ELSEVIER

International Journal of Pharmaceutics 159 (1997) 135–147

international journal of pharmaceutics

Research papers

Biodegradable nanoparticles as a sustained release system for the antigens/allergens of *Aspergillus fumigatus*: preparation and characterisation

Taruna Madan [a], Neeru Munshi [b], Tapas K. De [b], Amarnath Maitra [b], P. Usha Sarma [a,*], Shyam Sunder Aggarwal [c]

[a] *Centre for Biochemical Technology, Mall Road, Delhi, 7, India*
[b] *Department of Chemistry, University of Delhi, Delhi, 7, India*
[c] *College of Pharmacy, Pushp Vihar, Delhi, 17, India*

Received 6 March 1997; received in revised form 18 August 1997; accepted 20 August 1997

Abstract

Ultrafine monodisperse polyvinylpyrrolidone nanoparticles (PVP np) entrapping allergens/antigens of *A. fumigatus* were prepared by reverse micelles method. The entrapment efficiency of various formulations of PVP np varied from 74 to 92%. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), enzyme linked immunosorbent assay (ELISA) and western blot analysis showed intact integrity and immunoreactivity of the allergens/antigens after entrapment in nanoparticles. The effect of various factors such as size of the particles, ratio of monomer to antigen concentration, degree of cross-linking and schedule of immunisation were examined on the in vitro release rate and production of specific IgG and IgE antibodies in the mice. The studies showed that the formulation with 1% cross-linking agent with a mean particle size of 100 nm and a monomer: antigen ratio of 0.09:1 released encapsulated antigens in 9 weeks. The same formulation showed a sustained IgG level (>0.400 Abs) for approximately 12 weeks in comparison to IgG level (>0.400 Abs) for 7 days with free antigen. Increase in particle size or change in monomer concentration resulted in accelerated release rate. A sustained level of IgG antibodies achieved with three divided doses of entrapped antigen could also be achieved with same amount of entrapped antigens administered in single dose. The *A. fumigatus* specific IgE levels of nanoparticle entrapped allergens/antigens were lower than obtained with free allergens/antigens. It is concluded that PVP np provide a promising delivery system for proteins as it is biocompatible, conserves the integrity and biological activities of proteins, augments antibody response and provide a sustained antibody level with single step immunisation. The formulation may also be useful for hyposensitisation therapy for various allergens. © 1997 Elsevier Science B.V.

Keywords: *A. fumigatus*; Allergens; Antigens; Nanoparticle; Polyvinylpyrrolidone; Immunisation

* Corresponding address. Tel: +91 11 7257439; fax: +91 11 7257471; e-mail: cbt@delnet.ren.nic.in

PII S0378-5173(97)00278-0

1. Introduction

Aspergillus fumigatus is a well known pathogenic fungi causing a wide spectrum of clinical conditions such as invasive aspergillosis, allergic alveolitis, allergic bronchopulmonary aspergillosis (ABPA), chronic necrotising aspergillosis (Kurup and Kumar, 1991). Some of the antigens and allergens of *A. fumigatus* show cytotoxic, protease, elastase activities and are implicated as potential virulent factors in various *Aspergillus* related disorders (Kurup and Kumar, 1991; Banerjee et al., 1995; Madan et al., 1997). There are few reports indicating existence of protective acquired immunity against *A. fumigatus* when sublethal doses of conidia were administered to the animals before the challenge (Richard et al., 1982; Smith, 1972; Lehmann and White, 1976). However, at present there are no vaccines for aspergillosis as neither the nature of antigens nor the pathogenesis is well understood. Hyposensitisation therapy, carried out by administering small doses of allergens, is contraindicated in *Aspergillus* allergic disorders as allergens/antigens of *A. fumigatus* induce both type I and type III hypersensitivity reactions (Kurup and Kumar, 1991). In view of need for administration of the allergens/antigens of *A. fumigatus* to examine their role in protective immunity and in order to modulate the immune response to make them suitable for hyposensitisation therapy, a biocompatible, biodegradable sustained release polymeric system is required.

Biodegradable polymeric delivery systems are being used to deliver the emerging new class of therapeutic agents e.g., peptides, proteins and polynucleotides (Langer, 1981; Couvreur and Puisieux, 1993). Entrapment in the polymers maintains the integrity and activity of these biomolecules, augments the immunopotentiating effect of the antigens and sometimes modulates the type of antibody response as observed in the case of allergens entrapped in liposomes (Arora and Gangal, 1992), and hence, find application in vaccination and hyposensitisation strategies.

In order to achieve the desired therapeutic response with polymeric delivery of biomolecules it is important to control the particle size of polymeric matrix. Micron sized particles are rapidly cleared by reticuloendothelial system while submicron sized particles (usually known as nanoparticles) have prolonged circulation time and are efficient drug, enzyme and protein carriers by any route of administration (Gautier et al., 1992; Damge et al., 1988; Couvreur et al., 1986). Such formulations are proposed to have potential to improve the antibody response against antigens by acting as a regulated and sustained release system (Kreuter, 1985). The preparative methods for the production of nanoparticles from a variety of polymers: gelatin, dextran, polyacrylamide, polyacrylocyanate etc. have been extensively reviewed (Couvreur and Puisieux, 1993). Chemical polymerisation or solidification of the dispersed phase of the fine emulsion without any chemical reactions polydispersed nanoparticles with a mean particle diameter of 30–1000 nm (Allemann et al., 1993). Ultrafine nanoparticles with narrow size distribution can be prepared by polymerising the aqueous core of surfactant assemblies in a reverse micellar system (Speiser, 1984).

In the current study, various formulations of ultrafine nanoparticles entrapping *A. fumigatus* allergens/antigens were prepared by reverse micelle method. Nanoparticles were characterised by laser diffractometry and transmission electron microscopy. The integrity and immunoreactivity of the proteins after entrapment was established by SDS–PAGE, Western blot and ELISA. The effect of various factors such as the size of the particle, ratio of monomer to antigen, percentage of cross-linking agent and schedule of immunisation was examined on the in vitro release rate and production of *A. fumigatus* specific IgG and IgE antibodies in mice.

