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Altered immune response to liposomal allergens of *Aspergillus fumigatus* in mice

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Abstract

Aspergillus fumigatus has been implicated as the major pathogenic fungus causing *Aspergillus*-mediated disorders. It secretes complex glycoprotein antigens and allergens, which induce type I and type III mediated hypersensitivity reactions. The immune response to these allergens/antigens in allergic disorders is characterized by elevated levels of specific IgE, Th2 cytokines and eosinophilia. In the current study, the ability of negatively charged liposomes entrapped with glycoprotein antigens and allergens of *A. fumigatus* to modulate the immune response was studied. Immune response in mice was evaluated with both free and liposomal formulations. Liposome entrapped glycoprotein antigens/allergens of *A. fumigatus* elicited a Th1 type response with increased levels of TNF-α (5.5-folds), IFN-γ (four-folds), specific IgG (three-folds) and IgG2a (2.4-folds), low titers of specific IgG1 (2.2-folds decrease) and IgE (three-folds decrease), and decreased peripheral eosinophilia by four-folds in comparison to mice receiving free glycoprotein allergens/antigens of *A. fumigatus*. Histopathological examination of lung tissue sections clearly indicated reduced eosinophil infiltration in mice immunized with liposomal formulations. These results suggest potential of liposomal formulations for *A. fumigatus* allergens/antigens for exploration in immunotherapy. © 2002 Published by Elsevier Science B.V.

Keywords: Cytokines; Eosinophils; Glycoprotein antigens/allergens; Immune response; Liposomes

1. Introduction

Fungal allergies including rhinitis, allergic bronchopulmonary mycoses, allergic sinusitis and hypersensitivity pneumonitis are complex in nature (Kurup and Fink, 1993). *Aspergillus fumigatus* is the major causative agent of *Aspergillus*-mediated allergic disorders. It secretes a large number of antigens and allergens that induce type I-IgE and type III-IgG mediated hypersensitivity reactions. Immune response to these

Abbreviations: Af-3wcf, three week culture filtrate antigens/allergens of *A. fumigatus*; Af-gp, glycoprotein antigens/allergens of *A. fumigatus*; con A, concanavalin A; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffer saline.

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antigens in allergic disorders is characterized by elevated levels of specific IgE and IgG, high eosinophilia and up regulation of Th2 pathway with the secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines (Kurup et al., 2000).

Hyposensitization therapy carried out by native allergens is one of the therapeutic approaches being used for allergies due to pollen (grass and ragweed), cat, and mite allergens (Genin et al., 1994; Malling, 1998). However, hyposensitization therapy is contraindicated in *Aspergillus* induced allergic disorders as the IgE and IgG antibody response elicited against its antigens/allergens contributes to pathogenesis rather than protection (Kurup and Kumar, 1991). There are few reports indicating existence of protective acquired immunity against *A. fumigatus* when sublethal doses of conidia were administered to the animals before challenge (Smith, 1972; Lehmann and White, 1976; Richard et al., 1982). In view of this, examination of protective potential of *A. fumigatus* antigens/allergens and modulation of immune response to them through a biodegradable and biocompatible delivery system can be investigated.

Numerous formulations like liposomes, microspheres, lipid-based and polymer derived nanoparticles (Rhine et al., 1980; Gregoriadis, 1990; Couvreur and Puisieux, 1993; O'Hagen, 1997) have been used as antigen delivery systems for invoking protective host immune response against infectious diseases. Liposomal formulations are being extensively used as carriers of vaccines and drugs (Alving, 1991). The greater immunomodulatory activity of antigens delivered by liposomes may be attributed to their slow and sustained release, and slower clearance of the encapsulated protein (van Rooijen and Nieuwmegen, 1983; Childers et al., 2000). In addition, reduction or elimination of toxicity of antigens/drugs has also been observed with liposomal formulations (Saxena et al., 1998).

Immunoadjuvant action of liposomes has been shown to strengthen humoral and/or cell mediated immunity. Further, elicitation of protective immune response in mice using liposome entrapped antigens or peptides of *Leishmania donovani*, malarial sporozoite and hepatitis virus (Sanchez et al., 1980; Richards et al., 1988; Afrin and Ali, 1997; Lutsiak et al., 1998) has been established. The enhancement of the immune response to liposome entrapped antigens could be due to increased antigen uptake by antigen presenting cells and subsequent presentation to T-cells.

Liposomal formulations of pollen, cat and house dust mite allergens have resulted in modulation of antibody response and elicitation of Th1 immune response, thus can be investigated for vaccination and hyposensitization strategies in *A. fumigatus*-mediated allergic disorders (Wagner et al., 1984; Audera et al., 1991; Arora and Gangal, 1992; Genin et al., 1994; Malling, 1998). A shift of Th2 to Th1 type of cytokine response, reduction in levels of specific IgE antibodies and downregulation of eosinophilia in case of allergic disorders will facilitate alleviation of the disease (Sher and Coffman, 1992). In view of this, investigations on liposomal formulations of antigens/allergens of *A. fumigatus* are necessary.

Glycoproteins of *A. fumigatus* are the major immunodominant specific IgG and IgE antibody binding proteins and have been proposed as potential virulent factors (Kurup and Kumar, 1991; Sarma et al., 1998). In the present work, the immune response elicited in mice using free and liposomal formulations of glycoprotein antigens/allergens of *A. fumigatus* was evaluated using parameters such as levels of specific IgG and IgE antibodies, peripheral blood eosinophilia, cytokine profile of splenic cells and histological analysis of lungs for pulmonary eosinophilia.

2. Materials and methods

2.1. Experimental animals

About 5–6-week-old male Balb/c mice (body weight, 20 g) were obtained from National Institute of Nutrition, Hyderabad, India. They received mouse chow and acidified water ad libitum. Mice were randomized at the beginning of the experiments.

