

The encapsulation of enterotoxigenic *Escherichia coli* colonization factor CS3 in biodegradable microspheres enhances the murine antibody response following intranasal administration

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The aim of this study was to measure serum and mucosal antibody responses following intranasal administration of biodegradable poly(DL-lactide-co-glycolide) (PLGA) microspheres loaded with the CS3 colonization factor isolated from enterotoxigenic *Escherichia coli* (ETEC). The response was compared against that measured in mice similarly administered the native CS3 antigen and in mice co-administered, along with the CS3 antigen, a known mucosal adjuvant, the R192G mutant heat-labile enterotoxin (mLT). The integrity of the CS3 antigen released from the microspheres was maintained as determined by SDS-PAGE and immunoblotting. Native CS3 induced serum and mucosal (bronchoalveolar, small intestinal and faecal) IgG and IgA responses. The co-administration of the mLT mucosal adjuvant significantly enhanced ($P < 0.001$) serum and mucosal antibody responses to the CS3 protein. Likewise, the CS3-loaded PLGA microspheres induced significantly greater ($P < 0.001$) serum and mucosal antibody responses than native CS3, as well as inducing antibody responses superior to those of the CS3 plus mLT formulation. Following administration of CS3 plus mLT, the mice became distressed (loss of activity, increased huddling, ruffled fur), a situation not seen following administration of the CS3-loaded PLGA microspheres. The results in this trial show that the CS3-loaded PLGA microspheres when administered intranasally to mice caused no observable distress to the mice and significantly ($P < 0.001$) enhanced the immunogenicity of the CS3 protein.

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of diarrhoea in children in developing countries, with an estimated 800 000 deaths among those under the age of 5 years (Gaastra & Svennerholm, 1996). ETEC is also an agent of diarrhoea in travellers to high-risk areas (Black, 1990), and is of critical concern for military units assigned to these areas of operation (Hyams *et al.*, 1991). A safe and effective vaccine directed against ETEC would be of considerable public health benefit. However, at present, no licensed ETEC vaccine is available for at-risk individuals.

Administering antigen directly to the mucosa-associated lymphoid tissue (MALT), such as the nasal mucosa, can act to induce both local and systemic specific immune responses

(Eyles *et al.*, 1998a). This is particularly important considering that most pathogens enter the body via mucosal surfaces, and protection against such organisms most often requires local production of antibodies, most importantly, secretory IgA (sIgA) (Eyles *et al.*, 1998b). Native antigens can be weak immunogens when administered to mucosal surfaces as a result of adverse conditions and/or inadequate absorption (Eyles *et al.*, 1998b). However, mucosal delivery vehicles, such as PLGA microspheres, can act to protect antigens as well as increasing their absorption, thus enhancing the immunogenicity of the antigens (Byrd *et al.*, 2005; Eldridge *et al.*, 1991b).

In this study, the immune response in BALB/c mice intranasally administered CS3-loaded poly(DL-lactide-co-glycolide) (PLGA) microspheres was compared to that of mice similarly given native CS3 and CS3 co-administered with a known mucosal adjuvant, the R192G mutant heat-labile enterotoxin (mLT). We found that CS3-PLGA microspheres induced significantly higher ($P < 0.001$) levels of IgG and IgA systemic and mucosal antibodies than native CS3, and also induced antibody responses superior to those

Abbreviations: BALT, bronchus-associated lymphoid tissue; BCA, bichinchoninic acid; ETEC, enterotoxigenic *Escherichia coli*; GALT, gut-associated lymphoid tissue; MALT, mucosa-associated lymphoid tissue; mLT, mutant heat-labile enterotoxin (R192G); NALT, nasal-associated lymphoid tissue; PLGA, poly(DL-lactide-co-glycolide) microspheres; sIgA, secretory IgA.

of the CS3 plus mLT formulation. The mLT mucosal adjuvant significantly enhanced ($P < 0.001$) the immune response to the CS3 protein; however, signs of distress were seen in the mice, indicating that the mLT may have serious safety concerns when administered intranasally. In contrast, the CS3-PLGA microspheres when administered intranasally caused no observable distress to the mice.

METHODS

Mice. Female BALB/c mice (6–8 weeks of age) were obtained from Jackson Laboratories. The mice were housed in microisolator cages and provided with food and water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. All procedures were reviewed and approved by Walter Reed Army Institute of Research Animal Care and Use Committee, and performed in the same facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The mice were randomly placed in four different test groups and immunized intranasally (twice with a 2 week interval) with one of three different CS3 vaccine formulations: group 1, native CS3; group 2, CS3 plus mLT; group 3, CS3-PLGA; group 4, PBS control.

