Trivalent Human Papillomavirus (HPV) VLP vaccine covering HPV type 58 can elicit high level of humoral immunity but also induce immune interference among component types

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Both Human Papillomavirus (HPV) type 16/18 bivalent vaccine and type 16/18/6/11 quadrivalent vaccine have been proved to be safe and effective, and licensed for public use. However, these two vaccines do not quite match the distribution of HPV types in China, Southeast Asia and Latin America, where HPV 58 is highly prevalent. Here we produced three types of virus-like particles (VLPs) in baculovirus expression system, formulated a trivalent vaccine containing HPV 16, 18, and 58 L1 VLPs and examined its in vitro neutralizing titers. This vaccine could induce high level and long-term humoral immunity against the component types. But immune interference was observed when comparing type specific neutralizing antibody levels induced by trivalent vaccine to those by corresponding monovalent vaccines. This kind of interference would become more obvious when formulating more types of VLPs into multivalent vaccines, but could be greatly overcome by decreasing the antigen dosage and adding a proper adjuvant.

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1. Introduction

High risk types of Human Papillomavirus (HPV) have been proved to be the etiologic agents of cervical cancer [1], which ranks as the second most frequent cancer in women all over the world. Among all HPV types, HPV 16 and HPV 18 are two of the most prevalent types in cervical cancer worldwide. However, the distribution of other HPV types varies in different regions. In Asia, HPV 58 is the third most prevalent type in cervical cancer [2], especially in China, where the prevalence of HPV 58 is as high as 7.2% [3]. Besides, in South America and Oceania, the prevalence of HPV 58 in high-grade lesions patients are 8.4% and 10.4%, respectively, which makes HPV 58 as the second most prevalent type in those patients [4]. HPV58 is also the second most common type in both high-grade lesions and low-grade lesions in Central America and Asia [2,4].

The major capsid protein (L1) of HPV can self-assemble into virus-like particles (VLPs) [5,6]. L1 VLPs are highly immunogenic and are considered to be an ideal candidate for prophylactic vaccines. However, the neutralizing antibodies induced by L1 VLPs are predominantly type specific with the exception of the closely related types (about 85% L1 amino acid identity) which have weak cross-reactivities [7–13]. Furthermore, vaccination with VLPs or virions derived from one animal Papillomavirus type does not protect against experimental infections from different animal types [14–16]. Currently licensed HPV 16/18/6/11 quadrivalent and HPV 16/18 bivalent HPV L1 VLPs vaccines contained two most prevalent types in cervical cancer (HPV 16 and 18). The clinical trials of HPV 16/18 bivalent vaccine showed that this vaccine could partially protect against incident infection with HPV 45 and 31, but the vaccine efficacy against HPV 58 was very low [17,18]. Analysis of HPV 16/18/6/11 quadrivalent vaccine showed that it only had a 27% efficacy in preventing CIN 1–3 associated with nonvaccine types [19]. Thus, it is of great importance to develop prophylactic vaccines containing HPV 58 to meet the demands of HPV 58 prevalent regions.

Many reports demonstrated that immunization with multiple antigens could induce immune interference [20–29]. However, to our knowledge, it remains unclear whether the immune interfer-
ence happens on multivalent HPV L1 VLP vaccines. In this study, we developed HPV 16/18/58 trivalent L1 VLP vaccines and compared the type specific neutralizing antibody levels induced by the trivalent vaccine with those by corresponding monovalent vaccines. We found that the HPV 58 containing trivalent vaccine could induce high titers of HPV specific antibodies against all component types, and that the type specific neutralizing antibody levels were interfered by co-immunized antigens.

2. Materials and methods

2.1. Preparation of L1 genes

HPV 16, 18, 58 L1 genes were codon optimized according to the codon usage bias of Sf9 cells. All modification was made according to Table 1. Optimized genes were synthesized by Sangon Corp. (Shanghai, China) and constructed into pFastBac I (Invitrogen). Optimized genes were uploaded to Genbank, and the accession numbers are GU556964 (HPV 16 L1), GU556965 (HPV 18 L1) and GU556966 (HPV 58 L1), respectively. HPV 6 and 11 L1 genes were obtained by our lab previously [30–32].