2.2. Preparation of glycoprotein antigens/allergens of *A. fumigatus*

Glycoprotein antigens/allergens of *A. fumigatus* were prepared as reported by Banerjee et al. (1995). Briefly, strain 285 of *A. fumigatus*, isolated from the sputum of an ABPA patient, was grown in asparagine-synthetic medium as stationary culture for 3 weeks. The culture filtrate was obtained by filtration. Protein enriched fraction was made by precipitating the proteins with ammonium sulfate. After centrifugation the pellet was resuspended and dialyzed against distilled water until free from ammonium sulfate to get the 3 week culture filtrate antigens/allergens of *A. fumigatus* (Af-3wcf). The fraction containing glycoprotein antigens/allergens was obtained by Concanavalin A (con A)–Sepharose affinity chromatography. Briefly, the con A–Sepharose column was equilibrated with 10 mM Tris–Cl, pH 7.4 buffer containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. The unbound fraction was collected with equilibration buffer and con A bound proteins were eluted with 0.2 M methyl α-D-mannopyranoside in equilibration buffer. This was followed by overnight dialysis against distilled water. The con A bound fraction containing glycoprotein antigens/allergens of *A. fumigatus* (Af-gp) was lyophilized and used for the present study.

2.3. Preparation of liposome entrapped glycoprotein antigens/allergens

Liposomes were prepared as described by Arora and Gangal (1992). Negatively charged liposomes were prepared with phosphatidylcholine:cholesterol:phosphatidic acid (molar ratio 7:2:2). Briefly, a thin dry film of lipids (28.4 mg) was dispersed in 2.0 ml PBS (phosphate buffer saline 0.01 M, pH 7.4, 0.15 M NaCl) containing 10 mg Af-gp. The lipid suspension was sonicated (1 min) in probe type sonicator (Heat systems-Ultrasonics, New York). Liposomes formed were sedimented at $105\,000 \times g$ for 45 min at 4 °C in an ultracentrifuge. The unentrapped protein was removed by washing the liposome pellet twice with PBS. The percentage of protein entrapped in liposomes was estimated by the procedure of Lowry et al. (1951) after disrupting the vesicles with equal volume of 0.9% v/v sodium dodecylsulfate. The blank liposomes prepared with PBS were similarly disrupted with 0.9% SDS and used as blanks for protein estimation.

2.4. Immunization protocol

About 5–6-week-old BALB/c mice were exposed to free and liposomal formulations of Af-gp once a week for 7 weeks. Briefly, mice were lightly anaesthetized with ether, and 100 μg of Af-gp either free or entrapped in liposomes was administered subcutaneously. The study had four groups and each group consisted of 30 animals. Group I received Af-gp entrapped in liposomes, group II received free Af-gp suspended in normal saline, group III received empty liposomes suspended in sterile PBS, and group IV received sterile PBS. Immune responses were examined on 0th, 7th, 14th, 21st, 28th, 35th, 42nd and 49th day of the experiment in four mice from each group.

2.5. Antibody response

Mice were bled every week and the amount of *A. fumigatus* specific IgG and IgE antibodies in the sera was analyzed using Indirect ELISA assay (Madan et al., 1997). For ELISA Af-gp was coated at 1.0 μg/100 μl per well in carbonate buffer (0.05 M, pH 9.6) in a microtitre plate. The wells were washed with PBST (PBS-0.05% v/v Tween-20). Unoccupied sites were blocked with 3% bovine serum albumin (BSA) w/v in PBS at 37 °C for 1 h. The wells were again washed thrice with PBST and incubated overnight with serum dilutions (1:20 v/v for IgG and 1:10 v/v for IgE) at 4 °C. After washing thrice with PBST, the wells were incubated with the respective conjugates, anti-mouse IgG peroxidase 1:1000 v/v (Sigma, St. Louis, MO) and anti-mouse IgE peroxidase 1:1000 v/v (The Binding Site, Birmingham) for 2 h at 37 °C. The color was developed at room temperature with *o*-phenylenediamine (1 mg/ml) in 0.1 M citrate buffer, pH 4.6 (0.1 M citric acid and 0.1 M disodium hydrogen phosphate) and hydrogen peroxide 0.12% v/v. The reaction was stopped after 15 min with 50 μl of

H_2SO_4 and was read at 490 nm on SpectraMax 190 ELISA reader.

2.6. Isotype specific ELISA

Serum specific IgG1 and IgG2a levels were measured by ELISA as described above. The secondary antibodies were alkaline phosphatase coupled anti-mouse IgG1 (Sigma) and anti-mouse IgG2a (Sigma) and were used at 1:1000 v/v dilution. The color was developed with 4-nitrophenyl phosphate substrate (0.6 mg/ml) in glycine buffer (0.1 M glycine, 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$). The reaction was stopped after 15 min with 50 μl of NaOH. The color developed was read at 405 nm on ELISA reader (SpectraMax 190).

2.7. Peripheral blood eosinophilia

The eosinophils were estimated in total blood (1.0 μl heparanised) by counting on a haemocytometer after staining with Dunger's reagent (volume of blood made up to 20 μl with the reagent), an aqueous solution containing eosin (0.1% w/v), acetone (10% v/v) and Na_2CO_3 (0.1% w/v).

