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Adsorption of recombinant poxvirus L1-protein to aluminum hydroxide/CpG vaccine adjuvants enhances immune responses and protection of mice from vaccinia virus challenge

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Abstract

The stockpiling of live vaccinia virus vaccines has enhanced biopreparedness against the intentional or accidental release of smallpox. Ongoing research on future generation smallpox vaccines is providing key insights into protective immune responses as well as important information about subunit vaccine design strategies. For protein-based recombinant subunit vaccines, the formulation and stability of candidate antigens with different adjuvants are important factors to consider for vaccine design. In this work, a non-tagged secreted L1-protein, a target antigen on mature virus, was expressed using recombinant baculovirus technology and purified. To identify optimal formulation conditions for L1, a series of biophysical studies was performed over a range of pH and temperature conditions. The overall physical stability profile was summarized in an empirical phase diagram. Another critical question to address for development of an adjuvanted-vaccine was if immunogenicity and protection could be affected by the interactions and binding of L1 to aluminum salts (Alhydrogel) with and without a second adjuvant, CpG. We thus designed a series of vaccine formulations with different binding interactions between the L1 and the two adjuvants, and then performed a series of vaccination-challenge experiments in mice including measurement of antibody responses and post-challenge weight-loss and survival. We found that better humoral responses and protection were conferred with vaccine formulations when the L1-protein was adsorbed to Alhydrogel. These data demonstrate that designing vaccine formulation conditions to maximize antigen-adjuvant interactions is a key factor in smallpox subunit vaccine immunogenicity and protection.

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Keywords

Adjuvants; Immunologic/*pharmacology; Adsorption; Aluminum Hydroxide/immunology/*pharmacology; Animals; Antibody Formation/immunology; Antigens/chemistry/*immunology; Phosphates/immunology/*pharmacology; Formulation; Vaccine

1. Introduction

In the U.S., aluminum salts remain the primary adjuvant used in human vaccines [1]. Through mechanisms still not entirely clear, the adjuvant creates an environment that allows development of immune responses to the vaccine antigen(s) [2]. It has long been believed that adsorption of antigen to aluminum was critical for the development of immune responses [3]. Such adsorption of antigen was thought mechanistically to provide a depot effect that permits prolonged exposure to the antigen, although immune cell recruitment to the injection site and enhanced antigen uptake are now known to play key roles [4]. While antigen adsorption to aluminum has long been considered to be a key step in vaccine immunogenicity, a growing body of literature has shown that the strength of antigen adsorption is important [5–8]. In fact, there are several examples where no differences in antibody-response have been seen between adsorbed and non-adsorbed protein formulated with aluminum [9–13]. This apparent paradox has been explained experimentally by the finding that interstitial fluids cause the release of proteins adsorbed to aluminum [6, 9, 10, 14–16]. Thus, while an antigen could be initially adsorbed to aluminum, after injection the antigen might quickly dissociate. Non-adsorbed protein could also be retained in void spaces of aluminum [12]. Additionally, detailed studies have shown that the more tightly an antigen is bound to aluminum, the poorer the immune response [5–8, 13].

We have been investigating a protein-based subunit-vaccine as a safer future alternative to live smallpox vaccines [17–19]. A multi-subunit vaccine that contains antigens from the two infectious forms of poxviruses provides the best protection [19–28]. Subunit smallpox vaccines target antigens on mature-virus (e.g., L1, A27) and extracellular-virus (e.g., A33, B5). A key component of these subunit-vaccines is the mature-virus envelope protein, L1. While vaccines containing L1 alone do not protect nonhuman primates challenged with monkeypox virus from severe disease [23], vaccinated mice are protected by some L1 vaccines [19–22, 25, 26, 28]. In the work described here, we perform biophysical studies of the L1-protein and examine whether L1 adsorption (with or without a second adjuvant, CpG) to aluminum hydroxide (AH) is necessary for optimal immune responses and protection from vaccinia virus (VACV) challenge. Our findings demonstrate that designing vaccine formulation conditions to maximize antigen-adjuvant interactions is a key factor in smallpox subunit-vaccine immunogenicity and protection.

