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Biodegradable microparticles as controlled release antigen delivery systems

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SUMMARY

A model but poor immunogen, ovalbumin (OVA), was entrapped in a novel antigen delivery system comprising poly (D,L-lactide-co-glycolide) (PLGA) microparticles. Both the primary and the secondary IgG antibody responses obtained with OVA in microparticles were compared to those obtained with OVA emulsified in Freunds' adjuvants by two routes of immunization, intraperitoneal (i.p.) and subcutaneous (s.c.) injection. Following single i.p. or s.c. injections, the IgG serum antibody responses to OVA in microparticles were significantly greater than the responses to OVA in Freunds' complete adjuvant (FCA) for up to 10 weeks. After s.c. booster doses of OVA, the secondary IgG antibody responses to OVA in microparticles remained greater than the secondary responses to OVA in Freunds', but not significantly so. Furthermore, the primary IgG responses to OVA in microparticles obtained 8–12 weeks after a single i.p. injection were greater than the secondary responses to OVA in Freunds' obtained by repeat s.c. injections at Weeks 0 and 6. These results demonstrate that microparticles can function as potent antigen delivery systems for an entrapped antigen. Due to their ability to degrade slowly *in vivo* and to release entrapped antigens, microparticles have considerable potential as controlled release antigen delivery systems for the induction of long-term immune responses.

INTRODUCTION

Classical vaccines generally consist of either whole inactivated or live attenuated micro-organisms, which are often sufficiently immunogenic to induce potent immune responses without the addition of an adjuvant. However, advances in biotechnology and chemical synthesis have resulted in new approaches to vaccine development involving the synthesis of protein or peptide sequences which are homologous with epitopes capable of inducing protective immunity against infectious organisms. This newer approach to vaccine development can be termed the 'subunit' approach. Subunit vaccines prepared by chemical synthesis or biotechnology have several advantages over more traditional vaccines; they are chemically well defined, can be prepared reproducibly and assayed readily and are usually inexpensive to manufacture. However, a general drawback is poor immunogenicity, resulting in the need for repeated immunizations.

Abbreviations: BSA, bovine serum albumin; FCA, Freund's complete adjuvant; FIA, Freunds' incomplete adjuvant; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; OVA, ovalbumin; PBS, phosphate-buffered saline; PLGA, poly (D,L-lactide-co-glycolide); SAL, physiological saline; s.c., subcutaneous; 120, Tween 20.

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One approach to overcome these problems involves the use of controlled release antigen delivery systems.[1] We have adopted this approach in the preliminary studies described here. We entrapped a model antigen ovalbumin (OVA) in microparticles prepared from a biodegradable polymer, poly (D,L-lactide-co-glycolide) (PLGA). The biocompatibility of microparticles prepared from PLGA has been demonstrated by Visscher *et al.*[2] These polymers undergo biodegradation by random, non-enzymatic scission to form the endogenous metabolites lactic and glycolic acids. As a result of their biodegradability and excellent tissue compatibility profiles, PLGA polymers have been used for many years as surgical sutures.[3] In addition, PLGA polymers have also been used to entrap many drugs in the preparation of various controlled release drug delivery systems, an example of which (Zoladex™; I.C.I.) has now been licensed for use in man both in Europe and the U.S.A.[4]

PLGA microparticles release entrapped drugs in a complex manner by one or more mechanisms as a function of time, but the release of macromolecules is mainly controlled by bulk degradation of the polymer.[5] Consequently, depending on polymer composition and molecular weight, PLGA microparticles can be prepared that release antigens over a period of days to more than 1 year. Furthermore, mixed populations of PLGA microparticles prepared from different polymeric compositions and molecular weights can be engineered to release 'pulses' of antigens at pre-determined intervals following a single immunization. Hence, advances in pharmaceutical technology may

allow the development of safe, single-dose vaccines against a number of infectious diseases and help to overcome the major disadvantage of subunit vaccines, i.e. the need for booster injections. It is clear that controlled release vaccines would be particularly advantageous in the Third World, where repeated contact with the vaccinee is often difficult to achieve.[1]

We describe here some preliminary observations concerning the use of PLGA microparticles as parenteral antigen delivery systems for immunization with a model antigen, OVA, in a small animal model, the mouse.

MATERIALS AND METHODS

Animals

Male BALB/c mice (Olac Ltd, Cirencester, U.K.), aged up to 6–8 weeks and weighing about 25 g, were used and maintained on a normal mouse diet throughout the study.

