CONTROLLED-RELEASE SYSTEMS FOR PROTEINS & PEPTIDES: 3RD-GENERATION INTRANASAL ANTHRAX VACCINE-DELIVERY SYSTEM

By S. Mohan Mohanraj, PhD, and Meir Kende, PhD

ABSTRACT

The objective was to develop microsphere-based delivery systems (MDS) for controlled and pulsed-release delivery of recombinant anthrax vaccine via intranasal immunisation. Microsphere-based recombinant protective antigen (RPA) delivery systems for intranasal immunisation were successfully developed, wherein the MDS formulations produced extremely high antibody titres (over 150,000) in mice after 65 days of immunisation compared with the aqueous RPA vaccine system (at 15,900).

Selected MDS systems were challenged with anthrax toxin. The MDS systems, on two intranasal doses, showed 100% protection against anthrax toxin challenge in mice, compared with <17% that were protected by the aqueous RPA vaccine system and none in the non-immunised control group.

OBJECTIVES

The general objective of the work was to develop and evaluate microsphere-based antigen delivery systems to enhance the efficacy of the RPA vaccine via intranasal immunisation. To demonstrate that effective protection against anthrax can be achieved by alternative needle-free vaccination, PolyMicrospheres developed and evaluated novel antigen-adjuvant delivery systems.

The efficacy of vaccination can be considerably improved not only by incorporating the antigen in a matrix, but also incorporating potent adjuvants in the matrix to provide long-term delivery of antigen together with an adjuvant for further potentiation of the immune response.

Depending on the composition, the matrix delivery system releases the incorporated RPA/adjuvant at many distinct time points, stimulating primary and many booster responses for better immunity and protection. The controlled-release kinetics and the consequent multiple antibody peaks assure a long-persisting immunity and protection for at least one year.

> "The platform technology of this intranasal delivery system is also suitable for other human and veterinary vaccines including simultaneous intranasal delivery of multiple immunogens."

BACKGROUND AND SIGNIFICANCE

The possibility of biological warfare and bioterrorism is an increasing threat in today's world. Among these weapons, anthrax has become the most prominent threat. It is only prudent to take steps to minimise the damage from such an act of bioterrorism. One of the most effective precautions will be a two-dose immunisation with an effective vaccine delivery system, which can be easily administered.

The *Bacillus anthracis* organism can be very easily produced in bulk quantities and disseminated as stable, long-lasting spores which can infect a large population via inhalation or contact.[1-2] The most serious route of infection is pulmonary. The spores germinate and quickly disseminate in the hilar and tracheal lymph nodes; the ensuing bacteraemia produces over 80% mortality within a short period. An RPA vaccine has been developed and immunisation with this protein has offered significant protection against pulmonary anthrax.[3-7]

In a combat situation, logistics of vaccine administration, compliance and time are of essence. Vaccination with the first

Dr. S. Mohan Mohanraj
Director of Technology
T: +1 317 549 3764
E: mohan@polymicrospheres.com

PolyMicrospheres
Division of Vasmo Inc.
4101 East 30th Street
Indianapolis
IN 46218
USA

www.polymicrospheres.com

Dr Meir Kende
Dept of Molecular Biology,
Integrated Toxicology Division

US Army Medical Research Institute of Infectious Diseases (USAMRIID)
1425, Porter Street
Fort Detrick
MD 21702
United States

www.usamriid.army.mil

Copyright © 2017 Frederick Furness Publishing Ltd

generation of anthrax vaccines, after the initial injection, requires five parenteral booster doses in 18 months. Furthermore, side effects occur, which can range from local soreness to fever and illness, with increased chances of occurrence after a booster injection. The second-generation vaccine, RPA with alum adjuvant, requires three or four vaccinations over an 18-month period.

Thus there is a need for a vaccine delivery system which is convenient and offers long-term protection without multiple boosters. In this applied research, we developed and evaluated microsphere-based antigen-adjuvant intranasal delivery systems to enhance the efficacy of RPA vaccine via two doses. This platform technology could be utilised not only against inhalation anthrax but also against other microbes.

Mucosal surfaces of the nasal passages and the gastrointestinal tract are the major portals of entry of infectious agents and microbial toxins. Therefore, the mucosal surfaces constitute the first line of defence. Intranasal vaccination strategies that enhance mucosal immunity have practical significance to protect military personnel and civilian populations against various microbes and toxins.

