Vaccine Technologies & the Rationale for New Nanoparticle Formulations

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INTRODUCTION

Particle Sciences, Inc. (PSI) has developed a stable, hydrophobic nanoparticles technology that has the attributes of virus-like particles but with a much simpler architecture. They can be used to formulate vaccine antigens as particulates and link them to immunemodulators, such as TLR agonists at variable ratios and doses. This technology provides an efficient formulation platform for antigens and immunemodulators that can be optimized for both vaccine potency and safety profiles. The rationale for this vaccine technology builds on several of the major vaccine developments of the past 20 years that leverage the physicochemical attributes that the immune system uses to recognize microbes, leading to robust protective immune responses.

IMMUNE RESPONSES TO MICROBES

Two important vaccine advances have been the development of nanoparticle-based vaccine formulations and the identification of unique microbial products that are agonists for immunologic receptors: virus-like particles (VLPs) or nanoparticles, and Toll-Like Receptor (TLR) agonists, respectively.[1-3] The immune system continuously screens for and interacts with microbes, identifying them as foreign and targeting them for immune responses. The immune system has evolved to use these unique physical and chemical attributes, TLRs for instance, to recognize microbes and specifically respond with protective responses.

Bacteria and viruses, in essence, are naturally occurring micro/nanoparticulates that produce and are decorated with TLR agonists and assorted molecules, some of which are protective antigens. When antigen-presenting cells (APCs) of the innate immune system interact with the microbes' particle/TLR agonist structures, the APCs are stimulated to internalize the particles into endocytic vesicles and process them for immune presentation. APCs far more efficiently recognize and internalize these particles compared soluble molecules. The presentation of internalized antigens by APC involves processing the protein antigens into peptide fragments that bind to Major Histocompatibility Complex (MHC) proteins expressed on their surfaces. APCs interacting with microbes are also directly stimulated by the microbes' TLR agonists and respond by producing immunestimulatory signals, like co-stimulatory cell surface molecules and secreted cytokines that stimulate surrounding T and B lymphocytes.

Antigen-specific T lymphocytes recognize the presented antigen fragments on APC via their T cell receptors and are stimulated by APCs' co-stimulatory signals and cytokines. These stimulated T lymphocytes then proliferate, expanding

their numbers, and differentiate to carry out cytotoxic or regulatory functions. Cytotoxic T lymphocytes kill surrounding cells that are acting as hosts for microbes, enabling the propagation of microbes, like viruses. The regulatory T lymphocytes express immunostimulatory molecules on their cell surfaces and secret lymphokines that simulate and regulate other cells, like B lymphocytes and other T lymphocytes.

B lymphocytes recognize the unprocessed forms of antigens on the surface of the microbes via their antigen-specific antibodies expressed on their cell surfaces. Microbes, which are particulate, can be especially stimulatory for B cells because they have multiple copies of antigens arrayed on their surfaces, which will cross-link the antigen-specific antibodies on the surface of B lymphocytes. This antigen-mediated cross-linking of B cell surface antibodies, the T cell-produced lymphokines and cell-surface signals, and the APC-produced cytokines cooperatively stimulate B lymphocytes. These stimulated B cells respond by proliferating to expand their numbers and to differentiate in plasma cells: the cells responsible for secreting antigen-specific antibodies that protect us from these infecting microbes.

These fundamental, natural immunological processes have evolved to efficiently interact with and recognize microbes, and they explain why the original crude vaccines composed of killed or attenuated whole cell bacteria and viruses have been very potent immunogens/vaccines: they are essentially particulate vaccines.

MODERN VACCINE FORMULATION TECHNOLOGIES

Understanding and taking advantage of these natural processes has become increasingly important as vaccine development has progressed toward creating biopharmaceutical products composed of highly defined recombinant proteins and synthetic molecules as vaccine antigens. Though these well-characterized, purified molecules are known protective antigens, they have routinely been poor immunogens/vaccines, lacking immune potency and immunogenicity because they no longer have the physical architecture of particles and *in situ* TLR agonists that differentiate and identify foreign microbes for the immune system. It has become important that modern biopharmaceutical vaccines be rationally designed to have the chemical and physical attributes that distinguish microbes for immune responses.

