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A Surface Immunodeterminant of Human Papillomavirus Type 16 Minor Capsid Protein L2

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We used human papillomavirus type 16 (HPV-16) particles composed of capsid proteins L1 and L2 (L1/L2 capsids) as an antigen to produce mouse monoclonal antibodies (MAbs). Of 18 MAbs recognizing surface epitopes of L1/L2 capsids, 1 was an anti-L1 MAb and 17 were anti-L2 MAbs. Seven of 11 anti-L2 MAbs recognizing linear epitopes were found to bind to a synthetic peptide with an HPV-16 L2 sequence of amino acids (aa) 69–81, which is within a highly conserved region among different HPVs. The synthetic peptide reacted with the human sera that had been shown to be positive for an antibody against HPV-16, -18, -58, or -6b capsids composed of L1 alone. The data suggest that the HPV-16 L2 region of aa 69–81 contains a type-common immunodeterminant exposed on the surface of HPV virions. © 1998 Academic Press

Key Words: HPV-16; anti-L2 antibodies; a surface epitope of L1/L2-capsid.

INTRODUCTION

Human papillomaviruses (HPVs), agents causing a variety of epithelial lesions of the skin and genital tract, are classified into more than 70 types, based on the nucleotide sequence homology of viral DNA (Schiffman *et al.,* 1993). Despite overall similarity in the genomic organization, HPVs have rather diverse tissue specificity and malignant potential. Among the HPVs associated with the lesions in an anogenital tract, HPV type 16 (HPV-16) is predominantly found in cervical cancers and high-grade intraepithelial neoplasias (Lorincz *et al.,* 1991; Fisher, 1994).

An HPV capsid (an icosahedral particle with a diameter of 55 nm) consists of 72 pentameric capsomeres composed of the major (L1) and minor (L2) structural proteins (Crawford and Crawford, 1963; Baker *et al.,* 1991). The molar ratio of capsid proteins L1 and L2 is estimated to be 30 to 1 (Hagensee *et al.,* 1993; Kirnbauer *et al.,* 1993). Characterization of surface epitopes of these proteins is important to define neutralization epitopes as well as to analyze topographical location in the viral capsid. The surface epitopes on HPV-16 virus-like particles composed of L1 alone (L1 capsids) have been defined using mouse monoclonal antibodies (MAbs) (Christensen *et al.,* 1996) and antisera from patients with HPV infections (Wang *et al.,* 1997). These studies have shown that both linear and conformational epitopes are on the surface of L1 capsids and that at least three L1 regions [amino acids (aa) 111 to 130, 174 to 185, and 261 to 280] contain linear epitopes. On the other hand, HPV-16 L2 regions of aa 32–51, 62–81, 212–231, 279–291, and 362–381 have been located on the surface of L1/L2 capsids, from the observation that antisera against the peptides containing aa sequences of the regions react with HPV-16 L1/L2 capsids (Heino *et al.,* 1995). Studies of the other papillomaviruses identified a conformational L2 epitope of HPV-33 (Volpers *et al.,* 1995) and linear L2 epitopes of HPV-1 (Yaegashi *et al.,* 1991) and BPV-1 (Wen *et al.,* 1997).

In this study we examined epitopes that could be recognized by a series of newly produced mouse anti-L2 MAbs reactive with HPV-16 particles produced in insect Sf9 cells and composed of L1 and L2 (L1/L2 capsids). A strong surface immunodeterminant was located in the L2 sequence of aa 69 to 81, which is in a highly conserved region among different HPVs.

RESULTS

HPV-16 L1/L2 capsids

Composition of the HPV-16 L1/L2 capsids produced in insect cells was verified by cosedimantation of L1 and L2 in centrifugation through a sucrose gradient. The purified capsids (20 μg) were layered on the top of 5 ml of a 10 to 60% sucrose gradient in PBS and centrifuged in an SW50.1 rotor (BECKMAN) at 40,000 rpm for 45 min at 4°C. An aliquot (50 μl) of each fraction (0.3 ml from the bottom of the tube) was incubated in a carbonate buffer (pH 9.6) for 16 h at 4°C to disrupt capsids before measurement of L1 and L2 by ELISA using a mouse monoclonal antibody