Most importantly, mucosal immunisation elicits a broader immune response, and an enhanced systemic and topical protection. The efficacy of the vaccine is substantially enhanced by a mucosal adjuvant; consequently, a powerful systemic and mucosal immunoglobulin response is stimulated, thereby providing a very potent first-line protection against intranasal entry of microbes and toxins.

Controlled release of antigens from polymer microparticles has been of particular interest in the development of vaccine delivery systems.[8-10] 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 to enhance immune response to these antigens is an attractive method for improving their immunogenicity. Such adjuvants include CpG motifs, lipopolysaccharide, polyIC and monophosphoryl lipid A.[11-12] Other potent adjuvants include LTR72 and LTK63.[13-14] The biological activity of LTR72 and LTK63 is by cytokine-stimulated robust enhancement of both the humoral and the cellular immune response.

METHODS

The MDS were designed and developed as follows: in order to achieve full protection by a two-dose intranasal immunisation, the delivery system needs to be optimally designed using appropriate microencapsulation methods and finely tuned release kinetics.

A correct combination consists of the following: the second-generation RPA, most potent mucosal adjuvant (Adj), RPA-Adj ratio, poly(lactide/glycolide) ratio for the right half-life, microsphere particle diameter to have the proper drug load and to penetrate into the mucosal epithelial cells, and the process parameters to achieve the stability and integrity of the conformation of RPA (see Figure 1).

The matrix of the antigen/Adj-incorporated microspheres may be further coated with a bioadhesive to promote adhesion to the antibody producing M cells in the mucosal membranes of the respiratory tract. For these reasons and due to the extreme high cost of RPA, it was essential to develop, evaluate and refine the delivery systems in multiple stages. We were able to develop, test and refine the formulations as we progressed through each stage with the results from the animal studies.

The novel RPA/Adj delivery systems were designed to provide controlled- and pulsed-release delivery of the recombinant anthrax vaccine equivalent to multiple immunisations. A mucosal adjuvant such as LTK-63 was also incorporated into the microsphere matrices to provide a long-term delivery of a vaccine adjuvant for further potentiation of the immune system.

In these MDS, both the RPA and the Adj are incorporated into the same microsphere matrices. 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 microencapsulation methods and process techniques, the content is released in a controlled manner at multiple time-points stimulating antibody peaks several weeks apart, thereby a long-lasting (at least one year) immunity and protection is stimulated.

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.

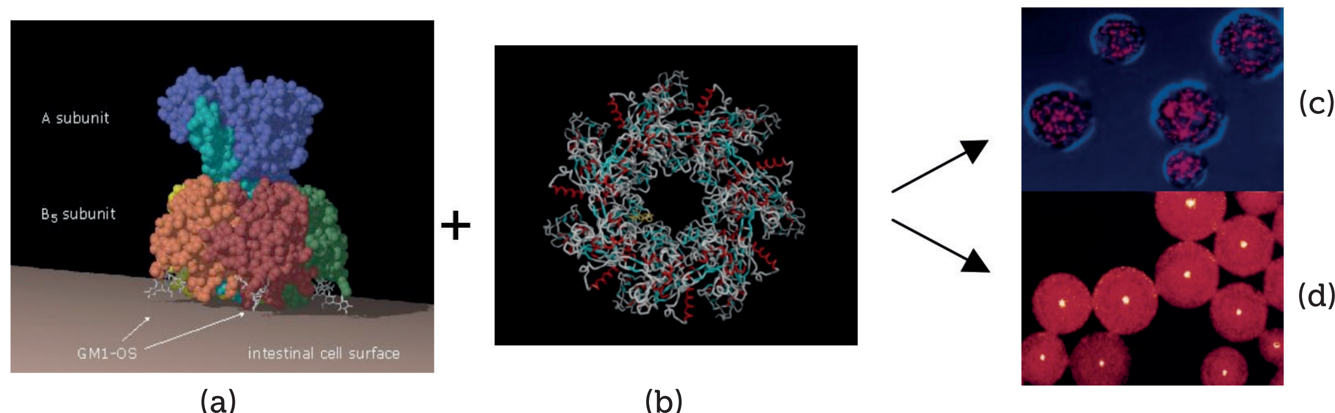

Figure 1: RPA (a) bound to an adjuvant (b) incorporated into heterogeneous microspheres (c) or homogeneous microspheres (d) with controlled-release kinetics over a period of 15 weeks stimulating antibody response at many distinct time points. The released RPA/adjuvant binds to the cell surface by the B subunit of the adjuvant. After entering the antibody producing cells, a long-lasting and enhanced immune response and protection is stimulated.