Intranasal immunization of mice. Mice were anaesthetized with a 25 μ l mixture of xylazine (Rompun, 20 mg ml⁻¹) (Bayer) and ketamine (Ketaset, 100 mg ml⁻¹) (Fort Dodge Animal Health) given intramuscularly in the right caudal muscle. Four micrograms of CS3, 4 μ g CS3 plus 2 μ g mLT or 0.364 mg solid CS3-encapsulated PLGA microspheres (4 μ g CS3 protein) were administered in a 10 μ l volume drop-wise to the external nares of each mouse using a 2–20 μ l Pipetteman (Ranin Instrument). Immediately prior to immunization, the vaccines were diluted with PBS to a concentration of 4 μ g CS3 protein 10 μ l⁻¹. Control mice were likewise administered 10 μ l PBS. The mice were observed for signs of distress for 14 days following immunization.

CS3. CS3 was purified from ETEC strain E9034A (O8:H9:CS3:LT⁺:ST⁺). Bacteria were grown on colonization factor antigen agar plates overnight at 37 °C. The cells were suspended in PBS and heated to 60 °C for 30 min. The bacterial suspension was then centrifuged at 9000 g for 30 min and the supernatant filtered (0.2 μ m) (Amicon; Millipore) to remove bacteria. The supernatant containing the CS3 was subjected to sequential ammonium sulfate precipitation (20% and 45% saturation) and the protein was recovered by centrifugation at 37 000 g for 30 min. The protein was dialysed extensively against PBS and stored at -40 °C. Approximately 10⁷ endotoxin units per ml were measured in the CS3 preparation by the *Limulus* amoebocyte lysate Pyrotell assay (gel-clot method) (Associates of Cape Cod).

mLT. The mutant form (R192G) of the heat-labile enterotoxin (mLT) was generously supplied by Dr Shahida Baqar, Department of Enteric Infections, Walter Reed Army Institute of Research (Dickinson & Clements, 1995).

CS3 encapsulation. CS3 was microencapsulated in 50:50 poly(DL-lactide-co-glycolide) using a water/oil/water double emulsion ($w_1/o/w_2$) method as previously described (de Lorimier *et al.*, 2003). Approximately 10⁷ endotoxin units per ml were measured in the CS3-PLGA preparation as described above.

Particle size distribution. Ten milligrams of CS3-PLGA microspheres was suspended by vortexing in 1 ml deionized water in a 1.5 ml microfuge tube. The resultant suspension was observed under an Olympus BH-2 optical microscope having a calibrated ocular

micrometer at 1000 $\times$ magnification. The diameters of 150 randomly chosen microspheres were measured (repeated three times) and the microsphere size calculated.

CS3 protein content determination. Forty milligrams of CS3-PLGA microspheres was dissolved in a solution of 0.25% SDS and 0.5 M sodium hydroxide (NaOH) (2.5 ml) with vortexing and incubated at 37 °C in a warm water-bath for 24 h (in triplicate). The solution was neutralized (pH 7) using NaOH and protein content measured by the bicinchoninic acid (BCA) method (Pierce), compared against a BSA standard (Pierce) and adjusted against PLGA microspheres without encapsulated protein (Jones, 2003). The CS3 protein loading was calculated as the amount of encapsulated CS3 antigen in μ g per mg PLGA microspheres, and expressed as percentage protein per unit weight.

Measurement of CS3 protein released and changes in pH. Ninety-one milligrams of CS3-PLGA microspheres (1000 μ g CS3 protein) was incubated in 1 ml PBS solution (0.1 M, pH 7.4, 0.02% sodium azide) at 37 °C in 1.5 ml microfuge tubes with constant end-over-end rotary motion for 56 days (in triplicate). At 1 week intervals, the solutions were centrifuged at 10 000 g for 10 min in a tabletop microfuge, and 1 ml samples stored at -40 °C until protein content was determined. The collected samples were neutralized (pH 7) using NaOH prior to protein content determination by the BCA method (Pierce), compared against a BSA standard (Pierce) and adjusted against PLGA microspheres without encapsulated protein (Jones, 2003). Following removal of the 1 ml samples, 1 ml fresh PBS solutions (pH 7.4) were immediately added to the microfuge tubes, microspheres suspended and incubation continued as before. The pH of the CS3 release solutions was measured daily for the first 2 weeks and weekly thereafter with a pH miniprobe.