2.8. Cytokines in spleen cultures

Spleens from animals sacrificed every week from each group were removed aseptically. Organs were minced and cells were suspended in culture medium (2×10^6 cells per well) and allowed to proliferate in RPMI 1640 medium containing 10% foetal calf serum. The supernatant

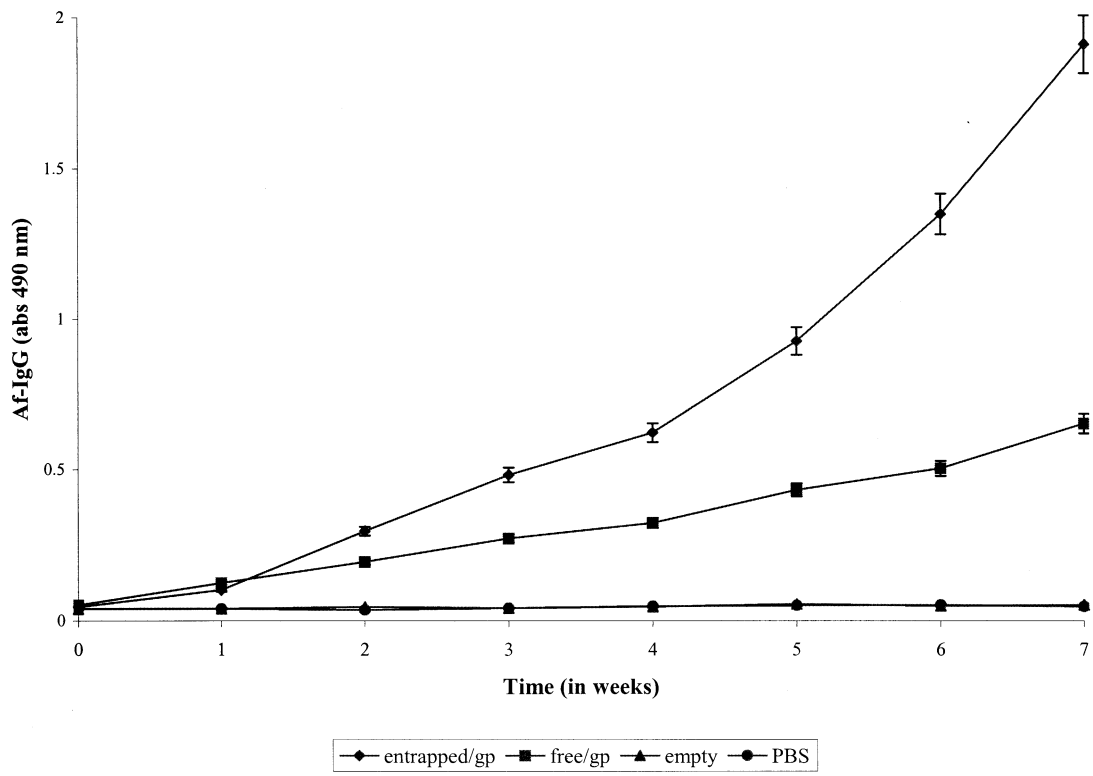

Fig. 1. *A. fumigatus* specific-IgG (Af- IgG) levels in mice immunized with free Af-gp (free Af-gp), liposomal formulations entrapping Af-gp (entrapped Af-gp), empty liposomes (empty) and sterile PBS (PBS). Each group had 30 Balb/c mice (5–6-week-old). One-hundred micrograms per mice of Af-gp either free or entrapped in liposomes (final volume 100 μl) was administered once a week subcutaneously for total of 7 weeks. The respective control groups were administered with 100 μl per mice of empty liposomes and PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

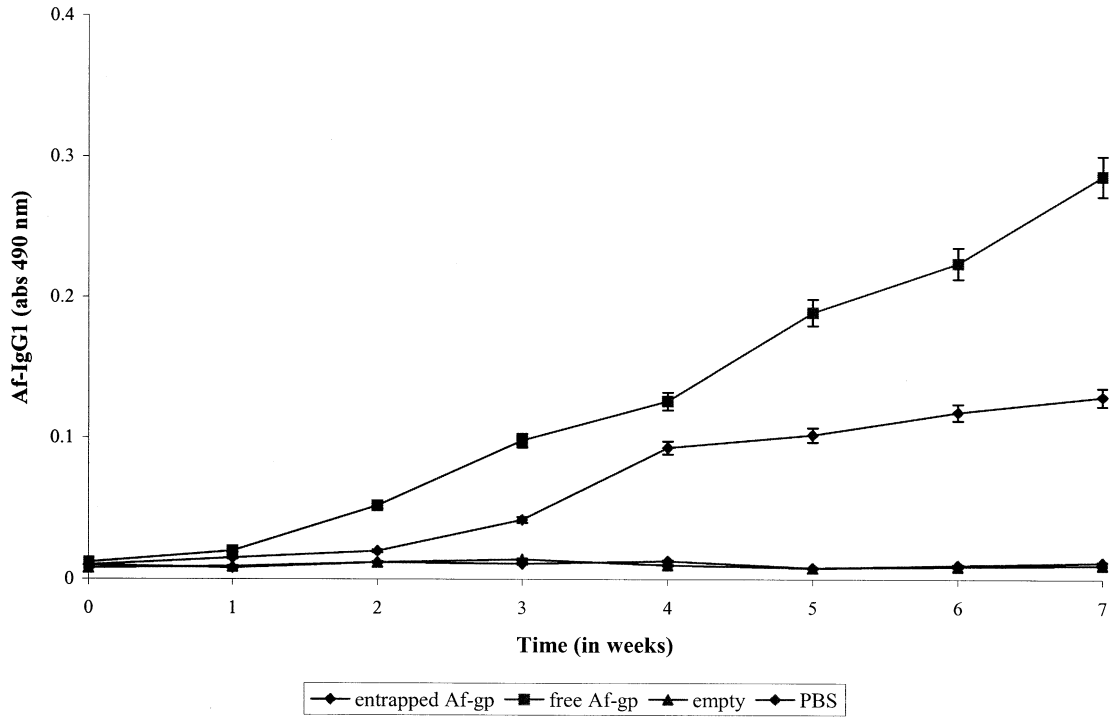

Fig. 2. *A. fumigatus* specific-IgG1 (Af- IgG1) levels in mice immunized with free Af-gp (free Af-gp), liposomal formulations entrapping Af-gp (entrapped Af-gp), empty liposomes (empty) and sterile PBS (PBS). Each group had 30 Balb/c mice (5–6-week-old). One-hundred micrograms per mice of Af-gp either free or entrapped in liposomes (final volume 100 μl) was administered once a week subcutaneously for total of 7 weeks. The respective control groups were administered with 100 μl per mice of empty liposomes and PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

