Humoral Immune Response Recognizes a Complex Set of Epitopes on Human Papillomavirus Type 6 L1 Capsomers†

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Although epitope mapping has identified residues on the human papillomavirus (HPV) major capsid protein (L1) that are important for binding mouse monoclonal antibodies, epitopes recognized by human antibodies are not known. To map epitopes on HPV type 6 (HPV6) L1, surface-exposed loops were mutated to the corresponding sequence of HPV11 L1. HPV6 L1 capsomers had one to six regions mutated, including the BC, DE, EF, FG, and HI loops and the 139 C-terminal residues. After verifying proper conformation, hybrid capsomers were used in enzyme-linked immunosorbent assays with 36 HPV6-seropositive sera from women enrolled in a study of incident HPV infection. Twelve sera were HPV6 specific, while the remainder reacted with both HPV6 and HPV11 L1. By preadsorption studies, 6/11 of these sera were shown to be cross-reactive. Among the HPV6-specific sera there was no immunodominant epitope recognized by all sera. Six of the 12 sera recognized epitopes that contained residues from combinations of the BC, DE, and FG loops, one serum recognized an epitope that consisted partially of the C-terminal arm, and three sera recognized complex epitopes to which reactivity was eliminated by switching all five loops. Reactivity in two sera was not eliminated even with all six regions swapped. The patterns of epitope recognition did not change over time in women whose sera were examined 9 years after their first-seropositive visit.

Human papillomavirus (HPV) infection of the genital tract is one of the most common sexually transmitted diseases (6). From 50 to 75% of sexually active individuals will be infected by genital HPVs in their lifetime (14). HPV infects the epithelium and causes aberrant cellular proliferation. This can potentially lead to benign genital warts, as seen with the low-risk HPV type 6 (HPV6) and HPV11, or to cervical cancer, seen with high-risk HPV types 16 and 18. Given that cervical cancer is still a leading cause of cancer deaths for women worldwide, eliminating genital HPV infections would have a significant public health impact.

Although HPVs cannot be easily cultured because infectious virus production is linked to epithelial cell differentiation, virus-like particles (VLPs) can be purified from the expression of the major capsid protein (L1) in eukaryotic cells (18, 25, 28, 31, 41). The major capsid protein self-assembles into a T = 7 icosahedral VLP composed of 72 L1 pentamers (capsomers). VLPs are structurally and immunologically similar to infectious virus as gauged by electron microscopic imaging studies, and their ability to bind type-specific, conformation-dependent monoclonal antibodies (MAbs). Consequently, experimental vaccines have tested the efficacy of immunizing with VLPs in animal models of papillomaviruses (2, 29, 45) and in humans (19, 32).

Type-specific, conformation-dependent antibodies made in response to VLP vaccination do indeed protect animals against infectious viral challenge (27, 29, 45) and neutralize virus in *in vitro* assays (27). Protection against infection has been attributed to the humoral immune response since passive transfer of serum from immunized animals to untreated animals protects the recipient against infectious viral challenge (2). Immunizing with capsomers also protects against infectious viral challenge, since capsomers have been shown to contain the epitopes found on VLPs that are recognized by neutralizing monoclonal antibodies (MAbs) (42, 54). A clinical trial of an HPV16 VLP-based vaccine was shown to be 100% effective in protecting women from persistent HPV16 infection and pathology (32). Another recent clinical trial of bivalent VLP vaccine also showed impressive efficacy in protecting against infection and associated pathology from HPV16 and HPV18 (19).

Despite the ongoing vaccine trials, little is known about the epitopes on the virus or VLPs that are recognized in response to natural infection or following vaccination. Initial epitope mapping used type-specific MAbs to define regions of L1 critical for MAb binding. Some studies suggest the existence of type-specific immunodominant epitopes. Residues 131 to 132 of HPV11 L1 confer type specificity (34) and are thought to be immunodominant as these residues had to be altered to further uncover additional HPV11 L1 regions critical for binding MAbs (35, 36). Similar studies with HPV6 L1 also support the existence of an immunodominant epitope, as altering HPV6 L1 residues 49 and 54 obliterates binding of the majority of HPV6 L1 type-specific MAbs (37, 48). Yet it is not known if residues critical for binding MAbs are also the regions recog-

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nized by human antibodies, or whether the human antibody response also targets a single immunodominant epitope.

One study concluded that human antibodies recognized a single immunodominant epitope because incubating a type-specific mouse MAb with HPV16 VLPs prevented binding of human antibodies on HPV16 VLPs (49). However, the role steric interference has in preventing additional antibody binding was not explored in that study. Another study has shown that type-specific human reactivity could be redirected from HPV16 to HPV11 by substitution of the C-terminal 334 amino acids (47). However, most of the reactivity to the C-terminal portion did not appear to be directed to the epitope recognized by the MAb described in the previous study. Thus, the epitope(s) important for human antibody recognition of HPV capsids remains poorly defined.

The recently solved crystal structure of a small T = 1 HPV16 VLP (7) showed that the dimensions of the exposed surface of the capsomer can be spanned by an antibody F(ab)₂ fragment that can potentially block access to other antibodies. Molecular modeling of other HPVs showed that residues deemed important for binding of HPV6, HPV11, and HPV16 monoclonal antibodies comprised loops on the accessible surface of the capsomer. These regions, although not sequential in primary structure, are juxtaposed upon L1 folding into capsomers, potentially defining complex, noncontiguous conformation-dependent epitopes. An atomic model of the full-size HPV VLP, made using cryo-electron microscopic data of bovine papillomavirus VLPs and the coordinates from HPV16 L1, suggested that the C terminus of L1 is surface exposed in intercapsomeric connections (38). This potentially implicates the C terminus as an immunogenic target, as recently supported by MAb epitope mapping (5).

