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Highly effective generic adjuvant systems for orphan or poverty-related vaccines[☆]

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Abstract

Safe and effective adjuvants are needed for many vaccines with limited commercial appeal, such as vaccines to infrequent (orphan) diseases or to neglected and poverty-related diseases. Here we found that three nonproprietary liposome formulations containing monophosphoryl lipid A each induced 3-fold to 5-fold increased titers of binding and neutralizing antibodies to anthrax protective antigen compared to aluminum hydroxide-adsorbed antigen in monkeys. All vaccinated monkeys were protected against lethal challenge with aerosolized Ames strain spores.

Keywords

Adjuvants; Liposomes; Monophosphoryl lipid A; Bacteriophage T4; Anthrax; Non-human primates

1. Introduction

A major challenge in current vaccinology is to develop new adjuvants to enhance the ability of highly purified recombinant protein and peptide antigens to induce adaptive immunity in humans [1]. Numerous commercial adjuvants and “adjuvant systems” have been tested as constituents of vaccines against various diseases [2–4] but these are often not easily accessible for many vaccine developers due to commercial or proprietary reasons [5].

[☆]The views expressed are those of the authors and should not be construed to represent the positions of the U.S. Department of Defense.

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Adjuvants are a priority for vaccines against biothreat agents such as anthrax and plague, and for diseases associated with poverty such as malaria, HIV/AIDS, and tuberculosis [5]. Anthrax vaccine is an example of a limited use vaccine which, because of policy and safety issues and highly specialized use, is essentially an “orphan” vaccine, as defined by U.S. law and federal regulations [6]. Current approved anthrax vaccines have many real and perceived shortcomings [7], and new types of less reactogenic and more potent adjuvants are needed for larger scale deployment of an improved vaccine [8,9].

A further challenge in vaccinology pertains to those vaccines for which human efficacy studies are not ethical or feasible. Regulatory approval for marketing such vaccines allows for efficacy testing under certain conditions in non-human animal species, the so-called “animal rule” [10], but it has been suggested, based on comparative adjuvant studies, that the relative effects observed in many commonly used animal models, such as mice, rats, guinea pigs, or rabbits, do not reliably predict adjuvant efficacy in humans [2,11,12]. Non-human primates (NHPs) are thought to be a valid surrogate model to predict the efficacies of many vaccines in humans, including recombinant anthrax protective antigen (PA) adsorbed to aluminum hydroxide (AH) [8]. However, the question still remains whether NHPs are attractive as a primary alternative to rodents to select new and superior adjuvants. Approaches designed to address this question could lead to more rapid and reliable prediction of relative potencies of vaccine adjuvants for a variety of orphan and poverty-related diseases than by comparisons of adjuvant effects in smaller animals.

2. Materials and methods

2.1. Animals and reagents

Thirty-nine Indian-origin Rhesus macaques (5–7 years old) that were negative for simian retroviruses (SRV, SIV and STLV), herpes B virus, and pre-screened for antibodies to anthrax protective antigen to ensure no prior exposure, were obtained from the Walter Reed Army Institute of Research (WRAIR) primate pool. The study was conducted in compliance with the animal welfare act and adhered to the principles in the guide for care and use of laboratory animals. The investigators used facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The WRAIR Animal Care and Use Committee approved all animal experiments. Animals were housed under ABSL-2 conditions and transferred to BSL-3 facilities at the WRAIR one week prior to challenge with *Bacillus anthracis* Ames strain spores.

Lipid A detoxified (*Salmonella minnesota* R595) which contains predominantly monophosphoryl lipid A (MPLA), 1,2 dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2 dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), and cholesterol were purchased from Avanti Polar Lipids, Inc. Recombinant PA produced in *B. anthracis* strain BH445 and purified as described earlier [13] was a gift from Dr. Stephen Leppla (NIH). *Escherichia coli* heat-labile enterotoxin was a gift from Dr. John Clements (Tulane University, New Orleans, LA). Alhydrogel[®] (AH) was purchased from E.M. Sergeant Pulp and Chemical Co., Inc.

