J. Pharm. Pharmacol. 2000, 52: 1195–1201
Received March 27, 2000
Accepted June 9, 2000

Biodegradable Microparticles with Different Release Profiles: Effect on the Immune Response After a Single Administration via Intranasal and Intramuscular Routes

IAN D. SPIERS, JIM E. EYLES, LES W. J. BAILLIE*, E. DIANE WILLIAMSON* AND H. O. ALPAR

*Pharmaceutical Sciences Institute, Aston University, Birmingham, B4 7ET and *DERA (Chemical and Biological Defence Sector), Porton Down, Salisbury, Wilts, SP4 0JQ, UK*

Abstract

In the development of single-dose microparticulate vaccines, identification of the type of protein release profile required to elicit high and sustainable immune responses is important. Microparticles exhibiting different protein release profiles (continuous, pulsatile and plateau) were made by solvent evaporation or solvent extraction methods from biodegradable polymers encapsulating the model antigen, bovine serum albumin (BSA). The immune responses obtained after a single intranasal or intramuscular administration of microparticles were determined, and also after a subcutaneous boost after 11 months.

Microparticles were manufactured with acceptable protein loading and average volume size ranging from 1 to 10 μm. The integrity of BSA extracted and released from microparticles after 2 months incubation was retained. Microparticulate preparations administered by either intranasal or intramuscular routes, evoked rapid, high titre and long-lived (up to 11 months after priming) specific serum IgG responses which were significantly greater than for free BSA. The type of protein release from microparticles had no significant effect on the systemic immune responses. Interestingly, a formulation exhibiting a plateau-release profile was the only microparticulate system capable of inducing significantly greater IgA responses than free BSA after intranasal immunization.

This study shows the benefit of microencapsulation in inducing high and long-lasting systemic immune responses after a single dose by both parenteral and mucosal delivery. We conclude that of the microparticles tested, the longevity and magnitude of humoral responses was not effected by the type of in-vitro protein release profile.

Advances in biotechnology have led to a new generation of potential vaccines called subunit antigens. These highly purified proteins are less toxigenic than current vaccines produced from deactivated pathogens and toxins. However, subunit antigens suffer from poor immunogenicity which necessitates the use of adjuvants and repeated dosing to induce the desired level of immunity. Ideal vaccine formulations should provide high and long-lasting immune responses but also be pharmaceutically acceptable. A promising approach to achieving this is the use of biodegradable polymeric microparticles which have been shown to be effective adjuvants for many proteins including the model antigen bovine serum albumin (BSA) (Conway et al 1997), tetanus toxoid (Alpar & Almeida 1994) and the *Yersinia pestis* antigens (Eyles et al 1998a,b, 2000). Microparticles can also be used as single-dose vaccines which offer significant advantages over conventional immunization schedules (Cleland 1999; O'Hagan et al 1998). An important consideration in the use of microparticles as single-dose vaccines, is establishing the type of release profile required to induce sustainable immune responses after parenteral and mucosal delivery.

The aim of this study was to compare the mucosal and systemic immune responses elicited after a single intranasal or intramuscular administration of protein-loaded polymeric microparticles with different protein release profiles. Microparticles were made from biodegradable polymers encapsulating the model protein BSA using either a

Correspondence: H. O. Alpar, Pharmaceutical Sciences Institute, Aston University, Birmingham, B4 7ET, UK.
E-Mail: H.O.Alpar@Aston.ac.uk

double emulsion solvent evaporation or solvent extraction method. Microparticles were characterized for the following properties: particle size, protein loading, zeta potential and protein release. The integrity of both released and encapsulated protein was also assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Immune responses after a single intranasal or intramuscular administration to BALB/c mice were measured for 320 days before a subcutaneous challenge with BSA.

Materials and Methods

Materials

Poly(DL-lactide-co-glycolide 50 : 50) (PLGA; MW 3 kDa) (resomer RG502) was obtained from Boehringer Ingelheim (Germany). Poly-L-lactide (PLLA; MW 100 kDa) was from Polysciences (PA). Polyvinyl alcohol (PVA; MW 13–23 kDa; 88% hydrolysed), sodium hydroxide and HPLC-grade dichloromethane, methanol, ethanol and isopropanol were supplied by Aldrich Chemical Co. Inc. (Dorset, UK). SDS and BSA were supplied by Sigma Chemical Company (Dorset, UK). Bicinchoninic acid (BCA) protein assay reagent A and cupric sulphate 4% w/v reagent B were supplied by Pierce (Rockford, IL) and halothane was supplied by RMB Animal Health (UK).