The goal of this work was to define regions of the viral capsid recognized by human antibodies. This is the first study to attempt to finely map immunodominant epitopes on HPV capsids using human sera in a systematic manner. We focused on HPV6b and HPV11 L1 because these proteins are 92% identical at the amino acid residue level (11, 44) yet are differentiated by type-specific MAbs (8, 9) and polyclonal sera from rabbits immunized with HPV6 or HPV11 VLPs (17) and condyloma patients (23). In the study of polyclonal rabbit sera, HPV6/HPV11 cross-reactivity was detected, but it was much weaker than the type-specific response. In this study, we finely map the targets of the human humoral immune response to individual loops on an HPV viral capsomer as opposed to previous studies that have mapped epitopes to large portions of L1. Though it has been thought that there is an immunodominant epitope on the viral capsid, these results argue against an immunodominant epitope hypothesis, as sera from different individuals targeted different loops of L1. Clearly these studies have favorable implications for strategies being designed for the implementation of VLP vaccination programs, since these results suggest that it is less likely that vaccination efforts will provide immune selection and spread of variant viruses.

MATERIALS AND METHODS

Production of capsomers with mutagenized loops. To increase capsomer yields from bacterial cultures, two cysteines (171 and 423) in HPV6 L1 in pET19b were mutated to serines, to generate HPV6 L1M (43). The forward primers used for

the cysteine to serine alteration were Cys171Ser, GG GGT AAA GGT AAA CAG **AGT ACT** AAT ACA CCT G, and Cys423Ser, G CAG TCA CAG GCC ATT **ACT AGT** CAA AAG CCC ACT CC. This double cysteine HPV6 L1M was used as the backbone to mutate various loops. All mutations substituted HPV11 sequence for the corresponding HPV6 sequence to create hybrid HPV6 L1M with various loops mutated. HPV6 L1M:FG (HPV6 L1M with loop FG [residues 263 to 289] mutated to the HPV11 L1 sequence) was cloned into pET19b from a baculovirus clone received from Steve Ludmerer (Merck Research Laboratories, West Point, PA). The L1 gene was PCR amplified from baculovirus DNA, subcloned into pGEM-T, and then excised and ligated into pET19b. Sequencing found two amino acid residues (272 and 273) had the HPV6 L1 sequence. These two amino acid residues were mutagenized to the corresponding HPV11 L1 sequence. This clone also had cysteines 171 and 423 mutated to serines for improved capsomer yield.

Starting with HPV6 L1M or HPV6 L1M:FG in pET19b, clones with one to five loops mutated to the HPV11 L1 sequence were sequentially built one region at a time following the QuikChange mutagenesis strategy (Stratagene, La Jolla, CA). The loops altered were loop BC (residues 49 to 54), loop DE (residues 131 to 133), loop EF (residues 169 to 179), and loop HI (residues 345 to 348). (see Table S1 in the supplemental material for the primers used). The loop FG sequence was derived from a clone provided by Steve Ludmerer, Merck Research Labs. To make hybrid capsomers with the C-terminal swaps, HPV6 L1 clones were mutagenized with forward primer 6Ct, AAT TCT GAT TAT AAA GAG TAC ATG CGT **CAC GTG** GAA GAG T, while HPV11 clones used forward primer 11Ct, AAT TCA GAT TAT AAG GAA TAC ATG CGC **CAC GTG** GAG GAG T, to introduce PmlI restriction sites (bold base pairs are silent restriction sites). This facilitated digestion of constructs to generate fragments which could be swapped and ligated to construct the C-terminal hybrids. Following mutagenesis, the entire L1 open reading frame was verified by sequencing in three overlapping segments.

The method used for capsomer preparation was a modification of the method described by Li et al. (33). *E. coli* DE3 cells were transformed with mutated L1 genes in pET19b. From a single colony, an overnight culture in LB with ampicillin (100 µg/ml) was grown at 37°C and then used to inoculate 50 ml of fresh LB/ampicillin (100 µg/ml). When 50-ml cultures reached an optical density (OD) of approximately 0.5 the culture was used to inoculate 2 liters. Before inoculating the larger volumes, each culture was softly pelleted to remove potentially secreted β-lactamases and resuspended in fresh medium to inoculate the larger volume culture. At an OD of 0.6, 2 liters of bacterial cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) and grown overnight at 30°C. Bacterial cultures were then pelleted and frozen at -20°C. Bacterial pellets were thawed in 200 ml of buffer A (50 mM Tris-HCl, pH 7.9, 5% glycerol, 2 mM EDTA, 15 mM β-mercaptoethanol, 250 mM NaCl) containing protease inhibitors (Complete, Roche, Indianapolis, IN). Lysozyme was then added (final concentration, 200 µg/ml) and incubated on ice for 20 min. Triton X-100 was added (final concentration, 0.05%) before sonicating three to six times for 45 seconds per burst at 1-minute intervals.

