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Neutralization of Bovine Papillomavirus by Antibodies to L1 and L2 Capsid Proteins

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We have generated four mouse monoclonal antibodies (MAbs) to bovine papillomavirus virions that bound type-specific, adjacent, and conformationally dependent epitopes on the L1 major capsid protein. All four MAbs were neutralizing at ratios of 1 MAb molecule per 5 to 25 L1 molecules, but only three effectively blocked binding of the virus to the cell surface. Therefore, antibodies can prevent papillomavirus infection by at least two mechanisms: inhibition of cell surface receptor binding and a subsequent step in the infectious pathway. The neutralizing epitopes of the bovine papillomavirus L2 minor capsid protein were mapped to the N-terminal half of L2 by blocking the neutralizing activity of full-length L2 antiserum with bacterially expressed peptides of L2. In addition, rabbit antiserum raised against amino acids 45 to 173 of L2 had a neutralizing titer of 1,000, confirming that at least part of the N terminus of L2 is exposed on the virion surface.

Bovine papillomavirus type 1 (BPV) has been used as a model system for studying the immune response to papillomaviruses because, in contrast to human papillomaviruses (HPVs), the infectivity of BPV may be measured by a quantitative in vitro assay (8). Immunization of rabbits with authentic BPV virions or with in vitro-generated BPV viruslike particles (VLPs) composed of the L1 major capsid protein alone or of L1 plus the L2 minor capsid protein induces high titers of type-specific neutralizing antibodies, while immunization with denatured particles fails to do so (9, 11, 15). These observations indicate that the major neutralizing epitopes are predominantly conformational and located on L1 (11). Similar results have been reported for virions of HPV type 11 (HPV-11) and cottontail rabbit papillomavirus (CRPV), although it is unclear whether the neutralizing epitopes are located on L1, L2, or both (2). Furthermore, monoclonal antibodies (MAbs) that only recognized native, but not denatured, papillomavirus virions were strongly neutralizing (3–5). Immunization with denatured L2 can also induce low levels of neutralizing antibodies and protection from infection (10, 17).

While previous studies have described some characteristics of neutralizing antibodies, they have not addressed the mechanisms by which the antibodies may prevent infectivity, the number of antibody molecules that must bind to a virion to neutralize its infectivity, or the genetic localization of neutralizing epitopes. For this report, we have used the BPV system to initiate an analysis of these issues.

Generation and characterization of BPV L1 MAbs. Using two different methods, we generated a total of four MAbs from BALB/c mice that had been immunized twice at 14-day intervals with 50 μg of purified BPV virions. To generate MAb 5B6, mice were sacrificed 33 days after the initial immunization, their splenocytes were fused to Ag8.653 myeloma cells, and hybridomas were hypoxanthine-aminopterin-thymidine selected by conventional techniques (13). The hybridomas were screened by using an enzyme-linked immunosorbent assay (ELISA) for BPV L1/L2 VLPs, prepared as previously described (12).

To generate MAbs 3, 6, and 9, mice were inoculated with 10^5 focus-forming units (FFU) of helper-free ABL-MYC recombinant retrovirus 4 days after the second immunization (18, 19). Plasmacytomas developed as malignant ascites 30 to 45 days after infection. Ascites were withdrawn twice, and the plasmacytoma cells were harvested by centrifugation (5 min, $600 \times g$) and viably frozen. Approximately 10^6 cells from each plasmacytoma were transplanted intraperitoneally into pristane-primed, unimmunized BALB/c mice. Tumors developed in 20 to 35 days, ascites fluids were drawn, and the plasmacytoma cells were collected for Southern blot analysis to determine the number of κ light-chain rearrangements by probing with a κ light-chain probe. The MAbs present in ascites containing only one κ light-chain rearrangement, i.e., monoclonal tumors, were isotyped. MAb 3 was determined to be an IgM, 6 and 9 were IgG2a, and 5B6 was an IgG2b. Immunoglobulin G (IgG) antibodies were purified by protein A-Sepharose chromatography, and IgM antibody was purified by maltose-binding protein affinity chromatography (Pierce), using the protocols recommended by the supplier.

The nature of the epitopes recognized by the MAbs was investigated by using ELISAs with various baculovirus-expressed papillomavirus VLP preparations as antigens with 300 ng per well as previously described (2). All of the MAbs reacted equally strongly with intact BPV L1 and L1/L2 VLPs (prepared as previously described [12]) but did not bind denatured BPV L1/L2, indicating that each MAb bound a conformational epitope on L1. None of the MAbs recognized L1/L2 VLPs of HPV-16, HPV-6b, CRPV (12), rhesus papillomavirus type 1, or BPV-4 (unpublished data) in this assay, indicating that all the MAbs recognize type-specific epitopes (data not shown). These results are consistent with evidence from earlier studies of BPV, CRPV, and HPV-11 indicating that the immunodominant neutralizing epitopes are contained within type-specific conformational determinants on the virion

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(2, 3, 5), and previous analysis of BPV L1 VLPs that localized these epitopes to L1 (11).

