Journal of Microbiology (2019) Vol. 57, No. 9, pp. 821–827
DOI 10.1007/s12275-019-9308-z
eISSN 1976-3794
pISSN 1225-8873

IgG and IgM responses to human papillomavirus L1 virus-like particle as a function of dosing schedule and vaccine formulation

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(Received Jun 20, 2019 / Revised Jun 29, 2019 / Accepted Jul 1, 2019)

Most commercialized virus-like particle (VLP) vaccines use aluminum salt as adjuvant, even though VLPs provoke adequate antibody responses without adjuvant. We do not have detailed knowledge of how adjuvant affects the profile of anti-VLP antibodies. Meanwhile, there is evidence that differences between vaccination protocols influence the glycosylation of antibodies, which may alter their effector functions. In the present study a murine model was used to investigate the effects of dosing schedule and adjuvant on the antibody profiles and glycosylation levels of antigen-specific antibody responses to human papillomavirus type 16 L1 (HPV16 L1) VLPs. Mice received subcutaneously 2,000 ng of antigen divided into 4 or 7 doses. The HPV16 L1 VLPs elicited > 4 $\log_{10}$ anti-HPV16 L1 IgG titers without adjuvant, and aluminum hydroxide as adjuvant increased IgG titers 1.3- to 4-fold and reduced the anti-HPV16 L1 IgG2a / anti-HPV16 L1 IgG1 ratio value (use of aluminum hydroxide reduced the ratio of the IgG2a). Immunization with HPV16 L1 VLPs in combination with Freund's adjuvant enhanced IgG titers 5- to 12-fold. Seven-dose immunization markedly increased anti-HPV16 L1 IgM titers compared to four-dose immunization, as well as increasing the proportion of glycosylated antibodies. Our results suggest that antibody glycosylation can be controlled immunologically, and IgG and IgM profiles and glycosylation profiles of the vaccine-induced antibodies can be used as indicators reflecting the vaccine characteristics. These results indicate that the HPV16 L1 VLP dosing schedule can affect the quality of antigen-specific antibody responses. We suggest that dosing schedules should be noted in vaccination protocols for VLP-based vaccines.

Keywords: virus-like particles, human papillomavirus, glycosylation, antibody profile, adjuvant

Introduction

Virus-like particles (VLPs) are produced by recombinant technology and mimic the shape of the virus (Fuenmayor *et al.*, 2017; Huang *et al.*, 2017). It is difficult to maintain the original antigenic properties of the virus in vaccines produced by traditional methods using the virus itself as antigen, because the virus undergoes inactivation or weakening to destroy its pathogenicity (Wang and Roden, 2013); this may distort tertiary epitope structure, which has an important role in inducing neutralizing antibodies. Well-constructed VLPs can provoke excellent immune responses because they do not need to be inactivated or attenuated. Therefore, VLPs are recognized as an ideal form of vaccine, and are attracting much attention as next-generation vaccine platforms (Kushnir *et al.*, 2012; Rohovie *et al.*, 2017).

Our immune system has evolved to efficiently recognize structures such as viruses (Xiang *et al.*, 2006; Gao *et al.*, 2018). This recognition mechanism is a great advantage when using VLPs as vaccine antigens. They are easily detected by antigen presenting cells (APCs) and their repetitively arranged epitopes cause strong B-cell activation, which provokes more powerful humoral immune responses than those obtained from split antigens (Riitho *et al.*, 2017). The most important advantage of VLPs is that, unlike split antigens, they can display neutralizing epitopes dependent on their tertiary structure (Kim and Kim, 2017). There is thus increasing evidence that VLP-based vaccines are able to induce adequate levels of antibody response without adjuvant because of their superior immunogenicity (Kim *et al.*, 2012, 2018).

Despite these advantages, currently licensed VLP-based vaccines such as those against human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis E virus (HEV), hepatitis A virus (HAV), and influenza A use aluminum salts as adjuvant (Cimica and Galarza, 2017). The use of these vaccines along with aluminum-based adjuvant has historically been recognized as an important factor in the development of vaccines. Aluminum salts promote the adsorption of antigens and their efficient transfer to APCs; indeed they were the first adjuvants to be developed (HogenEsch *et al.*, 2018). Since the 1920s, numerous doses of vaccines have been administered to humans and animals, and much data on the effectiveness and safety of aluminum salts has been accumulated (Clapp *et al.*, 2011). Given this long history of the use of aluminum-based adjuvant, it is natural that VLP vaccine developers consider using it in their vaccine formulations even if VLPs might not need such adjuvant. We actually have little knowledge of how aluminum salts affect the antibody profile resulting from immunization with VLPs.

The glycosylation of antibodies affects their structural stability and function (Zheng *et al.*, 2011), and recent studies suggest that vaccine formulations and dosing schedules have an important influence on the glycosylation of antibodies as

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well as on their isotype profiles (Isa *et al.*, 2002; Selman *et al.*, 2012). Nevertheless studies of the immunogenicity of VLP-based vaccines tend not to distinguish between adjuvant-containing formulations and adjuvant-free formulations. In the present study, the anti-HPV type 16 L1 (HPV16 L1) VLP antibody responses to formulations containing and not containing adjuvant were compared in mice. Four mouse groups were designed to investigate the anti-HPV16 L1 VLP antibody responses. V1 and V2 group received seven and four doses of HPV16 L1 VLP in the absence of adjuvant, respectively (Fig. 1). V3 and V4 group received four doses of the VLP in combination with aluminum hydroxide and Freund's adjuvant, respectively (Fig. 1). In the present study, IgG and IgM profile of the anti-HPV16 L1 antibody as well as IgG2a and IgG1 profile of that were investigated. In addition, changes in glycosylation pattern as a function of vaccine formulation and dosing schedule were investigated.

