Human papillomavirus (HPV) is the etiological agent for all cervical cancers, a significant number of other anogenital cancers, and a growing number of head and neck cancers. Two licensed vaccines offer protection against the most prevalent oncogenic types, 16 and 18, responsible for approximately 70% of cervical cancer cases worldwide and one of these also offers protection against types 6 and 11, responsible for 90% of genital warts. The vaccines are comprised of recombinantly expressed major capsid proteins that self-assemble into virus-like particles (VLPs) and prevent infection by eliciting neutralizing antibodies. Adding the other frequently identified oncogenic types 31, 33, 45, 52, and 58 to a vaccine would increase the coverage against HPV-induced cancers to approximately 90%. We describe the generation and characterization of panels of monoclonal antibodies to these five additional oncogenic HPV types, and the selection of antibody pairs that were of high affinity and type specific and recognized conformation-dependent neutralizing epitopes. Such characteristics make these antibodies useful tools for monitoring the production and potency of a prototype vaccine as well as monitoring vaccine-induced immune responses in the clinic.

MATERIALS AND METHODS

Generation of monoclonal antibodies. Mouse hybridomas were developed following traditional methods as previously described (12). Briefly, BALB/c mice (Taconic, Germantown, NY) received two intraperitoneal injections of 20 µg of highly purified HPV 31, 33, 45, 52, or 58 VLPs adsorbed on Merck aluminum adjuvant. A final boost of 20 µg VLP was administered intravenously 3 days prior to fusion. A separate fusion was performed for each VLP type. Spleens were removed from sacrificed mice, and lymphocytes were fused with mouse myeloma partner SP2/0-Ag14 (ATCC 1581) by polyethylene glycol 1500 (Roche) at a ratio of 3:1. Fused hybridomas were isolated through hypoxanthine-aminopterin-thymidine medium (Sigma, Atlanta, GA) selection, and supernatants were screened by a direct enzyme-linked immunosorbent assay (ELISA) for reactivity. Positive wells were cloned by limiting dilution, grown in ascites medium (Sigma), selected, and supernatants were titered by ELISA.

Screening ELISA for HPV type-specific and conformation dependent binding. (i) OD₄₅₀ and ELISA titrations. As a first screen, MAbs were tested at several dilutions from 10 µg/ml to 0.4 µg/ml as either purified MAbs or tissue culture supernatants. Immulon 4B microtiter plates were coated with VLP antigens of different HPV types under intact or disrupted conditions. Intact VLPs for types 6, 11, 16, 18, 31, 33, 45, 52, and 58 were solubilized in 50 mM histidine, 0.5 M NaCl, pH 6.2, and incubated overnight at 2 to 8°C. VLPs were disrupted by incubation in 0.2 M sodium carbonate buffer, pH 10.6, with 10 mM dithiothreitol (DTT) and dried on the plates overnight at 37°C. The diluted antibodies were incubated on blocked plates for 2 h at room temperature.
temperature. The plates were washed, and horseradish peroxidase-conju-
gated goat anti-mouse IgG (heavy plus light chain [H+L]) (Invitrogen) (1: 10,000 in assay diluent) was added and incubated for 1 h at room tem-
perature. Plates were washed and developed with TMB (tetramethyl benzidine; Pierce, Rockland, IL). The reaction was stopped with 2.0 N H2SO4 and the
optical density at 450 nm (OD450) was read. Background wells contained only
the conjugate and no MAb.

Type-specific MAbs were identified as antibodies that reacted to only
one VLP type. The following antibodies developed by Neil Christensen
(Penn State University) were used to confirm specific reactivity: H16.V5, H18.J4, H6.M48, H31.A6, H33.B6, and H45.N5. K11.B2, developed at
Merck Research Laboratories, was used as a control for type 11. H16.J4
(from Neil Christensen) was used as a positive control for types 52 and 58.
The data for the heat maps were generated at a MAb concentration of 0.1
µg/ml. HPV VLP conformation-recognizing antibodies were identified as
those with a signal to intact antigen and not to disrupted antigen.