A competition ELISA was developed to investigate the spatial relationships of the epitopes recognized by the MAbs. Each MAb was purified and biotinylated with sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce), and binding was detected using streptavidin peroxidase (Boehringer Mannheim) and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] substrate. BPV L1/L2 VLPs bound to the plates were preincubated (1 h at ambient temperature) with increasing concentrations of each nonbiotinylated MAb (up to 100 μg/ml) or polyclonal antiserum (up to 1:3 dilution), followed by a biotinylated MAb and streptavidin peroxidase. Rabbit polyclonal antisera to BPV L1 VLPs, but not control sera, blocked binding of all four MAbs to L1, and nonbiotinylated 5B6 competed with all MAbs for binding to L1 (not shown). These results suggest that there is a cluster of immunodominant neutralizing epitopes on L1.

In a similar assay, BPV L1 VLPs on a microtiter plate were preincubated with increasing concentrations of 5B6 prior to binding polyclonal antiserum raised against either BPV virions or L1 VLPs. Bound polyclonal antibodies were detected by using peroxidase-linked anti-rabbit IgG antiserum (Boehringer Mannheim) that did not recognize 5B6. At saturating amounts of 5B6 for virion binding, only 10 to 20% of the total VLP binding activity of either polyclonal serum was inhibited (data not shown). These results suggest that, although the mouse MAbs appear to recognize the same or adjacent epitopes, additional epitopes are recognized by the rabbit polyclonal antisera.

BPV neutralizing and cell surface binding inhibition activity of the MAbs. Various amounts of purified MAb were preincubated for 1 h with 60 ng of infectious BPV virions (200 to 300 FFU) prior to plating onto monolayers of C127 mouse fibroblasts (8). The cells were maintained for 3 weeks, and the reduction in number of foci in the presence of each MAb was scored (Fig. 1A). For negative controls, we confirmed that MAbs AU-1 and 1H8, which were generated previously to denatured BPV L1 and have been reported to lack neutralizing activity (7), also lacked neutralizing activity in our assays (data not shown). By contrast, all four of the MAbs were neutralizing with titers greater than 10^4. The ratios of MAb molecules to L1 molecules required to obtain a 50% reduction in the number of foci were 1:25 for 5B6, 1:12 for MAb 3, 1:5 for MAb 6, and 1:10 for MAb 9. Assuming that each virion contains 360 L1 molecules (i.e., 72 L1 pentamers per virion), that all virions are in the native conformation, and that all antibody molecules bind L1 protein, these results correspond to averages of 14, 30, 72, and 36 molecules of MAbs 5B6, 3, 6, and 9, respectively, per virion.

The effect of these MAbs upon binding of radiolabeled BPV virions to C127 monolayers was also examined to determine whether neutralization correlated with inhibition of cell surface binding by the virions. For the binding assays, 10 μg of BPV virions was radiolabeled with 500 μCi of diiodo-Bolton and Hunter reagent (~4,000 Ci/mmol) and dialyzed extensively in phosphate-buffered saline (PBS) (1). The iodinated BPV (~60 ng per sample, ~10^6 cpm/μg of L1) was incubated with the antibodies for 1 h in 1 ml of medium containing 10% fetal calf serum at ambient temperature. The samples were added to confluent monolayers of C127 cells in 48-well plates and incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. The cells were washed twice with PBS and harvested in 0.1 M NaOH–1% sodium dodecyl sulfate (SDS), and the amount of cell-associated radiolabel was determined with a Beckman γ-counter (17a). Binding in the presence and binding in the absence of 0.1% (wt/vol) NaN_3 were similar, and