Materials and Methods

Preparation of HPV16 L1 VLPs

HPV16 L1 VLPs were prepared as described previously. The L1 protein was produced in *Saccharomyces cerevisiae*, purified by heparin chromatography and dialyzed against phosphate buffer containing 0.325 M NaCl pH 7.2. The HPV16 L1 was stored at -75°C until use (Kim *et al.*, 2010).

Immunization with HPV16 L1 VLPs

Four different formulation of HPV16 L1 VLPs were prepared, and mice received four or seven doses (see Fig. 1 and Table 1). Six-week-old female mice were purchased from Orientbio (Korea), divided into groups of 10 mice and acclimated for 1 week prior to immunization. Mouse experiments were performed in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals and with the Chung-Ang University Guidelines for Animal Experiments. Experiments were approved by the University Committee for animal experiments (approval no. 2018-00075). Mouse sera were collected on days 39 and 52 (Fig. 1) and stored at -75°C. A phosphate-buffered saline (PBS) control group received 0.1 ml PBS, and its dosing schedule was the same as that of groups V2, V3, and V4. Aluminum hydroxide and complete and incomplete Freund's adjuvant were purchased from Sigma.

Titration of anti-HPV16 L1 IgG and IgM

Antibody titers were determined by end-point titration based on the enzyme-linked immunosorbent assay (ELISA) used in our previous study (Kim *et al.*, 2018) with modifications. 96-well immune plates (SPL, South Korea) were coated overnight with purified HPV16 L1 VLPs in PBS (50 ng/well) at 4°C and blocked with 5% skim milk (Sigma) in PBS containing 0.1% Tween 20 (PBST). Mouse sera were serially diluted (two- or three-fold) and incubated for 2 h at 37°C. Anti-HPV16 L1 IgG or IgM bound to the coated HPV16 L1 VLPs was detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Bethyl laboratories) or HRP-conjugated anti-mouse IgM (Bethyl Laboratories). Color reactions were developed with *o*-phenylenediamine (Sigma).

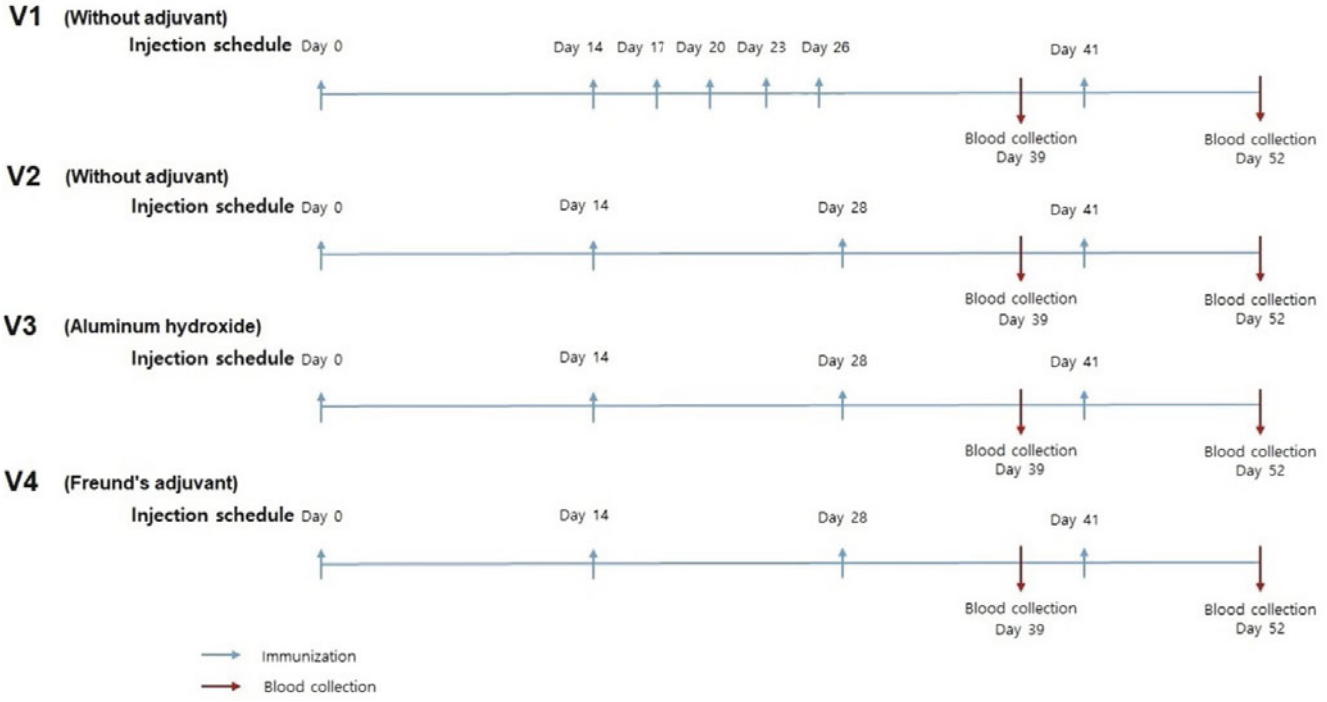

Fig. 1. Schematic diagram of dosing schedules. The V1 group received subcutaneously the HPV16 L1 VLPs without adjuvant on days 0, 14, 17, 20, 23, 26, and 41. The other groups received the VLPs with or without adjuvant on days 0, 14, 28, and 41. Blood was collected on days 39 and 52. Details of formulations, dosages and dosing schedules are given in Table 1.

