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Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region

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Abstract

Neutralizing antibody against human papillomavirus (HPV) minor capsid protein L2 can cross-neutralize different HPV genotypes in vitro. To identify the segments containing the cross-neutralization epitopes of HPV16 L2, we characterized antisera obtained by immunizing two rabbits with each of the ten synthetic peptides of 14 to 20 amino acids (aa) long, which represents a part of the HPV16 L2 sequence from aa 14 to 144. The antisera against the peptides within the region from aa 18 to 144 efficiently bound to HPV16 L1/L2-capsids and neutralized HPV16 pseudovirions, indicating that the region is displayed on the surface of the capsids and contains several neutralization epitopes. Antiserum against the peptide from aa 18 to 38 (anti-P18/38) cross-neutralized HPV18. Anti-P56/75 cross-neutralized HPV18, 31, and 58. Anti-P61/75 and anti-P64/81 cross-neutralized HPV18 and 58. Anti-P96/115 and the antiserum induced by a mutant P96/115 (S and T at aa 101 and 112 were replaced with L and S, respectively) cross-neutralized HPV31 and 58. The mixture of equal volumes of three antisera, anti-P18/38, anti-P56/75, and anti-mutant P96/115, neutralized HPV16, 18, 31, and 58 more efficiently than anti-P56/75 alone, suggesting that there is a synergistic effect of antibodies on the cross-neutralization. The cross-neutralization appears to be correlated with conserved aa sequences among HPV types. The data in this study provide a basis for designing vaccine antigens effective against a broader spectrum of the high-risk HPVs.

Keywords: HPV16; Minor capsid protein L2; Neutralization epitope

Introduction

Human papillomavirus (HPV) is a small nonenveloped virus having an 8-kb double-stranded circular DNA. To date more than 100 HPV genotypes, classified based on the homology of genomic DNA, have been identified in proliferative lesions of skin or mucosa (Stoler, 2000). HPVs that infect the genital mucosal epithelia are divided into two groups: low-risk types (such as types of 6 and 11) found mainly in benign condyloma and 15 high-risk types (types of 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73) (Munoz et al., 2004) found in cervical cancer, the most frequent gynecological cancer in the world (Ferlay et al., 1998; Jones, 1999). Although the distribution of high-risk HPVs in humans slightly varies from region to region, type 16 (HPV16) accounts for about 50% of the cases worldwide (Munoz et al., 2004).

An icosahedral HPV capsid is composed of major capsid protein L1 and minor capsid protein L2. Since it is difficult to obtain a large amount of HPV particles by using conventional cell cultures, surrogate systems capable of expressing L1 and L2 have been developed to obtain HPV capsids for structural and immunological analysis. Expression of L1 either alone or together with L2 in cultured cells results in production of L1 capsids (also called virus-like particle; VLP) (Kirnbauer et al., 1992) or L1/L2-capsids (Zhou et al., 1991). The L1 capsid is composed of 360 L1 molecules arranged as 72 pentameric capsomeres (Baker et al., 1991; Crawford and Crawford, 1963), and the L1/L2-capsid contains additional 12 L2 molecules whose N-terminal region is displayed on the surface of

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the capsids (Heino et al., 1995; Liu et al., 1997). These particles are morphologically indistinguishable by electron microscopy from HPV virions extracted from the lesions (Kirnbauer et al., 1992).

Expression of L1 and L2 in cells harboring episomal copies of BPV1 genome or an expression plasmid results in packaging of the episomal DNA into the L1/L2-capsids to produce infectious pseudovirions (Buck et al., 2004; Roden et al., 1996; Stauffer et al., 1998; Unckell et al., 1997; Zhao et al., 1998; Zhou et al., 1993). Although it is not clear whether the pseudovirions are assembled in the same way as the authentic HPV virion, the pseudovirions are used as a surrogate virus to detect neutralizing activity of anti-HPV antibodies.

