Human Vaccines

ISSN: 1554-8600 (Print) 1554-8619 (Online) Journal homepage: https://www.tandfonline.com/loi/khvi19

Effect of Alternative Aluminum Adjuvants on the Absorption and Immunogenicity of HPV16 L1 VLPs in Mice

Michael J. Caulfield, Li Shi, Su Wang, Bei Wang, Timothy W. Tobery, Henryck Mach, Patrick L. Ahl, Jayme L. Cannon, James C. Cook, Jon H. Heinrichs & Robert D. Sitrin

To cite this article: Michael J. Caulfield, Li Shi, Su Wang, Bei Wang, Timothy W. Tobery, Henryck Mach, Patrick L. Ahl, Jayme L. Cannon, James C. Cook, Jon H. Heinrichs & Robert D. Sitrin (2007) Effect of Alternative Aluminum Adjuvants on the Absorption and Immunogenicity of HPV16 L1 VLPs in Mice, Human Vaccines, 3:4, 139-145, DOI: 10.4161/hv.3.4.4309

To link to this article: https://doi.org/10.4161/hv.3.4.4309

[Human Vaccines 3:4, 139-146, July/August 2007]; ©2007 Landes Bioscience

Research Paper

Effect of Alternative Aluminum Adjuvants on the Absorption and Immunogenicity of HPV16 L1 VLPs in Mice

Michael J. Caulfield[1,*]
Li Shi[2]
Su Wang[1]
Bei Wang[2]
Timothy W. Tobery[1,†]
Henryck Mach[2]
Patrick L. Ahl[2]
Jayme L. Cannon[2]
James C. Cook[1]
Jon H. Heinrichs[1]
Robert D. Sitrin[3]

[1]Vaccine & Biologics Research; [2]Vaccine Pharmaceutical Research; [3]Bioprocess & Bioanalytical Research; Merck Research Laboratories; West Point, Pennsylvania

[†]Current address: Clinical Research and Biometrics; The Procter & Gamble Company; Cincinnati, Ohio USA

*Correspondence to: Michael J. Caulfield; Vaccine & Biologics Research; Merck Research Laboratories; 770 Sumneytown Pike; West Point, PA 19486; Tel.: 215.652.3402; Fax: 215.652.2142; Email: michael_caulfield@merck.com

Original manuscript submitted: 02/16/07
Manuscript accepted: 04/05/07

Previously published online as a *Human Vaccines* E-publication:
http://www.landesbioscience.com/journals/vaccines/abstract.php?id=4309

KEY WORDS

HPV, vaccine, aluminum adjuvant

ACKNOWLEDGEMENTS

The authors thank Dr. Stanley Hem for kindly providing assistance with TEM measurement and for valuable discussions and Joseph Antonello for statistical analyses. We also thank Janine Bryan, Marian Wentworth, Richard Haupt, Marie-Liesse Le Corfec and Eliav Barr for critical review of the manuscript and Jean Luo, David S. Thiriot, L Kareen Porter, for help with the characterization experiments.

ABSTRACT

Aluminum adjuvants are commonly used in prophylactic vaccines to enhance antigen immunogenicity through induction of high-titer antibody responses. Three major forms of aluminum adjuvants with substantially different physical and chemical properties have been described: aluminum phosphate ($AlPO_4$), aluminum hydroxide (AlOH) and amorphous aluminum hydroxyphosphate sulfate (AAHS). Here we describe the effect of these different aluminum adjuvants on the formulation and subsequent immunogenicity in mice of virus-like particles (VLPs) consisting of the L1 protein of Human Papillomavirus (HPV) Type 16. Electron microscopy demonstrated that the physical appearance of the phosphate-containing aluminum adjuvants was markedly different from that of aluminum hydroxide. All three aluminum adjuvants were found to display unique surface charge profiles over a range of pH, while AAHS demonstrated the greatest inherent capacity for adsorption of L1 VLPs. These differences were associated with differences in immunogenicity: anti-HPV L1 VLP responses from mice immunized with AAHS-formulated HPV16 vaccine were substantially greater than those produced by mice immunized with the same antigen formulated with aluminum hydroxide. In addition, HPV L1 VLPs formulated on AAHS also induced a substantial interferon-gamma secreting T cell response to L1 peptides indicating the potential for an enhanced memory response to this antigen. These results indicate that the chemical composition of aluminum adjuvants can have a profound influence on the magnitude and quality of the immune response to HPV VLP vaccines.

INTRODUCTION

Cervical cancer is among the most common carcinomas in women worldwide,[1] causing approximately 274,000 deaths annually.[2] Human papillomavirus (HPV) infection causes virtually all cases of cervical cancer. HPV infection also causes earlier dysplastic lesions of the cervix, vagina and vulva, as well as, in women and men, genital warts.[1,3,4] Of the 40 genital HPV types, four cause the majority of HPV-related disease: HPV 16 and HPV 18 cause 70% of cervical cancer and its immediate precancerous precursors, cervical intraepithelial neoplasia grade 3 (CIN 3) and adenocarcinoma in situ (AIS), as well as a similar proportion of HPV-related vulvar and vaginal cancers; HPV 6 and HPV 11 cause 90% of genital warts cases. Altogether, HPV6/11/16/18 cause 35–50% of early cervical lesions (CIN1) that often lead to abnormal pap smears and subsequent follow-up.

Universal prophylactic vaccination represents the most efficient way to eradicate infectious diseases. For HPV, an effective prophylactic vaccine that targets the four most important HPV types (HPV 6, 11, 16 and 18) will substantially reduce cervical cancer rates and the general disease burden associated with HPV infection.