Measurement of CS3 protein stability and antigenicity. The CS3 protein stability and antigenicity were examined by SDS-PAGE and immunoblotting (Western blotting). CS3 samples (native and released from PLGA microspheres) were loaded and run on 16% Tricine mini-gels (denaturing, 1 mm thickness) (Novex; Invitrogen) using the Invitrogen XCell SureLock Mini-Cell at constant 125 V. The gels were stained using the Invitrogen SimplyBlue Safe Stain (Coomassie G-250). The CS3 protein was blotted onto 0.2 μ m nitrocellulose membranes (Invitrogen) using the Invitrogen XCell II Blot Module at constant 25 V. The bound CS3 was reacted with mouse anti-CS3 IgG monoclonal antibody and the Invitrogen WesternBreeze Chromogenic Immunodetection System was used to visualize the CS3 bands. The CS3-release samples were desalted and concentrated 10-fold by the Microcon Centrifugal Filter Device (10 kDa molecular mass limit) (Millipore) prior to loading into mini-gel wells.

ELISA for CS3 serum and mucosal antibodies. Serum was collected and antibodies against CS3 measured by the use of a previously described ELISA (Byrd & Cassels, 2003). Concentrations of IgG subclasses were determined from standard curves using mouse Immunoglobulin Isotype Panel (Southern Biotechnology), with values expressed as μ g ml⁻¹. Faecal pellet elutions and bronchoalveolar and small intestinal lavages were collected and antibodies measured by an ELISA as described by VanCott *et al.* (1998). The mucosal sample concentrations were determined from standard curves using purified mouse IgA and IgG antibody (Southern Biotechnology) and the CS3-specific antibody concentrations were normalized based on total IgA and IgG content, with values expressed as μ g CS3-specific IgA or IgG (mg total IgA or IgG)⁻¹.

ELISA for total serum IgE. Total IgE antibody was measured in serum samples using the Pharmingen OptEIA Mouse IgE Set. Serum concentrations were determined from standard curves using purified mouse IgE antibody, with values expressed as ng ml⁻¹.

Capture ELISA for measurement of released CS3 protein. A modified four-layer sandwich capture ELISA was used to measure CS3 proteins released from PLGA microspheres (Berdal *et al.*, 1981). Microtitre plates were coated with optimal concentration of mouse anti-CS3 IgG monoclonal antibody. All washes were done with PBS and 0.05 % Tween-20 (PBS-T), and the diluent for all samples was PBS-T and 0.1 % gelatin. Test samples (native CS3 and CS3 released from PLGA microspheres) were added and incubated for 2 h, wells washed, and rabbit anti-CS3 IgG affinity purified antibody added and incubated for 2 h. Following wash, goat anti-rabbit IgG (γ) heavy-chain specific alkaline phosphatase conjugate (Kirkegaard & Perry) was added and incubated for 2 h. The *p*-NPP substrate (Sigma) was added and the plates read at 405 nm using a microplate reader. Sample concentrations were determined from standard curves using purified CS3 protein, with the values expressed as $\mu\text{g ml}^{-1}$.

Statistical analysis. Antibody titres were log transformed for all analysis. Standard deviations (SD) were calculated using GraphPad Prism version 4.0b for Macintosh. A two-sided Student's *t* test for independent samples (GraphPad Prism) was used to compare the mean serum and mucosal antibody values of different groups of immunized mice, and of different groups of immunized mice with those of preimmune and non-immune mice, where differences in *P* values of <0.01 were considered to be significant.

RESULTS

Characterization of CS3-PLGA

The CS3 protein core loading of the PLGA microspheres was 1.1 % (w/w) (SD 0.07 %). Fig. 1 shows the size frequency of the CS3-PLGA microsphere samples: particle size distribution (percentage frequency) is plotted against particle size diameter (μm). The number mean diameter of CS3

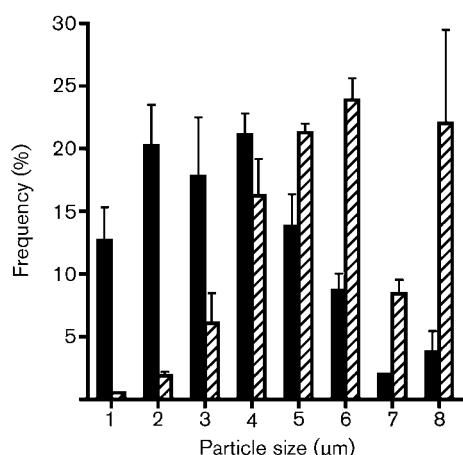


Fig. 1. Particle size distribution of CS3-PLGA microspheres. Values are percentage frequency of number (solid black bars) or volume (hatched bars) versus distribution of particle size (diameter in μm). Number and volume mean diameters were 3.56 μm and 4.46 μm , respectively. By volume: 24.9 % were 1–4 μm and 75.1 % were 5–8 μm . Error bars represent ± 1 SD of three counts (150 CS3-PLGA microspheres per count).