2.2. Preparation of seven anthrax protective antigen (PA)-vaccine adjuvant formulations

(Formulation 1) Recombinant PA was adsorbed to AH to give a final dose of 200 µg/ml of PA on AH having 2.4 mg of aluminum. (Formulation 2) For transcutaneous immunization (TCI) [14,15], PA (50 µg/dose) was mixed with *E. coli* heat-labile enterotoxin (100 µg/dose) just before application on the surface of the skin on the arms of the Rhesus macaques (PA + HLT).

(Formulations 3 and 4) PA was over-expressed in *E. coli* and purified as a fusion protein with bacteriophage T4 proteins, highly antigenic outer capsid protein (Hoc) and small outer capsid protein (Soc) and then displayed on the surface of hoc⁻soc⁻ bacteriophage T4 nanoparticle (T4-PA) [16–18]. For each animal, about 1.2×10^{12} of purified hoc⁻soc⁻ 4 phage particles were centrifuged at 15,000 rpm for 30 min using Lobind Eppendorf tubes. The phage pellet was then resuspended in 200 µl of PBS buffer, pH 7.4. A total of 1.15 mg PA-Hoc was first incubated with phage particles at 37 °C for 45 min in a total reaction volume of 1 ml. The reaction mixture was cooled to 8 °C followed by the addition of 0.57 mg of Soc-PA for 45 min. The phage particles loaded with PA-Hoc and Soc-PA were then sedimented by high-speed centrifugation at 15,000 rpm for 30 min. The supernatant containing the unbound PA-Hoc and Soc-PA antigens was discarded and the phage pellet was washed twice with excess PBS buffer to remove any remaining unbound antigen. The pellet was resuspended in 500 µl of PBS buffer and transferred to a new Eppendorf tube and stored at 4 °C. The copy number of displayed PA-Hoc and Soc-PA per capsid was quantified by SDS-PAGE and laser densitometry (PDSI, GE Healthcare) as described earlier [16,17]. The T4-PA antigen (+/- HLT) used for immunizations contained 155 copies of PA-Hoc and 200 copies of Soc-PA per phage capsid, which is equivalent to ~50 µg of PA/ animal.

(Formulation 5) PA was encapsulated in liposomes containing monophosphoryl lipid A [L(PA + MPLA)] to give a final concentration of 125 mM phospholipids, 200 µg/ml PA, and 400 µg/ml of MPLA. Detailed procedures for the preparation of liposomes and liposomal emulsion were described previously [19]. Briefly, multilamellar liposomes composed of DMPC, cholesterol, and DMPG (9:7.5:1) with MPLA were prepared by dispersion of lyophilized mixtures of lipids at a phospholipid concentration of 125 mM in Dulbecco's PBS either containing or lacking PA. (Formulation 6) Alternatively, PA was added to preformed liposomal MPLA [PA + L(MPLA)]. (Formulation 7) Liposome-stabilized oil-in-water emulsion was formulated with L(PA + MPLA) and 20% light mineral oil. The final phospholipid concentration of the emulsion [L(PA + MPLA)-emulsion] (also referred to as PA-emulsion) was 125 mM. The emulsion was formulated by mixing the liposomes and the oil just before use as previously described [20].

2.3. Immunization and challenge

Four Rhesus macaques were used for 6 of the 7 adjuvant groups while 8 animals were used with [L(PA + MPLA)] and 7 for the naive group. The immunization and analysis scheme is shown in Fig. 1A. Rhesus macaques were immunized under ABSL-2 conditions by the intramuscular (250 µl dose/animal) or transcutaneous routes (500 µl dose/animal) at weeks 0, 4, and 8 with PA-adjuvant formulations described above. Each group consisted of 4

animals, except the naive group which had 7 animals, and the [L(PA + MPLA)] group, which had 8 animals. Each animal received 50 µg of PA and 100 µg of MPLA or 100 µg of *E. coli* heat-labile enterotoxin. TCI (on the arm) was carried out as previously described [14,15]. T4-PA was injected by the intramuscular route and in one of the groups HLT (100 µg/animal) was applied on the surface of the arm at the site of injection.