Preventing HPV related disease is therefore a major public health objective and research on vaccines has been very active over the past 15 years. Experiments using species-specific papillomaviruses demonstrated that neutralizing serum antibodies against the L1 major capsid protein protect animals from infection.[5,6] These data suggested that vaccines that induce potent anti-HPV L1 antibody responses may protect humans against HPV infection and disease.

Vaccines consisting of virus like particles (VLPs) that are composed of the L1 major capsid protein of individual HPV types have been developed. Prophylactic administration of a quadrivalent HPV (Types 6, 11, 16, 18) L1 VLP vaccine (GARDASIL®, Merck & Co., Inc., Whitehouse Station, NJ, USA) has been shown to induce type-specific immunity[7,8] and to protect young women from development of cervical cancer, CIN 2/3, AIS, and genital warts caused by vaccine HPV types.[9] This vaccine has been licensed in several countries.

Because the risk for HPV infection peaks in the late teens or early twenties and remains as long as individuals are sexually active, prophylactic HPV vaccines must preferably be administered in early adolescence and confer long-term protection. To date, the protective efficacy of the HPV L1 VLP vaccines has been shown to persist for at least five years. The longer term duration of protection generated by these vaccines has not been defined. While the protective efficacy of HPV L1 VLP vaccines is associated with the development of potent neutralizing anti-HPV responses, the high efficacy of the vaccines has precluded definition of the minimum anti-HPV levels that confer protection from infection and disease caused by vaccine HPV types. Nevertheless, it has been hypothesized that high levels of anti-HPV antibody in serum may translate into prolonged persistence of protection.

Antibody responses to HPV antigens can be enhanced using adjuvants. More than 100 compounds or formulations exhibiting adjuvant properties have been described;[10] however, all adjuvants in widespread use in humans are mineral-based compounds containing aluminum. A key element of the mechanism by which aluminum adjuvants enhance immune responses is their ability to provide a depot effect to concentrate antigens for slow release to antigen presenting cells.[11] Aluminum adjuvants have also been used to deliver costimulatory molecules such as interleukin-12.[12,13] Three major forms of aluminum adjuvants with substantially different physical and chemical properties are in clinical use. The first type, aluminum hydroxide (AlOH) carries a net positive charge at neutral pH.[14] The second, aluminum phosphate ($AlPO_4$) has a net negative charge at neutral pH,[15,16] and the third type, which is proprietary to Merck, is amorphous aluminum hydroxyphosphate sulfate (AAHS) and carries approximately zero charge at neutral pH.

Here we examine immune responses to HPV L1 VLP type 16 when adsorbed onto three different aluminum-containing adjuvants, AAHS, AlOH and $AlPO_4$. We demonstrate that AAHS has unique properties that result in a higher capacity for adsorption of HPV L1 VLPs under physiological formulation conditions. In addition, mice immunized with HPV 16 L1 VLPs adsorbed onto AAHS generated significantly higher antibody titers than mice immunized with VLPs adsorbed to aluminum hydroxide. Furthermore, AAHS-formulated HPV vaccine induced a substantial cellular response as measured by IFNγ secreting T cells reactive with L1 peptides.

MATERIALS AND METHODS

Adjuvants. Aluminum hydroxide (Alhydrogel) and aluminum phosphate (Adju-Phos®) were purchased from Benntag Biosector A/S (Frederikssund, Denmark). The aluminum hydroxide and aluminum phosphate for EM studies were obtained from E.M. Sergeant Co. (Clifton, NJ). Amorphous aluminum hydroxyphosphate sulfate adjuvant (AAHS) was obtained from the Merck Manufacturing Division, West Point, PA. It is prepared by precipitation of alum with sodium hydroxide. Adjuvant concentration was calculated on the basis of aluminum (Al^{3+}) content. The aluminum concentration in each of the vaccine formulations was 450 μg/ml. The final aluminum dose per injection in animal studies was 22.5 μg in a volume of 50 μl.

Electron microscopy of aluminum adjuvants. Aluminum adjuvants were diluted using double-distilled water and then sprayed on a grid surface for freeze drying prior to electron microscopy (EM). Each sample was prepared in duplicate and a broad area of each grid was scanned. Photomicrographs were obtained from representative microscopic fields and the microstructures of the different aluminum adjuvants were evaluated visually. Duplicate samples displayed similar microscopic features, therefore, only one photomicrograph for each sample is shown.

Aluminum adjuvant surface charge analysis. Surface charge of the aluminum adjuvants were determined by measuring the voltage values of the particle-surface-zeta potential using a Malvern Zetasizer 3000 instrument (Malvern Instruments, Inc., Southborough, MA). The adjuvant samples were diluted with buffers of fixed salt concentration or ionic strength with pH ranging from pH 4 to pH 9 adjusted by a combination of acetic acid and Tris buffer or Tris buffer alone. Each profile reported is a representative curve of multiple measurements at different times using separate batches of aluminum adjuvants. Each zeta potential value reported is the average of five replicates.

HPV16 L1 VLPs. The major capsid protein, L1, of HPV type 16 was expressed intracellularly at high levels as a recombinant protein in a galactose-inducible *Saccharomyces cerevisiae* expression system, as described previously.[17] The purified product (greater than 97% L1 protein purity), was subjected to a disassembly/reassembly processes to generate uniform VLPs with improved antigenicity and storage stability.[18] HPV16 L1 VLPs, 4 μg/ml, were formulated with each of the three aluminum adjuvants at 450 μg/ml in 0.3 M NaCl, 10 mM histidine (pH ~6.5), 0.015% polysorbate 80.

