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Antigenic and Immunogenic Epitopes Shared by Human Papillomavirus Type 16 and Bovine, Canine, and Avian Papillomaviruses

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All types of papillomaviruses (PV) share common, so-called group-specific epitopes. To identify the major group-specific epitopes, we immunized 26 guinea pigs or rabbits with purified bovine PV type 1 (BPV), canine PV, or avian PV from the common chaffinch. The resulting hyperimmune sera, as well as a commercially available rabbit antiserum to BPV and seven monoclonal antibodies to BPV, were tested in an enzyme-linked immunosorbent assay with a set of 66 overlapping 20-amino-acid peptides representing the complete sequence of the major capsid proteins (L1 and L2) of human PV type 16 (HPV 16). Sera from the same animals before immunization were used as controls. The minimal reactive epitopes within each peptide were further characterized by testing of truncated peptides. The cross-reactive epitopes were clustered in two regions of L1, an internal region (at positions 171 to 235), which contained three epitopes, and the more reactive region at the carboxy terminus (at positions 411 to 475), which contained six epitopes. The most reactive of the HPV 16 broadly cross-reactive epitopes was a carboxy-terminal epitope which had the sequence DTYRF and which reacted with nine of the antisera to BPV, canine PV, or avian PV, with the commercially available rabbit antiserum to BPV, and also with a mouse monoclonal antibody to BPV. Antipeptide antisera to all of the HPV 16 L1 peptides and to the most antigenically reactive of their truncated analogs were made in guinea pigs. Antipeptide antisera reactive with BPV were obtained for three of the cross-reactive epitopes, and one of these antisera allowed highly sensitive detection of group-specific PV antigen by immunoperoxidase staining.

The papillomavirus (PV) capsid contains a major 57-kDa protein encoded by the L1 open reading frame (ORF) (25, 35, 40) and a minor 72-kDa protein encoded by the L2 ORF (22, 39). The L1 ORF is the most highly conserved of the PV ORFs, with approximately 40% of the amino acid residues being identical in distantly related PVs (1). The L2 ORF is highly conserved only in a short stretch at the amino terminus (1). Antisera against intact virions are mostly type specific and show a weak cross-reaction or no cross-reaction with viruses from different species (32), whereas antisera against virions treated with sodium dodecyl sulfate (SDS) (21) or carbonate buffer (pH 10) (32) cross-react strongly. A rabbit antiserum raised against SDS-treated bovine PV type 1 (BPV) is commercially available (Dako, Copenhagen, Denmark) and is routinely used in histopathological studies to detect PV group-specific antigens in virus-producing tissue (21, 23, 28, 41). In immunoblotting, this antiserum reacts with the L1 protein but not detectably with the L2 protein (2, 20, 38–40). Using synthetic peptides, Lim et al. (26) found the Dako serum to be reactive with more than 15 epitopes within the BPV L1 ORF. Using a nested set of bacterial fusion proteins, Jenison et al. (20) reported that only a single epitope within L1 of human PV (HPV) type 6b (HPV 6b) was cross-reactive with the Dako serum. An HPV 6b type-specific epitope reactive with human sera was also identified (20). Antisera against HPV 16 L1 fusion proteins that reacted with different HPV types as well as type-specific monoclonal antibodies reactive with an internal region of HPV 16 L1 have been produced (4, 34). Antigenic sites within the L1 protein have also been identified with antisera to HPV 16 fusion proteins (29). Seven monoclonal antibodies, designated AU1 to AU6 and IH8, against SDS-treated BPV have been extensively characterized (6, 26, 30). AU1, AU2, AU6, and IH8 were found to be cross-reactive with PVs from several other species, whereas AU3, AU4, and AU5 were found to be reactive only with BPV. AU1 identifies the epitope TYRY in the carboxy terminus of BPV L1, whereas AU2 and IH8 identify the epitope FGAAN in the middle of BPV L1. The AU6 epitope could not be identified (26).

We have previously shown that patients with cervical intraepithelial neoplasia (CIN) have immunoglobulin A (IgA) antibodies against disrupted BPV in both cervical secretions (11) and serum (12). The reactivities of human sera to overlapping synthetic peptides representing all of the HPV 16 ORFs have been mapped (8, 10), in some cases to regions that are highly conserved among PVs (10). The number of different HPV types is presently over 60 (7) and is increasing steadily. There is therefore a need for a monospecific antibody broadly reactive with a variety of both known and so-far-uncharacterized PVs. Previous attempts to produce a group-specific antiserum by immunization with fusion proteins or synthetic peptides have failed (5). The identification of the group-specific antigenic and immunogenic epitopes could thus be important for both the serological study and the immunohistochemical study of genetically divergent PVs.

MATERIALS AND METHODS

Peptide synthesis. Peptides were synthesized by the solid-phase method as described previously (10, 18, 19). For short peptides, a GGC linker was added to the carboxy terminus to

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facilitate binding to enzyme-linked immunosorbent assay (ELISA) plates. The sequence of HPV 16 (36) was corrected as described by Parton (33).

Virus purification and treatment. Virus isolation and purification were done as previously described (11). In brief, cutaneous warts were purified by two cycles of CsCl equilibrium density gradient centrifugation. A lighter band with a buoyant density of 1.29 g/ml and consisting of empty virus particles, as determined by electron microscopy, and a heavy band with a buoyant density of 1.34 g/ml and consisting of complete virus particles, as determined by electron microscopy, were pooled and stored at −20°C. Before immunization, the preparations were thawed and again analyzed by electron microscopy. Although many intact particles were present, empty and partially disrupted virions were also seen. In SDS gel electrophoresis, two different preparations of purified BPV were found to contain a major protein with a molecular mass of 56 kDa. In one preparation, a minor 70-kDa protein was also seen. The canine PV (CPV) preparation consisted of a major 56-kDa protein, and the avian PV (APV) preparation consisted of a major 60-kDa protein. The different preparations of BPV, CPV, and APV were each divided into four parts and treated in four different ways: five cycles of freezing-thawing, boiling for 5 min in 10% SDS, incubation with carbonate buffer (pH 9.6) overnight at 4°C, or no treatment.