Anti-L1 antibodies obtained by immunizing mice or rabbits with the L1 capsids have been shown to have primarily type-specific neutralizing activity. Limited cross-neutralizing activity has been observed between closely related types such as HPV18 and 45, and HPV6 and HPV11 (Giroglou et al., 2001). Anti-L1 antibodies can protect animals against challenge with animal papillomaviruses (Breitburd et al., 1995; Suzich et al., 1995). The L1 capsids of HPV6, 11, 16, and 18 were used in the recent clinical trials as prophylactic vaccines, which successfully induced type-specific neutralizing antibodies in recipients (Harper et al., 2006; Villa et al., 2005).

Anti-L2 antibodies have cross-neutralizing activity in vitro. Roden et al. (2000) showed that the antisera obtained by immunizing sheep with bacterially produced L2s of HPV6, 16, and 18 neutralize the pseudovirions of homologous HPV types and cross-neutralize those of the heterologous HPV types. Kawana et al. (1999) showed that a mouse monoclonal antibody recognizing a linear epitope within HPV16 amino acids (aa) 108–120 inhibits infection with HPV16 and 6 pseudovirions, which were produced by an in vitro packaging method. Recently, Pastrana et al. (2005) reported that antibodies to bovine papillomavirus type 1 (BPV1) L2 aa 1–88 neutralize HPV16, 18, and BPV1 pseudovirions and HPV11 native virions efficiently and HPV31, 6 and cottontail rabbit papillomavirus pseudovirions less efficiently.

Vaccination of animals with L2 protects animals from animal papillomavirus challenge similarly to vaccination of animals with the L1 capsids. Embers et al. (2002) showed that immunization of rabbits with the peptides having amino acid sequences of rabbit oral (ROPV) and cutaneous (CRPV) papillomavirus L2 segments corresponding to HPV16 L2 aa 108–120 protect the rabbits from challenge with ROPV and CRPV, respectively. The level of protection induced by these peptides is comparable to that with L1 capsid vaccination. These data, together with the cross-neutralization activity of anti-L2 antibody in vitro, suggest that L2-vaccine may induce antibody protecting against a broad spectrum of the high-risk HPVs. The detailed characterization of L2-neutralization epitopes is required to design L2-vaccine antigens.

In this study we produced antisera by immunizing rabbits with synthetic peptides representing segments of the HPV16 L2 surface region and examined their neutralizing activity against HPV16 and cross-neutralizing activities against HPV18, 31, and 58.

Results and discussion

Binding of anti-L2-peptide rabbit antisera with L1/L2-capsid

We searched for potentially immunogenic segments within the N-terminal region (amino acids [aa] 1–150) of HPV16 L2 by using a computer program (Lasergene Soft ver.6, DNA Star Inc., Madison, WI). The peptides representing the aa sequences of the predicted antigenic segments and three mutant peptides (Fig. 1) were synthesized and conjugated with keyhole limpet hemocyanin (KLH). Numbering of aa in L2 of HPV 16 is deduced from the revised sequence of HPV16 (HPV16R) registered in the HPV Sequence Database (Los Alamos National Laboratory, NM). The peptide having aa sequence corresponding to HPV16 L2 aa 14 to 27 was designated as P14/27. The other peptides were designated similarly. Peptides P28/42(32V, 39T) (D at aa 32 and K at aa 39 of P28/42 were replaced with V and T, respectively), P61/75(73V) (I at aa 73 of P61/75 was replaced with V), and P96/115(101L, 112S) (S at aa 101 and T at aa 112 of P96/115 were replaced with L and S, respectively) were designed to convert the original aa into those of majority of the oncogenic HPVs. The immunization of two