Naive and immunized animals were transferred to BSL-3 facilities at the Walter Reed Army Institute of Research on week-13 and challenged with aerosolized *Bacillus anthracis* Ames strain spores (9.6–86.9 LD₅₀; see Table 1) on week-14 using a class III glove box under strict ABSL-3 conditions. The challenge consisted of $5.5\text{--}7 \times 10^4$ cfu delivered as an aerosol between 5 and 15 min using a head-only exposure system and the plethysmography data collected real-time using a Skornik plethysmography system with the animal contained in a sealed and head inside-only exposure chamber. The animals were observed at 4-h intervals daily following exposure for 7 days and then twice a day for 28 days for signs of illness or morbidity. Blood and fecal material were collected every second day after challenge for the first 7 days and then at weekly intervals until the end of the study. All surviving animals were humanely euthanized at the end of the study. All of the animals that either succumbed to the challenge or were euthanized at the end of the study were necropsied for both gross and fine histological changes. All organs were immersion fixed in neutral-buffered 10% formalin for at least 21 days before being removed from ABSL-3 containment for histology processing.

2.4. PA-specific IgG antibodies

PA-specific IgG antibodies were analyzed in individual NHP serum samples collected on weeks 0, 4, 8, 12, and 14 by ELISA as previously described [21]. Human reference serum AVR801 (pooled serum derived from AVA-vaccinated human donors, a gift from Dr. Conrad Quinn, CDC) was used to generate a standard curve, which was used to quantitate the PA-specific IgG antibodies (µg/ml).

2.5. Lethal toxin neutralizing antibodies

Lethal toxin (LTx) neutralizing antibody titers were determined by the ability of individual serum samples to neutralize the cytotoxicity of LTx in a J774A.1 macrophage cell line as described previously [15,22]. A 4-parametric sigmoid regression curve was used to determine the dilution of antiserum that resulted in 50% reduction in toxicity (ED₅₀) of anthrax LTx. Each plate contained control wells that had either toxin alone (no antiserum) or cells alone (no toxin and no antiserum). Polyclonal rabbit anti-PA-specific antibody and human reference serum AVR801 were used as positive controls.

2.6. Statistical analysis

PA-specific IgG concentrations and LTx ED₅₀ titers between the different PA-adjuvant vaccine formulations were analyzed using 2-way ANOVA with Bonferroni posttests.

3. Results

3.1. Challenge of immunized animals with aerosolized anthrax spores

Seven test adjuvant formulations were compared, each with anthrax PA as a model antigen, using the immunization scheme shown in Fig. 1A. The animals were challenged with 100 LD₅₀ of *Bacillus anthracis* Ames strain spores at week-14, 6 weeks after the second boost (Table 1). All of the 7 naive animals died by day 7 (Fig. 1B and Table 1). However, all the animals in each of the adjuvant groups survived (Fig. 1B).

The observed gross and histopathologic lesions in naive animals, examined first and unblinded for comparative purposes, were consistent with documented lesions of inhalation anthrax infection in Rhesus macaques, although there were some slight variations in the lesions among the 7 naive animals (Table 1). Bacilli were noted in vessels and other vascular spaces in the following tissues: tongue, mandibular lymph node, mandibular salivary gland, aorta, trachea, lung, mediastinal lymph node, liver, heart, pancreas, stomach, duodenum, jejunum, ileum, cecum, colon, kidney, adrenal gland, urinary bladder, inguinal lymph node, testicle, epididymis, prostate gland, skeletal muscle, pituitary gland, brain, and eye. Six of the 7 naive animals were positive for bacteremia at day 4 (Table 1) and for one of the animals the sample was not available. Blinded examinations of all of the animals in the 7 adjuvant formulation groups revealed that, when compared with the unblinded naive animals, none of the immunized animals showed any bacteremia, bacilli in the tissues, or anthrax lesions (Table 1). This demonstrated that PA-AH and the other formulations were highly protective for preventing lethal effects of aerosolized anthrax, but the challenge results could not distinguish between the relative effects of the different adjuvants.