Adsorption capacity of aluminum adjuvants for HPV L1 VLPs. HPV L1 VLPs were adsorbed on aluminum adjuvants by mixing the antigen with aluminum adjuvants at a desired mass ratio in 0.5 M NaCl, 20 mM histidine (pH ~6.5), and 0.02% Polysorbate 80. The aluminum adjuvanted VLPs were allowed to completely settle at 4°C. The resulting supernatant was analyzed using UV absorption against a control VLP sample without aluminum adjuvant to determine unbound protein content. UV absorbance spectra were measured at ambient temperature using an HP 8452A diode array spectrophotometer and a cuvette with a pathlength of 1 cm. The protein concentration was calculated using a multi-component analysis of second derivative near-UV spectra as reported previously.[19]

Animals. Mice were purchased from Charles River Laboratories (Raleigh, NC) and housed in Microisolator cages in the animal facility at Merck Research Laboratories (MRL), West Point, PA. Mice were generally 6–8 weeks of age at the start of experiments. Mice, under anesthesia, were immunized intramuscularly into the anterior tibialis muscle with 50 μl volumes of formulated vaccine containing 22.5 μg of aluminum and either 2 or 0.2 μg of protein per dose on days 0 and 21. Serum samples were collected on days 28 and 42 for analysis of anti-HPV16 L1 VLP antibody (ELISA) and spleen cells were collected from a subset of mice for ELISPOT assays. All studies were approved by the Institutional Animal Care and Use Committee.

DNA vaccine construction. The gene for the major capsid protein L1 of HPV type 16 was subcloned into the V1Jns expression vector as previously described.[20] Plasmid DNA used for immunization was purified from *E. coli* DH5α cells by a modified alkaline lysis procedure, and DNA was further purified by ultracentrifugation on two sequential $CsCl_2$ gradients.

Anti-HPV16 L1 ELISA (total and isotype-specific antibodies). Ninety-six well MaxiSorp™ plates (Nunc, Rochester, NY) were coated with 50 μl/well of the yeast cell derived HPV16 L1 VLPs in phosphate-buffered saline (PBS, pH 7.5) overnight at 4°C. Plates were washed 3x with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO) and then blocked with 200 μl/well of 1% BSA (Sigma) in PBS (blocking buffer) for 2 h at room temperature. Five-fold serial

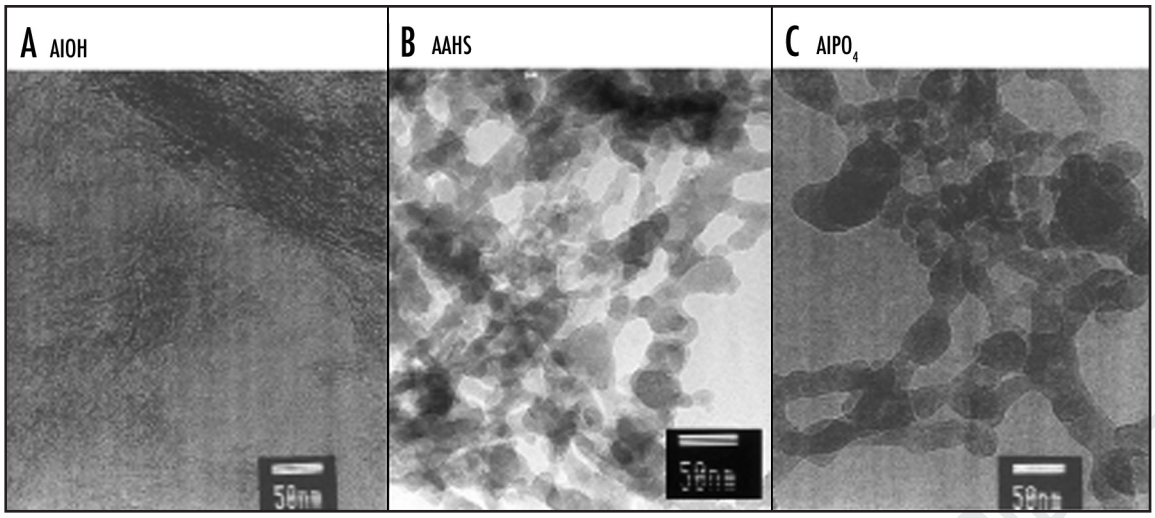

Figure 1. Electron micrographs of aluminum adjuvants AlOH, AAHS and $AlPO_4$. Bar indicates 50 nm scale.

dilutions were made for each serum sample (in duplicate) in eight consecutive wells of the plate and these were then incubated overnight at 4°C. After three wash cycles with PBS (using a TiterTech plate washer), horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin reagents specific for total mouse IgG, or for mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) were diluted in blocking buffer and added to the plate (50 µl/well). Following incubation at room temperature for 2 h, the plates were washed 6 times and then 60 µl/well of TMB substrate (Pierce, Rockford, IL) was added to each well for color development. After 30 min incubation at room temperature, the reaction was stopped by the addition of 60 µl/well of 2N H_2SO_4. Optical densities were read at 450 nm using a Molecular Devices microplate reader. Data were analyzed using the Softmax computer program (version 2.32) (Molecular Devices, Menlo Park, CA). Endpoint antibody titers were calculated as described previously.[13] The three aluminum adjuvants were statistically compared with regard to their ability to elicit anti-HPV 16 L1 IgG responses in mice. An ANOVA model was applied to the log transformed anti-HPV 16 L1 IgG titers. The log transformation was applied to better satisfy the normality and homogeneity of variance assumptions. Pairwise comparisons among the adjuvants were performed using Tukey's studentized range test.