(ii) HPV pseudovirion-based neutralization assay. Neutralization
experiments were performed by some of us at Deutsches Krebsforschun-
gscentrum (DKFZ) using a well-characterized assay (15). Prior to receipt
d at DKFZ, samples were dialyzed against phosphate-buffered saline (PBS)
to remove sodium azide, concentrations were confirmed by absorbance
at 280 nm, and vials were recoded to blind the experimenters to the expected
type specificity of the MAbs. Two neutralization experiments were per-
formed. The first experiment determined the specificity of the MAbs
against a panel of pseudovirions. All MAbs were diluted to a nominal
concentration of 0.56 ng/ml. In the first assay, each MAb was further
diluted 1:300 and 1:1,500. These two dilutions were tested for neutraliza-
tion activity against a panel of pseudovirions (HPV types 31, 33, 45, 18, 52,
38, 35, 59, and 16). A positive control MAb, K18.L2 (reactive to HPV
L2), was included on each plate.

The second experiment was a full titration of each MAb against its
reactive HPV type using a nonreactive HPV type as a control for each
assay. Each MAb was titrated in duplicate over 8 points with 3-fold dilu-
tions at a starting concentration of either 2,800 ng/ml, 280 ng/ml, or 28
ng/ml. A 50% effective concentration (EC50) was determined for each
MAb using Prism 5.04 (GraphPad Software).

Dilutions of monoclonal antibodies were prepared in supplemented
Dulbecco’s modified Eagle medium (DMEM), and 50 µl of the dilutions
was added to each well of a 96-well plate (in triplicate). The outside wells
of the plate were excluded and filled with 150 µl medium to avoid poten-
tial plate effects. Next, 50 µl of the pseudovirus (16) (diluted in DMEM)
was added to each sample well. The antibody-pseudovirus mixture was
incubated for 20 min at room temperature before the addition of 50 µl
HeLaT K4 cells (15) to each well (2.5 x 105 cells/ml). Plates were incu-
bated for 48 h at 37°C (5% CO2). The amount of secreted Gaussia lucif-
erase was determined in 10 µl of cell culture medium using the coelen-
terazine substrate and Gaussia glow juice (PKK, Germany) according to
the manufacturer’s instructions. A microplate luminometer (Victor; Perki-
Elmer) was used to measure culture medium-associated lumines-
cence 15 min after substrate addition.

(iii) Sequence alignment. Sequences were translated from nucleotides
to amino acids, if necessary. Amino acid sequences were multiply aligned
using the default parameters in ClustalX 2.1 or VectorNTI Advance 11.5.1
AlignX. Sequence homologies were calculated from the resultant align-
ments.

RESULTS

Panels of MAbs were successfully prepared against VLPs of each
HPV type: 31, 33, 45, 52, and 58. The MAbs were generated and
developed from mouse hybridomas according to standard tech-
niques, as detailed in Materials and Methods. We took additional
steps during screening to confirm clonality and determine the
antibody isotypes for each at an early stage. Once MAb clones were
identified, the MAbs were characterized for four quality criteria.
First, MAbs were checked for specificity to bind only to the cog-
nate HPV VLP type against which they were generated. Second,
the specificities of MAbs to intact or disrupted VLPs were deter-
mined. Third, the EC50 for binding of each MAb to its VLP (or
cross-reactive VLP) was measured. Fourth, the type-specific neu-
tralization pattern of each MAb was determined, and the neutral-
ization EC50 was quantified. Ultimately, MAbs that best satisfied
these criteria were selected for further development and se-
quenced to determine variable heavy and light regions.

Type-specific MAb binding to L1 VLPs. To understand the
binding specificity of each MAb, we performed a series of ELISAs.
Each set of MAbs raised against a specific HPV VLP type was tested
against that same VLP type and other VLP types found in cur-
rently marketed HPV vaccines and vaccine candidates. These in-
cluded the oncogenic types HPV16 and HPV18 (included in Gar-
dasil and Cervarix), HPV31, HPV33, HPV45, HPV52, HPV58, and
two types implicated in anogenital warts, HPV6 and HPV11
(included in Gardasil). For each type, we tested intact VLPs and
disrupted VLPs to determine if the MAbs could discriminate be-
tween the two. This is an important criterion for product release
and clinical assays, as intact VLPs have been shown to and are
expected to be stable and closely model the HPV virions (17, 18).

