**Quantitative and epitope-specific antigenicity analysis of the human
papillomavirus 6 capsid protein in aqueous solution or when adsorbed on
particulate adjuvants**

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Abstract

Human papillomavirus (HPV) 6 is a **human pathogen which causes genital warts**. Recombinant virus-like particle (VLP) based antigens are the active components in prophylactic vaccines to elicit functional antibodies. The binding and functional characteristics of a panel of 15 murine monoclonal antibodies (mAbs) against HPV6 was quantitatively assessed. Elite conformational indicators, recognizing the conformational epitopes, are also elite viral neutralizers as demonstrated with their viral neutralization efficiency (5 mAbs with neutralization titer below 4 ng/mL) in a pseudovirion (PsV)-based system. The functionality of a given mAb is closely related to the nature of the corresponding epitope, rather than the apparent binding affinity to antigen. The epitope-specific antigenicity assays can be used to assess the binding activity of PsV or VLP preparations to neutralizing mAbs. These mAb-based assays can be used for process monitoring and for product release and characterization to confirm the existence of functional epitopes in purified antigen preparations. Due to the particulate nature of the alum adjuvants, the vaccine antigen adsorbed on adjuvants was considered largely as “a black box” due to the difficulty in analysis and visualization. Here, a novel method with fluorescence-based high content imaging for visualization and quantitating the immunoreactivity of adjuvant-adsorbed VLPs with neutralizing mAbs was developed, in which antigen desorption was not needed. The facile and quantitative *in situ* antigenicity analysis was amendable for automation. The integrity of a given epitope or two non-overlapping epitopes on the recombinant VLPs in their

adjuvanted form can be assessed in a quantitative manner for cross-lot or cross-product comparative analysis with minimal manipulation of samples.

Keywords: human papillomavirus; monoclonal antibody; neutralization epitope; conformational sensitivity; pseudovirion; *in situ* antigenicity analysis.

Abbreviations: HPV, human papillomavirus; VLP, recombinant virus-like particle; mAb, monoclonal antibody; PsV, pseudovirus; HRP, horseradish peroxidase; GFP, green fluorescent protein; HAI, hemagglutination inhibition; CSI, conformational sensitivity index.

1. Introduction

Human papillomavirus (HPV) is a causative agent of genital warts, cervical cancer and other genital lesions [1]. Until now, more than 170 types of HPV have been identified. According to their virulence, different HPV serotypes were classified as low-risk types (e.g., HPV6 and 11) and high-risk types (e.g., HPV16 and 18) [2-5]. HPV6 and 11 are responsible for almost 90% of genital wart cases. Gardasil®-4, first licensed HPV vaccine, contains both HPV 6 and 11 types as active vaccine antigens to prevent viral infection which could lead to genital warts. For the vaccination with Gardasil®-4, HPV6/11 infection has been reduced, which was responsible for low degree precancerous lesions due to a markedly decreased re-check rate of the abnormal Pap smears [6].

HPV virus-like particles (VLPs) can be assembled from the recombinant major capsid protein L1 alone or L1 together with the minor capsid protein L2 [7, 8]. VLPs faithfully mimic conformational epitopes on the virion surface, especially surface loops, which are critical for eliciting the response of a variety of protective antibodies [9, 10]. The quadrivalent vaccine Gardasil®-4 and, more recently, the nine-valent vaccine Gardasil®-9 was developed using the recombinant VLP approach and licensed for human use by Merck [11]. Both vaccines contain the L1 antigens of low-risk serotypes HPV6/11 as active components to maximize the effectiveness of adolescent vaccination for girls and boys. For antigenicity analysis, conformational and type-specific antibodies played a vital role in identifying the key epitopes on the viral capsid of native virions, pseudovirions (PsVs) or VLPs. In addition, well-

characterized mAbs with known epitope information could also be used in competition assays to analyze the quality and quantity of neutralizing antibodies elicited by immunization of animals and to assess vaccine-induced immune responses in clinical studies [12-15].