was collected after incubation of 72 h at 37 °C and was assayed for different cytokines (IL-4, IFN-γ, and TNF-α) as described by the protocols supplied with endogen kits (Endogen, Inc., MA). In brief, antimouse cytokine precoated plate was incubated with plate reagent, standards and samples for 2 h at 37 °C. After washing, antimouse cytokine peroxidase was added and incubated for 1 h at 37 °C. After second washing, tetramethylbenzidine (TMB) substrate was added and kept in dark for 30 min. The reaction was stopped with the provided stop solution. The absorbance of the plate was read at 450 nm. A standard curve was made for each cytokine and the serum values for different groups of mice were estimated from the standard curve (Saxena et al., 1999).

2.9. Histological examination

The lungs were removed from the sacrificed animals 3 days after 7 weeks of immunization. The lungs were fixed in 10% formaldehyde (v/v) and stored at 4 °C. Sections were cut with a microtome and stained with haematoxylin and eosin stain. The stained specimens were observed at a magnification of × 400.

2.10. Statistical analysis

Differences in serum antibody titers between immunized groups were analyzed by Student's *t*-test. $P < 0.05$ were considered to represent statistically significant differences.

3. Results

3.1. Route of immunization

The subcutaneous route of injection was selected as it naturally provides a slow release of proteins. Animals subcutaneously injected with free Af-gp showed tissue damage at the site of injection indicating the cytotoxic and proteolytic effect of Af-gp. However, this was not seen with liposomes encapsulated with Af-gp. Phospholipids used were non-irritating, as the site of injection showed no visible inflammatory reaction during the study.

3.2. Level of A. fumigatus specific antibodies in different mice groups

All the animals immunized with either liposome entrapped or free Af-gp developed high titers of specific IgG and IgE antibodies in the serum. The *A. fumigatus* specific IgG (Af-IgG) antibody levels in the sera from different mice groups at different time intervals is shown in Fig. 1. The group that received liposome entrapped formulations of Af-gp showed elevated levels of Af-IgG compared with the group that received free antigen ($P < 0.05$). The levels increased significantly after 4th week of immunization. The Af-IgG levels in the group injected with liposomal formulations were three-folds higher at the end of the 7th week. Analysis of sera from both the control groups (injected with either PBS or empty liposomes) consistently showed insignificant antibody titers. Increased levels of *A. fumigatus* specific IgG1 (Af-IgG1) were observed in animals exposed to free Af-gp (Fig. 2), whereas animals exposed to

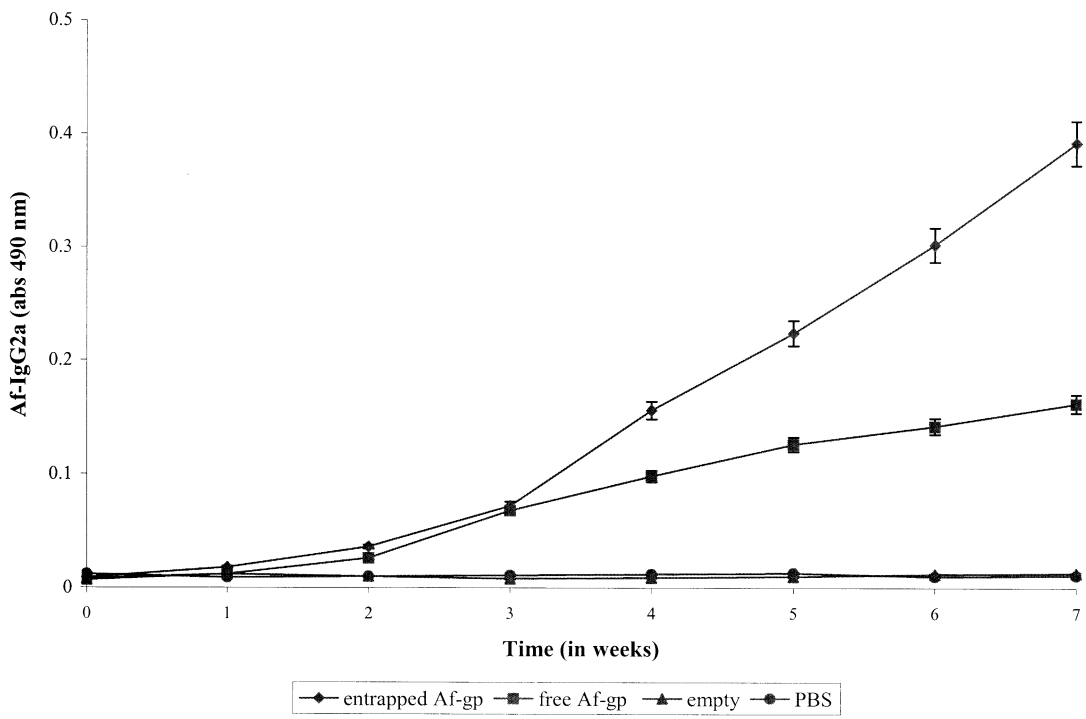

Fig. 3. *A. fumigatus* specific-IgG2a (Af-IgG2a) levels in mice immunized with free Af-gp (free Af-gp), liposomal formulations entrapping Af-gp (entrapped Af-gp), empty liposomes (empty) and sterile PBS (PBS). Each group had 30 Balb/c mice (5–6-week-old). One-hundred micrograms per mice of Af-gp either free or entrapped in liposomes (final volume 100 μl) was administered once a week subcutaneously for total of 7 weeks. The respective control groups were administered with 100 μl per mice of empty liposomes and PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