2. Materials and methods

2.1. Experimental animals

Six to eight week old male BALB/c mice were obtained from National Institute of Nutrition, Hyderabad, India. They were pathogen free and received Purina chow and acidified water ad libitum. Mice were randomised before initiation of experiments.

2.2. Allergens and antigens of *A. fumigatus*

Allergens and antigens of *A. fumigatus* were prepared as described by Banerjee et al., 1990. Briefly, strain 285 of *A. fumigatus*, isolated from the sputum of an ABPA patient, was grown in synthetic medium (*l*-asparagine, Sigma, USA) for three weeks in stationary culture. The culture filtrate was obtained by filtration. Protein enriched antigenic fraction was prepared by precipitating the protein in the culture filtrate with ammonium sulfate (Sigma, USA). The fraction obtained was extensively dialysed and lyophilised for use in the present study.

2.3. Patient sera

Sera of patients with ABPA were received from the VP Chest Institute, Delhi (Banerjee et al., 1995). Sera from healthy donors without any indication of pulmonary disease were taken as controls.

2.4. Preparation of nanoparticles

The polyvinylpyrrolidone (PVP) nanoparticles entrapping antigenic fraction were prepared according to the method described in a patent application by Maitra et al., 1996. PVP was selected as polymer of choice since it is biocompatible, biodegradable and has been used frequently as a plasma expander in human beings. Briefly, the freshly distilled vinyl pyrrolidone (monomer) (Sigma, USA), tetramethyl ethylene diamine (TEMED) (Sigma, USA), methylene bis acrylamide (BIS) (cross linking agent; CLA) (Sigma, USA) was taken in 40 ml of 0.03 M Aerosol OT (AOT) (Sigma, USA) in *n*-hexane (SDS, India) and sonicated to form a clear reverse micellar solution. The mixture was purged with nitrogen gas. The polymerisation was started by adding ammonium per sulfate (Sigma, USA) solution and the reaction was allowed to continue for about 6–8 h in nitrogen atmosphere at 35°C. The *n*-hexane was evaporated off and then white mass of AOT surfactant and nanoparticles was dissolved in appropriate amount of phosphate buffer (0.01 M, pH 7.4) and then dialysed with 12 kDa cutoff dialysis membrane (Sigma, USA). The particles were suspended in phosphate buffer (0.01 M, pH 7.4) before characterisation and immunisation.

2.5. Characterisation of nanoparticles

2.5.1. Size determination

Dynamic (quasi-elastic) laser light scattering measurement for determining the size of the encapsulated antigens dispersed in aqueous buffer were performed using Brookhaven 9000 USA instrument with BI 200 SM goniometer as described by Munshi et al., 1995. Argon ion air cool laser was operated at 488 nm as a light source. Measurement was carried out for 3 samples of each formulation and mean values were taken.

2.5.2. Surface morphology

The surface morphology of the nanoparticles was examined by observation of the nanoparticle under transmission electron microscopy. Samples were prepared by resuspending lyophilised particles in acetone and placed the suspension on a 10×10 mm aluminium stub allowing it to air dry. The samples were gold coated for 2 min and then viewed using a Jeol 6400 scanning electron microscope (Jeol, Japan).

2.5.3. Efficiency of entrapment

The amount of *A. fumigatus* allergenic/antigenic protein entrapped in nanoparticles was determined by assaying the amount of free protein using a bicinconinic acid (BCA) (Sigma, USA) protein assay (Smith et al., 1985). The samples containing nanoparticles and unentrapped antigens were passed through a 100 kDa filter (Millipore, USA) which retained the nanoparticles and free protein came out in filtrate. Two samples were kept for each formulation and mean values have been plotted.

2.5.4. Protein integrity

The integrity of *A. fumigatus* antigens/allergens following entrapment in PVP nanoparticles was investigated by SDS–PAGE according to the method of Laemmli, 1970. Samples of allergens/antigens released from PVP nanoparticles in phosphate buffer saline (0.01 M phosphate buffer, pH

Table 1
ELISA absorbance values for specific IgG and IgE antibodies in pooled ABPA patient sera with free and entrapped *A. fumigatus* allergens/antigens

Sera sample	Abs for specific IgG		Abs for specific IgE	
	Free	Entrapped	Free	Entrapped
Pooled sera of ten ABPA patients	0.843	0.786	0.268	0.225
Pooled sera of five normals	0.043	0.036	0.068	0.059

Abs: Absorbance at 490 nm.
Free: antigen released from Formulation I (free antigen in normal saline).
Entrapped: antigen released from Formulation II (1% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm)
The values given are mean values of three tests with each sample in duplicate.

7.4, 0.15 M NaCl (PBS), native allergens/antigens and molecular weight markers were solubilised with sample buffer loaded onto a vertical slab gel (10%) and subjected to electrophoresis at 200 mV. Following electrophoresis the gels were either stained with Coomassie brilliant Blue (0.1% w/v) (Sigma, USA) in water:methanol:acetic acid (45:45:10) (Qualigens, India) to visualise the protein or subjected to Western blot. The immunoreactivity of the entrapped antigens/allergens was investigated by Western blot according to the method of Towbin et al., 1979 and ELISA as per the method of Banerjee et al., 1995 with sera of ABPA patients. For western blot, the electrophoressed allergen/antigen samples were transferred to a nitrocellulose (0.45μ, Schleicher and Schuell, Germany) by electroblotting and the membrane was blocked with Tris–buffered saline (TBS) (0.01 M Tris (Sigma, USA), pH 7.4, 0.15 M NaCl (Qualigens, India)). The blot was incubated with pooled sera of ABPA patients diluted 1:100 for IgG and 1:25 for IgE in TBS for 3 h at 37°C. After washing with TBS containing 0.3% w/v sorbitan monolaurate (Sigma, USA) (TBS/T20) the blot was incubated with antihuman IgG peroxidase (Sigma, USA) (1:2000) for IgG and antihuman IgE peroxidase (Sigma, USA) (1:3000) for IgE. The ability of allergen/antigen to bind anti-*A. fumigatus*-IgG/IgE was shown colorimetrically using diamino benzidine (1 mg/ml) (Sigma, USA) in TBS and H_2O_2 (Qualigens, India) 0.05% v/v. For ELISA the allergens/antigen samples released from nanoparticles were coated on wells of a microtitre plate (Nunc, Denmark) in carbonate/bicarbonate buffer (0.05 M, pH 9.6) (Qualigens, India) at a concentration of 1 μg of protein/well for 3 h at 37°C. The remaining sites in the wells were blocked with 3% BSA (Sigma, USA) in phosphate buffer saline (0.01 M, pH 7.4) (PBS) for 1 h at 37°C. After washing the wells with PBS containing 0.3% w/v sorbitan monolaurate (Sigma, USA) (PBS/T20) the wells were incubated with pooled sera of ABPA patients diluted 1:100 for IgG and 1:25 for IgE in PBS. After washing with PBS/T20, the wells were incubated with anti-human IgG peroxidase (1:2000) for IgG and anti-human IgE peroxidase (1:3000) for IgE. The ability of allergen/antigen to bind anti-*A. fumigatus*-IgG/IgE was shown colorimetrically using *o*-phenylene diamine (1 mg/ml) (Sigma, USA) in PBS and H_2O_2 0.05% v/v. The absorbance was measured at 490 nm using NUNC immunoreader (Table 1).

