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Neutralizing Antibodies against Human Papillomavirus Types 16, 18, 31, 52, and 58 in Serum Samples from Women in Japan with Low-Grade Cervical Intraepithelial Neoplasia[▽]

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We have very limited information on serum neutralizing antibody in women naturally infected with the human papillomaviruses (HPVs) that are causally associated with cervical cancer. In this study, serum samples collected from 217 Japanese women with low-grade cervical intraepithelial neoplasia were examined for their neutralizing activities against HPV16, -18, -31, -52, and -58 pseudovirions. Eighty-four patients (39%), 35 patients (16%), 17 patients (8%), and 1 patient were positive for neutralizing antibodies against one, two, three, and four of these types, respectively. Presence of neutralizing antibody did not always correlate with detection of HPV DNA in cervical swabs collected at the time of blood collection. The neutralizing titers of the majority of sera, ranging between 40 and 640, were found to be conserved in the second sera, collected 24 months later, independently of emergence of HPV DNA in the second cervical swabs. The data strongly suggest that HPV infection induces anti-HPV neutralizing antibody at low levels, which are maintained for a long period of time.

Human papillomavirus (HPV) is a small, nonenveloped virus with a circular, double-stranded DNA of 8 kbp packaged in icosahedral capsids composed of two capsid proteins, L1 (major) and L2 (minor) (2, 3). Of the more than the 100 genotypes classified for DNA homology (4), 15 types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, and -73) are called high-risk HPVs because they are causally associated with cervical cancer (15), the second most frequent gynecological cancer in the world (17).

HPV infects the basal cells of the stratified epithelia through small epithelial lesions. In the infected cells, the viral DNA is maintained as episomes, which start to be transcribed and replicate with the onset of terminal cell differentiation (5). HPV progeny virions are produced in the upper layers of the epidermis or mucosa and released from them. The HPV DNA detectable in cervical swabs is likely to originate from these virions and thus to be considered a sign of HPV propagation, probably because it seems difficult to collect HPV DNA by swabbing the episomal HPV DNA maintained in the infected basal cells. The high-risk HPVs occasionally induce low-grade cervical intraepithelial neoplasia types 1 and 2 (CIN1 and CIN2), which usually regress spontaneously (18). A small fraction of the lesions progress to high-grade CIN (CIN3), the precursor of cervical cancer (18).

Expression of L1 in insect cells or in *Saccharomyces cerevisiae* results in the spontaneous formation of virus-like particles (VLPs). VLPs are highly immunogenic in animals and humans (6, 8, 9, 11) and induce predominantly type-specific neutralizing antibodies (7, 19). A prophylactic vaccine using HPV6, -11, -16, and -18 VLPs as antigens has been developed and shown to successfully induce type-specific neutralizing antibodies in recipients in large-scale clinical trials (8, 12, 21). These studies strongly suggest that type-specific neutralizing antibody may be induced in women naturally infected with an HPV.

Although cell cultures supporting efficient HPV replication are not available, coexpression of L1 and L2 from codon-modified L1 and L2 genes in cultured animal cells harboring episomal DNA with a reporter results in packaging of the reporter DNA into L1/L2 capsids to produce infectious pseudovirions (PVs) (1). PVs are used as a surrogate virus to detect the neutralizing activities of anti-HPV antibodies (14, 16).

In this study, we collected serum samples and cervical swabs from 217 Japanese women with CIN1 or CIN2 at diagnosis (first samples) and 24 months later (second samples) and examined these samples for the presence of anti-HPV neutralizing antibody and HPV DNA, respectively. The results suggest that even at low levels, HPV infection induces persisting neutralizing antibody.