2. Materials and Methods

2.1. Generation and purification of non-histidine tagged variola virus L1 (L1V)

Non-tagged L1 protein (amino acids 1 to 185; gene bank accession number AAA60821.1) was expressed using recombinant baculovirus technology. These residues are identical for variola and vaccinia viruses, but to distinguish this protein from the prior histidine-tagged “VACV” protein we have worked with in the past [17–19], we call this non-tagged “variola” protein L1V. The baculovirus that expresses the secreted protein was constructed as previously described [29]. Insect larvae were infected and proteins recovered from frozen larvae resuspended in buffer containing reducing agent, salt, and protease inhibitors. After centrifugation and filtration, material was treated with detergent to inactivate virus. The protein was purified to 99%-homogeneity using an ion-exchange capture step (BioRad

High-Q Resin), followed by affinity (Blue sepharose), and then orthogonal ion-exchange chromatographic methods. A buffer exchange to PBS was carried with G-25 column followed by filter-sterilization.

2.2. Biophysical characterization of L1V-protein

To study the thermal stability of the protein as a function of pH, the L1V-protein stock was dialyzed against 20-mM citrate phosphate-buffers at varying pH. All the buffer solutions had a constant ionic strength of 0.15 adjusted with NaCl. Far-UV circular dichroism (CD) was used to study the secondary structure of L1V using a Jasco J815-spectropolarimeter equipped with a Peltier temperature-controller. Static light scattering at 275-nm was studied using a Photon Technology International spectrofluorometer (Lawrenceville) equipped with a Peltier temperature controller. 8-anilino-1-naphthalene sulfonate (ANS) fluorescence was used to study the protein tertiary structural changes as a function of pH and temperature using a PTI spectrofluorometer. See figure legends for additional details. The thermal melt data acquired from far-UV CD, ANS fluorescence emission intensity, and scattered light intensity were used to construct an empirical phase diagram (EPD) using pH and temperature as independent variables. Details concerning EPD preparation and interpretation are described elsewhere [30, 31].

2.3. Binding of CpG to Alhydrogel

The binding state of CpG-10104 (5'-TCGTCGTTTCGTCGTTTTGTCGTT-3' from Coley Pharmaceutical, now part of Pfizer) to the 2%-Alhydrogel (Brenntag) was studied in formulations containing Alhydrogel (2-mg/mL based on aluminum-ion) or phosphate-treated Alhydrogel (PTAH) at two L1V concentrations (0.04- and 0.2-mg/mL). To prepare PTAH, Alhydrogel was mixed with 20-mM phosphate-buffer and incubated at 5°C for 1-hr with shaking. The phosphate-buffer was prepared from a KH_2PO_4 stock solution in 10-mM histidine buffer at pH7 with 150-mM NaCl.

2.4. Vaccine formulation

L1V-protein for animal studies was dialyzed against 10-mM histidine buffer (pH7) containing 150-mM NaCl and sterile filtered through a 0.22- μm filter (Millex). The vaccines (50- μl /dose) were formulated on the day of vaccination. Initially, Alhydrogel (100- μg /dose based on aluminum-ion) was incubated with mixing for 1-hr at 5°C in histidine buffer in the presence or absence of phosphate-buffer forming phosphate-treated Alhydrogel (PTAH) and Alhydrogel (AH), respectively. Then L1V alone or with CpG at two different concentrations was added and incubated with mixing for another hour at 5°C. In the final formulations, the concentrations were 2-mg/mL (100- μg /dose) for Alhydrogel (PTAH or AH), 0.04-mg/mL (2- μg /dose) for L1V, and 0.4- or 1.0-mg/mL (20- or 50- μg /dose) for CpG. Formulations were loaded into a syringe, the remaining vaccine was centrifuged to pellet the Alhydrogel, and 5- μl of the supernatant was loaded on a Mini-Protean TGX Any kD precast gel (Bio-Rad) and electrophoresed under reducing-denaturing conditions. Protein bands were visualized by silver-staining (Invitrogen Silverquest Staining Kit) following the manufacturer's instructions.