Table 1. Descriptions for mouse immunization groups. All mouse groups received total 2000 ng of HPV16 L1 VLPs divided into 4 or 7 doses.

Group	Number of mice	Route of immunization	Number of immunization	Adjuvant	Dosing amount of HPV16 L1 VLP	Dosing schedule	Blood collection
V1	10	Subcutaneous	7	None	1st: 1000 ng 2nd – 6th: 100 ng 7th: 500 ng	1st: day 0 2nd-6th: day 14, 17, 20, 23 and 26 7th: 41	Day 39 Day 52
V2	10	Subcutaneous	4	None	1st – 4th: 500 ng	1st: day 0 2nd: day 14 3rd: day 28 4th: day 41	Day 39 Day 52
V3	10	Subcutaneous	4	Aluminum hydroxide: 200 μg / dose	1st – 4th: 500 ng	1st: day 0 2nd: day 14 3rd: day 28 4th: day 41	Day 39 Day 52
V4	10	Subcutaneous	4	Freund's adjuvant[a]	1st – 4th: 500 ng	1st: day 0 2nd: day 14 3rd: day 28 4th: day 41	Day 39 Day 52

[a] Complete and incomplete adjuvant were used for prime and boost immunization, respectively. The adjuvants were prepared according to the manufacturer's direction

End-point titers were set at optical densities (OD) two-fold the OD of the control serum (PBS-immunized mice)

Titration of anti-HPV16 L1 IgG1 and IgG2a

The procedures for determining anti-HPV16 L1 IgG1 and IgG2a were basically the same as those for the ELISAs above. To detect anti-HPV16 L1 IgG1 and IgG2a bound to the coated HPV16 L1 VLPs, HRP-conjugated anti-mouse IgG1 (Bethyl Laboratories) and IgG2a (Abcam), respectively, were used as secondary antibodies.

Determination of relative levels of glycosylated anti-HPV16 L1 antibodies

The enzyme-linked lectin assay (ELLA) described in a previous report (Jin *et al.*, 2016) was used with modification. 96-well immuno plates (SPL) were first blocked with 10% regular fetal bovine serum (rFBS, GenDEPOT) in PBS for 2 h at 37°C. The rFBS was then oxidized with 20 mM sodium metaperiodate (Sigma), prepared as previously described (Kim *et al.*, 2008), at room temperature (RT) for 1 h. The plates were further blocked with 1% oxidized bovine serum albumin (oBSA) prepared in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). HPV16 L1 VLPs were prepared in 1% oBSA in TBS-T, and added to the plates blocked with rFBS and 1% oBSA. Mouse sera were serially diluted in 1% oBSA in TBS-T and reacted with the HPV16 L1 VLPs at 37°C for 2 h. Thereafter biotinylated Concanavalin A (1 μg/ml, ConA, Vector Laboratories) or lectins prepared in TBS-T were added to detect glycosylation of the anti-HPV16 L1 antibodies and the reactions were incubated at RT for 1 h. ConA was detected with HRP-conjugated streptavidin (Thermo) at 37°C for 40 min. Color reactions were developed as described above. End-point titers detected with ConA were set at an OD of 1.5-fold the OD of the control serum. Relative levels of glycosylated anti-HPV16 L1 antibody were calculated as: titer determined by ConA ELLA x 100 / anti-HPV16 L1 IgG titer determined by ELISA.

Statistical analysis

The Mann-Whitney U test was used to evaluate differences in glycosylation level of anti-HPV16 L1 antibodies or anti-HPV16 L1 antibody titers between groups, and $P<0.05$ was considered significant. Statistical analyses were performed using the Graphpad prism 5 program.

Results

Use of adjuvant enhances anti-HPV16 L1 IgG response and repetitive doses enhances anti-HPV16 L1 IgM responses

Previous studies have shown that repeated immunization of VLPs more clearly demonstrates antibody response characteristics (Kim *et al.*, 2012; Lee *et al.*, 2013). Therefore, four groups of mice (V1-V4) received subcutaneously 2,000 ng of antigen divided into four or seven doses. V1 received seven doses of HPV16 L1 VLPs without adjuvant, and V2 received four doses (Fig. 1 and Table 1). V3 received four doses of the HPV16 L1 VLPs in combination with aluminum hydroxide and V4 received four such doses in combination with Freund's adjuvant (Fig. 1 and Table 1). Freund's adjuvant is a water-in-oil emulsion that contains heat-killed mycobacteria, the most widely used and effective adjuvant for experimental antibody production (Stills, 2005). Generally, Freund's adjuvant is regarded to gold standard for assessing the antigen-specific antibody response. Anti-HPV16 L1 IgG and anti-HPV16 L1 IgM titers were preferentially measured because IgG and IgM are the primary antibodies that provide protective immunities. Anti-HPV16 L1 antibody titers were analyzed in sera collected on days 39 and 52 (Fig. 2 and Table 2). Overall, the anti-HPV16 L1 IgG titers of V1 and V2 were lower than those of V3 or V4 (Fig. 2A), V4 having the highest titers (Fig. 2A).