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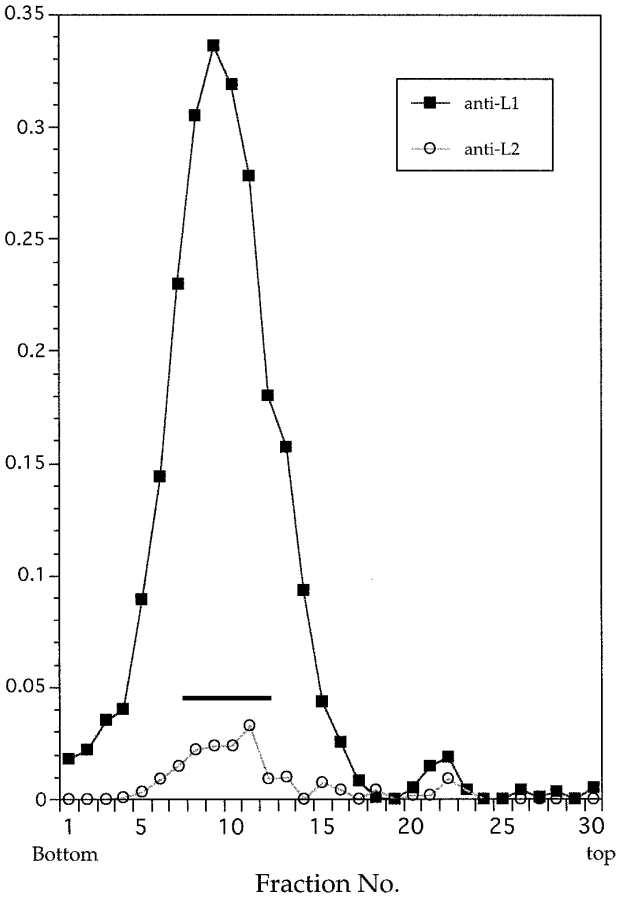

FIG. 1. Sucrose gradient sedimentation of HPV-16 L1/L2 capsids. L1/L2 capsids purified by CsCl equilibrium density gradient centrifugation were sedimented in a sucrose gradient [10–60% (wt/vol) in PBS] by centrifugation and fractionated (0.3 ml/fraction) from the bottom. L1 and L2 proteins in each fraction were denatured in carbonate buffer (pH 9.6) and measured by ELISA using anti-L1 and anti-L2 antibodies. Fractions 8 to 12 (indicated by a bold bar) were pooled and used for further characterization.

against HPV-16 L1 and the anti-L2 mouse antiserum (Fig. 1). Both L1 and L2 were detected in the same fractions (fractions from 8 to 12). Spherical particles with a diameter of 50–60 nm were observed exclusively in the pool of the rest of these fractions by electron microscopy (Fig. 2A). The proteins in these fractions were electrophoresed in SDS–polyacrylamide gels followed by silver staining (Fig. 2B) and analyzed by Western blotting (Fig. 2C). The proteins with expected sizes (56 kDa for L1 and ~90 kDa for L2) (Kirnbauer *et al.,* 1993) were detected. The molar ratio of L1 to L2, although it could not be calculated from electrophoresis, appeared to be reasonable, as expected of HPV virions. These data indicate that the purified particles were composed of L1 and L2.

Characterization of MAbs against the HPV-16 L1/L2 capsids

Eighteen stable hybridoma cell lines secreting MAbs reactive with intact HPV-16 L1/L2 capsids were obtained from mice immunized with L1/L2 capsids (Table 1). Since further purification of antigen (L1/L2 capsids) for ELISA by sedimentation in a sucrose gradient did not alter the reactivity of MAbs (data not shown), we concluded that the MAbs did not react with minor contaminants of disrupted capsids. Although the sera from the immunized mice reacted with L1 capsids (virus-like particles composed of only HPV-16 L1 protein) strongly, only one MAb (No. 32) was anti-L1. The other 17 MAbs which did not react with L1 capsids were anti-L2 antibodies recognizing epitopes displayed on the surface of L1/L2 capsids.

Reactivities of the anti-L2 MAbs with His-L2/330 (His-tagged truncated L2 protein containing aa 1 to 330, expressed in Sf9 cells), His-L2/173 (aa 1 to 173), bacterially expressed MBP-L2 (the C-terminal 155-amino-acid region fused with maltose binding protein), and GST-L2 [internal region (aa 141–243) fused with GST protein] were examined by ELISA (Table 1 and Fig. 3). Eleven MAbs (Nos. 17, 2, 4, 5, 6, 7, 9, 10, 11, 12, and 13) bound to both His-L2/330 and His-L2/173. None of MAbs bound to MBP-L2 and GST-L2. Therefore, it was indicated that these MAbs recognized linear epitopes in the N-terminal region of 140 amino acids. Six MAbs (Nos. 15, 16, 18, 24, 35, and 36) were not reactive with any of the four antigens, indicating that their recognition epitopes are probably conformational.

