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# Biodegradable microparticles as a delivery system for the allergens of *Dermatophagoides pteronyssinus* (house dust mite): I. Preparation and characterization of microparticles

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**Abstract**

The encapsulation of allergens into biodegradable microparticles may offer the potential for novel forms of hyposensitization therapy. The use of microparticles for hyposensitization therapy may offer the potential advantages of a reduced number of doses, through controlled release of allergens, and the possibility of oral delivery. Nevertheless, a possible concern is the effect of the microencapsulation process on the integrity and activity of the entrapped allergens. Therefore, in the current studies, an allergen, *Dermatophagoides pteronyssinus* (D.Pt.), was entrapped in poly(lactide-co-glycolide) (PLG) microparticles and several established techniques were used to investigate the integrity of the entrapped allergen. Isoelectric focusing indicated that all the protein components of the allergen were successfully entrapped in the microparticles. A radioallergosorbent test (RAST) inhibition assay indicated that there was a minor reduction in the binding of specific IgE to the entrapped allergen, but this was thought unlikely to affect the ability of the allergen to act as a hyposensitizing agent. Finally, the IgG binding activity of the major allergenic component of D.Pt. (Der P1) was shown to be unchanged following microencapsulation. Hence, the current studies indicated that the allergen D.Pt., was not adversely affected following encapsulation into PLG microparticles. Therefore, microparticles may have potential as delivery systems for hyposensitization therapy.

*Keywords:* Poly(lactide-co-glycolide); Microparticle; *Dermatophagoides pteronyssinus*; Hyposensitization therapy

## 1. Introduction

Hyposensitization therapy, also known as desensitization or allergen specific immunotherapy, has been used in humans for more than 70 years since it was first described by Noon (1911). Therapy involves injections of small doses of the offending allergen in increments up to a tolerated maximum dose. The injections are then given either as a short extension course in pre-seasonal therapy, or once or twice monthly for several years, in perennial therapy. The mechanisms through which hyposensitization therapy exerts a clinical effect are still not fully understood, but the aim of therapy is to induce tolerance to the offending agent. Several mechanisms of tolerance induction to allergens have been proposed, in-

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cluding altered regulation of IgE synthesis, production of blocking antibodies and decreased reactivity of effector mechanisms (Rocklin, 1989). There are several limitations associated with the current methods of hyposensitization therapy, including the inconvenience of the large number of injections required for the therapy to be effective. In addition, the efficacy and safety of hyposensitization therapy has been questioned (Kay, 1989; Norman, 1989). The issue of safety was highlighted in the UK by the CSM (1986). The CSM recommended that patients should be monitored for adverse effects for at least 2 h following treatment and that full resuscitation equipment should be available. Not surprisingly, this resulted in a considerable reduction in the use of hyposensitization therapy in the UK. Nevertheless, hyposensitization therapy is still widely available in Europe and in the USA. Moreover, in the absence of effective alternative therapies for a condition which is increasing in incidence, it is believed that hyposensitization therapy still has a valuable contribution to make in the treatment of allergic disorders (Durham et al., 1991). However, there is a need for safer and more effective forms of hyposensitization therapy.

Many alternative approaches have been investigated in attempts to achieve safer and more effective hyposensitization therapy, with varying degrees of success (WHO/IUIS Report, 1989). Approaches have varied from the use of adjuvants such as aluminium hydroxide (Sledge, 1938) and tyrosine (Wheeler et al., 1982), to the chemical modification of allergen structure using glutaraldehyde (Moran and Wheeler, 1976) and formaldehyde (Haddad et al., 1973), or the coupling of allergen to carriers, to render the allergen more tolerogenic (Wheeler et al., 1985). Also under active investigation is the use of allergen delivery systems such as liposomes (Arora and Gangal, 1992).