2.6. Schedule of immunisation

In order to test the effectiveness of various formulations in inducing an immune response, 6–8 week old BALB/c mice were exposed to free and entrapped *A. fumigatus* allergens/antigens once a week for three weeks. Mice were anaesthetised with ether, their backs were shaved and 350 μg of *A. fumigatus* antigen suspended in 100 μl of normal saline were administered subcutaneously. Each group contained fifteen animals, five of which received the antigen entrapped in nanoparticle formulations, five received free antigen in normal saline and five controls received

empty nanoparticles suspended in normal saline. In another experiment, the immune response elicited by single immunisation of the mice with 1 mg of entrapped antigens (1% CLA, mean particle size: 100 nm) was compared with immune response obtained with three equally divided immunisations of 1 mg of same formulation once a week.

2.7. Antibody response

Mice were bled every week and amount of *A. fumigatus* specific IgG and IgE antibody in the mice sera was analysed using indirect ELISA assay. For ELISA the protein enriched antigenic fraction of *A. fumigatus* was coated on wells of a microtitre plate and serum was obtained from immunised mice (1:20 for IgG and 1:15 for IgE).

2.7.1. Statistical analysis

Differences in serum antibody titres between immunised groups were analysed by Student's *t*-test. *p* values less than 0.05 were considered to represent statistically significant differences.

$$t = \frac{\chi_1 - \chi_2\sqrt{n_1 \cdot n_2}}{S\sqrt{n_1 + n_2 - 2}}$$

3. Results

3.1. Nanoparticle characterisation

Fig. 1 shows that size distribution pattern obtained by laser diffractometry for the PVP nanoparticles entrapping *A. fumigatus* allergens/antigens with 1% CLA, monomer: antigen ratio as 0.09:1 and water pool size $w_0 = 8$ (Formulation II) is very narrow. The mean diameter of the nanoparticles of formulation II was 100 nm. These nanoparticles were prepared in the water pool of reverse micellar solution which has a size range of few nanometers. However, the size of these nanoparticles was always found to be higher than the size of the water pool in which the polymeric reaction was carried out. This may be due to the interdroplet interaction between the water pools which increases the particle size by promoting secondary growth of the particles.

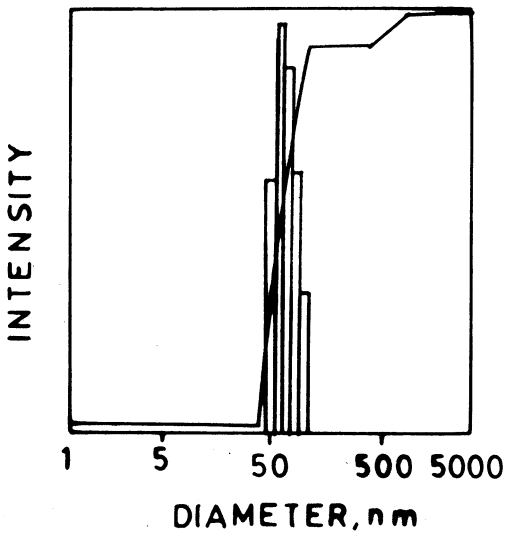

Fig. 1. Quasi elastic laser light scattering data of nanoencapsulated *A. fumigatus* allergens/antigens (Formulation II; 1% CLA, 0.09:1 Mon:Ag ratio p.size < 50 nm).

A transmission electron micrograph of nanoparticle formulation II is shown in Fig. 2 having a mean size of 100 nm. The shape of the particles appear to spherical. The formulations with a bigger water pool size of 16 and particles obtained with emulsion polymerisation had parti-

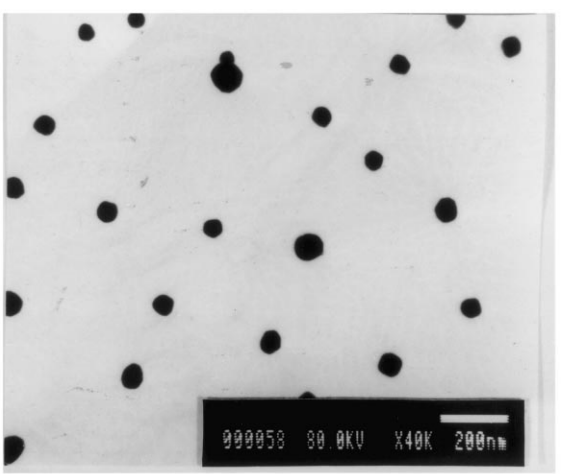

Fig. 2. Transmission electron micrograph of nanoparticles (Formulation II; 1% CLA, 0.09:1 Mon:Ag ratio p.size < 50 nm) with entrapped *A. fumigatus* allergens/antigens.