Peptides. Peptides consisting of 20 amino acid residues in length (20-mers), that overlapped the adjacent peptide sequences by 10 amino acids, and represented the entire amino acid sequence of the HPV16 L1 protein were purchased from Chiron Technologies (Raleigh, NC). A nine amino acid peptide (YYHAGTSLR) representing a recently identified CD8 epitope[21] was purchased from SynPep (Dublin, CA). Each peptide was solubilized in 100% DMSO at 20 mg/ml. Stocks of individual peptides or peptide pools were made in 100% DMSO at a final concentration of 400 µg/ml for each peptide. Peptides and peptide pools were used at a final concentration of 2 µg/ml for each peptide in the ELISPOT assay.

ELISPOT assay for IFNγ production. Spleen cells from immunized mice were assayed for their ability to secrete IFNγ during in vitro restimulation with antigenic peptides using a modified ELISPOT assay.[22] Briefly, 96-well polyvinylidine difluoride (PVDF)-backed plates (MAIP NOB 10; Millipore, Bedford, MA) were coated with antibody to IFNγ, (clone R4-6A2; Pharmingen), washed three times with PBS, and then blocked with RPMI-1640 medium containing 10% heat-inactivated FBS. Cells were cultured at 5 x 10^5 per well in 0.1 ml of medium for restimulation with the newly identified 9-mer CD8 peptide, or with a pool of overlapping 20mer peptides covering the entire amino acid sequence of HPV16 L1 as described previously.[21] As a control, an identical concentration of DMSO was added to medium not containing stimulatory peptides. After 18–24 hr incubation at 37°C, the plates were washed 6x with PBS containing 0.005% Tween 20. Plates were then incubated with biotinylated anti-mouse IFNγ (clone XMG1.2; Pharmingen). The plates were washed six additional times before the addition of Streptavidin-AP conjugate (Pharmingen). After three washes with PBS-Tween and three washes with PBS, spots were developed with a one-step NBT/BCIP reagent (Pierce). Spots were counted using a stereomicroscope.

RESULTS

Physical properties of three types of aluminum adjuvants. Preparations of aluminum hydroxide (AlOH), amorphous aluminum hydroxyphosphate sulfate (AAHS), and aluminum phosphate ($AlPO_4$) were analyzed by electron microscopy to evaluate differences in the micro-structural appearance that may affect adjuvant performance in vaccine formulations. Transmission electron micrographs (Fig. 1) demonstrate a profound structural and morphological difference between aluminum hydroxide, which is crystalline in nature and forms linear strands, and the remaining two adjuvants (AAHS and $AlPO_4$), which form an amorphous mesh. The unique crystalline morphology of aluminum hydroxide is seen with different source materials, including Alhydrogel® and Rehydrogel®.[15,16]

The three aluminum formulations were also evaluated for surface charge properties across a wide range of pHs as shown in Figure 2. All three aluminum adjuvants differ significantly in the point of zero charge (PZC) with values of approximately 5, 7 and 10 ($AlPO_4$, AAHS and AlOH, respectively). As a result of the inherent PZC differences, the surface charge of these adjuvant particles are also dramatically different when prepared in a neutral (pH 7) solution; aluminum phosphate bears a negative charge and aluminum hydroxide a positive charge (approximately -20 and +30 mVolts, respectively) while AAHS has essentially no charge under these conditions.

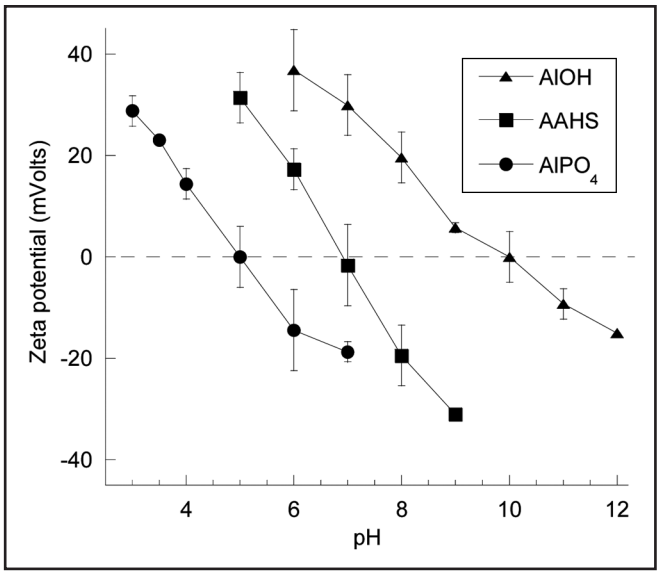

Figure 2. Surface charge profiles of different aluminum adjuvants. Aluminum adjuvant particles were diluted to 15 µg/ml and then analyzed by measuring the voltage values of the particle surface zeta potential under conditions of fixed total salt concentration. The pH of each sample was pre-adjusted with a buffer of the desired pH with an identical salt concentration.

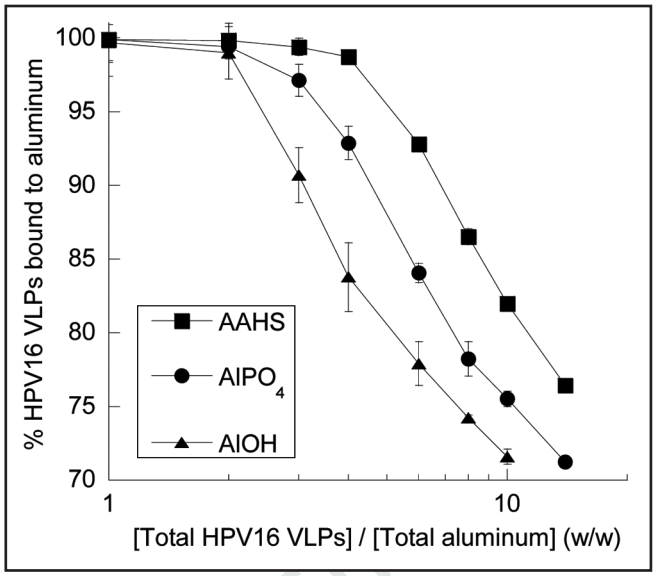

Figure 3. Binding capacity of different aluminum adjuvant to HPV16 VLPs. HPV16 VLPs were slowly added to aluminum adjuvant solutions with the desired VLPs/aluminum (w/w) ratio as indicated. All final formulations for each data point were prepared under a single solution condition with 0.5 M NaCl, 20 mM histidine, and 0.02% Polysorbate 80 at pH ~6.5.