microspheres was 3.56 μm , and volume mean diameter was 4.46 μm with 24.9 % being 1–4 μm and 75.1 % being 5–8 μm . The pH of the solution in which the CS3 protein was released upon PLGA microsphere degradation remained above 7 to at least day 10; thereafter, there was a steady decrease to pH 3–4 by 42–56 days (Fig. 2).

Approximately 40 % of the CS3 protein was released from the PLGA microspheres within 24 h of *in vitro* incubation, which is indicative of CS3 bound on and near the surface of the PLGA microspheres. Thereafter, there was a steady release of the CS3 protein from the degrading PLGA microspheres. Approximately 90 % of the CS3 was released from the degraded PLGA microspheres by day 56 when total protein was measured by the BCA assay, whereas about two-thirds this amount was measured by capture ELISA (Fig. 2). The CS3 appears to have been adversely affected as a result of microencapsulation and/or during *in vitro* release from the PLGA microspheres. However, SDS-PAGE (Fig. 3a) and immunoblotting (Fig. 3b) indicated absence of any major fragments of CS3 (as compared to the unencapsulated native CS3), with the CS3 subunit protein remaining intact and retaining its antigenicity.

Effect of the vaccines on the mice

The mice that were intranasally administered native CS3 and the CS3-PLGA microspheres showed no signs of distress. However, the mice that were intranasally administered the mLT showed loss of activity with increased huddling, with their fur becoming ruffled, lasting for 24–48 h, indicating an adverse reaction to the mLT adjuvant.

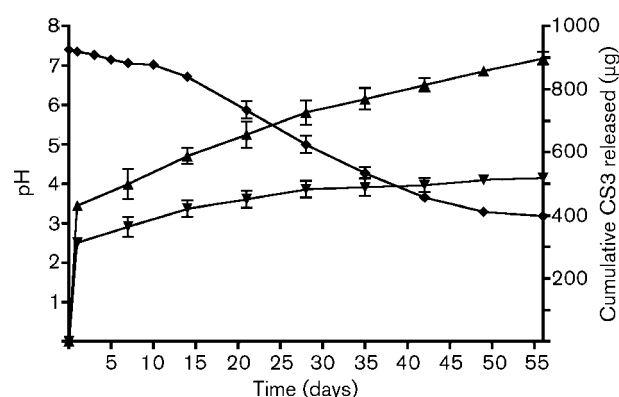


Fig. 2. Cumulative release of CS3 protein from PLGA microspheres as determined by BCA (CS3-BCA) or capture ELISA (CS3-ELISA) versus change in pH over a 56 day period. Release medium: 0.1 M PBS, pH 7.4, 0.02 % sodium azide. The data represent the mean ± 1 SD of three separate CS3 release assays for pH (◆) and BCA protein measurements (▲), and of two separate assays for capture ELISA protein measurements (▼).