Antisera to BPV, CPV, and APV. Rabbits or guinea pigs were injected subcutaneously with 100 μg of virus or keyhole limpet hemocyanin-conjugated synthetic peptides in Freund's complete adjuvant and then with two injections of 100 μg of virus or keyhole limpet hemocyanin-conjugated synthetic peptides in Freund's incomplete adjuvant at biweekly intervals. The animals were bled 10 days after the third injection. Two different batches of rabbit antiserum against SDS-disrupted BPV were purchased from Dako. Thirty-two guinea pigs were immunized with the purified viral preparations of BPV, CPV, and APV. For BPV, four guinea pigs were immunized for each of the four different treatments, whereas for CPV and APV, two guinea pigs each were used. Two rabbits were immunized with BPV disrupted by freezing-thawing. Twenty-five of the guinea pigs and 1 of the rabbits responded with high titers (1:10,000 to 1:100,000) of antibodies to the immunizing antigen, as measured by ELISA. The anti-BPV titers of both batches of the Dako serum were exceptionally high (>1:100,000). Sera from 5 nonimmunized rabbits and from 32 nonimmunized guinea pigs did not react with any of the purified PVs in ELISA.

Forty-one guinea pigs were immunized with an overlapping set of 35 synthetic peptides corresponding to the deduced amino acid sequence of the L1 ORF of HPV 16 and with 6 peptides corresponding to the major group-specific epitopes (see below). Thirty-six animals responded with an antipeptide antibody titer of >1:2,000, as measured by ELISA. Seven monoclonal antibodies against BPV (AU1 to AU6 and IH8) were the kind gift of A. B. Jenson, Georgetown University, Washington, D.C. (6, 26, 30).

ELISA. The synthetic peptides were diluted to 20 μg/ml and PV was diluted to 3 μg/ml in 10 mM carbonate buffer (pH 9.6) or in phosphate-buffered saline (PBS), and the dilutions were added to half-area (50 μl per well) microtiter plates (Costar, Cambridge, Mass.) and kept at room temperature overnight. After being washed once with PBS–0.05% Tween 20 (PBS-T), the plates were blocked with 10% lamb serum in PBS (LS-PBS) for 60 min at 37°C; rabbit and guinea pig sera in four different dilutions were added to duplicate wells in the plates and allowed to react for 120 min at 37°C. Monoclonal antibodies were allowed to react for 60 min at 37°C. After the plates were washed five times with PBS-T, either a peroxidase-conjugated swine antibody against rabbit immunoglobulins (Dako), diluted 1:1,000 in LS-PBS, or a peroxidase-conjugated rabbit antibody against guinea pig immunoglobulins (Dako), diluted 1:2,000 in LS-PBS, was incubated on the plates for 120 min at 37°C. For the monoclonal antibodies, we applied a peroxidase-conjugated goat antibody to mouse IgG (Southern Biotechnology), diluted 1:1,000 in LS-PBS, for 60 min at 37°C. The plates were washed five times with PBS-T and developed with 0.4 mg of 2,2′-azino-di(3-ethylbenzthiazolinsulfonate)deammonium salt per ml in 0.1 M citrate buffer (pH 4) with 0.9% hydrogen peroxide. The A_{415} values were recorded after 60 min. In the case of anti-PV ELISAs, endpoint titers were calculated as the last of serial twofold dilutions to yield an absorbance half the maximum absorbance. Titers for antipeptide ELISAs were calculated as single-dilution optical density (OD) titers with the formula dilution × absorbance × 10 (17). OD titers have been shown to correlate strongly with endpoint dilution titers ($r = 0.98$) (3, 17). We also used the criterion postulated by Kurstak (24) that OD titers should only be calculated with absorbances in the OD interval from 0.2 to 1.0. Prior to the calculation of OD titers, both the absorbance on uncoated wells of the same serum and the absorbance on the same peptide of preimmune serum from the same animal were subtracted. In the case of the Dako serum, for which preimmune sera were not available, serum samples from three normal nonimmunized rabbits were used as controls.

Immunohistocytochemistry. The peroxidase-antiperoxidase method was used to stain formalin-fixed, paraffin-embedded sections of human warts and CIN lesions. The sections were deparaffinized and rehydrated, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS. After incubation with 5% milk for 60 min, the sections were incubated with guinea pig antipeptide antiserum or with rabbit anti-BPV antiserum (Dako) overnight at 4°C. In between PBS washes, the sections were incubated with rabbit anti-guinea pig immunoglobulins (Dako), diluted 1:150, for 1 h, with swine anti-rabbit immunoglobulins (Dako), diluted 1:100, for 1 h, and with a rabbit peroxidase-antiperoxidase complex (Dako), diluted 1:100, for 1 h. After a final PBS wash, the antibody complex was visualized by the addition of 3-amino-*s*-ethylcarbazole in acetate buffer (pH 4). Counterstaining was performed with Mayer's hematoxylin.

RESULTS

Reactivity of antisera to BPV, CPV, and APV with overlapping HPV 16 L1 and L2 peptides. The complete amino acid sequences of the L1 and L2 proteins of HPV 16 have been synthesized as a set of 66 synthetic peptides 20 amino acids long and with a 5-residue overlap (10). These peptides were tested in an ELISA with the Dako serum against BPV, 26 antisera against BPV, CPV, or APV, and seven monoclonal antibodies against BPV.

The major epitopes cross-reacting with the Dako serum were from the carboxy terminus of the HPV 16 L1 protein: peptide 30 (mean titer of two batches of Dako serum, 1:250,000) and peptide 33 (mean titer, 1:49,000) (Fig. 1A). Minor epitopes were peptides 16 (mean titer, 1:4,100) and 17 (mean titer, 1:1,200) from the middle of HPV 16 L1 (Fig. 1A). The peptides deduced from the L2 protein were not reactive with the Dako serum (Fig. 1B).

