JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 769–774
© 2006 The Authors
Received November 10, 2005
Accepted December 20, 2005
DOI 10.1211/jpp.58.6.0007
ISSN 0022–3573

An investigation into the adjuvanticity and immunogenicity of zein microspheres being researched as drug and vaccine carriers

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Abstract

We have determined whether zein microspheres could act as vaccine adjuvants i.e. increase the immune responses to co-administered immunogens. Ovalbumin (model antigen)-loaded zein microspheres, blank zein microspheres and ovalbumin solution were intramuscularly administered to mice and the sera antibody levels were determined by ELISA. Another group of mice was orally dosed with blank zein microspheres, and serum and faecal antibody levels were determined. As expected, negligible antibody titres were obtained with the ovalbumin solution. Surprisingly, intramuscular administrations of blank zein microspheres elicited high levels of serum IgG which bound to the ovalbumin antigen coated on ELISA microtitre plates. This indicated that anti-zein antibodies had been elicited by blank zein microspheres and that these antibodies were cross-reacting with ovalbumin antigen coated onto ELISA plates. Such cross-reactivity inhibited the determination of the adjuvant activity of zein microspheres, if any. Additional ELISA assays, where zein was used as the coating antigen, confirmed the generation of anti-zein antibodies by blank zein microspheres i.e. zein microspheres were immunogenic following intramuscular administration. Upon oral administration of blank zein microspheres, serum IgG levels remained low but intestinal IgA levels increased following booster doses i.e. systemic tolerance, but not mucosal tolerance, to oral zein particles was achieved. Zein microspheres were immunogenic when administered intramuscularly and orally.

Introduction

Microspheres – defined as monolithic polymeric matrix systems (Couvreur & Puisieux 1993) – have been extensively investigated as sustained/controlled release drug and vaccine carriers. Sustained release of entrapped active entity enables a reduced dosing frequency and enhanced patient compliance. The use of microspheres for vaccine delivery also has the advantage of presenting the vaccine in a particulate form, which facilitates vaccine recognition and uptake by antigen presenting cells, resulting in enhanced immune responses (Storni et al 2005). Consequently, a variety of particulate vaccine delivery systems have been studied (Bramwell & Perrie 2005). Polylactide-co-glycolide (PLGA) has been the polymer of choice for the formulation of microspheres as it is already used in biomedical devices in man. However, PLGA particles do suffer from certain disadvantages, such as instability of encapsulated proteins (Putney & Burke 1998; Schwenderman 2002; Jiang et al 2005), and a large number of alternative polymers, synthetic and natural, have been investigated.

One such polymer is zein, a naturally occurring hydrophobic plant polymer, which is biodegradable, has GRAS (Generally Regarded as Safe) status (Anon 1985) and has been used extensively in the pharmaceutical, food, agricultural and other industries. Zein – the prolamin of maize (*Zea mays* L.) found in maize germ and endosperm – consists of a mixture of peptides, which may be divided into several classes. Each class differs in solubility, molecular weight, amino acid composition, immunological properties etc. The classification of the different classes of zein protein has led to confusion and many nomenclatures have been proposed (Esen 1986, 1987, 1990; Landry & Guyon 1984a, b; Wilson 1985). In this study, the nomenclature proposed by Esen (1986, 1987, 1990) has been used. According to this nomenclature, zein is composed of four classes of protein (α, β, δ and γ). α-Zein is the most abundant, consists of two polypeptides of estimated MW 22

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Acknowledgements: The authors thank Satyanarayana Somavarapu and Steve Coppard for their help during the conduct of the animal work, and David McCarthy for doing the scanning electron microscopy.

and 24 kDa, and its amino acid composition is similar to that of whole zein (Paulis et al 1969; Paulis & Wall 1977; Esen et al 1981). β-Zein, a methionine-rich polymer of 17 kDa, and δ-zein, a minor fraction of MW 10 kDa, have similar solubility profiles to α-zein. γ-Zein is composed of two components: one with MW 27 kDa and the other with MW 18 kDa. It is believed, however, that the 18 kDa polypeptide is a truncated version of the 27 kDa peptide. Zein microspheres have been formulated as carriers of anti-cancer agents (Matsuda et al 1989; Suzuki et al 1989; Mathiowitz et al 1993), the parasiticide ivermectin (Liu et al 2005), the pesticide abamectin (Demchak & Dybas 1997) and essential oils (Parris et al 2005). Films composed of zein microspheres have been investigated as a biomaterial in tissue engineering and have shown good biocompatibility (Dong et al 2004). The drug release from the microspheres was reported to be low, and indicated the possibility of sustained-release of antigens entrapped within zein microspheres.

