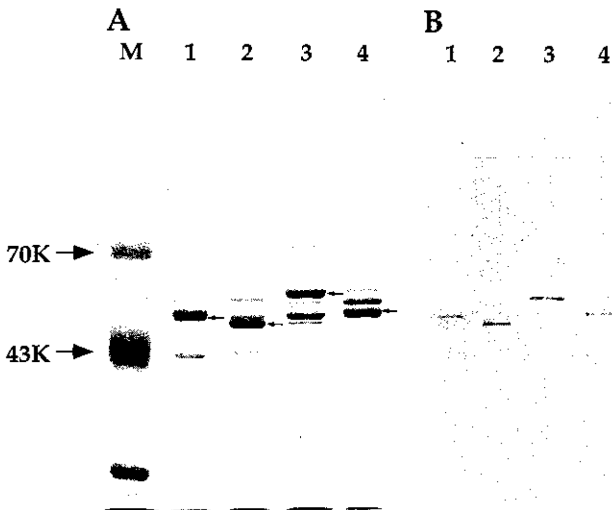

Fig. 1. Proteins in the purified VLP preparations. VLPs were lysed by boiling in Laemmli sample buffer and electrophoresed in 8% SDS-polyacrylamide gels. Proteins were either stained with 0.2% Coomassie blue (A) or analyzed by western blotting with the anti-HPV 16 L1 MAb (PharMingen), which has broad reactivities to HPV L1 proteins (B). VLPs of HPV 6b (lane 1), HPV 16 (lane 2), HPV 18 (lane 3), HPV 58 (lane 4), protein size markers (M). Arrows directed to the left indicate proteins stained with the antibody by western blotting.

particles resembled those of VLPs of HPV 1,[9] HPV 11,[4,7] HPV 16,[5,7] HPV 18[7] and HPV 33[6] in morphology.

The VLP preparations were electrophoresed and stained with Coomassie brilliant blue (Fig. 1A) or analyzed by western blotting using anti-16 L1 MAb (Fig. 1B). The major protein bands recognized by the antibody were located at positions corresponding to 55K to 64K. The estimated molecular weights were consistent with those expected from the amino acid compositions deduced from the nucleotide sequences (56K for HPV 16, 64K for HPV 18, 59K for HPV 58, and 56K for HPV 6b). The concentrations of L1 proteins in VLP preparations were estimated by comparison with known amounts of BSA in SDS-polyacrylamide gels stained with Coomassie brilliant blue (data not shown).

Mouse antibodies against L1 VLPs of HPVs 16, 18, 58, and 6b We examined the immunogenicity of HPV VLPs in mice. The mouse sera (pools of sera from 10 immunized animals) contained antibodies to the VLPs used for immunization as assayed by ELISA (Fig. 2A), in which 0.50 μg of L1 VLPs per well was used, because 0.25 μg per well of L1 VLPs was required to obtain the maximum titer of the anti-L1 mouse antibodies (data not presented). Unlike the anti-16 L1 MAb, these antibodies did not react with the SDS-denatured antigens (Fig. 2A). Although all of the mouse antisera cross-reacted with the heterotypic L1 VLPs, the levels of reactivity with the heterotypic L1 VLPs were much lower than those with homotypic L1 VLPs (Fig. 2B). Thus, immunization with HPV VLPs induced in mice antibodies recognizing conformational epitopes specific to the type of HPV.

Human antibodies against L1 VLPs of HPVs 16, 18, 58, and 6b We examined 338 human sera (201 sera from the healthy donors and 38 from patients with condyloma, 22 from patients with CIN, and 67 from patients with cervical cancer) by ELISA for IgG antibodies against L1 VLPs of HPVs 16, 18, 58, and 6b. Representative results of three independent assays are summarized in four histograms in Fig. 3. The frequency distributions of the great majority of the samples from the healthy donors appeared to be normal and to peak near a specific absorbance of 0.1. The samples with specific absorbance values higher than the cut-off value were considered to be seropositive. Thirty-six selected sera (9 samples from each seropositive group for anti-HPV 16, 18, 58 or 6b) did not react with either SDS-denatured L1 proteins or bacterially expressed L1 proteins (data not presented). The data indicate that the anti-HPV L1 antibodies in human sera recognize mainly conformational epitopes.