In Fig. 1, the ELISA binding of MAbs to VLPs is plotted as
heatmaps, where OD450 values are represented in a false-color
scale. VLPs from different HPV types were coated onto microtiter
plates either as intact or disrupted particles. Intact VLPs were eval-
uated by electron microscopy and biophysical analysis to ensure
no or low frequencies of partially formed VLPs. We review each of
the HPV VLP types in turn. Binding of MAbs to HPV31 VLPs is
shown in Fig. 1a and b. MAB H31.1H1 binds to both disrupted
(Fig. 1b) and intact particles (Fig. 1a) and therefore recognizes a
linear epitope. MAB H31.5E5 binds to a conformational epitope
on both HPV31 and HPV52 intact VLPs. The remaining HPV31
MAbs are strictly type specific and recognize conformational
epitopes. From this set, MAB H31.0F12 (IgG1) and MAB
H31.5D10 (IgG2b) were chosen for further evaluation. These
MAbs recognize only intact particles and are type specific.

Binding of MAbs to HPV45 VLPs is shown in Fig. 1c and d. All
of the anti-HPV45 MAbs recognize conformation-dependent
epitopes, as shown by the binding to intact HPV45 VLPs (Fig. 1c)
and lack of binding to disrupted VLPs (Fig. 1d). Four of the MAbs
also recognize intact HPV18 VLPs, a type closely related to
HPV45. HPV45 and HPV18 are in the same α7 phylogenetic
and have a high amino acid sequence homology (88%). It is
therefore not surprising that four of the eight MAbs recognize
HPV18 as well as HPV45. All of the MAbs recognize intact
HPV45 VLPs and are posited to map to conformational epitopes.
MAbs HG5.6G6 (IgG2b) and HG5.10B4 (IgG1) were chosen for
subsequent study. MAB HG5.6G6 is cross-reactive with HPV18;
the implications of this for assays are discussed below.

In Fig. 1e and f, the binding of four HPV52 MAbs to HPV52
VLPs ELISA is graphed. Despite there being only four MAbs, they are
all type specific and conformational. We chose H52.8D11 (IgG2b)
and H52.9F7 (IgG2a) for further testing. All but one MAB (H58.3A8)
generated against HPV58 recognize intact VLPs, as shown in Fig. 1g
and h. MAB H58.12F4 slightly recognize intact VLPs, and two differ-
ent MAbs (H58.10A7 and H58.10A9) recognize disrupted HPV58
and, to a lesser extent, HPV52. H58.2C3 (IgG1) and H58.6E11
(IgG2b) were selected for assay development.

Hybridomas were subsequently generated and screened against
HPV33 VLPs at a later phase but following the same methods as for

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the previous types. All but one MAb (H33.1E7) recognize intact VLPs. H33.1E7 recognizes a well-conserved continuous epitope on all 9 HPV types (Fig. 1j). H33.3B2, H33.7A11, H33.2C12, and H33.4B6 recognize both continuous and conformational epitopes of HPV31 VLPs. H33.4B6 also recognizes continuous and conformational epitopes on VLPs from HPV31, HPV52, and HPV58. While all but one MAb bound to intact HPV33 VLPs, a few MAbs exhibited binding to other types. H33.7A11 recognized types 16, 52, and 58. H33.4B6 recognized types 31, 52, and 58 and had higher binding to 31 and 58 than to its cognate type, HPV33. Interestingly, MAb H33.1E7