TLR AGONIST AND ADJUVANTS

To improve the immunological potency of modern prophylactic and therapeutic vaccine antigens, alternative adjuvants and formulations have been pursued for more than 20 years. Many, if not most, of the active components of these adjuvants have been found to be TLR agonists.[2,3] These agonists are, or mimic, the components of microbes that are not produced by eukaryotic cells and so act as distinguishing markers for microbes. These components are, among others: LPS for which a modified form known as monophosphoryl Lipid A (MPL) has been developed and approved as a human vaccine adjuvant; flagellin, which is the monomeric protein that multimerizes to form the bacterial motility organ flagella; unmethylated CpG sequences, which are typical of prokaryotic DNA, single-stranded RNA sequences that are viral-like; and PAM_3cys/PAM_2cys, which is a lipid structure attached to some proteins exclusively in prokaryotes. However, when simply mixed with soluble purified antigens, these adjuvants/agonists typically require high adjuvant doses to be effective (Figure 1). At these high adjuvant

doses, TLR agonists disperse and systemically activate APC to secret cytokines, which results in an increased frequency of fevers and serious adverse responses in vaccinated individuals. In natural microbe-immune system interactions, the TLR agonists are present in very low doses and are physically associated with the particulate structure of the microbes. In this circumstance, TLR agonists interact and stimulate immune responses in a very localized and microbe-specific fashion. Thus, it's not surprisingly that the physical linkage of these TLR agonists to the vaccine antigens has been demonstrated to significantly reduce the required dose of TLR agonists needed as an adjuvant (Figure 1).

Linkages between TLR agonists and antigens have to-date been covalent in nature.[4] As an example, CpG adjuvants, a TLR9 agonist, has been chemically conjugated to a number of antigens.[5-7] Similarly, agonists for TLR7/8, which mimic to single stranded viral RNA sequences, have increased adjuvant potency when conjugated to antigens.[8] PAM_3cys, a TLR1/TLR2 agonist, has been linked at the N-terminus to protein antigens by their recombinant bacterial expression and demonstrated to be critical to those antigens' immunogenicity as vaccines, namely to OspA vaccine for Lyme disease.[9] In support of this mechanistic explanation, non-responding patients in a vaccine clinical trial of the Lyme vaccine were demonstrated to have deficiencies in TLR1/TLR2 function.[10] Similarly, the TLR5 agonist, flagellin, has been expressed as a fusion protein with several different microbe antigens, including recombinant West Nile virus antigen and influenza HA and M2e antigens, and has significantly improved the immunogenicity of those vaccines.[11-13]

These covalent linkage methods complicate formulation preparation, create significant limitations, require genetic or post-production modifications, and add to the regulatory burden. When agonists are linked by recombinant engineering, the ratio of linked antigen to agonist is fixed, typically at a one-to-one ratio. To the vaccines' detriment, the threshold dose of the adjuvant, above which adverse reactions are observed, becomes the limiting factor in the antigen dose that can be achieved. Chemical conjugation can be more flexible but is complicated by the difficulty of reproducibly conjugating the components at precise, desired ratios. Both recombinatory and conjugation approaches result in new chemical entities and carry with them the significant associated regulatory hurdles. Moreover, the conjugation process chemically alters the antigens, which can denature the critical protective epitopes of