The sonicated suspension was then homogenized with a Dounce homogenizer (B pestle, 20 strokes). The homogenate was centrifuged at 12,000 × g for 20 min, after which the supernatant was subjected to precipitation with ammonium sulfate to 35% saturation. The pellet was resuspended in 100 ml of buffer B (10 mM Tris-HCl, pH 7.9, 5% glycerol, 2 mM EDTA, 15 mM β-mercaptoethanol, 1 M NaCl) with a Dounce homogenizer as above. The homogenate was again centrifuged at 10,000 × g for 15 min and the supernatant was precipitated overnight with ammonium sulfate (35% saturation). The ammonium sulfate precipitation was centrifuged at 12,000 × g for 20 min, and the pellet was resuspended in 20 ml of buffer C (10 mM Tris-HCl, pH 7.2, 5% glycerol, 2 mM EDTA, 15 mM β-mercaptoethanol, 100 mM NaCl) and dialyzed overnight at 4°C against the same buffer C. The suspension was centrifuged at 10,000 × g for 20 min and the supernatant was subjected to affinity chromatography for L1 purification. The supernatant was applied to a DE-52 cellulose column, and the flowthrough was loaded onto a P11 phosphocellulose column (both from Whatman). The P11 column was successively washed with buffer C with 0.1 M NaCl and then buffer C with 0.5 M NaCl. L1 was eluted with buffer C with 1 M NaCl. L1-containing fractions were stored at -20°C until use. Capsomer purification steps were followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis with Coomassie blue staining and immunoblotting for L1.

Assessing mutagenized capsomer folding by trypsin digestion. A trypsin digest previously used to assess viral capsid folding was followed with some modifications (5, 26, 33). Briefly, capsomers were diluted into digestion buffer (10 mM Tris-HCl, 2 mM EDTA, 15 mM 2-β-mercaptoethanol, 100 mM NaCl, 100 mM NaHCO₃, pH 7.8) to a final concentration of 30 µg/ml. Dithiothreitol (10 mM

final concentration) was added to capsomers to be trypsinized and then incubated at 37°C for 20 min. Sequencing-grade trypsin (Roche, Indianapolis, IN) was solubilized in 1 mM HCl to 1 µg/ml and serially diluted by thirds; 1 µl of the various concentrations was then added to 15 µl of L1 capsomer aliquots and digested at 37°C for 30 min before stopping the reaction with the addition of phenylmethylsulfonyl fluoride (to 1 mM) from a 100 mM stock. Samples were analyzed on 10% polyacrylamide gels and transferred to nitrocellulose for immunoblotting with the CAMVIR-1 MAb for L1 detection.

Assessing mutagenized capsomer folding by ELISAs with MAbs. Mutagenized capsomers were diluted in cold phosphate-buffered saline (PBS) to a concentration of 3.5 µg/ml and plated in triplicate (50 µl/well) onto Immulon 2 HB flat-bottomed microtiter plates (Thermo Labsystems, Beverly, MA). Capsomers were allowed to bind for 1 h at room temperature on a rotating shaker, after which they were washed with PBS; 120 µl blocker solution (5% goat serum, 0.05% Tween in PBS) was added per well, incubated at room temperature for 1 h, and washed as before.

HPV6- and HPV11-specific MAbs characterized previously (9, 48) were diluted 1:5,000 in blocker solution, and 50 µl was added per well to the plated capsomers and allowed to bind for 1 hour at 37°C. Plates were washed, and anti-mouse immunoglobulin G-alkaline phosphatase (Roche) diluted 1:3,000 in blocker solution was added and incubated for 1 hour at 37°C before washing and addition of 100 µl/well of developer [43.3 mg of Sigma 104 alkaline phosphatase substrate (*p*-nitrophenyl phosphate disodium hexahydrate) (Sigma Chemical, St. Louis, MO) per 10 ml of substrate buffer (100 mM NaHCO₃, 10 mM MgCl₂ · 7H₂O, pH 9.5)]. Enzyme-linked immunosorbent assay (ELISAs) performed with MAbs were read after 30 min incubation, and OD readings for empty (no-antigen) wells were subtracted from OD readings of test wells. Antibody binding and substrate steps were performed on a rotating shaker incubator at 37°C to minimize diffusion limitation effects.

ELISAs with human sera. The human sera used in this study came from a cohort of university women enrolled in a study of the natural history of genital HPV infections (52). HPV6-seropositive samples identified previously by capsid or capture ELISAs were used (4). Capture MAbs H6.N8 and H11.A3 were used in combination at a dilution of 1:10,000 each in carbonate buffer (0.1 M NaHCO₃, pH 9.5), and 50 µl per well was applied to Immulon 2 HB plates and incubated for 1 hour at room temperature. After washing with PBS and blocking with 120 µl block solution (5% goat serum and 0.05% Tween-20 in PBS) per well for 1 hour at room temperature, 50 µl of test capsomers diluted to 3.5 µg/ml were added to plate wells in triplicate to be used as antigen, and incubated at 37°C for 1 hour. Plates were then washed before the addition of 50 µl of sera (diluted 1:100 in block solution) and incubated for 1 hour at 37°C in a rotating incubator. Plates were washed again and then incubated with 100 µl of alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G (Roche) diluted to 1:3,000 in block solution for 1 hour at 37°C in a rotating incubator. After the final wash, plates were incubated with 100 µl of developer per well, and OD readings for empty (no-antigen) wells were subtracted from OD readings of test wells.

Preadsorption assays. Capsomers were extensively dialyzed against PBS in 4°C prior to coupling to agarose beads. Coupling reaction to Amino Link Coupling Gel (Pierce Biotechnology, Rockford, IL) generally followed the manufacturer's protocol. Briefly, capsomers with or without altered loops were diluted in PBS to 200 µg/ml in 2 volumes of gel resin bed volume. The manufacturer's protocols for coupling at pH 7.2 in PBS were followed overnight at 4°C in rotating tubes. Agarose beads were used within 3 days to avoid storage with sodium azide.