Preparation of microparticles

All microparticles were made using a modification of a double emulsion method (Conway & Alpar 1996) using either solvent evaporation or extraction. Briefly, 6 mg BSA was dissolved in 0·5 mL of an aqueous solution of PVA (2·5% w/v) which was subsequently added to 5 mL dichloromethane containing 250 mg polymer. The mixture was probe sonicated (Soniprep 150, MSE; UK) for 2 min at 60 W in an ice bath. The primary emulsion formed was added dropwise into 75 mL PVA (5% w/v) and further emulsified using a Silverson homogenizer at 16 000 rev min^{-1} (Silverson Machines; Bucks, UK) for 8 min. The resultant w/o/w emulsion was gently stirred until the dichloromethane had evaporated (except for preparations using solvent extraction or rotary evaporation). The particles were collected by centrifugation (100 000 *g*, 25 min), washed three times with distilled water and freeze-dried. Microparticles intending to have continuous protein release were prepared by solvent extraction with the addition of methanol (A1), ethanol (A2) or isopropanol (A3) to the w/o/w emulsion or by rotary evaporation of the dichloromethane (A6). Physical blends of PLLA and PLGA were used to produce microparticles with the potential of pulsatile type release: 90% PLLA/10% PLGA (A4) and 50% PLLA/50% PLGA (A5). Plateau-releasing microparticles (A7) were made using 100% PLLA.

Characterization of microparticles

Particle sizes were determined by laser diffraction (MasterSizerE, Malvern Instruments Ltd; Malvern, UK). The zeta potentials of microparticles suspended in 1 mM potassium chloride (pH 5·55) were studied using a Malvern Zetasizer (Malvern Instruments Ltd; Malvern, UK). The protein loading of microparticles was determined in triplicate by digestion in 1 M sodium hydroxide until the medium lost all turbidity, neutralization of the solution to pH 7 with 10 M hydrochloric acid and then submitting samples and standards (treated in the same manner) to BCA analysis (Smith et al 1985).

In-vitro release studies

The release of BSA from microparticles was determined by incubation of particles (approx. 5 mg) in 1 mL phosphate-buffered saline (PBS) containing 0·01% w/v sodium azide and 5 mM SDS at 37°C. For each time point, triplicate samples were used and the protein concentration of the supernatants was determined by BCA assay after centrifugation of the microparticulate suspension (15 000 rev min^{-1}).

Protein integrity

Protein release samples after 2 months incubation were assessed by SDS-PAGE 10% w/v as previously described (Spiers et al 1999). Entrapped protein within microparticles after 2 months incubation was extracted using dichloromethane and PBS containing 5% w/v SDS and were also assessed by SDS-PAGE 10% w/v.

Animals

BALB/c female mice (25 g, 6 weeks old) were used. All experimentation strictly adhered to the 1986 Scientific Procedures Act.

Immunization

Groups of 5 mice received primary immunizations with either microparticles or protein equivalent to 15 μg BSA in sterile PBS by intranasal or intra-

muscular administration. For intranasal immunization, mice were lightly anaesthetized with an inhaled gaseous mixture of 3% (v/v) halothane in oxygen ($300\,cm^3\,min^{-1}$) and nitrous oxide ($100\,cm^3\,min^{-1}$). Each mouse received a 50 μL volume (25 μL per nostril) of suspension/solution, administered with a micropipette. For intramuscular immunization mice were injected with 50 μL of suspension/solution using a $0{\cdot}5 \times 16$-mm needle and 1 mL volume syringe, into the left hind leg muscle. After 326 days all mice were subcutaneously boosted in the scruff of the neck with 100 μL sterile PBS containing 1 μg BSA using a $0{\cdot}45 \times 10$ mm needle and 1 mL volume syringe.

Body fluid and tissue sampling

To assess primary humoral responses in serum, tail-vein blood samples were taken from all mice throughout the experiment. On day 340, all the mice were terminally anaesthetized with an inhaled gaseous mixture of 3% (v/v) halothane in oxygen ($300\,cm^3\,min^{-1}$) and nitrous oxide ($100\,cm^3\,min^{-1}$). Cardiac puncture blood samples were removed from the anaesthetized mice before killing by cervical dislocation. Broncho-alveolar (BAL) washings were collected as described by Eyles et al (1998b).