IgM is an antibody isotype produced at the beginning of the B-cell immune response and its production decreases as the production of other isotypes including IgG increases. With the exception of V1, whose dosing schedule differed from those of the other groups (Fig. 1), the relative levels of the anti-HPV16 L1 IgM titers of the other groups were the same as those of the anti-HPV16 L1 IgG titers: the IgM titer was high in the order V4, V3, and V2 (Fig. 2B). Therefore when the same dosing schedule is employed the use of ad-

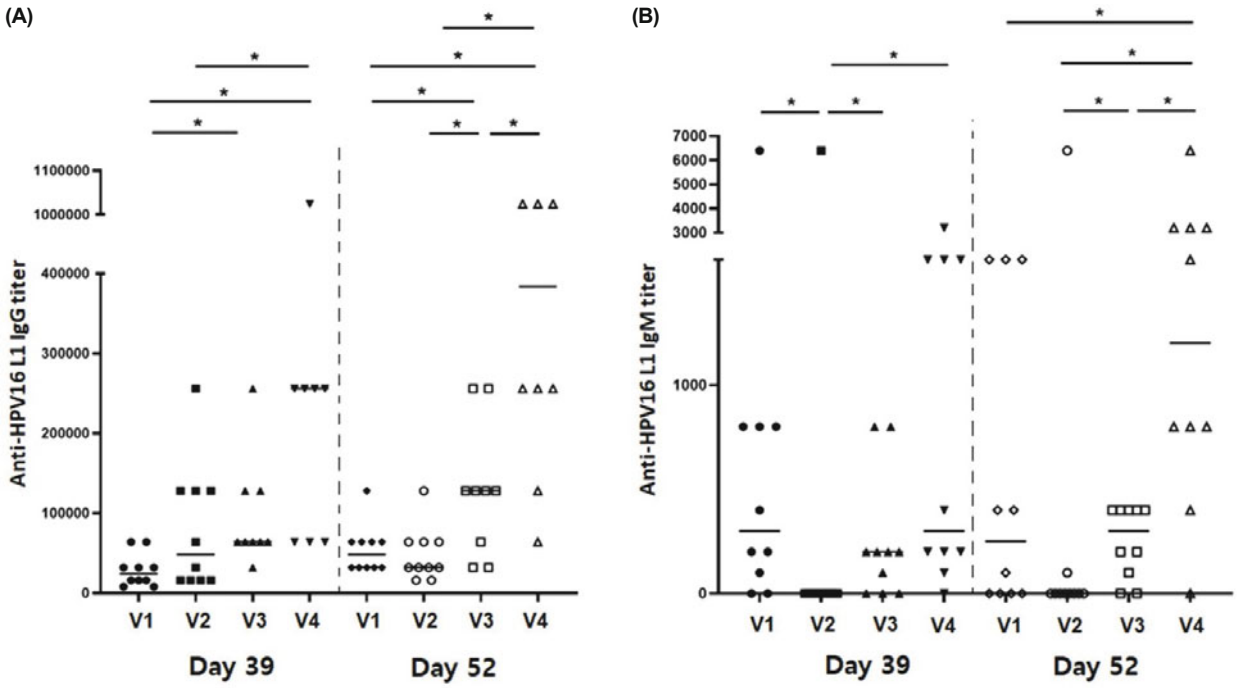

Fig. 2. Anti-HPV16 L1 IgG and IgM titers on days 39 and 52. (A) and (B) are anti-HPV16 L1 IgG and HPV16 L1 IgM titers, respectively. Horizontal bars are median values. Details of anti-HPV16 L1 IgG and IgM are presented in Table 2. * $P < 0.05$ (Mann-Whitney U test). n=10 for all 4 groups.

juvant significantly stimulates anti-HPV16 L1 IgG and anti-HPV16 L1 IgM responses. Meanwhile, the IgM response of V1 appeared to be higher than that of V2 (Fig. 2B), indicating that the dosing schedule has an impact on the IgM response. Details of the anti-HPV16 L1 IgG and IgM titers are shown in Table 2. These results indicate that use of adjuvant is effective to enhance the antibody response to HPV16 L1 VLP and repetitive doses enhance the IgM response to that.

Anti-HPV16 L1 IgG2a and IgG1 responses are affected by use of adjuvant

IgG2a and IgG1 response reflect T helper type 1 (Th1) and T helper type 2 (Th2) response to the antigen (Firacative *et al.*, 2018). In this experiments, therefore, antibody titers to two types of IgG isotypes were measured. Anti-HPV16 L1 IgG2a and IgG1 titers and IgG2a / IgG1 ratios are summarized in Table 3. Overall, anti-HPV16 L1 IgG1 responses were stronger than anti-HPV16 L1 IgG2a responses. V1 and V2 had significantly higher IgG2a / IgG1 ratios (V1, 0.3; V2, 0.3) than V3 (0.037) on day 39, and the IgG2a / IgG1 ratio of V3 was lower than that of V4 (0.1) on day 39 as well as day 52 (V1, 0.1; V2, 0.07; V3, 0.012; V4, 0.07). These results indicate that the antibody response to HPV16 L1 VLPs consists mainly of IgG1, and the use of aluminum hydroxide as adjuvant strengthens this response.