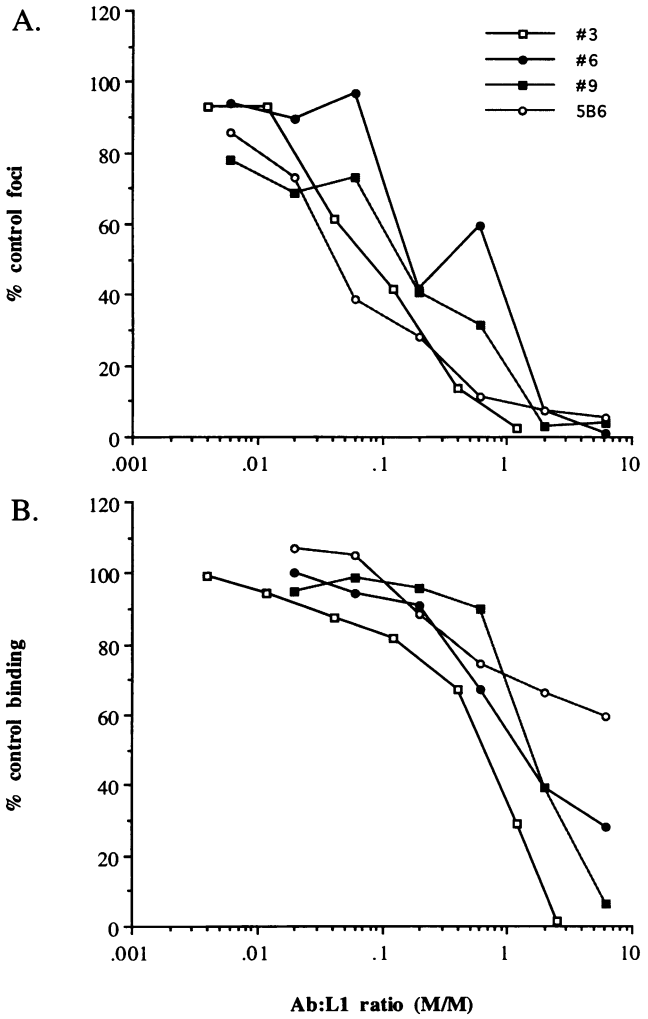

FIG. 1. Titration of the effect of the MAbs on BPV infectivity and binding to C127 cells. (A) BPV virions were incubated with MAbs at the ratio of MAb to L1 molecules indicated for 1 h at room temperature prior to plating onto C127 cells. The C127 cells were cultured for 3 weeks and then stained, and the foci were counted and calculated as a percentage of control infectivity obtained in the absence of antibody. (B) Radiolabeled BPV virions were incubated with MAbs at the ratio of MAb to L1 molecules indicated for 1 h at room temperature. The mixture was incubated with C127 cells for 4 h at 37°C. The cells were washed with PBS, and the cell-associated radiolabel was counted. The specific binding was calculated as a percentage of the difference in the cell-associated counts in the presence of a control serum and the counts bound in the presence of an excess of neutralizing serum.

specific binding was measured as the difference in cell-associated counts in the presence of a control polyclonal serum and the counts in the presence of excess neutralizing serum (17a). Binding of radiolabeled BPV virions to C127 cells was inhibited by MAbs 3, 6, and 9 at ~2 MAbs per L1 molecule (Fig. 1B). However, even at a ratio of 5 MAbs per L1 molecule, MAb 5B6 inhibited binding only by 40%, although this antibody efficiently neutralized BPV infectivity at a ratio of 0.1 MAb per L1 molecule. This poor inhibition of cell surface binding was surprising since the ability of 5B6 to compete for binding of the other MAbs to VLPs in the competition ELISA

TABLE 1. Expression of BPV L2 and six overlapping peptides of L2 as 6His fusion proteins in *E. coli*[a]

Construct	Amino acids of BPV L2	Oligonucleotides	Apparent molecular mass (kDa)
Whole L2	1–469	4186–4201 5592–5567	78
Peptide A	1–88	4186–4201 4449–4427	16
Peptide B	45–173	4318–4338 4704–4684	24
Peptide C	130–257	4573–4595 4956–4936	23
Peptide D	216–340	4831–4850 5205–5185	21
Peptide E	300–425	5083–5104 5460–5441	27
Peptide F	384–469	5335–5355 5592–5567	20

[a] Overlapping regions of L2 and full-length L2 were amplified by PCR from *Bam*HI-cut and religated BPV-pML DNA. The oligonucleotides used for these PCRs contained a GC clamp, a *Bgl*II site followed by the nucleotides indicated (numbering taken from the BPV sequence; the 5′ oligonucleotide sequence is described first). The fusion proteins were induced with IPTG, affinity purified with Ni^{2+}-NTA agarose, separated by SDS-polyacrylamide gel electrophoresis, and Coomassie stained, and their molecular masses were determined by comparison to standards.