2.2. Production and purification of VLPs

L1 genes were expressed in baculovirus expression system and purified by CsCl ultracentrifugation as described previously [33]. The purity of L1 was evaluated by SDS-PAGE with Coomassie blue staining. VLPs were further verified by transmission electron microscopy (TEM) [31].

2.3. Vaccine formulations

We formulated pentavalent, trivalent, bivalent and monovalent vaccines with high and low dose of antigens, with or without Aluminium adjuvant according to Table 2. High dose vaccines contained 5 μg VLPs of each type, while low dose vaccines contained 0.1 μg VLPs of each type. The adjuvant we used here is Aluminium hydroxide (Sigma–Aldrich). All the vaccines were formulated in a total volume of 100 μl in PBS. The control vaccine contained 100 μl PBS only.

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2.4. Immunization of Balb/c mice

Balb/c mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, and kept in the animal facility of the Institute of Basic Medical Science, Chinese Academy of Medical Sciences. All experimental protocols were approved by the Institutional Animal Care and Use Committee. Experiment groups immunized with different vaccine formulations were listed in Table 2. Briefly, for the long-term experiments, mice (n = 4 per group) were immunized intramuscularly with Trivalent-1 vaccine, Mono 16, 18, 58 vaccines or PBS, respectively at week 0, 2, 4, and were given an extra boost at week 52. Serum samples were collected at 2 week’s interval for first 12 weeks and then at 10 week’s interval until week 52. Samples were also collected 2 weeks after the extra boost. All samples were analyzed by ELISA for type specific antibody responses [30]. Serum samples collected at week 4 and 6 were analyzed for neutralizing antibody level (pseudo-neutralization assay). For dose adjustment experiments, mice (n = 4 per group) were immunized intramuscularly with Trivalent-1, Trivalent-2, Mono 16, Mono 18 and Mono 58 vaccines, respectively at week 0, 2, 4. Serum samples collected at week 4 and 6 were analyzed by pseudo-neutralization assay. For low dose experiments, mice were immunized with Pentavalent-low, Trivalent-low, Bivalent-low and corresponding low dose monovalent vaccines with or without Aluminium adjuvant at week 0 and 2. Serum samples collected at week 4 were examined by pseudo-neutralization assay. For separate inoculation experiments, mice (n = 4 per group) were immunized intramuscularly with Trivalent-1, Separate 16, Separate 18, Separate 58 and corresponding monovalent vaccines, respectively. Trivalent-1 vaccine and monovalent vaccines were inoculated at one site, while “Separate” vaccines were inoculated at two sites. “Separate 18” meant that HPV 18 L1 VLPs were injected at left leg, while other two types at right leg. “Separate 16” indicated that HPV 16 L1 VLPs were injected at left leg separately, while HPV 18 L1 VLPs and HPV 58 L1 VLPs were mixed and injected at right leg. “Separate 58” also had similar meaning.

![Fig. 2. The ELISA titer of anti-HPV 16 (A), anti-HPV 18 (B) and anti-HPV 58 (C) total IgG antibodies in trivalent and corresponding monovalent groups during 1-year observation. Mice were immunized at week 0, 2, 4 and 52. The arrows indicated the immunization time points. The serum IgG titers were calculated from log_{10} transformed values. Results were expressed as the mean ± SE (n = 4).](image URL)
Fig. 3. Neutralizing antibodies against HPV 16, HPV 18 and HPV 58 induced by pentavalent, trivalent and monovalent vaccines. Groups of mice (n = 4) were immunized with pentavalent, trivalent, and corresponding monovalent vaccines at 0, 2 and 4 weeks. The neutralizing antibody titers were detected 2 weeks after the second immunization (A) and 2 weeks after the third immunization (B). The neutralizing antibody titers were calculated from log10-transformed values. Percent infection inhibition against PsV of component types were detected 2 weeks after the second injection at a dilution of 1:10,000 (C) and 2 weeks after the third injection at a dilution of 1:50,000 (D). The statistically significant differences (using one-way ANOVA) were indicated by: *P < 0.05; **P < 0.01.