MATERIALS AND METHODS

Patients and samples. Serum samples and cervical swabs were collected from 217 Japanese women (20 to 49 years old) with CIN1 or CIN2 with informed consent. The first samples were obtained at diagnosis, and the second ones were obtained 24 months later. Three of the second samples were collected at 10, 14,

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TABLE 1. Neutralization of HPV16, -18, -31, -52, and -58 PVs with rabbit antisera obtained by immunization with HPV16, -18, -31, -52, and -58 VLPs

Antiserum	Neutralizing titer of antibody against:				
	HPV16	HPV18	HPV31	HPV52	HPV58
Anti-HPV16 VLP	204,800	<40	800	<40	<40
Anti-HPV18 VLP	<40	1,638,400	<40	<40	<40
Anti-HPV31 VLP	200	<40	1,638,400	<40	<40
Anti-HPV52 VLP	<40	<40	<40	409,600	400
Anti-HPV58 VLP	<40	<40	<40	25,600	409,600

TABLE 2. Numbers of CIN-afflicted women negative and positive for neutralizing antibody

HPV type	No. of women							
	Neutralization		Neutralizing titer					
	−	+	40	80	160	320	640	≥1,280
HPV16	167	50	11	14	8	8	6	3
HPV18	194	23	8	4	5	2	2	2
HPV31	187	30	9	6	4	6	4	1
HPV52	174	43	12	18	6	3	2	2
HPV58	155	62	10	11	13	9	12	7

and 11 months, however, because the lesions that progressed to CIN3 were surgically removed.

Rabbit antisera against HPV16, -18, -31, -52, and -58 VLPs. The recombinant baculovirus expressing HPV16, -18, -31, -52, or -58 L1 was inoculated into Sf9 cells (five flasks containing 175 cm^2 culture) and incubated for 72 h at 27°C. The cells were collected and suspended in 5 ml of phosphate-buffered saline (PBS) containing 0.5% NP-40. After incubation for 10 min at room temperature, the cells were centrifuged at 10,000 × *g* at 4°C for 15 min to precipitate nuclei. The nuclei were suspended in PBS containing CsCl (1.28 g/ml) and lysed with brief sonication (Sonifier250; Branson, Germany). The solution was centrifuged at 34,000 rpm for 20 h at 20°C in an SW50.1 rotor (Beckman Coulter Inc., Fullrerion, CA). The fractions with buoyant densities around 1.28 g/ml were pooled and dialyzed against PBS supplemented with 0.5 M NaCl at 4°C to remove CsCl. Then, the solution was layered on the top of a discontinuous sucrose density gradient (5% and 60%) in PBS and centrifuged at 31,000 rpm for 2 h at 4°C in an SW50.1 rotor. The particles between the 5% and 60% sucrose layers were collected and dialyzed against PBS supplemented with 0.5 M NaCl at 4°C to obtain purified VLPs.

Japanese white rabbits (2.3 to 3.0 kg in weight) were subcutaneously injected with a mixture of each purified VLP and Freund's complete adjuvant (injection performed by Scrum Inc., Tokyo, Japan). Immunization was repeated four times at 2-week intervals, and serum samples were obtained 1 week after the last immunization. The antisera were filtered (Steradisc25; Kurabo Inc., Osaka, Japan) before use for the assays in this study.

Preparation of PVs. Five plasmids (pYSEAP, expressing secreted alkaline phosphatase [SEAP]; p16L1h, expressing HPV16 L1; p16L2h, expressing HPV16 L2; peL1fB, expressing HPV18 L1; and peL2bhb, expressing HPV18 L2) were gifts from J. T. Schiller. Six plasmids (p31L1h, expressing HPV31 L1; p31L2h, expressing HPV31 L2; p58L1h, expressing HPV58 L1; p58L2h, expressing HPV58 L2; p52L1h, expressing HPV52 L1; and p52L2h, expressing HPV52 L2) had previously been constructed (10, 11).

293FT cells (Invitrogen Corp., Carlsbad, CA), which had been seeded in two 10-cm culture dishes (6 × 10^6 cells/dish) 16 h before transfection, were transfected with a mixture of an L1 plasmid (13.5 μg), an L2 plasmid (3 μg), and pYSEAP (13.5 μg) by using Fugene HD (Rosch Diagnostics GmbH, Mannheim, Germany). Sixty hours later, PVs were extracted and purified from the cells as described previously (11). The infectivity of the purified PV was estimated from the SEAP activity of the culture medium of cells infected with PVs by a colorimetric SEAP assay (http://home.ccr.cancer.gov/lco/ColorimetricSEAP.htm).