To determine the potential impact of adjuvant-property differences on antigen-binding capacity, adsorption titrations were carried out for all three aluminum adjuvants using L1 VLPs from HPV16 as the test antigen. The results (Fig. 3) revealed that they differ substantially in their capacity for adsorption of HPV16 L1 VLPs at neutral pH (typically used in vaccine formulations) with AAHS exhibiting a substantially higher binding capacity than the other two aluminum-containing adjuvants. All three adjuvants bound 100% of the VLP protein at lower HPV/aluminum ratios. However, as the HPV/aluminum ratio increased, an apparent separation between AAHS and the other two adjuvants was observed. The AlOH and $AlPO_4$ preparations were rapidly saturated with protein at HPV/aluminum ratios of approximately 2 and 2.5, respectively, whereas the AAHS adjuvant did not approach saturation until approximately twice this level was achieved. Presumably, these differences are due to surface charge dissimilarities. These results demonstrate a clear advantage in the binding capacity of AAHS for HPV16 L1 VLPs when compared to both AlOH and $AlPO_4$ formulations.

Aluminum adjuvant effect on total anti-HPV16 L1 VLP responses. HPV16 L1 VLP vaccines formulated with AAHS, $AlPO_4$ or AlOH were then evaluated for their ability to induce potent antibody responses in mice. Three weeks after the second dose of vaccine, geometric mean antibody titers (GMT) to L1 were found to be 3.2-fold higher in serum from mice immunized with L1 formulated with AAHS, and 2.2-fold higher in mice immunized with L1 formulated with $AlPO_4$, compared with mice that had received the same VLPs formulated with AlOH (Fig. 4; $p < 0.01$ and $p = 0.06$, respectively). The serum antibody responses induced by immunization of mice with AAHS or $AlPO_4$ formulated antigen were not significantly different.

We next examined the relative titers of IgG1 and IgG2a in sera from mice immunized with AAHS-adjuvanted HPV16 L1 vaccine, as the ratio of these antibody isotypes reflects the induction of a T_H2 versus a T_H1 immune response. As shown in Table 1, a dose

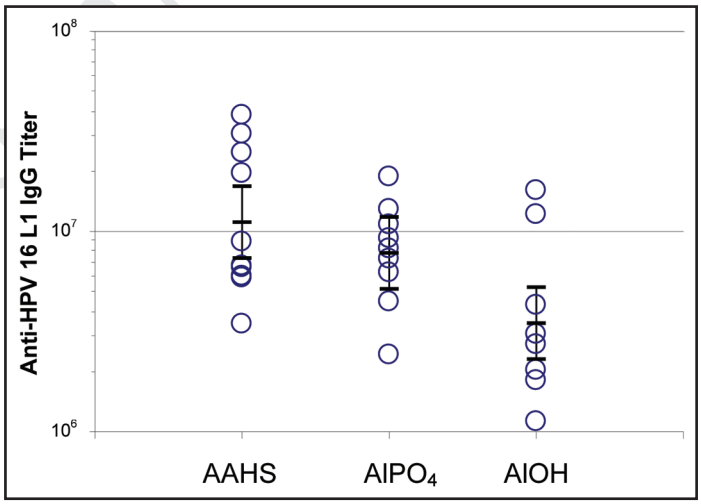

Figure 4. Antibody response to HPV16 L1 formulated on different aluminum adjuvants. BALB/c mice were injected intramuscularly on days 0 and 21 with 0.2 µg of HPV16 L1 VLPs formulated with aluminum hydroxyphosphate sulfate (AAHS), aluminum phosphate ($AlPO_4$) or aluminum hydroxide (AlOH). Sera collected from 10 mice per group at day 42 (three weeks post dose two) were tested by ELISA for IgG antibody titers to HPV16 L1 VLPs. Individual titers are indicated by the open circles. Geometric mean titers for each adjuvant are indicated by the cross bars. The intervals encompassing the GMTs correspond to Tukey's studentized range test in that non-overlapping intervals between adjuvants indicate statistically significant differences between adjuvants at the 0.05 significance level. Compared to the AlOH group, titers were statistically significantly higher for the AAHS group ($p < 0.01$) and nearly statistically significantly higher for the $AlPO_4$ group ($p = 0.06$).

dependent response was observed with approximately a 2.6-fold higher total IgG geometric mean antibody titer in the 2 µg group compared to the 0.2 µg group. This effect was independent of adjuvant concentration as the aluminum adjuvant dose was consistent

Table 1 **Effect of HPV16 L1 VLP dose on the isotype distribution of the IgG antibody response to HPV16 L1 VLPs[a]**

		Anti-HPV16 L1 Antibody (GMT)			
HPV16 L1 VLP	Adjuvant	Total IgG	IgG1	IgG2a	Ratio IgG2a/IgG1
0.2 μg	AAHS	1,322,275	20,492	54,707	2.7
2.0 μg	AAHS	3,494,667	907,102	5,028	0.01

[a]BALB/c mice were injected intramuscularly on days 0 and 21 with the indicated dose of HPV16 L1 VLPs formulated with AAHS (22.5 μg per dose). Antibody titers were measured by ELISA from sera collected at week 6 (three weeks post-dose 2) to detect total IgG or isotype-specific responses (IgG1 or IgG2a).