3.2. Comparative effects of adjuvants on induction of PA-specific immunity

In contrast to the pathologic findings after challenge, substantial differences were observed between adjuvants in the levels of PA-specific IgG antibody concentrations (Fig. 2A) and lethal toxin neutralizing antibody titers (Fig. 2B). When compared to PA-AH, PA - emulsion and [PA + L(MPLA)] each induced significantly higher levels of PA-specific IgG antibodies at weeks 8, 12, or 14, as indicated by asterisks in Fig. 2. PA-emulsion and [L(PA + MPLA)] also induced significantly higher levels of lethal toxin neutralizing antibodies. However, PA + L(MPLA) induced the highest levels of toxin neutralizing antibodies ($p < 0.001$), nearly 4-fold and 5-fold greater than PA-AH at weeks 12 and 14, respectively, the highest toxin neutralizing antibody titers to PA yet reported in NHPs [23–25]. Interestingly, after only a single injection of [PA + L(MPLA)], the PA-specific neutralization titer at week-4 was equivalent to that after injection of PA-AH at week-4.

4. Discussion

Although aluminum salt adjuvants are deemed relatively safe, they are sometimes associated with local reactions, including subcutaneous nodules, erythema, induration, and contact hypersensitivity [26], and new generic adjuvant formulations that exhibit a high level of safety, lower reactogenicity, but superior immunopotency would be preferred. The safety and lack of reactogenicity of liposomal MPLA has been previously demonstrated in human phase I trials with several candidate vaccine formulations for malaria, HIV, and cancer [11].

Our results with liposome formulations in NHPs are consistent with similar relative potencies observed in humans, but contrast with effects of similar formulations in mice and rabbits. For example, the best performing adjuvant system in this study, [L(MPLA)] mixed with PA, failed to show any adjuvant activity in mice when [L(MPLA)] was mixed with malaria antigen [27], and with HIV antigen in mice it induced antibody titers only 16% of those induced by antigen encapsulated in L(MPLA) [28]. Although [L(MPLA) + antigen] has never been tested in humans, it is a major constituent of both AS01b and AS15, which consist of empty liposome formulations that contain both MPLA and QS21 (AS01b), or MPLA, QS21, and CpG (AS15), that are simply mixed with antigen [3,29]. AS01b and AS15 are both in late stage (phase 3) clinical testing in humans [29]. Furthermore [L(PA + MPLA) – emulsion] had much less adjuvant activity than PA-AH in mice and rabbits (unpublished data), but the liposome-stabilized emulsion was the best performing adjuvant for inducing immune responses to prostate specific antigen in elderly and immunosuppressed late stage prostate cancer patients [11].

Our approach provides evidence that comparative adjuvant studies in NHPs may be a useful alternative approach to the use of other animal models for primary studies to identify safe, generic, easily manufactured, and potent adjuvants that could substitute for aluminum salts in candidate human vaccines, including anthrax vaccine. In the case of anthrax vaccine, the protective effects of PA adsorbed to AH are consistent with the presumed efficacies of licensed anthrax vaccines. However, because of perceptions and issues related to current vaccines [7], new generations of anthrax vaccine may have greater public acceptance. Although aluminum salts are relatively inexpensive, they are also sometimes weak adjuvants, and they do have a history of side effects [26]. The present results in macaques, that compare PA as a model antigen in combination with different 7 adjuvants and adjuvant systems, suggest that generic adjuvant systems containing liposomal MPLA might provide a safe and more potent alternative to AH for an anthrax vaccine.