Neutralization test. The serum samples were diluted with a neutralization medium (Dulbecco's modified Eagle's medium [without phenol red] containing 10% FBS, 1% nonessential amino acids, and 1% GlutaMax-I). Fifty microliters of a serum sample was mixed with 50 μl of neutralization medium containing an aliquot of the PV stock sufficient to induce an optical density at 405 nm of approximately 1.0 of SEAP under our assay conditions (400 pg L1 for HPV16, 800 pg L1 for HPV18 and HPV52, 200 pg L1 for HPV31, and 125 pg L1 for HPV58) and incubated for 1 h at 4°C. The numbers of PVs used were in the linear range of dose-response relation. Then, the mixture was inoculated into 293FT cells, which had been seeded in a well of a 96-well plate (1 × 10^4 cells) 16 h before the inoculation. The culture medium was harvested after incubation of the cells for 4.5 days at 37°C, and the SEAP activity of the culture medium was measured. The neutralization titer was presented as the reciprocal of the maximum dilution of serum that reduced the SEAP level to half of that for the sample not treated with serum.

HPV DNA detection and typing. HPV DNA was detected in the cervical swabs by a PCR-based method as described previously (23). In brief, total cellular DNA was extracted from each specimen by the standard procedure. HPV DNA was amplified by PCR using the consensus primers for the HPV L1 region. HPV types were identified on the basis of restriction fragment length polymorphism, which has been shown to identify at least 26 types of genital HPVs (23).

RESULTS

Neutralization of HPV16, -18, -31, -52, and -58 PVs with rabbit antisera obtained by immunization with HPV16, -18, -31, -52, or -58 VLPs. The rabbit anti-VLP serum prepared in this study was found to type-specifically and efficiently neutralize the PV used for immunization (Table 1). The neutralizing activity of the antiserum was evaluated from the reduction of infectivity of the PV that had been incubated with the diluted serum. Although anti-HPV18 VLP did not cross-neutralize the PVs of the heterologous types tested, the other antisera showed very limited cross-neutralization between phylogenetically closely related HPV16, -31, -52, and -58 PVs (4). The cross-neutralizing titers of anti-HPV16 VLP against HPV31 PV, anti-HPV31 VLP against HPV16 PV, anti-HPV52 VLP against HPV58 PV, and anti-HPV58 VLP against HPV52 PV were 1/256, 1/8,192, 1/1,024, and 1/16, respectively, of the titers neutralizing the homologous PVs. The data strongly suggest that, like VLPs, natural HPV virions have highly type-specific dominant neutralization epitopes (as revealed in the rabbit experiments), which are likely to be recognized by humans in the natural infection.

Neutralization of HPV16, -18, -31, -52, and -58 PVs with serum samples from women with CIN1 or CIN2. Diluted serum samples from 217 women with CIN1 or CIN2 were each mixed with one of the PVs, and the resulting reductions of infectivity were measured. Serum samples that did not reduce the infectivity to less than half at a dilution of 1:40 were tentatively defined as negative for neutralization. Under this cutoff condition, 137 samples (63%) were found to neutralize PVs of one or more of the following: HPV16, -18, -31, -52, and -58.

Table 2 shows the numbers of women negative and positive for neutralization. Neutralizing antibodies against HPV16, -18, -31, -52, and -58 were detected in 50 (24%), 23 (11%), 30 (19%), 43 (21%), and 62 (30%) samples, respectively. The titers of the majority (94% for HPV16, 91% for HPV18, 98% for HPV31, 95% for HPV52, and 90% for HPV58) of the neutralizing sera ranged between 40 and 640.

