Formulation and Immunogenicity of a Potential Multivalent Type III Secretion System-Based Protein Vaccine

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ABSTRACT: The virulence of many pathogenic Gram-negative bacteria is dependent upon their type III secretion (TTS) systems. Here, we discuss initial formulation studies of five TTS needle tip proteins IpaD (*Shigella flexneri*), BipD (*Burkholderia pseudomallei*), SipD (*Salmonella* spp.), LcrV (*Yersinia* spp.), and PcrV (*Pseudomonas aeruginosa*) as targets for subunit vaccines. Excipient screening and subsequent assays lead to the selection of 10% sucrose and 5% dextrose as an optimal stabilizer combination for all five proteins. All of the proteins adsorb to aluminum hydroxide adjuvant, although the mechanisms of adsorption may vary. The proteins are physically stable when adsorbed to the adjuvant for at least 3 months at room temperature and chemical stability is enhanced in the presence of excipients. The ability of the IpaD and SipD proteins to elicit strong humoral immune responses was also tested in a murine model in the presence and absence of their needle counterparts MxiH and PrgI (see previous paper in this issue). Both proteins produce high antibody titers regardless of dose. While the IpaD titer is boosted slightly in the presence of its needle protein, MxiH, SipD titers appear to be reduced when administered in the presence of its needle counterpart, PrgI. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:4497–4509, 2010

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INTRODUCTION

Despite modern advances, Gram-negative bacterial pathogens continue to persist as serious health risks for humans, particularly in the developing world. These agents cause diseases such as bacillary dysentery (*Shigella flexneri*), septicemia (*Salmonella typhi*, *Burkholderia pseudomallei*, and *Yersinia pestis*), gastroenteritis (*Salmonella typhimurium* and *Yersinia enterocolitica*) and chronic infections in compromised individuals (*Pseudomonas aeruginosa*). *Shigella* alone is estimated to cause 165 million infections each year worldwide with over a million deaths.[1] The severity and frequency of such infections highlights the need for safe, efficacious vaccines that can be inexpensively produced and administered anywhere in the world. This need has increased in the last 20 years with the emergence of antibiotic resistant strains for many of these pathogens.[2–5]

We have elected to develop proteins from the type III secretion system (TTSS) of important pathogens for the development of targeted subunit vaccines. The TTSS plays an integral role in the pathogenesis of these bacteria either by facilitating host cell invasion or by preventing macrophage phagocytosis.[6–8] The type III secretion apparatus (TTSA) comprises a basal body and needle, which together span the inner and outer bacterial membrane and extend into the extracellular space.[7–9] The overall complex is composed of more than 20 proteins and functions to translocate effector proteins from the bacterial cytoplasm into the membrane and cytoplasm of a host cell.

A specific class of class of proteins has recently been shown to localize to the extracellular end of the TTSA needle, where it functions to regulate the secretion of other proteins passing through it.[10,11] Due to the location and function of these so called tip proteins, it is anticipated that they might be effective antigens in a subunit vaccine. The five tip proteins selected for this vaccine formulation study are IpaD from *S. flexneri*, BipD of *B. pseudomallei*, SipD of *Salmonella*

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spp., LcrV of *Yersinia* spp., and PcrV of *P. aeruginosa*. These proteins, ranging in size from 32 to 37 kDa, possess primarily α-helical secondary structure and are dumb-bell shaped with a central coiled-coil separating the N- and C-terminal domains.[12,13–16] It has previously been established in animal models that several of these prospective antigens are highly immunogenic and provide at least a degree of protection.[17–20] Furthermore, in the case of *Shigella*, experiments have demonstrated that *in vitro*, anti-IpaD IgG neutralized host cell invasion in model systems.[10]

While the tip proteins show promise in eliciting an appropriate, protective immune response, it is important that any formulation involving these antigens be sufficiently stable to permit distribution to regions of the world lacking reliable refrigerated storage.[21] This requirement demands any such product possess greater stability than is often expected for use in developed countries. Until now, little attention has been given to formulation considerations for these tip proteins. In this work, we describe studies to systematically ascertain optimal formulation compositions and conditions. This has been done with the ultimate goal of combining these antigens into a single multivalent vaccine. As such, we have sought a single formulation which stabilizes all of the antigens.

An adjuvant is often necessary for subunit vaccines to increase their immune response.[22] This boost in immunogenicity is accomplished by a variety of mechanisms ranging from activation of Toll-like receptors on antigen presenting cells to creating an antigen depot.[22–24] The adjuvant selected for this project is the aluminum salt Alhydrogel®. The mechanism of action of this crystalline substance has not been fully elucidated.[25–29] Nevertheless, due to its excellent safety record, inclusion in many FDA approved commercial vaccines and the pI's of the tip proteins (all between ~5 and 5.5 which facilitates electrostatic interactions with the positively charged adjuvant), it is a facile choice.[29–32] Adsorption and desorption studies were conducted to elucidate the nature of protein–Alhydrogel® interactions.

The antigenic potential of two of the TTSS tip proteins introduced by intramuscular injection, IpaD and SipD was also examined in a murine model in the presence and absence of Alhydrogel® as well as in combination with their corresponding needle proteins $MxiH^{\Delta 5}$ and $PrgI^{\Delta 5}$, respectively (see previous paper in this issue). It is thought that antigens with repeating epitopes are more immunogenic than those possessing only a single recognition site and that an increased antigen size results in increased immunogenicity.[33] Therefore, a polymerized version of MxiH was also used in combination with its tip protein, IpaD, to create a vaccine with multi-valent and multi-epitope properties.

MATERIALS AND METHODS

Materials

Recombinant proteins were expressed and purified as described previously.[15,16] All proteins were dialyzed into 20 mM citrate/phosphate buffer at pH 7 and stored at −80°C until use. For the final formulation, a 10 mM histidine buffer was selected containing 5 mM phosphate. Phosphate was included to exchange some of the exposed oxyhydroxide in the aluminum hydroxide adjuvant with phosphate, thus lowering the pH microenvironment at the adjuvant/protein interface (unpublished data). Samples for immunogenicity studies were prepared according to the preceding paper in this issue. In bivalent formulations, the solution materials were mixed, and the adsorption step was performed subsequent to mixing. Alhydrogel® (2%) and Adjuphos® were acquired from E.M. Sergeant Pulp and Co., Inc. (Clifton, NJ).

Excipient Screening

A high throughput screening method was devised to identify compounds that could serve as stabilizers in a pharmaceutical formulation. Previous work indicated that all of the putative antigens aggregated significantly at pH 5 and 6 at elevated temperatures.[34] This physical degradation pathway was selected because it could be easily adapted for high throughput screening and reflects a major pathway for the alteration and loss of activity of many proteins.[34] An assay was developed employing 96-well plates at 55°C using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) which measured the time-dependent optical density increase at 360 nm. Samples were analyzed in duplicate with a negative control containing buffer and excipient on the same plate. Measurements were taken at 2 min intervals for a period of 2 h. The effectiveness of each additive was quantified by percent inhibition of aggregation. The value of this parameter was calculated using the following equation:

$$\%\text{Inhibition} = \left(1 - \frac{\Delta \text{OD}_{360}(E)}{\Delta \text{OD}_{360}(A)}\right) \times 100$$

where ΔOD_{360} (E) is the change in optical density over the course of the experiment with excipient present and ΔOD_{360} (A) represents the same measurement without excipient. To create the appropriate sample conditions, protein stock solutions were diluted to 0.2 mg/mL in 20 mM citrate phosphate buffer at pH 5. All excipients tested belong to the generally regarded as safe (GRAS) library of compounds.