The recent development of potentially effective malaria vaccines, which has been partly based on demonstration of preclinical effectiveness in macaques of novel strong and safe commercial adjuvant systems that yield better efficacies than those obtained with AH, further illustrates the potential usefulness of testing of new types of adjuvant systems in macaques [3]. Although PA was used as a model antigen in this study, encapsulation of a variety of antigens in liposomal adjuvant systems containing MPLA have demonstrated both safety and effectiveness in macaques and humans [3,11,29]. For vaccines to important poverty-related diseases, such as malaria, HIV/AIDS, tuberculosis, and many other difficult diseases that could benefit from strong and safe nonproprietary adjuvant systems, three generic liposome formulations containing MPLA are identified in this study that may have utility.

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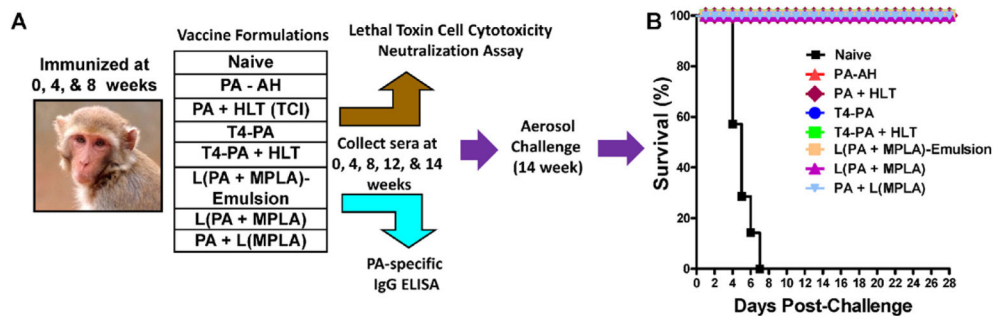
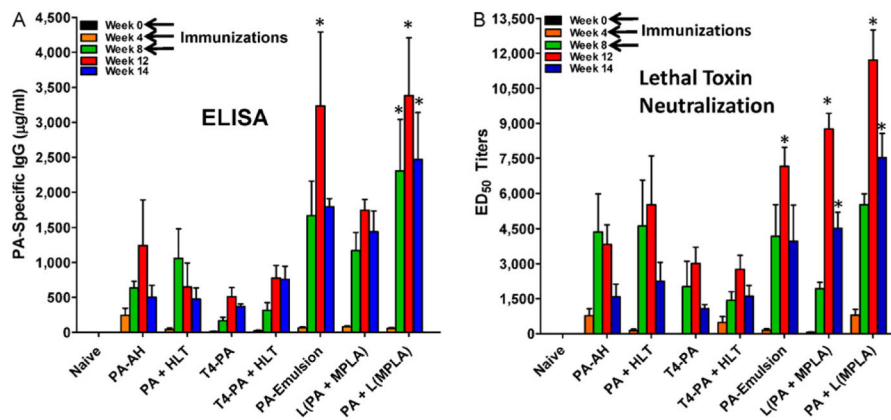


Fig. 1. Immunization scheme and challenge with aerosolized *Bacillus anthracis* spores in Rhesus macaques. (A) Immunization scheme for various vaccine formulations. Each animal received 50 µg of PA/animal/immunization. (B) At week-14, animals were challenged with aerosolized *B. anthracis* Ames strain spores (9.6–86.9 LD₅₀, Table 1) and the data are shown as a Kaplan Meyer survival curve. All of the 7 naive animals succumbed to disease by day 7. All of the 32 immunized animals remained healthy and survived through the 28-day post-challenge period.