For preadsorption studies, bead coupled to capsomers were blocked twice with block solution in rotating tubes for 30 min at room temperature to ensure proper nonspecific blocking of excess beaded capsomers. They were washed with cold PBS, and resuspended in block solution in 2 resin volumes. Five ml of diluted serum (1:100 in block solution) was incubated with 1 ml of resin volume (or 2 ml of 50/50 resin/block solution) for each plate tested. Samples were preadsorbed for 1 hour at room temperature in rotating tubes before centrifuging to pull down bound antibodies on beads.

Supernatants were tested for residual binding activity in direct ELISAs. Direct ELISA plates had 50 µl of antigen (diluted in cold PBS to 3.5 µg/ml) plated directly onto Immulon 2 HB plates, incubated for 1 hour, washed with PBS before blocking for 1 hour, and washed again before the addition of preadsorption supernatants. Supernatants were tested in triplicate wells against each test antigen. Subsequent ELISA steps were the same as described above.

RESULTS

HPV6 L1 capsomers with mutagenized loops. To assess the role of the loops and C-terminal region of HPV6 L1 in binding

human antibodies, capsomers were generated in which one to five loops on the HPV6 L1 backbone, with or without the C terminus, were mutagenized to the corresponding amino acid residues of HPV11 L1. The FG loop had the most amino acid changes (nine) and extended over 28 residues, while the DE loop changed one residue and inserted another. In addition, capsomers (HPV6:Ctrm and HPV11:Ctrm) in which the last 139 residues were swapped between HPV6 L1 and HPV11 L1, were generated to probe the immunogenicity of the C-terminal portion of L1 (Fig. 1A).

Molecular modeling of the loops using the crystal structure solved for the HPV16 L1 capsomer showed that these regions were on the surface of the capsomer, accessible to circulating antibodies. More importantly, the structure showed that upon folding into capsomers, loops not contiguous in primary sequence became juxtapositioned to potentially combine into complex epitopes (Fig. 1B).

Mutagenized L1 clones were expressed in bacteria and purified by affinity chromatography to yield capsomers. The major band on final elution corresponded to the 55 kDa of L1 as seen by Coomassie staining (Fig. 1C), and Western blot analysis probing for L1 with CAMVIR-1 confirmed the purification of L1 (Fig. 1D). Thus, capsomers with altered loops were efficiently produced in bacterial cultures.

To ascertain that capsomers with mutagenized loops were properly folded after purification from bacterial cultures, the capsomers were assayed by trypsin digestion (5, 26, 33). Properly folded capsomers have only peripheral trypsin cleavage sites exposed, yielding L1 fragments of 42 kDa. Improperly folded capsomers normally expose inaccessible trypsin sites and are completely proteolyzed. Exposure to trypsin resulted in reduction to a 42-kDa trypsin-resistant fragment for the HPV6 and HPV11 wild-type L1 capsomers (Fig. 2A), whereas a negative control (16:F50L, a construct of HPV16 L1 known not to fold properly because of a phenylalanine to leucine substitution) (5) showed trypsin sensitivity. Additionally, all of the loop substitutions or C-terminal alterations yielded capsomers that were trypsin resistant (Fig. 2A).

Another method to verify that the bacterially derived capsomers folded properly was to test the ability of type-specific, conformation-dependent monoclonal antibodies to bind the hybrid capsomers. Direct plating of capsomers and assaying by ELISA with HPV6- and HPV11-specific MAbs showed that all of the capsomers were able to bind efficiently to either H6.N8 or H11.A3 (Fig. 2B). Only capsomers that had HPV11 L1 amino acids 131 to 132 in loop DE were able to bind H11.B2, as predicted (34). Similarly, only capsomers that had HPV6 L1 loops BC and EF in particular were able to bind MAb H6.M48, as expected (37, 48). These results also showed that H11.A3 targeted a bipartite epitope, involving residues in loops BC and EF, as previously reported (37).

Furthermore, other residues may be implicated in stabilizing the epitope, as the presence of these two loops yielded less MAb binding than the wild-type HPV11 L1 capsomers. MAb H6.N8 was shown to be partially dependent on residue 53 of loop BC, but loop EF was not required for binding, agreeing with earlier observations (48). Loops FG and DE contributed to binding only when loop BC was altered, as capsomers with loops FG and DE substituted were able to bind H6.N8 in the context of an HPV6 L1 BC loop (Fig. 2B). The binding of

FIG. 1. Capsomers with mutagenized loops are efficiently produced and purified from bacterial cultures. Linear model of HPV6 L1 loops mutated, showing amino acid residues targeted and changed (A). These regions are color coded in the molecular model of HPV6 L1 (B), after aligning HPV6 and HPV16 L1 sequences and modeling the crystal structure of HPV16 L1. Capsomers with different loop changes can be purified from bacterial cultures, as seen in the Coomassie gel that shows the capsomer purification scheme (C) where the high-salt elution contains a relatively pure band of 55 kDa corresponding to L1 lane 1: insoluble fraction; 2: NH_4^+ supernatant; postdialysis 3: pellet and 4: supernatant; column load flowthrough for 5: DE52 and 6: P11; resin residue on 7: DE52 and 8: P11; 9: 250 mM, 10: 500 mM, and 11: 1 M (L1 fraction) elutions. Immunoblotting for L1 with Camvir (D) following the purification of L1 shows that fainter lower-molecular-weight bands seen on coomassie staining are L1 breakdown products lane 3: insoluble fraction; 4: NH_4^+ supernatant; postdialysis 5: pellet and 6: supernatant; column load flowthrough for 7: DE52 and 8: P11; resin residue on 9: DE52 and 10: P11; 11: 250 mM, 12: 500 mM, and 13: 1 M (L1 fraction) elutions.