Table 2
Various polyvinylpyrrolidone formulations entrapping *A. fumigatus* allergens/antigens

Formulation	% CLA	Particle size (nm)	Monomer:antigen	% entrapment
I (Free Antigen in normal saline)				
II	1	<50	0.09:1	90
III	0.6	<50	0.09:1	82
IV	0.3	<50	0.09:1	79
V	1	<50	0.18:1	92
VI	1	<50	0.045:1	85
VII	1	<1000	0.09:1	74
VIII	1	>1000	0.09:1	69
IX (Empty nanoparticles in normal saline)				

Particle size: Mean particle diameter obtained by laser diffractometry.
Entrapment efficiency:

$$\frac{\text{Amount of antigen entrapped}}{\text{Total amount of antigen}} \times 100.$$

The values given are mean values of two tests with each sample in duplicate.

cle size ranging from hundreds of nm to thousands of nm, respectively (data not shown). With change in % of cross-linking agent (CLA) or monomer antigen ratio, the mean particle size was not altered (data not shown). The entrapment efficiency of the various formulations of the nanoparticles ranged from 74 to 92% as determined by BCA assay (Table 2). Efficiency of entrapment was enhanced with increase in percentage of CLA and with decrease in particle size.

SDS–PAGE analysis of protein which had been entrapped, showed that the bands of various allergenic/antigenic proteins released from nanoparticles coincided with the bands obtained with native *A. fumigatus* allergenic/antigenic mixture (Fig. 3). There were no additional bands present to indicate the presence of aggregates or fragments. This would suggest that the structural integrity of *A. fumigatus* allergens/antigens was not significantly affected by the entrapment process.

The western blot showed that pooled patient sera containing anti-Af-IgG/IgE antibodies recognised similar antigens and allergens in native as well as allergenic/antigenic proteins released from the nanoparticles, thus providing further evidence that the allergens/antigens were intact conformationally and retained their immunoreactivity (Fig. 4).

3.2. In vitro release rate

The cumulative percent release of *A. fumigatus* allergens/antigens from various formulations of nanoparticles at different time intervals has been plotted in Fig. 5. The in vitro release from various formulations never reached 100%. It may be due to degradation of allergenic/antigenic proteins at 37°C or some of the protein may not have been released from the matrix. The duration of in vitro release of entrapped allergens/antigens was prolonged with increase in the amount of CLA (Formulation II: 9 weeks; Formulation III: 8 weeks; Formulation IV: 7 weeks). The release rate was enhanced with increase in nanoparticle size and with a change in monomer antigen ratio (Formulation VII: 5 weeks; Formulation VIII: 3 weeks; Formulation V: 6 weeks; Formulation VI: 8 weeks).

3.3. Antibody response

The subcutaneous route of administration was selected as it naturally provides a slow release of proteins. The mice subcutaneously injected with free antigen showed extensive tissue damage at the site of injection indicating cytotoxic and proteolytic effect of *A. fumigatus* allergens/antigens.

However, the tissue damage was not seen with encapsulated *A. fumigatus* allergens/antigens. The polymer polyvinylpyrrolidone was non irritating and site of injection showed no visible inflammatory reaction. Analysis of sera from control mice given empty nanoparticles consistently showed insignificant antibody titres.

3.3.1. Effect of percentage of cross-linking agent (CLA)

The *A. fumigatus* specific IgG antibody levels (Af-IgG) induced in the mice with various formulations of nanoparticles are shown in Fig. 6. All the polymeric formulations had a prolonged Af-IgG response in comparison to free antigen ($p < 0.05$, Student's t-test). Similar to the in vitro release, increased amount of CLA resulted in prolonged sustenance of Af-IgG level (> 0.4 Abs).

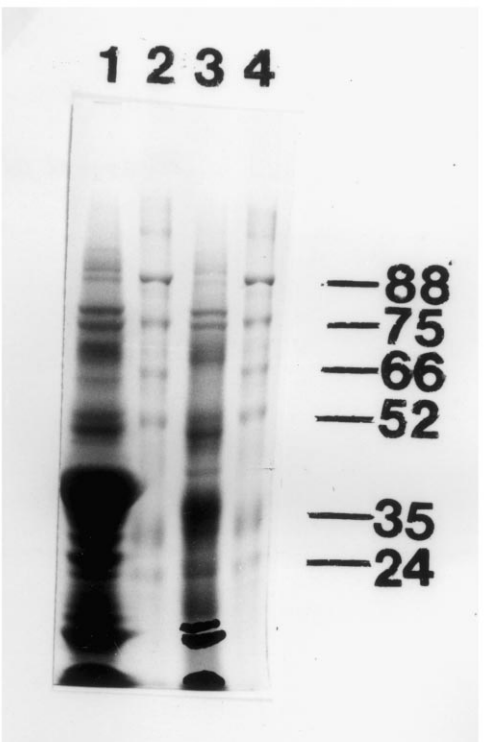

Fig. 3. SDS–PAGE analysis of *A. fumigatus* allergens/antigens released from nanoparticles (Formulation II; 1% CLA, 0.09:1 Mon:Ag ratio p.size < 50 nm). Lane 1: native culture filtrate allergens/antigens. Lane 2 and 4: MW markers. Lane 3: *A. fumigatus* allergens/antigens released from nanoparticles.