Repetitive doses increase the proportion of glycosylated anti-HPV16 L1 antibody

The glycosylation of antibody has critical roles for their effector functions (Jennewein and Alter, 2017). Therefore, the glycosylation levels of anti-HPV16 L1 antibodies as a function of vaccine formulation and dosing schedule were investigated. We used a modification of our previous method for detecting glycosylation of serum anti-HPV16 L1 antibodies (Jin *et al.*, 2016), and confirmed the specificity of the reaction and its linearity as shown in Fig. 3A and B, respectively. ConA

Table 2. Anti-HPV16 L1 IgG and IgM titers following immunization with HPV16 L1 VLPs

Blood collection	Group	IgG		IgM		IgG : IgM ratio[a]
		Median	25–75 percentile	Median	25–75 percentile	
Day 39	V1	24,000	16,000–32,000	300	125–800	98,765 : 1,235
	V2	48,000	16,000–128,000	0	0–0	100,000 : 0
	V3	64,000	64,000–128,000	200	25–200	99,688 : 312
	V4	256,000	112,000–448,000	300	200–1,600	99,883 : 117
Day 52	V1	48,000	32,000–64,000	250	0–1,300	99,482 : 518
	V2	32,000	32,000–64,000	0	0–0	100,000 : 0
	V3	128,000	80,000–224,000	300	125–400	99,766 : 234
	V4	384,000	256,000–896,000	1,200	800–3,200	99,688 : 312

[a] Sum of IgG and IgM titers was set at 100,000

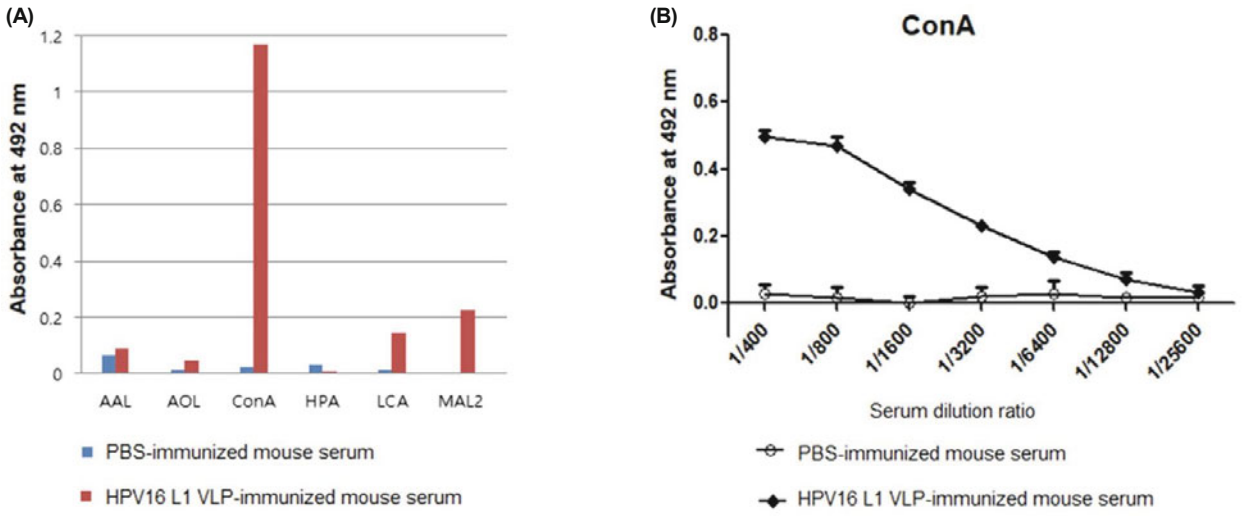

Fig. 3. Specificity and linear response of ConA ELLA. Sera of PBS-treated or HPV16 L1 VLP-immunized mice were reacted with coated HPV16 L1 VLPs, and 6 lectins were applied to detect glycosylations of the anti-HPV16 L1 antibodies. The sera of the PBS or HPV16 L1 VLP-immunized mice were pooled from PBS group (n=10) and V4 group (n=10), respectively. (A) shows the binding specificity of ConA for anti-HPV16 L1 antibodies (a mixture of 10 mouse sera from V4 group was used). Serum of PBS-treated control mice was used as a negative control (a mixture of 10 mouse sera from PBS group was used). (B) illustrates the response of ConA ELLA as a function of serum dilution. A linear response was obtained only in the reaction with serum of HPV16 L1 VLP-immunized mice. AAL, *Aleuria aurantia* Lectin [fucose linked (α-1,6) to *N*-acetylglucosamine or fucose linked (α-1,3) to *N*-acetyllactosamine]; AOL, *Aspergillus oryzae* l-fucose-specific lectin (α1,6-linked fucose); ConA, Concanavalin A (α-linked mannose or high mannose type glycans); HPA, *Helix pomatia* agglutinin (*N*-acetylgalactosamine); LCA, *Lens culinaris* lectin (α-linked mannose α-linked fucose attached to the *N*-acetylchitobiose); MAL2, *Maackia Amurensis* Lectin II (sialic acid attached to galactose in α-2,3 linkage)

gave the strongest response of the six lectins to the mouse anti-HPV16 L1 antibodies (Fig. 3A). Lectins are proteins that recognize specific glycosylation structure, and ELLA is a high throughput strategy which uses 96-well plate platform to detect the glycosylation (Thompson *et al.*, 2011). ConA recognizes α-linked mannose which is abundant in N-linked glycosylation (Maupin *et al.*, 2012). Therefore, ConA can provide superior sensitivity to detect the glycosylation level. As shown in Fig. 4, the V1 anti-HPV16 L1 antibody had a substantially higher level of glycosylation than those of the other groups. Glycosylation of antibody has been suggested to have key functions for antibody-dependent cellular cytotoxicity (ADCC) as well as complement-dependent cytotoxicity (CDC) (Jennewein and Alter, 2017). Therefore, it is thought that the anti-HPV16 L1 antibodies produced by the repetitive doses potentially have superior effector functions. In conclusion, our results indicate that dosing schedule is a critical factor affecting the glycosylation level.