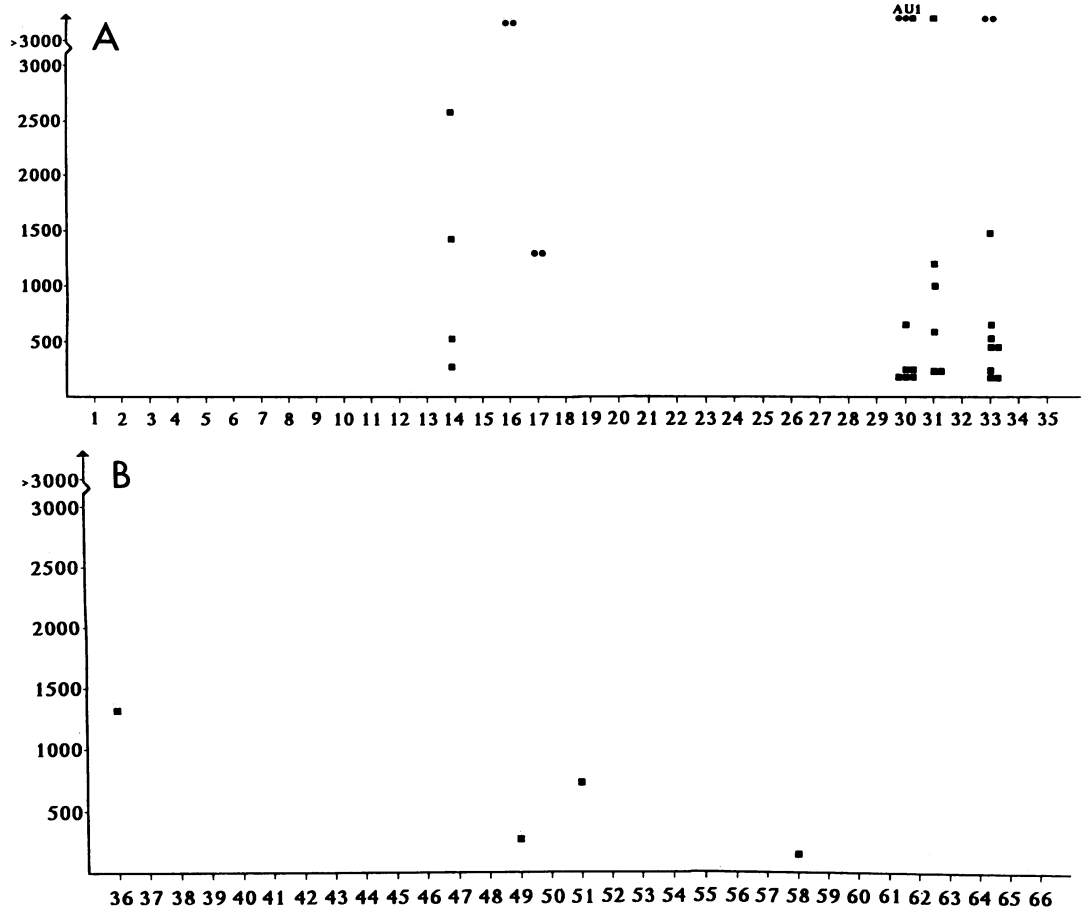

FIG. 1. ELISA immunoreactivity to the HPV 16 L1 and L2 ORFs of 25 guinea pig antisera to BPV, CPV, or APV and a rabbit serum to BPV (■), two batches of Dako serum to BPV (●), and seven monoclonal antibodies to BPV (e.g., antibody AU1). Each dot represents the ELISA OD titer of a serum when reacted with the peptide whose number is given below the abscissa. The absorbance values for the same serum when reacted with uncoated wells and for preimmune guinea pig or rabbit serum when reacted with the same peptide have been subtracted. The peptides are 20 residues long, overlap by 5 residues, and contain the entire amino acid sequence of the L1 (35 peptides) (A) and L2 (31 peptides) (B) ORFs of HPV 16. Peptides 1 and 36 represent the amino termini of the L1 and L2 proteins, respectively, and each consecutive peptide is positioned 15 amino acids closer to the carboxy terminus, which corresponds to peptide 35 for the L1 protein and peptide 66 for the L2 protein. Peptides 65 and 66 are 21 residues long and thus differ in their positions by 16 amino acids instead.

Four of 25 guinea pig anti-PV antisera reacted with peptide 14 (Fig. 1A). Seven of 25 guinea pig antisera reacted with peptide 30, and 6 reacted with peptide 31 (Fig. 1A). The reactive sera were from guinea pigs immunized with BPV, CPV, or APV (either untreated or disrupted by SDS, freezing-thawing, or carbonate buffer). The rabbit serum to BPV disrupted by freezing-thawing also reacted with peptide 31 (Fig. 1A). Eight of 25 guinea pig antisera reacted with peptide 33 (Fig. 1A). These sera were from guinea pigs immunized with either BPV or CPV disrupted by SDS, freezing-thawing, or carbonate buffer. The L2 protein was much less reactive, with only single reactivities being found (Fig. 1B).

Characterization of epitopes with peptide analogs. To define the epitopes within the most reactive peptides, we synthesized truncated peptide analogs of the original peptide sequences. The first shortened peptide (a) contained the first 15 (1 to 15) residues from the original 20-residue peptide, the second (b) contained the first 10 (1 to 10) residues, the third (c) contained the last 15 residues (5 to 20), and the fourth (d) contained the last 10 residues (10 to 20). Dako serum reactivity to peptide 16 was slightly reduced with shortened peptide 16a (titers, 1:4,000 versus 1:2,000) but was not detectable with the other shortened peptides (Fig. 2A). None of our 26 antisera to BPV, CPV, or APV reacted with peptide 16 or any of the analogs. The previously described Dako serum-reactive 28-residue peptide (20) from the L1 ORF of HPV 6b and the corresponding peptide from the L1 ORF of HPV 16 were also synthesized. Since these peptides overlap our peptides 15, 16, and 17, they are here designated 15–17:HPV 6 and 15–17:HPV 16. Both of these peptides were reactive with the Dako serum at high titers (>1:10,000) (Fig. 2A). Only 1 of our 26 antisera against BPV, CPV, or APV reacted with the 15–17:HPV 6 peptide, and 2 reacted with the 15–17:HPV 16 peptide (Fig. 2A).

The Dako serum was equally reactive with peptide 30b and with peptide 30 (titer, 1:240,000). It was somewhat less reactive with peptides 30a (titer, 1:120,000) and 30c (titer, 1:28,000) and did not react with peptide 30d (Fig. 2B). For the Dako serum, the minimal epitope in this region thus appears to be contained within amino acids DTYRF (Fig. 2B). Reactivity in 9 of our 26 anti-PV antisera was detected