In this paper, a panel of murine anti-HPV6 mAbs were developed and characterized in details. Their binding and functional parameters were quantitatively assessed using a combination of different analytical methods. Parameters relevant to their binding affinity, conformational sensitivity, pseudovirion (PsV)-based neutralization efficiency and hemagglutination inhibition activity were assessed. Top performing mAbs were used in the binding assaying for analyzing PsVs and recombinant VLPs. More importantly, the feasibility of a novel epitope-specific immunofluorescence assay was demonstrated for *in situ* antigenicity assay of antigen when adsorbed on particulate alum adjuvants without the need of antigen desorption.

2. Materials and Methods

2.1 Recombinant HPV6 VLPs

The recombinant HPV6 L1 protein expressed by *E. coli* was previously reported to be capable of self-assembly into VLPs, which were produced and purified using previously published procedures [16].

2.2 Monoclonal antibodies (mAbs)

A panel of monoclonal antibodies (mAbs) was obtained using standard hybridoma technology to fuse HPV6 VLP-immunized mouse splenocytes with SP2/0 cells (This study was approved by the Animal Welfare Ethics Committee of Xiamen University). Finally, a panel of representative 15 murine mAbs against HPV6 VLPs were obtained and quantified by the UV 2000 (GE, Healthcare).

2.3 Direct binding ELISA

A panel of murine mAbs was characterized in an antigen-coated microwell plate with a serially diluted mAb in solution. The binding affinity to native and denatured VLP was reflected as the antibody concentration to achieve 50% of the maximal signals (or EC₅₀ values).

2.4 PsV-based neutralization assay

The PsV-based neutralization assay was used to assess the neutralization efficiency of different mAbs [12, 17]. The median inhibitory concentration (IC₅₀, ng/ml) for a given mAb was defined as the antibody concentration for achieving 50% inhibition of PsV.

2.5 Hemagglutination inhibition (HAI) assay

The erythrocytes from five species (mouse, rabbit, guinea pig, chicken and pig) were screened for the hemagglutination phenomenon caused by the HPV 6 VLPs. The rabbit erythrocytes showed most prominent hemagglutination, thus being used for the subsequent hemagglutination study by different mAbs. The minimal concentration of a mAb that could inhibit VLP-induced hemagglutination was defined as the HAI titer (μg/ml).

2.6 Sandwich ELISA for antigenicity analysis of PsV and VLP

The concentration of L1 on HPV6 PsV was determined by the sandwich ELISA composed of two linear mAbs (18E4: Ag: 2D4-HRP) through a standard curve of HPV6 VLP-L1. Seven neutralizing mAbs were used with different pairings in the sandwich ELISA to evaluate the antigenicity of the HPV6 VLP and HPV6 PsV.

2.7 In situ antigenicity analysis assay for adjuvanted VLP antigen

To assess the antigenicity of HPV6 VLP adsorbed on particulate adjuvants, a mixture of HPV6 VLP antigen was adjuvanted with an amorphous Aluminum based adjuvants sized at 10-20 um. The high content imaging using the fluorescence signals of the antigen-on-Adjuvants by labeled antibody was achieved using the Opera Phenix™ (Perkin Elmer, Boston, USA) High Content Analysis imaging system. The total fluorescence intensity of in each well was measured using a multifunction microplate reader (Paradigm™, Beckman-Coulter, Brea, CA).

A detailed description on the methods including the statistical methods was provided in *Supplementary Materials*.