Discussion

In this study we investigated humoral immune responses to HPV L1 VLPs on their own, using different dosing schedules, and to HPV L1 VLPs when aluminum salt or an oil-based adjuvant was used. It was found that use of adjuvant (aluminum hydroxide or Freund's adjuvant) is effective to enhance antibody response to HPV16 L1 VLP and use of aluminum hydroxide strengthens the IgG1 response to that. In particular, we found that repeated doses increase the production of antigen-specific IgM and glycosylated antibodies of the antigen-specific antibody. This findings suggest that the profiles of IgG and IgM and the proportion of the glycosylated antibody can be used as indicators of vaccine characteristics.

Table 3. Anti-HPV16 L1 IgG2a and IgG1 titers following immunization with HPV16 L1 LVP

Blood collection	Group	IgG2a		IgG1		IgG2a /IgG1 ratio	
		Median	25–75 percentile	Median	25–75 percentile	Median	25–75 percentile
Day 39	V1	8,100	675–12,150	36,450	12,150–36,450	0.3 ($P < 0.05^a$)	0.05–0.3
	V2	4,050	450–12,150	12,150	6,075–30,375	0.3 ($P < 0.05^b$)	0.037–1
	V3	900	37.5–4,050	36,450	36,450–109,350	0.037 ($P < 0.05^c$)	0–0.037
	V4	36,450	18,225–36,450	109,350	109,350–328,050	0.1	0.05–0.3
Day 52	V1	12,150	1,350–12,150	72,900	18,225–109,350	0.1	0.037–0.83
	V2	4,050	675–12,150	24,300	6,075–91,125	0.07	0.037–2.27
	V3	750	38–4,050	109,350	54,675–109,350	0.012 ($P < 0.05^c$)	0.000343–0.09
	V4	12,150	6,075–255,150	328,050	164,025–328,050	0.07	0.037–0.83

[a] Compared to V3
[b] Compared to V3
[c] Compared to V4

The structure of glycosylation on antibody is heterogeneous (Higel *et al.*, 2016). Therefore, further study to investigate the glycosylation change as a function of dosing schedule or dosing amount will bring new insights to vaccination strategy.

Meanwhile, no anti-HPV16 L1 IgM activity was detected in V2, which received the same formulation as V1 but with a different dosing schedule (Fig. 2B and Table 2). In a previous study, repeated doses of vaccine prolonged the IgM response (Turner, 1978), and V1 indeed received more repeated doses of HPV16 L1 VLPs than the other groups (Fig. 1). Therefore, the higher relative IgM titer of V1 is thought to be associated with prolonged IgM responses.

It is thought that IgG2a and IgG1 reflect Th1 and Th2 responses, respectively (Firacative *et al.*, 2018). Previously, IgG1 was observed to be the predominant subclass not only after HPV infection but also after vaccination with Cervarix, while IgG2 was a minor response (Scherpenisse *et al.*, 2013). Similarly in our case, IgG1 predominated and there was little IgG2a in any of the groups (Table 3). At the same time the IgG2a / IgG1 ratio in V3 was significantly lower than in V1, V2, and V4, indicating that the use of aluminum hydroxide enhances the Th2 response. This result is consistent with a previous suggestion that the use of aluminum hydroxide shifts the $CD4^+$ T cell response towards Th2 (Marrack *et al.*, 2009).

In attempts to develop an Alzheimer's disease vaccine, Th1/Th2 immune responses to amyloid beta appeared to cause systemic inflammatory autoimmunity that would seriously hinder successful vaccination (Marciani, 2016), and it was suggested that a Th2-biased immune response might be effective in inducing a protective antibody response against amyloid beta (Marciani, 2016). HPV L1 VLPs carries foreign epitopes and our results and previous results indicate that VLPs can elicit Th2-biased antibody responses. Therefore, the use of HPV L1 VLPs in combination with aluminum salt might be an alternative way to develop a prophylactic Alzheimer's disease vaccine. In fact papillomavirus VLPs have been remarkably effective as vaccine platforms for Alzheimer's disease (Chackerian *et al.*, 2006; Zamora *et al.*, 2006).

The glycosylation of antibodies is a critical factor affecting their effector functions, such as binding with complement and Fc receptor (Jennewein and Alter, 2017). Recent evidence indicates that antibody glycosylation can be controlled immunologically (Jennewein and Alter, 2017). It has been suggested that antibodies induced by pathogens or vaccine antigens have glycosylation profiles distinct from those of total circulating antibodies (Mahan *et al.*, 2016). However, how the glycosylation of antibodies elicited by vaccination or pathogen-specific antigens is controlled is largely unknown. In the present study, the dosing schedule was found to be a critical factor affecting the glycosylation profile of the elicited antibody (Fig. 4). Selman *et al.* also examined changes in glycosylation of vaccine antigen-specific IgG upon influenza and tetanus vaccination in Caucasian adults and African children and found that the glycosylation profile of the IgG differed depending on the vaccination time point (Selman *et al.*, 2012), and Guo *et al.* (2005) noted that repeated immunization with ovalbumin increased the fucosylation of ovalbumin-specific IgG in the mouse. Recently it was proposed that different regimens of human immune deficiency virus vaccination induce antigen-specific IgG with different glycosylation profiles, and that the glycosylation of vaccination-induced antibody is programmable and modulated by a variety of inflammatory signals during B cell priming (Mahan *et al.*, 2016). Clearly our results and those of others indicate that the vaccination protocol affects the glycosylation profile of the resulting antibody.