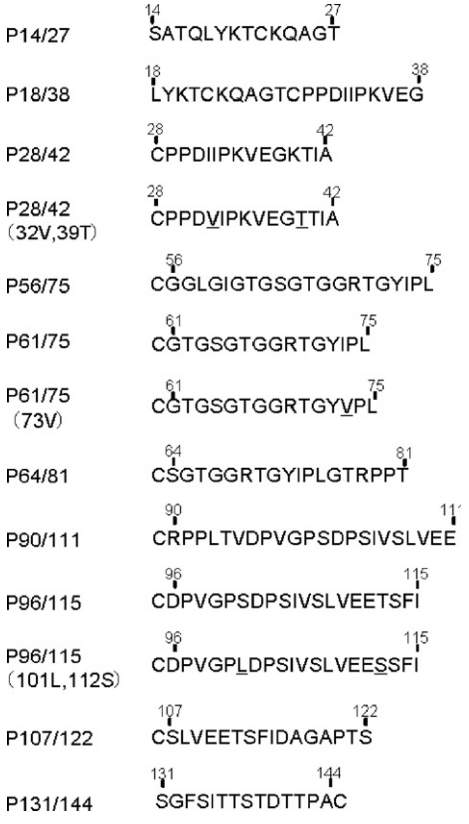

Fig. 1. Synthetic peptides used as antigens for immunizing rabbits. The potentially immunogenic segments were searched for within the HPV16 L2 surface region. The peptides having the amino acid sequences of the predicted antigenic regions were synthesized and conjugated with keyhole limpet hemocyanin (KLH). The numbers above the amino acid sequences are amino acid number of HPV16 L2, which consists of 473 amino acids. The N-terminal Cs of P56/75, P61/75, P61/75(73V), P64/81, P90/111, P96/115, P96/115(101L, 112S), and P107/122 and the C-terminal C of P131/144 were added for the C-mediated conjugation reaction. Two rabbits were immunized with each peptide.

rabbits with each of the peptide antigens induced antibodies with binding titers of 8000 to 64,000. The binding titer was expressed as a reciprocal of the maximum dilution of serum that induced higher OD than that the corresponding preimmune serum diluted at 1 to 100 did (data not shown).

Table 1 shows the bindings of the antisera that were diluted at 1 to 500 to the L1 capsids of HPV16, the L1/L2-capsids of HPV16, 18, 31, and 58 by ELISA. The antiserum from rabbit #1 immunized with P-14/27 (anti-P14/27#1) did not react to the L1/L2-capsids, but anti-P14/27#2 reacted to the L1/L2-capsids. Because both antisera were highly reactive to P14/27, P14/27 probably contained at least two epitopes. The epitope recognized by anti-P14/27#1 appears not to be displayed on the surface of the L1/L2-capsid, suggesting that the N-terminal border between the inside and surface region of L2 is within the segment from aa 14 to 27. The other sera against the peptides having the authentic aa sequences of HPV16 L2 were found to bind to HPV16 L1/L2-capsids, indicating the L2 region from aa 18 to 144 is displayed on the surface of the L1/L2-capsids.

Anti-P56/75#1 and #2, anti-P61/75#1 and #2, anti-P64/81#1 and #2, anti-P90/111#1, anti-P96/115#1and #2 cross-bound to the L1/L2-capsids of HPV18, 31, and 58. Anti-P90/111#2 did not bind to the L1/L2-capsids of HPV18 and 58. It is possible that epitope(s) recognized by anti-P90/111#2 may be different from those recognized by anti-P90/111#1.

Although anti-P28/42 sera bound to HPV16 L1/L2-capsids efficiently and to HPV58 L1/L2-capsids less efficiently, anti-P28/42(32V, 39T) bound to HPV16 L1/L2-capsids with a low efficiency and did not bind to the L1/L2-capsids of HPV18, 31, and 58. The result strongly suggests that D at aa 32 and K at aa 39 are associated with the epitope.

Anti-P61/75(73V)#1 and #2 and anti-P96/115(101L, 112S) #2 bound to the L1/L2-capsids of HPV16, 18, 31, and 58, suggesting that P61/75(73V) and P96/115(101L, 112S) have potential to induce antibodies capable of binding to the 15 oncogenic HPVs.