The N-terminal unique regions (A: aa 1–12 and B: aa 56–81 of HPV-16 L2) in which amino acids sequences are highly conserved among L2 of different HPVs (Fig. 4) attracted our attention. As to the immunological cross-reactions, it is important to know whether the conserved region contains the immunodeterminants. Thus, 11 MAbs that recognized linear epitopes were examined for reactivity with synthetic peptides with amino acid sequences corresponding to those of the conserved regions (Table 2). None of the MAbs reacted with P-1/12 and P-56/68. However, 6 MAbs (Nos. 2, 4, 6, 7, 9, and 10) bound to P-69/81. Since P-69/81 inhibited binding of these MAbs to L1/L2 capsids in ELISA competitively (data not shown), we concluded that the reactivities of the MAbs with P-69/81 were specific. MAb 17 reacted with both P-63/75 and P-69/81. The data indicated that the epitopes recognized by the six MAbs contained, at least partly, the region of aa 69–81 and that the epitope for 17 contained the region of aa 69–75. Epitopes for the other MAbs (Nos. 5, 11, 12, and 13) may be located in the region of aa 13–55 or 82–140.

Reactivity of human sera with P-69/81

To know the immunogenicity of the region of aa 69–81 to humans, reactivities of human sera to P-1/12, P-56/68, and P-69/81 were examined by ELISA using the serum samples selected from those previously

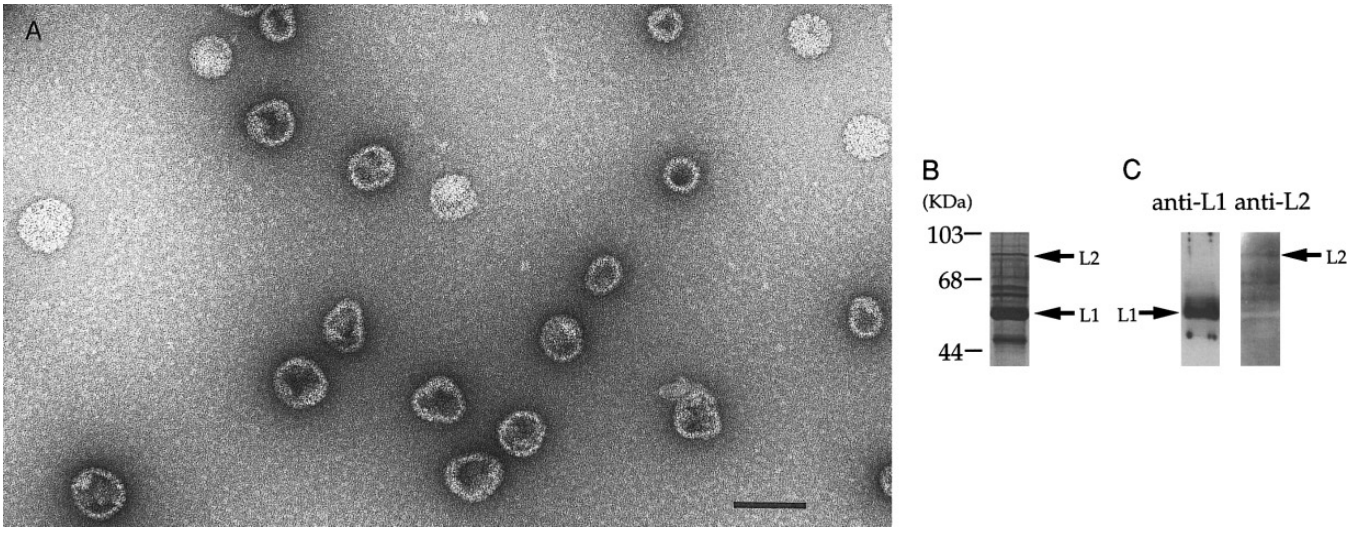

FIG. 2. HPV-16 L1/L2 capsids purified through sucrose gradient sedimentation. (A) Electron microscopy. The bar represents 100 nm. (B) Proteins were lysed by boiling in sample buffer, electrophoresed in 10% SDS–polyacrylamide gels, and stained by the silver stain method or (C) analyzed by immunoblotting with the anti HPV-16 L1 and antisera to GST-L2. Molecular size standards are on the left.