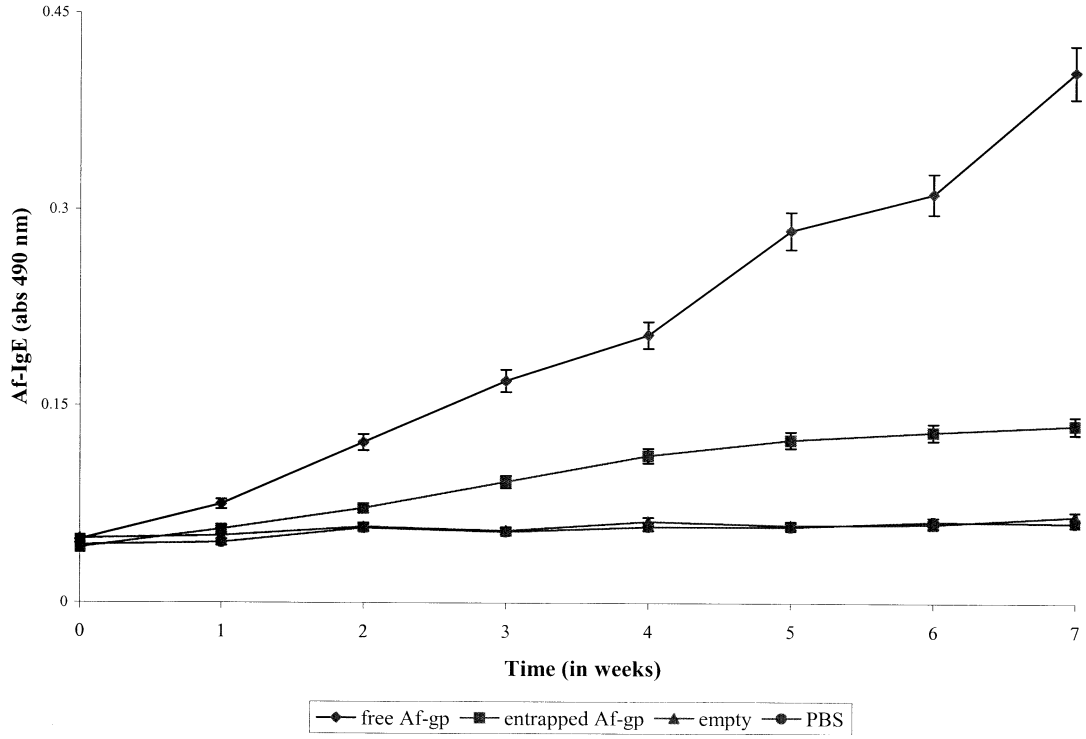

Fig. 4. *A. fumigatus* specific-IgE (Af-IgE) levels in mice immunized with free Af-gp (free Af-gp), liposomal formulations entrapping Af-gp (entrapped Af-gp), empty liposomes (empty) and sterile PBS (PBS). Each group had 30 Balb/c mice (5–6-week-old). One-hundred micrograms per mice of Af-gp either free or entrapped in liposomes (final volume 100 μl) was administered once a week subcutaneously for total of 7 weeks. The respective control groups were administered with 100 μl per mice of empty liposomes and PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

liposome entrapped Af-gp showed elevated levels of *A. fumigatus* specific IgG2a (Af-IgG2a) (Fig. 3). Af-IgG1 level was 2.2-fold higher in mice group exposed to free Af-gp in comparison to the group receiving liposomal formulations. In contrast, levels of Af-IgG2a were 2.4-fold higher in animals exposed to liposomal formulations when compared with those receiving free Af-gp. *A. fumigatus* specific IgE (Af-IgE) levels obtained with liposomal formulations constantly remained lower than obtained with free antigen ($P < 0.05$). The increase in Af-IgE level in mice receiving liposomal formulations was not significant from the 4th week (Fig. 4). At the end of the 7th week, the Af-IgE level in mice injected with free Af-gp was three-fold higher than observed in mice group receiving liposomal formulations. Sera from control groups had insignificant antibody levels.

It was observed that the ratio of Af-IgG to Af-IgE in animals challenged with liposome entrapped Af-gp was always higher than those in animals challenged with free Af-gp (Fig. 5). The difference in antibody titers was statistically significant ($P < 0.05$). The IgG/IgE ratio has been reported to be a better indicator of clinical improvement than IgG or IgE measured separately (Uhlin et al., 1987).

3.3. Peripheral eosinophilia

An increase in the number of blood eosinophils occurred in all animals exposed to Af-gp. However, a reduction in the peripheral eosinophilia was observed in the mice group immunized with liposomal formulations. Fig. 6 shows the ratio of peripheral blood eosinophil counts of the experi-

mental mice groups to their respective control groups during the study. The ratio was significantly higher (four-folds) when calculated for the mice group receiving free Af-gp compared with the group receiving liposomal formulations. The eosinophil count in both the control groups was not significantly different.

3.4. Cytokine profile in splenic supernatants

Levels of IL-4, IFN-γ and TNF-α cytokines were studied in all mice group every week. At the end of 7th week, ratios of levels of IL-4, IFN-γ and TNF-α of the group immunized with free Af-gp to the respective control group were 21, 3 and 2, respectively. On the other side, ratios of these cytokines in the group challenged with liposomal formulations to the respective control group were observed to be 10, 12 and 11, respectively (Fig. 7). The levels of IFN-γ and TNF-α showed higher concentrations in the splenic supernatants of mice group receiving liposomal formulations.

3.5. Histologic examination of the lungs

Animals exposed to free Af-gp at the end of the 7th week showed extensive infiltration by eosinophils in comparison to that of its control group (Fig. 8). The number of eosinophils in mice challenged with liposomal formulations was considerably reduced. Histopathological examination of control group receiving empty liposomes had no conspicuous cellular infiltrates.

The results with the free and liposomal formulations of Af-gp have been summarized in Table 1.