Analysis of blood samples

Titration of IgG antibody in serum samples from immunized mice was achieved using a reported procedure for an indirect enzyme linked immunosorbent assay (Conway et al 1997). End-point titre was estimated as the maximum dilution of the serum giving an absorbance reading greater than the maximum optical density of titrated naive serum. IgG subclass analysis was performed on pooled serum samples representative of the immunization group.

Analysis of BAL washings

Assay of specific IgG and IgA content in BAL washings were performed as described for serum. Final optical density readings were derived from the optical density of BAL washings minus the optical density of naive washes.

Statistical analysis

Analysis of variance and parametric multiple comparison tests were used to analyse titre data from the different groups. $P < 0{\cdot}05$ was considered significant.

Results and Discussion

All the microparticles had acceptable protein loading and average volume size ranging from 1 to 10 μm (Table 1). The protein loading efficiency was reduced when the percentage of PLGA in the microparticles was increased to 50% (A5). This is probably due to a reduction in viscosity of the organic phase when using greater proportions of low molecular weight PLGA which would lead to a greater loss of protein from the internal phase to the external phase. There was also very little difference in the zeta potentials of the different preparations (Table 1).

The integrity of BSA entrapped and released from microparticles after 56 days incubation at 37°C was assessed by SDS-PAGE. BSA extracted from all the microparticles gave similar bands to free BSA (native) and thus the integrity of the entrapped BSA was retained after microencapsulation and incubation (results not shown). Therefore the formulation method used to prepare the microparticles had no adverse effects on protein integrity.

Table 1. Particle characteristics of manufactured microparticles.

Microparticle	Volume mean (μm)	Number mean (μm)	Zeta potential (mV)	Protein loading (% w/w)
A1	$5{\cdot}86 \pm 2{\cdot}33$	$4{\cdot}07 \pm 1{\cdot}42$	$-7{\cdot}5 \pm 0{\cdot}5$	$2{\cdot}06 \pm 0{\cdot}03$
A2	$5{\cdot}54 \pm 2{\cdot}40$	$3{\cdot}38 \pm 1{\cdot}49$	$-7{\cdot}7 \pm 0{\cdot}6$	$1{\cdot}98 \pm 0{\cdot}17$
A3	$5{\cdot}65 \pm 2{\cdot}74$	$3{\cdot}32 \pm 1{\cdot}48$	$-4{\cdot}9 \pm 0{\cdot}2$	$1{\cdot}98 \pm 0{\cdot}07$
A4	$4{\cdot}06 \pm 1{\cdot}28$	$2{\cdot}57 \pm 1{\cdot}15$	$-4{\cdot}0 \pm 0{\cdot}4$	$2{\cdot}10 \pm 0{\cdot}10$
A5	$2{\cdot}59 \pm 1{\cdot}05$	$1{\cdot}82 \pm 0{\cdot}52$	$-9{\cdot}2 \pm 0{\cdot}6$	$1{\cdot}31 \pm 0{\cdot}15$
A6	$5{\cdot}49 \pm 2{\cdot}08$	$3{\cdot}87 \pm 1{\cdot}37$	$-3{\cdot}5 \pm 0{\cdot}4$	$2{\cdot}13 \pm 0{\cdot}18$
A7	$2{\cdot}65 \pm 0{\cdot}81$	$2{\cdot}12 \pm 0{\cdot}54$	$-4{\cdot}8 \pm 0{\cdot}3$	$2{\cdot}25 \pm 0{\cdot}03$

Data are mean ± s.d., n = 3. Microparticles with continuous protein release were prepared by solvent extraction with the addition of methanol (A1), ethanol (A2) or isopropanol (A3) to the w/o/w emulsion or by rotary evaporation of the dichloromethane (A6). Physical blends (%) of PLLA and PLGA were used to produce microparticles with pulsatile-type release: 90 : 10 PLLA : PLGA (A4) and 50 : 50 PLLA : PLGA (A5). Plateau-releasing microparticles (A7) were made using 100% PLLA.

Table 2. Protein release profiles for microparticles after incubation at 37°C over 56 days.

