CAN WE ACHIEVE EFFECTIVE ORAL DELIVERY OF VACCINES? EVALUATING A NEW MICROSPHERE BASED CONTROLLED-RELEASE DELIVERY SYSTEM

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ABSTRACT

In recent years, therapeutic proteins, such as vaccines, antigens, and hormones, have made significant advances using sophisticated biotechnological techniques like recombinant technology. However, the mode of administration has been a limiting factor. Frequent injections and low patient acceptability make even the simplest parenteral administration of these drugs problematic, thus there is a need for new delivery systems to deliver these drugs more effectively. Oral delivery of proteins and peptides has long been hailed the holy grail of drug delivery for obvious reasons (i.e. ease and cost of administration, patient compliance and acceptability) but has remained a challenge due to enzymatic degradation in the gastro-intestinal (GI) tract and low bioavailability.

Here we evaluate a microsphere based controlled-release delivery system that appears to have promising results. PolyMicrospheres successfully developed an oral delivery system for a recombinant vaccine that resulted in an antibody titre count three times higher than parenteral delivery. The microsphere based delivery system greatly enhanced immunity: 100% survival of mice against the toxin and 88% survival of rabbits against the live bacterial spores, compared with 0% survival with the aqueous recombinant protective antigen (RPA) system.

OBJECTIVES

The objective of this work was to develop novel antigen-adjuvant delivery systems to enhance the efficacy of RPA via oral immunisation against anthrax. To demonstrate that effective protection against anthrax spores can be achieved by alternative oral, needle-free vaccination, PolyMicrospheres developed and evaluated the efficacy of microsphere based delivery systems. The efficacy of vaccination can be considerably improved, not only by incorporating the antigen in a matrix, but also by incorporating potent adjuvants in the matrix to provide long-term delivery of antigen together with an adjuvant for further potentiation of the immune response. The goal was to design and develop an oral RPA delivery system with controlled-release kinetics over a period of months, stimulating an enhanced antibody response at many distinct time points for long lasting protection.

BACKGROUND & SIGNIFICANCE

In response to anthrax being the most prominent threat in biological warfare, a recombinant PA vaccine was developed offering significant protection.[1,2,3] Vaccination with the first generation of anthrax vaccine, after the initial injection

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in the presence of alum adjuvant, requires five booster doses in 18 months. Additionally side-effects can occur, ranging from local soreness to fever and illness, with increased chance of occurrence after a booster injection. Recombinant protective antigen with alum adjuvant (a second-generation vaccine) still requires three to four vaccinations over an 18 month period, thus there is an obvious need for a safe and effective self-administered oral vaccine delivery system which can elicit full protection after a few weeks of vaccination. In this applied research we developed and evaluated novel microsphere based antigen-adjuvant oral delivery systems to enhance the efficacy of RPA vaccine. This platform technology could be utilised not only against inhalation anthrax but also against other microbes and toxins.

OVERCOMING THE CHALLENGES OF ORAL DELIVERY

Mucosal surfaces of the GI tract and the nasal passages are the major portals of entry of infectious agents and microbial toxins, therefore the mucosal surfaces constitute the first line of defence. Oral vaccination strategies that enhance mucosal immunity have practical significance to protect military personnel and civilian populations against biological weapons and other microbes. Vaccination by the oral mucosal route stimulates sIgA in the GI tract and lungs, while also stimulating systemic immunity,[4] thus mucosal vaccination elicits a broader immune response, as well as enhanced systemic and topical protection. The efficacy of the vaccine is substantially enhanced by a mucosal adjuvant; consequently a powerful systemic and mucosal immunoglobulin (IgG and IgA) response is stimulated, thereby providing a very potent first-line protection against oral and intranasal entry of microbes and toxins.

Oral administration of a vaccine possesses all the prerequisites for a simple, safe and effective route of vaccination. Although the mucosal surfaces of the GI tract represent a large area, only the ileum with its neutral pH has the proper environment for effective presentation of orally administered vaccine. Numerous studies have demonstrated that for oral stimulation of high immunoglobulin levels frequent administration of high doses of the aqueous vaccine is required.[5] However while oral vaccination with multiple doses of aqueous vaccine can be rendered more effective in the presence of a mucosal adjuvant, even then several doses will likely be required for vaccination. Also one drawback of oral administration of protein antigens is that they are broken down in the upper GI region by digestive enzymes and hence lose their immunogenicity.