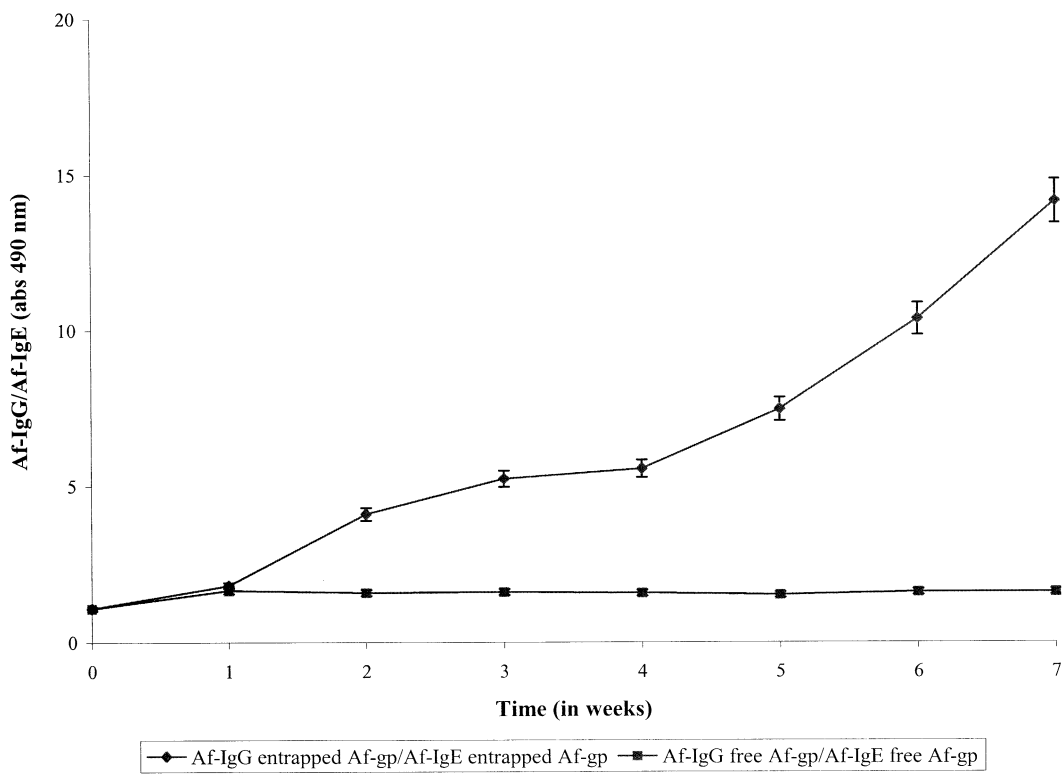

Fig. 5. Ratio of Af-IgG to Af-IgE in mice groups immunized with free Af-gp and liposomal formulations encapsulating Af-gp. Each reading is a mean ± S.D. of 12 reading (triplicate values from four animal of each group).

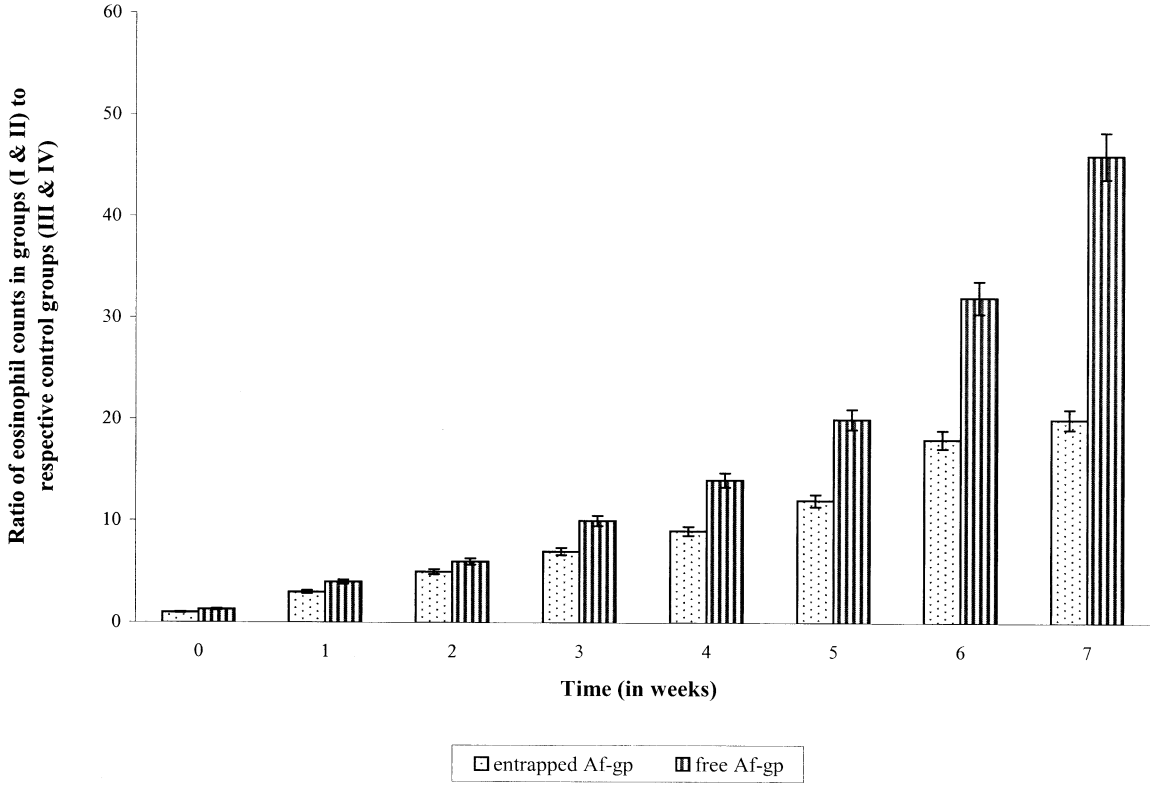

Fig. 6. Ratios of eosinophil counts in the peripheral blood of the immunized groups (I & II) to their respective control groups (III & IV). Group I received Af-gp entrapped in liposomes; group II, free Af-gp suspended in normal saline; group III, empty liposomes suspended in sterile PBS; group IV, sterile PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