2.5. Pseudovirus neutralization assay

2.5.1. Preparation of pseudoviruses

Production of pseudoviruses were produced according to previous studies [34–36]. To be specific, 293TT cells (provided by Prof. John Schiller) were co-transfected with L1, L2 expression vectors (p16SHELL and p18SHELL, provided by Prof. John Schiller; p58SHELL, provided by Prof. Tadahito Kanda) and reporting plasmid (pEGFP-N1, Clonetech). Cells were harvested 48 h after transfection, lysed with cell lysis buffer [0.5% Brij58 (Sigma–Aldrich), 0.2% Benzonase (Merck), 0.2% Plasmid Safe ATP-Dependent DNase (EPICENTRE Biotechnologies) DPBS-Mg solution], and incubated at 37 °C for 24 h. The cell lysate was extracted with 5 M NaCl solution, and then examined for the titers. The titers of pseudoviruses were defined as the dilution factors at TCID50 (tissue culture infective dose). 2000 TCID50/50 μl pseudoviruses were determined as the inoculating dose for neutralization assay.

2.5.2. Detection of sera neutralizing antibodies

293TT cells were incubated at 37 °C in 96-well plate at a density of 1.5 × 10⁴ cells per well for 6 h. Sera were diluted according to a 5-fold dilution. Pseudoviruses were diluted to 2000 TCID50/50 μl. 60 μl pseudoviruses diluent and 60 μl serially diluted sera were mixed thoroughly and incubated at 4 °C for 1 h in a dilution plate. The negative control was prepared by mixing of 60 μl pseudoviruses diluent and 60 μl culture media. 100 μl of mixture per well were added to the cell culture plate and incubated at 37 °C for 72 h. Cells were digested with trypsinase and transferred to cell sorting tube. The fluorescent cells were detected by FACS (fluorescence activated cell sorting). The percent infection inhibition was calculated with following formula:

\[
\text{Percent infection inhibition(%) = } \left(1 - \frac{\text{the proportion of fluorescent cells in the sera incubated sample}}{\text{the proportion of fluorescent cells in the negative control sample}}\right) \times 100
\]

The endpoint titers were calculated as the base 10 logarithm of the highest sera dilution with percent infection inhibition higher than 50%.

Every sample was detected at least three times, and the values presented here were calculated as the means of three repeated tests.

2.6. Statistical analysis

Statistical analysis was performed by one-way ANOVA using SPSS software. Values were compared between different groups. P values <0.05 were considered to be statistically significant.
Fig. 4. Neutralizing antibody titers and percent infection inhibition in dose-depending experiments. Groups of mice (n = 4) were immunized with trivalent 1, trivalent 2, and three monovalent vaccines at 0, 2 and 4 weeks. The neutralizing antibody titers were detected 2 weeks after the second immunization (A) and 2 weeks after the third immunization (B). Percent infection inhibition against PsV of component types were detected 2 weeks after the second injection at a dilution of 1:10,000 (C) and 2 weeks after the third injection at a dilution of 1:50,000 (D). The statistically significant differences (using one-way ANOVA) were indicated by: *P < 0.05; **P < 0.01; ***P < 0.001.

3. Results

3.1. Production of HPV 16, 18 and 58 L1 VLPs

The codon optimized L1 genes were expressed efficiently in Sf9 cells, and the expression levels were about 2-fold higher than those of the wild type genes (data not shown). The L1 containing fractions of CsCl ultracentrifugation were examined under electron microscopy, and were confirmed to be fully assembled VLPs (Fig. 1A–C). The purities of HPV 16, 18, 58 L1 VLPs were analyzed by SDS-PAGE with Coomassie blue staining, and only one band was observed when 10 μg of VLPs were loaded each lane (Fig. 1D).