Microparticle preparation

Microparticles with entrapped OVA (Grade V; Sigma, Poole, Dorset, U.K.) were prepared using PLGA obtained from Boehringer Ingelheim KG (Resomer RG503; Ingelheim, Germany). The microparticles were prepared by solvent evaporation from an oil-in-water emulsion as previously reported by Beck *et al.*[6] and adapted by Jeffery *et al.* (unpublished results) for entrapment of macromolecules. Briefly, the antigen was suspended in a volatile organic solvent in which the polymer was also dissolved, this formed the dispersed phase of the emulsion. The disperse phase was then emulsified into an aqueous continuous phase in the presence of polyvinyl alcohol (PVA; 88% hydrolysed; Aldrich Chemical Company, Poole, Dorset, U.K.) using a Silverson mixer (Silverson Machines Limited, Chesham, Bucks, U.K.). The microparticles were then formed by the controlled evaporation of the volatile solvent. Full details of the microparticle preparation method will be published elsewhere, along with details of the extensive microparticle characterization that was undertaken. Following preparation, the microparticles were collected by centrifugation, washed three times in 0·1% PVA to remove non-entrapped OVA and freeze-dried. The protein content of the microparticles was determined in a Bicinchoninic acid protein assay (Sigma), after dissolution of an aliquot of the microparticles in dichloromethane (HPLC Grade; Aldrich). The microparticles contained an average of 1% w/w OVA. The volume mean diameter of the microparticles, as measured by laser diffractometry, was 5·34 μm (Malvern laser sizer 2600D).

Immunization protocols

Immediately before administration, the required dose of freeze-dried microparticles (~10 mg PLGA microparticles per mouse containing 100 μg OVA) was weighed and resuspended in the appropriate volume of physiological saline (SAL).

Primary immunization. Two groups of 10 mice were each immunized i.p. with a single dose of 100 μg OVA either entrapped in PLGA microparticles or emulsified in FCA (Sigma).

An additional group of 10 mice was immunized i.p. with 100 μg of OVA in SAL on two occasions. This group of animals received the primary immunization at the same time as the two groups above, but also received a booster i.p. immunization of 100 μg OVA in SAL 6 weeks after the primary immunization.

Repeat immunization. Three groups of 10 mice were each immunized s.c. with 100 μg OVA either entrapped in PLGA microparticles, emulsified in FCA or dissolved in SAL. Booster s.c. immunizations of OVA in identical vehicles were administered to each study group 6 weeks after the primary immunization, except the FCA group, which received a booster s.c. immunization of 100 μg OVA in Freunds' incomplete adjuvant (FIA).

In each study, sera samples were collected from the tail veins of the mice at 2-week intervals for 12 weeks following primary immunization.

Measurement of IgG by ELISA

The specific anti-OVA IgG antibody content of each serum sample was determined in an established ELISA and standardized against a positive control antiserum obtained by hyper-immunization of mice with OVA in FCA. The hyper-immunization schedule involved primary and secondary i.p. immunization, separated by 1 week, of OVA (100 μg in FCA) and the serum sample was taken from the tail vein 1 week after the second immunization. The ELISA was performed as follows: microtitre plates (Dynatech M 129B; Billingshurst, Sussex, U.K.) were coated overnight with 100 μl per well of OVA 4 μg/ml, they were washed three times in phosphate-buffered saline (PBS) and blocked for 2 hr at 37° with 0·5% bovine serum albumin (BSA) and 0·05% Tween 20 (T20) in PBS 200 μl per well. Serum samples (100 μl) at four separate dilutions from 1/200 in BSA/T20/PBS were added to the wells and incubated overnight at 4°. The plates were washed three times in PBS and 100 μl sheep anti-mouse IgG (Evai-Bio, Betworth, West Sussex, U.K.) diluted 1/2,000 in BSA/T20/PBS were added to the wells and incubated at 37° for 2 hr. The plates were washed three times in PBS and 100 μl anti-sheep IgG alkaline phosphatase conjugate (Sigma) were added to the wells at 1/300 in BSA/T20/PBS and incubated at 37° for 1 hr. The plates were washed with PBS and 100 μl of p-nitrophenyl phosphate (1 tablet in 5 ml of diethanolamine buffer; Sigma) were added to each well. The reaction was stopped after 20 min by the addition of 50 μl 3M NaOH per well and the plates were read at 405nm in an ELISA reader (Biorad, Hemel Hempstead, Herts, U.K.). The results are expressed as antibody units calculated from the standard curve obtained from the hyperimmune mouse serum diluted between 1/2,000 and 1/64,000, the value for each serum sample dilution falling in the standard curve and the value for the sample taken as the mean of the four separate dilutions of that sample.

Statistical analysis

The results are expressed as mean ± SE for 10 mice. An unpaired Student's *t*-test was used to compare the means for each study group at the different sample times and to assess statistical significance.