Copyright © 2017 Frederick Furness Publishing Ltd

A. Preparation of the MDS formulations:

- Polymers poly(dl-lactide-co-glycolide) and poly(dl-lactide)
- Microsphere Mean Diameter range: 9-16 µM
- RPA of anthrax (from List Biological) Loading: 0.8-1%
- Adjuvant (LTK63 from Chiron) Loading: 0.04-0.08%

MDS formulations were prepared using established protocols currently in use at PolyMicrospheres.[15] A modified complex coacervation process was used.

Processes include heterogeneous (Process I leading to Type I products) and homogeneous (Process II leading to Type II products) water-in-oil primary emulsions leading to different matrix-formulations to release the antigen and adjuvant at different rates.

Preparation of MDS coated with a bioadhesive:
These formulations were prepared using established protocols currently in use at PolyMicrospheres. Briefly, a dispersion of MDS microspheres was coated with a solution of a bioadhesive polymer. The coated microspheres were centrifuged or filtered, and dried under vacuum or lyophilised.

B. Analytical methods for the characterisation:
The MDS formulations developed were characterised as to mean particle diameter, size distribution, antigen and adjuvant loadings using established protocols currently in use at PolyMicrospheres.[15] Briefly, the analytical instruments and techniques are summarised as follows:

a) Particle size and size distribution analysis using Shimadzu Laser Diffraction Particle Size Analyzer SALD-1100 and/or Coulter N4MD Multi-angle Sub-Micron Particle size Analyzer.
b) Particle aggregation, uniformity, shape, and surface morphology analysis using a Nikon-Diaphot high resolution inverted microscope.
c) Measurement of antigen/adjuvant loadings in the microparticle delivery systems: A known weight of the microparticle sample was dissolved in a suitable volume of solvent and extracted with a known volume of PBS. The concentration of protein was determined using a Bio-Rad Protein Assay Kit.

C. Protocols for vaccination of mice and efficacy:
Selected formulations were tested for their ability to induce a systemic antibody response in mice following a two-dose intranasal administration. The immune response was assessed by an ELISA assay of periodic test bleeds for anti-RPA antibodies.

Mouse immunisation protocol:
Groups of 8 AJ mice were immunised with 3-3.4 mg each of MDS incorporated with both RPA and Adj by intranasal administration. The second dose was administered after 20 days of 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 under light anesthesia over a 15-week period after second immunisation. Serum was prepared and assayed for anti-PA antibodies by ELISA.

ELISA assay for mouse anti-PA antibodies:
Individual serum samples bled at various time points (10, 30, 65 and 108 days after immunisation) were assayed for anti-PA IgG, IgG1, IgG2a, and IgG2b immunoglobulins using standard ELISA protocols. Horseradish peroxidase-labelled anti-mouse antibody directed against the appropriate Ig class of interest 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 were determined.

Anthrax-toxin challenge studies in mice:
Selective MDS formulations were tested for efficacy in inducing protection against a lethal challenge of anthrax toxin. Control groups include an aqueous RPA system and a non-immunised group. Mice were bled as described above from the retro-orbital sinus for 15 weeks. The serum was tested for antibody titre to assure that animals receiving the vaccine have responded. The anthrax-toxin challenge was performed after 110 days of the second immunisation. This challenge consists of iv mixture of lethal factor (1.5 mg/kg) and protective antigen (3 mg/kg) in a combination equivalent of approximately five LD_{50} in non-immunised mice. On day 42 after toxin challenge, experiments were terminated.

MDS Product code	Mean Diameter	Drug loading		Type (see Methods)
		RPA	Adj	
MDS-I	9 µM	0.96%	0%	I, coated w/bioadhesive
MDS-V	12 µM	0.88%	0.08%	I
MDS-VI	13 µM	0.87%	0.08%	I, coated w/bioadhesive
MDS-VII	9 µM	0.82%	0.04%	I
MDS-VIII	10 µM	0.81%	0.04%	I, coated w/bioadhesive
MDS-X	16 µM	0.81%	0.04%	II, coated w/bioadhesive

Table 1: MDS incorporated with both RPA and ADJ in the same microspheres.

