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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.

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 HPV6 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,

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.

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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 [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, 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 *Escherichia 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 serilly 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 EC_{50} 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 (IC_{50}, 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 HPV6 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

L1 protein 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 based adjuvants sized at 10–20 μm [18–21]. 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 HPV6 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 b). 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 S(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 epitopes on the viral capsid to prevent the viral infection in the assay.

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