Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is an allergic disorder caused by the opportunistic fungal pathogen, Aspergillus fumigatus (Afu). Lung surfactant proteins SP-A and SP-D can interact with the glycosylated antigens and allergens of Afu, inhibit specific IgE binding to these allergens, and block histamine release from sensitized basophils. We have now examined the therapeutic effect of exogenous administration of human SP-A, SP-D, and a recombinant fragment of SP-D (rSP-D), in a murine model of pulmonary hypersensitivity induced by Afu antigens and allergens, which resembles human ABPA immunologically. The ABPA mice exhibited high levels of Afu-specific IgG and IgE, blood eosinophilia, extensive infiltration of lymphocytes and eosinophils in the lung sections, and a Th2 cytokine response. Treatment with SP-A, SP-D, and rSP-D lowered blood eosinophilia, pulmonary infiltration, and specific Ab levels considerably, which persisted up to 4 days in the SP-A–treated ABPA mice, and up to 16 days in the SP-D– or rSP-D–treated ABPA mice. The levels of IL-2, IL-4, and IL-5 were decreased, while the level of IFN-γ was raised in the splenic supernatants of the treated mice, indicating a marked shift from Th2 to Th1 response. These results clearly implicate pulmonary SP-A and SP-D in the modulation of allergic reactions.
binding of allergen-specific IgE to mite allergens (9), suggesting that these surfactant proteins may be involved in modulating allergic reactions.

Previously, we have demonstrated that human SP-A, SP-D, and a recombinant fragment of SP-D (rSP-D), composed of trimeric neck and CRD regions, could bind to the Afu 3-week culture filtrate (3wcf) and two immunodominant glycoprotein allergens, gp55 and gp45, in a carbohydrate-specific and calcium-dependent manner, inhibit the ability of Afu-IgE to bind these allergens, and block Afu allergen-induced histamine release from sensitized basophils isolated from ABPA patients (10). SP-A and SP-D have been reported to reduce the proliferation of PBMCs isolated from mite-sensitive asthmatic children (11), and SP-D, in particular, has a suppressive effect on the secretions of IL-2 by PBMCs (12). To further dissect the protective roles of SP-A and SP-D in the pathogenesis of ABPA, we have now examined the therapeutic effect of exogenous administration of purified preparations of human SP-A, SP-D, and rSP-D in a murine model of fungal hypersensitivity caused by allergens and antigens of Afu (which mimic immunological parameters of human ABPA). Our results strongly implicated the involvement of SP-A and SP-D in protection against allergen-mediated immune reactions.

Methods

Mice. Specific-pathogen-free, 6- to 8-week-old BALB/c mice were obtained from the National Centre for Laboratory Animal Sciences (National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania, Hyderabad, India) and Harlan-OLAC, Shaw’s Farm (Bicester, Oxfordshire, United Kingdom). They received Purina chow and acidified water ad libitum. Mice were randomized before experiments were performed.

Antigens. The 3wcf (protein-enriched antigenic fraction, 27 mg/ml) of Afu (strain 285, isolated from sputum of an ABPA patient visiting the V. Patel Chest Institute, Delhi, India) was used to sensitize the mice. Culture filtrate antigens were prepared by growing the organism in a synthetic broth (L-asparagine medium) and block Afu allergen-induced histamine release from sensitized basophils isolated from ABPA patients (13), and were judged to be pure by using SDS-PAGE (Figure 1b), Western blot analysis, and amino acid composition. They were found to be free from IgG, IgM, and IgE contamination with ELISA using anti-human IgG, anti-human IgM, and anti-human IgE peroxidase conjugates, respectively.

SP-A and SP-D preparations were also examined for endotoxin levels using the QCL-1000 Limulus amebocyte lysate system (BioWhittaker Inc., Walkersville, Maryland, USA). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin). The amount of endotoxin was found to be 16 pg/µg of SP-A and 56 pg/µg of SP-D.