FIG 1 ELISA reactivity of MAbs generated against specific HPV types. HPV VLPs from five types were used to coat microtiter plates as either intact VLPs (left panels) or disrupted VLPs (right panels). (a) Intact HPV31 VLPs, (b) disrupted HPV31 VLPs, (c) intact HPV45 VLPs, (d) disrupted HPV45 VLPs, (e) intact HPV52 VLPs, (f) disrupted HPV52 VLPs, (g) intact HPV58 VLPs, (h) disrupted HPV58 VLPs, (i) intact HPV33 VLPs, and (j) disrupted HPV33 VLPs. In the heatmaps, the OD_{450} for binding of individual MAbs (y axis) versus HPV VLP types (x axis) are displayed on a false-color scale. Vertical bars of color indicate that many MAbs are type specific; horizontal bars indicate MAbs that recognize conserved epitopes on multiple tested VLPs.
recognized all nine VLPs when disrupted (Fig. 1j) and had little or no binding to any of the intact VLPs (Fig. 1i), including HPV33. From these data, we hypothesize that MAb H33.1E7 recognizes a continuous epitope that is normally buried or occluded in intact VLPs. We chose MAb H33.5D4 (IgG1) and MAb H33.6G9 (IgG2b) for subsequent characterization. Both of these MAbs recognize conformational epitopes and are type specific. Finally, the MAb pairs (two for each HPV type) selected from prior hybridomas (Fig. 1) were tested for cross-reactivity to HPV33 and shown to be negative. The selected MAb pairs are listed in Table 1.

Affinity of MAbs to HPV VLPs. To quantify the binding affinities of the MAbs to the specific HPV VLPs, we conducted an extensive series of ELISAs, detailed in Materials and Methods. Briefly, HPV VLPs were used to coat plates, and MAbs were run in dilution series and detected with an anti-mouse secondary antibody. Data were analyzed by the typical four-parameter logistic equation. All of the VLP-MAb ELISA data are plotted in Fig. 1 in the supplemental material, with overlays of fits to the analytical function. The EC50 binding parameters determined from fits are listed in Table 2. All type-specific MAbs EC50s were excellent, ranging from 1.7 to 4.9 ng/ml. These are more than sufficient for a robust and high-quality ELISA. The one cross-reactive MAb, H45.6G6, had an EC50 of 13 ng/ml to HPV18 VLP, a 7-fold increase from its EC50 of 1.7 ng/ml to HPV45.

HPV type-specific pseudovirus neutralization by MAbs. The monoclonal antibody pairs (Table 1) for HPV31, HPV33, HPV45, HPV52, and HPV58 were tested for neutralization activity in a well-characterized in vitro pseudovirus neutralization assay (15). Two experiments were performed. The first experiment examined the specificity of the MAbs against a broad panel of HPV pseudovirions, and the second experiment quantitatively determined the EC50 of each monoclonal antibody (MAb) against the HPV type or types against which it had neutralizing activity. These experiments were conducted in a blinded fashion; the experimenters did not know the HPV type against which the MAbs were generated or the specificity according to binding assays (Fig. 1; also, see Fig. S2 in the supplemental material).

For vaccine development, measuring neutralization ensures that the assays are monitoring relevant epitopes. Each MAb was tested for neutralization against all five HPV types upon which they were derived (HPV31, HPV33, HPV45, HPV52, and HPV58), the types found in the quadrivalent HPV vaccine Gardasil (HPV6, HPV11, HPV16, and HPV18), and an additional type, HPV59 (in the same phylogenetic group α7 as HPV18 and HPV45). Each MAb was tested against the panel of HPV pseudoviruses at two concentrations, 1.8 μg/ml and 0.37 μg/ml (see Materials and Methods). Each MAb that bound to HPV VLPs in a type-specific manner also completely neutralized its respective HPV pseudovirion at both concentrations tested (Fig. 2). In addition, the cross-reactive binder H45.6G6 could also neutralize HPV18 pseudovirions. As might be expected, H45.6G6 neutralized HPV45 more efficiently than HPV18. With this exception, none of the MAbs exhibited significant cross-neutralization of other HPV pseudovirion types.