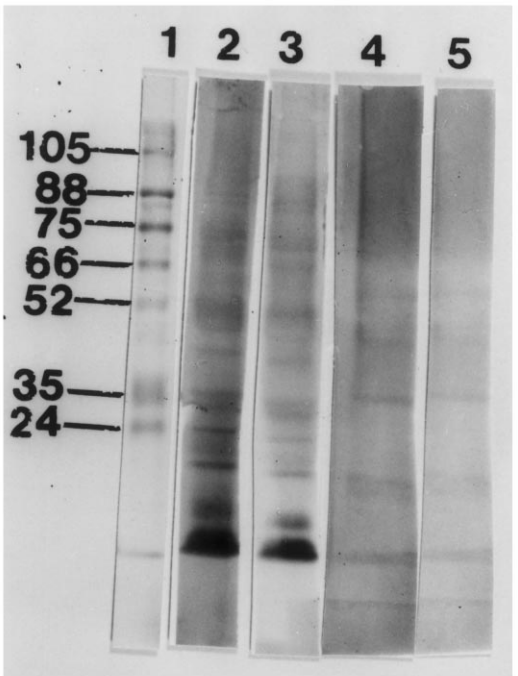

Fig. 4. IgG and IgE binding analysis of *A. fumigatus* allergens/antigens released from nanoparticles (Formulation II; 1% CLA, 0.09:1 Mon:Ag ratio p.size < 50 nm). Lane 1: MW markers. Lane 2: IgG binding of native culture filtrate allergens/antigens. Lane 3: IgG binding of culture filtrate allergens/antigens released from nanoparticles. Lane 4: IgE binding of native culture filtrate allergens/antigens. Lane 5: IgE binding of culture filtrate allergens/antigens released from nanoparticles.

The duration of significant Af-IgG levels ($>$ 0.4Abs) for formulations I, II, III, IV, V was 7 days (18–25), 70 days (15–85), 60 days (15–75) and 40 days (10–50) respectively. The *A. fumigatus* specific IgE antibody levels (Af-IgE) obtained with different nanoparticle formulations were always lower than obtained with free antigen (Formulation I) (Fig. 7). Formulation II with 1% CLA had the lowest Af-IgE which was three fold less than Formulation I ($p < 0.00001$, Student's t-test).

3.3.2. Effect of monomer:antigen ratio

Three different monomer:antigen ratios were used to prepare nanoparticles entrapping *A. fumi-*

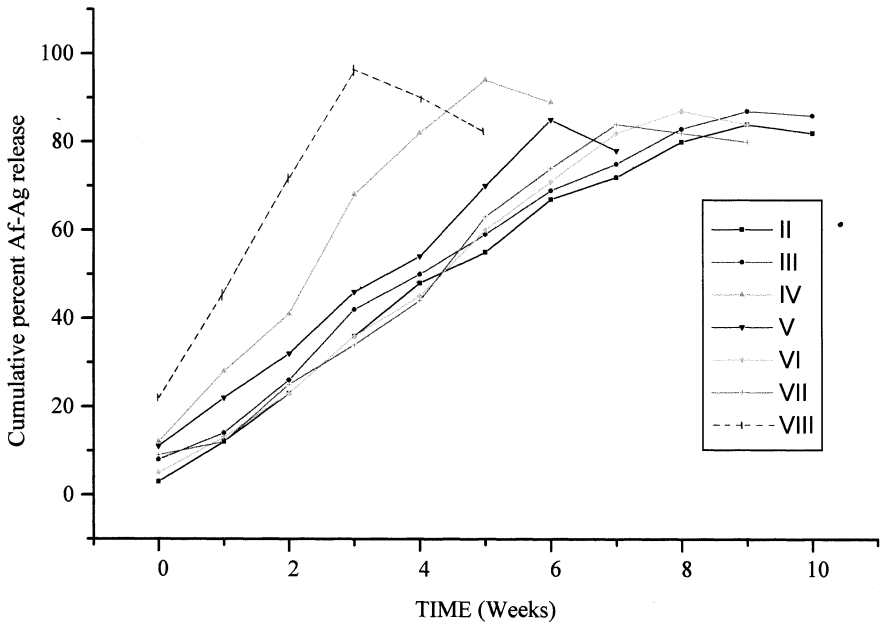

Fig. 5. In vitro release of *A. fumigatus* culture filtrate allergens/antigens at pH 7.4 and 37°C from various formulations of PVP nanoparticles (Formulation: II, 1% CLA. 0.09:1 Mon:Ag ratio, p.size < 50 nm; III, 0.6% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; IV, 0.3% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; V, 1% CLA, 0.18:1 Mon:Ag ratio, p.size < 50 nm; VI: 1% CLA, 0.045:1 Mon:Ag ratio, p.size < 50 nm; VII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size < 1 μm; VIII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size > 1 μm).

gatus allergens/antigens (Formulation II, V, VI). Formulation II had a longer duration of significant Af-IgG level than formulations V (49 days) and VI (56 days) (Fig. 6). Af-IgE levels for formulations V and VI were almost similar and were higher than formulation II (Fig. 7) ($p < 0.001$, Student's t-test).

3.3.3. Effect of particle size

Particles of formulations II, VII and VIII had mean diameters of < 50, < 1000 and > 1000 nm. Fig. 6 shows that significant Af-IgG level (> 0.4 Abs) was longer for formulation II (70 days) than formulation VII (56 days) and formulation VIII (35 days). However the difference between formulation II and VIII was significant at $p < 0.05$ and between formulation II and VII was significant only at $p < 0.5$ (Student's t-test). Further formulation VIII with biggest particle size showed the highest Af-IgG antibody level (> 0.9 Abs). Af-IgE levels increased with increase in particle size and were significant with formulation VIII (> 0.2 Abs) (Fig. 7).

3.3.4. Effect of immunisation interval

Fig. 8 shows the Af-IgG/IgE levels obtained with formulation II administered in two modes; three immunisations of 350 μg once a week or one single immunisation of 1 mg (IIa). The duration of significant Af-IgG levels obtained with formulations II (70 days) and IIa (62 days) were almost similar (II and IIa were statistically different only at $p < 0.8$, Student's t-test). The Af-IgE levels obtained with single immunisation were lower than those obtained with multiple immunisations of formulation II, however, statistically they were different only at $p < 0.5$ (Student's t-test).