An alternative approach which may overcome some of the current problems of hyposensitization therapy is the use of poly(lactide-co-glycolide) (PLG) microparticles as delivery systems (O'Hagan et al., 1991a). The entrapment of allergens into PLG microparticles would protect the allergens from degradation following oral administration and promote their uptake into the Peyer's patches (O'Hagan et al., 1991b). Alternatively, PLG microparticles would enhance delivery of allergens to macrophages (O'Hagan et al., 1993) and allow controlled release. A reduction in the total number of doses required, or a reduction in the dose of allergen per administration, would be beneficial and might lead to better compliance and wider acceptability. Furthermore, oral administration may reduce the side effects in comparison to parenteral delivery and may facilitate home therapy, which would be convenient for parents, and less traumatic for young children.

PLG microparticles are prepared from biocompatible and biodegradable polymers (Visscher et al., 1987), which have been used for many years as resorbable sutures (Wise et al., 1979). PLG undergoes degradation by non-enzymatic hydrolysis to yield lactic and glycolic acids, which are normal body metabolites. Therefore, PLG microparticles with entrapped allergens are an attractive candidate delivery system for hyposensitization therapy.

However, the microparticle preparation process exposes the allergens to potentially damaging conditions, including exposure to organic solvents and shear forces from high speed agitation. Therefore, in the current studies, the effect of the microencapsulation process on the integrity of an allergen extract from *Dermatophagoides pteronyssinus* (house dust mite) was investigated.

## 2. Materials and methods

### 2.1. Allergen extract

*D. pteronyssinus* (D.Pt.) freeze-dried extract (batch no. X1116) was supplied by SmithKline Beecham Pharmaceuticals (SB), Great Burgh, Surrey.

### 2.2. Preparation of poly(lactide-co-glycolide) microparticles

Microparticles with entrapped D.Pt. were prepared using the method described by Jeffery et

al. (1993). The microparticles were prepared by emulsifying a 2% w/v aqueous solution of D.Pt. with a 6% w/v solution of PLG (co-polymer composition 50:50, Resomer RG 505, Boehringer Ingelheim, Germany) in dichloromethane (DCM, HPLC grade, May and Baker Ltd, Essex) using a Silverson homogeniser (Silverson Machines, Chesham, Bucks). The resulting water-in-oil emulsion was then emulsified with a 10% w/v aqueous solution of polyvinyl alcohol (PVA) (13-23 kDa, 87-89% hydrolysed, Aldrich Chemical Co., Dorset) to produce a water-in-oil-in-water emulsion. The emulsion was then stirred overnight under ambient temperature and pressure to allow solvent evaporation and microparticle formation. The microparticles were centrifuged, washed in double-distilled water and freeze dried.

### 2.3. Microparticle characterization

The size distribution of the microparticles was determined by laser diffractometry using a Malvern MasterSizer S3.00 (Malvern Instruments, Malvern, UK) and the size was expressed as volume mean diameter ($V_{md}$) (in $\mu$m). The surface morphology of the microparticles was observed using a scanning electron microscope (Jeol 6400, Jeol Instruments, Welwyn, Herts).

The level of entrapment of D.Pt. in microparticles was assessed as described by Hora et al. (1990). Briefly, 10-15 mg of freeze-dried microparticles, accurately weighed, were shaken overnight with 1 ml of 0.1 M sodium hydroxide (NaOH) (Sigma Chemical Co., Dorset) containing 5% w/v sodium dodecyl sulphate (SDS) (Sigma). The samples were centrifuged and the protein content of the supernatant was determined in triplicate using a bicinchoninic acid (BCA) protein assay (Sigma) (Smith et al., 1985), with known dilutions of D.Pt. as standards.

### 2.4. Assessment of the integrity of D.Pt. in microparticles

The integrity of D.Pt. following entrapment in PLG microparticles was investigated using isoelectric focusing (IEF), a radioallergosorbent test inhibition assay (RAST) and a quantitative assay for the major allergen, Der P1.

