Formulation and Immunogenicity Studies of Type III Secretion System Needle Antigens as Vaccine Candidates

BROOKE S. BARRETT,[1] AARON P. MARKHAM,[1] REZA ESFANDIARY,[1] WENDY L. PICKING,[2] WILLIAM D. PICKING,[2] SANGEETA B. JOSHI,[1] C. RUSSELL MIDDAUGH[1]

[1]Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, 2030 Becker Drive, Kansas 66047

[2]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma 74078

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ABSTRACT: Bacterial infections caused by *Shigella flexneri*, *Salmonella typhimurium*, and *Burkholderia pseudomallei* are currently difficult to prevent due to the lack of a licensed vaccine. Here we present formulation and immunogenicity studies for the three type III secretion system (TTSS) needle proteins MxiH$^{\Delta 5}$, PrgI$^{\Delta 5}$, and BsaL$^{\Delta 5}$ (each truncated by five residues at its C terminus) as potential candidates for vaccine development. These antigens are found to be thermally stabilized by the presence of carbohydrates and polyols. Additionally, all adsorb readily to aluminum hydroxide apparently through a combination of hydrogen bonds and/or Van der Waals forces. The interaction of these proteins with the aluminum-based adjuvant changes with time resulting in varying degrees of irreversible binding. Peptide maps of desorbed protein, however, suggest that chemical changes are not responsible for this irreversible association. The ability of MxiH$^{\Delta 5}$ and PrgI$^{\Delta 5}$ to elicit strong humoral immune responses was tested in a murine model. When administered intramuscularly as monomers, the needle components exhibited dose dependent immunogenic behavior. The polymerized version of MxiH was exceptionally immunogenic even at low doses. The responses of both monomeric and polymerized forms were boosted by adsorption to an aluminum salt adjuvant. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:4488–4496, 2010

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INTRODUCTION

Shigella flexneri, *Salmonella typhimurium*, and *Burkholderia pseudomallei* are three pathogens responsible for an extensive number of potentially preventable diseases. For example, *Shigella* is one of the leading causes of infant mortality in developing countries, and is responsible for the infection of more than 165 million people each year worldwide.[1] *Salmonella* is best known for its high-profile outbreaks in the developed world and is associated worldwide with more than 200 million cases every year.[2–4] *Burkholderia* is endemic to tropical regions, and is listed as a Category B Bioterrorism Agent by the CDC due to the severity of acute disease, the potential for aerosol delivery and worldwide availability.[5] In all of these cases, there are no licensed vaccines for prevention of infection by these pathogens, which are also becoming increasingly resistance to antibiotic treatment.[6–9] The need for vaccine development in this area is widely recognized and the work presented here targets this effort.

S. flexneri, *S. typhimurium*, and *B. pseudomallei* each relies upon a type three secretion system (TTSS) as an essential virulence component. The type III secretion apparatus (TTSA) is appropriately referred to as a molecular "injectisome" and is composed of more than 20 proteins which assemble to form basal and extracellular components.[10] This macromolecular conduit allows the bacteria direct physical contact with a host cell and is responsible for the transport of effector proteins from the bacterial cytoplasm directly into target cells where they subvert normal cellular functions.[11–13] The surface exposed "needle" portion of the structure is composed of a defined number of monomeric subunits

Additional Supporting Information may be found in the online version of this article.

Brooke S. Barrett and Aaron P. Markham contributed equally to this work.

Correspondence to: C. Russell Middaugh (Telephone: 785-864-5813; Fax: 785-864-5814; E-mail: middaugh@ku.edu)

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polymerized into a hollow helical array with an inner pore diameter of approximately 1.5–3.0 nm.[14–17] This appendage is required for virulence and is intimately involved in the initial stages of an infection.[10,18] Therefore we have explored the use of these surface exposed proteins as potential vaccine antigens.