3. Results

3.1 Conformational sensitivity of the anti-HPV6 mAbs

To assess the conformational sensitivity of the mAbs against HPV 6 L1 protein, native VLPs and denatured L1 protein were used. The VLP morphology and the loss of conformation in denatured L1 protein was shown or illustrated (**Fig. 1a and 1b**). The representative binding

profiles of different mAbs (namely 10H1, 6G7 and 3F3) to *native* and *denatured* HPV6 VLPs were presented in **Fig. 1c**. The change in binding affinity upon the loss of protein conformation, or relative effective concentration or rEC_{50} (ratio of EC_{50} values obtained with native and denatured antigen) quantitatively reflects the conformational sensitivity of that given mAb. According to conformational sensitivity index (CSI, or rEC_{50}) values, mAbs were ranked and divided into 4 categories: *very sensitive* to conformation, *sensitive* to conformation, *insensitive* to conformation and preferring to the denatured antigen (**Fig. 1d** and **Table 1**).

3.2 Viral neutralizing activity of the anti-HPV6 mAbs

Virus neutralizing activity is a functional characteristic of the mAbs. A PsV-based neutralization assay was used to demonstrate the viral neutralization activity. The neutralizing titer as reflected by median inhibitory concentration (NT- IC_{50} , ng/ml) was defined as the antibody concentration needed to achieve 50% inhibition of PsV (**Supplementary Fig. 1S(a) and 1S(b)**). The NT- IC_{50} (in the unit of ng/ml) of each antibody was tabulated (**Table 1**). In general, elite conformational indicators were also elite neutralizers (**Supplementary Fig. 1S(c)**), such as those mAbs in group I in **Table 1** which showed high CSI values (>10) and low NT- IC_{50} values (<20 ng/ml). High neutralizing efficiency of these mAb indicate that they bind to important important epitopes on the viral capsid to prevent the viral infection in the assay.

3.3 Hemagglutination inhibition activity

Erythrocytes from a certain species such as rabbit or mouse were found to be agglutinated in the presence of multimeric and highly ordered viral assemblies such as HPV VLPs. In addition to the above-mentioned neutralization activity of mAbs, hemagglutination inhibition (HAI) test [22-24] was used to evaluate the ability of mAbs to inhibit the binding of PsV and VLPs to the surface of erythrocytes. Most of the 15 mAbs exhibited HAI activity with a mAb concentration dependence (**Table 1**), as illustrated in **Fig. 2a** with 11B10, 10H1 and 12H4 as examples. Those top conformation indicators with high neutralization efficiency also showed higher HAI activity, indicating the relatedness of these functional characteristics (**Fig. 2b**). The correlation between neutralization activity and HAI activity was illustrated in **Fig. 2c** by grouping the mAbs based on their neutralization efficiency.

Based on the mAb characteristics (**Table 1**), 12H4 and 17D5 were selected as the primary choices for the antigenicity analysis of HPV PsVs or VLPs due to their high neutralizing ability and high conformational sensitivity (**Fig. 2b** and **Table 1**).

3.4 Quantitative analysis of HPV6 VLPs and PsVs with functional mAbs

Both PsV and VLP spherical particles mimic the native virions structure, with PsV composed of L1 + L2, and VLP composed of only L1 in the viral capsid [25]. To approximate the L1 content in PsV preparation, a ‘mass ELISA’ based on two mAbs (18E4 and 2D4, **Table 1**) recognizing linear epitopes was performed with VLP lot as a reference. Subsequently, to compare the degree of the likelihood in the surface epitopes in VLPs and PsVs, the antigenicity of PsVs was assessed in ‘Antigenicity ELISA’ using conformational sensitive mAbs as

compared to VLP preparations. Sandwich ELISA assays with different mAb pairings were performed with EC₅₀ values and slopes derived and tabulated in **Supplementary Fig. 2S(a)** and **Supplementary Table 1S**. For better illustration, the profiles for VLP vs. PsV pairs were plotted in the same plot **Supplementary Fig. 2S(b)**. The relative functional activity based on rEC₅₀ values (tabulated in **Supplementary Table 1S**) was in the range of 1.2 to 2.6, reflecting comparable antigenicity between HPV6 VLP and PsV preparations based on the normalization of L1 content. The slopes for PsVs were generally higher ($p < 0.05$) than those obtained with VLPs (**Supplementary Fig. 2S(b)** and **Supplementary Table 1S**), suggesting a higher affinity between neutralizing mAbs and PsVs. The higher affinity could be indicative of bearing more resemblance to native virions with L2 being part of the viral capsid and some DNAs packed inside for PsVs as compared to L1 only VLPs.