3.2. Antibody responses to trivalent and monovalent vaccines

To investigate whether co-immunization of different types of VLPs will have some influence on serum antibody levels, we immunized mice with Trivalent-1 vaccine and corresponding monovalent vaccines. Mice sera were collected and tested by VLP-ELISA and pseudovirus neutralization assay. The results of VLP-ELISA (Fig. 2) showed that trivalent vaccine and monovalent vaccines could induce high level of circulating antibodies against component types. The antibody titers could reach to $4 \times 10^4$ to $8 \times 10^4$ 2 weeks after the third immunization. No statistical differences were observed between trivalent group and corresponding monovalent groups ($P > 0.05$ using one-way ANOVA). The type specific antibody level gradually declined with time, but still could remain above $10^3$ for at least 1 year. At week 52, mice were boosted with an extra injection. Two weeks after that, the serum antibodies increased to or exceeded the highest level after previous three injections.

To evaluate the protection ability of multivalent vaccines, we tested the in vitro neutralizing antibody titers of the sera collected 14 days after the second and the third injections by pseudovirus neutralization assay. As illustrated in Fig. 3, the neutralizing antibody levels of trivalent and monovalent vaccine immunized groups could reach to $2 \times 10^3$ to $10^4$ after the second injection and $10^4$ to $2.5 \times 10^5$ after the third injection, respectively. Different from the results of ELISA, we observed that there were significant differences between the anti-HPV 58 neutralizing antibody levels of trivalent group and HPV 58 monovalent group ($P < 0.05$, using one-way ANOVA) after the second injection (Fig. 3A), and also between the anti-HPV 18 neutralizing antibody levels of trivalent group and HPV 18 monovalent group ($P < 0.05$, using one-way ANOVA) after the third injection (Fig. 3B). To analyze the differences between groups more intensively, we also compared percent infection inhibition of sera after second and third injections at dilutions of 1:10,000 and 1:50,000, respectively. At 1:10,000 dilution, the HPV 18 pseudovirus infection inhibition of trivalent group was significantly lower than that of HPV 18 L1 monovalent group (Fig. 3C); there was also a significant difference between the HPV 58 pseudovirus infection inhibition of trivalent group and that of HPV 58 L1 monovalent group (Fig. 3C). Similarly, at 1:50,000 dilution, the infection inhibitions of trivalent group against all three types were significantly
lower than those of corresponding monovalent groups (Fig. 3D). From these results we can conclude that VLPs of one HPV type can interfere with the induction of neutralizing antibodies to VLPs of other types.

Then we investigated whether adding new types of VLPs will induce more obvious immune interference. We formulated a pentavalent vaccine containing HPV 16, 18, 58, 6, 11 L1 VLPs, and compared the neutralizing antibody levels of pentavalent group with trivalent and monovalent groups. We observed that HPV 16, 18, 58 specific neutralizing antibody titers were even lower in pentavalent group than in trivalent group both after the second and third injections (Fig. 3A and B), and the interference on percent infection inhibition was also more severe in pentavalent group (Fig. 3C and D).

3.3. Influence of vaccine formulation on immune interference

To examine whether the immune interference can be compensated by adjusting the amount of antigens in vaccine, we formulated two types of trivalent vaccines. Trivalent-1 vaccine contained same amount of all three types of VLPs (5 μg of each type), while in Trivalent-2 vaccine the dose of HPV 58 VLPs was doubled (Table 2). Mice were injected with these two types of trivalent vaccines and corresponding monovalent vaccines, respectively. As demonstrated in Fig. 4A and B, significant differences were observed between the anti-HPV 16 neutralizing antibody levels of Trivalent-2 group and Mono 16 group; and also between the anti-HPV 18 neutralizing antibody levels of Trivalent-2 group and Mono 18 group. But there were no statistically significant differences between the anti-HPV 58 neutralizing antibody levels of Trivalent-2 group and Mono 58 group. We also compared the percent infection inhibition of sera from different groups at different time and dilutions. The sera collected 2 weeks after the second and third injections were detected at dilutions of 1:10,000 and 1:50,000, respectively (Fig. 4C and D). We observed that as for percent infection inhibition of HPV 16 and HPV 18 pseudovirus, the differences between Trivalent-1 group and corresponding monovalent groups were less significant than those between Trivalent-2 group and monovalent groups. However, when comparing percent infection inhibition of HPV 58 pseudovirus, difference between Trivalent-1 group and Mono 58 group was more significant than that between Trivalent-2 group and Mono 58 group. These results indicated that the strategy of increasing the amount of HPV 58 VLPs in trivalent vaccine would have some influence on antibody production against all three component types, and by that strategy the immune interference on HPV 58 could only be partially compensated, while the immune interference on other types would be magnified consequentially.