In conclusion, the use of aluminum-based adjuvant influenced IgG2a and IgG1 profiles, and differences in dosing schedule affected IgM profiles and the glycosylation of antigen-specific antibody, upon vaccination with HPV16 L1 VLPs. Changes in the dosing schedule may have a significant effect on the effector functions of the resulting antibodies, and this should be further addressed in future studies, together with antibody glycosylation patterns as a function of vaccination protocol. Such studies should increase our understanding of the immunological mechanisms controlling antibody glycosylation, and may help to develop vaccines of higher quality.

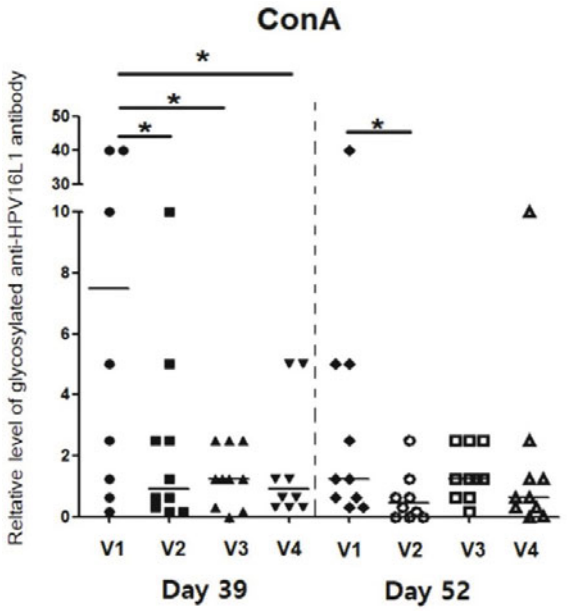

Fig. 4. Relative levels of glycosylated anti-HPV16 L1 antibodies on days 39 and 52. Glycosylation levels were determined using ConA ELLA as described in Materials and Methods. Horizontal bars are median values. * $P < 0.05$ (Mann-Whitney U test). n=10 for all groups.

Acknowledgements

This study is supported by the Graduate Research Scholarship 2018 of Chung-Ang University.

Conflicts of Interest

The authors declared no conflict of interest.

References

Chackerian, B., Rangel, M., Hunter, Z., and Peabody, D.S. 2006. Virus and virus-like particle-based immunogens for Alzheimer's disease induce antibody responses against amyloid-beta without concomitant T cell responses. *Vaccine* **24**, 6321–6331.

Cimica, V. and Galarza, J.M. 2017. Adjuvant formulations for virus-like particle (VLP) based vaccines. *Clin. Immunol.* **183**, 99–108.

Clapp, T., Siebert, P., Chen, D.X., and Braun, L.J. 2011. Vaccines with aluminum-containing adjuvants: Optimizing vaccine efficacy and thermal stability. *J. Pharm. Sci.* **100**, 388–401.

Firacative, C., Gressler, A.E., Schubert, K., Schulze, B., Muller, U., Brombacher, F., von Bergen, M., and Alber, G. 2018. Identification of T helper (Th)1- and Th2-associated antigens of *Cryptococcus neoformans* in a murine model of pulmonary infection. *Sci. Rep.* **8**, 2681.

Fuenmayor, J., Godia, F., and Cervera, L. 2017. Production of virus-like particles for vaccines. *N Biotechnol.* **39**, 174–180.

Gao, Y., Wijewardhana, C., and Mann, J.F.S. 2018. Virus-like particle, liposome, and polymeric particle-based vaccines against HIV-1. *Front. Immunol.* **9**, 345.

Guo, N., Liu, Y., Masuda, Y., Kawagoe, M., Ueno, Y., Kameda, T., and Sugiyama, T. 2005. Repeated immunization induces the increase in fucose content on antigen-specific IgG N-linked oligosaccharides. *Clin. Biochem.* **38**, 149–153.

Higel, F., Seidl, A., Sorgel, F., and Friess, W. 2016. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. *Eur. J. Pharm. Biopharm.* **100**, 94–100.

HogenEsch, H., O'Hagan, D.T., and Fox, C.B. 2018. Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want. *NPJ Vaccines* **3**, 51.

Huang, X., Wang, X., Zhang, J., Xia, N., and Zhao, Q. 2017. *Escherichia coli*-derived virus-like particles in vaccine development. *NPJ Vaccines* **2**, 3.

Isa, M.B., Martinez, L., Giordano, M., Passeggi, C., de Wolff, M.C., and Nates, S. 2002. Comparison of immunoglobulin G subclass profiles induced by measles virus in vaccinated and naturally infected individuals. *Clin. Diagn. Lab. Immunol.* **9**, 693–697.

Jennewein, M.F. and Alter, G. 2017. The immunoregulatory roles of antibody glycosylation. *Trends Immunol.* **38**, 358–372.

Jin, Y., Kim, S.C., Kim, H.J., Ju, W., Kim, Y.H., and Kim, H.J. 2016. A lectin-based diagnostic system using circulating antibodies to detect cervical intraepithelial neoplasia and cervical cancer. *Glycobiology* **26**, 100–107.