We have investigated zein microspheres as vaccine carriers/adjuvants, using ovalbumin as a model antigen. The need for research into vaccine adjuvants remains despite much work in this field, due to the poor immunogenicity of newer and purer subunit vaccines, the actual and possible future toxicity of many adjuvants currently in research, and the fact that only a handful of adjuvants are currently used in commercial vaccines. The formulation and characterization of zein microspheres, loaded with ovalbumin, have been reported (Hurtado-López & Murdan 2005, 2006). The spherical particles were smooth with a non-porous surface, and had a diameter of approximately 600 nm, and the loaded ovalbumin was released slowly when incubated in phosphate-buffered saline.

The aim of this study was to determine the adjuvanticity of zein microspheres. Vaccine carriers should only increase the immune responses to the desired antigen, not to non-vaccine proteins, such as the host, food or to itself. Therefore, the generation of immune responses to blank zein microspheres following intramuscular and oral administrations were investigated.

Materials and Methods

Animals

Female BALB/c mice, obtained from B&K Ltd, UK, were maintained on a normal mouse diet, which contained maize germ (a source of zein). Water was freely available. The School's Ethical Review Committee approved all procedures, which were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The experiments were started when the mice were eight-weeks old and weighed approximately 20 g.

Materials

Ovalbumin (Grade II), zein, bovine serum albumin (BSA), povidone MW 360 000 (polyvinylpyrrolidone; PVP 360), phosphate-buffered saline (PBS) tablets, Tween 20, potassium chloride, anti-mouse IgA (a-chain specific) peroxidase conjugate, anti-mouse IgG (whole molecule) peroxidase conjugate, hydrogen peroxide (30%, v/v, aqueous solution), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) tablets, heparin and sodium lauryl sulfate (sodium dodecyl sulfate) were obtained from Sigma, UK. Dibasic sodium phosphate (disodium hydrogen orthophosphate), citric acid, potassium hydrogen orthophosphate and sodium chloride were purchased from BDH Laboratory Supplies, UK. Other chemicals were of reagent grade and were used as received. ELISA 96-well microtitre plates were purchased from Dynatech, UK. Double distilled water was used throughout.

Preparation of blank and ovalbumin-loaded zein microspheres

Zein microspheres were prepared by a coacervation method based on the solubility properties of zein, as described by Hurtado-Lopez & Murdan (2005). Briefly, to prepare blank (i.e. without ovalbumin) zein microspheres, zein (62.5 mg) was dispersed in 10 mL 100% ethanol with gentle stirring. This produced a coarse dispersion. Tween 20 (5 mL; 2.5%, v/v) and povidone (5 mL; PVP 40, 4%, w/v) aqueous solutions were added to the ethanolic dispersion. Consequently, a fine aqueous–alcoholic zein suspension was formed. Ethanol was removed by rotary evaporation at 90 mbar and 40°C for 10–15 min and a fine aqueous suspension of zein microspheres was produced. The suspension was stored until needed. The average diameter of the blank microspheres was found to be 1356 ± 36.4 nm, with a polydispersity of 0.662 ± 0.218 (Hurtado-Lopez & Murdan 2005).

Ovalbumin-loaded zein microspheres were produced as above, except for the addition of ovalbumin (50 mg) with zein (62.5 mg) into 10 mL 100% ethanol (which gave rise to a coarse dispersion upon stirring), and the addition of 0.3 mL 1% w/v aqueous sodium hydroxide solution. The latter was added as it was found to prevent the aggregation of ovalbumin and zein that otherwise occurred upon the addition of the two proteins into 100% ethanol. Following the addition of Tween 20 and povidone solutions and the removal of ethanol, ovalbumin-loaded zein microspheres were produced. The average particle diameter was 607.47 ± 48.3 nm, with a polydispersity of 0.386 ± 0.166. The experimental loading of ovalbumin in the microspheres was 23.90%; the loaded ovalbumin was expected to be entrapped within the microsphere matrix. The unloaded ovalbumin was present either as non-uniform aggregates or dissolved in the aqueous medium. The unloaded ovalbumin was not removed from the microsphere suspension before administration to experimental animals; thus the mice received some free and some entrapped ovalbumin.