Table 3 shows the numbers of women positive for neutralizing antibodies against one or multiple HPV types. One woman had antibodies against four types (HPV16, -18, -31, and -58). Seventeen women had antibodies against three types, and 35 women had antibodies against two types. Titers of antibodies against HPV16 and 52 did not correlate with titers of

TABLE 3. Numbers of CIN-afflicted women positive for neutralizing antibody

HPV type(s)	No. of women
16, 18, 31, 52, 58	0
18, 31, 52, 58	0
16, 31, 52, 58	0
16, 18, 52, 58	0
16, 18, 31, 58	1
16, 18, 31, 52	0
16, 18, 31	2
16, 18, 52	2
16, 18, 58	0
16, 31, 52	1
16, 31, 58	4
16, 52, 58	3
18, 31, 52	1
18, 31, 58	0
31, 52, 58	4
16, 18	3
16, 31	5
16, 52	3
16, 58	6
18, 31	1
18, 52	3
18, 58	3
31, 52	2
31, 58	3
52, 58	6
16	21
18	6
31	6
52	18
58	33
None	80

antibodies against HPV31 and -58, respectively (data not shown), strongly suggesting that the titers were not influenced by the cross-neutralization found by the hyperimmune rabbit antisera (Table 1).

Table 4 shows the numbers of women positive for both serum neutralizing antibody and corresponding HPV DNA by the cervical swabbing assay, positive for neutralizing antibody but negative for HPV DNA, and negative for neutralizing antibody but positive for HPV DNA. The cervical swabs, which were collected at the time of serum collection, were examined for the presence of HPV DNA by PCR amplifying the L1 region with the consensus primers. Although there were women positive for both neutralizing antibody and HPV DNA, approximately half (HPV16 and -52) or more than half (HPV18, -31, and -58) of the women were positive for neutralizing antibody without detection of HPV DNA. The neutralizing titers of the serum samples from the DNA-positive women were not statistically different from those from the DNA-negative women. Furthermore, some serum samples from the women positive for HPV DNA were not neutralizing. The data indicate that the presence of HPV DNA, which is clear evidence of HPV replication shortly prior to serum collection, did not always correlate with the presence of neutralizing antibody or with a boost of the level of neutralizing antibody.

TABLE 4. Numbers of CIN-afflicted women with and without neutralizing antibody and HPV DNA

HPV type	Presence of HPV DNA	No. of women	
		With neutralizing antibody	Without neutralizing antibody
HPV16	+	24	14
	−	23	
HPV18	+	5	4
	−	15	
HPV31	+	4	3
	−	26	
HPV52	+	24	16
	−	19	
HPV58	+	23	7
	−	39	

TABLE 5. Fluctuation of HPV-neutralizing titers between serum samples collected after a 24-month interval

Antibody	Neutralizing titer for indicated group		
	Descent[a]	Conservation[b]	Ascent[c]
Anti-HPV16	12	37	10
Anti-HPV18	5	17	8
Anti-HPV31	10	20	4
Anti-HPV52	7	34	12
Anti-HPV58	9	51	11

[a] For the descent group, neutralizing titers decreased by more than half or converted from positive to negative.

[b] For the conservation group, neutralizing titers fluctuated between half and twofold.

[c] For the ascent group, neutralizing titers increased more than twofold or converted from negative to positive.

Neutralizing titers of serum samples collected 24 months later. The majority of women conserved their levels of serum neutralizing antibodies for 24 months. The second samples were collected 24 months after the first collection. Nine, 7, 4, 10, and 9 women converted from negative to positive for serum neutralizing antibodies against HPV16, -18, -31, -52, and -58, respectively. Eight, three, six, seven, and three women lost neutralizing activity against HPV16, -18, -31, -52, and -58, respectively. The titers of the majority of the serum samples fluctuated between half and twofold (Table 5).

The ascent of the neutralizing titer was not associated with detection of HPV DNA in the cervical swabs. Table 6 shows the neutralizing titers of the 21 paired serum samples collected from women whose first cervical swabs were negative for HPV DNA and whose second swabs were positive for HPV DNA. Although the neutralizing titers for 6 women rose slightly, those for 15 women did not. The data, in agreement with the data shown in Table 3, indicate that the presence of cervical HPV DNA does not necessarily boost the level of serum neutralizing antibody.