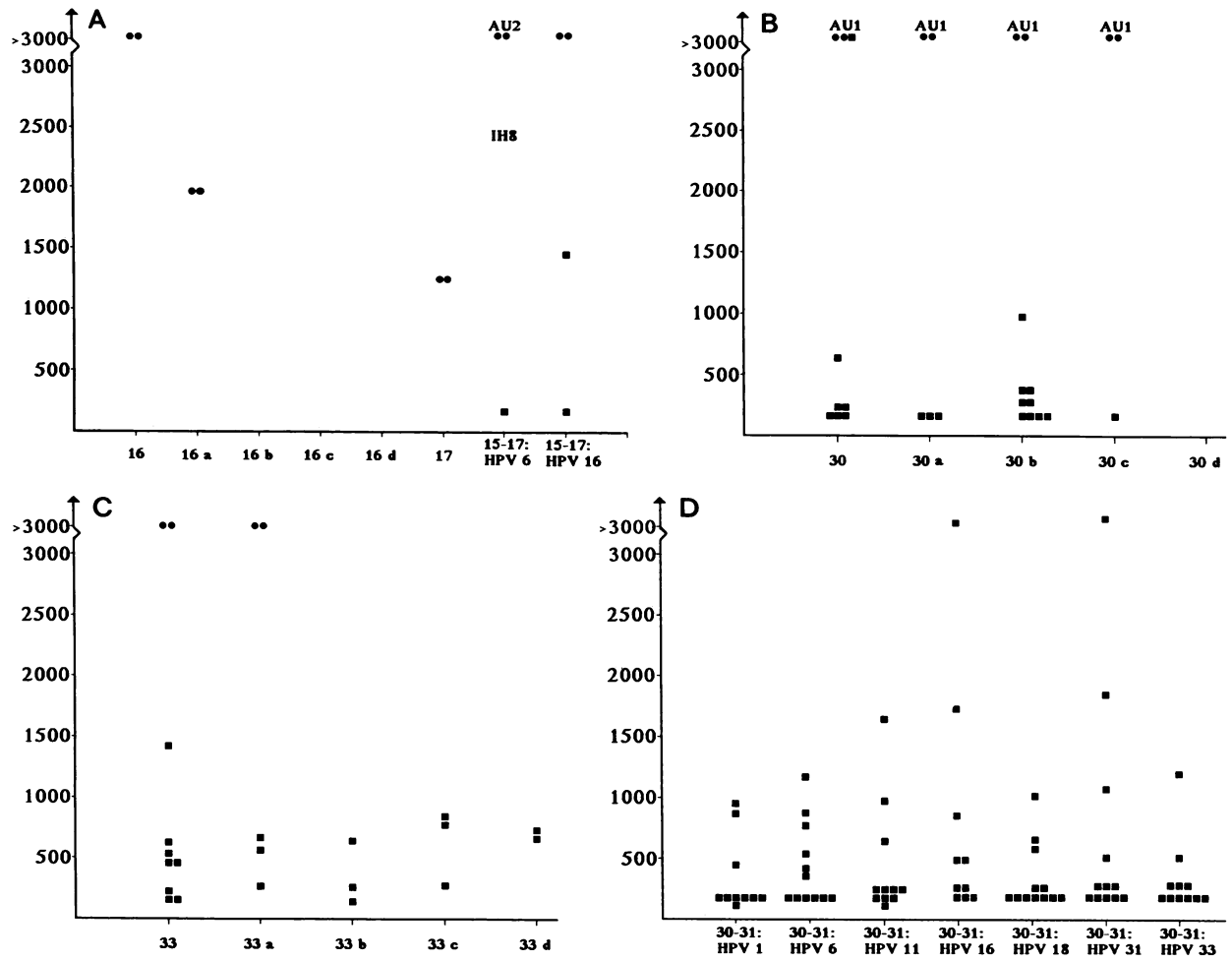

FIG. 2. ELISA immunoreactivity to cross-reactive HPV 16 L1 peptides and some of their truncated analogs in sera from 25 guinea pigs immunized with BPV, CPV, or APV and a rabbit serum to BPV (■), the Dako serum to BPV (●), and seven monoclonal antibodies to BPV, notably antibodies AU1, AU2, and IH8. The same ELISA and the same sera as those used in Fig. 1 were used here. (A) The analogs of peptide 16 (VHTGFGAMDFTTLQANKSEV), peptides 16a to 16d, had the sequences VHTGFGAMDFTTLQA, VHTGFGAMDF, GAMDFTTLQANKSEV, and TTLQANKSEV, respectively. The previously described Dako serum-reactive peptide from HPV 6b (DGDMVD TGFGAMNFADLQTNKSDVPIDI) (20) (here designated 15–17:HPV 6) and the corresponding peptide from HPV 16 (DGDMVH TGFGAMDFTTLQANKSEVPLDI) (15–17:HPV 16) were also tested. (B) The analogs of peptide 30 (GGTLEDTYRFVTSQAIACQKH) had the sequences GGTLEDTYRFVTSQAI (30a), GGTLEDTYRF (30b), DTYRFVTSQAIACQKH (30c), and VTSQAIACQKH (30d). (C) For peptide 33 (SADLDQFPLGRKFLLQAGLK), the analogs had the sequences SADLDQFPLGRKFLL (33a), SADLDQFPLG (33b), QFPLGRKFLLQAGLK (33c), and RKFLLQAGLK (33d). (D) Immunoreactivity to a previously described HPV 6b type-specific peptide (QSQAITCQKPTPEKEKPDPYK [30–31:HPV 6]) and the corresponding peptides from HPV 1, 11, 16, 18, 31, and 33 (LGSSLAAKCPEQ APPEPQTDPY [30–31:HPV 1], QSQAITCQKPTPEKEKQDPYK [30–31:HPV 11], TSQAIACQKHTPPAPKEDPLK [30–31:HPV 16], QS VAITCQKDAAPAENKDPYD [30–31:HPV 18], TSQAITCQKTAPQKPKEDPFK [30–31:HPV 31], and TSQAITCQKDAAPAENKDPYD [30–31:HPV 33]).

with peptide 30b; and 3 of these 26 antisera also reacted with peptide 30a, and 1 reacted with peptide 30c (Fig. 2B).

A rabbit serum to frozen-thawed BPV was reactive with peptide 31 (ACQKHTPPAPKEDPLKKYT), peptide 31a (ACQKHTPPAPKEDP), and peptide 31b (ACQKHTPPAP) but not with peptide 31c (TPPAPKEDPLKKYT) or peptide 31d (KEDPLKKYT) (data not shown). One guinea pig antiserum reacted with all four peptide 31 analogs, and one antiserum reacted with peptide 31b (data not shown).

The Dako serum reacted with peptide 33a and peptide 33 at similar titers (1:45,000 versus 1:49,000) but not at all with the other shortened peptide 33 analogs (Fig. 2C). Three of the eight guinea pig antisera that reacted with peptide 33 also reacted with peptides 33a, 33b, and 33c, and two reacted with peptide 33d (Fig. 2C).