Intramuscular immunization

Mice were divided into groups of five animals each. To determine the adjuvanticity of zein microspheres, the mice

were intramuscularly dosed with either ovalbumin-loaded zein microsphere suspension (150 μg ovalbumin in 30 μL suspension), ovalbumin dissolved in saline (150 μg ovalbumin in 30 μL solution), or blank zein microsphere suspension (30 μL).

The microsphere suspensions were vortexed immediately before injection and a single injection in the quadriceps was used to administer the dose. Booster intramuscular administrations were given twelve weeks after the primary immunization, in an identical way. To determine immune responses, the animals were bled from the tail vein, seven weeks after the first dose and again, one, four and seven weeks after the booster dose. The blood samples were allowed to clot overnight in a refrigerator, after which they were centrifuged at 21 000 rev min^{-1} for 10 min in a table-top centrifuge. Sera were collected and stored at -70°C until assayed.

Oral immunization

To determine whether oral administration of zein microspheres gave rise to immune responses against zein, mice were orally dosed with 100 μL blank zein microsphere suspension, containing 625 μg zein protein, on three consecutive days following overnight fasting. Identical booster doses were administered on days 29, 30 and 31 of the study. The animals were bled four weeks after priming and again four weeks after boosting, and serum samples were obtained as described above. At the same time intervals, fresh faecal samples were collected from individual mice placed in metabolic cages. The faeces were added to PBS pH 7.4, the mixtures were homogenized, centrifuged at 21 000 rev min^{-1} for 15 min in a table-top centrifuge, and the supernatants were collected and stored at -70°C until assayed. A control group of non-immunized animals was used to obtain control faecal and blood samples.

Determination of antibody levels by ELISA

Serum and faecal samples were analysed for anti-ovalbumin and anti-zein IgG and IgA antibodies, respectively, by a standardized ELISA method. Microtitre ELISA plates were coated with 100 μL antigen solution per well. This was either ovalbumin 1% w/v in PBS pH 7.4 or zein 1% w/v in 60% v/v ethanol. Zein, a water-insoluble protein, was dissolved in 60% ethanol as the adsorption of zein to polystyrene microtitre plates when dissolved in aqueous alcohols has been demonstrated, as has the subsequent quantitative measurement of the protein following a regular ELISA assay (Conroy & Esen 1984; Chirdo et al 1995).

Following overnight incubation at 4°C, the plates were washed three times using PBS–Tween 20 (0.05% Tween 20), then once again with double distilled water. The plates were blocked with 100 μL aqueous blocking solution (povidone 1% w/v when ovalbumin was the antigen or bovine serum albumin 1% w/v when zein was the antigen) per well at 37°C for 1 h. Plates were washed as described. Serum or faecal samples (100 μL/well, diluted 16 times) were placed in the top row of ELISA plates, serial double dilutions were conducted in the other rows and the plates were incubated for 1 h at 37°C. Following washing, 100 μL anti-mouse IgA or IgG peroxidase conjugate (diluted 1:1 000 in PBS pH 7.4) per well was added to the ELISA plates, which were incubated at 37°C for 1 h. Plates were washed, freshly prepared ABTS/hydrogen peroxide solution was added (50 μL/well) and the plates were incubated at 37°C for 30 min. The reaction was stopped by the addition of 1% w/v sodium lauryl aqueous solution (50 μL/well) and the plates were read at 405 nm in an ELISA reader (Opsys MR, Dynex Technologies). To compare the levels of antibodies generated by the different formulations, the optical density readings obtained for the serum that had been diluted 16 times was used.

Statistical analyses

One-way analysis of variance, followed by post-hoc Tukey HSD tests were used to compare the anti-ovalbumin IgG titres generated by intramuscularly administered ovalbumin solution, and ovalbumin-loaded and blank zein microspheres. Kruskal–Wallis, followed by Nemenyi tests were conducted to compare the primary and secondary anti-zein IgG titres generated by intramuscularly administered ovalbumin-loaded and blank zein microspheres. Mann Whitney U tests were conducted to compare the primary and secondary immune responses to orally administered blank zein microspheres. Results were considered statistically significant when $P < 0.05$.