3.5 In situ antigenicity analysis of antigen adsorbed on particulate adjuvants

For recombinant VLP based vaccines, the final step in vaccine production is the adsorption onto a particulate adjuvant for antigen stabilization and immune potentiation. Very limited number of methods are available to assess the activity or conformation of the adsorbed antigen in adjuvanted vaccines. Here, using a high content imaging platform, we successfully showed the visualization of binding with neutralizing mAbs of HPV6 VLPs adsorbed on particulate adjuvants (**Fig. 3a**). Furthermore, the full binding profiles can be obtained of the formulated antigen with serially diluted vaccine preparations to derived the quantitative information, such as median effective concentration for a give batch (**Fig. 3b**). This was

enabled with fluorescently labeled mAbs. More importantly, in this novel *in situ* antigenicity analysis, two non-overlapping epitopes, such as those recognized by mAbs 12H4 and 17D5, can be assessed simultaneously in a single assay by labeling two mAbs with two different fluorescent dyes (such as Alexa Fluor 488 and 594) as illustrated in **Fig. 3**. Both the red fluorescence (mAb 12H4) and green fluorescence (mAb 17D5) intensity on adjuvants showed concentration dependence. The sigmoidal binding curves were obtained from both platforms (**Fig. 3b**). While high content imaging system is a method of choice for visualization of antibody binding activity of adsorbed antigen, the plate reader-based assay for total fluorescence intensity measurement is a good method for antigen quantitation. The EC₅₀ values (~ 1.0 to 4.0 ug/mL, **Fig. 3**) were calculated from a sigmoidal curve with a laboratory formulated vaccine batch (**Fig.3**). In addition, there was no mutual interference between the red fluorescence and green fluorescence, indicating that these two neutralizing epitopes recognized by 12H4 and 17D5 are not fully overlapping.

Similar to reported *in vitro* relative potency assay with antigen desorption, by curve fitting with a 4-parameter logistic fit equation, EC₅₀ values for different batches could be calculated using the fluorescence intensity. The advantage for the *in situ* antigenicity assay does not need sample pretreatment for antigen desorption or adjuvant dissolution. As a practical note, the concentration of HPV6 VLP used in this feasibility study was similar to the commercial vaccine (40 µg/ml HPV L1 protein in Gardasil®-4). Therefore, this method could be applied to

vaccine antigenicity analysis for cross-lot or cross-product comparison in the final formulations without any needs of sample pretreatment.

4. Discussion

Monoclonal antibodies have become essential tools for analyzing various biomolecular interactions. Binding to neutralizing antibodies for VLPs could be a surrogate marker for vaccine potency. Comparable binding profiles for VLP and PsV in different mAb-based sandwich ELISAs (**Supplementary Fig. 2S**) confirmed the similar surface structures or the presence of critical epitopes, analogous to those in authentic virions. Therefore, type-specific and conformation sensitive mAbs with high affinity to VLPs and PsVs are useful analytical tools for the study of HPV pathogenesis or in the quality analysis of vaccine antigens in aqueous solutions as well as in formulated products [26-29].

For vaccine potency analysis, mouse potency assay is still the gold standard for many vaccines. But *in vitro* relative potency assay (IVRP) for vaccine quality had gradually replaced animal assays in recent years due to advantages such as low cost, better precision and short test period. The correlation between IVRP and animal based potency assay (ED₅₀) had been verified on several VLP based vaccines such as HPV vaccine Gardasil® [38]. Analysis of critical immuno dominant and neutralizing epitopes is essential as the *in vitro* assessment of the intended function (immunogenicity) of recombinant VLP based vaccines. *In vitro* relative potency assay for HPV6 vaccine antigen will be established based on the results presented here.