The previously described HPV 6b type-specific epitope (20) from the L1 carboxy terminus was synthesized together with the analogous peptides from HPV 1, 11, 16, 18, 31, and 33. These peptides overlap with our peptides 30 and 31 and are referred to here as peptides 30–31:HPV 1, 30–31:HPV 6, and so on. The Dako serum did not react with any of these peptides (Fig. 2D). Ten of our samples of guinea pig sera reacted with peptides 30–31:HPV 1 and 30–31:HPV 16, 11 reacted with peptides 30–31:HPV 11 and 30–31:HPV 33, and 12 reacted with peptides 30–31:HPV 6, 30–31:HPV 18, and

TABLE 1. L1 epitopes cross-reactive between HPV 16 and BPV, CPV, or APV

Peptide	Epitope contained within the sequence	Reactivity with 26 samples of sera to BPV, CPV, or APV (no. of sera reactive at an OD titer of >100)	Reactivity with Dako serum (titer)[a]	Reactivity with anti-BPV MAbs[b] (titer)	Further definition of epitope
Internal region					
14	KGSPCTNVAVNPGDCPPLEL	4	0		Not attempted
15–17:HPV 6	FGAAN[c]	0	NK[d]	AU2 (>100,000) IH8 (2,400)	N residue critical
16a	VHTGFGAMDFTTLQA	0	4,100		Not possible to shorten peptide
17	NKSEVPLDICTSICKYPDYI	0	1,200		Not attempted
Carboxy-terminal region					
30b	DTYRF	9	>100,000	AU1 (>100,000)	Neighboring sequences increase affinity
31b	ACQKHTPPAP	3	0	Some critical residues within ACQKH	
30–31	VTSQAIACQKHTPPAPKEDLPL	10	0		Reactivity to corresponding peptides from other HPV types (see Fig. 2D)
33a	SADLDQFPLGRKFLL	3	49,000		Not possible to shorten peptide
33b	SADLDQFPLG	3	0		
33d	RKFLLQAGLK	2	0		

[a] Mean titer for two batches of Dako serum. NK, not known.
[b] MAbs, monoclonal antibodies. Obtained from A. B. Jenson, Georgetown University, Washington, D.C. AU3, AU4, AU5, and AU6 were not reactive.
[c] BPV sequence. Not present in HPV 16.
[d] Dako serum was probably reactive, since the titer to peptide 15–17:HPV 6 exceeded the titer to peptide 15–17:HPV 16 by more than fourfold.

30–31:HPV 31 (Fig. 2D). None of 37 prebleed sera were reactive.

Reactivity of monoclonal anti-BPV antibodies. All of the HPV 16 L1 and L2 peptides were also tested for reactivity with seven monoclonal antibodies to BPV (6, 26, 30). The AU1 monoclonal antibody reacted with peptide 30 at high titers (>1:100,000) (Fig. 1A and 2B). AU1 also reacted with shortened peptides 30a to 30c (titers, >1:100,000, 1:6,000, and 1:8,500, respectively) but not with peptide 30d, resembling the reactivity of the Dako serum with these peptides (Fig. 2B). The IH8 and AU2 monoclonal antibodies reacted with peptide 15–17:HPV 6 at titers of 1:2,400 and >1:100,000, respectively (Fig. 2A) but were not at all reactive with the corresponding peptide from HPV 16 (15–17:HPV 16) or with any of the HPV 16 peptides (Fig. 1 and 2). The epitope for IH8 and AU2 has been mapped to the sequence FGAAN (26). The N residue is probably important for reactivity, since it is present in the 15–17:HPV 6 peptide but not in the 15–17:HPV 16 peptide. Monoclonal antibodies AU3, AU4, AU5, and AU6 were not reactive with any of our peptides. The results of the epitope mapping are summarized in Table 1.

Influence of treatment on epitope exposure. We treated our purified BPV by boiling it in 10% SDS, by incubating it overnight with carbonate buffer (pH 10), or by freezing-thawing it in multiple cycles, in comparison with untreated virus. The virus preparations thus treated and in PBS were coated onto ELISA plates and tested for reactivities with monoclonal antibodies which were raised against BPV and which were known to react only with disrupted BPV (6, 26, 30). Guinea pig sera against the major group-specific epitopes were also tested. The reactivities of these antibodies indicated that disruption with 10% SDS and disruption with carbonate buffer were equally effective in disrupting the virions and exposing internal epitopes (Table 2). Freeze-thaw treatment seemed less effective (Table 2). Low-titer reactivities of the monoclonal antibodies against untreated virions were also seen, probably because some partially disrupted virions were present in the untreated virus preparations as well.

Since the high-pH carbonate buffer used for disrupting BPV has also been used to coat native PV onto ELISA plates (37), we also investigated the effect of coating untreated BPV onto ELISA plates with carbonate buffer. Coating in carbonate buffer (i.e., diluting untreated virus in high-pH carbonate buffer and immediately applying it to ELISA plates) was also an effective method of disruption (Table 2).

TABLE 2. Influence of treatment on epitope exposure

Treatment of purified virions	Result obtained with the following buffer used for coating of virions onto ELISA plates[a]:	
	PBS	Carbonate buffer
None (untreated)	0.093	0.642
10% SDS	1.108	ND
Carbonate buffer (pH 10)	1.179	ND
Freezing-thawing	0.248	ND

[a] Purified BPV virions were left untreated or treated by being boiled in 10% SDS, by overnight incubation with carbonate buffer (pH 10), or by five cycles of freezing-thawing. The virus preparations in PBS or in carbonate buffer (pH 9.6) were coated onto ELISA plates and tested in an ELISA with an antipeptide serum to one of the major cross-reactive epitopes, peptide 30b. Values are ODs after subtraction of the ODs for the blank and for the prebleed sera. Testing with monoclonal antibodies to BPV (IH8, AU1, AU3, AU4, and AU5) and with the antipeptide serum to peptide 16a yielded similar results. ND, not done.