MABs and trypsin digests of bacterially derived capsomers independently showed that capsomers with mutagenized loops were able to fold properly and appropriately present conformation-dependent epitopes.

Human sera were heterogeneous in reactivity to HPV6. The human sera tested came from a prospective cohort of university women enrolled in a study to characterize the natural history of HPV infection (52). The immunoglobulin A re-

FIG. 2. Capsomers with mutagenized loops fold properly. Capsomer preparations with different loop changes yielded a substantial fraction of the capsomer preparation resistant to trypsin because of proper folding, as evidenced by the 42-kDa L1 fragment (A). When assayed by ELISA with type-specific, conformation-dependent mouse monoclonal antibodies H11.A3, H11.B2, H6.N8, and H6.M48, as indicated, capsomers with different loop changes bound MAbs, as expected (B). Capsomer loop changes are shown on the x axis, and optical density is shown on the y axis.

FIG. 3. Overview of HPV6 and HPV11 seroreactivity of sera used. Overview of serum reactivity by capture ELISA against HPV6 L1 (solid bars) and HPV11 (open bars) for all HPV6-specific sera (A) and some sera that are reactive to both HPV6 and HPV11 (B). Optical density is on the y axis and serum samples are along the x axis.

sponse in incident HPV infection was recently characterized using a capsomer-based ELISA that showed good correlation with VLP-based ELISAs, in both direct and capture ELISA formats (39). A capture ELISA method was used because a comparison of direct versus capture capsomer plating revealed that the capture ELISA gave more HPV6 L1-specific responses with less HPV11 L1 cross-reactivity (data not shown).

Thirty-six women from the cohort study were identified as being HPV6 seropositive and HPV6 DNA positive, albeit not necessarily free of concomitant infection with other HPV types. By capture ELISA, 12 women were found to have HPV6-specific antibodies, defined as binding HPV11 L1 capsomers to levels less than one-third that of wild-type HPV6 L1 (Fig. 3A), while the remaining HPV6-seropositive samples also reacted with HPV11 L1 to various extents (Fig. 3B). Analysis of HPV DNA status showed that a higher percentage of serum samples that were HPV6 L1 specific were from individuals infected with HPV6 DNA only, while 6/11 seropositive samples came from women that were more likely to be infected with multiple HPV types. However, given the small sample size, these associations did not reach statistical significance (data not shown).

All 12 HPV6-specific sera were tested by capture ELISA against various capsomers with different loops mutagenized and scored as either positive (if the serum bound that particular capsomer with given loop alterations to levels within 1 standard deviation of wild-type HPV6 L1 binding levels) or negative (if antibody binding levels for that test capsomer were one-eighth or less that of HPV6 L1 levels) or as partial if antibody binding levels were intermediate.

Of the 12 HPV6-specific samples, four individuals had sera for which epitopes could be defined by binding to capsomers with different loops mutagenized. The targeted epitopes differed among these individuals. In the simplest case, one serum lost antibody binding when loop DE was altered (Fig. 4A). In another sample reactivity was lost when either loop DE or FG was altered, suggesting that the epitope consisted of residues from each loop (Fig. 4B). Another serum sample required both loops BC and DE to be altered to eliminate antibody binding (Fig. 4C). In this case each single alteration resulted in a partial loss of binding, suggesting a polyclonal response, i.e., two types of antibodies, one that targeted the BC loop, and another that targeted the DE loop. A fourth serum lost reactivity when both

the DE and FG loops were lost, though the single alterations had little effect (data not shown).

A fifth individual's serum had reactivity that was eliminated when the C terminus of HPV6 L1 was replaced with the C terminus of HPV11 L1 (Fig. 4D). However, reactivity was not restored by the reciprocal swap, indicating that residues in addition to those in the C terminus of HPV6 L1 comprised the epitope. Three sera had reactivity that could only be eliminated when all five loops were eliminated, indicating a complex and likely polyclonal response to infection. An example is shown in Fig. 4E. For the remaining four women with HPV6-specific reactivity, seroreactivity was reduced to some of the mutagenized capsomers, but was not eliminated by replacing the five loops and the C terminus (data not shown), suggesting that additional type-specific residues scattered throughout L1 may contribute to type-specific seroreactivity. As expected when sera that had reactivity to both HPV6 and HPV11 were tested for binding, they showed reactivity, to greater or lesser extents, to all of the mutagenized capsomers (Fig. 4F).

Validation of epitope mapping. Because ELISA reactivity can be forced by excessive antigen or antibody, combined antigen titrations and antibody dilutions were done for a subset of five HPV6-seropositive sera. The five serum samples were selected to represent the spectrum from highly HPV6 L1-specific samples to HPV6- and HPV11-cross-reactive sera, as well as low to high OD values in the initial capture ELISA results. Four of the five serum samples were tested against all test capsomers at various antigen concentrations, from 64 $\mu\text{g/ml}$ to 0.001 $\mu\text{g/ml}$, and with serum dilutions from 1:50 to 1:200,000 on the same 96-well plate. The remaining sample was tested in the same fashion but within a narrower antigen window (8 $\mu\text{g/ml}$ to 0.125 $\mu\text{g/ml}$). These antigen titration/serum dilution studies showed that for each serum sample, all mutated capsomers had similar affinities for each serum sample based on their 50% effective concentration (EC_{50} , the antigen concentration at which 50% of maximum binding is achieved). More importantly, the EC_{50} s of all five sera were within the same order of magnitude. This validated that the initial ELISA conditions were within the linear range of reactivity, and neither capsomers nor antibodies were in excess (see Fig. S1 and Table S2 in the supplemental material).