RESULTS

Primary immunization

The serum IgG antibody responses to OVA encapsulated in PLGA microparticles were significantly greater than those for equivalent amounts of OVA emulsified in FCA for 10 weeks following single i.p. injections. At 12 weeks the IgG responses remained greater, but not significantly so. The IgG antibody

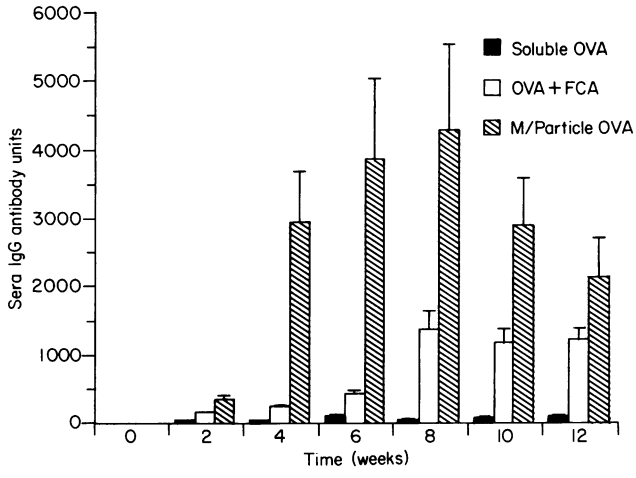

Figure 1. The sera primary IgG antibody responses to intraperitoneal injections of 100 μg ovalbumin administered to two groups of animals encapsulated in PLGA (M/Particle OVA) microparticles or emulsified in FCA (OVA+FCA) and the sera IgG response to 100 μg OVA dissolved in physiological saline (Soluble OVA) injected into a separate group at the same time, but boosted at six weeks. Each column represents the mean response ± SE of 10 animals.

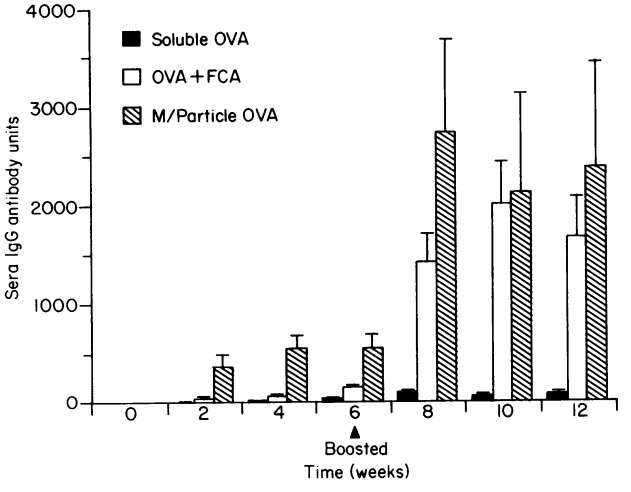

Figure 2. The sera IgG antibody responses to primary and secondary subcutaneous injections of 100 μg of ovalbumin injected into three groups of animals either entrapped in PLGA microparticles (M/Particle OVA), emulsified in FCA (OVA + FCA) or dissolved in physiological saline (Soluble OVA). The soluble antigen group and the microparticle group were boosted after 6 weeks with 100 μg OVA in the same vehicles as the primary injection, while the FCA group was boosted with OVA ins FIA. Each column represents the mean response ± SE of 10 animals.

responses to single i.p. injections of OVA in PLGA microparticles were significantly greater than both the primary and secondary responses to OVA in SAL for the full 12-week duration of the study (Fig. 1).

Similarly, significantly greater serum IgG antibody responses to OVA in PLGA microparticles, compared to OVA in SAL or FCA, were induced for 6 weeks after single s.c. immunizations (Fig. 2).

Repeat immunization

Following booster injections for each group of animals previously immunized s.c., the IgG responses to OVA in PLGA microparticles remained greater than the responses to OVA in Freunds', but the differences were not significant. However, the responses to OVA in PLGA were significantly greater than the responses to OVA in SAL for the full 12 weeks of the study (Fig. 2).

DISCUSSION

In accordance with recommendations for the testing of new adjuvants,[7] we have assessed both the primary and the secondary immune responses to OVA entrapped in our novel antigen delivery system, PLGA microparticles. The results obtained were compared with the responses obtained with the same antigen in Freunds' adjuvants. The results demonstrate that the entrapment of the soluble protein antigen OVA, normally a poor immunogen, in PLGA microparticles results in the induction of potent serum IgG antibody responses following parenteral immunization by two different routes. Throughout both studies, the IgG responses induced by OVA in PLGA microparticles were significantly greater than or comparable to those induced with Freunds', the most potent adjuvant available for small animal models.[7] Furthermore, while FCA is unsuitable for human or veterinary use due to toxicity, and its use in experimental animals is being discouraged for similar reasons, PLGA polymers have been safely used in man as drug delivery systems for several years.[5]