The vaccine antigens studied here are the monomeric subunits of the oligomerized needle appendage from each bacterial system. These small (~10 kDa), acidic (p$I < 5$) proteins are characterized by distinct patches of both positive and negative surface charge, which probably contribute to their intrinsic polymerization properties.[19] When some of these proteins are expressed recombinantly, their propensity to oligomerize results in a viscous solution of highly associated products; however, it is possible to prepare them as soluble monomers when five residues are deleted from the C terminus.[20] The resulting recombinant proteins, MxiH$^{\Delta5}$, PrgI$^{\Delta5}$, and BsaL$^{\Delta5}$, from the gram-negative bacteria *S. flexneri*, *S. typhimurium*, and *B. pseudomallei*, respectively, have been shown to retain native structure.[20]

Previously, we characterized the solution stability of these mutant monomeric needle antigens, and found them to be pH sensitive, thermally labile proteins with reversible transitions and molten globule-like behavior in the physiological temperature range.[21] Here we describe formulation studies of these vaccine candidates accompanied by examination of the immunogenicity of MxiH$^{\Delta5}$ and PrgI$^{\Delta5}$ in a murine model. BsaL$^{\Delta5}$ was not studied due to material limitations. We have also formulated a recombinant polymeric needle construct of MxiH which mimics the actual polymerized TTSS needle structure. In many cases, antigens with repeating epitopes display greater immunogenicity than those possessing only a single recognition site. Increased antigen size (as occurs upon polymerization) has also been shown to likewise increase immunogenicity.[22] It should be emphasized that the intention of this work is not to propose a final clinical formulation, but rather to examine aspects of potential future formulations which may be useful for further development of these and related vaccines based on the needle proteins.

MATERIALS AND METHODS

Materials

C-terminal truncated proteins were expressed in *E. coli* with a C-terminal His_6 tag as described previously.[17,20] Affinity purification was performed using nickel chelation chemistry, and the samples were stored in 20 mM isotonic pH 6 citrate phosphate buffer and stored at −80°C until use. For immunogenicity studies, MxiH was also expressed in its full-length form, resulting in largely polymerized material referred to as "Needle" hereafter (data not shown). Alhydrogel® (2%) and Adjuphos® were acquired from E.M. Sergeant Pulp and Co., Inc. (Clifton, NJ).

Screening for Excipients

Potential excipients were screened for stabilizing effects using far-UV circular dichroism spectroscopy. Secondary structure was used as the stability indicating parameter in this case because of the lack of other useable temperature sensitive signals (i.e., Trp fluorescence, static light scattering, etc.) exhibited by these proteins.[21] Thermal melts were conducted using a Jasco J-720 spectropolarimeter equipped with a six position sample holder and a Peltier temperature control device (Easton, MD). Initially, individual far-UV CD spectra were recorded for each of 6 samples at 10°C from 260 to 190 nm in a 0.1 cm path length cell. The cell holder temperature was then increased from 10 to 85°C in increments of 0.5°C. At each temperature step the cell holder was incubated for 5 min to ensure thermal equilibration of the samples before a spectrum was obtained. For each thermal melt, a control protein sample containing 0.25 mg/mL protein in 20 mM isotonic citrate phosphate buffer at pH 6.0 was run simultaneously with 5 samples containing test compounds at the indicated concentrations. Spectra of the compounds alone were also collected and subtracted from the protein spectra when necessary. Using the instrument software, the resulting data were converted to molar ellipticity as a function of temperature. Midpoints of thermal transitions (T_ms) were determined using Microcal Origin® sigmoidal fit graphing tools.

Adsorption Isotherms

Protein was dialyzed into isotonic 10 mM histidine buffer at pH 6 overnight at 4°C using 3500 MWCO Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Rockford, IL). Upon completion, the protein concentration was determined by UV absorbance spectroscopy using an Agilent 8453 UV/Vis spectrophotometer equipped with a diode array detector (Agilent Technologies, Santa Clara, CA). Extinction coefficients of 9970, 11,460, and 12,950 $M^{-1}\,cm^{-1}$ at 280 nm were used for MxiH$^{\Delta5}$, PrgI$^{\Delta5}$, and BsaL$^{\Delta5}$, respectively.[21] Protein was then adsorbed to Alhydrogel by combining a predetermined volume of Alhydrogel stock, protein stock and histidine buffer to produce a range of protein concentrations (0–1.25 mg/mL) and a constant aluminum concentration (0.5 mg/mL), and allowing it to rotate end-over-end at 4°C for 1 h. Samples were then centrifuged at 14,000*g* for 30 s, and the resulting supernatant was removed and assayed for protein content by UV absorbance spectroscopy. The amount