As an additional approach to validate the ELISA results we turned to preadsorption with HPV6 or HPV11 capsomers to

FIG. 4. Defined epitopes are different for different individuals. Four of the HPV6-specific sera had epitopes defined as indicated (A to D), where altering the HPV6 L1 loop(s), shown on the x axis, diminished OD reactivities, shown on the y axis. Altering loop DE obliterates binding in serum I (A), while altering either loop DE or FG does away with binding in serum II (B). Serum IV (C) targets both loops BC and DE, while serum VI targeted an epitope involving the C terminus (D). Three sera required altering all five loops to obliterate antibody binding (E). The majority of the HPV6-seropositive sera were cross-reactive (F), binding HPV11 L1 to significant levels such that they could not be used to define epitopes.

demonstrate that the reactivity to the mutated capsomers seen with the HPV6-specific sera could be specifically competed away by preadsorption with HPV6 capsomers but not with HPV11 or HPV6-FRM:C-term (HPV6 L1 capsomers with all five loops and the C terminus altered to the corresponding HPV11 L1 sequence). Serum samples were preadsorbed with

excess capsomers coupled to beads, and the unbound antibodies in the supernatant were assayed in direct ELISA against capsomers with different loop changes. To ensure that the coupling process preserved proper capsomer structure, capsomers coupled to beads were tested with type-specific, conformation-dependent HPV6 and HPV11 MAbs. Preadsorption

FIG. 5. Preadsorption studies validated by MAbs and HPV6-specific human serum. Either HPV6 L1, HPV11 L1, HPV6-FRM, or HPV6L1:Cterm capsomers were covalently coupled to agarose beads and incubated with MAb H6 M.48 (A) or H11.B2 (B) after blocking. Unbound supernatants were subsequently reacted against HPV6 L1 (A) or HPV11 (B) capsomers in direct ELISAs, and competition of reactivity by preadsorbing measured by OD (y axis). HPV6-specific

with HPV6 L1 or 6:Cterm beads but not other bead conjugates competed away the reactivity of MAb H6.N8 to HPV6 L1 in subsequent ELISAs. Preadsorption with HPV11 L1 or HPV6 L1 capsomers with all five loops altered (6-FRM) did not significantly reduce reactivity to HPV6 L1 (Fig. 5A). Conversely, only preadsorbing with HPV11 L1- or HPV6-FRM-coupled beads competed away the reactivity of MAb H11.B2 to HPV11 L1 in subsequent ELISAs (Fig. 5B). Thus, the coupling process maintained proper folding of the capsomers. Preadsorbing HPV6-specific sera with HPV6 L1 or HPV6:Cterm capsomers coupled to beads significantly reduced binding to HPV6 L1 capsomers, whereas preadsorbing with beads coupled to HPV11 capsomers did not significantly reduce reactivity to HPV6 L1 in subsequent ELISAs (Fig. 5C), validating the ELISA results.

We did however find two sera for which the preadsorption results further clarified the ELISA results. Both of these sera had shown type-specific responses, but the epitopes could not be mapped by ELISAs. One serum had strong and type-specific binding to HPV6 L1M but much weaker and cross-reactive binding to the 6:BC+DE capsomers (Fig. 5D). This indicated that the type-specific epitope recognized by this serum included the BC and DE loops. A second serum exhibited a similar pattern of reactivity to capsomers containing the DE and FG loop substitutions (not shown). In both of these sera, binding to capsomers with other loop substitutions could not be competed away by other capsomers, indicating that the observed binding was indeed specific to those altered capsomers. Because residual binding to capsomers with those specific loop substitutions was nonspecifically competed away, it suggests that residual binding was nonspecific and hiding a targeted response to those loops. Thus, the preadsorption studies identified two other sera for which simple epitopes could be defined.

Reactivity to HPV6 and HPV11 is cross-reactive. Preadsorption of sera with capsomer-bound beads could also be applied to the sera that had reactivity to both HPV6 and HPV11 capsomers to distinguish between sera composed of cross-reactive antibodies and sera composed of antibodies type-specific to both HPV types. Preadsorption of cross-reactive sera with HPV6 or HPV11 beads should eliminate reactivity to both HPV6 and HPV11. Preadsorption of serum with specific reactivities to HPV6 and HPV11 with HPV6 beads would eliminate binding to HPV6 and not significantly affect binding to HPV11. Ten sera that reacted with both HPV6 and HPV11 were tested by preadsorption (see representative example in Fig. 6A). The similar reactivity to HPV6 and HPV11 capsomers seen on ELISA after preadsorption with either HPV6 or HPV11 cap-

sera were also used to validate covalently linked capsomers (C). The serum sample for which the epitope was elucidated via capture ELISAs was preadsorbed onto HPV6 L1, HPV11 L1, HPV6-FRM:Cterm, or blank beads and subsequently reacted against capsomers with different loop mutations (x axis) and residual antibody binding was measured by optical density (y axis). A human serum sample for which the epitope could not be defined by capture ELISAs was preadsorbed onto HPV6 L1, HPV11 L1, HPV6-FRM:Cterm, or blank beads and subsequently reacted against HPV6 L1M capsomers or HPV6:BC+DC capsomers.