Table 1
Binding of antibody to the L1/L2-capsids of HPV16, 18, 31 and 58 (absorbency in ELISA with serum diluted at 1 to 500)

Antiserum/Antigen		HPV16 L1	HPV16 L1/L2	HPV18 L1/L2	HPV31 L1/L2	HPV58 L1/L2
Anti-P14/27	#1	0.066	0.066	0.044	0.053	0.061
	#2	0.064	0.112	0.055	0.057	0.138
Anti-P18/38	#1	0.069	0.744	0.057	0.067	0.124
	#2	0.160	0.457	0.065	0.090	0.100
Anti-P28/42	#1	0.085	0.255	0.055	0.113	0.159
	#2	0.084	0.547	0.061	0.091	0.156
Anti-P28/42 (32V, 39T)	#1	0.093	0.106	0.058	0.075	0.053
	#2	0.089	0.135	0.059	0.070	0.070
Anti-P-56/75	#1	0.068	0.951	0.717	0.807	0.514
	#2	0.085	1.022	0.565	0.336	0.462
Anti-P61/75	#1	0.066	0.954	0.634	0.444	0.271
	#2	0.109	0.884	0.612	0.343	0.553
Anti-P61/75 (73V)	#1	0.075	0.700	0.379	0.421	0.170
	#2	0.075	0.694	0.269	0.427	0.153
Anti-P64/81	#1	0.226	1.033	0.838	0.693	0.340
	#2	0.112	1.029	0.647	0.717	0.205
Anti-P90/111	#1	0.104	1.049	0.367	0.561	0.265
	#2	0.078	0.879	0.085	0.459	0.096
Anti-P96/115	#1	0.087	0.984	0.436	0.491	0.172
	#2	0.102	1.049	0.808	0.757	0.570
Anti-P96/115 (101L, 112S)	#1	0.075	0.840	0.070	0.808	0.430
	#2	0.043	0.841	0.421	0.672	0.571
Anti-P107/122	#1	0.086	1.034	0.068	0.149	0.309
Anti-P131/144	#1	0.072	0.497	0.048	0.077	0.052
	#2	0.119	0.921	0.056	0.064	0.072

Neutralization of HPV16, 18, 31, and 58 pseudovirions with the rabbit antisera

Neutralizing activities of the antisera were measured by inhibition of infection of 293TT cells, a human fibroblast cell line expressing a high level of SV40 T-antigen, with infectious HPV16, 18, 31, and 58 pseudovirions containing the SEAP expression plasmid having the SV40 replication origin. Serum was mixed with the pseudovirion stock and then inoculated to 293TT cells. Seventy-two hours later the SEAP activity of the culture medium was measured. The neutralizing titer was expressed as a reciprocal of the maximum dilution of serum that reduced the level of SEAP activity to half of the sample not treated with serum (Table 2).

Anti-P14/27#1 and #2 did not neutralize the pseudovirions tested in agreement with the lack of their efficient binding to the L1/L2-capsids (Table 1).

Anti-P18/38 neutralized HPV16 and cross-neutralized HPV18. Although the antisera diluted at 1 to 500 did not bind to the HPV18 L1/L2-capsids efficiently (Table 1), the antisera bound to HPV18 pseudovirions (data not presented), suggesting that the capsid containing DNA may be somewhat different from the empty capsid conformationally.

Anti-P28/42#1 did not neutralize HPV16 nor cross-neutralized HPV18, 31, and 58. Anti-P28/42#2, which bound to the HPV16 L1/L2-capsids more efficiently than anti-P28/42#1 (Table 1), neutralized HPV16 and cross-neutralized HPV58. Because anti-P28/42#1 and anti-P28/42#2 showed a similar binding efficiency to the HPV58 L1/L2-capsid, antibodies in these antisera probably recognized different epitopes.