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Administering the vaccine in a micro-encapsulated delivery system protects against the acidic environment in the stomach, thereby its native form is preserved, which is critical for antibody maturation and immunogenicity.[6] The uptake of microspheres is enhanced by the endocytic M-cells lining the intestine. Microspheres can be taken up from the intestine into the immune-inductive environment of the Peyer's patches, where they can induce both mucosal and systemic immune responses. These microsphere based delivery systems can be designed in such a way that they not only protect their content from the digestive enzymes but also deliver it at desired intervals. Controlled release of antigens from polymer microparticles has been of particular interest in the development of vaccine delivery systems.[6,7]

The efficacy of vaccination can be improved not only by incorporating the antigen in the polymer matrix, but also by incorporating potent adjuvants in the matrix to provide long-term delivery of antigen together with a vaccine-adjuvant for further potentiation of the immune response. Many modern vaccines are composed of highly purified or recombinant proteins or synthetic peptides. The use of potent adjuvants (such as CpG motifs, lipopolysaccharide, polyIC, monophosphoryl lipid A,[8,9] LTR72 and LTK63[10]) to enhance immune response to these antigens is an attractive method for improving their immunogenicity.

Vaccine delivery by the oral route is not easy to achieve, but if it is possible the preparation can be packaged in a stable form that is easy to administer.

METHODOLOGY

The microsphere based delivery systems (MDSs) were tailor-made to suit the oral administration route. In many stages PolyMicrospheres has designed, developed, and evaluated third-generation anthrax vaccine delivery systems specifically for oral administration. Each stage consists of a group of optimal formulations with various diameters, polymer matrices and/or with different loadings to provide controlled (or pulsatile) release of the recombinant anthrax vaccine.

In order to achieve full protection by oral immunisation the delivery system needed to be designed to perfection and developed with optimal micro-encapsulation methods and release kinetics. The MDSs comprised a combination of the following: the second-generation RPA, a potent mucosal adjuvant (ADJ), optimal RPA-adjuvant ratio, proper drug loading, poly(lactide/glycolide) ratio for the ideal half life, microsphere particle diameter to penetrate into and be retained by the mucosal epithelial cells of the intestinal tract, and the process parameters to achieve the stability and integrity of the conformation of RPA.

We focused significantly on the design and development of the RPA- and ADJ-incorporated MDSs. A mucosal adjuvant such as LTK-63 was incorporated with the recombinant protective antigen into biodegradable polymer microspheres to provide a long-term delivery of a vaccine adjuvant for further potentiation of the immune system. Selected RPA- and ADJ-encapsulated microsphere matrices were further coated with a bio-adhesive

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(MDS-BioAd) to promote the adhesion, retention and uptake of the microparticles into the mucosal membranes of the intestine.

The RPA/adjuvant delivery systems were designed to provide controlled- and/or pulsatile-release delivery of the recombinant anthrax vaccine and the adjuvant. Depending on the optimal combination of the RPA/ADJ in the polymer matrix, the molar ratio of lactide-glycolide, drug loading, particle diameter and the micro-encapsulation methods and process techniques, the contents are released in a controlled manner at multiple time points stimulating antibody peaks several weeks apart, thereby providing long-lasting immunity and protection. The delivery systems were designed such that the antigen can reach the mucosal antigen processing cells in its native conformation for induction of an effective protective immunity. The MDS formulations were prepared with the following parameters:

- Matrix materials: poly(dl-lactide-co-glycolide) and poly(dl-lactide)
- Microsphere mean diameter range: 6-20 µm
- RPA of anthrax (from List Biological Laboratories, Campbell, CA, US) Loading: 0.5-1%
- Adjuvant (LTK63 from (Chiron, Italy) Loading: 0.04-0.13%
- MDS products were prepared using established protocols currently in use at PolyMicrospheres.[11] A modified complex coacervation process was used. Selective MDS microspheres were further coated with a solution of a bio-adhesive polymer.

Analytical Methods for the Characterisation & Stability Studies

The MDS products developed were characterised as to mean particle diameter, size distribution, antigen and adjuvant loadings using established protocols currently in use at PolyMicrospheres.