2.5. Mice vaccinations and challenge

Six-week-old female BALB/c mice, purchased from Charles River and allowed to acclimate to the ABSL2-facility at UPenn, were vaccinated and challenged as previously described [17, 19]. Briefly, mice (5- to 10-mice/group) were intramuscularly vaccinated. Two-weeks after the initial vaccination, mice were boosted and bled 3-weeks later. The following day, mice were challenged intranasally with VACV (strain-WR) in 20- μL PBS. Mice were monitored and weighed daily. Mice that reached end-point criteria (e.g., appearance,

posture, mobility) and/or had 30%-weight-loss were humanely euthanized. The animal use and care were performed in accordance with institutional guidelines. We and others [17, 19, 25, 27, 32] have used this type of vaccination to challenge timeline to demonstrate the ability to generate rapid protection from orthopoxvirus challenge.

2.6. Antibody ELISA, sodium thiocyanate elution, and in-vitro virus neutralization

Groups of mice were bled 3 weeks after the boost vaccination and equal volumes of sera from individual mice were mixed. Antibody titers in sera were assayed by direct ELISA and in-vitro virus neutralization were performed as previously described [17, 19]. We measured the relative binding-affinity of equimolar amounts of antibody using a sodium thiocyanate elution method [33]. Equimolar amounts of sera from groups were based on standard ELISA data. After initial binding of antibody to wells coated with L1V (in triplicate), the plates were washed and incubated at room temperature for 20 minutes with a range of concentrations of sodium thiocyanate (0.0625 to 4 M) and antibody that remained bound was detected as described previously [17, 19]. The relative percentage of antibody binding was calculated as (OD with thiocyanate/OD without thiocyanate) $\times$ 100.

2.7. Statistical analysis

Area-under-the-curve was calculated for each set of ELISA data and Kruskal-Wallis one-way analysis of variance (ANOVA) was used to determine that statistical differences existed between groups. ANOVA was also used to show that statistical differences existed between neutralization activities and differences in maximum weight-loss. Tukey's multiple comparison test was then used to compare the various groups. Kaplan-Meier survival plots were compared by the Cox-Mantel test. Statistics were performed using GraphPad Prism™ (Version-5).

3. Results

3.1. L1V expression and purification

Our prior work on a future generation smallpox vaccine used recombinant histidine-tagged VACV proteins. To move the subunit-vaccine strategy forward toward development, we began to work with non-tagged variola virus proteins. The amino acid sequence of the expressed ectodomain of L1 (residues 1–185), however, is identical for vaccinia and variola viruses. To distinguish the non-tagged protein used in these studies, we call the protein L1V. Based on the densitometric scan of an SDS-PAGE stained with coomassie-blue, the L1V-protein was estimated to be 99%-pure and reacted with anti-L1 polyclonal and monoclonal antibodies (data not shown).

3.2. Biophysical characterization of L1V-protein and construction of an empirical phase diagram (EPD)

The development of successful protein-based subunit-vaccines does not just rely on identification of antigens that confer protection. Antigen stability is an important factor to consider. To begin to address this concern we performed a series of biophysical studies with the L1V-protein over a range of pH and temperature conditions. The overall stability profile of the antigen was presented in the form of an EPD constructed from the biophysical data. Fig. 1A shows the CD spectra of L1V at different pH-values. These data reveal that the expressed L1V-protein does not show substantial variation over a wide pH-range at low temperature and contains primarily alpha-helical secondary structure. This result is consistent with the published L1 crystal structure [34]. The helical structure of the protein appears to be maintained when temperature is below ~65°C under most of the pH-conditions (Fig. 1B). Compared to the unfolding curves obtained at other pH-values, sharper transitions

are observed at pH4 to 6, indicating different unfolding processes for L1V at different pH-values. Protein tertiary structural changes as a function of pH and temperature were studied using ANS as an extrinsic fluorescence probe. As a protein unfolds, ANS-binding to exposed apolar regions usually results in increased fluorescence emission. Fig. 1C shows that at low pH, the protein is less stable with transitions occurring at or above 40°C. At pH $\geq$ 7 no such sharp transitions are observed. Similar results were found using static light scattering, which shows the protein begins to aggregate at temperatures above 40°C at pH $\leq$ 5 (Fig. 1D). These data were used to generate EPD that provides a visual representation of the overall thermal stability of L1V (Fig. 2). The color changes represent structural transitions between different physical states of the protein. The EPD shows L1V-protein is most stable between pH6 to 8 and at temperatures below 65°C. Based on these results, formulation studies with the protein were carried out at pH7.