Since 5 μg is a relatively large VLP dose for a mouse, we formulated pentavalent, trivalent, bivalent and monovalent vaccines with only 0.1 μg VLPs of each type (Table 2), and examined the serum samples collected at 2 weeks after second injection to determine whether immune interference still happened. As illustrated in Fig. 5A, no significant difference was observed between neutralizing antibody titers of multivalent groups and corresponding monovalent groups, but mean titers dropped slightly with the increase of valency. When comparing percent infection inhibition of these groups, similar results were also observed (Fig. 5B). Thus we could conclude that immune interference between co-immunized types of VLPs would become less significant when lower doses were used, but it would be boosted up with the increase of vaccine valency.

3.4. Influence of separate site inoculation on immune interference

To determine whether immunizing different types of VLPs at different sites would overcome the interference among types, mice were injected with one type of VLPs on one leg and two types on the other. Then the neutralizing antibody titers and percent infection inhibition were detected 2 weeks after second and third injections. When comparing the neutralizing antibody titers, we did not see much effect of immunization at multiple sites (Fig. 6A and B). However, when comparing percent infection inhibition, we found that the immune interference was decreased to some extent, but still could not be avoided completely (Fig. 6C and D).

3.5. Effect of adjuvant on overcoming immune interference

Since certain adjuvants are formulated into current commercial VLP vaccines, it is important to determine whether interference observed here could be overcome by adding a proper adjuvant to vaccines. In this study, we produced pentavalent, trivalent, bivalent and three monovalent low dose vaccines (containing 0.1 μg VLPs of each type) adjuvanted with Aluminium hydroxide (Table 2) and vaccinated mice intramuscularly. Neutralizing antibody titer and percent infection inhibition were examined. As presented in Fig. 7,
T. Zhang et al. / Vaccine 28 (2010) 3479–3487

Fig. 6. Neutralizing antibody titers and percent infection inhibition in separate immunization experiments. Groups of mice (n = 4) were immunized with three types of VLPs mixed together or separated at two sites intramuscularly as described in Section 2. The neutralizing antibody titers were detected 2 weeks after the second immunization (A) and 2 weeks after the third immunization (B). Percent infection inhibition against PsV of component types were detected 2 weeks after the second injection at a dilution of 1:10,000 (C) and 2 weeks after the third injection at a dilution of 1:50,000 (D). The statistically significant differences (using one-way ANOVA) were indicated by: * P < 0.05; ** P < 0.01; *** P < 0.001.

HPV16 neutralizing antibody titers of all groups were almost the same, and the immune interference on HPV 16 pseudovirus infection inhibition was not observed either. As for HPV 18 and HPV 58, no significant differences were observed among neutralizing antibody levels of all groups, but mean titers and mean percent infection inhibition of multivalent groups were slightly lower than those of monovalent groups (Fig. 7).