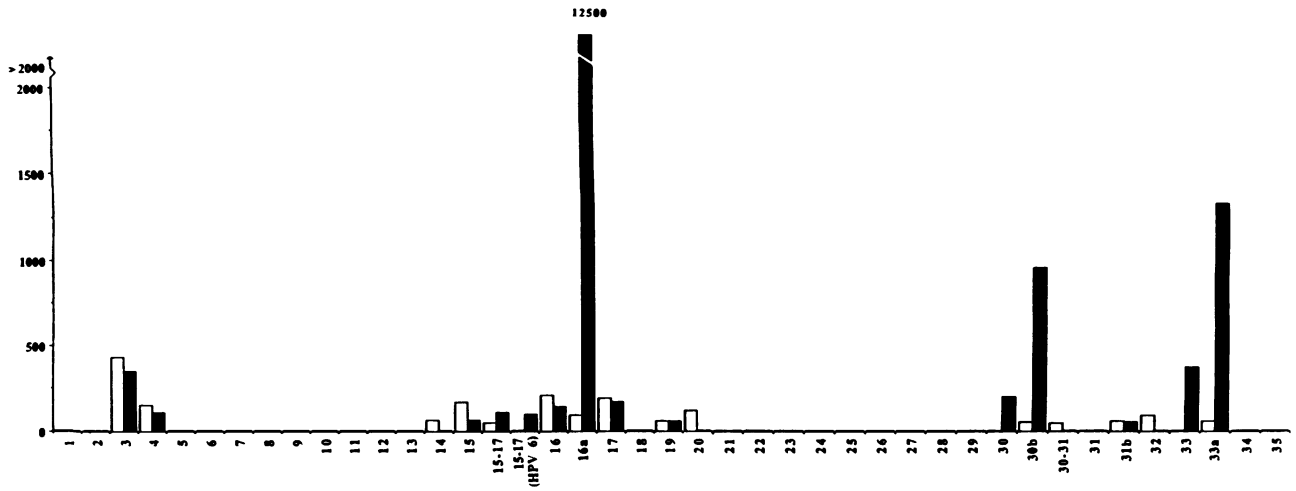

FIG. 3. Group-specific antipeptide antisera. Forty-two samples of guinea pig antisera against synthetic peptides derived from the L1 ORF of HPV 16 were tested in an ELISA with untreated BPV coated in PBS (open bars) or with disrupted BPV (untreated BPV coated in carbonate buffer; filled bars). Numbers below the abscissa are the designations of the immunizing synthetic peptides (see the text for an explanation of the designations). The numbers on the ordinate are OD titers in the ELISA. The figure "12,500" above the bar for peptide 16a denotes the OD titer of this serum.

Anti-BPV reactivity of antibodies to HPV 16 peptides. Guinea pig antipeptide sera against the entire set of overlapping HPV 16 L1 peptides as well as against peptides 15–17:HPV 16, 15–17:HPV 6, 16a, 30b, 30–31:HPV 16, 31b, and 33a were tested for reactivity in ELISA with untreated BPV coated in PBS or with disrupted BPV (untreated BPV coated in carbonate buffer). The highest titer was found for the antiserum to 15-residue peptide 16a, which had a titer of 1:12,500 against disrupted BPV (Fig. 3). The antisera against 10-residue peptide 30b and against 15-residue peptide 33a were also reactive with disrupted BPV, at titers of 1:900 and 1:1,200, respectively (Fig. 3). Interestingly, the reactivities of antisera against the longer, 20- or 28-residue peptides, which also contained these group-specific epitopes (peptides 15–17:HPV 16, 16, 30, and 33) were much lower; e.g., the antiserum to peptide 15–17:HPV 16 had an anti-BPV titer of 1:120, 2 orders of magnitude lower than that of the antiserum to peptide 16a, although these sera had similar titers against the immunizing peptide.

A few antisera (e.g., against peptides 3, 4, 15, 16, and 17) were more reactive with untreated virions than with disrupted virions (Fig. 3). These reactivities were rather low; the most reactive antiserum, the serum against peptide 3, had a titer of 1:450.

Immunohistocytochemistry. To investigate whether the broadly cross-reactive antiserum against peptide 16a was useful for immunohistocytochemistry, we also tested this serum in immunoperoxidase staining of formalin-fixed, paraffin-embedded human skin warts and CIN lesions. Preimmune serum from the same guinea pig did not produce any staining (Fig. 4A), whereas the antiserum against peptide 16a produced strong nuclear staining, primarily localized to the upper layer of the epithelium (Fig. 4B) but not seen in histologically normal areas adjacent to the lesions. The antiserum could be diluted to 1:20,000 without a significant reduction in the strength of the staining. The commercially available anti-BPV serum (Dako) produced typical nuclear staining in the upper layer of the epithelium (Fig. 4C). The staining showed a gradual reduction in sensitivity at dilutions from 1:1,000 to 1:16,000 (Fig. 4C). At a 1:1,000 dilution, the staining of the Dako serum resembled the staining of the antiserum to peptide 16a at a 1:20,000 dilution (data not shown). We also performed immunoperoxidase staining with the antiserum to peptide 16a at a 1:8,000 dilution in the presence of 1 mg of the immunizing peptide per ml. The staining could be completely blocked by the addition of free peptide. Four human skin warts and 12 human CIN lesions were tested, and the antiserum to peptide 16a was found to stain all 4 skin warts and 4 of 12 CIN lesions, whereas the Dako serum stained the 4 skin warts but only 2 of 12 CIN lesions (data not shown).

DISCUSSION

Synthetic peptides that can mimic antigenic sites present in the native protein are now used for the diagnosis of many viral infections (15, 27, 31). The first reported synthetic peptide of HPV 16 that was reactive with human sera was derived from the E2 ORF (9). Several additional HPV 16 peptides that were reactive with human sera were identified by the synthesis of overlapping synthetic peptides representing the entire coding capacity of HPV 16 (8, 10). In this study, these overlapping HPV 16 L1 and L2 peptides were used to map antigenic and immunogenic sites cross-reactive between HPV, BPV, CPV, and APV. Although we only tested for epitopes cross-reactive between HPV 16 and BPV, CPV, and APV, HPV 16 and BPV are among the least related in the PV group (1), and it is therefore likely that the broadly cross-reactive epitopes described here are cross-reactive among most viruses of the PV group.