3.3. Formulation of L1V-protein with adjuvants

Prior studies of a recombinant protein-based smallpox vaccine showed that while adjuvanting the protein antigens with Alhydrogel provided partial protection, optimal protection was obtained with the inclusion of CpG-oligonucleotides [17–19, 25]. A critical question that was not answered was whether immunogenicity and protection could be affected by the binding states of the protein (and CpG) to Alhydrogel. To begin to address this, we initially measured the zeta-potential of Alhydrogel in the presence of various concentrations of phosphate-buffer (Supplementary Fig. 1). The data revealed that the surface charge of Alhydrogel changed from positive ($\sim$ 35-mV) to negative ($\sim$ -30-mV), as the concentration of phosphate-buffer increased. Increasing the incubation time of Alhydrogel and phosphate from 1-hr to 5-days had no apparent effects on the zeta-potential of Alhydrogel. As the surface charge of Alhydrogel becomes negative, binding of L1V decreases (data not shown). Eventually, 20-mM phosphate was selected to generate vaccine formulations with >80%-of L1V-protein not bound to Alhydrogel. We next examined the adsorption of CpG to Alhydrogel or phosphate-treated Alhydrogel (PTAH). The studies were performed in the absence or presence of L1V at either 0.04-mg/ml (2- μ g/dose) or 0.2-mg/ml (10- μ g/dose) (Fig. 3). In the absence of phosphate-buffer, CpG remained completely bound to Alhydrogel up to a concentration of 0.8-mg/ml (Fig. 3A). In the presence of 20-mM-phosphate-buffer, however, CpG remained bound to Alhydrogel only up to concentrations of 0.4-mg/ml (Fig. 3B). The presence or absence of L1V-protein did not alter the binding characteristics of the CpG-oligonucleotide.

Based on previous studies [17], the dosage of L1V was selected as 0.04-mg/mL (2- μ g/dose) for all the vaccine formulations used for the mouse studies. A concentration of 2-mg/mL (100- μ g/dose) Alhydrogel was used to ensure binding of L1V to AH (without phosphate-treatment). The same Alhydrogel concentration was used for PTAH-containing formulations. Based on the above studies, 20-mM phosphate was used to generate PTAH, resulting in vaccine formulations with unbound L1V (formulations #2, 4, and 6, Fig. 4); while L1V was adsorbed to the aluminum-adjuvant in the formulations containing AH (formulations #1, 3, and 5, Fig. 4). According to the results shown in Fig. 3B, 0.4- and 1.0-mg/mL (20- and 50- μ g/dose, respectively) of CpG was chosen to generate formulations with CpG bound and primarily unbound to PTAH. At these two concentrations, CpG was expected to be mainly bound to AH (Fig. 3A). Two formulations (#7 and 8, Fig. 4) without L1V were also included as negative-controls.

Silver-stained SDS-PAGE of the supernatants from the vaccine formulations (Fig. 4) directly demonstrated the binding states of L1V and CpG to AH and PTAH. One sees that AH (without phosphate-buffer) adsorbs most of the protein (and CpG) (Fig. 4, formulations 1, 3, and 5). Phosphate-treated Alhydrogel (PTAH) results in little adsorption of L1V and/or CpG(50- μ g/dose) (formulations 2 and 4). At the lower dose of CpG(20- μ g/dose), PTAH

adsorbed the CpG, but not L1V (formulation 6). The efficacies of these formulations were then tested in animal studies.