Microparticle	BSA released (%)				
	2 h	7 days	14 days	28 days	56 days
A1	20·17 ± 2·71	27·50 ± 6·28	36·11 ± 1·50	48·52 ± 3·42	65·21 ± 9·05
A2	21·91 ± 4·11	27·82 ± 4·16	39·29 ± 7·03	46·20 ± 1·16	55·49 ± 7·23
A3	26·56 ± 1·32	30·92 ± 3·54	40·97 ± 8·72	62·89 ± 4·32	69·44 ± 6·59
A4	14·97 ± 5·89	15·36 ± 3·25	20·32 ± 1·98	42·79 ± 7·03	45·70 ± 16·36
A5	29·84 ± 3·72	30·12 ± 4·81	28·20 ± 0·81	65·05 ± 3·71	60·74 ± 6·36
A6	19·47 ± 7·54	19·86 ± 2·00	28·01 ± 3·85	44·99 ± 4·67	51·27 ± 4·25
A7	28·41 ± 6·76	33·65 ± 0·98	25·80 ± 4·71	35·71 ± 6·90	20·92 ± 9·95

Data are mean ± s.d., n = 3. Microparticles with continuous protein release were prepared by solvent extraction with the addition of methanol (A1), ethanol (A2) or isopropanol (A3) to the w/o/w emulsion or by rotary evaporation of the dichloromethane (A6). Physical blends (%) of PLLA and PLGA were used to produce microparticles with pulsatile-type release: 90 : 10 PLLA : PLGA (A4) and 50 : 50 PLLA : PLGA (A5). Plateau-releasing microparticles (A7) were made using 100% PLLA.

The release of BSA from microparticles after 56 days is shown in Table 2. The fast removal of organic solvent during microparticle formation by solvent extraction (A1, A2, A3) and rotary evaporation (A6) lead to microparticles that showed continuous protein release on incubation. Microparticles made from different physical blends of PLLA and PLGA (A4 and A5) gave a pulsatile release profile with a second release of protein occurring between 14 to 28 days. This release profile was predicted because on incubation, PLGA quickly erodes compared with PLLA leading to the release of protein. Microparticles made from 100% PLLA (A7) gave a plateau-release profile with the level of BSA remaining relatively constant after the initial release (protein released after 2 h and 56 days was not significantly different).

Mean specific serum IgG immune titres to a single nasal administration of microparticles, followed by a subcutaneous booster administration of 1 μg free BSA on day 326, are shown in Figure 1. The only particulate recipients that showed any significant variation in IgG titres after nasal priming and subcutaneous boosting was treatment group A3 (PLLA microparticles formed by solvent extraction with isopropanol). Boosting in this group showed a significant increase in the systemic immune response when compared with the initial priming data (day 28), but not when compared with later priming time points (days 187 and 320). For the particulate formulations, this indicates that once the immune responses reached optimal priming via this dosing route, the titres were maintained for nearly a year and boosting with BSA itself had little effect. In contrast, the free BSA group significantly increased after boosting. All the microparticle preparations elicited serum IgG titres that were significantly greater than for free BSA. Thus microencapsulation of BSA is of clear benefit in inducing higher IgG titres than free BSA. There was no significant variation in IgG titres between microparticulate groups which indicates that the different protein release patterns did not influence the systemic immune response once primed. The BAL IgA and IgG responses are shown in Table 3 as optical density values. The microparticle preparation A7 had BAL IgA levels significantly greater than free BSA, whereas all the microparticulate preparations had BAL IgG levels greater than free BSA. The reason for the improved mucosal antibody response with A7 (plateau-release profile) requires further investigation. The higher IgA levels achieved by A7 could be attributed to the retention of protein within the particles for a longer period of time, allowing presentation of unadulterated protein to antigen-presenting cells rather than release of the protein into the harsh external environment.

The serum IgG immune titres to a single muscular administration of the microparticles are shown in Figure 2, followed by a subcutaneous booster administration of 1 μg free BSA on day 326. Apart from free BSA, any significant increase in IgG titres occurred in the first 6 months after which there were no significant changes in titres even after boosting. Free BSA itself did increase significantly on boosting. For the particulate formulations, this shows that again once the immune responses reached optimal priming the titres were maintained for up to 320 days and boosting with BSA had little effect.