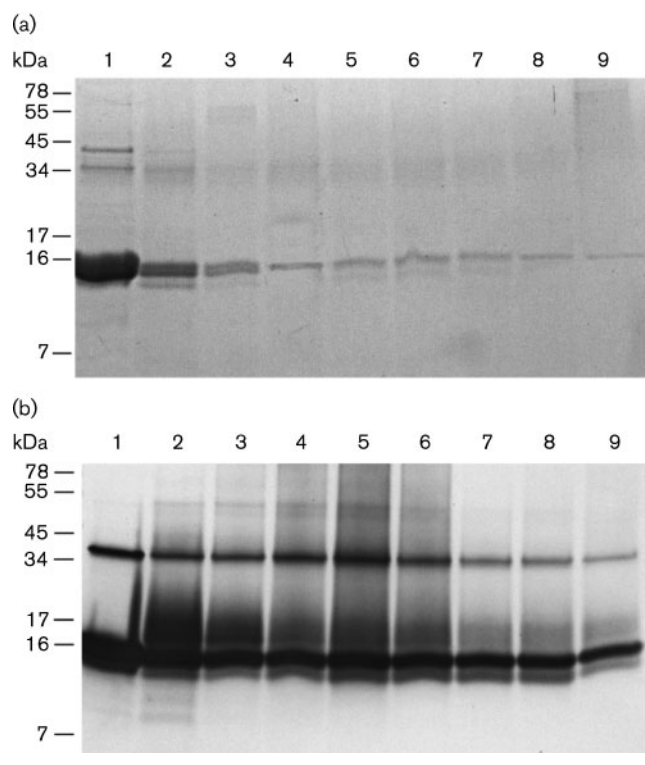


Fig. 3. SDS-PAGE (a) and Western blot (b) analysis of native CS3 and released CS3. kDa, standards. Lanes: 1, native CS3; 2–9, released CS3, days 7, 14, 21, 28, 35, 42, 49 and 56, respectively. 16% Tricine-gel, 20 μ l sample per well.

Serum and mucosal antibody responses following intranasal immunization

Native CS3 administered intranasally to the mice induced serum and mucosal (bronchoalveolar, small intestinal and faecal) IgG, IgA and IgM antibody responses (Table 1). The CS3-PLGA microspheres significantly enhanced ($P < 0.001$) both serum and mucosal IgG and IgA antibody responses compared to native CS3. Likewise, the mLT mucosal adjuvant significantly enhanced ($P < 0.001$) the serum and mucosal IgG and IgA antibody responses to the CS3 antigen. The CS3-PLGA serum and mucosal antibody responses were greater than those of CS3 plus mLT; however, the differences were not significant.

CS3 administered by the intranasal mucosal route induced an IgG response dominated by the IgG1 subclass, followed by IgG2a, which is indicative of a mixed Th1/Th2 with the Th2-type predominant (IgG1 > IgG2a) (Fig. 4). The encapsulation of CS3 or the co-administration of mLT did not alter the mixed Th1/Th2 profile.

IgE antibody response following intranasal immunization

No increase in the levels of total serum IgE were detected in mice intranasally immunized with native CS3 or the CS3-PLGA microspheres compared to preimmune and non-immune levels (Table 1). However, total serum IgE was significantly elevated ($P = 0.005$) in the mice administered the mLT mucosal adjuvant.

Table 1. ELISA values to CS3 following immunization with the vaccine formulations

Serum and mucosal samples were collected 14 days after the second intranasal immunization.

	CS3	CS3 + mLT	CS3-PLGA
Serum IgG*	4.3 (3.9–4.4)	5.4§ (5.4–5.7)	5.5§ (5.4–5.7)
Serum IgA	2.4 (2.0–2.5)	3.3 (3.0–3.5)	3.4 (3.0–3.5)
Serum IgM	2.1 (2.0–2.3)	2.4 (2.3–2.6)	2.3 (2.3)
Lung IgA†	439.4 (364.9–475.3)	765.8§ (594.9–966.8)	974.0§ (662.2–1337.2)
Lung IgG	307.8 (210.8–370.9)	599.0 (416.1–840.0)	850.9§ (710.3–953.0)
Intestinal IgA	51.2 (41.7–68.8)	334.7§ (177.7–461.8)	426.4§ (303.7–571.7)
Intestinal IgG	194.9 (106.0–251.5)	559.0§ (438.7–689.7)	822.8§ (495.4–1289.8)
Faecal IgA	5.7 (3.2–7.4)	46.6§ (37.2–59.5)	74.8§ (50.1–101.8)
Faecal IgG	182.7 (92.4–285.4)	449.9 (325.5–531.5)	508.2§ (455.3–589.8)
Total serum IgE‡	221.9 (131.5–275.9)	2515.3¶ (1000.1–3908.8)	282.7 (156.8–330.8)

*Serum IgG and IgA values are expressed as geometric mean titres (GMT) (range of antibody values) ($n = 7$ mice).

†Lung, small intestinal and faecal values are expressed as μ g CS3-specific IgA or IgG ($\text{mg total IgA or IgG}^{-1}$) (range of antibody values) ($n = 6$ mice for lung and intestinal lavages and 7 mice for faecal pellet elutions). The mean total IgA and IgG antibody concentrations ($\mu\text{g ml}^{-1}$): lung IgA (0.98 ± 0.56), lung IgG (0.89 ± 1.02), intestinal IgA (33.45 ± 13.98), intestinal IgG (1.30 ± 0.64), faecal IgA (136.85 ± 56.77), faecal IgG (0.13 ± 0.04); similar to those reported by VanCott *et al.* (1998).

‡Total serum IgE values are expressed as ng ml^{-1} (range of antibody values) ($n = 6$ mice).