3.4. L1V/CpG adsorbed to Alhydrogel induces better antibody responses in mice than non-adsorbed protein and CpG

The adsorbed versus non-adsorbed formulations resulted in statistically significant differences in antibody responses. With Alhydrogel as the only adjuvant, there is higher antibody titers and mature-virus (MV)-neutralization activity in the formulation that adsorbed L1V to AH (Fig. 5; group 1 vs. 2). The inclusion of CpG (1-mg/ml; 50- μ g/dose) to the vaccine enhances antibody responses (Fig. 5). Total IgG, IgG-isotypes, and MV-neutralization activities are greatly increased in the presence of CpG. Also, in the presence of CpG, an IgG2a-isotype antibody is generated (Fig. 5C). Furthermore, when compared to non-adsorbed formulations, formulations in which both the L1V-protein and CpG are primarily adsorbed to Alhydrogel, the antibody responses are greatly enhanced (Fig. 5A, solid vs. open symbols). Similar differences are seen with formulations at the lower dose of CpG (0.4-mg/ml; 20- μ g/dose), but overall responses are lower than with CpG at 1-mg/ml (50- μ g/dose). To determine if the relative affinity of the antibodies generated to adsorbed (sera from group 3) vs. non-adsorbed (sera from group 4) L1V differed, we used sodium thiocyanate at various concentrations to elute bound antibody. Using equimolar amounts of antibody (group 3 at 1:3200 and group 4 at 1:800), we found that the antibodies had similar elution curves (Fig. 5D & E).

3.5. L1V/CpG adsorbed to Alhydrogel induces better protection in mice than non-adsorbed protein and CpG

Three weeks after boost vaccinations, mice were intranasally challenged with a lethal dose of VACV (Fig. 6). Control mice vaccinated with adjuvant(s) alone (groups 7 and 8) all died or needed to be humanely sacrificed. Mice vaccinated with L1V and CpG alone also do not survive challenge (data not shown). Groups vaccinated with L1V and Alhydrogel (groups 1 and 2) lost a significant amount of weight and had an overall survival of 60%. At a non-lethal challenge dose, mice vaccinated with L1V/AH lost significantly less weight than mice vaccinated with L1V/PTAH (data not shown). Groups vaccinated with L1V/AH/CpG (groups 3 and 5) had 100%-survival and significantly less maximum weight-loss when compared to non-adsorbed formulations (L1V/PTAH/CpG; groups 4 and 6). This result indicates that mice vaccinated with formulations that adsorbed the L1V-protein and CpG to Alhydrogel had significantly less disease (as measured by weight-loss) than formulations that did not adsorb L1V to aluminum or did not include CpG in the formulation.

4. Discussion

Subunit-vaccines are important alternatives to live-attenuated or whole-organism killed vaccines. This is not only because of improved safety profiles and the advantage of focusing the immune response to specific protective antigen(s), but because of the negative public perception of some whole-organism vaccines. Hem and Hogenesch [6] predicted that an important area of research to improve the performance of aluminum-adsorbed vaccines would include an emphasis on preformulation studies including characterization of the adsorption of the antigen on to aluminum. There has been a growing body of literature that has compared aluminum-adsorbed vaccine formulations and how protein antigens adsorb to aluminum, the effect of interstitial fluid on protein de-adsorption, and the resulting immune responses [5–16]. These carefully performed studies have raised some important new hypotheses on the formulation of aluminum-based vaccines. This type of preformulation characterization work led to the recommendation that antigen stability and

antigen-adjuvant interactions should be more carefully considered during vaccine design and preclinical development [35, 36].

The purpose of this work is thus to investigate L1V-protein stability in solution, and to determine if adsorption of the poxvirus L1-protein, a key antigenic component of a future generation multi-subunit smallpox vaccine, to aluminum adjuvant was required for optimal antibody responses and protection from VACV-challenge. First, it was determined by different biophysical techniques, combined with an EPD approach to data analysis [30, 31], that the L1V-protein best maintained a native conformation during exposure to temperature stress in the pH-range of 6–8. Based on these data, a solution pH of 7 was selected for preparation of vaccine formulations. As a next step, the role of aluminum adjuvant in the formulation was examined by a combination of in-vitro and animal studies.