In the future, IVRP will reduce animal use, improved precision and shorter turn-around time, which can be used for process control, vaccine batch release and vaccine stability assessment.

The vaccine antigen, once formulated on aluminum based amorphous particulate adjuvant (largely considered as ‘a black box’), could be hardly characterized or with its activity visualized. Recently, there are reports on the use of flow cytometry or an o-phthalaldehyde fluorescent protein assay or near-infrared transmittance spectroscopy to analyze the antigens adsorbed to aluminum hydroxide adjuvant for antibody binding or for total protein quantitation [39-43]. In most of other methods for analyzing adjuvanted vaccines, the antigens were desorbed from adjuvants prior to analysis. The feasibility of a novel *in situ* antigenicity analysis assay to qualitatively and quantitatively assess the antigenicity of adjuvanted antigens. This was enabled using a high content fluorescence imaging platform with well-characterized mAbs. Information and knowledge on nature of the corresponding epitopes is critical to interpret the data for antigenicity analysis. Therefore, vaccine final products, such as filled vials for Gardasil®, could be analyzed using these platforms for the antigenicity analysis of the antigen adsorbed on adjuvants without the need of antigen desorption. With no or limited sample pre-treatment, this method could also be used to analyze vaccine final containers (vials or prefilled syringes) for product release and for products undergone real-time/under actual conditions storage or accelerated stability studies (such as thermal or shearing stress studies). In contrary to polyclonal antibodies still being used in different binding assays, mAbs offer the advantages of consistent supply, uniformity and epitope specificity. This is important in the

process development and process understanding when making recombinant protein based antigens as vaccine candidates. Knowledge of the critical epitopes and the availability of these molecular probes would facilitate the development of assays for tracking epitopes in antigens during vaccine bioprocessing [30-32]. Furthermore, these mAbs recognizing neutralizing and immuno dominant epitopes could also be used to better characterize the immune response elicited by VLP-based vaccines [33-35]. Improved understanding of critical conformational epitopes through the use of these mAbs described here would aid in the design of next-generation vaccines. The key epitopes for a given type such as those for HPV 6 could be grafted to another base VLP to make a chimeric VLPs with cross-type antigenic determinants [29, 36, 37].

In summary, a panel of anti-HPV 6 mAbs were characterized in details with respect to their functional characteristics. These mAbs are useful molecular tools for the development of quality analysis methods, as the immune reactivity to these functional antibodies can serve as surrogate markers of vaccine efficacy. Based on these functional mAbs, an *in vitro* antigenicity (or potency) assay for HPV6 antigen will be established to replace the animal based potency assay. A novel *in situ* antigenicity assay would enable the direct visualization of the antibody-antigen interaction on adjuvants. The *in situ* (or ‘on Alum’) antigenicity analysis could be a favorable method for the quality control of vaccine products including real time or accelerated stability of final products with minimal sample pretreatment.

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Figure legends

Figure 1. Conformational sensitivity of a panel of murine anti-HPV 6 VLP mAbs determined by a binding assay. (a) Transmission electron micrograph of recombinant HPV 6 L1 VLPs showing virion-like assembly. The scale bar is 100 nm. (b) Schematic diagram of denatured and reduced L1. (c) Representative binding profiles of anti-HPV 6 VLP mAbs with different levels of sensitivity to the protein conformation in the antigen: very sensitive (e.g., 10H1), sensitive (e.g., 6G7), and preference for denatured HPV 6 VLP (e.g., 3F3). (d) Conformational sensitivity ranking of anti-HPV 6 VLP mAbs based on rEC50 values. All mAbs were ranked according to the conformational sensitivity indices (rEC50), which is an indicator of the loss (or gain) of binding activity when the VLP conformation had been disrupted to a great extent on the solid surface after harsh treatment.