FIGURE 1

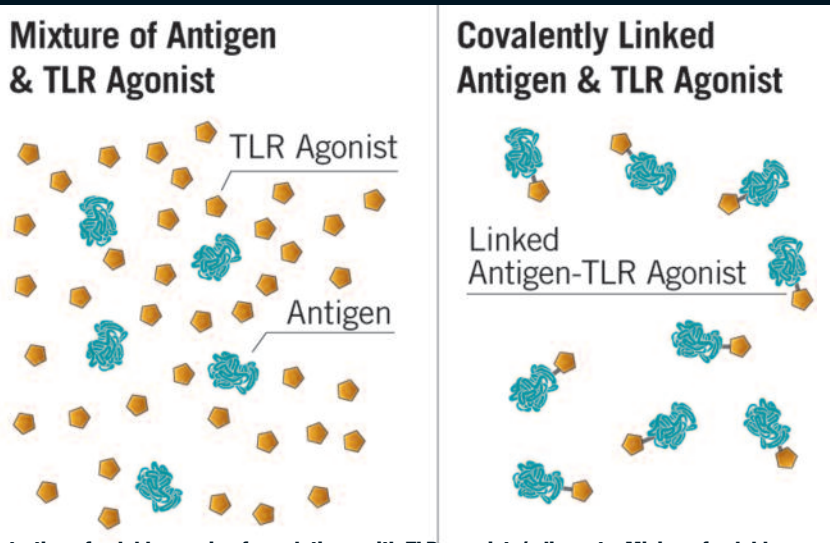

Illustration of soluble vaccine formulations with TLR agonists/adjuvants. Mixing of soluble antigens and TLR agonists can improve vaccine potency but typically requires high molar doses of the TLR agonist in comparison to antigen. The resulting high dose of adjuvant can lead to adverse reactions. Covalent linkages of antigen to TLR agonist can reduce the dose of the TLR agonist (by conjugation, fusion, protein or PAM_3cys-lipidation) in formulations and further improve the potency of these formulations. When using covalent linkages between soluble antigens and TLR agonists however, it is difficult to reduce the ratio of linked agonists to antigens below one which may limit the further optimization of the formulation's potency and safety.

the antigens and can involve conjugation linkers with their own safety concerns. What has been needed is a formulation technology that can readily link antigens and agonists at desired, varying ratios in a quick, reproducible, and flexible fashion.

VIRUS-LIKE PARTICLE & NANOPARTICLE VACCINES

Particle-based formulations have been advanced in recent years by the development of VLP and pseudovirus-based technologies, which use recombinant expression vectors in host cells to incorporate protein antigen into non-infectious VLP complexes.[14] VLP vaccine formulations have been successfully developed for human HepB and HPV vaccines, and other VLP vaccines are in clinical development for, among others, influenza and RSV.[15-16] The technology has been especially applicable for the formulations of viral vaccines using viral components that are protective antigens that self-associate/assemble into VLP structures. It has been demonstrated that these VLP vaccines often carry nucleotide-based, virus-related nucleotide sequences that are TLR agonists (TLR 7, 8, and 9 agonists) and benefit the potency of these