Similar to work by us and others [17, 25], the inclusion of Alhydrogel (AH) in the vaccine formulation was critical for the development of an immune response to the antigen. We found that the L1V-protein without AH (L1V/CpG) did not generate measurable antibody responses and did not protect from challenge (data not shown). We then compared vaccine formulations with AH, with fully adsorbed L1V (with or without CpG), to vaccine formulations with phosphate-buffer treated aluminum hydroxide (PTAH), which did not significantly adsorb L1V. Similar to other studies that investigated the immune response to adsorbed antigens like recombinant protective antigen from anthrax [11] or recombinant antigens from *Streptococcus pneumonia* [37], we too consistently found that the L1V antigen adsorbed to an aluminum salt gave enhanced antibody-responses and better protection after VACV-challenge when compared to formulations that had L1V free in solution in the presence of AH (i.e., unbound L1V). The mechanism of why L1V gives enhanced antibody responses when adsorbed to AH is not known and will require further investigation. It appears not to be something specific to pox antigens, since preliminary studies with the other antigens in our multi-subunit vaccine show the adsorption of A27V to AH is not required for enhanced antibody responses (Xiao & Isaacs, unpublished). It is also not unique to L1V since preliminary data indicate that responses to A33V or B5V are enhanced when it is adsorbed to AH (Xiao & Isaacs, unpublished). Based on measuring the relative affinity of the antibodies generated in the presence of CpG, the total IgG and IgG2a responses appear similar (Fig. 5D & E) indicating that the antibody maturation is similar in mice vaccinated with the adsorbed and non-adsorbed formulations. It will be interesting to see if a potential mechanism for higher antibody titers when L1V is adsorbed to AH is due to an altered tertiary structure that makes the protein more vulnerable to proteolytic processing. This conformational destabilization was seen previously for some model protein antigens [38, 39], but antibody responses were not ascertained. Alternatively, Levesque et al. hypothesized that the differences in antibody responses that they saw with recombinant antigens from *Streptococcus pneumonia* may have been due to initially higher localized concentration of antigen in proximity with adjuvant when antigen was adsorbed to AH [37].

The inclusion of CpG adjuvant in our vaccine is critical for optimal protection from VACV-challenge (Fig. 7, groups that included CpG vs. groups with Alhydrogel only). The inclusion of CpG, however, does not supersede the importance of L1V adsorption to AH in the generation of improved antibody-responses and protection. This effect can best be seen when comparing the antibody titers (Fig. 5) and weight-loss after challenge (Fig. 6A) of group 5 (L1V/AH/CpG(20-μg)) versus group 6 (L1V/PTAH/CpG(20-μg)). At this CpG dose, both formulations adsorb the CpG, but differentially adsorb L1V to the Alhydrogel (Fig. 4, lanes 5 and 6). The antibody titers (Fig. 5A–C), neutralization-activity (Fig. 5F), and protection after challenge (Fig. 6A) are all enhanced in group 5, the vaccine formulation with L1V adsorbed to the AH.

As mentioned, we are in the process of performing similar studies with the other protein antigens that will be part of the recombinant protein-based subunit smallpox vaccine. For A33 and B5, the development of the IgG2a-isotype is important for protection [18, 19, 25, 40, 41]. A vaccine formulation that further enhances the Th1-type antibody-response to these components of the vaccine, will likely generate more protective vaccine. As a potent neutralizing target on the MV envelope, the generation of anti-L1 IgG2a-isotype responses is theoretically less important. Our data support this supposition. As shown in Fig. 5, the vaccine formulations that generate the highest anti-L1 titers (groups 3 and 5) provide the most protection after challenge (Fig. 6). The IgG2a response developed in group 4 is statistically better than the response generated in group 5 (Fig. 5C). Yet, protection after challenge is much better in group 5 compared to group 4, indicating that the IgG2a response to L1 is less critical.

For this work we focused only on antibody responses since antibody responses are the key protective mechanism of protection upon secondary orthopoxvirus challenge (for review see [42]). However, we do not know if T-cell responses are different between the adsorbed and non-adsorbed formulations. This will be an interesting line of future research and may provide insight into the mechanism(s) of the enhanced antibody responses we found to formulations that adsorbed the L1V antigen.

Conclusions

In conclusion, this work demonstrates that designing vaccine formulation conditions to maximize antigen-adjuvant interactions is a key factor for immunogenicity and protection of recombinant L1-protein-based smallpox vaccines. We hope to translate these findings to improve immune responses in vaccinated non-human primates and protection from monkeypox virus challenge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AH	Alhydrogel without phosphate treatment
EPD	empirical phase diagram
PTAH	phosphate treated alhydrogel
VACV	vaccinia virus

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Highlights

- !! Biophysical characterization of the orthopoxvirus L1 vaccine antigen is presented (83)
- !! L1-protein adsorbed to Alhydrogel generated better IgG responses than non-adsorbed (84)
- !! Addition of CpG to Alhydrogel significantly enhances the anti-L1 antibody responses (85)
- !! L1-adsorbed to Alhydrogel resulted in better protection from vaccinia challenge (81)
- !! Vaccine formulation design is key to maximize vaccine immunogenicity and protection (83)

Fig. 1.