§ $P < 0.001$ comparing CS3 + mLT and CS3-PLGA to CS3.

|| $P < 0.005$ comparing CS3 + mLT and CS3-PLGA to CS3.

¶ $P = 0.005$ comparing total serum IgE of preimmune, CS3 and CS3-PLGA to CS3 + mLT. Preimmune total serum IgE was 236.3 ng ml^{-1} ($149.0\text{--}262.9$).

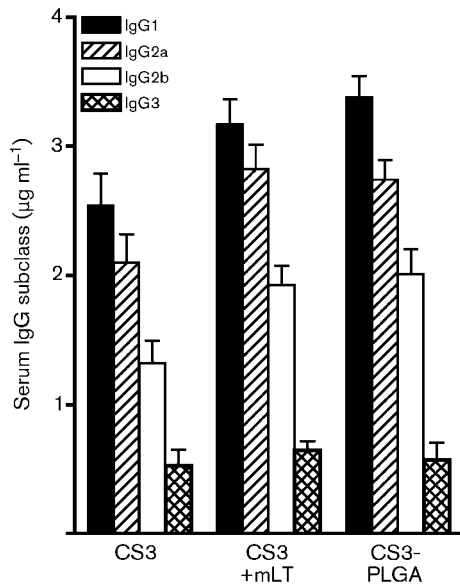


Fig. 4. Serum IgG subclass antibody responses to CS3 14 days following the second intranasal immunization with CS3, CS3+mLT and CS3-PLGA. Values are expressed as $\mu\text{g ml}^{-1} \pm 1$ SD (mean of seven mice), as determined from standard curves of purified mouse IgG subclass antibody. At all values, $P < 0.01$ compared to the preimmune and non-immune (PBS control) sera.

DISCUSSION

This study demonstrates that encapsulation of CS3 into PLGA microspheres acts to enhance the immune response to CS3 when administered to the nasal mucosa of mice. The PLGA microspheres acted in an adjuvant capacity, enhancing both systemic and mucosal antibody responses to the CS3 protein antigen. The mechanism through which the PLGA microspheres act to increase immunogenicity is not completely understood at present. However, the sustained release of antigen as the PLGA microspheres slowly degrade coupled with uptake of the antigen-loaded PLGA microspheres by macrophages and antigen-presenting cells, which then continuously target peripheral lymph nodes and MALT, is most likely responsible (Eldridge *et al.*, 1991a). The PLGA microspheres are composed of biodegradable polymers, lactide and glycolide, which degrade over time *in vivo* to normal metabolic products, being ultimately excreted from the body via the Krebs cycle (Lewis, 1990). They have a long safety record of use in humans, having been utilized as resorbable sutures, implants and drug delivery vehicles (Lewis, 1990).

The nasal and pulmonary regions of the respiratory tract are inviting target areas for the delivery of vaccines (Eyles *et al.*, 1998a). Inhalation or introduction of an immunogen into the nose is relatively simple, allowing for greater patient compliance. The large surface areas of the nasal epithelium (being covered with microvilli) and the lungs, in which the

epithelial barriers are reasonably permeable to macromolecules, make these regions very attractive for mucosal antigen delivery. Antigen delivery to nasopharyngeal passages, which contain nasal-associated lymphoid tissue (NALT) and associated M-cells, allows for induction of serum and mucosal immune responses. In addition, reduced levels of proteolytic enzymes and pH extremes as compared to the gastrointestinal tract further make the respiratory tract more advantageous for vaccine delivery. Encapsulated antigens (such as CS3-PLGA microspheres) administered to the respiratory tract may be less efficiently cleared by mucociliary activity compared to the soluble protein. Thus, intranasally administering the CS3-PLGA microspheres may allow for increased uptake via antigen-processing cells by prolonging the time in which the CS3-loaded microspheres contact the mucosal surface. This may in turn enhance the immune response to the encapsulated CS3 protein. Therefore, for this trial, the intranasal route was chosen as the most advantageous for delivery of the vaccine formulations to the mice.

MALT is the major B and T cells' inductive site and supplies the mucosa regularly with sIgA (Kiyono *et al.*, 1992). The gut-, nasal- and bronchus-associated lymphoid tissues (GALT, NALT and BALT) are specific MALT inductive sites giving rise to local mucosal responses involving most importantly IgA-committed cells (Sminia & Kraal, 1999). The importance of sIgA in protection is that it is the primary immunoglobulin providing immune exclusion of bacteria and viruses at local surfaces (Mestecky *et al.*, 1986). It has been shown that sIgA in mucosal secretions has the ability to agglutinate bacteria, neutralize bacterial toxins and block adherence of bacteria to mucosal epithelial cells (Kilian *et al.*, 1988; McGhee *et al.*, 1992). Also, sIgA is enhanced in blocking bacterial adherence as compared to IgG due to its charge, extensive glycosylation and resistance to proteolysis (McGhee *et al.*, 1992).