FIGURE 2

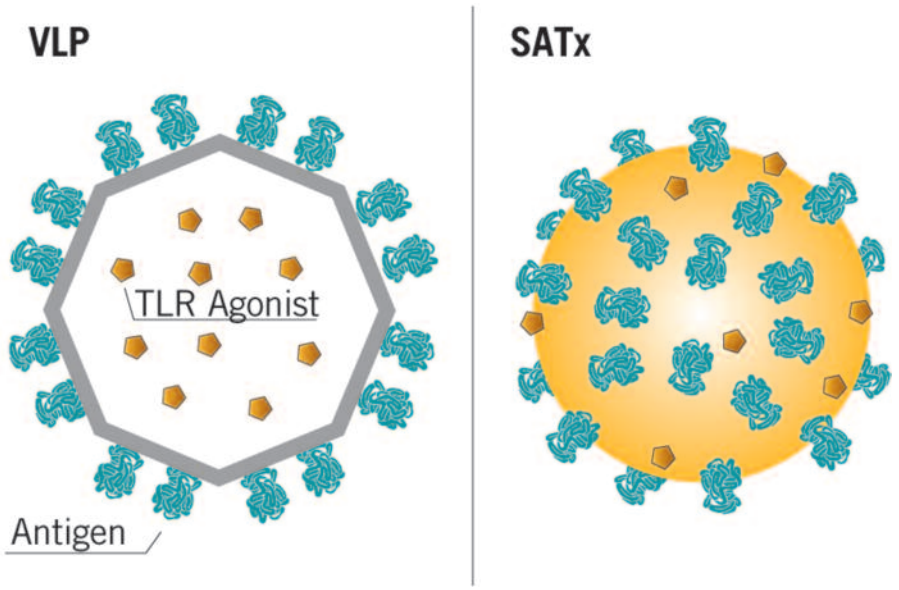

Illustration of nanoparticle based formulations. VLP products have been a successful strategy for making potent and safe viral vaccines, such as HPV and HepB. The protective viral antigen assembles into pseudoviron particles, which typically and naturally contain nucleotide-based TLR agonists, which adjuvant the product. SATx formulations are hydrophobic nanoparticles that have integrated surfactants that customize the particles' surface charges. Various antigens and TLR agonists are efficiently bound to the surfaces by electrostatic and hydrophobic binding. The dose and ratio of antigen and TLR agonists can be readily modified and optimized for both vaccine potency and safety. Results of animal studies have demonstrated that dose and ratio of TLR agonist can be decreased significantly compared to soluble formulations (Figure 1), improving the safety profile.

formulations (Figure 2).

One of the biggest challenges in developing VLP vaccines is that they depend upon the incorporation of protein antigens' genes into expression vectors that when expressed in cells as proteins, assemble into virus-like particles. This is particularly a challenge when not working with self-assembling viral protein antigens. For these non-viral targets, a portion of the non-viral protein antigen needs to be engineered into a self-assembling viral protein as a fusion protein. This limits the size of the protein antigen that can be inserted, and it depends upon the newly engineered protein antigen maintaining its protective confirmation. It takes significant development time and effort to create versions of these formulations, and they lack flexibility and control of TLR agonist content. The VLP products at the end of the manufacturing process are complex and often diverse populations of VLP structures that can be challenging to consistently manufacture, characterize, test, and release as a vaccine products.

Approaches that efficiently formulate existing purified antigens, whether proteins or other biological molecules, into nanoparticles would be useful formulation

platforms. Liposome-based formulations have been in development for many years for this purpose. The liposomes have been designed in some applications to contain and deliver vaccine antigens and immunostimulatory molecules like cytokines, lymphokines, and TLR agonists.[17] The greatest challenges for these formulations has been the long-term instability of liposomes because they are based on rather fluid, lipid membranes, and the method of linking antigens to the liposomes has often depended again on covalent-conjugation.

Particulate vaccine formulations have also be been developed and evaluated using poly(lactic-co-glycolic acid) (PLGA)-based particles. These polymer-based particles can be used to entrap vaccine formulations within the particles, which are then released as the PLGA biodegrades.[18] Vaccine antigens have also been conjugated to their surfaces for external display, though this can be a difficult process to control and reproduce. These particles also have significant limitations resulting from the necessity in using organic solvents and chemical conditions, which can denature protein antigens, making them useless as vaccines. Moreover, the PLGA particles are not stable for long-periods of time in aqueous suspensions and must be lyophilized and stored dry to prevent their natural hydrolysis before being administered as vaccines.

SURFACED ARRAYED THERAPEUTICS (SATx)

The nanoparticles developed by PSI are based on stable, hydrophobic nanoparticles that have integrated charge modifiers that give their surfaces desired electrostatic characteristics. The charge attributes of these particle can be readily modified by using different positively and negatively charged surfactants in their formulation. To these charged and lipophilic surfaces, antigens and TLR agonists can be associated by electrostatic and hydrophobic binding. The technology has now been used to successfully formulate several antigens and TLR agonists for both parenteral and mucosal vaccines.