All the microparticulate formulations gave IgG titres that were significantly greater than free BSA on intramuscular immunization. This confirms that microencapsulation of BSA is beneficial in eliciting high IgG titres. Formulation A5 gave IgG titres significantly greater than A2 at day 28, whereas the other microparticulate preparations showed no

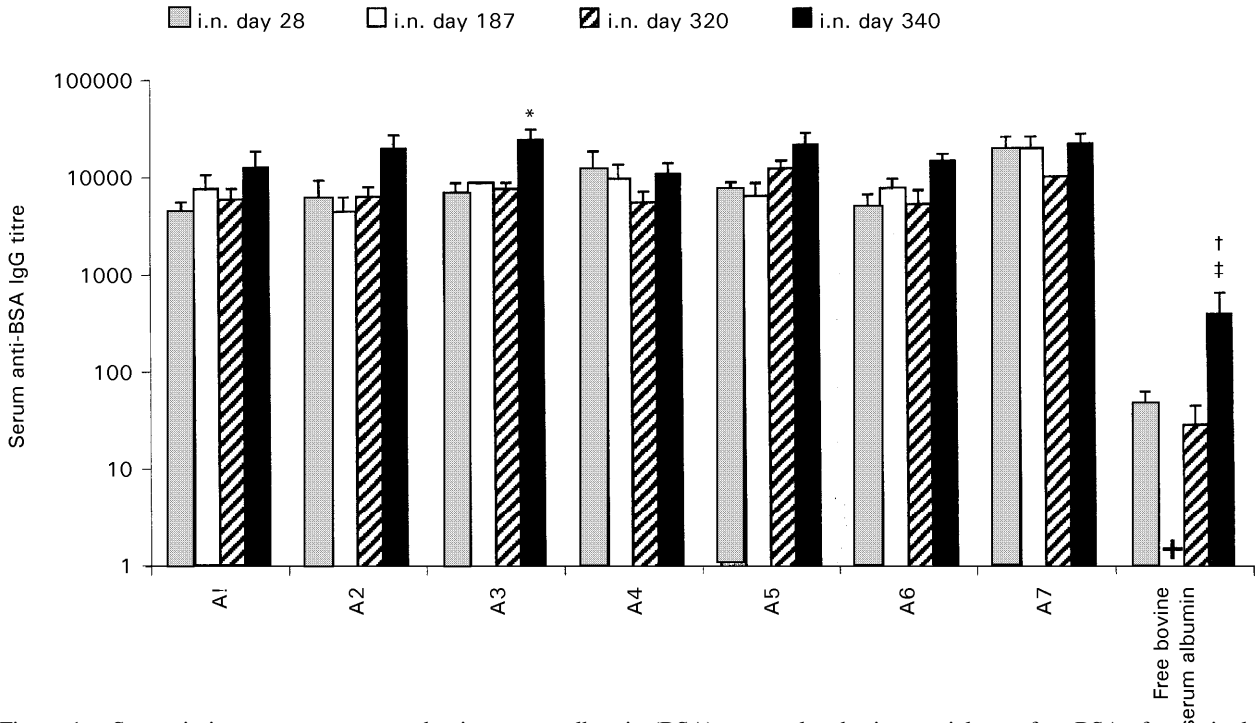

Figure 1. Systemic immune responses to bovine serum albumin (BSA)-encapsulated microparticles or free BSA after a single intranasal dose (15 μg BSA) and a subcutaneous boost (1 μg BSA) on day 326. *$P < 0{\cdot}05$ significantly different compared with day 28, †$P < 0{\cdot}05$ significantly different compared with day 187, ‡$P < 0{\cdot}05$ significantly different compared with day 320. Values are mean ± s.d., n = 5.

Table 3. Mucosal immune responses to bovine serum albumin (BSA)-encapsulated microparticles or free BSA after a single intranasal dose (15 μg BSA) and a subcutaneous boost (1 μg BSA) on day 326.

	Optical density	
	Anti-BSA IgA	Anti-BSA IgG
Free BSA		0·038 ± 0·032
A1	0·136 ± 0·035	0·467 ± 0·039
A2	0·149 ± 0·052	0·369 ± 0·136
A3	0·253 ± 0·104	0·512 ± 0·032
A4	0·311 ± 0·123	0·382 ± 0·123
A5	0·178 ± 0·075	0·467 ± 0·026
A6	0·207 ± 0·126	0·331 ± 0·032
A7	0·479 ± 0·078	0·505 ± 0·071

Values are mean ± s.d., n = 5.

significant difference. After 6 months, none of the particulate groups showed any significant difference in IgG titre. In a separate experiment, A7 was shown to have IgG titres not significantly different to when alhydrogel or Freund's incomplete adjuvant were used with the same dose of BSA after a single intramuscular administration (results not shown). Once optimal priming titres are achieved, it seems that the type of microparticulate protein release has little effect on the immune response elicited by the formulations used in this study. The BAL IgA and IgG responses are shown in Table 4 as optical densities after intramuscular injection. None of the preparations gave significantly greater anti-BSA IgA titres than free BSA in the BAL washings, whereas all preparations except A6 gave anti-BSA IgG titres significantly greater than free BSA. The poor IgA response to parenterally delivered microparticles was expected and highlights the significance of the results obtained by nasal administration.