The antibody in the sera from the patients and the age-matched healthy donors are summarized in Table I. The prevalence of antibodies to L1 VLPs of any of HPVs 16, 18, and 58 in the sera from the patients with CIN (22 to 61 years old) or cervical cancer (26 to 85 years old) was significantly higher than that in the sera from the age-matched healthy donors (for CIN, 25 to 60 years old; for cervical cancer, 35 to 85 years old). Similarly, the prevalence of anti-HPV 6b antibody in the sera from the patients with condyloma acuminatum (16 to 64 years old) was higher than that in the sera from the age-matched healthy females (16 to 32 years old).

The antibodies in the sera from the patients who were positive for HPV 16 DNA [HPV DNAs from biopsy specimens from 73 cases of CIN and cervical cancer patients were previously examined by using consensus primers for the L1 region[22]] are summarized in Table II. Anti-HPV 16 antibody was not always detected in these sera; it was detected in 3 out of 6 sera from the patients with CIN and 9 out of 13 sera from the patients with cervical cancer. Among anti-HPV 16-negative sera, samples #302 and #284 were positive for anti-HPVs 18, 58, and 6b, and anti-HPV 18, respectively.

Sera reacting with more than one type of L1 VLP were also found among anti-HPV 16-positive sera (Table II). Sample #459 reacted with four VLPs. Samples #302, #447, and #280 reacted with three VLPs (the combinations of VLPs were different). Two sera (#465 and #505) were positive for anti-HPVs 16 and 58. Four sera were positive for anti-HPVs 16 and 6b.

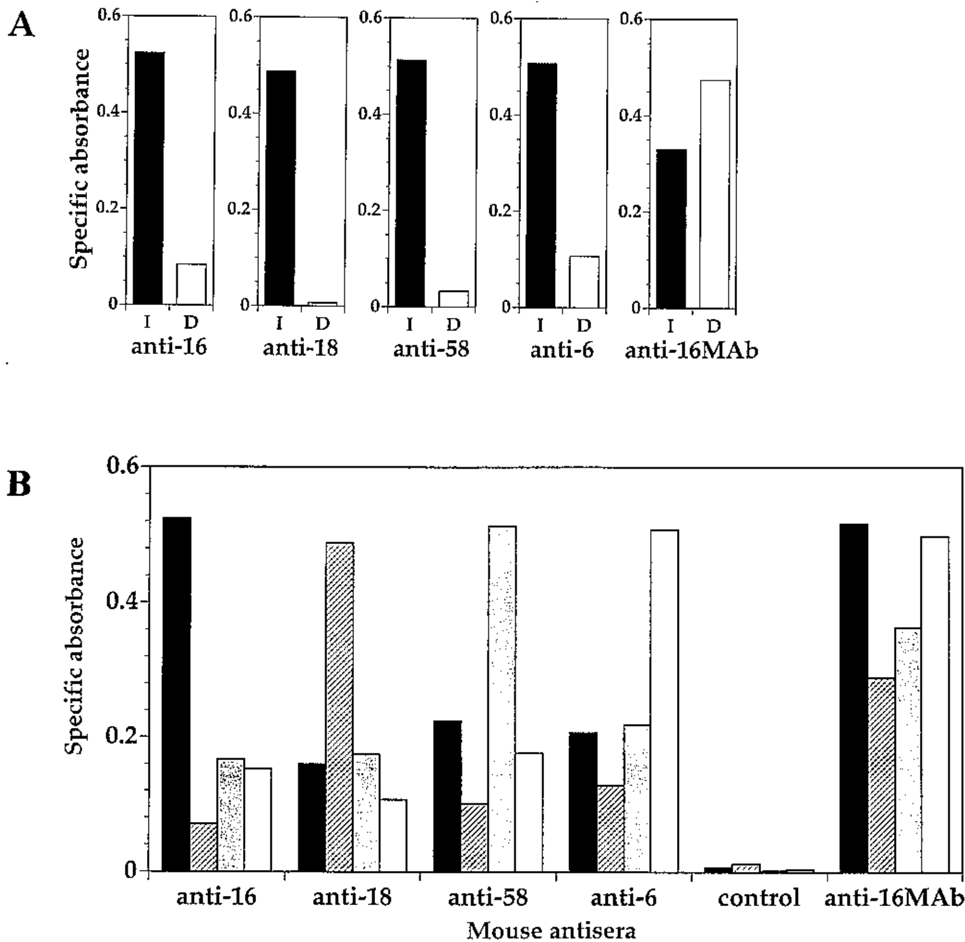

Fig. 2. Immunogenicity of HPV 16, 18, 58, and 6b VLPs. A, Reactivities of mouse antisera to native or SDS-denatured L1 proteins measured by ELISA. Titers (specific As) to intact (I) and denatured (D) L1 protein are shown. Anti-16MAb was the mouse anti-HPV 16 L1 MAb (PharMingen). B, Cross-reactivities of the mouse antibodies against L1 VLPs of HPV types 16, 18, 58, and 6b measured by ELISA. The antigens used were VLPs of HPV 16 (■), 18 (▨), 58 (▩), and 6b (□). Control and anti-16MAb were preimmune sera and the mouse anti-HPV 16 L1 MAb, respectively.