Researchers at St. Georges University working with Particle Sciences have formulated recombinant vaccines for HIV and tuberculosis using SATx.[19,20] The binding of recombinant monomeric antigens on the surface of the nanoparticles multimerizes the antigen on the particles, which can be a strong stimulant for B cells (as previously discussed). Protective immune responses were readily induced with these formulations when the formulations were mucosally administered in mouse animal models. Particle formulations and microbes can be superior mucosal immunogens because these mucosal tissues have specialized cells, M cells, which transport particles and microbes across the mucosal membrane and into the underlying lymphoid tissues.[21] Similar vaccine formulations have also been developed as vaccines to other infectious diseases for parenteral administration.

The potency of the SATx formulations have been significantly improved by the inclusion of TLR agonists, like CpG and other agonists, on the surface of the nanoparticles. *In vitro* human lymphocyte responses to tetanus toxoid were significantly enhanced by the co-formulation of antigens and CpG on SATx.[18] These SATx formulations can be flexibly, rapidly developed to optimize the dose and ratio of antigens and TLR agonist. Results of formulations using vaccine antigens and TLR agonists have proven to significantly improve the vaccines' potencies and significantly reduced the

necessary dosage of TLR agonists in the formulations, in comparison to mixing or covalently linking these antigens and agonists. This reduction in the necessary dosage of TLR agonists thereby improves the vaccines' adverse reaction profile.

PSI has demonstrated that the technology can also be used to efficiently develop formulations that link different vaccines antigens via nanoparticles. In addition to typical protein-based vaccines, this approach can be used to develop conjugate-like vaccines that link non-proteinaceous antigens, such as polysaccharides, with both protein carrier molecules and as well as TLR agonists. To date, conjugate vaccines covalently link polysaccharide antigens to protein antigens. These conjugate vaccines provide polysaccharide antigen-specific B lymphocytes a means to bind the polysaccharide via their antibody and also interact with associated protein antigens, which facilitates needed T lymphocyte signaling. Nanoparticles carrying both the polysaccharides and carrier proteins on their surfaces should provide a more bio-mimetic function to the B lymphocytes. A number of critical infectious disease targets have important polysaccharide-based protective antigens, like S. pneumonia, H. influenza, and N. menningiditis. The development of conjugate vaccines has been key to preventing these diseases in infants because they can't immunologically respond to polysaccharide antigen alone.[22] However, conjugate vaccines are difficult and expensive to consistently manufacture. SATx could provide a new, flexible, and cost-effective alternative to covalent conjugation in developing these vaccines for human and veterinary applications.

SATx technology provides a new flexible formulation platform for the generation of nanoparticulate vaccine formulations without the limitations of previous formulation technologies that have inhibited the optimization of vaccine potencies and safety profiles. The SATx formulation platform is available to PSI clients for collaborative development projects and subsequent licensure. ◆

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BIOGRAPHIES

Dr. Robert S. Becker graduated from the University of Kansas with a PhD in Microbiology and Immunology, and Columbia University Business School with an MBA. He worked academically at the University of Illinois Chicago and Loyola University Chicago for several years in the fields of cellular and molecular immunology. He subsequently worked 21 years in what is today Sanofi Pasteur and the biotechnology company VaxInnate in leading research/development and business development roles, and an additional several years in the medical device field. In that time, he has worked on the development of numerous vaccines, formulation technologies, and drug delivery devices, including vaccines of influenza, pneumococci, meningococci, Lyme, Malaria, HPV, Dengue, pediatric combination vaccines, and intradermal delivery. He has responsibility at Particle Sciences for the growth and management of the biopharmaceutical and vaccine formulation business.

Dr. Mark A. Mitchnick founded Particle Sciences in 1991 and has been its CEO since that time. Prior to that, he was a practicing physician in New York and in a number of developing countries. In addition to his role as CEO, Dr. Mitchnick has been extensively involved in global health endeavors. He is an active member of the life sciences community, a consultant for several private equity firms, and has served as a Director for multiple therapeutic and diagnostic companies. Dr. Mitchnick holds over 20 patents related to drug delivery, diagnostics, and physiologic monitoring. He earned his BSc in Animal Sciences from Purdue University and his MD from Georgetown University Medical School. He trained in Pediatrics at The New York hospital, Cornell Medical Center, and completed the OPM program at Harvard Business School.