We found the group-specific epitopes to be clustered in internal and carboxy-terminal regions of L1. Within these two antigenic regions there were several closely spaced, sometimes overlapping epitopes. At least nine different epitopes were identified; two of them (15–17:HPV 6 and DTYRF) have also been reported to be broadly cross-reactive by others (20, 26). A peptide that has been reported to be a type-specific epitope (20) (our peptide 30–31) was found by us to contain the most broadly cross-reactive region of all, apparently shared by HPV 1, 6, 11, 16, 18, 31,

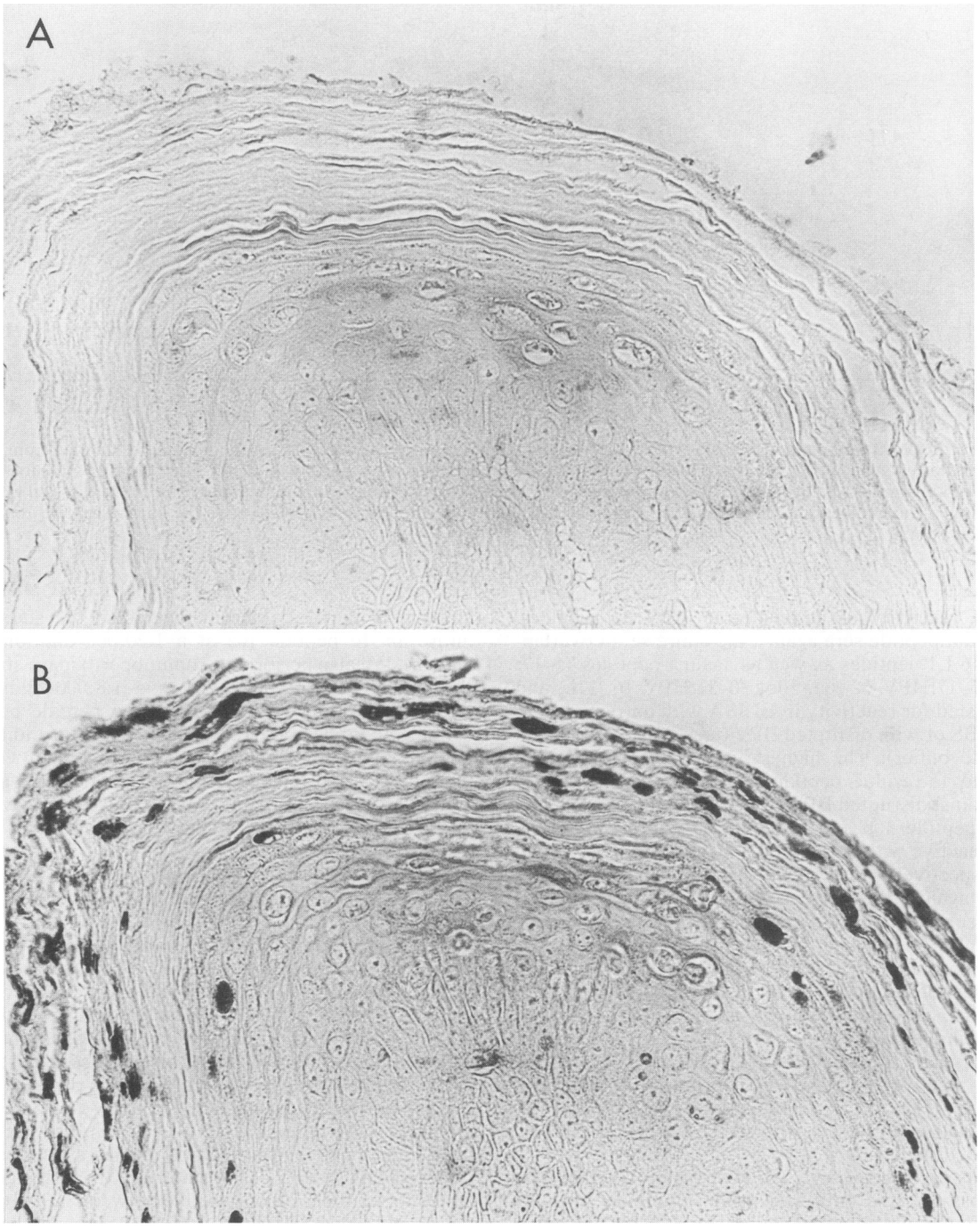

FIG. 4. Immunohistocytochemical staining of a human cutaneous wart (peroxidase-antiperoxidase method). Sections (4 μm) were stained with a guinea pig preimmune serum, diluted 1:16,000 (A); a guinea pig antiserum to peptide 16a (VHTGFGAMDFTTLQA), diluted 1:16,000 (B); and the commercially available anti-BPV serum (Dako), diluted 1:16,000 (C).

and 33 as well as by BPV, CPV, and APV. Although our conclusion is at variance with the conclusion of Jenison et al. (20), we confirmed their results; the single anti-BPV serum (Dako) tested in their study was not reactive with this peptide.

Two of the broadly cross-reactive epitopes (16a and 33a) could not be shortened to less than 15 amino acids without losing reactivity with the Dako serum, even though the stretch of critical residues in linear epitopes is usually only 4 to 8 amino acids (16). There is evidence that even short peptides can assume some three-dimensional structure (13, 14), and it is possible that the amino acids surrounding the critical epitope are essential for the conformation of the epitope. Alternatively, the peptide might form a small loop bringing noncontinuous critical residues of the epitope into proximity. For one of the major cross-reactive epitopes,