Along with sIgA, it is important to note the presence of IgG in mucosal immunity. Following intranasal immunization with the CS3 vaccines, IgG was measured at elevated levels in the mucosal samples. It is known that IgG is found at high levels in the deep lung, and that pulmonary IgG, in addition to transudating from serum, is produced locally in BALT (Eyles *et al.*, 2000). Also, IgG antibody-producing cells have been shown to be present at high levels in nasal mucosa (Brandtzaeg, 1985; Heritage *et al.*, 1997), with IgG and IgA being found in comparable amounts in nasal secretions (Rudin *et al.*, 1998). Serum IgG is found in the lungs and intestines comparable to locally secreted IgA (sIgA) (Pierce & Reynolds, 1974; Robbins *et al.*, 1997). The high level of secreted and/or transudated IgG present coupled with the substantially decreased enzymic degradation of IgG in the respiratory tract as compared to the intestinal tract support the importance of IgG in protection in this tissue (Brandtzaeg, 1995; Manzanec *et al.*, 1992; Steinmetz, 1997). Others have reported that following intranasal immunization, IgA responses dominate throughout the respiratory tract, whereas IgG responses contribute to immunity in lungs but not in

nasal passages (Hodge *et al.*, 2001). Results seen in several bacterial and viral vaccine trials indicate that serum IgG is important in immunity on epithelial surfaces, and specific IgG in sufficient amounts is able to prevent enteric infections (Robbins *et al.*, 1992, 1995, 1997).

An important factor of mucosal immunity is the trafficking of lymphocytes among distant mucosal sites. This communication between the mucosal lymphoid tissues, GALT, BALT and NALT, known as the common mucosal immune system, allows IgA-committed cells from one mucosal site to seed distant mucosal sites, where they differentiate into antibody-producing plasma cells, resulting in antigen-specific antibody mucosal sites throughout the body (Sminia & Kraal, 1999). The anti-CS3 IgA mucosal antibody response measured in the intestinal and faecal samples of the mice immunized intranasally is a result of the trafficking of anti-CS3 antibody-secreting cells between the two mucosal sites, respiratory and gastrointestinal. Swallowing of some of the antigen administered intranasally appears not to be responsible as the swallow reflex of deeply anaesthetized mice is essentially eliminated, and in addition, only a small inoculating volume was administered to the mice (Byrd & Cassels, 2003).

The CS3 vaccines administered intranasally induced primarily an IgG1 subclass response followed by IgG2a, with the IgG2b subclass measured at moderate levels (Fig. 4). This is in agreement with the response seen in previous mouse trials involving CFA/I and CS6 (Alves *et al.*, 1998; Byrd & Cassels, 2003). The IgG subclass responses induced by CS3 nasal vaccination strongly suggest that CS3 activates a mixed Th1/Th2 immune response, while PLGA encapsulation or co-administration of the mLT adjuvant did not alter the mixed Th1/Th2 profile. These results as well as others (Eyles *et al.*, 1998b) indicate that intranasal administration of native and microencapsulated antigens is Th2 skewed (IgG1 dominant). This indicates that antigens given by mucosal routes are processed by both antigen presenting cells and T lymphocytes.

It has been noted that the size of the microspheres can have an effect on the degree and time of an antibody response. Eldridge *et al.* (1991a, b) have suggested that the adjuvant effect of PLGA microspheres was the result of their size being < 10 µm in diameter. The adjuvanticity of microspheres of < 10 µm resides in their ability to allow phagocytosis of a relatively large quantity of antigen by macrophages and accessory cells followed by directed migration of these cells into draining lymph nodes at the injection site (Eldridge *et al.*, 1991b). Therefore, the size of the PLGA microspheres used in this mouse trial was < 10 µm in diameter (Fig. 1).