### 2.5. Isoelectric focusing

IEF using agarose pre cast gels (FMC Bioproducts, Rockland, USA) was performed according to manufacturers instructions. D.Pt. was released overnight from PLG microparticles into phosphate-buffered saline (PBS), the microparticles were removed by centrifugation and the supernatant was concentrated by filtration (Ultra free low binding cellulose 5 kDa cut off, Millipore, Japan). The samples were resuspended in PBS and native D.Pt. extract and the D.Pt. extracted from microparticles were loaded onto the pre-cast agarose gel, along with p*I* markers (pH from 3 to 10, Pharmacia, Sweden). The gels were fixed, silver stained and air dried.

### 2.6. Radioallergosorbent test (RAST) inhibition assay

The RAST inhibition assay was performed in triplicate on the same batch of microparticles. D.Pt. was released from the microparticles into PBS overnight and the samples were centrifuged. The protein content of the supernatants were then determined using a BCA assay. The supernatant was concentrated by filtration (Ultra free low binding cellulose 5 kDa cut off, Millipore, Japan) and resuspended in BPTA (1% BSA in PBS containing 1% Tween 20). Serial dilutions of the D.Pt recovered from the microparticles, native D.Pt. and a laboratory standard of D.Pt. (SB) were performed in BPTA on microtitre plates (NUNC, Denmark). The laboratory standard of D.Pt. is a fully characterized batch of D.Pt. which is used as an in-house standard at SB for assessing each new batch of the allergen. Pooled serum from patients sensitive to D.Pt. (specific human serum pool with high levels of specific IgE antibody to D.Pt.) was added to all the sample wells. The microtitre plates were agitated and incubated (2 h; 37°C).

For the assay, D.Pt. was conjugated to cellulose discs following cyanogen bromide activation of the discs. The activated discs were conjugated

with D.Pt. as described by Ceska et al. (1972). After incubation, a disc with D.Pt. conjugated was added to each well and the microtitre plates were shaken and incubated (3 h; 37°C). The fluid was aspirated from each well, without removing the discs and the plates were washed five times. Finally, $^{125}$I-anti IgE (Pharmacia) was added to each well, the plates were shaken and incubated overnight at 4°C. The discs were again washed and transferred to test tubes and counted in a gamma counter (Canberra, Packard, Berks, UK). The data were analysed and the results were plotted as percentage of inhibition of IgE binding against protein concentration.

### 2.7. Der P1 assay

The samples were prepared as described for the RAST inhibition assay, but without concentration. A determination of the binding of specific IgG to Der P1 was then performed. Each sample was assayed in duplicate using serial dilutions. $^{125}$I-Der P1 (SB) was added to test tubes containing the D.Pt. recovered from the microparticles, native D.Pt. and the laboratory standard D.Pt. (SB). Rabbit serum specific for Der P1 (SB) was then added, the tubes were capped, vortex mixed and incubated (2 h; 37°C). After incubation, protein A-Sepharose suspension (Pharmacia) was added to the samples and they were roller mixed (1 h; room temperature). The tubes were then centrifuged at 4000 rpm and the supernatants were aspirated leaving the protein A sepharose in the tubes. The protein A-Sepharose was washed four times with PBS-Tween (0.5% v/v). The tubes were then transferred to the gamma counter (Packard) and each tube was counted. The data were analysed and the results were plotted as the percentage inhibition of specific IgG binding to Der P1 against protein concentration.

## 3. Results

### 3.1. Microparticle characterisation

The microparticles with entrapped D.Pt. had a mean size of 1.0 μm, with a classical Gaussian size distribution (Fig. 1). The microparticles were smooth and spherical, with no obvious pits or pores present (Fig. 2). The entrapment level of D.Pt. in the microparticles was 2.8% w/w, as determined by the BCA assay.