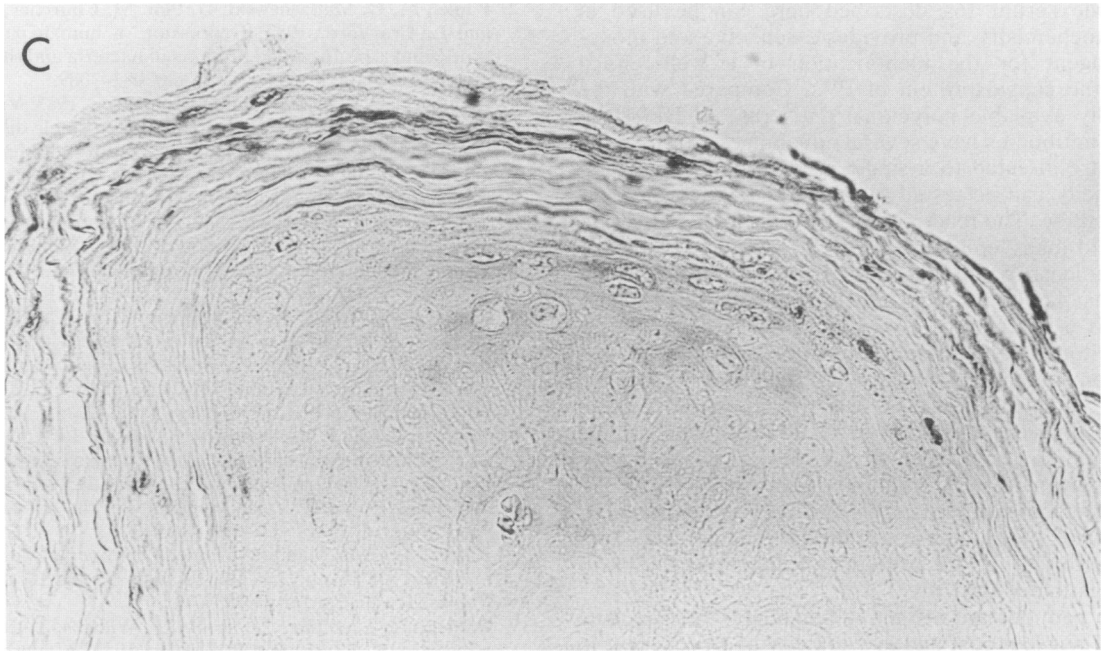

FIG. 4—*Continued.*

shortened peptide 30b, the antigenic reactivity was similar to that of the original peptide, 30. However, there were decreases in the titers of the Dako serum and of our anti-BPV, -CPV, or -APV sera to the other analogs of peptide 30 (30a and 30c), even though peptide 30b is contained within peptide 30a. The likely explanation is that the epitope in peptides 30 and 30b is exposed better or assumes a more favorable conformation than in peptides 30a and 30c. In contrast, anti-BPV monoclonal antibody AU1 had much higher titers against peptides 30 and 30a than against peptides 30b and 30c, implying that the epitope recognized by AU1 (although also contained within DTYRF) was not completely identical to the epitope recognized by the Dako serum. Also, in this case it is possible that the surrounding amino acids in peptides 30 and 30a might have influenced the affinity of the antibody by affecting the conformation of the DTYRF epitope. It is also conceivable that they might have had a weak direct interaction with the antibody.

The fact that a synthetic peptide reacts with antibodies from animals immunized with the whole protein does not necessarily imply that immunization with this peptide will induce an antibody response to the protein. For instance, peptide 30–31 from the carboxy terminus of L1 was the most reactive peptide with our sera to BPV, CPV, and APV in ELISA. However, when these peptides were used for immunization, the resulting antipeptide sera did not react with purified BPV in ELISA. Similarly, previous attempts to immunize with synthetic peptides containing group-specific epitopes (e.g., peptide 15–17:HPV 6) or fusion proteins to produce a group-specific serum were not successful (5). We also found that immunization with peptide 15–17:HPV 6 or with the corresponding peptide, 15–17:HPV 16, did not induce any significant group-specific reactivity. However, immunization with a 15-residue truncated analog (peptide 16a), contained within peptide 15–17:HPV 16, resulted in an antipeptide serum reactive at high titers with BPV. A likely explanation for why only the shorter peptide works as a broadly cross-reactive immunogen is that the longer peptides may also expose other, immunodominant immunogenic sites that are not group specific. The same phenomenon was seen for peptides 30 and 33; shortened peptides 30b and 33a were better immunogens for inducing group-specific antibodies than were their longer counterparts. It thus seems that the optimization of synthetic immunogens frequently requires the removal of residues that are not contained in the immunogenic epitope.

The mapping of broadly cross-reactive PV epitopes with synthetic peptides serves several purposes. (i) Peptides derived from the group-specific immunogenic sites could be useful in the serological diagnosis of infection with PVs. (ii) The peptides could be used as immunogens to produce antibodies against the PV capsid that might be useful in immunohistocytochemistry and in other immunological assays for the detection of infection with viruses in the PV group. (iii) The mapping and characterization of these epitopes could be useful for the understanding of the immunobiology of PV infection and could provide information useful for the engineering of PV vaccines.

Both serum and cervical secretions from CIN patients contain antibodies, mainly of the IgA class, that cross-react with BPV (11, 12). We also reported that the set of HPV 16 L1 peptides used in this report contained several peptides that were regularly reactive with IgA antibodies in the sera of patients with HPV 16-carrying cervical carcinomas (10). It is therefore most interesting that two of the main L1 peptides reactive with human IgA antibodies, peptides 16 and 31, were now also shown to contain broadly cross-reactive epitopes. It is possible that these immunogenic sites could be exploited for peptide-based group-specific PV serology. However, the broadly cross-reactive antibodies resulting from hyperimmunization of experimental animals with purified PV are not necessarily directed against exactly the same epitopes within these two peptides as are the antibodies from humans with in vivo PV infections.

Antipeptide serum 16a described here can be used in immunohistochemistry and provides a sensitive and monospecific reagent for the identification of a well-defined epitope in the capsid protein of PVs. Compared with the commercially available polyclonal antiserum to BPV, the antipeptide antibodies have several advantages. Antipeptide antibodies are directed to a single predetermined site, and their specificity can be tested by blocking of the reactivity with free peptide. The reactivity of the polyclonal antiserum is directed to many epitopes; the Dako serum is known to react with at least 15 epitopes in the BPV L1 protein (26), of which at least 4 epitopes have now been found to be broadly cross-reactive. Furthermore, we found that the response to different epitopes is highly variable among polyclonal anti-PV sera from different animals. The improved sensitivity, consistency, and specificity of our broadly cross-reactive anti-peptide antisera are likely to facilitate the search for previously unknown PV types in various tissues and may also be important for the immunological diagnosis of PV infections. Detection of virus capsid proteins by immunological assays is of special interest in the PV system, since it is not possible to test for the presence of infectious PV by in vitro propagation of the virus.

Synthetic peptides are attractive candidates for vaccines because of their low cost and low toxicity and because it is possible to engineer the exact chemical composition of the vaccine in detail. The study of broadly cross-reactive epitopes is important in the PV system, since the number of different PV types is so large that type-specific PV vaccines are likely to be only of limited value. Previous data have shown that both natural infection and hyperimmunization with intact virions almost exclusively give rise to type-specific antibodies but that group-specific epitopes are exposed on disrupted virions (21, 32). The preferential reactivity with disrupted BPV of our antisera to the main cross-reactive epitopes (16a, 30b, and 33a) is in agreement with these conclusions. In view of the high degree of conservation of the major capsid proteins (46% sequence homology between BPV and HPV 16 [1]), it is highly likely that at least some conserved amino acid residues are exposed on the virion surface, e.g., residues that are functionally important for virion adherence. A likely explanation for this apparent discrepancy is that conserved residues may be exposed on the virion surface but are not immunogenic. However, by hyperimmunization with synthetic peptides it might be possible to induce antibodies against amino acid residues that are not immunogenic in the context of the whole protein. Our peptide 3 in the amino terminus of HPV 16 L1 is an example of a region to which no group-specific antibodies were raised in the context of the whole protein but which could be used to generate antipeptide antibodies also reactive with intact BPV. The generation of this broadly cross-reactive response, although of a low titer, suggests that the design of peptide-based broadly cross-reactive PV vaccines may ultimately be possible.

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