D. Safety & histopathology studies:
Four to six weeks after the second immunisation of selected MDS formulations at the dose used in the immunisation protocol, five mice 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 for routine serum chemistry, including liver and kidney function tests, creatinine kinase, and for complete haematology parameter determinations. Evaluation criteria were to compare the histopathology, and serum chemistry including kidney and liver function tests with normal untreated animals.

 Copyright © 2017 Frederick Furness Publishing Ltd

RESULTS AND DISCUSSION

The RPA-adjuvant delivery systems were designed, developed and crafted at PolyMicrospheres in many stages. Each stage consists of a group of optimal formulations with various diameters, polymer matrices and drug loadings to provide controlled and pulsed-release of the anthrax vaccine. As the formulations were developed, they were tested systematically in mice (as described above).

The antibody titres of immunity stimulated with MDS were determined in mice. Based on the results, formulations were refined, redesigned and the process conditions were altered to obtain formulations with the desired integrity, antigen/adjuvant content and release kinetics required to stimulate full protection against the anthrax toxin challenge. Development and evaluation were done side-by-side and stage-by-stage. We developed more than 40 MDS formulations using established protocols currently in practice at PolyMicrospheres to achieve the best release rates. Only selective MDS formulations with significant results are reported here.

A. Development and optimisation of MDS for RPA vaccine

Significant effort was focused on the design and development of the RPA- and Adj- incorporated MDS, wherein both the RPA and the Adj were incorporated into the same microspheres (Table 1). We have also developed MDS systems coated with a mucoadhesive to promote the adherence and retention of the microparticles into the mucosal membranes of the respiratory tract.

B. Studies on immunisation of mice with MDS via intranasal administration

We have immunised mice via two-dose intranasal administration with the MDS systems. The second dose was administered after 20 days of the first immunisation. The MDS products were tested for their efficacy to induce an antibody response in mice.

Figure 2 shows the antibody (IgG) response in mice immunised with selected MDS systems via two-dose intranasal administration. Figure 3 shows the antibody IgG Subclass (IgG1, IgG2a, and IgG2b) titres in mice immunised with selected MDS systems.

The MDS systems, MDS-V to MDS-X, all produced high antibody titres. MDS-V and MDS-X showed extremely high antibody titres on days 30-108. Thus, it is very likely that the MDS immunised mice were already fully protected 30 days after the second immunisation.

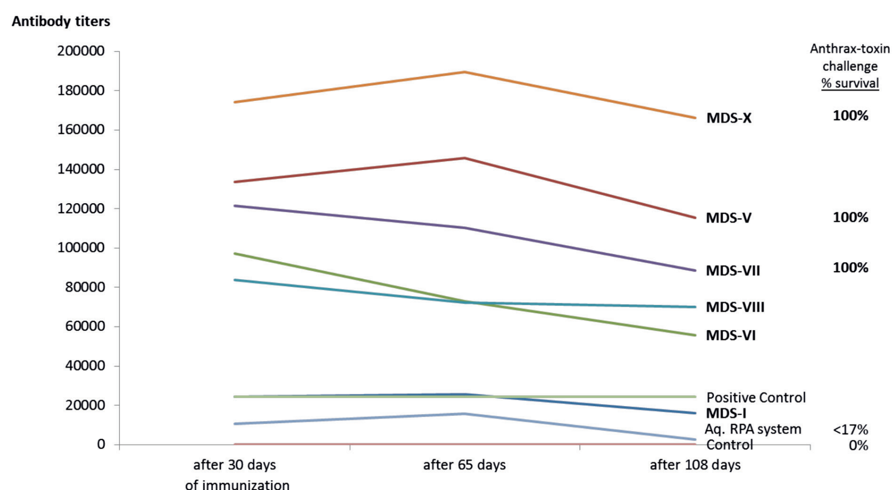

Figure 2: Immune response to selected MDS systems in mice via two-dose intranasal immunisation: antibody (IgG) titres (at dilution X).