Biophysical characterization of L1V-protein antigen. (A) Far-UV CD spectra of L1V at different pH-values. Thermal melt data of L1V at different pH-values by monitoring (B) CD signal at 208-nm, (C) ANS fluorescence emission intensity, and (D) static light scattering at 275-nm as a function of temperature. Far-UV CD spectra (A) were collected at a scanning speed of 20-nm/min from 190 to 260-nm using a 0.1-cm path length cuvette. Buffer spectra were subtracted from the sample spectra for baseline correction. The temperature dependent changes of the CD signal at 208-nm (B) were monitored over a temperature range of 10 to 85 °C, with a resolution of 0.5 °C and a heating rate of 15 °C/hr. Buffer spectra were subtracted from the sample spectra for baseline correction. For ANS fluorescence emission intensity (C), each sample contained ~0.1-mg/mL protein and a 15-fold molar excess of ANS. The samples were excited at 372-nm and emission spectra were collected from 400–600-nm with a 1-nm/sec data collection rate and 1-sec integration time. Emission spectra were collected every 2.5°C with 3-min equilibration over a temperature range of 10 to 85°C. The ANS-buffer baseline at each corresponding pH and temperature was subtracted from the sample emission spectra. ANS fluorescence emission peak positions and intensities were obtained by a “center of spectral mass” method using Origin software. This produces significant shifts in the actual peak position of approximately 12-nm, but produces more reproducible values of the wavelength maximum. Signals of the scattered light (D) were detected at a 90° angle from the incident light using a slit width of ~1-nm. A spectral window of 250- to 370-nm was used to collect light scattering data. The spectra were collected from 10 to 85°C at 2.5°C intervals and a 3-min equilibration time at each temperature. A buffer baseline was subtracted from each sample measurement.

Fig. 2.

Empirical phase diagram (EPD) of L1V-protein constructed with thermal melt data from the CD signal at 208-nm, ANS fluorescence peak intensity, and static light scattering at different values of solution pH. The colors have no absolute meaning but simply represent different structural states of the protein. . The data generated by the biophysical techniques described in Fig. 1 were initially sorted by pH and temperature using Microsoft Excel (Microsoft Corp, Seattle, WA) and Matlab (Math Works, Inc., Natick, MA) for phase diagram preparation. The discrete parameters are analogous to coordinates that are associated with a set of variables from each technique employed. These variables represent unit vectors that define an n-dimensional space with dimensions equal to the number of variables included in the data set. Individual unit vector projectors are then calculated and summed to obtain a density matrix. The eigenvalues and eigenvectors of the density matrix are calculated, and the three most influential components are represented as colors in a red, green, and blue scheme. Finally, the three components are summed to yield a final color that represents the physical (not necessarily equilibrium) state of L1V protein under each pH and temperature condition.

Fig. 3. Binding of CpG oligonucleotide to aluminum adjuvants, (A) Alhydrogel (AH) and (B) phosphate-treated Alhydrogel (PTAH). The CpG titration was then performed with six formulations containing either (A) AH or (B) PTAH with 0-, 0.04- or 0.2-mg/mL L1V protein. After 1-hr incubation at 5°C with shaking, the samples were centrifuged at 12,000-rpm for 3-min using an IEC Micromax centrifuge. The supernatant was separated from the pellet, and CpG in the supernatant was quantified by measuring the absorbance at 260-nm using a NanoDrop 2000 spectrophotometer (Thermo). The amount of measured free CpG in solution was then used to calculate the percentage of unbound CpG under each formulation condition.