Figure 2. Cell-based hemagglutination inhibition activity of mAbs and correlations among three different functional characteristics. (a) Hemagglutination inhibition activity of mAbs. Taking the hemagglutination inhibition phenomenon of three anti-HPV6 VLP mAbs, 11B10, 10H1 and 12H4, as examples, different mAbs showed varying levels of HAI activity. (b) Relatedness of conformational sensitivity, neutralization titer and HAI titer of 15 mAbs to HPV 6 VLPs. As conformational sensitivity of mAbs decreased, the mAbs also showed lower neutralization efficiency and HAI activity. The neutralization titer of 15F7, 8F4, 2D4 and 3F3 mAbs were all greater than 45 ng /ml. (c) Correlation between HAI activity and neutralization efficiency, grouped by neutralization titers of mAbs. The neutralization activity and the ability

to inhibit hemagglutination of mAbs showed reasonable agreement ($P < 0.05$). The red arrows indicate HAI titers of 45 $\mu\text{g/ml}$ or higher.

Figure 3. *In situ* antigenicity analysis of HPV6 VLP-L1 adsorbed on aluminum-based particulate adjuvant. (a) Pictures of fluorescence intensity from high to low based on the serially dilution of HPV6 VLP adsorbed on the adjuvant. (b) Sigmoidal curves of HPV6 VLP-L1 adsorbed on aluminum-based particulate adjuvant with mAbs 12H4 and 17D5, either added alone or added simultaneously, based on the fluorescence intensity. The EC_{50} values could be achieved by the software GraphPad Prism 5.0. Quantitative antigenicity analysis of the antigen on adjuvant could be performed specifically for a given epitope or two non-overlapping epitopes using the high content PhenixTM platform for visual analysis and ParadigmTM platform for fluorescence quantitative analysis.

Table 1. Conformational sensitivity and other functional characteristics of a panel of murine anti-HPV6 monoclonal antibodies

mAb*	Isotype	Binding to native VLPs EC ₅₀ , ng/mL	Binding to denatured VLPs EC ₅₀ , ng/mL	Conformational sensitivity index (CSI) †	Neutralization titer IC ₅₀ , ng/mL	Hemagglutination inhibition titer , µg/mL
I. Very sensitive (Relative EC₅₀ >15)						
18D2-3	IgG2b	1.4	>1000	>690	0.57	13
10H1	IgG1	9.0	>1000	>110	0.42	13
11B10	IgG1	8.8	>1000	>110	0.68	8.9
12H4	IgG1	16	>1000	>63	0.35	20
5D3	IgG2b	20	>1000	>51	3.5	8.9
17D5	IgG2a	54	>1000	>19	17	45
II. Sensitive (Relative EC₅₀, 2.0 ~ 15)						
1A2	IgG2b	70	>1000	>14	63	13
15F7	IgG1	130	>1000	>7.8	23	>45
3F8	IgG2b	190	>1000	>5.2	>1000	13
10G7	IgG1	280	>1000	>3.5	280	45
III. Insensitive (Relative EC₅₀, 0.5 ~ 2.0)						
6G7	IgG1	380	530	1.4	>1000	45
18E4	IgG1	44	44	1.0	>1000	20
8F4	IgG2a	5.8	3.0	0.50	>1000	>45
IV. Preferring denatured L1 (Relative EC₅₀ < 0.5)						

2D4	IgG2a	23	6.4	0.30	>1000	>45
3F3	IgG2a	120	15	0.12	>1000	>45

* These mAbs, recognizing different epitopes on the HPV capsid protein, were divided into four groups based on their sensitivity to the conformation of recombinant HPV 6 VLPs.

† The conformational sensitivity index (CSI) refer to the relative EC_{50} (rEC_{50}) for each mAb was calculated by dividing the EC_{50} obtained for binding the denatured HPV6 VLPs by that for the native HPV6 VLPs in a pair of direct binding ELISAs with different coating conditions for the same antigen.

Fig. 1

Fig. 2

(a)

(b)

Fig. 3