examined for antibodies against L1 capsids of HPV-6b, -16, -18, and -58 (Matsumoto *et al.,* 1997). The serum samples analyzed were 20 samples negative for anti- for HPV-6b, -16, -18, and -58, 22 samples positive for the anti-HPV-16 L1 capsid antibody and negative for the anti-HPV-6b, -18, and -58 L1 capsid antibodies, and 30 samples negative for anti-HPV-16 and positive for any of anti-HPV-6b, -18, and -58. Figure 5 shows the results obtained with the samples diluted in PBS (1: 50). Whereas the ELISA titers to P-1/12 and P-56/68 were low in all of the samples, those to P-69/81 were high in the sera positive for any one of the anti-HPV L1 antibodies. Means of the titers of sera positive for anti-HPV-16 and sera positive for any of anti-HPV-6b, -18, and -58 were significantly higher than that of sera negative for these antibodies. The data suggest that the HPV-16 L2 region of aa 69–81 contains one of immunodeterminants for humans as well as mice and suggest that antisera against virions of different HPVs contain antibodies cross-reactive to P-69/81.

TABLE 1

Characterization of 18 MAbs Reactive with L1/L2 Capsids

MAb No.	Subtype	ELISA titer (A_{450})			
		L1/L2 capsids	L1 capsids	His-L2/330	His-L2/173
17	IgM	0.242	0.000	0.133	0.102
2	IgM	0.156	0.000	0.154	0.113
4	IgG2a	0.115	0.000	0.141	0.101
6	IgG2a	0.161	0.000	0.128	0.102
7	IgM	0.181	0.055	0.111	0.122
9	IgM	0.102	0.000	0.235	0.164
10	IgM	0.125	0.000	0.171	0.118
5	IgG2a	0.204	0.000	0.137	0.094
11	IgM	0.133	0.000	0.115	0.136
12	IgG3	0.158	0.044	0.144	0.106
13	IgG3	0.213	0.000	0.139	0.119
15	IgG2a	0.228	0.000	0.021	0.022
16	IgG3	0.163	0.000	0.036	0.033
18	IgM	0.184	0.036	0.059	0.025
24	IgG2a	0.162	0.000	0.029	0.018
35	IgG3	0.167	0.000	0.021	0.017
36	IgM	0.174	0.000	0.027	0.029
32	IgG2a	0.246	0.128	0.024	0.025

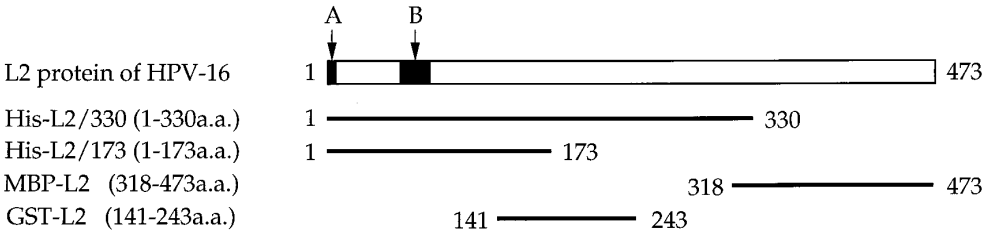

FIG. 3. Antigens used for characterization of MAbs. His-L2s, MBP-L2, and GST-L2 were expressed as fusion proteins (fused with His tag, maltose binding protein, or glutathione *S*-transferase, respectively). A (aa 1–12) and B (aa 56–81) indicate the regions of which amino acid sequences are conserved among L2 proteins of different HPVs (see Fig. 4).