FIG. 6. HPV6 and HPV11 reactivity is due to cross-reactive antibodies according to preadsorption studies. Human sera reactive to both HPV6 and HPV11 were preadsorbed onto HPV6 L1, HPV11 L1, or empty beads and subsequently reacted against HPV6 L1M or HPV11 L1M capsomers (x axis) and residual antibody binding was measured by optical density (y axis) after subtracting readings from blank wells. Equal reactivity to HPV6 and HPV11 capsomers after preadsorption to either HPV6 or HPV11 capsomer beads suggests that cross-reactivity is due to cross-reactive antibodies (A). One sample had a small amount of differential binding after preadsorption (B).

somers on beads suggests that the binding of HPV6 and HPV11 capsomers is due to cross-reactive antibodies.

All of these sera showed clear evidence of cross-reactivity. In only one case did we see that preadsorption with HPV6 beads removed reactivity to HPV6 capsomers more effectively (73.8% reduction compared to beads only, 95% confidence interval 69.6% to 78.0%) than did HPV11 beads (64.3%, 95% confidence interval 60.3% to 68.3%), and conversely that preadsorption with HPV11 beads more effectively eliminated reactivity to HPV11 (74.0%, 95% confidence interval 71.1% to 76.9%), than did preadsorption with HPV6 beads (64.4%, 95% confidence interval 60.6% to 68.6%) (Fig. 6B). Although this serum showed evidence of dual specificity most of its reactivity could be attributed to cross-reactive antibodies.

Antibody targets do not change over time. To look for evidence of changes in the specificity of antibody responses over time, serum samples from the same individual taken at different time points were tested against test capsomers with different loop changes. Sera from 25 individuals had samples available from different time points, with some individuals having samples up to 10 years after the initial seropositive sample. These sera were tested against the mutagenized capsomers, and antibody binding levels were normalized to antibody binding of HPV6 L1. Two samples from an individual whose serum

FIG. 7. HPV6 L1 targets do not change over time. Sera from individuals who had samples either 4 months apart (A and B) or 9 years apart (C) were tested against capsomers with different loop changes via capture ELISA, and optical density (y axis) was normalized to that of HPV6 L1.

had an epitope that could be defined showed no changes in capsomer binding at least within a 4-month interval (Fig. 7A). Studies with more broadly reactive serum showed that binding patterns changed little, if at all, over the course of 4 months (Fig. 7B) or over the course of 9 years (Fig. 7C). There is a slight increase in antibody binding levels in later serum samples for the two individuals shown here, but it is not consistent with all mutated capsomers and not seen with all individuals.

DISCUSSION

Phylogenetic analyses of various human papillomaviruses have shown a high degree of homology between the major capsid proteins (L1s) of different papillomaviruses (12). Despite the similarity, type-specific antibody responses have been well documented in studies of genital HPV infection (4, 24, 30) as well as animal model immunization studies (17, 50). There has been no evidence for cross-protection against related HPVs, and individuals can be infected with multiple HPV types (46). Thus, it was unexpected to find two-thirds of HPV6-

seropositive sera to be cross-reactive with HPV11 L1. Individuals with cross-reactive sera were not known to be infected with HPV11, although three individuals were included who were classified as HPV6 or HPV11 DNA positive, because early in the study not all samples were individually tested for HPV6 or HPV11 separately. Furthermore, when separate testing was done, only four individuals were found to have HPV11 DNA in the genital tract, and these were not included in the analysis. We did not have a sufficiently large number of HPV6-seropositive women to assess whether cross-reactivity was associated with infection with multiple HPV types or clinical sequelae. While these studies have shown that a large proportion of HPV6-seropositive sera can bind to HPV11 L1, it does not answer whether this binding is protective against HPV11 infection. The development of *in vitro* assays to neutralize HPV11 pseudovirions should answer this question.

These studies show that the humoral immune response against genital HPV6 infection targets a complex set of epitopes. By mutagenizing loops on HPV6 L1 to the homologous HPV11 L1 loops and assessing the hybrid capsomers for loss of antibody binding, we have shown that there is no single immunodominant epitope that is recognized by all HPV6-seropositive serum samples. Six sera had epitopes that could be defined by changing the BC, DE, and FG loops, either singly or in different combinations, and a seventh had an epitope that at least partially involved the C-terminal end. Three other sera had reactivity that was only eliminated when all five loops were swapped, suggesting very complex epitopes. More importantly, these results suggest that the antibody response against HPV is not focused on one common epitope, minimizing the likelihood that immunization programs will produce HPV L1 escape variants against which the vaccine will be ineffective.

Although there are other residues that differ between HPV6 and HPV11 L1 in addition to the six regions probed in this study, it is unlikely that these residues contribute to epitope formation because those residues are spatially isolated. Epitope mapping efforts with MAbs have shown that a stretch of residues has a bigger impact on binding of antibodies than single residues (34, 37). In fact, mutating the five divergent loops in HPV6 L1 and swapping the C-terminal 139 amino acid residues yields an L1 sequence that is almost identical to HPV11 L1 except for a few nonclustered single-residue differences between. These HPV6 L1:FRM+Ctrm capsomers have human antibody and MAb binding patterns similar to those of HPV11 L1, suggesting that the remaining unaltered residues had little if any impact on binding of antibodies. Although some HPV variants have been used to map MAb binding sites (40, 51), serologic studies have suggested that antibodies occurring in response to HPV infection are able to bind variants of the same HPV type, as inferred from the lack of differences in seroconversion among individuals infected with different variants (53). Thus, the role of additional single-amino-acid changes in altering antibody binding is expected to be minimal.