Fig. 4. Vaccination groups and Silver-stain SDS-PAGE analysis of different L1V vaccine formulations. Shown is a photograph of a silver-stained gel of the supernatants (5 μl) from eight vaccine formulations. Lane “m” is low-range molecular weight markers (Amersham) with the indicated apparent molecular weights in kDa. Lane “s1” and “s2” is the starting amount of L1V-protein (2 μg/dose; 0.04-mg/ml) in PBS or after dialysis against histidine buffer, respectively. Vaccine formulations number 1 to 8 are indicated at the top and bottom of the gel with the indicated amount of L1V-protein per dose (2-μg/dose; 0.04-mg/ml); whether the aluminum adjuvant was untreated Alhydrogel (AH) or phosphate treated Alhydrogel (PTAH); and the amount of CpG added (50-μg/dose; 1-mg/ml or 20-μg/dose; 0.4-mg/ml).

Fig. 5.

Antibody responses of groups of mice vaccinated with Alhydrogel +/- CpG containing L1V formulations. 96-well plates were coated at 4°C with L1V-protein (1-μg/ml) and probed with pooled heat-inactivated sera (obtained 3-weeks after the boost vaccination) followed by HRP-conjugated rabbit-anti-mouse IgG (1:4000) (Abcam), or conjugated rat-anti-mouse IgG1 (1:1000) or IgG2a (1:1000) (BD Biosciences). ELISA results shown are (A) total IgG, (B) IgG1 and (C) IgG2a isotypes, as well as sodium thiocyanate elution of (D) total IgG and (E) IgG2a. Solid symbols are formulations with Alhydrogel (AH); open symbols are formulations with phosphate treated Alhydrogel (PTAH). Triangles are groups 3 and 4 (formulations with CpG (50-μg/dose); Squares are groups 5 and 6 (formulations with CpG (20-μg/dose); Circles are groups 1 and 2 (no CpG). Diamonds are control formulations of AH only or AH+CpG. (F) Mature-virus (MV) neutralization. In-vitro virus neutralization was performed in triplicate with ~200-pfu of MV in a 100-μl final volume. Solid bars represent a serum dilution of 1:1,000; open bars represent serum dilution of 1:10,000. Graphs shows average values (μ standard deviation of the means). Statistical differences between groups were significant by Tukey's multiple comparison test except for the following comparisons:

ELISA IgG: 1 vs 2; 2 vs 7; 2 vs 8; 7 vs 8 (all other comparisons had $p < 0.003$)

ELISA IgG1: 2 vs 7; 2 vs 8; 3 vs 5; 4 vs 6; 7 vs 8 (all other comparisons had $p < 0.009$)

ELISA IgG2a: 1 vs 2; 1 vs 6; 1 vs 7; 1 vs 8; 2 vs 6; 2 vs 7; 2 vs 8; 6 vs 7; 6 vs 8; 7 vs 8 (all other comparisons had $p < 0.04$)

Affinity IgG: 3 vs 4

Affinity IgG2a: 3 vs 4

Neut (1:1,000): 2 vs 7; 2 vs 8; 3 vs 4; 3 vs 5; 4 vs 5; 7 vs 8 (all other comparisons had $p < 0.0002$)

Neut (1:10,000): 1 vs 2; 1 vs 6; 1 vs 7; 2 vs 6; 2 vs 7; 2 vs 8; 4 vs 5; 6 vs 7; 6 vs 8; 7 vs 8 (all other comparisons had $p < 0.006$)

Fig. 6.

Percent maximum weight loss and survival after challenge. Groups of mice vaccinated with L1V formulations containing Alhydrogel +/- CpG and then 3-weeks after the boost vaccination, groups of mice were intranasally challenge with 1.4×10^6 -pfu. (A) Maximum weight loss. The graph shows maximum weight loss of individual mice (μ standard deviation) of the means. Statistical differences between groups were significant ($p < 0.0004$) by Tukey's multiple comparison test for the following comparisons for maximum weight loss: 1 vs 3; 1 vs 5; 2 vs 3; 2 vs 5; 3 vs 4; 3 vs 6; 3 vs 7; 3 vs 8; 4 vs 5; 5 vs 6; 5 vs 7; 5 vs 8. . (B) Survival curves after challenge. Statistical differences ($p < 0.003$) in the survival curves (compared by Cox-Mantel test) were seen in all L1V vaccination groups compared to controls (groups 7 and 8). Statistical differences ($p < 0.03$) were also seen with groups 3 and 5 (100%-survival) compared to groups 1 and 2 (60%-survival).