Protocols for Vaccination Of Mice & Efficacy Studies

A/J mice were immunised with 7-8 mg of each MDS by a two-dose oral administration. The second dose was administered 20 days after the first immunisation with the same dosage of each MDS. Control groups include aqueous RPA system and non-immunised control. Mice were bled from the retro-orbital sinus over a period of 31-108 days after immunisation.

ELISA Assay for Mouse Anti-PA Antibodies

Individual serum samples bled at various time points after immunisation were assayed for anti-PA immunoglobulins using standard ELISA protocols. Horseradish peroxidase-labelled anti-mouse antibody directed against IgG was used. The amount produced was determined spectrophotometrically. A standard curve was prepared using known amounts of purified mouse anti-PA antibodies, obtained from USAMRIID as a positive control, and the amount of anti-PA antibodies in the samples was determined.

Anthrax Toxin Challenge Studies in Mice

Selective MDS formulations were tested for their efficacy in inducing protection against a lethal challenge of anthrax toxin. Control groups include an aqueous RPA system and a non-immunised group. The anthrax toxin challenge was performed 110 days after immunisation. This challenge consisted of intravenous injection of a mixture of lethal factor (1.5 mg/kg) and protective antigen (3 mg/kg) in a combination equivalent of approximately five LD50 in non-immunised mice. On day 42 after the toxin challenge the experiments were terminated.

Protocols for Immunisation of Rabbits & Efficacy Studies

New Zealand White rabbits were immunised with 30-32 mg of each MDS by a two-dose oral administration. The second dose was administered 21 days after the first immunisation with the same dosage of each MDS. Control groups include aqueous RPA system and non-immunised control. The rabbits were bled from the ear vein 18, 25, and 32 days after immunisation.

ELISA Assay for Rabbit Anti-PA Antibodies

The ELISA assay for the rabbit experiments was similar to that described above for measuring mouse anti-PA antibodies except that horseradish peroxidase-labelled anti-rabbit Ig antibodies were used.

Anthrax Spore Challenge Studies in Rabbits

The immunised rabbits were challenged after 42 days with a lethal aerosol exposure of live *Bacillus anthracis* (Ames) spores and monitored for survival. All animals were aerosol-challenged with a target dose of 200 LD50. Animals were observed twice daily for 14 days post-challenge for clinical signs of disease including lethargy and respiratory distress. All B anthracis challenges were performed in a BL-3 containment laboratory.

Protocols for the Safety & Histopathology Studies

Four to six weeks after immunisation with selected MDS formulations, mice/rabbits at each time point were sacrificed for complete organ histopathology to rule out toxic side effects. At these time points blood samples were collected prior to sacrificing the mice/rabbits for routine serum chemistry including liver and kidney function tests, creatinine kinase, and complete haematology parameter determinations.

RESULTS & DISCUSSION

With the expertise PolyMicrospheres has in this area we were able to develop, test, and refine the formulations as we progressed through each stage of the animal studies. As the formulations were developed, they were tested systematically in mice. Based on ELISA immunoglobulin titres in mice, prototype formulations were then developed for efficacy studies in rabbits. We developed more than forty MDS products to achieve desired release kinetics by using established micro-encapsulation process protocols currently in practice at PolyMicrospheres. Only very selective MDS products with significant results are reported in Table 1 and discussed here.

Product code	Mean Diameter (µm)	Drug Loading (%)		Type
		RPA	ADJ	
MDS for mouse studies:				
MDS-P	12	0.88	0.08	MDS
MDS-Q	13	0.87	0.08	MDS-BioAd
MDS-R	9	0.82	0.04	MDS
MDS-S	18	0.81	0.04	MDS-BioAd
MDS for rabbit studies:				
MDS-T	7	0.49	0.13	MDS
MDS-U	7	0.48	0.13	MDS-BioAd

Table 1: MDS incorporated with both RPA and ADJ for two-dose oral immunisation.