The entrapment efficiency was determined by calculating the actual entrapment of D.Pt. as a

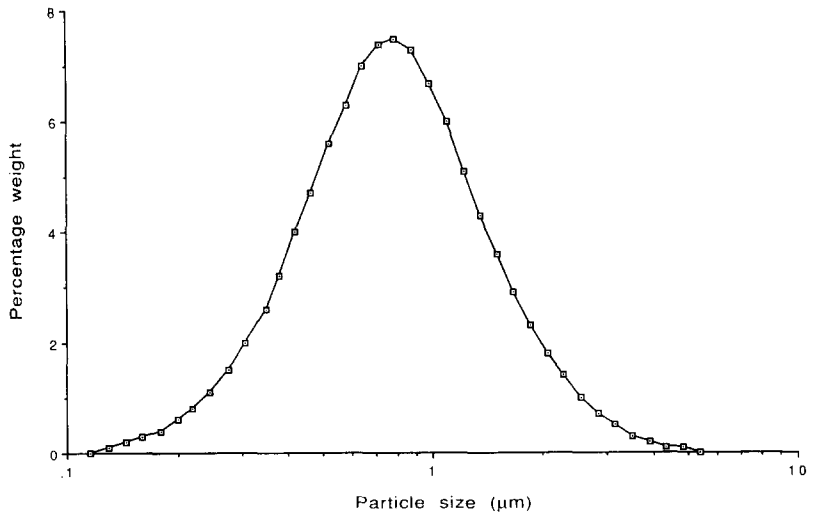


Fig. 1. Particle size distribution of a typical batch of PLG microparticles with entrapped D.Pt.

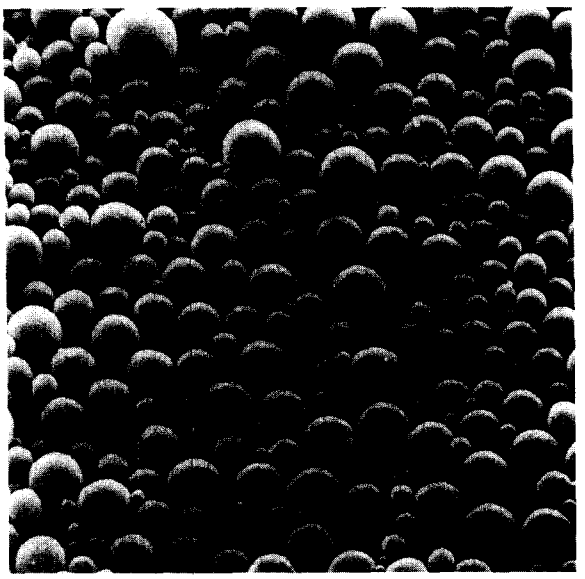

Fig. 2. Scanning electron micrograph of a typical batch of PLG microparticles with entrapped D.Pt. Magnification ×3900.

percentage of the theoretical entrapment. The theoretical entrapment is the % w/w that would be achieved if all the available D.Pt. was successfully entrapped in the microparticles. Therefore, the equation to calculate the percentage entrapment efficiency is as follows;

$$\frac{\text{actual D.Pt. entrapment (\% w/w)}}{\text{theoretical D.Pt. entrapment (\% w/w)}} \times 100$$

The entrapment efficiency of the D.Pt. in microparticles was 51%.

### 3.2. Allergen integrity

The profile obtained following IEF analysis of the D.Pt. extracted from the microparticles is shown in Fig. 3. Some alterations in the IEF profile of D.Pt. extracted from the microparticles in comparison to the native D.Pt. were observed. Minor changes in the IEF profile were observed at p*I* 3-5, since the released D.Pt. exhibited a more dense staining pattern than the original D.Pt. In addition, at p*I* 6.55 two faint bands which were present in the original material are absent from the material extracted from the microparticles.

### 3.3. RAST inhibition assay

The results obtained from the RAST inhibition assay are shown in Fig. 4. The results are expressed as the percentage inhibition of IgE binding activity plotted against protein concentration for the D.Pt. released from microparticles, native D.Pt. and the laboratory standard of D.Pt.. The results indicated that there was some loss of IgE binding activity following entrapment of D.Pt. into microparticles.