DISCUSSION

In this study we characterized mouse MAbs reactive with intact L1/L2 capsids of HPV-16. Although the sera from the immunized mice reacted with L1 capsids strongly, 17 of a total of 18 MAbs were anti-L2. The reason almost all of the hybridomas we obtained secreted anti-L2 MAbs is unclear at present. The similar result that all of 12 MAbs obtained from Balb/c mice immunized with intact virions of HPV-1 were anti-L2 has been reported (Yaegashi *et al.,* 1991). Since immunogenicity is different from antigen to antigen, one of the possible explanations is that we might have scarified mice at the time the production of anti-L2 antibodies was dominant. Eleven anti-L2 MAbs recognized linear epitopes located in the N-terminal region of L2 protein (aa 1–140). The 6 anti-L2 MAbs not reactive with any of the linear L2 antigens may recognize either conformational L2 epitopes or possibly L2-dependent conformational L1 epitopes. Seven MAbs reacted with P-69/81 (a synthetic peptide with an HPV-16 L2 sequence of aa 69–81), indicating that the region containing aa 69–81 is a surface immunodeterminant of L2.

The HPV-16 L2 region of aa 62–81 has been located on the surface of the L1/L2 capsids by Heino *et al.* (1995). They examined reactivities of a series of guinea pig and rabbit antisera raised by immunization with synthetic peptides having HPV-16 L2 sequences. Antibodies against peptides with aa 32–51, 62–81, 212–232, and 362–381 bind to intact L1/L2 capsids. The topographical location of the region close to the N-terminus of L2 displayed on the surface of the L1/L2 capsids is consistent with the case of BPV-1 virions (Wen *et al.,* 1997). Three anti-L2 MAbs recognizing epitopes in the region of aa 61–123 of BPV-1 L2 can access and bind to their epitopes on the intact virions. The topographical location of L2 seems to be common to papillomavirus capsids.

The human serum sample positive for anti-HPV-16, -18, -58, or -6b L1 capsids reacted with P-69/81. The data indicate that the region of aa 69–81 of HPV-16 L2 is an immunodeterminant for humans. It is likely that the regions of HPV-18, -58, and -6b L2 proteins containing amino acid sequences similar to aa 69–81 of HPV-16 L2 (aa 70–82 of HPV-18 L2, aa 72–84 of HPV-58 L2, and aa 68–80 of HPV-6b L2) (Fig. 4) are common immunodeterminants among HPVs. Besides type-common epitopes, the type-specific immunodeterminant regions of HPV-33 L2 (aa 117–130) (Volpers *et al.,* 1995) and HPV-1 L2 (aa 102–108) (Yaegashi *et al.,* 1991) have been reported to be detectable. The MAbs not binding to P-69/81 in this study may contain those recognizing type-specific epitopes of HPV-16 L2.

Although the function of L2 within the capsid has not

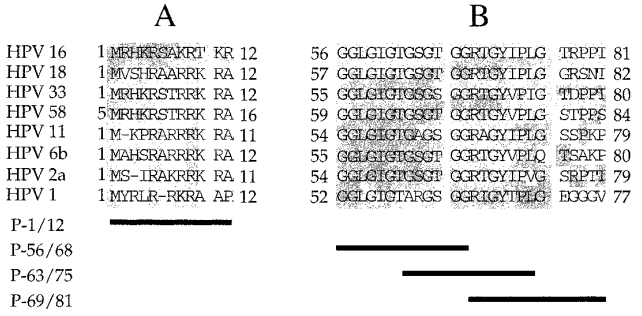

FIG. 4. Alignment of the HPV-16 L2 amino acid sequence of the homologous regions of L2 proteins from different HPVs. Amino acids identical to those of HPV-16 are shaded. Synthetic peptides (P-1/12, P-56/68, P-63/75, and P-69/81) contain the amino acid sequences of HPV-16 L2, indicated by bold lines.

TABLE 2

Reactivities of MAbs with Synthetic Peptides

MAb	ELISA titer (A_{450}) P-63/75	P-69/81
17	0.084	0.108
2	0.000	0.078
4	0.000	0.126
6	0.017	0.077
7	0.012	0.047
9	0.037	0.103
10	0.008	0.112
5	0.004	0.004
11	0.020	0.026
12	0.000	0.010
13	0.000	0.000