It was particularly encouraging that encapsulation of OVA in PLGA microparticles resulted in the induction of potent IgG antibody responses following a single injection, while FCA was unable to induce comparable responses. Furthermore, on comparing Figs 1 and 2, it should be noted that a single i.p. injection of OVA in PLGA microparticles gave a greater IgG antibody response at Weeks 8–12 post-immunization (Fig. 1), than was obtained following a booster s.c. injection of OVA in Freunds' at 6 weeks (Fig. 2). In addition, although these results confirm that Freunds' is a potent adjuvant system for OVA (Fig. 2), much greater immune responses were obtained from OVA in PLGA microparticles. This was a surprising finding, since with the microencapsulated OVA we had merely hoped to induce antibody responses that were significantly greater than those induced by soluble OVA. The level of antibody response actually induced by microparticles was considerably greater than expectations. Hence, these results indicate that PLGA microparticles can function as potent antigen delivery systems for induction of a primary immune response to entrapped antigens and additional adjuvants may not be required in the formulation of effective vaccines. Considering the poor record of adjuvant approval by the regulatory authorities, aluminium hydroxide remains the only adjuvant approved by the Food and Drugs Administration of the United States for administration to humans,[8] this is an encouraging finding.

An important issue not addressed in this paper is the effect of the formulation processing on the entrapped antigen, particularly the exposure to organic solvents. While many antigens may prove to be relatively 'robust' and not adversely affected by incorporation into microparticles, some antigens may prove more labile and may require more careful processing. Studies recently undertaken at Nottingham have indicated that micro-

particles can be prepared by solvent evaporation from a wide range of volatile solvents and solvent combinations (unpublished observations). The effect of exposure to organic solvents is likely to be both antigen and solvent dependent, and a range of solvents and combinations may need to be assessed for their suitability for each individual antigen. It should be noted that some of the currently available 'split' influenza vaccines are obtained after exposure of the virus to the organic solvent, ether. Therefore, depending on the antigen to be entrapped, exposure to organic solvents is not necessarily a deleterious step during vaccine development.

The use of polylactide pellets (1·0 cm diameter) as controlled release parenteral vaccines was discussed by Marcotte & Goosen,[9] but only *in vitro* release data on albumin as a model macromolecule was reported. Hora *et al.*[10] have recently shown that human serum albumin can be incorporated into PLGA microparticles of a similar composition to those used here and released intact *in vitro*. Isoelectric focusing and polyacrylamide gel electrophoresis data suggested that the model protein was not affected by encapsulation and was released in its pure form.[10] PLGA microparticles have previously been used as oral antigen delivery systems in mice for staphylococcal enterotoxin B.[11] Oral immunization with microspheres induced a disseminated mucosal IgA anti-toxin antibody response. The use of an alternative microparticle formulation as an oral antigen delivery system for OVA in rats has previously been reported by some of us.[12]

Extensive research experience with a range of microparticulate drug carrier systems indicates that if they are of an appropriate size (<6–7 μM), then they are efficiently phagocytosed by various macrophage populations both *in vivo*[13] and *in vitro*.[14] It seems likely that phagocytosis by macrophages probably accounts for the ability of PLGA microparticles to function as potent antigen delivery systems, since macrophages process and present antigens together with class II molecules to the T-helper cells to induce T-cell dependent immune responses.[15] The PLGA microparticles used in this study were manufactured within a desired size range (89% $<8·2$ μm and 41% $<5·0$ μm) which is appropriate for phagocytosis by antigen-presenting cells, e.g. macrophages. Phagocytosis of PLGA microparticles with entrapped OVA probably resulted in the delivery of relatively large amounts of undegraded antigen intracellularly to the cells responsible for immune response initiation and presumably resulted in more effective antigen presentation.

Nevertheless, the controlled release of OVA from PLGA microparticles may also have contributed to the potent immune responses observed. The PLGA polymer used in this study (molecular weight approximately 9000, 50:50 ratio of lactide/glycolide) was chosen to degrade relatively rapidly and to release the entrapped antigen over weeks rather than months. Alternative polymers with slower degradation kinetics could also be used for microparticle preparation which should result in the induction of potent long-term immune responses. Hence, the preliminary results obtained here are very encouraging and indicate that PLGA microparticles are effective antigen delivery systems that can induce potent primary and secondary immune responses. The exciting potential of these controlled release delivery systems to induce potent long-term immune responses to clinically relevant antigens remains to be assessed. In conclusion, PLGA microparticles are extremely flexible delivery systems capable of encapsulating a wide range of materials, formulations with various entrapped antigens prepared by several alternative techniques are currently being assessed as experimental vaccines by this group.

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