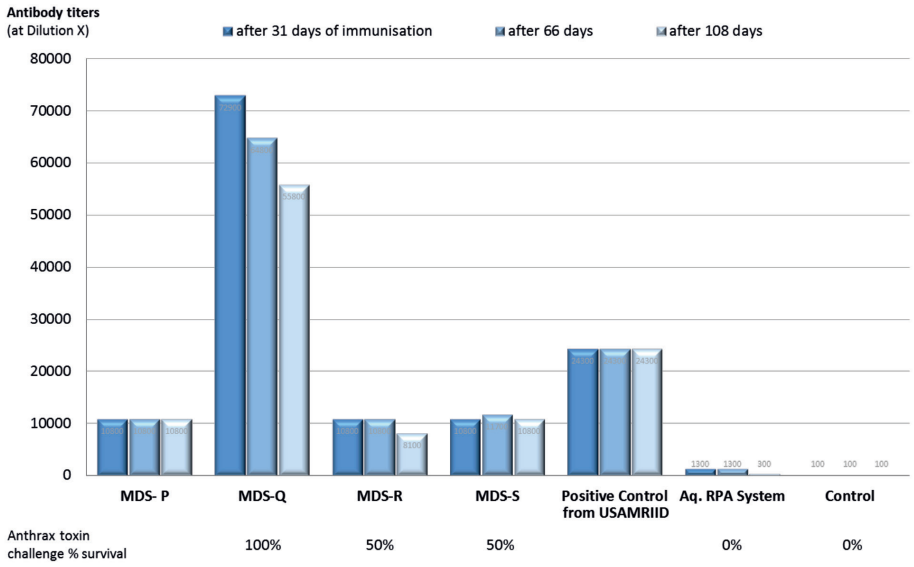

Figure 1: Efficacy of selected MDSs in mice via two-dose oral immunisation followed by anthrax toxin challenge.

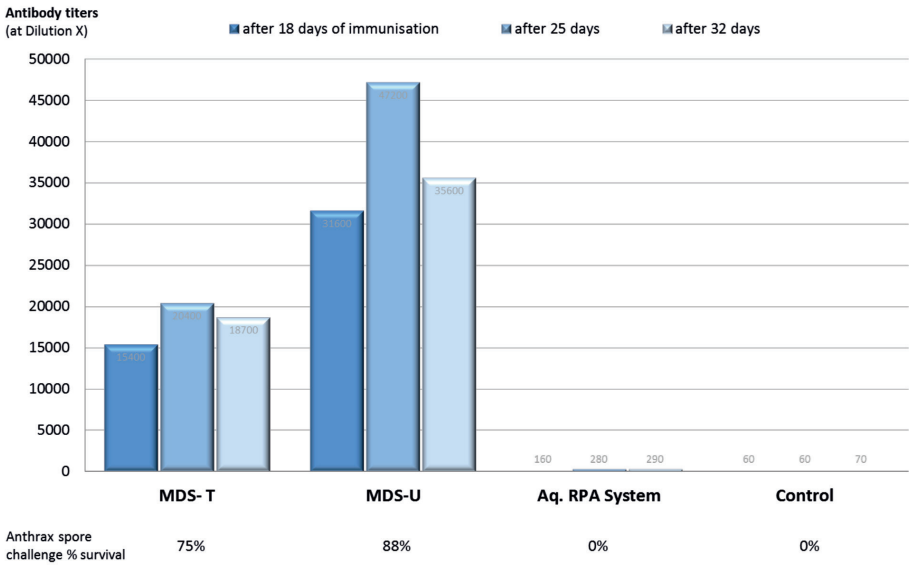

Figure 2: Efficacy of selected MDSs in rabbits via two-dose oral immunisation followed by live anthrax spore challenge.

Immunisation of Mice with MDS via Two-Dose Oral Administration

We evaluated these MDSs for their efficacy to elicit immune response in mice. Figure 1 shows the antibody (IgG) titres in mice immunised with selected MDSs via two-dose oral administration. Mice immunised with selected MDS products were challenged with anthrax toxin. Figure 1 includes toxin challenge studies on selected MDSs via the oral-route against anthrax toxin.

Our orally delivered MDS-Q showed extremely high IgG titres throughout the 108-day testing period, exhibiting a two- to three-fold increase over the parenterally delivered positive control (and a 50-fold increase over the aqueous RPA system). Since the MDS-Q exhibited 70000 IgG titres 31 days after immunisation, it is very likely that the MDS-immunised mice were already fully protected within 31 days after two-dose oral immunisation. The IgG titres induced by the MDSs remained high over the testing period of 108 days, indicating a continuous controlled or pulsatile release of the RPA and ADJ over that period.