4. Discussion

The reverse micelle method used in the current study has allowed the preparation of smooth, spherical, ultrafine, monodisperse, biodegradable and biocompatible nanoparticles encapsulating *A.*

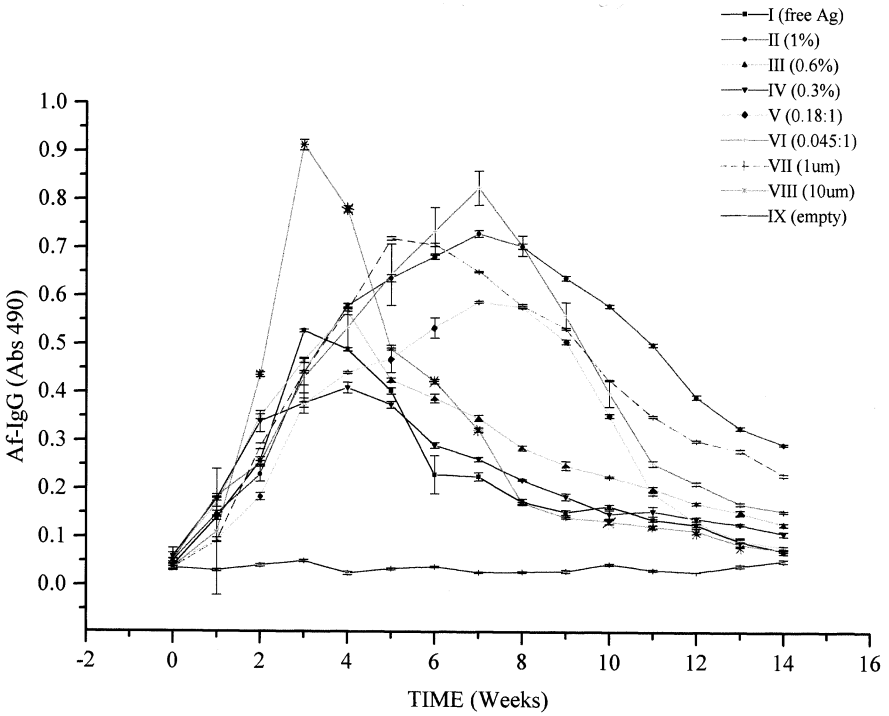

Fig. 6. Af-IgG raised in mice to free *A. fumigatus* allergens/antigens and various formulations of nanoparticles enacapsulating *A. fumigatus* allergens/antigens (Formulation: II, 1% CLA. 0.09:1 Mon:Ag ratio, p.size < 50 nm; III, 0.6% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; IV, 0.3% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; V, 1% CLA, 0.18:1 Mon:Ag ratio, p.size < 50 nm; VI: 1% CLA, 0.045:1 Mon:Ag ratio, p.size < 50 nm; VII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size < 1 μm; VIII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size > 1 μm). The data represent mean $\pm$ S.D. of ELISA values obtained (for five animals immunised with the same formulation) in two different ELISA assays.

fumigatus allergens/antigens. The nanoparticle preparation method exposes the allergens/antigens to potentially damaging conditions, including exposure to organic solvents and high speed shear. Nevertheless, the results obtained here indicated that nanoencapsulation appears to have only minor effects on the integrity and immunoreactivity of *A. fumigatus* allergens/antigens which is a complex mixture of more than 30 allergenic and antigenic proteins.

The effect of increase in CLA on release of antigens in vitro and duration of Af-IgG levels in vivo is probably due to formation of a densely cross-linked matrix. A similar observation was reported by Munshi et al., 1995 in release of enzymes from polyacrylamide nanoparticles. The use of variable monomer antigen ratio affects the pore size and hence, influences the in vitro release as well as in vivo antibody response.

No tissue damage observed with subcutaneous injection of encapsulated allergens/antigens indicates that polymeric encapsulation prevented the cell death and necrosis mediated by free allergens/antigens of *A. fumigatus*. Further, the slowly released allergen/antigen may have been easily cleared by the body defense mechanisms.

The maintenance of significant Af-IgG levels with nanoencapsulated *A. fumigatus* allergens/antigens for a longer time indicate that nanoparticles presumably act as a repository for the antigens, protecting them from rapid degradation and allowing small antigenic mass to be delivered continuously and effectively. It has been reported that molecules entrapped in the polymer are released in unaltered form at controlled rates and immune cells are given ample time for recognition of true antigenic determinants (Rhine et al., 1980).