4. Discussion

A number of groups have attempted to modulate the immune response to free antigens and allergens by polymerization using glutaraldehyde and formaldehyde (Akdis and Blaser, 2000), or, by entrapping in matrices like polyvinylpyrrolidone (Couvreur and Puisieux, 1993), lipids and nanoparticles (Madan et al., 1997) etc. Liposomes (lipid vesicles) are considered better carriers for allergens in immunotherapy as they are safe to administer and allergens can be easily incorporated in their natural form. Arora et al., have shown that liposome entrapped pollen allergens not only increased specific IgG levels and T suppressor cell subpopulation, but also diminished specific IgE, and plasma histamine levels (Arora and Gangal, 1990a,b, 1991, 1992, 1998). Elimination of anaphylactic reactions in ragweed sensitive guinea pigs after injection of liposome entrapped ragweed allergen has been observed (Wagner et al., 1984).

However, the immune response of fungal allergens entrapped in liposomes has not been examined so far. Af-3wcf exhibited an altered immune response when entrapped in nanoparticles. Madan et al. (1997) showed an increase in Af-IgG (1.3-folds) and a decrease in Af-IgE (three-folds) in mice receiving 350 μg of Af-3wcf entrapped in nanoparticles when compared with those receiving free Af-3wcf. However, this study did not include an enriched fraction of immunodominant antigens/allergens of *A. fumigatus*. In the current work attempt was made to decrease the antigenic

amount entrapped in liposomes by using immunodominant glycoprotein antigens/allergens of *A. fumigatus* and evaluate the immunomodulatory properties of liposomal formulations in mice. These glycoprotein allergens/antigens exhibit cytotoxic, protease and elastase activities. Their relevance in immunodiagnosis is well-documented (Kurup and Kumar, 1991).

The maintenance of higher levels of Af-IgG for 7 weeks with liposome-encapsulated Af-gp suggests that liposomes may act as a repository for the antigens and allergens of *A. fumigatus*. The lipid bilayers may also protect them from rapid degradation. Further small amounts of antigens and allergens are released continuously and effectively. High levels of Af-IgG2a and low levels of Af-IgG1 antibodies elicited with liposome entrapped with Af-gp indicated a shift to protective immune response.

The observed three-fold reduction in Af-IgE response with liposomal formulations may be a result of an altered response of immune cells. This may be attributed to the lipid bilayer that prevents direct antigen recognition by circulating B cells thus avoiding spontaneous generation of specific IgE antibodies in mice. The results of the present study are in coherence with various reports indicating the immunomodulating activity of liposome entrapped allergen(s) (Arora and Gangal, 1991, 1992, 1998). Also, a lower dose of *A. fumigatus* antigens/allergens encapsulated in liposomes could effectively reduce Af-IgE levels indicating that release may be slow and for longer duration, suggesting possible utility in hyposensitization therapy of *A. fumigatus* allergic disorders. A reduction in the level of peripheral blood eosinophils in mice immunized with liposomal formulations was observed. This was also corrob-

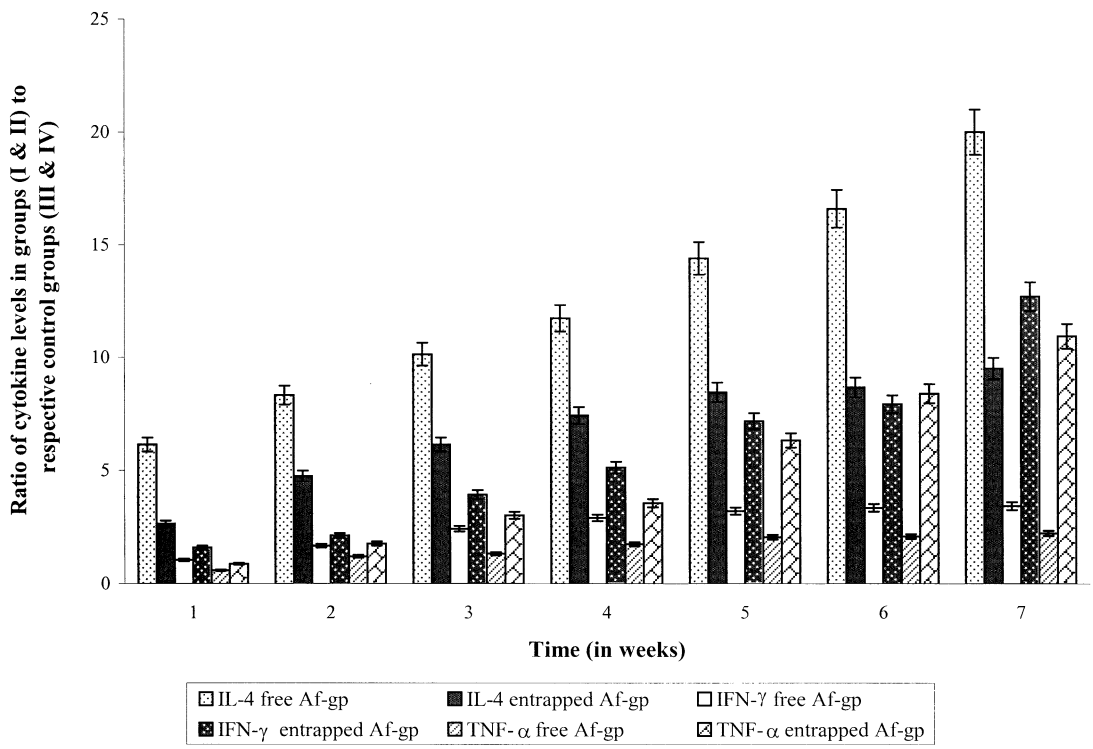

Fig. 7. Ratios of IL-4, IFN-γ, and TNF-α levels in splenic supernatants from immunized groups (I & II) to their respective control groups (III & IV). Group I received Af-gp entrapped in liposomes; group II, free Af-gp suspended in normal saline; group III, empty liposomes suspended in sterile PBS; group IV, sterile PBS. Each reading is a mean ± S.D. of 12 readings (triplicate values from four animals of each group).