### 3.4. Der P1 assay

The results for the Der P1 assay are shown in Fig. 5. The results are expressed as the percent-

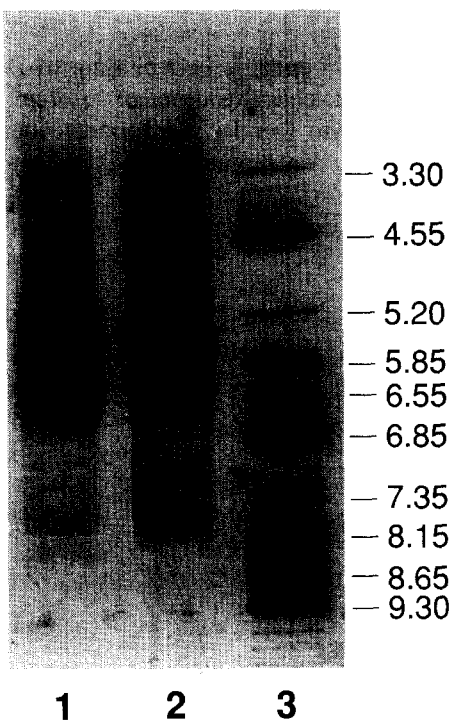


Fig. 3. An agarose IEF gel, silver stained, to show the isoelectric points of the proteins present in native D.Pt. and D.Pt. released from PLG microparticles. (1) Native D.Pt.; (2) D.Pt. released from PLG microparticles; (3) p*I* markers ranging from pH 3 to 10.

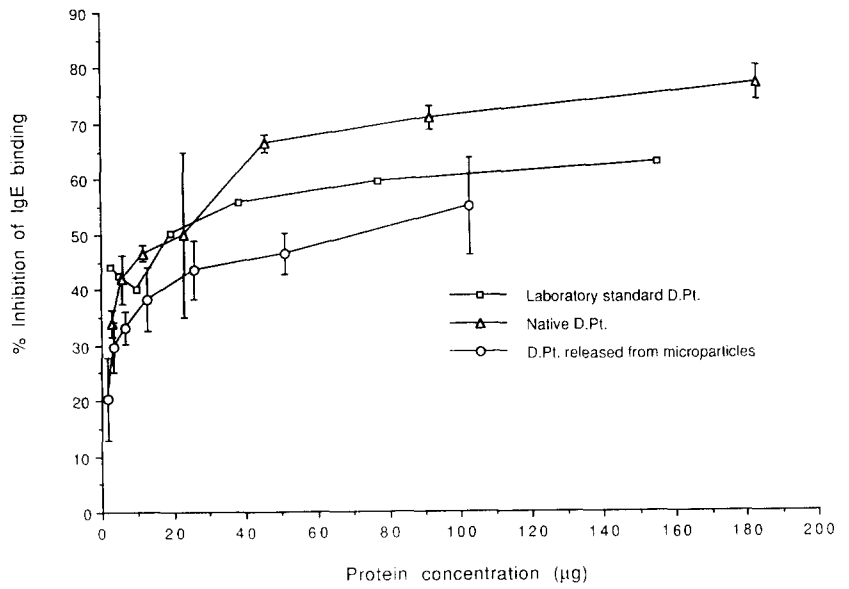


Fig. 4. RAST inhibition assay: % inhibition of IgE binding activity of D.Pt. released from PLG microparticles compared to native D.Pt.

age inhibition of specific IgG binding to Der P1 plotted against concentration of protein. The binding of IgG to Der P1 was found to be unaltered following entrapment of D.Pt. into microparticles.

## 4. Discussion

In the current study, we have assessed the effect of entrapment in PLG microparticles on the integrity of an allergen extracted from D.Pt.