With the establishment of the specificity of neutralization, the neutralization activity of each MAb was further quantified against its cognate antigen. A complete titration series of each MAb was performed in the neutralization assay against the pseudoviruses. The neutralization data were fitted with the four-parameter logistic equation and summarized by the EC50. The neutralization curves are plotted along with nonlinear least-square fits in Fig. S2 in the supplemental material. The cross-reactive HPV45.6G6 was tested against both HPV45 and HPV18. Consistent with the neutralization map (Fig. 2) and the differential in binding affinities (Table 2), the neutralization of HPV45 pseudovirion was considerably more potent than that of HPV18, approximately 750-fold. All neutralization EC50s are listed in the last column of Table 2. Type-specific neutralizations ranged from 0.35 to 12 ng/ml. These data demonstrate that the MAbs are specifically and potentially neutralizing in the in vitro assays and indicate that the MAbs recognize epitopes that are neutralization sensitive.

To identify the MAbs, ensure long-term availability, and facilitate sharing with the wider scientific community, the variable regions of the 10 MAbs were sequenced. Alignments of the amino acid sequences for the light chains and heavy chains are shown in Fig. 3. In each alignment, the complementarity-determining regions (CDRs) are highlighted. Although it is difficult to draw inferences from a limited set of sequences, it is interesting that pairs of MAbs with the same type specificity tend to have higher similarity than pairs of different types. For example, the light chains for HPV52 MAbs are identical with the exception of one amino acid in CDR1, one amino acid in CDR2, and three amino acids in CDR3. These cross-specific and type-specific patterns may indicate which CDRs are essential for binding to the HPV L1 epitopes and which regions determine the type specificity.

### Table 1: Characteristics of anti-HPV type-specific monoclonal antibodies

| MAb     | HPV type | Isotype  | Function in assays¹ |
|---------|----------|----------|---------------------|
| H31.5F12| 31       | IgG1, κ  | Capture             |
| H31.5D10| 31       | IgG2b, κ | Detection           |
| H33.5D4 | 33       | IgG1, κ  | Capture             |
| H33.6G9 | 33       | IgG2b, κ | Detection           |
| H45.6G6 | 45       | IgG2b, κ | Capture             |
| H45.10B4| 45       | IgG1, κ  | Detection           |
| H52.8D11| 52       | IgG2b, κ | Capture             |
| H56.2C3 | 58       | IgG1, κ  | Capture             |
| H58.6E11| 58       | IgG2b, κ | Detection           |

¹ Antibodies are used either to capture virus-like particles on the ELISA plate or to detect the bound VLP on the plate or bead.

### Table 2: Binding and neutralization EC50 concentrations for anti-HPV type-specific MAbs

| MAb     | HPV type(s) and epitope² | HPV type(s) neutralized | EC50 (ng/ml) | Binding | Neutralizing |
|---------|-------------------------|-------------------------|--------------|---------|--------------|
| H31.5F12| 31                      | 31                      | 4.1          | 5.2     |
| H31.5D10| 31                      | 31                      | 3.6          | 9.1     |
| H33.5D4 | 33                      | 33                      | 2.3          | 0.39    |
| H33.6G9 | 33                      | 33                      | 2.3          | 0.38    |
| H45.6G6 | 45, 18                  | 45, 18                  | 1.7 (HPV45), 13 (HPV18) | 0.35 (HPV45), 270 (HPV18) |
| H45.10B4| 45                      | 45                      | 4.9          | 12      |
| H52.8D11| 52                      | 52                      | 4.0          | 0.49    |
| H56.2C3 | 58                      | 58                      | 3.4          | 0.41    |
| H58.6E11| 58                      | 58                      | 2.2          | 1.1     |

² All are conformational.
Neutralizing MAbs for HPV Types 31, 33, 45, 52, and 58

FIG 2 Neutralization by MAbs is specific to HPV type. All 10 of the MAbs listed in Table 1 had type-specific neutralizing activity against their respective HPV types. In addition, H45.6G6.A8 had diminished but detectable neutralizing activity for HPV18, a closely related type.