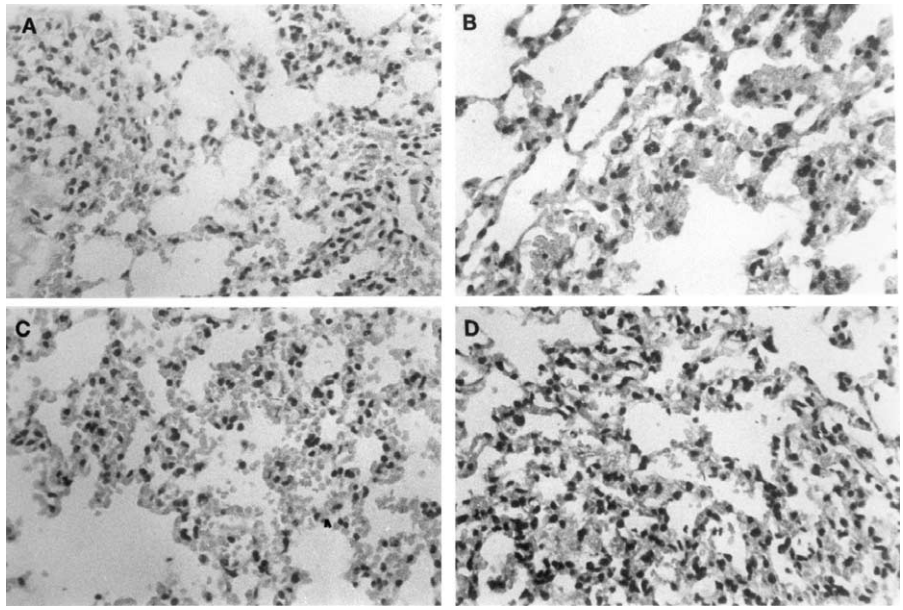

Fig. 8. Histopathological examination of the lung sections of mice from each group after the immunization schedule. The tissue sections stained with hematoxylin and eosin were observed at $\times 400$. The sections are A, control mice injected with empty liposomes; B, mice immunized with liposomal formulations showing few eosinophils; C, control mice injected with PBS; D, mice immunized with free Af-gp showing numerous eosinophils.

Table 1
Immune response elicited in mice with free and liposomal formulations of Af-gp

Preparation	Specific IgG	Specific IgE	Th1 cytokine levels	Th2 cytokine levels	Peripheral eosinophils
Liposome entrapped con A bound antigens/allergens of *A. fumigatus*	Increased by three-folds	Decreased by three-folds	Increased IFN-γ (by four-folds) and TNF-α (by 5.5-folds)	Decreased IL-4 by 2.1-folds	Decreased by four-folds
Free con A bound antigens/allergens of *A. fumigatus*	Decreased	Increased	Decreased IFN-γ and TNF-α	Increased IL-4	Increased

orated by the histologic findings in the lungs. Cellular infiltration consisting of eosinophils, seen in the lung sections, was markedly reduced in mice immunized with liposomal formulations.

Macrophages are implicated as the predominant antigen presenting cells for processing and presentation of liposomal antigens (Alving, 1991). Liposomes are engulfed by macrophages allowing liposomal processing of the antigen and presentation to T cells on MHC class II molecules. Additionally a significant portion of the liposome antigen is released into the cytosol where it can enter the class I MHC antigen processing pathway (Harding et al., 1991).

Liposome entrapped allergens of *A. scoparia* pollen have been shown to induce increase in specific IgG1, IgG2a, IgG2b, IgG3 and IgM and IFN-γ and decrease in specific IgE and IL-4 levels (Sehra et al., 1998). In the present work elevated levels of IFN-γ and TNF-α were observed, suggesting a Th1 type protective immune response using liposome based delivery system for Af-gp.

Free Af-gp receiving mice group augmented a Th2 type of cellular response (high levels of IL-4) similar to the previous reports by Kurup et al. (1994).

IFN-γ, TNF-α and granulocyte macrophage colony stimulating factors have been shown to enhance killing of *A. fumigatus* by macrophages and polymorphonuclear cells in the *in vitro* assays (Rollides et al., 1993a,b). TNF-α has been shown to play an important role in host defense against aspergillosis and neutralization of its activity results in increased susceptibility to aspergillosis (Roillides et al., 1998).

Based on the observations in the present study, such as, use of low amount antigens/allergens of *A. fumigatus* (100 μg), sustained high levels of Af-IgG, Af-IgG2a, low titers of Af-IgE, down regulation of IL-4, upregulation of IFN-γ and TNF-α and decreased eosinophilia suggest a positive therapeutic value of fungal allergens entrapped in liposomes. The change in immune response to liposomal preparations could be due to a change in the mode of antigen presentation by lipid entrapment.

An insight into the mechanism involved in the changed immune response may help in refining the liposome-system for obtaining prolonged high levels of specific IgG with a low antigenic dose. Also, this may decrease the time for the immunotherapy course. In conclusion, the present study suggests that liposomes are useful in delivery of antigens/allergens of *A. fumigatus*. Further, liposomal formulations of epitopic peptides of *A. fumigatus* may be useful for studying their protective potential and utility for immunotherapy.

Acknowledgements

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