Anti-P56/75#1 and #2 neutralized HPV16 and cross-neutralized HPV18, 31, and 58. Anti-P61/75#1 and #2 and anti-P64/81#1 and #2 neutralized HPV16 and cross-neutralized HPV18 and 58. Based on the comparison of the aa sequences of P56/75, P61/75, and P64/81, we speculated that P56/75 may induced antibody recognizing the portion from aa 56 to 61, of which aa sequences are common among the 15 oncogenic HPVs (Fig. 2). Because anti-P61/75 and anti-P64/81 did not cross-neutralize HPV31 despite the efficient binding to HPV31 L1/L2-capsids (Table 1), it is strongly suggested that the binding is necessary but not sufficient for the neutralization.

Both anti-P90/111#1 and #2 neutralized HPV16, and anti-P90/111#2 cross-neutralized HPV31. Anti-P96/115#1 and #2 neutralized HPV16. Anti-P96/115#2, which bound to the L1/L2-capsids more efficiently than anti-P96/115#1, efficiently cross-neutralized HPV31 and HPV58 but did not HPV18. Anti-P107/122#1 and anti-P131/144#2 neutralized HPV16 exclusively.

Table 2
Neutralization of HPV16, 18, 31, and 58 pseudovirions with the antisera

Antiserum		Neutralizing titer against			
		HPV16	HPV18	HPV31	HPV58
Anti-P14/27	#1	<50	<50	<50	<50
	#2	<50	<50	<50	<50
Anti-P18/38	#1	800	50	<50	<50
	#2	400	100	<50	<50
Anti-P28/42	#1	<50	<50	<50	<50
	#2	800	<50	<50	50
Anti-P28/42,	#1	<50	<50	<50	<50
32V, 39T	#2	<50	<50	<50	<50
Anti-P56/75	#1	400	200	200	400
	#2	200	50	100	200
Anti-P61/75	#1	400	100	<50	50
	#2	800	200	<50	100
Anti-P61/75,	#1	100	<50	<50	<50
73V	#2	100	<50	50	<50
Anti-P64/81	#1	3200	400	<50	100
	#2	800	200	<50	50
Anti-P90/111	#1	200	<50	50	<50
	#2	200	<50	<50	<50
Anti-P96/115	#1	200	<50	50	<50
	#2	400	<50	400	200
Anti-P96/115,	#1	100	<50	200	200
101L, 112S	#2	100	50	100	100
Anti-P107/122	#1	100	<50	<50	50
Anti-P131/144	#1	<50	<50	<50	<50
	#2	200	<50	<50	<50

Among antisera induced by the peptides having aa substitutions, anti-P96/115(101L, 112S) showed efficient cross-neutralization. Anti-P96/115(101L, 112S)#1 neutralized HPV16, 31, and 58. Anti-P96/115(101L, 112S)#2 neutralized the all HPVs tested.

We selected three antisera, anti-P18/38#2, anti-P56/75#1, and anti-P96/115(101L, 112S)#1, which showed the cross-neutralizing activity and the antigens used to obtain these antisera do not overlap each other, mixed equal volumes of these antisera, and the mixture's neutralizing activity was measured similarly. The mixture neutralized HPV16, 18, 31, and 58 efficiently. When anti-P18/38#2 and anti-P96/115(101L, 112S)#1 were replaced with preimmune serum, neutralization activity was lowered. The data suggest that the bindings of multiple antibodies to the L2 surface region enhance neutralization of HPVs.