Multi-reacting sera were also found in the samples not included in Table II (data not presented); from the healthy donors, from the patients with HPV DNAs other than type 16, and from the patients whose HPV DNA had been undetectable or unidentified. The sera positive for two to four different antigens were found more frequently in the seropositives from the patients with CIN [45% (5/11)], cervical cancer [51% (21/41)], and condyloma [36% (9/25)] than in those of the healthy donors [16% (11/68)].

DISCUSSION

The mouse antisera raised by immunization with HPVs 16, 18, 58, and 6b VLPs contained mainly antibodies recognizing conformational epitopes (Fig. 2A). Each antiserum reacted with the corresponding L1 VLP with the highest titer among HPV 16, 18, 58, and 6b L1 VLPs, although they all showed low-level cross-reactivities with heterotypic L1 VLPs (Fig. 2B). The data strongly suggest that antibodies recognizing conformationally dependent epitopes are type-specific.

The human antibodies did not react with SDS-denatured L1 proteins. The data are consistent with our previous failure to detect human anti-L1 antibodies by ELISA using bacterially expressed denatured L1 proteins as antigens (unpublished data) and with the report by Kirnbauer *et al.*,[11] who also observed that human antibodies reactive with HPV 16 VLPs did not bind to denatured L1 protein. It is therefore likely that human

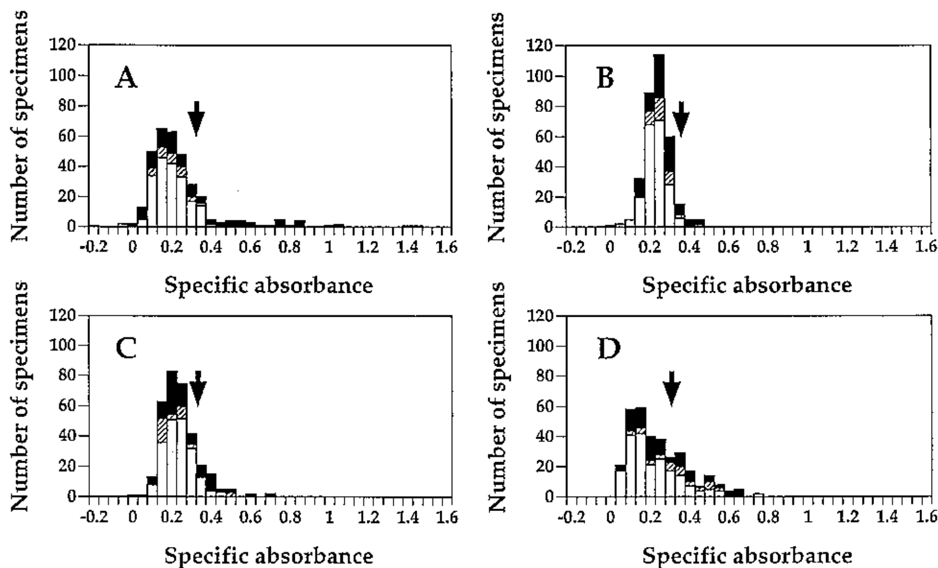

Fig. 3. Detection of IgG antibodies against VLPs of HPV 16 (A), HPV 18 (B), HPV 58 (C), and HPV 6b (D) by ELISA using VLPs (0.50 μg/well) as antigens. The number of samples is plotted against intervals of specific A. Arrows indicate the cut-off value determined statistically (0.319 for anti-HPV 16, 0.312 for anti-HPV 18, 0.304 for anti-HPV 58, and 0.273 for anti-HPV 6b). Serum samples from healthy donors (□), from patients with cervical cancer or cervical intraepithelial neoplasia (■), from patients with condyloma acuminatum (▨).