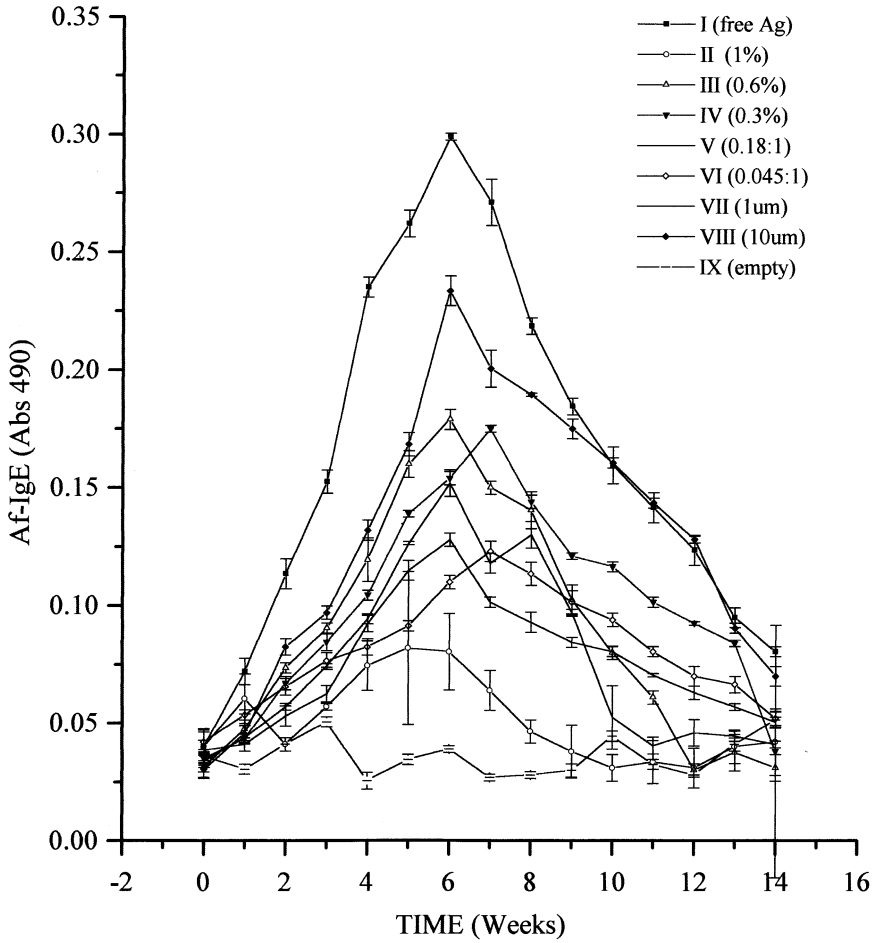

Fig. 7. Af-IgE raised in mice to free *A. fumigatus* allergens/antigens and various formulations of nanoparticles encapsulating *A. fumigatus* allergens/antigens. (Formulation: II, 1% CLA. 0.09:1 Mon:Ag ratio, p.size < 50 nm; III, 0.6% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; IV, 0.3% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm; V, 1% CLA, 0.18:1 Mon:Ag ratio, p.size < 50 nm; VI: 1% CLA, 0.045:1 Mon:Ag ratio, p.size < 50 nm; VII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size < 1 μm; VIII, 1% CLA, 0.09:1 Mon:Ag ratio, p.size > 1 μm). The data represent mean $\pm$S.D. of ELISA values obtained (for five animals immunised with the same formulation) in two different ELISA assays.

The rapid degradation of the molecule is compensated by the constant diffusion of the immunogen from the polymer, thus ensuring a sustained immune response by providing antigens to immune system for long periods.

The three fold reduction in Af-IgE response with nanoencapsulated *A. fumigatus* allergens/antigens in the present study may be a result of change in response of immune cells to particulate allergens/antigens. Repeated administrations of small doses of allergens/antigens is known to modulate the type of immune response usually obtained with normal delivery (Durham et al., 1991). These results further indicate that these formulations may find a potential use in hyposensitisation therapy of *A. fumigatus* mediated as well other allergic disorders. Liposomal allergenic formulations, currently in clinical use, are specifically designed to have reduced IgE but enhanced IgG inducing activity (Arora and Gangal, 1992).

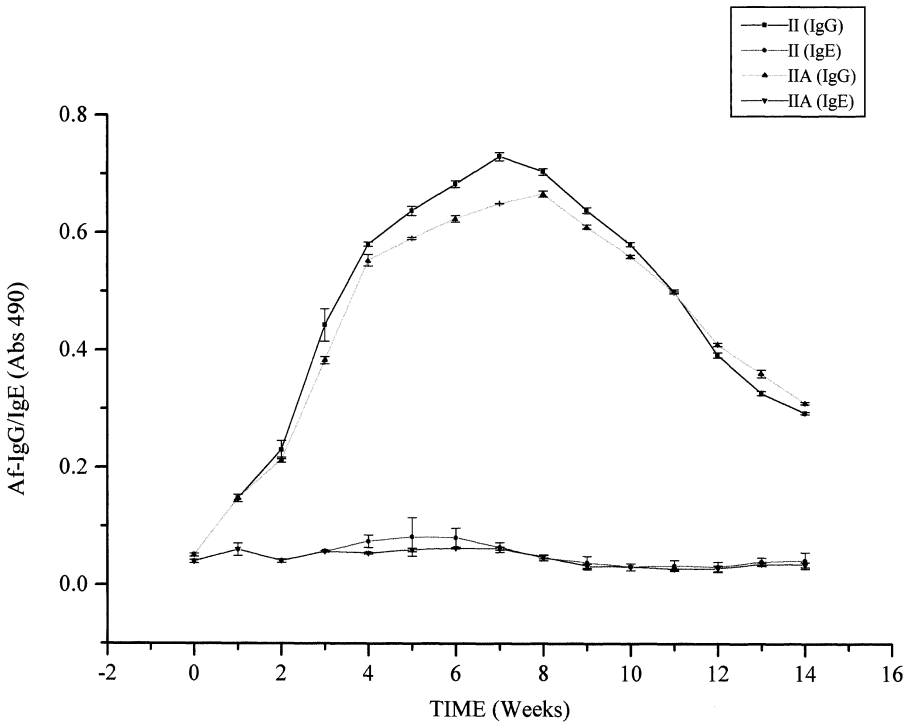

Fig. 8. Af-IgG and Af-IgE raised in mice to single and multiple immunisations of formulation II enacapsulating *A. fumigatus* allergens/antigens. II A: single immunisation of formulation II (1 mg) (1% CLA, 0.09:1 Mon:Ag ratio, p.size < 50 nm) and II, three weekly immunisations of formulation II (0.33 mg each). Total amount of antigen used was the same for both schedules of immunisation. The data represent mean ± S.D. of ELISA values obtained (for five animals immunised with the same formulation) in two different ELISA assays.

The similar antibody levels with single and multiple immunisations suggests that PVP nanoparticles could provide a simple, safe and effective single step immunisation. Excellent biocompatibility and single step introduction of antigen could make this method of immunisation as an alternative to immunological adjuvants such as Freund's adjuvant which cannot be used in humans.

In the current study exposure to soluble *A. fumigatus* antigen resulted in production of elevated levels of specific IgG and IgE in the sera of mice similar to the previous reports by Kurup et al., 1994. Nanoencapsulated *A. fumigatus* allergens/antigens however, showed a different immune response than reported by Kurup et al., 1994 using *A. fumigatus* antigen coated polystyrene beads. The particulate Af-antigen showed increased total IgE, decreased Af-IgG1 and eosinophilia in comparison to the decreased Af-IgE and prolonged significant Af-IgG levels by encapsulated Af-antigen. The interleukin profile of immune response with *A. fumigatus* antigens linked with polystyrene beads was of TH2 type (Kurup et al., 1994). A TH2 type of response has been associated with disease progression rather than protection while a TH1 type of response is considered to be protective in nature (Sher and Coffman, 1992). The low Af-IgE levels suggests a probable TH1 type of response with nanoencapsulated *A. fumigatus* allergens/antigens. Also, a V3 loop peptide of HIV-1 encapsulated in PVP nanoparticles showed raised IgG2a and IgG2b in mice indicating a protective response (Ahluwalia, 1996). Investigations on the interleukin profile of mice

immunised with nanoencapsulated *A. fumigatus* allergens/antigens are in progress that will indicate the type of T-helper cell response. The nanoencapsulated *A. fumigatus* allergens/antigens can be used for protective immunisation provided the T-helper response is favourable.