Results and Discussion

The zein microspheres (blank and ovalbumin-loaded) were prepared by a simple coacervation method. Spherical zein particles with a smooth surface were produced, as observed by scanning electron microscopy (Figure 1). The average diameter of the blank microspheres was found to be 1356 ± 36.4 nm, with a polydispersity of 0.662 ± 0.218, while that of ovalbumin-loaded particles was 607.47 ± 48.3 nm,

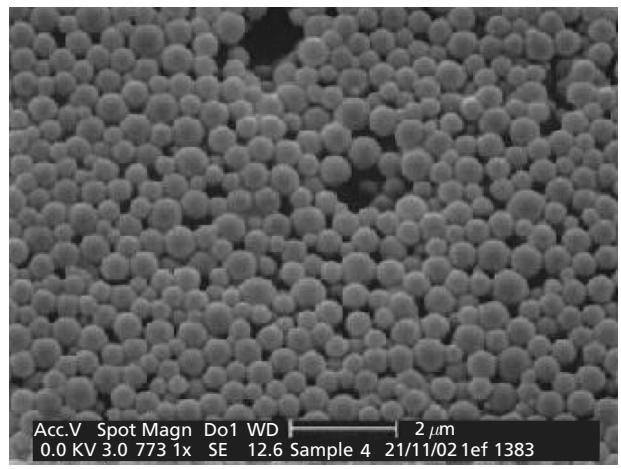

Figure 1 Scanning electron micrograph of ovalbumin-loaded zein microspheres.

with a polydispersity of 0.386 ± 0.166. The experimental loading of ovalbumin in the microspheres was 23.90% (Hurtado-Lopez & Murdan 2005).

Immune responses to intramuscularly administered formulations

To determine whether zein microspheres enhanced the immunogenicity of ovalbumin, thereby acting as an adjuvant, the serum anti-ovalbumin IgG levels generated following administration of 'free' and 'microsphere-loaded' ovalbumin were measured. Blank zein microspheres were used as controls. The primary and secondary (at weeks one, four and seven after the booster dose) IgG titres of the three preparations are shown in Table 1. To determine any statistical difference between the titres of the three preparations, one-way analysis of variance was conducted for each time-point. From Table 1 it can be seen that, as expected, administration of free, non-encapsulated ovalbumin gave rise to very low levels of primary and secondary antibodies, ovalbumin being poorly immunogenic in the free form. Booster doses of the free antigen significantly increased the levels of anti-ovalbumin IgG; these levels however decreased with time. At all time points, the antibody titres generated by the solution were significantly lower than those of the microsphere formulations. The administration of both blank and ovalbumin-loaded zein microspheres elicited a higher IgG response, especially following the booster administrations. Serum IgG levels remained high for several weeks following the booster administration. Interestingly, there was an IgG response to blank zein microspheres. The primary IgG levels were higher ($P < 0.05$) than those elicited by the ovalbumin-loaded zein microspheres, while the secondary IgG levels (at all time points) were not significantly different to those elicited by ovalbumin-loaded zein microspheres ($P > 0.05$).

The high antibody responses generated by blank zein microspheres indicated that anti-zein antibodies had been generated and that these antibodies were binding (non-specifically) to ovalbumin antigen coated onto the ELISA microtitre plate wells. The non-specific binding of anti-zein antibodies to ovalbumin antigen coated on ELISA microtitre plate walls meant that any possible increase in anti-ovalbumin IgG titres, due to microencapsulation of the antigen into zein particles, could not be detected. Thus, from this study, the adjuvant activity of zein particles could not be ascertained.

Generation of antibodies by blank zein microspheres was surprising, given that the experimental mice were fed a standard mouse diet which included zein. It is well known that feeding of dietary proteins predominantly induces tolerance i.e. feeding does not result in immune responses to the proteins; in addition when the same protein is subsequently administered via an immunogenic route, such as the intramuscular one, injurious local or systemic immune responses are not elicited (Mowat & Weiner 1999). To investigate further the immune responses generated to zein particles, additional ELISA assays, where the microtitre plate wells were coated with zein antigen, were conducted to measure the primary and secondary anti-zein IgG levels elicited by blank and ovalbumin-loaded zein microspheres. The serum IgG levels are shown in Table 2. To determine any significant difference between the primary and the secondary titres of the two formulations, Kruskal–Wallis followed by Nemenyi's tests were conducted. It was found that the anti-zein IgG titres generated by blank and by ovalbumin-loaded zein microspheres were not significantly different from each other, for both primary and secondary response ($P > 0.05$). In addition, for both formulations, the anti-zein IgG titres increased significantly following the booster dose ($P < 0.05$).