DISCUSSION

Accumulating data indicate that HPV infection induces highly type-specific neutralizing antibody. The recipients injected with the HPV vaccine containing HPV16 and -18 VLPs develop antibodies neutralizing HPV16 and -18 exclusively (7). The rabbits immunized with HPV16, -18, -31, -52, and -58

TABLE 6. Neutralizing titers of pairs of serum samples from women negative for HPV DNA at the first blood collection and positive for HPV DNA at the second blood collection

Neutralizing antibody	Neutralizing titer for indicated serum sample		No. of patients
	First (HPV DNA$^-$)	Second (HPV DNA$^+$)	
Anti-HPV16	<40	<40	3
	320	160	1
Anti-HPV18	<40	<40	1
	<40	40	2
	320	320	1
Anti-HPV52	<40	<40	4
	<40	80	1
	<40	160	1
	<40	640	1
	160	80	1
	80	40	1
Anti-HPV58	<40	320	1
	40	40	1
	80	80	1
	160	160	1

VLPs developed antibodies neutralizing homologous HPV efficiently with very limited cross-neutralization (Table 1). Thus, the past and the persistent infections of women with HPV could be traced by detection of anti-HPV type-specific neutralizing antibody. In this study, we examined serum samples from 217 Japanese women with CIN1 or CIN2 for neutralizing antibodies against HPV16, -18, -31, -52, and -58, the types common in CIN1 and -2 in Japan (13).

One hundred thirty-seven (63%) serum samples were positive for neutralizing antibodies against one or more HPV types tested (Table 2). The neutralizing titers of the majority of the serum samples, which ranged between 40 and 640 (Table 2), fluctuated within the narrow range, between half and twofold, after 24 months (Table 5). The data strongly suggest that women infected with an HPV develop a low-level anti-HPV neutralizing antibody and keep the level for a long period.

The titers for the serum samples from women positive for HPV DNA by the cervical swabbing assay were similar to those for women negative for HPV DNA. Detection of HPV DNA in the second swabbing did not correlate with a marked rise in neutralizing titers in the second serum samples (Table 6). The cervical swabs probably did not contain HPV propagated in the lower genital tract (22). Low-level propagation of HPV usually continues in CIN1 and -2 lesions. Therefore, a negative result for HPV DNA may be due, at least partly, to inappropriate sampling and to low sensitivity in the detection method and must be carefully interpreted. But a positive result for HPV DNA indicates that HPV propagated at a detectable level. The data strongly suggest that the level of anti-HPV neutralizing antibody is not boosted by HPV propagation.

The inefficient immune response of the women to the infecting HPV may be associated with the HPV life cycle: propagation in the terminally differentiating cells with a limited burst size. During the long history of virus and human, the life cycle may have evolved to escape the host immune systems. A low level of neutralizing antibody may not be protective against repeated infection with the homologous HPV type. Repeated infection may raise the risk of integration of the HPV E6/E7 oncogenes into cell DNA, leading to the initial step for cell immortalization by HPV.

The serum samples from 53 (24%) women were positive for neutralization against two or more HPV types tested (Table 3). Cross-neutralization does not seem to contribute to neutralization of multiple HPV types, because the anti-HPV16 titers did not correlate with the anti-HPV31 titers and the anti-HPV52 titers did not correlate with the anti-HPV58 titers. The data strongly suggest that the women whose sera neutralized multiple types were infected with those multiple HPVs.

The tentative cutoff used in this study probably grouped the serum samples containing very low levels of neutralizing antibody into the negative group, explaining at least partly why the 80 (37%) women with CIN1 and CIN2, the lesions caused by HPV infection, were negative for the neutralizing antibodies against the tested HPV types, which cover the major types detected in CIN1 and -2 in Japan (13). Enhancing the sensitivity of the neutralization assay would be desirable for further studies of natural HPV infection and the host immune response against it.

Recently, Steel et al. (20) measured neutralizing antibody in sera of young women by using HPV16 and -18 PVs produced similarly to those in this study. They found that most of the women seroconverted and kept their neutralizing activity for a long time, while some failed to seroconvert, which is consistent with the data obtained in this study.

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