Thus, we conclude that PVP nanoparticle formulation is useful for delivery of *A. fumigatus* related or any other purified proteins as well as antigenic/allergenic peptides and evaluation of their immune response in the animals. Further, it could be used to study the role of individual peptides in producing allergic responses and to select the appropriate peptides for immunotherapy.

References

Ahluwalia, A., 1996. Influence of novel delivery systems on the immunogenicity of the V3 loop (306–320) peptide of HIV-1 (M.Sc. Thesis). All India Institute of Medical Sciences, pp. 33–36.

Allemann, E., Gurny, R., Doelker, E., 1993. Drug loaded nanoparticles—preparation methods and drug targeting issues. Eur. J. Pharm. Biopharm. 39, 173–191.

Arora, N., Gangal, S.V., 1992. Efficacy of liposome entrapped allergen in down-regulation of IgE response in mice. Clin. Exp. Allergy 22, 35–42.

Banerjee, B., Chetty, A., Joshi, A.P., Sarma, P.U., 1990. Identification and characterisation diagnostically relevant antigens of *Aspergillus fumigatus*. Asian Pac. J. Allergy Immunol. 18, 13–18.

Banerjee, B., Madan, T., Sharma, G.L., Prasad, H.K., Nath, I., Sarma, P.U., 1995. Characterisation of glycoprotein antigen (45kD) of *Aspergillus fumigatus*. Serodiagn. Immunother. Infect. Dis. 7, 147–152.

Couvreur, P., Grislain, L., Lenaerts, V., Brasseur, F., Guiot, P., Biernacki, A., 1986. Biodegradable polymeric nanoparticles as drug carrier for antitumor agents. In: Guiot P., Couvreur P. (Eds.), Polymeric Nanoparticles and Microspheres. CRC Press, Boca Raton, FL, pp. 27–93.

Couvreur, P., Puisieux, F., 1993. Nano- and microparticles for the delivery of polypeptides and proteins. In: Pitt, C.G. (Ed.), Advanced Drug Delivery Reviews, vol. 10. Elsevier, Amsterdam, pp. 141–159.

Damge, C., Michel, C., Aprahamian, M., Couvreur, P., 1988. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. Diabetes 37, 246–251.

Durham, S.R., Varney, V., Gaga, M., Frew, A.J., Jacobson, M., Kay, A.B., 1991. Immunotherapy and allergic inflammation. Clin. Exp. Allergy 21 (s1), 206–210.

Gautier, J.C., Grangier, J.L., Barbier, A., DuPont, P., Dussossory, D., Pastor, G., Couvreur, P., 1992. Biodegradable nanoparticles for subcutaneous administration of growth hormone releasing factor h-(GRF). J. Control. Release 20, 67–78.

Kreuter, J., 1985. Poly (alkyl acrylate) nanoparticles. In: Widder, K.J., Green, R. (Eds.), Methods in Enzymology, vol. 112. Academic Press, Orlando, FL, pp. 129–138.

Kurup, V.P., Kumar, A., 1991. Immunodiagnosis of aspergillosis. Clin. Microbiol. Rev. 4, 439–456.

Kurup, V.P., Seymour, B.W.P., Choi, H., Coffmann, R.L., 1994. Particulate *Aspergillus fumigatus* antigens elicit a T_{H2} response in BALB/c mice. J. Allergy Clin. Immunol. 93, 1013–1020.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Langer, R., 1981. Polymers for the sustained release of macromolecules; their use in a single step method of immunisation. In: Langnone, J.J., Vunakis, H. (Eds.), Methods in Enzymology, vol. 73. Academic Press, Orlando, FL, pp. 57–75.

Lehmann, P.F., White, L.O., 1976. Acquired immunity to *Aspergillus fumigatus*. Infect. Immun. 13, 1296–1298.

Madan, T., Arora, N., Sarma, P. Usah, 1997. Identification and evaluation of a major cytotoxin of *A. fumigatus*. Mol. Cell. Biochem., 167, 89–97.

Maitra, A., Ghosh, P.K., De, T.K., Sahoo, S., 1996. A process for the preparation of highly monodispersed polymeric hydrophilic nanoparticles. Indian patent application no. 2350/DEL/96 dated 20 Dec. 1996.

Munshi, N., Chakraborti, K., De, T.K., Maitra, A.N., 1995. Activity and stability studies of ultrafine nanoencapsulated catalase and penicillinase. Colloid Polym. Sci. 273, 464–472.

Rhine, W., Hsieh, D.S.T., Langer, R., 1980. Polymers for sustained macromolecule release: procedures to fabricate reproducible delivery systems and control release kinetics. J. Pharm. Sci. 69, 265.

Richard, J.L., Thurston, J.R., Cutlip, R.C., Pier, A.C., 1982. Vaccination studies of aspergillosis in turkeys; subcutaneous innoculation with several vaccine preparations followed aerosol challenge exposure. Am. J. Vet. Res. 43, 488–492.

Sher, A., Coffman, R.L., 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Ann. Rev. Immunol. 46, 385–409.

Smith, G.R., 1972. Experimental aspergillosis in mice; aspects of resistance. J. Hygeine Camb. 70, 741–754.

Speiser, P., 1984. Hardened reverse micelles as drug delivery systems. In: Luisi, P.L., Straub, B.E. (Eds.), Reverse Micelle Technological and Biological Relevance. Plenum, New York, pp. 339–346.

Smith, P.K., Crohn, R.J., Hermanson, A.K., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Geoke, N.M., Olsen, B.J., Klenke, D.C., 1985. Measurement of protein using bicinconinic acid. Anal. Biochem. 150, 76–85.

Towbin, H., Stachalin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets, procedure and some applications. Proc. Nat. Acad. Sci. U.S.A. 76, 4350.