Table 2 **Effect of HPV16 L1 VLP dose on the T cell response (IFNγ ELISPOT) to HPV L1 peptides[a]**

			IFNγ SFCs / 106 spleen cells	
Immunogen	Adjuvant	Anti-HPV16 L1 (antibody GMT)	CD8 peptide	L1 Peptide pool (20 mers)
HPV16 L1 VLP				
0.2 μg	AAHS	106,779	2	13
2.0 μg	AAHS	251,220	12	100
HPV16 L1 DNA				
100 μg	none	4,092	302	310

[a]Spleen cells from BALB/c mice injected intramuscularly on days 0 and 21 with the indicated dose of HPV16 L1 VLPs or with a plasmid DNA vaccine encoding HPV16 L1 were harvested at day 28 (1 week post-dose 2) for evaluation in the IFNγ ELISPOT assay. Responses were elicited by stimulation with either a known CD8 peptide (L1 aa 34-42) or with 2 μg per ml of a pool of 20 mer HPV16 L1 peptides. ELISPOT results are expressed as the geometric mean responses from four mice per group tested individually whereas antibody titers (total IgG) are geometric-mean titers (GMTs) for sera collected from 10 mice per group at day 28. Spleen cells from mice immunized with the DNA vaccine serve as a positive control for the ELISPOT assay.

for both groups. At the high antigen dose, the responses to HPV16 L1 formulated with AAHS predominantly consisted of antibodies of the IgG1 isotype, indicative of a T_H2 response.

Induction of IFNγ T cell responses to HPV VLPs. The ability of AAHS-formulated HPV 16 L1 VLP vaccine to induce a cellular response was evaluated by measuring stimulation of IFNγ secreting T cells in an ELISPOT assay. As a positive control, we also immunized mice with a DNA construct encoding the L1 gene. DNA immunization is known to be a potent inducer of cellular immunity, although it only weakly stimulates antibody responses. The results shown in Table 2 demonstrate that HPV16 L1 VLPs formulated on AAHS were able to induce a substantial CD4 T cell response to L1 peptides as measured using the IFNγ ELISPOT assay. This result was somewhat unexpected since previous reports have indicated that induction of IFNγ T cell responses to VLPs occurred only in the absence of AlOH.[23] Only a modest ELISPOT response to the CD8 epitope was seen in mice immunized with VLPs whereas a strong response to this peptide was observed in the positive control group immunized with the HPV16 L1 DNA vaccine.

DISCUSSION

Despite over 70 years of use as vaccine adjuvants, the physicochemical properties underlying the immune-stimulating effects of aluminum-containing adjuvants have not been fully defined. Nevertheless, aluminum adjuvants remain the predominant immune modulators in use in approved vaccines. It is believed that immune stimulation caused by aluminum-containing adjuvants is predominantly due to their direct stimulation of antigen presenting cells, as well as their ability to form an antigen depot at the site of injection.[24] Recently however, the concept that antigen persists for long periods of time at injection sites has been challenged.[11] Regardless of the mechanism of adjuvanticity, it has long been recognized that aluminum adjuvants are strong inducers of T_H2, antibody-mediated, immune responses, but only poorly stimulate cell-mediated immunity.[25]

The ability of an antigen to induce a potent, long-lasting immune response is dependent on the form of aluminum adjuvant used for the selected antigen, probably due both to the adjuvant's relative ability to adsorb and slowly release the antigen, as well as its immunostimulatory properties with the selected formulation. Merck Aluminum Adjuvant (AAHS) is a proprietary aluminum hydroxyphosphate sulfate formulation that is both physically and functionally distinct from traditional aluminum phosphate and aluminum hydroxide adjuvants. At a macromolecular level, AAHS is structurally related to aluminum phosphate as it forms an amorphous mesh-like structure. In contrast, aluminum hydroxide adjuvants form highly crystalline structures. These structural differences might potentially impact the ability of an adjuvant to form an antigen depot as well as its intrinsic capacity to stimulate antigen presenting cells.

Adsorption of proteins to aluminum adjuvants has been described as involving at least two separate processes. One of these is a ligand-exchange interaction, primarily occurring between phosphate groups on protein antigens and the aluminum atom on the adjuvant. A second mechanism is an electrostatic interaction between proteins and charged groups on the adjuvants.[25] However, antigen adsorption on aluminum adjuvants is a highly complex process. Overall, hydrophobic interactions, hydrogen bonding, ionic charge, and van der Waals forces can all influence antigen binding to aluminum adjuvants. AAHS bears a nearly zero charge at neutral pH, whereas both aluminum phosphate and aluminum hydroxide are strongly charged at neutral pH. The net charge of these two adjuvants likely impacts both their ability to bind antigen as well as the release of that antigen following injection into the host and exposure to serum fluids which are likely to approximate a neutral pH. These adjuvants also differ in their rate of dissolution in interstitial fluid in vitro and in vivo.[26] Aluminum phosphate has been shown to dissolve relatively quickly whereas aluminum hydroxide is difficult to dissolve in vitro[27] and can persist for long periods of time in vivo.[28] Regardless of the mechanism of binding of antigen, it is clear that AAHS has an increased capacity for adsorption of L1 VLPs with essentially 100% of protein bound as formulated in these studies. In this study however, the HPV VLPs were completely bound to all three forms of adjuvant. Therefore, the differences observed in the immunogenicity of these three formulations is likely due to the inherent properties of these adjuvants rather than simply a function of their binding capacities. Nevertheless, the enhanced binding capacity displayed by AAHS would allow for higher concentrations of VLP proteins or additional serotypes of HPV to be included in a vaccine formulation without the delivery of additional aluminum. In addition, HPV VLPs have been shown to be highly stable following disassembly and reassembly when formulated with AAHS with essentially no loss in potency observed for HPV16 VLPs following three months of incubation at 37°C.[29]