Table 3
Neutralization of HPV16, 18, 31, and 58 pseudovirions with the mixture of three antisera

Mixture of antisera	Neutralizing titer			
	HPV16	HPV18	HPV31	HPV58
Anti-P18/38 #2 Anti-P56/75 #1 Anti-P96/115 (101L, 112S) #1	1600	800	800	400
Anti-P18/38 #2 Anti-P56/75 #1 Preimmune for P96/115 (10L1, 112S) #1	800	800	400	100
Preimmune for P18/38 #2 Anti-P56/75 #1 Preimmune for P96/115 (101L, 112S) #1	100	50	50	50
Preimmune for P18/38 #2 Preimmune for P56/75 #1 Preimmune for P96/115 (101L, 112S) #1	<50	<50	<50	<50

In summary, the data in this study indicate that multiple neutralization epitopes are present within the HPV16 L2 surface region, from aa 18 to 144. It was suggested that some of the peptide antigens having highly conserved aa sequences in the L2 surface region among oncogenic HPVS would have a potential to induce type common neutralization antibodies. From the data of the neutralization tests with the mixtures of three antisera (Table 3), it was suggested that the effective neutralization could be achieved through the binding of multiple antibodies to the L2 surface. It appears to be a good idea to

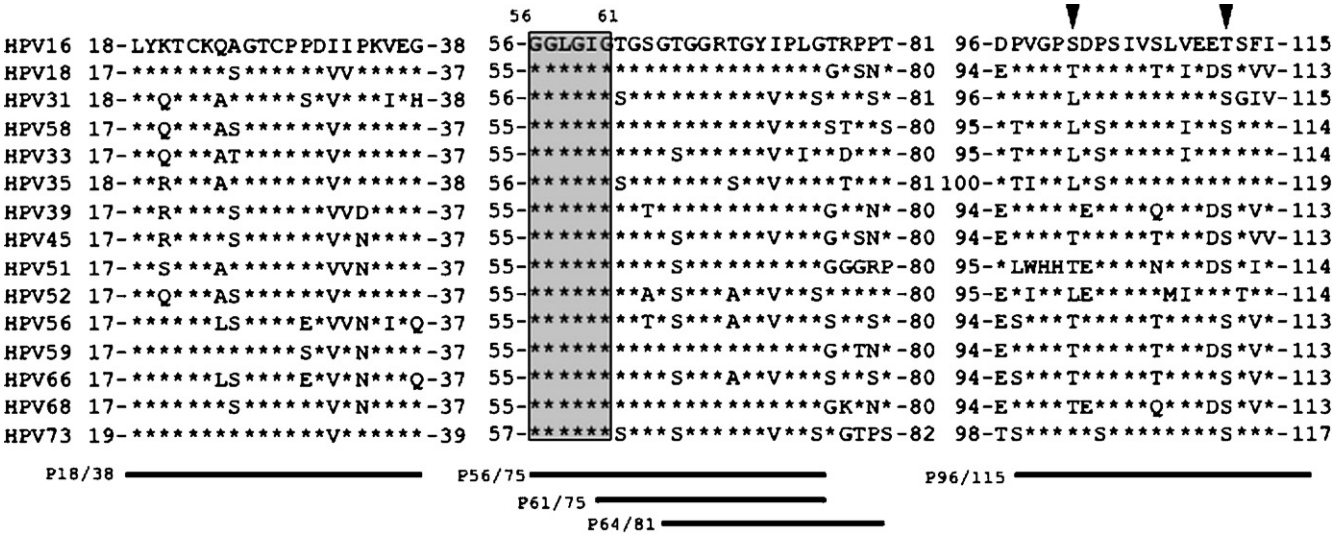

Fig. 2. Amino acids sequences of the L2 portions, aa 18 to 38, aa 56 to 81, and aa 96 to 115 of HPV16 and the corresponding portions of the other oncogenic HPVs. Amino acids identical to those of HPV16 were indicated by asterisk (*).

develop a vaccine antigen capable of inducing multiple antibodies binding to different epitopes on the L2 surface region. Since the aa sequences of the L2 surface regions of 15 oncogenic HPVs are largely similar, it seems possible to develop a vaccine antigen capable of inducing antibodies binding to the L2 surface regions of the multiple types of the oncogenic HPVs.