Kim, H.J., Cho, S.Y., Park, M.H., and Kim, H.J. 2018. Comparison of the size distributions and immunogenicity of human papillomavirus type 16 L1 virus-like particles produced in insect and yeast cells. *Arch. Pharm. Res.* **41**, 544–553.

Kim, H.J. and Kim, H.J. 2017. Yeast as an expression system for producing virus-like particles: what factors do we need to consider? *Lett. Appl. Microbiol.* **64**, 111–123.

Kim, H.J., Kim, S.Y., Lim, S.J., Kim, J.Y., Lee, S.J., and Kim, H.J. 2010. One-step chromatographic purification of human papillomavirus type 16 L1 protein from *Saccharomyces cerevisiae*. *Protein Expr. Purif.* **70**, 68–74.

Kim, H.J., Lee, S.J., and Kim, H.J. 2008. Antibody-based enzyme-linked lectin assay (ABELLA) for the sialylated recombinant human erythropoietin present in culture supernatant. *J. Pharm. Biomed. Anal.* **48**, 716–721.

Kim, H.J., Lim, S.J., Kwag, H.L., and Kim, H.J. 2012. The choice of resin-bound ligand affects the structure and immunogenicity of column-purified human papillomavirus type 16 virus-like particles. *PLoS One* **7**, e35893.

Kushnir, N., Streatfield, S.J., and Yusibov, V. 2012. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* **31**, 58–83.

Lee, J.Y., Xu, M.L., Kim, H.J., Kang, H.A., and Kim, H.J. 2013. A comparative study of the adjuvanticity of *Hansenula polymorpha*, *Saccharomyces cerevsiae* and *Yarrowia lipolytica* in oral and nasal immunization with virus capsid antigens. *Biotechnol. Lett.* **35**, 1881–1888.

Mahan, A.E., Jennewein, M.F., Suscovich, T., Dionne, K., Tedesco, J., Chung, A.W., Streeck, H., Pau, M., Schuitemaker, H., *et al.* 2016. Antigen-specific antibody glycosylation is regulated via vaccination. *PLoS Pathog.* **12**, e1005456.

Marciani, D.J. 2016. A retrospective analysis of the Alzheimer's disease vaccine progress - The critical need for new development strategies. *J. Neurochem.* **137**, 687–700.

Marrack, P., McKee, A.S., and Munks, M.W. 2009. Towards an understanding of the adjuvant action of aluminium. *Nat. Rev. Immunol.* **9**, 287–293.

Maupin, K.A., Liden, D., and Haab, B.B. 2012. The fine specificity of mannose-binding and galactose-binding lectins revealed using outlier motif analysis of glycan array data. *Glycobiology* **22**, 160–169.

Riitho, V., Walters, A.A., Somavarapu, S., Lamp, B., Rumenapf, T., Krey, T., Rey, F.A., Oviedo-Orta, E., Stewart, G.R., Locker, N., *et al.* 2017. Design and evaluation of the immunogenicity and efficacy of a biomimetic particulate formulation of viral antigens. *Sci. Rep.* **7**, 13743.

Rohovie, M.J., Nagasawa, M., and Swartz, J.R. 2017. Virus-like particles: Next-generation nanoparticles for targeted therapeutic delivery. *Bioeng. Transl. Med.* **2**, 43–57.

Scherpenisse, M., Schepp, R.M., Mollers, M., Meijer, C.J., Berbers, G.A., and van der Klis, F.R. 2013. Characteristics of HPV-specific antibody responses induced by infection and vaccination: cross-reactivity, neutralizing activity, avidity and IgG subclasses. *PLoS One* **8**, e74797.

Selman, M.H.J., de Jong, S.E., Soonawala, D., Kroon, F.P., Adegnika, A.A., Deelder, A.M., Hokke, C.H., Yazdanbakhsh, M., and Wuhrer, M. 2012. Changes in antigen-specific IgG1 Fc N-glycosylation upon influenza and tetanus vaccination. *Mol. Cell Proteomics* **11**, M111.014563.

Stills, H.F. Jr. 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J.* **46**, 280–293.

Thompson, R., Creavin, A., O'Connell, M., O'Connor, B., and Clarke, P. 2011. Optimization of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis. *Anal. Biochem.* **413**, 114–122.

Turner, G.S. 1978. Immunoglobulin (IgG) and (IgM) antibody responses to rabies vaccine. *J. Gen. Virol.* **40**, 595–604.

Wang, J.W. and Roden, R.B. 2013. Virus-like particles for the prevention of human papillomavirus-associated malignancies. *Expert. Rev. Vaccines* **12**, 129–141.

Xiang, S.D., Scholzen, A., Minigo, G., David, C., Apostolopoulos, V., Mottram, P.L., and Plebanski, M. 2006. Pathogen recognition and development of particulate vaccines: Does size matter? *Methods* **40**, 1–9.

Zamora, E., Handisurya, A., Shafti-Keramat, S., Borchelt, D., Rudow, G., Conant, K., Cox, C., Troncoso, J.C., and Kirnbauer, R. 2006. Papillomavirus-like particles are an effective platform for amyloid-beta immunization in rabbits and transgenic mice. *J. Immunol.* **177**, 2662–2670.

Zheng, K., Bantog, C., and Bayer, R. 2011. The impact of glycosylation on monoclonal antibody conformation and stability. *MAbs* **3**, 568–576.