An initial study suggested the existence of a single immunodominant epitope on HPV16. In that study (49), an HPV16-specific MAb bound to HPV16 VLPs blocked subsequent binding of human antibodies, while other MAbs tested did not diminish human antibody binding for as many sera. The dimensions of an antibody F(ab)₂ fragment (161 Å from one Fab hypervariable loop to the hypervariable loop on the other Fab)

(20) easily span the outer diameter range of an individual capsomer (110 to 120 Å) (1), such that a bound antibody could sterically inhibit access to additional binding sites. Thus, depending on the target of the MAb, the antibody may be positioned in a way where the F(ab)₂ or the F_c portion sterically inhibits access by other antibodies.

A subsequent serologic study supported the existence of an immunodominant C-terminal epitope because much of the antibody reactivity targeted the C-terminal portion of L1, using HPV L1 hybrids in which 70% of the protein had been swapped (48). Although type-specific MAbs were able to recognize those HPV16/HPV11 L1 hybrids, sera from children were also found to be reactive to hybrid VLPs but not the control, wild-type VLPs, suggesting that less than optimal folding could have exposed normally buried, conserved, cross-reactive epitopes. The approach pursued in this study swapped smaller regions of L1 to map epitopes and produced properly folded hybrid capsomers.

While most vaccine studies have used VLPs as the immunogen (15, 21, 28), the VLP subunits, capsomers, have also been shown to be effective at inducing type-specific, protective antibodies (16, 42, 54). In addition, capsomer-based ELISAs were shown to correlate well with VLP-based ELISAs in both direct and capture formats (39). Capsomer-based vaccines and serologic assays would be less costly than more involved eukaryotic expression-based approaches. Additionally, it was easier to perform mutagenesis on bacterially expressed capsomers.

Having confirmed that they were properly folded by two assays, trypsin sensitivity and binding to conformation-dependent MAbs, the capsomers were suitable antigens for the ELISAs. Nonetheless, it is not possible to exclude small differences in folding between capsomers and VLPs. Of interest is the C-terminal arm, which is thought to be important for intercapsomeric junctions in VLPs. The crystal structure of T = 1 L1 particles showed that the C-terminal arms were involved in intercapsomeric junctions but not surface exposed (7), while a more recent atomic model blending cryo-electron microscopic data and the crystallographic structure of HPV16 L1 resulted in a reassessment, suggesting the C-terminal arm is surface exposed in intercapsomeric junctions, as is seen in polyomavirus capsids (38). Recently, mapping of the epitope recognized by MAb H16.U4 identified a region within the C-terminal arm, supporting the model of a surface-exposed C-terminal arm (5).

Although it is difficult to accurately assess folding of the C-terminal arm in individual capsomers, an unordered C-terminal arm in individual capsomers may have compromised epitopes present on the C terminus of L1 in VLPs. This could explain the minimal loss of antibody binding seen with the C-terminal hybrids. Though it can be argued that significant epitopes were lost by using capsomers rather than VLPs, the fact that the majority of sera were still able to bind to C-terminal hybrid capsomers suggests that the C-terminal arm is not a major target of the humoral immune response. Supporting that conclusion was the earlier study which noted that all HPV16-seropositive samples that bound to HPV16 VLPs were able to bind to HPV16 capsomers (39). Chen also examined the L1 sequence of 49 HPVs, and although there is a hot spot of variability in the C terminus, the majority of the C terminus

seems to be conserved, likely for structural reasons (7). One study characterizing HPV6 L1 variation among 17 clinical isolates showed that despite genetic variations clustering in three regions of L1, a nonsilent mutation, Glu→Gln at residue 431 of the C terminus, was the most frequently found (3). If the C terminus played a significant role in antibody binding it would be expected to have a greater degree of divergence resulting from evolutionary pressures selecting residue changes that evade the host immune response.

That the humoral immune response targets many regions on the viral capsid is not unique to HPVs. Various studies have shown that antibodies recognize various epitopes of human immunodeficiency virus, including the V2 loop, the V3 loop, the C1 region, a C1/C5 epitope, a C1/C2 epitope, and the CD4 binding domain of the surface glycoprotein gp120 (13). The lymphocytic choriomeningitis virus mouse model has shown that if the cellular immune function is intact, no lymphocytic choriomeningitis virus escape mutants are observed despite the high mutation rates of the RNA virus, suggesting there are antibodies monitoring several portions of the capsid (10). Even in the case of hepatitis B virus, where the a determinant region of hepatitis B virus surface antigen has been deemed immunodominant because of the high rate of vaccine escape mutants observed with altered sequence in this 20-amino-acid-residue stretch (55), antibodies that target multiple regions of the capsid have been postulated. Indeed, when sera from health care workers immunized with various commercially available hepatitis B virus vaccines were assayed against three different HBsAg variants, induced antibodies were able to bind heterologous HBsAg proteins, albeit at reduced levels, as seen in this study, suggesting that antibodies which recognize other regions of hepatitis B virus were present (22).

In conclusion, we have shown that individuals naturally infected with HPV6 produce antibodies against HPV6 major capsid protein L1 that target a complex set of epitopes, both type specific and cross-reactive. Although some sera recognize simple epitopes focusing on one or two loops of HPV6 L1, many individuals have antibodies that react with five surface-exposed loops and perhaps with other regions as well. These results lessen the concern for escape variants from widespread immunization. It will be important to compare the reactivity seen in naturally infected women with the response that is generated by vaccination.

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