In the anthrax toxin challenge studies, all control (non-immunised) mice died within the first three days, demonstrating that the challenge was correctly administered to the mice. Aqueous RPA system group mice had 0% survival (all died within four days), while the MDS-R and MDS-S groups each showed 50% protection. Our MDS-Q group showed 100% survival and protection against the anthrax toxin challenge on the 42nd day when the experiments were terminated.

Immunisation of Rabbits with MDS via Two-Dose Oral Administration

The MDSs were then tested for their efficacy to induce an antibody response in rabbits. Only selective MDS systems showing high efficacy in rabbit studies are reported here. Figure 2 shows the antibody titres in rabbits immunised with selected MDSs via two-dose oral administration. Rabbits immunised with selected MDS products were challenged after 42 days with live anthrax spores. Figure 2 includes the summary of the results of live anthrax spore challenge studies on rabbits.

These MDS systems produced high antibody titres in rabbits over the testing period of 18-32 days after the two-dose oral immunisation. Our MDS-U exhibited very high antibody titres in rabbits (35000-47000 during the 32-day testing

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period), a 100-fold increase over the aqueous RPA system. The IgG titres induced by the MDSs remained high throughout the testing period indicating a continuous controlled (or pulsatile) release of the RPA and ADJ over the testing period. Since the MDS-U exhibited >45000 IgG titre 25 days after immunisation, it is likely that the MDS-immunised rabbits were already protected within those 25 days.

In the anthrax spore challenge studies, all control (non-immunised) rabbits died within the first four days, demonstrating that the challenge was correctly administered to the rabbits. The aqueous RPA system group also had no survival. Our MDS-T and MDS-U groups protected 75% and 88% of New Zealand White rabbits against live anthrax spores. Preliminary safety and histopathology studies indicated that the heart, lungs, liver, spleen, kidneys, and small intestine were normal in rabbits immunised with the delivery systems MDS-T and MDS-U and did not show any toxic effects.

These results from the mouse and rabbit studies indicate that we have successfully developed a viable oral delivery system for recombinant anthrax vaccine. By a two-dose oral administration, the MDS-Q afforded a 100% protection of mice against anthrax toxin and the MDS-U afforded an 88% protection of rabbits against live anthrax spores.

Given the high antibody titres and survival rate against exposure, our oral delivery system is approaching the same efficacy as parenteral delivery. The implications for this are enormous, especially as we begin to apply this platform to other vaccines, antigens, and hormones.

CONCLUSION

PolyMicrospheres successfully developed a viable MDS offering effective oral delivery of recombinant anthrax vaccine. Our delivery system MDS-Q produced extremely high antibody titres (55000-72000 through 108 days) in mice, compared with the parenterally delivered positive control (24300 titres). In rabbits our delivery system MDS-U exhibited over 35000 antibody titres through 32 days (a 100-fold increase over the aqueous RPA system), proving the value of a microsphere-based system. In addition our MDS-Q showed 100% protection in mice against lethal anthrax toxin challenge, while the aqueous RPA system was completely ineffective. Our MDS-U also protected 88% of rabbits against live anthrax spores whereas the aqueous RPA system again protected none. The IgG titres induced by the MDS systems remained high throughout the testing period indicating a continuous controlled (or pulsatile) release of the RPA and ADJ. Preliminary histopathology studies did not show any toxic effects.

Oral vaccination with an MDS was an immense advancement. Even the three week immunisation time with two doses of our MDS is a significant reduction from the current parenteral immunisation protocol requiring 3-4 doses of RPA-alum adjuvant spread over 18 months. The MDS delivery system protects its load against enzymatic degradation in the acidic environment of the stomach, and the bioadhesive properties of the microspheres increase the adhesion and residence time in the small intestine, thereby providing increased time to stimulate full immune response and protection against anthrax spores.

Oral vaccination with an MDS offers several key advantages over parenteral administration: it reduces immunisation time, can be self-administered, and can be packaged in a stable form as a pill (with a long shelf-life). On a macro level it simplifies the logistics of immunisation to large populations by increasing compliance and eliminating the need for medical personnel, thus increasing availability and lowering the system cost.

We are still optimising various parameters but are certainly moving in the right direction: towards the efficacious oral delivery of vaccines and other therapeutics. This microsphere-based delivery technology can be easily translated to the oral delivery of other proteins, peptides, vaccines and hormones. The societal implications are very exciting, as we are beginning thinking about clinical trials and delivering this to large populations.

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