A second HPV vaccine is currently in development (GlaxoSmithKline, Rixensart, Belgium) which contains just two HPV types rather than the four types in GARDASIL®. This vaccine is formulated with a modified aluminum based adjuvant, ASO4. This adjuvant is a combination of aluminum hydroxide (AlOH) and a detoxified salmonella endotoxin, monophosphoryl lipid A (MPL). A recent publication by Giannini et al.[19] compared the immune response to L1 VLPs from types 16 and 18 formulated with ASO4 versus VLPs formulated on aluminum hydroxide alone. The results of the study showed differences in immune response to L1 VLPs when MPL was added to aluminum hydroxide, but did not address the question as to whether a different aluminum adjuvant might elicit different (i.e., better) responses to HPV L1 VLPs than VLPs formulated with AlOH.

In this study we show that immunization of mice with AAHS-formulated HPV 16 L1 VLP vaccine induced a 3.2 fold increase in the anti-L1 antibody titers (measured three weeks post dose two) compared to the same antigen formulated with AlOH. In both groups of animals, 22.5 μg of aluminum adjuvant was used along with 0.2 μg of L1 VLPs. Similarly, Giannini et al.[30] compared the adjuvant effects of AlOH and ASO4, an AlOH adjuvant also containing MPL, on VLP immunogenicity. In their murine study, 2 μg each of HPV16 and HPV18 VLPs were formulated with either 50 μg of AlOH or 50 μg of AlOH plus 5 μg MPL. They reported that on days 14 and 37 post dose two, the ASO_4 adjuvant induced an increased anti-VLP response (5.1–2.8 fold increase and 8.5–3.5 fold increase [HPV16 and HPV18, respectively]) relative to the AlOH group. Although these two studies differed in both the times at which immunogenicity was assessed and the quantity of vaccine components (HPV VLPs and aluminum adjuvant) used, the relative differences observed between AlOH and either ASO4 or AAHS were similar in both experiments. Study design differences aside, it is clear from both studies that AlOH is not the optimum adjuvant for generating high-titer antibody responses to HPV VLPs. The inclusion of either an alternative aluminum adjuvant with unique physico-chemical properties, such as AAHS, or the addition of a second immune-stimulating molecule such as MPL, can significantly increase antibody titers to HPV L1 VLPs.

Over seventy years of experience with aluminum adjuvants in vaccine formulations have established their safety and efficacy in human use.[11] In addition, the durability of the immune response for AAHS-formulated HPV VLPs has been demonstrated both in rhesus macaques,[31] and in humans, where geometric mean titers through five years of follow up remained at or above those following natural infection.[32] This sustained immune response correlated with prolonged efficacy in human clinical trials, as this vaccine was demonstrated to be 100% effective in preventing precancerous cervical dysplasia and genital warts throughout the five year study period.[32]

Prevention of HPV-induced cervical carcinoma is likely to be primarily antibody mediated, however a sustained memory response requires T-cell involvement. Therefore, we examined the ability of AAHS to stimulate a T_H2 (antibody mediated) rather than a T_H1 (cell mediated) response through evaluation of antibody isotype profiles and IFNγ production by T cells following immunization of mice. Not surprisingly, our data indicate that the major immune response induced by AAHS was an IgG1-predominant, T_H2 response, although, surprisingly, a substantial IFNγ producing T cell stimulation was obtained at the 2 μg dose of protein. We have previously shown that the addition of IL-12 to AAHS allows for the selective targeting of T_H1 responses to hepatitis B antigen.[13] Similar studies performed with HPV L1 VLPs (data not shown) indicate that a similar shift in immune responses, from a predominantly T_H2 response to a more balanced T_H1/T_H2 response, can also be induced by including IL-12 with AAHS.

An optimal immune response is achieved when the chemical and physical properties of both the antigen and the aluminum adjuvant are considered. Based on the study reported here, aluminum hydroxide (AlOH) was the least, and amorphous aluminum hydroxyphosphate sulfate (AAHS) the most, effective aluminum adjuvant tested with HPV 16 L1 VLP antigen. The increased capacity of AAHS to bind L1 VLPs may contribute to the ability of this adjuvant to induce an enhanced antibody response following immunization of mice. The chemical, physical and immunogenicity data presented and discussed here demonstrate the utility of AAHS as an optimal and safe adjuvant for HPV L1 VLP vaccines and indicate that AAHS is the best choice among available aluminum adjuvants for generating immune responses to HPV VLPs.