4. Discussion

Based on the results we have, we can conclude that HPV trivalent VLP vaccine could induce high level of humoral immunity against component types. There was no significant difference between trivalent group and monovalent groups when comparing their ELISA antibody titers against corresponding types, but when comparing their neutralizing antibody levels measured by in vitro pseudovirus neutralization assay, there were significant differences between trivalent group and monovalent groups. ELISA could detect all sera antibodies binding to coating antigens, including neutralizing and non-neutralizing antibodies. Neutralizing antibodies are mainly against conformational epitopes on virus surface, and are usually type specific; while non-neutralizing antibodies are mostly against linear epitopes on virus surface, and some of them have broad cross-reactivity[37–45], even between distantly related types such as HPV 16 and 18[35]. This kind of non-neutralizing cross-reactivity would provide some portion of positive signals in ELISA when detecting sera from multivalent immunized groups[46]. This might give an explanation of the difference between ELISA and neutralizing assay.

Neutralizing antibody titer detection is discontinuous and gaps between detecting points increase with sera dilutions. On the contrary, percent infection inhibition at a certain dilution is a continuous parameter, which provides a more detailed result when comparing two groups at a proper dilution. In our results, percent infection inhibition and neutralizing antibody titer reflected almost the same trend: multiple VLPs co-immunization could elicit high level of neutralizing antibodies, but the neutralizing antibody levels or percent infection inhibition of trivalent groups were lower than those of corresponding monovalent groups.

A clinical study from Garland and Steben showed that HPV 16/18/6/11 quadrivalent vaccine and HPV 16 monovalent vaccine could induce same level of anti-HPV 16 antibodies[47]. Since the vaccines they used were formulated with relatively low dose of VLPs and were adjuvanted with Aluminium salts, these results were in accordance with our observation in adjuvant experiments. In another study, Gasparic et al. co-immunized different types of Papillomavirus (PV) L1 DNA vaccines in mice, and observed interference between types, however, the interference they observed was due to differences of expression level[48]. In our study, VLPs were used as antigens and influences at expression level could be ruled out, so the interference we observed indeed occurred after antigens contacted with immune system.

Immune interference has been reported in many other vaccines. A lot of studies in co-immunization revealed that
immune interference could happen in both antigen specific T cell responses and B cell responses [20–27,29,46,49–55]. Immune interference could occur between different variants of homologous epitopes [24,26,27]: and it could also happen when heterogenous antigens were immunized together [25,54]. The mechanism of immune interference is unclear yet. Different antigens may be interfered at different degree. A study on co-immunization of recombinant hepatitis B surface antigen (HBsAg) and inactivated hepatitis A virus (HAV) suggested that a stronger immunostimulant might be interfered less [25]. Many studies on polysaccharide conjugated vaccines showed that the extent of reduction of immune response was related to the amount of protein analogues contained in vaccines [24,26]. Researches on foot rot vaccines, dengue vaccines and to the amount of protein analogues contained in vaccines that the extent of reduction of immune response was related [24,26]. Researches on foot rot vaccines, dengue vaccines and to the amount of protein analogues contained in vaccines that the extent of reduction of immune response was related [24,26].

In our study, three HPV types all suffered from immune interferences at different degree. We increased the amount of HPV 58 VLPs, and the immune interference on HPV 58 was partially overcome. However, the antibody responses to HPV 16 and 18 were reduced obviously. These results suggested that increasing the dosage of one antigen could reduce immune interference on it but increase immune interference on other co-immunized antigens. Immune interference could be diminished when one of the three antigens was inoculated separately, suggesting that increasing dosage or types of antigens at one site of injection might lead to more severe immune interference between component types. Besides, we found that the pentavalent group had relatively more severe immune interference than trivalent group, and that the immune interference would be decreased when decreasing the dosage of each VLP component and adding Aluminium adjuvant. Taken together, our results might provide possible strategies for developing multivalent VLPs vaccines covering more HPV types.

Acknowledgements

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References


Fig. 7. Neutralizing antibody titers and percent infection inhibition in low dose vaccination with Aluminium adjuvant. Groups of mice (n = 4) were immunized with pentavalent-low-Al, trivalent-low-Al, bivalent-low-Al, and three mono-low-Alum vaccines at 0 and 2 weeks. The neutralizing antibody titers were detected 2 weeks after the second immunization (A) and percent infection inhibition against PsV of component types were detected 2 weeks after the second injection at a dilution of 1:2000 (B).