**Fig. 2.**

Immune responses to PA-adjuvant formulations in Rhesus macaques. (A) PA-specific IgG antibodies in sera of naive and immunized animals as determined by ELISA. Shown are the average concentrations of PA-specific IgG \pm S.D. from each of the groups on weeks 0, 4, 8, 12, and 14, respectively. PA-emulsion induced significantly higher ($p < 0.01$) PA-specific IgG on week-12 compared to PA-AH. [PA + L(MPLA)] induced significantly higher PA-specific IgG on weeks 8 ($p < 0.05$), 12 ($p < 0.001$), and 14 ($p < 0.01$), respectively than PA-AH. (b) Lethal toxin neutralizing antibody titers (ED_{50}) were determined in individual serum samples. At week 12, PA-emulsion immunized animals had significantly higher titers ($p < 0.05$) compared to PA-AH, while animals immunized with [L(PA + MPLA)] had significantly higher titers on weeks 12 ($p < 0.001$) and 14 ($p < 0.05$). The highest titers were induced in animals immunized with [PA + L(MPLA)] and ED_{50} titers were significantly higher than the titers induced by PA-AH at weeks 12 ($p < 0.001$) and 14 ($p < 0.001$).

Table 1

Summary of analyses related to the challenge of vaccinated Rhesus macaques.

Formulation	Monkey no.	Antibodies (week-14, pre-challenge)		Aerosol challenge dose (LD ₅₀)	Bacteremia blood (CFU/ml)	Bacteria in feces (CFU/g)
		PA-specific IgG (µg/ml)	Neutralizing antibody titer (ED ₅₀)			
Naive	D88Z	<1	0	22	50	70
	DAIP	<1	0	19.9	130	124
	DA83	<1	0	15.9	2000	60
	DA91	<1	0	16.8	20	90
	03D085	<1	0	26.1	24	0
	03D135	<1	0	14.3	No sample	No sample
PA-AH	CL26	<1	0	21.8	24	0
	A0X74	360	859	22	0	0
	CJ3J	842	2651	22.7	0	0
	DA98	604 ^a	5121 ^a	17.9	0	0
	DB10	299	1222	85.6	0	0
	D117	364	1459	19	0	6
PA + HLT	CX5G	811	4353	25.3	0	0
	DA2P	641	2613	20.1	0	26
	DA2W	83	534	86.9	0	0
	DB4T	909	1604	15.9	0	0
	04D256	1146	2809	21.3	0	0
	03D116	718	1439	9.6	0	0
T4-PA + HLT	04D110	274	566	13.7	0	0
	DA9V	445	1354	85.3	0	0
	DA9H	340	755	12.3	0	14
	D151	317	1111	23.3	0	0
	CL1T	571 ^a	3404 ^a	18.3	0	20
	D060	599	4196	17.4	0	0
L(PA + MPLA)	DA1E	1005	6081	15.3	0	6
	CX5A	671	1297	64.5	0	0

Formulation	Monkey no.	Antibodies (week-14, pre-challenge)				Aerosol challenge dose (LD ₅₀)	Bacteremia blood (CFU/ml)	Bacteria in feces (CFU/g)
		PA-specific IgG (µg/ml)	Neutralizing antibody titer (ED ₅₀)	PA-specific IgG (µg/ml)	Neutralizing antibody titer (ED ₅₀)			
PA + L(MPLA)	DA64	808	1930	808	1930	53.2	0	0
	04C073	2918	6018	2918	6018	18.9	0	0
	04D145	1811	5323	1811	5323	18.7	0	0
	03D096	1604	5901	1604	5901	15.7	0	0
	04D233	2109	5344	2109	5344	15	0	0
	DB6V	3653	10,463	3653	10,463	17.6	0	0
	04D109	1229	7497	1229	7497	18.8	0	0
	M001	1375	6012	1375	6012	16.3	0	0
	04D085	3614	6155	3614	6155	12.5	0	0
	04D060	1881	8249	1881	8249	20.4	0	0
L(PA + MPLA) - emulsion	04D283	2075	4054	2075	4054	13.8	0	0
	04D081	1574	2099	1574	2099	9.7	0	0
	DB98	1640	1448	1640	1448	14.7	0	0

^aValues shown were obtained at week-12.