This study shows that particles which display a continuous protein release in-vitro offer no immunological benefit over particles with plateau-type release for a reasonably stable protein, such as BSA, administered intranasally or intramuscularly. Timing of the booster dose is important as highlighted by a recent study with cytomegalovirus glycoprotein B subunit vaccine (Frey et al 1999). Delivery systems that release protein at later booster times need to be investigated before a definitive statement can be made on the effects of pulsatile release. In this study, pulsatile release did not enhance the immune response. When considering situations that require high immune responses in the shortest period of time, as arise when individuals have to enter high-risk environments with little warning, rushing the immune response seems to be difficult and none of the systems investigated were better than the plateau-release system in inducing a fast response. In fact

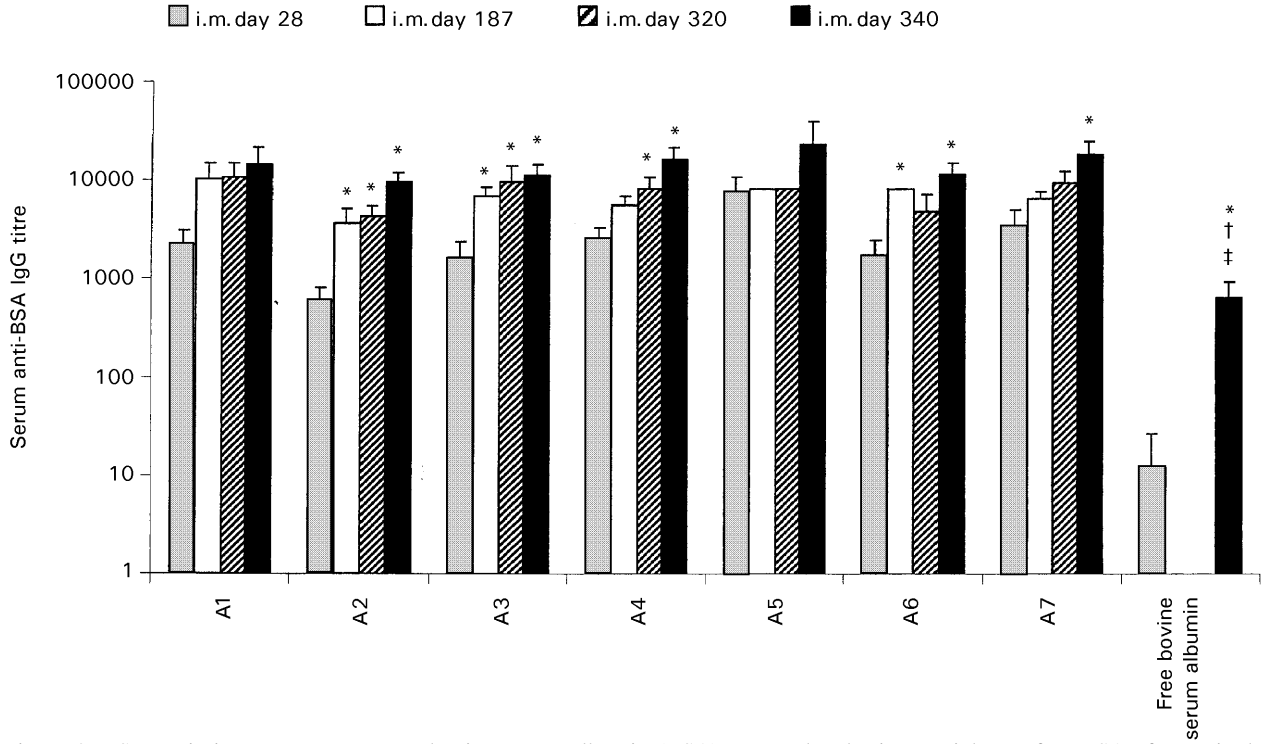

Figure 2. Systemic immune responses to bovine serum albumin (BSA)-encapsulated microparticles or free BSA after a single intramuscular dose (15 μg BSA) and a subcutaneous boost (1 μg BSA) on day 326. *$P < 0.05$ significantly different compared with day 28, †$P < 0.05$ significantly different compared with day 187, ‡$P < 0.05$ significantly different compared with day 320. Values are mean ± s.d., n = 5.