The results confirmed that feeding of dietary zein did not induce tolerance to zein particles when the latter were administered intramuscularly. This could be due to the fact that zein was administered in the particulate form, which is known to be more immunogenic than the soluble state. The generation of antibodies by zein particles showed that zein, when administered intramuscularly in a particulate form, was immunogenic and raised concern over the use of these particles as drug and vaccine delivery systems. Drug carriers should be non-immunogenic to avoid concern about their biocompatibility (Hillery 2001). For the same reason, vaccine delivery systems (some of which, such as microspheres, also act as adjuvants) should be non-immunogenic.

Table 1 The primary and secondary anti-ovalbumin IgG titres generated following the intramuscular administration of ovalbumin solution, ovalbumin-loaded zein microspheres and blank zein microspheres in mice

Formulation	Anti-ovalbumin IgG titre			
	Primary response	Secondary response (week(s) after booster dose)		
		1 week	4 weeks	7 weeks
Ovalbumin solution	0.11 ± 0.01	0.89 ± 0.09	0.38 ± 0.04	0.34 ± 0.13
Ovalbumin-loaded zein microspheres	0.15 ± 0.03	1.74 ± 0.32	1.36 ± 0.37	1.36 ± 0.25
Blank zein microspheres	0.21 ± 0.03	1.59 ± 0.21	1.64 ± 0.34	1.11 ± 0.09

Mean $\pm$ s.d. are shown, $n = 5$.

Table 2 The primary and secondary anti-zein IgG titres generated following the intramuscular administration of blank and of ovalbumin-loaded zein microspheres in mice

Formulation	Anti-zein antibody titre	
	Primary response	Secondary response
Ovalbumin-loaded zein microspheres	0.25 ± 0.03	0.91 ± 0.08
Blank zein microspheres	0.24 ± 0.03	0.82 ± 0.13

Mean $\pm$ s.d. are shown, $n = 5$.

Immune responses to orally administered zein microspheres

Oral tolerance (partial or complete immunological non-responsiveness to orally administered antigen) to zein particles would allow their use as oral drug/vaccine delivery vehicles. To determine the possibility of oral tolerance to zein microspheres, a group of five mice was orally dosed with blank zein microspheres and the immune responses (serum IgG and intestinal mucosal IgA to zein antigen) were monitored. Any significant difference between the primary and secondary response was determined using Mann Whitney U tests.

The primary and secondary serum IgG titres were low and similar to those in naïve mice. The serum IgG titres could therefore be assigned to the presence of zein in the mouse feed, rather than to the zein microspheres. Anti-zein IgG titres did not increase following the booster dose ($P > 0.05$) i.e. orally administered zein particles did not induce a systemic immune response, and instead systemic tolerance to oral zein particles was induced.

In contrast, the intestinal mucosal IgA response did not show the induction of tolerance. The primary IgA levels were low and similar to the levels in naïve mice. However, following the booster dose, IgA levels increased significantly ($P < 0.05$; titre doubled from 0.32 ± 0.001 to 0.62 ± 0.05). The generation of IgA antibodies to zein particles was expected to limit the application of the latter as oral drug/vaccine carriers, as the secretory IgA molecules will bind to zein particles, and thereby prevent the latter's adherence to the mucosal surface and subsequent uptake by Peyer's patches.

Our results – the absence of a strong serum IgG response concomitantly with the presence of a significant IgA response – reflected reports by other researchers (Challacombe & Tomasi 1980; McGhee et al 1999) of systemic unresponsiveness, concomitant with mucosal responsiveness to oral antigens. IgA responses seemed to be more resistant to the induction of oral tolerance (McGhee et al 1999).

Conclusions

We have investigated the potential of zein microspheres as a vaccine delivery system using ovalbumin as a model antigen. The adjuvanticity of zein microspheres could not be ascertained; however, their immunogenicity was demonstrated. Following the intramuscular administration of blank and ovalbumin-loaded zein microspheres, a significant IgG response to zein was elicited. Following oral administration of blank zein microspheres, a significant anti-zein IgG response was not observed, but the intestinal anti-zein IgA titres was found to double i.e. oral administration induced systemic, but not mucosal, tolerance to zein particles. Thus, this study showed the immunogenicity of zein particles, and raised questions about their potential application as drug and vaccine delivery vehicles.

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