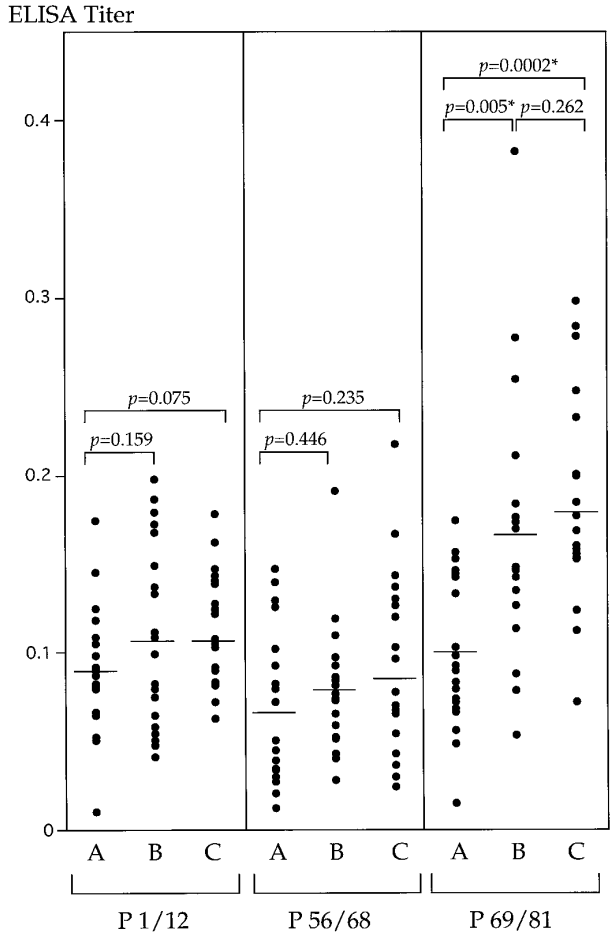

FIG. 5. Reactivity of 72 human serum samples to P-1/12, P-69/81, and P-56/68. (A) 20 samples negative for anti-HPV-6b, -16, -18, and -58 L1 capsid antibodies. (B) 22 samples positive for anti-HPV-16 L1-capsid and negative for anti-HPV-6b, -18, and -58 L1 capsids. (C) 30 samples negative for anti-HPV-16 L1 capsid and positive for any of the anti-HPV-6b, -18, and -58 L1 capsids. The bars represent the means of ELISA titers (A_{450}) and the significance of differences between means is indicated by *P* values (*t* test).

been fully elucidated (Roden *et al.,* 1996; Day *et al.,* 1998), antibodies against the N-terminal region of L2 proteins [aa 45–173 for BPV-1 (Roden *et al.,* 1994a,b), aa 11–200 for BPV-4 (Chandrachud *et al.,* 1995; Gaukroger *et al.,* 1996), and aa 131–151 for BPV-4 (Campo *et al.,* 1997)] have been demonstrated to contain neutralizing activities. The surface region of L2 may play a role in the step of viral entry to cells. Analyses of neutralizing activities of the MAbs described in this study are currently in progress.

MATERIALS AND METHODS

Recombinant baculovirus expressing HPV-16 L1 and L2 genes

DNA fragments containing HPV-16 L1 ORF (nucleotides 5637 to 7154) and L2 ORF (4237 to 5658) (Seedorf *et al.,* 1985) were subcloned, by PCR-primed introduction of appropriate restriction sites, from an HPV-16 DNA clone with nucleotide substitution of G for C at nucleotides 6240 (Matsumoto *et al.,* 1997). The L1 and L2 ORFs were inserted into pFastBacDUAL donor plasmid (GIBCO BRL, New York, NY) between *Not*I and *Xba*I sites (for expression from the polyhedrin promoter) and between *Sma*I and *Bam*HI sites (for expression from p10 promoter), respectively. The structure of the recombinant plasmid was confirmed by DNA sequencing analysis and introduced into *Escherichia coli* DH10Bac competent cells that contain Bacmid DNA with a mini-attTn7 target site and the helper plasmid (GIBCO BRL). The resultant recombinant Bacmid DNA was purified and transfected into Sf9 cells, which were grown in suspension culture at 27°C in SFM medium (GIBCO BRL) supplemented with 3% fetal calf serum (FCS). Recombinant baculovirus clones were purified by plaque isolation as described previously (Matsumoto *et al.,* 1997).

Antisera to HPV-16 L1 and L2 proteins

A mouse monoclonal antibody against HPV-16 L1 was a commercial product (PharMingen, San Diego, CA). For production of antisera to L2, an internal part of HPV-16 L2 (aa 141 to 243) was bacterially expressed as a fusion protein with glutathione *S*-transferase (GST-L2) and purified by affinity column chromatography using glutathione–Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). Balb/c mice were immunized with a mixture of purified GST-L2 (50 μg per mouse) and Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) followed by three injections of the antigen (25 μg per mouse) mixed with incomplete adjuvant (DIFCO Laboratories) at intervals of 2 to 4 weeks. Sera were collected 7 days after the final injection.