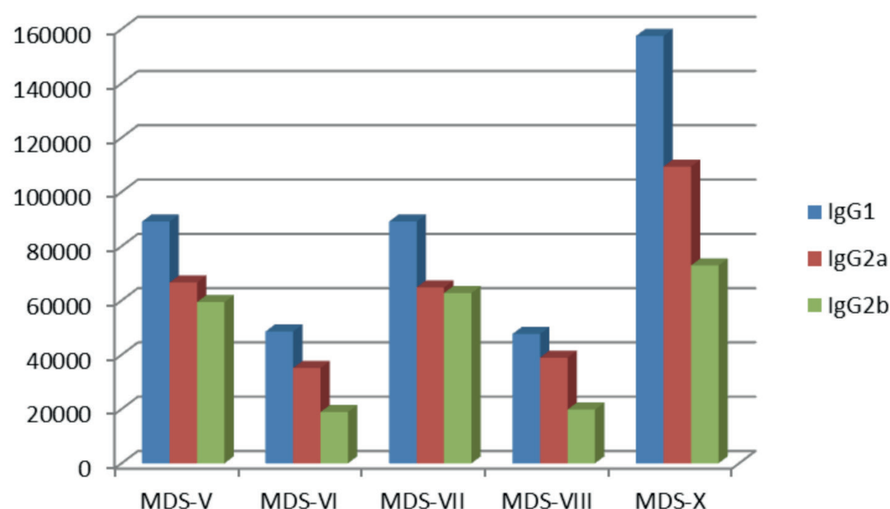

Figure 3: Antibody IgG subclass titers of selected MDS systems in mice via two-dose intranasal immunisation after 65 days (at dilution X).

Among the Type I (Table 1) systems, MDS-V and VII are the most effective. Among all of the delivery systems, MDS-X (Type II) is by far the best, exhibiting a 12-fold increase (on 65 days) over the aqueous RPA system. The IgG titres induced by the MDS systems remained high over the testing period of 108 days, indicating a continuous controlled release of the RPA and Adj over 3.6 months, while the IgG titre of the aqueous RPA system declined almost completely by 108 days.

The MDS systems, MDS-V to MDS-X, all produced high IgG subclass titres. MDS-V, MDS VII and MDS-X showed very high IgG subclass titres.

Among the Type I systems, MDS-V and VII are the most effective. MDS-V and MDS-VII, with different Adj loadings, exhibited similar IgG1, IgG2a, and IgG2b titres.

Among all of the delivery systems, MDS-X (Type II) is by far the best, exhibiting extremely high IgG Subclass titres.

Although IgG correlated well with protection (Figure 2), it is important to identify the protective antibody subclasses. The IgG2a response (which is believed to be the principal protective antibody against tumour cells and tumours, trypanosoma and hepatitis B virus infection) correlates well with the resistance to anthrax toxin challenge. IgG1, IgG2a and IgG2b effector activity is complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). CDC and ADCC are directed against tumour or viral antigen expressed on the cell surface.

Copyright © 2017 Frederick Furness Publishing Ltd

C. Challenge of immunised mice against anthrax toxin

Mice immunised with selected MDS products showing high antibody titres were challenged with anthrax toxin. Figure 2 includes toxin challenge studies on selected MDS systems against anthrax toxin. Anthrax-toxin challenge was performed after 110 days of the second immunisation. On day 42 after the toxin challenge, experiments were terminated. All control (non-immunised) mice died within the first three days, demonstrating that the challenge was correctly administered to the mice.

The median survival time of mice immunised with two-dose of aqueous RPA system is <12 days (5 died in <6 days, and one survived), showing <17% protection against the toxin challenge. Mice immunised with MDS-V, MDS-VII and MDS-X groups had all survivors. The MDS systems MDS-V, MDS-VII, and MDS-X showed 100% protection against the anthrax toxin challenge on the 42nd day when the experiments were terminated. These vaccine delivery systems afforded good protection against anthrax toxin while the aqueous vaccine system did not.

These results indicate a viable intranasal delivery system for anthrax vaccine. Microsphere-based intranasal RPA/Adj delivery system inoculated to mice substantially augmented the RPA-specific ELISA IgG titres for more than 108 days compared with the aqueous RPA system titres. Unlike with the aqueous RPA system, mice vaccinated intranasally with two doses of MDS system resisted challenge with anthrax toxin.

D. Histopathology studies on immunised mice

Preliminary histopathology studies indicate that the heart, lungs, liver, spleen, kidneys and small intestine are normal in mice immunised with the delivery systems MDS-V and MDS-X, and they did not show any toxic effects.