References

1. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995; 87:796-802.
2. Parkin DM, Bray F. Chapter 2: The burden of HPV-related cancers. Vaccine 2006; 24: S11-25.
3. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189:12-9.
4. Munoz N. Human papillomavirus and cancer: The epidemiological evidence. J Clin Virol 2000; 19:1-5.
5. Jansen KU, Rosolowsky M, Schultz LD, Markus HZ, Cook JC, Donnelly JJ, Martinez D, Ellis RW, Shaw AR. Vaccination with yeast-expressed cottontail rabbit papillomavirus (CRPV) virus-like particles protects rabbits from CRPV-induced papilloma formation. Vaccine 1995; 13:1509-14.
6. Christensen ND, Reed CA, Cladel NM, Han R, Kreider JW. Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. J Virol 1996; 70:960-5.
7. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, Jansen KU. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 2002; 347:1645-51.
8. Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, Zahaf T, Innis B, Naud P, De Carvalho NS, Roteli-Martins CM, Teixeira J, Blatter MM, Korn AP, Quint W, Dubin G. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: A randomised controlled trial. Lancet 2004; 364:1757-65.
9. Mao C, Koutsky LA, Ault KA, Wheeler CM, Brown DR, Wiley DJ, Alvarez FB, Bautista OM, Jansen KU, Barr E. Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: A randomized controlled trial. Obstet Gynecol 2006; 107:18-27.
10. Vogel FR, Powell MF. A compendium of vaccine adjuvants and excipients. Pharm Biotechnol 1995; 6:141-228.
11. HogenEsch H. Mechanisms of stimulation of the immune response by aluminum adjuvants. Vaccine 2002; 20:S34-9.
12. Jankovic D, Caspar P, Zweig M, Garcia-Moll M, Showalter SD, Vogel FR, Sher A. Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. J Immunol 1997; 159:2409-17.
13. Wang S, Liu X, Caulfield MJ. Adjuvant synergy in the response to hepatitis B vaccines. Vaccine 2003; 21:4297-306.
14. Hem SL, White JL. Structure and properties of aluminum-containing adjuvants. In: Powell M, Newman M, eds. Vaccine Design: The subunit and Adjuvant Approach. New York: Plenum Press, 1995:249-76.
15. Shirodkar S, Hutchinson RL, Perry DL, White JL, Hem SL. Aluminum compounds used as adjuvants in vaccines. Pharm Res 1990; 7:1282-8.
16. al-Shakhshir R, Regnier F, White JL, Hem SL. Effect of protein adsorption on the surface charge characteristics of aluminium-containing adjuvants. Vaccine 1994; 12:472-4.
17. Cook JC, Joyce JG, George HA, Schultz LD, Hurni WM, Jansen KU, Hepler RW, Ip C, Lowe RS, Keller PM, Lehman ED. Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein L1 from *Saccharomyces cerevisiae*. Protein Expr Purif 1999; 17:477-84.
18. Mach H, Volkin DB, Troutman RD, Wang B, Luo Z, Jansen KU, Shi L. Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). J Pharm Sci 2006; 95:2195-206.
19. Mach H, Volkin DB, Burke CJ, Middaugh CR. Ultraviolet absorption spectroscopy. Methods Mol Biol 1995; 40:91-114.

20. Tobery TW, Smith JF, Kuklin N, Skulsky D, Ackerson C, Huang L, Chen L, Cook JC, McClements WL, Jansen KU. Effect of vaccine delivery system on the induction of HPV16L1-specific humoral and cell-mediated immune responses in immunized rhesus macaques. Vaccine 2003; 21:1539-47.
21. Tobery TW, Caulfield MJ. Identification of T-cell epitopes using ELISpot and peptide pool arrays. Methods Mol Med 2004; 94:121-32.
22. Lalvani A, Brookes R, Hambleton S, Britton WJ, Hill AVS, McMichael AJ. Rapid effector function in CD8+ memory T cells. J Exp Med 1997; 186:859-65.
23. Wagner R, Deml L, Schirmbeck R, Niedrig M, Reimann J, Wolf H. Construction, expression, and immunogenicity of chimeric HIV-1 virus-like particles. Virology 1996; 220:128-40.
24. Lindblad EB. Aluminium adjuvants-in retrospect and prospect. Vaccine 2004; 22:3658-68.
25. Brewer JM. (How) do aluminium adjuvants work? Immunol Lett 2006; 102:10-5.
26. Flarend RE, Hem SL, White JL, Elmore D, Suckow MA, Rudy AC, Dandashli EA. In vivo absorption of aluminum-containing vaccine adjuvants using Al. Vaccine 1997; 15:1314-8.
27. Seeber SJ, White JL, Hem SL. Solubilization of aluminum-containing adjuvants by constituents of interstitial fluid. J Parenter Sci Technol 1991; 45:156-9.
28. Gherardi RK, Coquet M, Cherin P, Belec L, Moretto P, Dreyfus PA, Pellissier JF, Chariot P, Authier FJ. Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. Brain 2001; 124:1821-31.
29. Shi L, Sings HL, Bryan JT, Wang B, Wang Y, Mach H, Kosinski M, Washabaugh MW, Sitrin R, Barr E. GARDASIL (R): Prophylactic human papillomavirus vaccine development - From bench top to bed-side. Clinical Pharmacology and Therapeutics 2007; 81:259-64.
30. Giannini SL, Hanon E, Moris P, Van Mechelen M, Morel S, Dessy F, Fourneau MA, Colau B, Suzich J, Losonksy G, Martin MT, Dubin G, Wettendorff MA. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. Vaccine 2006; 24:5937-49.
31. Ruiz W, McClements W, Jansen K, Esser M. Kinetics and isotype profile of antibody responses in rhesus macaques induced following vaccination with HPV 6, 11, 16 and 18 L1-virus-like particles formulated with or without Merck aluminum adjuvant. J Immune Based Ther Vaccines 2005; 3:2.
32. Villa LL, Costa RLR, Petta CA, Andrade RP, Paavonen J, Iversen OE, Olsson SE, Hoye J, SteinWall M, Riis-Johannessen G, Andersson-Ellstrom A, Elfgren K, von Krogh G, Lehtinen M, Malm C, Tamms GM, Giacoletti K, Lupinacci L, Railkar R, Taddeo FJ, Bryan J, Esser MT, Sings HL, Saah AJ, Barr E. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. Br J Cancer 2006; 95:1459-66.