Table I. Prevalence of Antibodies in the Sera from the Patients and from the Age-matched Healthy Donors

Diagnosis	No.[a]	Age[b]	Number of seropositive samples				
			anti-HPV 16	anti-HPV 18	anti-HPV 58	any of anti-HPV 16, 18, 58	anti-HPV 6
CIN	22	43.9	4 (18%)	5 (23%)	3 (13%)	10 (45%)	5 (23%)
Healthy donor	98	43.2	2 (2%)	1 (1%)	10 (10%)	12 (12%)	24 (24%)
			$P<0.01$	$P<0.01$	NS	$P<0.01$	NS
Cervical cancer	67	53.5	23 (34%)	9 (13%)	19 (28%)	33 (49%)	24 (36%)
Healthy donor	102	53.7	4 (4%)	1 (1%)	11 (11%)	14 (14%)	19 (19%)
			$P<0.01$	$P<0.01$	$P<0.01$	$P<0.01$	NS
Condyloma	38	27.0	6 (5%)	4 (11%)	6 (16%)	13 (34%)	21 (55%)
Healthy donor	48	25.4	5 (10%)	1 (2%)	6 (13%)	12 (25%)	12 (25%)
			NS	NS	NS	NS	$P<0.01$

a) No., number of serum samples.
b) Age, mean of age of patients or healthy donors.
The P value was obtained by means of the χ^2 test.
NS, not significant ($P>0.01$).

serum anti-HPV antibodies are type-specific like the mouse antibodies, since they recognize conformational epitopes.

The antibodies against L1 VLPs of high- and intermediate-risk HPVs (HPVs 16, 18, and 58) were associated with CIN and cervical cancer (Table I) as reported previously.[11-14] The results are consistent with the reported association of the presence of viral DNA of these HPV types with cervical neoplasia.[1-3] The association of anti-HPV 6b antibody with condyloma acuminatum (Table I) is also consistent with the presence of HPV 6b DNA in patients with the disease.[1-3]

The antibody positivity in the sera from the patients with CIN (45%) or cervical cancer (49%) (Table I) is

Table II. Antibodies in the Sera from the HPV 16 DNA-positive Patients with CIN or Cervical Cancer

Patient	Age	Diagnosis	ELISA titer (A at 450 nm) against			
No.			HPV 16L1	HPV 18L1	HPV 58L1	HPV 6L1
#465	28	CIN[a]	0.548	—[b]	0.357	—
#298	37	CIN	0.325	—	—	—
#468	38	CIN	0.447	—	—	0.375
#302	41	CIN	—	0.413	0.443	0.569
#284	41	CIN	—	0.362	—	—
#270	50	CIN	—	—	—	—
#464	30	cancer	0.629	—	—	—
#459	34	cancer	1.378	0.367	0.669	0.609
#502	43	cancer	—	—	—	—
#445	45	cancer	—	—	—	—
#505	45	cancer	0.738	—	0.319	—
#473	50	cancer	1.040	—	—	0.373
#447	52	cancer	0.726	0.336	—	0.308
#444	53	cancer	0.715	—	—	—
#28	54	cancer	—	—	—	—
#471	58	cancer	0.576	—	—	0.395
#280	63	cancer	0.802	0.418	0.351	—
#449	65	cancer	0.822	—	—	0.331
#275	72	cancer	—	—	—	—

HPV DNAs were previously examined by PCR using consensus primers for the L1 region.[22]
a) CIN, cervical intraepithelial neoplasia.
b) —, negative (below cut-off value).

lower than the HPV DNA positivity for these diseases (more than 90%).[1-3] The discrepancy between the antibody and DNA data may be explained, at least partly, by the finding that some of the HPV 16-DNA positive patients with CIN or cervical cancer were negative for antibody against L1 protein of HPV 16 (Table II). This result, which agrees with previous reports,[11, 13, 14] suggests that HPV infection does not always induce production of detectable antibodies, or that the level of antibody declined to an undetectable level before the development of cancer.

Some of the serum samples reacted with L1 VLPs of more than one type of HPV. The multi-reactivities of the sera may be explained partly by the presence of cross-reactive antibodies. Another possible explanation is that individuals carrying multi-reacting serum may have been infected with various different HPVs and probably have different antibodies; this seems more probable, because i) these human antibodies recognizing conformational epitopes are very likely to be type-specific, and ii) different sera reacted with different combinations of antigens. The type-specific anti-HPV L1 VLP antibodies raised by the first infection with one HPV may not be able to protect the host from infection with other HPVs. The reason why patients with CIN, cervical cancer, or condyloma might be infected with various HPVs more frequently than healthy females is not clear at present. The sensitivity of individuals to infection with HPVs or the opportunity of individuals to be exposed to HPVs may vary.

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