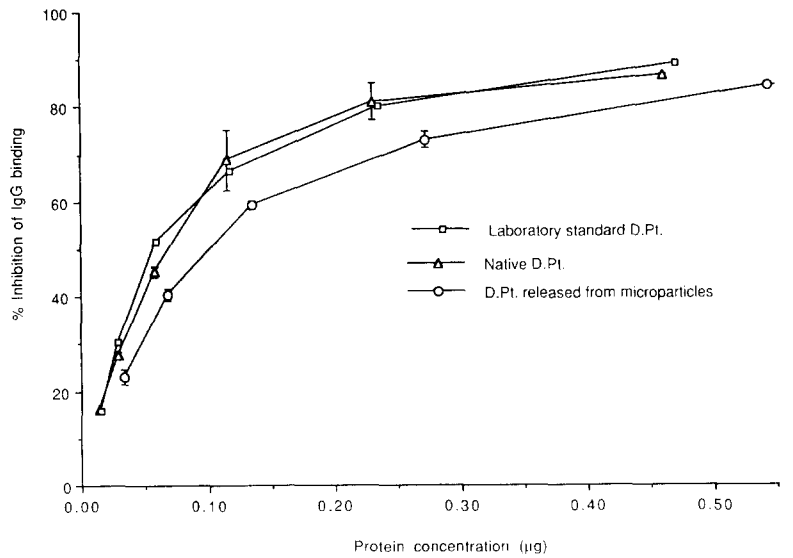


Fig. 5. Der P1 assay: % inhibition of IgG binding to D.Pt. released from PLG microparticles compared to native D.Pt.

The microparticles were produced by a water-in-oil-in-water emulsion solvent evaporation technique as previously described by Jeffery et al. (1993). The microparticle preparation method exposes the allergens to potentially damaging conditions, including exposure to DCM and high speed shear. Nevertheless, the results obtained here indicated that microencapsulation appeared to have only minor effects on the integrity of the D.Pt., which is a complex mixture of materials containing at least four major allergenic components as well as many other protein components which could produce immunogenic responses. At present, it is unknown whether or not the minor changes observed in IgE binding activity in the RAST inhibition assay following microencapsulation, would affect the biological activity of the allergen extract.

The IEF profile of the D.Pt. released from microparticles indicated that some changes had occurred in the allergen. This may be due to selective entrapment of components in the microparticles or possibly due to an interaction between the allergen components and the polymer during or following entrapment in the microparticles which prevented release of these components. Nevertheless, the IEF findings indicated that most of the protein components of D.Pt. were successfully entrapped into the microparticles and that no obvious degradation of specific components had occurred. The RAST inhibition assay showed that there was a minor reduction in the binding capacity of IgE to D.Pt. following entrapment into microparticles. Since the IEF profile indicated that some loss of allergen components had occurred during the entrapment procedure, the loss of activity in the RAST inhibition assay may be due to the loss of these specific components or it may be due to a reduction in the IgE binding activity of a number of the individual components of D.Pt. during the preparation of microparticles. Nevertheless, the loss of IgE binding activity is unlikely to be a significant factor in relation to the potential efficacy of D.Pt., since current theories of the mechanisms of hyposensitization therapy do not require the maintenance IgE binding activity. Indeed, some products currently in clinical use, allergoids, were specifically designed to have reduced IgE binding activity, but retained IgG inducing activity (Moran and Wheeler, 1976).

Therefore, D.Pt. entrapped in microparticles may still be effective for hyposensitization therapy, even if IgE binding activity were completely lost. In relation to the IgG binding activity of one of the major allergens of D.Pt. (Der P1), there appeared to be no loss in binding activity of the material released from the microparticles, in comparison to native D.Pt. This indicated that the structure of this major allergen component has been maintained during the microencapsulation process.

The results from the current studies are encouraging and indicate that the microparticle preparation process has minimal effects on the integrity of the entrapped allergen, D.Pt. Hence, the current findings indicate that PLG microparticles may be suitable candidates for hyposensitization therapy. In more general terms, the current findings also indicate that since proteins are minimally affected following entrapment in microparticles, then microparticles also possess considerable potential as delivery systems for protein antigens and therapeutic agents. Future studies will be designed to investigate the responses following immunization with D.Pt. entrapped in microparticles, these studies will be reported separately.

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