CONCLUSION

In this applied research, PolyMicrospheres has successfully developed a viable intranasal microsphere-based delivery system (MDS) offering an effective delivery of recombinant anthrax vaccine via two-dose immunisation. MDS-V and MDS-X systems delivered over 100,000 antibody titres throughout 108 days, compared with less than 3,000 for the aqueous RPA system. In addition, the novel MDS showed 100% protection against lethal anthrax toxin challenge, while the aqueous RPA system was ineffective.

Preliminary histopathology studies of the MDS did not show any toxic effects. Even three weeks immunisation time with two doses of our MDS is a significant reduction of the current parenteral immunisation protocol of 18 months with the RPA-alum adjuvant, plus the added benefits of reducing the cost of the immunisation and logistics.

The platform technology of this intranasal delivery system is also suitable for other human and veterinary vaccines including simultaneous intranasal delivery of multiple immunogens.

ACKNOWLEDGEMENT

Research Support: US Army Medical Research, Contract Number: DAMD17-02-C-0018

REFERENCES

1. Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Hauer J, McDade J, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK and Tonat K, *"Anthrax as a biological weapon: Medical and public health management". Journal of the American Medical Association, 1999, Vol 281, pp 1735-1745.*
2. Scorpio A, Blank TE, Day WA and Chabot DJ, *"Anthrax vaccines: Pasteur to the present". Cellular and Molecular Life Sciences". 2006, Vol 63, pp 2237-2248.*
3. Gu ML, Leppla SH and Klinman DM, *"Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen". Vaccine, 1999, Vol 17, pp 340-344.*
4. Demicheli V, Rivetti D, Deeks JJ, Jefferson T and Pratt M, *"The effectiveness and safety of vaccines against human anthrax: a systematic review". Vaccine, 1998, Vol 16, pp 880-884.*
5. Friedlander AM, Pittman PR and Parker GW, *"Anthrax vaccine: Evidence for safety and efficacy against inhalational anthrax". Journal of the American Medical Association, 1999, Vol 282, pp 2104-2106.*
6. Friedlander AM and Little SF, *"Advances in the development of next-generation anthrax vaccines". Vaccine, 2009, Vol 27, pp D28-D32.*
7. Robert J, Cybulski J, Sanz P and O'Brien AD, *"Anthrax vaccination strategies". Molecular Aspects of Medicine, 2009, Vol 30, pp 490-502.*
8. Hanes J, Cleland JL and Langer R, *"New advances in microsphere-based single-dose vaccines". Advanced Drug Delivery Reviews, 1997, Vol 28, pp 97-119.*
9. Igartua M, Hernandez RM, Esquisabel A, Gascon AR, Calvo MB and Pedraz JL, *"Enhanced immune response after subcutaneous and oral immunization with biodegradable PLGA microspheres". Journal of Controlled Release, 1998, Vol 56, pp 63-73.*
10. Yan C, Rill WL, Malli R, Hewetson J, Naseem H, Tammariello R and Kende M, *"Intranasal stimulation of long-lasting immunity against aerosol ricin challenge with ricin toxoid vaccine encapsulated in polymeric microspheres". Vaccine, 1996, Vol 14, pp 1031-1038.*
11. Klinman DM, Klaschik S, Tomaru, K, Shirota H, Tross, D., Ikeuchi, H., *Immunostimulatory CpG oligonucleotides: effect on gene expression and utility as vaccine adjuvants". Vaccine, 2010, Vol 28, pp 1919-1923.*
12. Baldridge JR, Yorgensen Y, Ward JR and Ulrich JT, *"Monoposphoryl lipid A enhances mucosal and systemic immunity to vaccine antigens following intranasal administration". Vaccine, 2000, Vol 18, pp 2416-25.*
13. Giuliani NM, Del Giudice G, Gianelli V and Dougan G, *"Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of Eschericia coli heat-labile enterotoxin with partial knockout of ADP-rybosyltransferase activity". Journal of Experimental Medicine, 1998, Vol 187, pp 1123-32.*
14. Kende M, Del Giudice G, Rivera N and Hewetson J, *"Enhancement of intranasal vaccination in mice with deglycosylated chain A ricin by LTR72, a novel mucosal adjuvant". Vaccine, 2006, Vol 14, pp 2213-2221.*
15. March KL, Mohanraj S, Ho PPK, Wilensky RL and Hathaway DR, *Circulation, 1994, Vol 89, pp 1929-1933.*

  Copyright © 2017 Frederick Furness Publishing Ltd