DISCUSSION

We have developed and characterized panels of monoclonal antibodies against HPV L1 VLPs of oncogenic types HPV31, HPV33, HPV45, HPV52, and HPV58. From each panel, two MAbs were selected for type specificity, sensitivity to VLP conformation, potent neutralization, and differing isotypes. Binding affinities of intact VLPs were confirmed by a surface plasmon resonance (SPR) method that we published previously (14). The MAbs were selected for use in two assays, one assay for vaccine production and one assay for vaccine trial clinical serology. The use of the same MAbs in both assays ensures that we detect the same epitopes and characteristics (correlates of neutralization and correct VLP conformation) in both phases.

The in vitro release potency (IVRP) assay (19) is used to monitor both product quality and consistency for release; the in vitro nature of the assay is in contrast to the historical in vivo mouse potency assays. Within each pair, one MAb is used to capture the VLP on the ELISA plate, and the second MAB is used for detection in the sandwich format. The role of each MAB is listed in the final column of Table 1. The final readout is through a conjugated anti-mouse reagent specific to one mouse isotype. Instead of having to generate and qualify labeled conjugates for each type-spe-

FIG 3 Alignment of amino acid sequences of the light- and heavy-chain regions of MAbs shown in Tables 1 and 2. Complementarity-determining regions (CDRs) are shaded.

| mAb      | Light Chain Sequence | CDR1                                      | CDR2                                      | CDR3                                      |
|----------|----------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| H31.5F12 | D1VWMQSPSGLAVSGEKTVMQ 序列 1 | H31.5F12 序列 1                           | H31.5F12 序列 1                           | 序列 1                                     |
| H31.5D10 | D1VMTIRSHKFMSTGTVSGLRTSI      | 序列 2                                      | 序列 2                                      | 序列 2                                     |
| H33.5D4  | D1VLTQSPSLAVGLATGRATISCRASESVD 序列 3 | 序列 3                                      | 序列 3                                      | 序列 3                                     |
| H33.6G9  | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 4                                      | 序列 4                                      | 序列 4                                     |
| H45.6G6  | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 5                                      | 序列 5                                      | 序列 5                                     |
| H45.10B4 | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 6                                      | 序列 6                                      | 序列 6                                     |
| H52.8D11 | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 7                                      | 序列 7                                      | 序列 7                                     |
| H52.9F7  | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 8                                      | 序列 8                                      | 序列 8                                     |
| H58.2C3  | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 9                                      | 序列 9                                      | 序列 9                                     |
| H58.6E11 | D1VMTQPSASLSAVGTTVIITIASTN1Y | 序列 10                                     | 序列 10                                     | 序列 10                                    |

| mAb      | Heavy Chain Sequence | CDR1                                      | CDR2                                      | CDR3                                      |
|----------|----------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
| H31.5F12 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 1 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 1       | 序列 1                                      | 序列 1                                     |
| H31.5D10 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 2 | 序列 2                                      | 序列 2                                      | 序列 2                                     |
| H33.5D4  | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 3 | 序列 3                                      | 序列 3                                      | 序列 3                                     |
| H33.6G9  | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 4 | 序列 4                                      | 序列 4                                      | 序列 4                                     |
| H45.6G6  | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 5 | 序列 5                                      | 序列 5                                      | 序列 5                                     |
| H45.10B4 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 6 | 序列 6                                      | 序列 6                                      | 序列 6                                     |
| H52.8D11 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 7 | 序列 7                                      | 序列 7                                      | 序列 7                                     |
| H52.9F7  | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 8 | 序列 8                                      | 序列 8                                      | 序列 8                                     |
| H58.2C3  | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 9 | 序列 9                                      | 序列 9                                      | 序列 9                                     |
| H58.6E11 | E1VQLQSQFLSVKSGAVVMSCKA-GVYTF 序列 10 | 序列 10                                     | 序列 10                                     | 序列 10                                    |
pecific detection MAb, the unlabeled MAbs can be used with a secondary detection MAb. For instance, MAb H31.5D10 can be detected through anti-IgG2b MAbs that do not bind to the capture MAb, MAb H33.5D4 (IgG1). In this way, only three reagents must be labeled and qualified (anti-IgG1, anti-IgG2a, and anti-IgG2b) instead of custom modification of five HPV-specific MAbs. These three reagents are available from many commercial suppliers, which also simplifies assay development.