Preparation of HPV-16 L1 capsids and L1/L2 capsids

Purified HPV-16 L1 capsids were prepared as described previously (Matsumoto *et al.,* 1997). HPV-16 L1/L2 capsids were produced in Sf9 cells infected with the recombinant baculovirus capable of expressing HPV-16 L1 and L2. Approximately 1.5×10^9 Sf9 cells were infected with the recombinant baculovirus at a m.o.i. of 10 to 30 for 1.5 h at a room temperature and incubated for 2–3 days at 27°C. The infected cells collected by centrifugation were resuspended in 15 ml of phosphate-buffered saline (PBS, pH 7.2), disrupted by brief sonication, layered onto 15 ml of 40% (wt/vol) sucrose in PBS, and centrifuged in a BECKMAN SW28 rotor at 25,000 rpm for 2 h at 4°C. The pellets were resuspended by gentle sonication in 35 ml of 27% (wt/wt) CsCl in PBS and centrifuged in a BECKMAN SW28 rotor at 27,500 rpm for 20 h at 20°C. The capsid band (at a density of 1.28 g/ml) was collected and dialyzed extensively against PBS.

Immunization of mice with HPV-16 L1/L2 capsids

Purified HPV-16 L1/L2 capsids (20 μg per mouse) were mixed with complete Freund's adjuvant and injected subcutaneously into Balb/c mice. The antigen (15 μg per mouse) mixed with incomplete Freund's adjuvant and the antigen (15 μg per mouse) alone were injected to mice subcutaneously at 4 weeks after the first immunization and intraperitonially at 6 weeks, respectively. Five days after the final injection, spleen cells of the immunized mice were fused to myeloma cell line X63-Ag8.653 (grown in RPMI medium containing 10% FCS) using the standard method (Galfre *et al.,* 1977). Hybridomas were selected with hypoxanthine–aminopterin–thymidine (Dainippon Pharmaceutical Co., Osaka, Japan). Antibody in culture medium of each hybridoma clone was screened by ELISA using intact L1/L2 capsids as the antigen.

Purification of antigens used for epitope mappings

Histidine-tagged truncated L2 proteins of HPV-16 (His-L2/330 and His-L2/173 contained aa 1 to 330 and 1 to 173, respectively) were expressed in Sf9 cells by recombinant baculoviruses constructed with pFastBacHT (GIBCO BRL). These proteins were purified from cell lysates using an Ni^{2+} column (GIBCO BRL) by the manufacturer's standard procedure. MBP-L2 [C-terminal region containing aa 318 to 473 was fused with maltose binding protein (MBP)] was bacterially expressed and purified by affinity column chromatography (NEB, Beverly, MA). Purities of antigen preparations were verified by SDS–polyacrylamide gel electrophoresis. P-1/12, P-56/68, P-63/75, and P-69/81 were BSA-conjugated synthetic peptides with the HPV-16 L2 amino acid sequences corresponding to aa 1 to 12, 56 to 68, 63 to 75, and 69 to 81, respectively. Syntheses and conjugation to BSA were performed by Sawady Technology (Tokyo, Japan).

ELISA

The antigens [1 μg/well for L1/L2 capsids and L1 capsids in PBS (pH 7.2), 2 μg/well for His-L2/330, His-L2/173, MBP-L2, and GST-L2 (pH 9.6), and 50 μg/well for BSA-conjugated peptides in carbonate buffer (pH 9.6)] were incubated in wells of an ELISA plate (Dynatech Laboratories, Chantilly, VA) for 14 to 16 h at 4°C. Wells were blocked with 0.2% gelatin in PBS for overnight at 4°C. After three washings with PBS containing 0.05% NP-40 and 0.05% Tween 20, cell culture supernatants of hybridomas or the samples to be tested (300 μl/well) were added to wells and incubated for 1 h at room temperature. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (DAKO Corp., Carpinteria, CA) (1:2000 in 1% BSA in PBS) was used as a secondary antibody. 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 and the absorbancy at 450 nm (A_{450}) was measured. Specific absorbancy was calculated by subtracting the absorbancy of mock using wells covered with gelatin and a part of fusion proteins (MBP and glutathione *S*-transferase) or BSA conjugated with synthetic peptides.

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