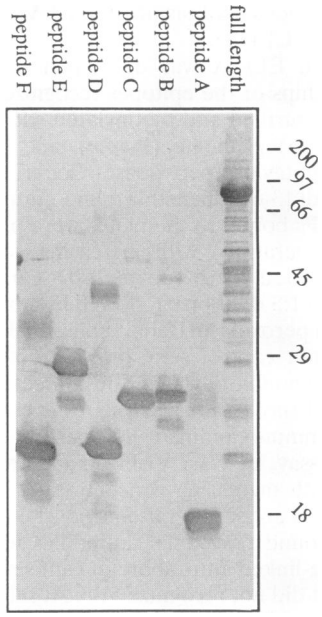

FIG. 2. Expression of BPV L2 and six overlapping peptides of L2 as 6His fusion proteins in *E. coli*. The affinity-purified fusion proteins (Table 1, footnote *a*) were separated on an SDS–15% polyacrylamide gel, Western blotted (immunoblotted), and probed with rabbit antisera to glutathione *S*-transferase–BPV L2.

strongly suggests that it binds to an overlapping or adjacent epitope on the virion.

The ability of MAb 5B6 to compete for binding of the other MAbs to VLPs, as demonstrated above, argues that low affinity is not responsible for its weak inhibition of virion binding to the C127 cells. To demonstrate by a complementary approach that 5B6 recognizes BPV, cryoelectron microscopy of BPV L1 VLPs was carried out in the presence or absence of excess 5B6. The results showed that the VLP surface was coated with antibody molecules that generally obscured the pentameric subunit structure seen in control particles, supporting the conclusion that this antibody binds efficiently to BPV L1 (data not shown).

The findings described for MAb 5B6 indicate that for this MAb inhibition of cell binding is not required for its neutralizing activity. In addition, quantitative comparison of the other three MAbs for their capacity to neutralize infectivity and inhibit binding indicated that Ab:L1 ratios that were sufficient for effective neutralization by each MAb were lower than those required for its inhibition of cell surface binding (compare Fig. 1A and B). These observations imply that the inhibition of virion binding to the cell surface cannot fully account for the neutralizing activity of any of the L1 MAbs and that one or more steps not measured by the cell surface binding appear to be more sensitive to the activity of the neutralizing antibodies.

Identification of linear neutralizing epitopes on BPV L2. Since none of the MAbs generated against BPV virions recognized L2, we used a different approach to study neutralization by antibodies to this viral protein. As noted earlier, antisera generated to denatured L2 have been shown to have low levels of neutralizing activity. Therefore the full-length BPV *L2* gene was amplified by PCR amplification using the primers listed in Table 1 and was cloned into the *Bam*HI site of pQE-12 (Qiagen) that encodes a C-terminal six-histidine tag (L2-6His). Following transfer of the recombinant plasmid into *Escherichia coli* HB101, expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 37°C, and the recombinant L2-6His protein was solubilized in 6 M guanidine-HCl and purified on an Ni^{2+}-nitrilotriacetic acid (NTA) agarose column under denaturing conditions according to the manufacturer's instructions (Qiagen). An antiserum was generated to L2-6His by injecting 330 μg of the purified protein into New Zealand White rabbits three times at 2-week intervals, using complete Freund's adjuvant for the initial inoculation and incomplete Freund's adjuvant for the booster injections. This serum was neutralizing for BPV infection of C127 cells with a titer of 33. Antiserum generated to an *E. coli* fusion protein of glutathione *S*-transferase and BPV L2 was also neutralizing, demonstrating that the neutralizing epitopes are not present in the 6His tag (data not shown) (12, 16).

In an effort to determine the region(s) of L2 that contains the neutralizing epitopes, six overlapping peptides of L2, which together contained all residues of the full-length protein, were expressed with C-terminal 6His tags in *E. coli* and affinity purified as described above (Table 1). All of the peptides were recognized by rabbit antiserum to glutathione *S*-transferase–L2 in a Western blot (immunoblot), and their migration rates were similar to those expected (Table 1 and Fig. 2).

To analyze the ability of the peptides to competitively bind the L2 neutralizing serum, 10 μg of each L2-6His peptide was incubated with 100 μl of anti-full-length L2 serum for 1 h and then with 60 ng of BPV virions for 1 h, and the mixture was added to a monolayer of C127 cells. The cells were cultured for 3 weeks, and the number of foci was scored. As expected, the antiserum to full-length L2 neutralized infection, and infectivity was restored by preincubation of this antiserum with the full-length L2-6His protein. However, preincubation of L2 antiserum with any single L2 peptide failed to effectively block neutralization (Table 2). Therefore, various combinations of L2 peptides were preincubated with anti-full-length L2 sera. Pairs of the N-terminal three peptides restored some infectivity. However, infectivity was only completely restored by preincubation of the anti-L2 sera with a combination of all three N-terminal peptides (A+B+C). None of the peptides