The adjuvant effect of microencapsulation on the CS3 antigen was compared to that of a known mLT mucosal adjuvant. The co-administration of the mLT was shown to induce greater antibody responses than CS3 given alone, but not when compared to those induced by the PLGA microspheres. Concerns have been raised as to potential problems

that could be encountered with the use of cholera and heat-labile bacterial toxins and toxoids when administered intranasally (van Ginkel *et al.*, 2000). Involvement of nasal and respiratory surfaces with these adjuvants has the potential of inducing adverse inflammatory responses (Simecka *et al.*, 2000; van Ginkel *et al.*, 2000). Total serum IgE was elevated in mice intranasally administered mLT as a mucosal adjuvant, with no elevated levels of total serum IgE measured in mice intranasally administered native CS3 or the CS3-PLGA microspheres. These results are consistent with those seen by others (Beignon *et al.*, 2002; Boyaka *et al.*, 2003; Takahashi *et al.*, 1996), which have shown elevated levels of total serum IgE following administration of LT or toxoid forms of LT. Even though the exact role of IgE is still uncertain, there is concern that increased levels of IgE may be associated with risk of anaphylaxis (Beignon *et al.*, 2002). IgE is believed to be involved in human and mouse allergic airway inflammation (Coyle *et al.*, 1996; Zuberi *et al.*, 2000). The mice in this study showed definite signs of distress (loss of activity, increased huddling, ruffled fur) following intranasal administration of the mLT adjuvant, which is consistent with the mLT retaining some toxicity (Komase *et al.*, 1998; Rappuoli *et al.*, 1999). Thus, a critical factor in the use of the PLGA microspheres in nasal delivery is their documented safety. We did not detect a significant increase in IgE responses in the mucosal samples of the mice administered the mLT adjuvant. Likewise, Takahashi *et al.* (1996) detected only low faecal IgE antibody responses following oral administration of 25 µg of ETEC LT. However, we feel that the measurement of total IgE in serum was sufficient to note that mLT can indeed induce this type of antibody response in the mice when administered to the nasal mucosa.

CS3 protein was released from the PLGA microspheres by a combination of pore diffusion and polymer erosion (Fig. 2) (Kissel *et al.*, 1997). Upon exposure of the CS3-loaded microspheres to the aqueous environment, water diffused into the polymeric matrix, resulting in swelling of the microspheres along with the formation of pores through which the CS3 antigen was released. The porous core structure of the PLGA microspheres contained large cavities, resulting in a high initial burst release of the CS3 protein. A significant amount (approx. 40%) of CS3 was released over a short period (24 h) with this initial burst release of antigen corresponding to a priming immunization dose. This initial burst effect is thought to be due to the rapid release of antigen located on or near the surface of the microspheres and poorly entrapped antigen (McGee *et al.*, 1994). The subsequent rate of CS3 release was a result of degradation of the polymer matrix. Therefore, following the initial burst release of CS3, the PLGA microspheres degraded over time, with the entrapped CS3 protein being slowly released. In addition, the acidic monomers, lactic acid and glycolic acid, were continuously released into the medium as the microspheres slowly degraded, significantly reducing the pH. Release studies conducted for this study (Fig. 2) and by others (Kersten & Gander, 1996) have shown that the pH can drop to 3 or lower towards the end of the degradation of

the microspheres, even if the buffer is exchanged with fresh medium on a regular basis. The core itself of PLGA microspheres becomes increasingly more acidic ($\text{pH} < 3$) as a result of hydrolytic chain scission of the polymer (Kissel *et al.*, 1997). The drop in pH can adversely affect the immunogenicity of an antigen.

The microencapsulation process itself as well as the degradation of the PLGA microspheres can result in antigen denaturation, fragmentation and aggregation (Jones, 2003). The capture ELISA (Fig. 2) showed a decrease in measurement of the CS3 protein that may indicate some level of denaturation and/or aggregation while the overall immunogenicity of the CS3 protein was not destroyed (Desai *et al.*, 2000; Sasiak *et al.*, 2001). We found that the released CS3 protein retained its antigenicity as determined by capture ELISA (Fig. 2) and immunoblotting (Fig. 3b), as well as serum and mucosal antibody responses (Table 1) measured in mice following immunization with the CS3-PLGA microspheres, thus showing that the encapsulation procedure and release of CS3 following microsphere degradation were not detrimental to the CS3 antigenicity.

The intranasal administration of the CS3-PLGA microspheres induced systemic and mucosal responses in mice that were significantly greater ($P < 0.001$) than those induced by native CS3. Importantly, the CS3-encapsulated PLGA microspheres caused no distress to the mice as well as no elevation of total serum IgE. Encouraged by these results, we plan to test other ETEC colonization factors encapsulated in PLGA microspheres in the intranasal mouse model and administered via other routes, such as intragastric and transcutaneous. The results from this and other such studies will aid in supporting future primate and human vaccine trials directed against ETEC.

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