Materials and methods

Cell

293TT cells, a cell line expressing a high level of SV40 T antigen, was a kind gift from J. T. Schiller (National Cancer Institute, USA). The cells were cultured with in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Invitrogen Corp. Carlsbad, CA), 1% GlutaMax-I (Invitrogen Corp.), and hygromycine B (400 μg/ml) (Invitrogen Corp.).

Plasmids

Five plasmids; pYSEAP expressing SEAP, p16L1h expressing HPV16 L1, p16L2h expressing HPV16 L2, peL1fB expressing HPV18 L1, and peL2bhb expressing HPV18 L2 were gifts from J.T. Schiller. Four plasmids; p31L1h expressing HPV31 L1, p31L2h expressing HPV31 L2, p58L1h expressing HPV58 L1, and p58L2h expressing HPV58 L2 were newly constructed by the replacement of the HPV16L1 gene in p16L1h with the codon-modified HPV31L1, HPV31L2, HPV58L1, and HPV58L2 genes, respectively. The codons were changed to those used most frequently in human mRNAs; Ala: GCC; Cys: TGC; Asp: GAC; Glu; GAG; Phe: TTC; Gly: GGC; His: CAC; Ile: ATC; Lys: AAG; Leu: CTG; Asn: AAC; Pro: CCC; Gln: CAG; Arg; AGG; Ser: AGC; Thr: ACC; Val: GTG; Trp: TGG; Tyr: TAC.

Synthetic peptides

Peptides (Fig. 1) were synthesized by Fmoc method (SCRUM Inc., Tokyo, Japan). C was added to the N-terminus of P56/75, P61/75, P61/75(73V), P64/81, P-96/115, P96/115 (101L. 122S), and P-107/122 and to the C-terminus of P131/144, respectively. The carrier protein, keyhole limpet hemocyanin (KLH), was conjugated with P14/27 and P18/38 by bisimide-ester method. KLH was conjugated with the other peptides at the N- or C-terminus Cs of the peptides by the m-maleimidobenzoyl-*N*-hydroxysuccinimide-ester method.

Rabbit anti-peptide serum

The peptide was conjugated with KLH at the cysteine residue of each peptide. Japanese white rabbits (2.3–3.0 kg of weight, 2 animals for each antigen) were subcutaneously injected with the KLH conjugated peptide antigens mixed with Freund's complete adjuvant (SCRUM Inc., Tokyo, Japan). Immunization was repeated 4 times at 2-week interval, and serum was obtained at 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 capsids

The recombinant baculoviruses capable of expressing HPV16L1, HPV16L1/L2, HPV18L1/L2, HPV31L1/L2, HPV58L1/L2 were produced by using Bac-to-Bac baculovirus expression system (Invitrogen Corp., Carlsbad, CA), following the manufacturer's instruction. The transfer vectors pFastbac1 was used for the cloning of the L1 gene, and pFastbac dual was used for the cloning of both L1 and L2 genes. The recombinant baculovirus was inoculated to Sf9 cells (5 bottles of 175 cm^2 culture flask) and incubated for 3 days at 27 °C. The cells were collected and suspended in 5 ml of PBS containing 0.5% NP-40. After 10-min incubation at room temperature (RT), 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. The solution was centrifuged at 34,000 rpm at 20 °C for 20 h in an SW50.1 rotor (BECKMAN COULTER Inc., Fullrerion, CA). The fractions around a buoyant density of 1.28 g/ml were pooled and dialyzed against phosphate buffer (pH7.4) containing 0.5 M NaCl at 4 °C to remove CsCl.