The second important assay for a vaccine program is a clinical assay to monitor serological responses in vaccinees. Similarly to the serological assay for Gardasil (6, 7), a multiplexed competitive Luminex immunoassay (cLIA) has been developed for the non-avalent vaccine. The same requirements for MAb that recognize neutralizing epitopes apply. Due to the competitive nature of the assay, it is even more important that the MAbs be strictly type specific. The cLIA requires one MAb specific to each VLP type. For each type, we chose the “detection” MAb (Table 1) from the pair used in IVRP, and this MAb had to be strictly type specific to maintain the type specificity of the assay results. This is important because the potential for vaccine-induced cross-reactivity has been established. Sera from women immunized with Cervarix (HPV16/HPV18) can neutralize HPV31 and HPV45 in vitro (20, 21), presumably due to HPV16 and HPV18 cross-reactivity, respectively. Unlike same-type neutralization, the cross-reactive neutralization is apparent only after three vaccinations at months 0, 1, and 6, and the neutralization activity is reduced by more than a factor of 100. Similarly, sera from women immunized with Gardasil (HPV6, HPV11, HPV16, and HPV18) can neutralize HPV45 in vitro (22), presumably due to HPV18 cross-reactivity. The clinical examples demonstrate the possibility for cross-reactive neutralizing MAbs but also reinforce the need for additional new HPV types in a vaccine to reach high levels of type-specific neutralization of new oncogenic HPV types.

In general, when developing and selecting MAbs for vaccine release and clinical assays, we seek to satisfy four criteria. First, each MAb must be specific to one of the genotypes in the vaccine. Second, two or more MAbs that will be used in the same assay must not mutually compete or interfere. Third, the MAbs must have sufficient affinity to bind to the antigen and not rapidly dissociate. Fourth, the MAbs must represent critical quality attributes of the vaccine. These may include specificity to conformational epitopes (e.g., to discriminate between intact VLPs or properly folded antigens), recognition of neutralizing epitopes (to correlate with functional assays), and sensitivity to thermal or chemical stress upon the vaccine.

In the specific case of the IVP, an intact sandwich of both MAbs in the pair binding to the VLP is required, so only one MAb must be type specific for the assay itself to be type specific. Furthermore, the second requirement for a lack of competition is not crucial due to the highly multivalent character of VLPs. The same epitope is expressed many times across the VLP surface; binding of a MAb to a single site does not significantly limit the binding of a second MAb.

The pattern of cross-reactivity of the monoclonal antibody panels (Fig. 1) is consistent with the amino acid homology (see Table S1 in the supplemental material) between the HPV L1 proteins and phylogenetic grouping (23, 24). HPV18 and HPV45 are closely related (88% homologous, group α7) compared to their homology of only 62% to 65% with the other L1 proteins considered here. HPV6 and HPV11, which are responsible for anogenital warts (92% homologous, group α10), had lower homology (64% to 68%) to the other L1 sequences. Accordingly, none of the 39 antibodies raised to the five new VLP types bind to HPV6 or HPV11 VLPs, with the exception of H33.1E7, which binds to all disrupted VLPs. The remaining types (HPV16, HPV31, HPV53, HPV52, and HPV58) all belong to group α9, with intragroup L1 homology of 77% to 90%. The exhibited cross-reactivity of some MAb within group α9, i.e., that between 31 and 52 and that between 33 and 16, 31, 52, and 58, is consistent with their relatively high homology (see Table S1 in the supplemental material).

In summary, high-affinity and strongly neutralizing antibodies were generated, characterized, and used to develop sandwich immunoassays that identify and quantify type-specific VLP antigens. These MAb have utility in research assays (14), vaccine product release assays (19), and clinical serological assays (6, 7) to broaden the coverage to new oncogenic HPV strains.

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