TABLE 2. Localization of the neutralizing epitopes within BPV L2[a]

BPV/serum/peptide[b]	No. of foci	% Control
−/−/−	0	0
+/−/−	324	100
+/−/buffer	330	102
+/prebleed/−	290	90
+/anti-L1 VLPs/−	0	0
+/anti-L2/buffer	14	4
+/anti-L2/whole L2	259	80
+/−/A+B+C	292	90
+/−/D+E+F	312	96
−/−/A+B+C	0	0
−/−/D+E+F	0	0
+/anti-L2/A	34	10
+/anti-L2/B	62	19
+/anti-L2/C	61	19
+/anti-L2/D	18	6
+/anti-L2/E	26	8
+/anti-L2/F	12	4
+/anti-L2/A+B	148	46
+/anti-L2/A+C	154	48
+/anti-L2/A+D	58	18
+/anti-L2/A+E	48	15
+/anti-L2/A+F	22	7
+/anti-L2/B+C	93	29
+/anti-L2/B+D	99	31
+/anti-L2/B+E	76	23
+/anti-L2/B+F	45	14
+/anti-L2/C+D	89	27
+/anti-L2/C+E	96	30
+/anti-L2/C+F	69	21
+/anti-L2/D+E	21	6
+/anti-L2/D+F	22	7
+/anti-L2/E+F	31	10
+/anti-L2/A+B+C	314	97
+/anti-L2/B+C+D	149	46
+/anti-L2/C+D+E	79	24
+/anti-L2/D+E+F	30	9

[a] Antiserum to full-length BPV L2 was incubated for 1 h with 10 μg of the affinity-purified peptides indicated, mixed with BPV virions (~300 FFU) for an hour, and plated onto C127 cells. The cells were cultured for 3 weeks, and then stained, and the numbers of foci were determined.

[b] +, presence of element; −, absence of element.

had any effect upon infectivity in the absence of antibody, nor did they generate foci without the virus (Table 2).

The findings described above suggested that regions in the N terminus of L2 may be exposed on the surface of BPV and contain linear neutralizing epitopes. To obtain additional evidence, antisera to each of the three purified L2 peptides A, B, and C were generated as described for the full-length L2. When the reactivity of the antisera and prebleeds was examined by ELISA, all three sera, but not the prebleeds, reacted with both native and denatured BPV L1/L2 VLPs but not with BPV L1 VLPs (Table 3). However, quantitative differences were noted in the ELISA titers of the sera.

When the neutralizing properties of the sera were studied, the antisera raised against peptides A and C were only weakly neutralizing, with titers of 3 and 10, respectively (Table 3). However, antiserum to peptide B, which had the highest ELISA titers, exhibited a neutralizing titer in excess of 1,000. Furthermore, antibody from this serum, which had been affinity purified on an L2 (amino acids 45 to 174)-6His column, was also neutralizing (titer of >200).

Although the C-terminal portion of CRPV L2 has been reported to contain neutralizing epitopes (6, 14), our results strongly suggest that the major neutralizing epitopes of BPV

TABLE 3. Analysis of polyclonal antiserum to full-length L2 and peptides of L2[a]

Antiserum to L2	Amino acids of L2	Titer[b]			
		BPV L1 VLPs	BPV L1/L2 VLPs	Denatured BPV L1/L2	Neutralizing
Full length	1–468	<100	1,000	10,000	33
Peptide A	1–88	<100	100	1,000	3
Peptide B	45–173	<100	100,000	100,000	1,000
Peptide C	130–257	<100	1,000	10,000	10

[a] Rabbit antisera were raised against 1 mg of affinity-purified full-length L2 and peptides of L2 fused to a 6His tag.

[b] Titers of each polyclonal antiserum were determined in an ELISA against the following VLP preparations: BPV L1 VLPs, BPV L1/L2 VLPs, or BPV L1/L2 VLPs that were disrupted by high pH in the presence of dithiothreitol (denatured BPV L1/L2). Neutralization of BPV was assessed by mixing 200 to 300 FFU of the virus with 3.3-fold dilutions of each antiserum for 1 h prior to plating onto C127 cells. Foci of transformed C127 cells were counted after a 3-week incubation.

L2 are located within the N-terminal half of L2. These observations imply that at least some of the N-terminal region of BPV L2 is on the virion surface. In addition, immunization with the amino acid 45 to 173 peptide induced a much higher neutralizing antibody titer than the full-length L2 protein (1,000 versus 33, respectively). This finding raises the possibility that a small L2 peptide might have a greater potential for inducing immunity to papillomavirus infection than would the full-length L2.

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