ELISA with the L1 capsid of HPV16 and the L1/L2-capsids of HPV16, 18, 31, and 58 as antigens

A well of the ELISA plate was coated with 100 μl of PBS (pH 7.4) containing the purified L1 capsids (1 μg) or the L1/L2-capsids (1 μg) by incubation for 14 to 16 h at 4 °C. The well was blocked with 5% skim milk in PBS containing 0.1% Tween 20 for 2 h at 37 °C. After washing with PBS containing 0.05% Tween 20 and 0.05% NP-40 three times, 100 μl of the serum sample was added to the well and incubated for 1 h at RT. The secondary antibody was horseradish peroxidase-conjugated anti-rabbit IgG goat serum (SC-2030, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A mixture of 0.01% H_2O_2 and o-phenylenediamine (2 mg/ml) in 0.1 M citrate buffer (pH4.7) was added to the wells and the absorbency at 450 nm was measured after incubation for 30 min at 22 °C.

Preparation of pseudovirions

293TT cells, which had been seeded in a 10-cm culture dish (4×10^6 cells) at 16 h before the transfection, were transfected with a mixture of an L1-plasmid, an L2-plasmid, and pYSEAP by using Optifect (Invitrogen Corp.). For HPV16, 31, and 58 pseudovirion production 13.5 μg of the L1-plasmid, 3 μg of the L2-plasmid, and 13.5 μg of pYSEAP were used. For HPV18 pseudovirion production 14.5 μg of the L1-plasmid, 1 μg of the L2-plasmid, and 14.5 μg of pYSEAP were used. Sixty hours later the cells were harvested with trypsin. The cells were suspended in 0.5 ml of lysis buffer (PBS containing 1 mM $CaCl_2$, 10 mM MgCl2, 0.35% Brij58 [Sigma-Aldrich Inc., St. Louis, MO], 0.1% Benzonase [Sigma-Aldrich Inc.],

0.1% Plasmid Safe ATP dependent-DNase [EPICENTRE Corp. Madison, WI]) and incubated for 30 h at 37 °C with slow rotation. The lysate was cooled on ice for 5 min, mixed with 5 M NaCl solutions to adjust to concentration of NaCl to 0.85 M, and further kept on ice for 10 min. Then, the lysate was centrifuged at 5000 × *g* for 10 min at 4 °C. The supernatant was laid on an Optiprep gradient (from top to bottom, 27%, 33%, and 39% in PBS containing 1 mM $CaCl_2$, 10 mM $MgCl^2$, and 0.8 M NaCl) and centrifuged at 50,000 rpm for 3.5 h at 16 °C with SW55Ti rotor (Beckman Coulter Inc. Fullerton, CA). Fractions (300 μl each) were obtained by puncturing the bottom. An aliquot (1 μl) of each fraction was inoculated to 293TT cells (2 × 104) in 96-well flat-bottom tissue culture treated plates (Corning Costar Corp., New York, NY). SEAP activity of the culture medium was measured by the colorimetric SEAP assay (NCI home page: http://home.ccr.cancer.gov/lco/colorimetricseap.htm). The fraction induced the highest SEAP activity in the culture medium of the cells was used for the neutralization test as a stock of the infectious pseudovirions. To remove aggregates the pseudovirion stock was filtered (Ultrafree-MC centrifugal Filter Devices, MILIPORE corp., Bedford, MA) before use for the neutralization assay.

Neutralization test

The serum was diluted with the neutralization medium (DMEM [without phenol red] containing 10% FBS, 1% non-essential amino acids, 1% GlutaMax-I). Fifty μl of a serum sample was mixed with 50 μl of the neutralization medium containing an aliquot of the pseudovirion stock (0.05 μl of HPV16, 31, and 58 pseudovirions and 0.2 μl of HPV18 pseudovirions) and incubated for 1 h at room temperature. Then, the mixture was inoculated to 293TT cells (2×10^4) that had been seeded with 100 μl of the neutralization medium in 96-well flat-bottom tissue culture plates 6 h prior to the inoculation. The culture medium was harvested after incubation of the cells for 66 h at 37 °C, and SEAP activity of the culture medium was measured by the colorimetric SEAP assay. The neutralization titer was presented as the reciprocal of maximum dilution of serum that reduced SEAP level to half of the sample not treated with serum.

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