Table 4. Mucosal immune responses to bovine serum albumin (BSA)-encapsulated microparticles or free BSA after a single intramuscular dose (15 μg BSA) and a subcutaneous boost (1 μg BSA) on day 326.

	Optical density	
	Anti-BSA IgA	Anti-BSA IgG
Free BSA	0	0·039 ± 0·032
A1	0·101 ± 0·032	0·499 ± 0·039
A2	0·053 ± 0·045	0·337 ± 0·136
A3	0·001 ± 0·104	0·512 ± 0·032
A4	0·013 ± 0·119	0·382 ± 0·123
A5	0·016 ± 0·104	0·467 ± 0·029
A6	0·001 ± 0·126	0·363 ± 0·032
A7	0·013 ± 0·078	0·473 ± 0·071

Values are mean ± s.d., n = 5.

the plateau-releasing profile formulation (A7) was able to induce high local IgA responses after intranasal delivery, which could be an added bonus especially if the invading bacteria enters mucosally. Picogram quantities of protein have been reported to constitute sufficient stimulus for a secondary response (Khan et al 1994) and because of the difficulty in detecting very small amounts of protein trickling from delivery systems in an in-vivo situation, the plateau-type releasing system is probably achieving the ideal requirement of long lasting immunity.

References

Alpar, H. O., Almeida, A. J. (1994) Identification of some of the physico-chemical characteristics of microspheres which influence the induction of the immune response following mucosal delivery. Eur. J. Pharm. Biopharm. 40: 198–202

Cleland, J. L. (1999) Single-administration vaccines: controlled release technology to mimic repeated immunisations. Trends Biotechnol. 17: 25–29

Conway, B. R., Alpar, H. O. (1996) Double emulsion microencapsulation of proteins as model antigens using polylactide polymers: effect of emulsifier on the microsphere characteristics and release kinectics. Eur. J. Pharm. Biopharm. 1: 42–48

Conway, B. R., Eyles, J. E., Alpar, H. O. (1997) A comparative study on the immune responses to antigens in PLA and PHB microspheres. J. Controlled Release 49: 1–9

Eyles, J. E., Sharp, G. J. E., Williamson, E. D., Spiers, I. D., Alpar, H. O. (1998a) Intra nasal administration of polylactic acid microspheres co-encapsulated *Yersinia pestis* subunits confers protection from pneumonic plague in the mouse. Vaccine 16: 698–707

Eyles, J. E., Spiers, I. D., Williamson, E. D., Alpar, H. O. (1998b) Analysis of local and systemic immunological responses after intra-tracheal, intra-nasal and intra-muscular administration of microsphere co-encapsulated *Yersinia pestis* sub-vaccines. Vaccine 16: 2000–2009

Eyles, J. E., Williamson, E. D., Spiers, I. D., Stagg, A. J., Jones, S. M., Alpar, H. O. (2000) Generation of protective immune responses to plague by mucosal administration of microsphere coencapsulated recombinant subunits. J. Controlled Release 63: 191–200

Frey, S. E., Harrison, C., Pass, R. F., Yang, E., Boken, D., Sekulovich, R. E., Percell, S., Izu, A. E., Hirabayashi, S., Burke, R. L., Duiliège, A.-M. (1999) Effects of antigen dose and immunisation regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine. J. Infect. Dis. 180: 1700–1703

Khan, M. Z. I., Opdebeeck, J. P., Tucker, I. G. (1994) Immunopotentiation and delivery systems for antigens for single-step immunisation: recent trends and progress. Pharm. Res. 11: 2–11

O'Hagan, D. T., Singh, M., Gupta, R. K. (1998) Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. Adv. Drug Deliv. Rev. 32: 225–246

Smith, P. K., Krown, R. I., Hermanson, G. T., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76–85

Spiers, I. D., Alpar, H. O., Eyles, J. E., Bozkir, A., Miller, J., Williamson, E. D. (1999) Studies on the co-encapsulation, release and integrity of two subunit antigens: rV and rF1 from *Yersinia pestis*. J. Pharm. Pharmacol. 51: 991–997