Expression and purification of rSP-D. A recombinant homotrimer, composed of eight Gly-Xaa-Yaa repeats from the collagen region, α-helical coiled-coil neck region, and CRD of human SP-D, was expressed in Escherichia coli and purified by a procedure involving denaturation and renaturation of the inclusion bodies, ion-exchange, affinity, and gel-filtration chromatography. The recombinant preparation was judged to be pure by using SDS-PAGE (Figure 1a), immunoblotting, and amino-terminal sequencing. The purified recombinant protein was assessed for correct folding using disulfide mapping and its crystallographic structure complexed with maltose in the carbohydrate-binding pocket (A.K. Shrive et al., unpublished data). The rSP-D was also examined for its binding to simple sugars, phospholipids, and maltosyl-BSA, as described previously (14). The amount of endotoxin present in the rSP-D preparations was estimated as described above for native SP-A and SP-D preparations and found to be 42 pg/µg of rSP-D.

Immunization of mice. Mice were divided into ten groups, with eight mice in each group (untreated ABPA mice, untreated control mice, SP-A-treated ABPA mice, SP-A-treated control mice, SP-D-treated ABPA mice, SP-D-treated control mice, rSP-D-treated ABPA mice, rSP-D-treated control mice, BSA-treated ABPA mice, and BSA-treated control mice). A murine model of pulmonary hypersensitivity was prepared as described previously (15) and called “ABPA mice” for descriptive convenience. Briefly, animals in the ABPA mice groups were lightly anesthetized with ether, and 50 µl (100 µg) of the antigen mixture per mouse was slowly applied to the nostrils using a micropipette with a sterilized disposable tip. After inoculation, the animals were held upright for a few minutes, until all antigen applied to the nostril was completely inhaled. These mice also received 100 µl (200 µg) of the same antigen mixture per mouse intraperitoneally. Intranasal instillation and intraperitoneal injections were given twice a week to each mouse for 4 weeks (28 days). The last immunization with antigen was carried out on 28th day (named day 0 for the treatment study), followed by treatment with surfactant proteins or BSA (Sigma Chemical Co., St. Louis, Missouri, USA), for the next 3 days (day 1, 2, and 3 of the treatment study). Mice in the control groups were immunized identically, but with sterile PBS.
Administration of SP-A, SP-D, and rSP-D. Groups of untreated ABPA mice and untreated control mice were intranasally administered 50 µg of PBS on days 1, 2, and 3. Groups of mice receiving treatment were named after respective proteins being administered. Human SP-A (3 µg in 50 µl of PBS per mouse) was intranasally administered to SP-A–treated ABPA mice and SP-A–treated control mice on days 1, 2, and 3. Human SP-D (1 µg in 50 µl of PBS per mouse) was intranasally administered to the SP-D–treated ABPA mice and SP-D–treated control mice on days 1, 2, and 3. Ther SP-D (1 µg in 50 µl of PBS per mouse) was intranasally administered to the groups of rSP-D–treated ABPA mice and rSP-D–treated control mice on days 1, 2, and 3. BSA (3 µg in 50 µl of PBS per mouse) was intranasally administered to BSA–treated ABPA mice and BSA–treated control mice groups on days 1, 2, and 3. The selected dose of SP-A and SP-D was based on the physiological concentrations of these proteins reported in rodent lung lavage: the SP-A concentration in the rat lavage was 7.3 ± 0.8 µg/ml and the SP-D concentration in the lavage from C57Bl/6 mice 6–8 weeks of age was observed to be 552 ng/ml (16, 17). For human lung lavage, the SP-A concentration ranges from 1 to 10 µg/ml and the SP-D concentration varies between 300 ng and 600 ng/ml (7, 18).

Afu-IgG and Afu-IgE Ab’s in mice. The Afu-IgG and Afu-IgE levels in the serum were measured by ELISA, as described previously (19). The serum dilutions used for IgG and IgE estimation were 1:50 (vol/vol) and 1:25 (vol/vol), respectively. Protein A peroxidase (for IgG) conjugate and anti-mouse IgE peroxidase conjugate (for IgE) were used at 1:1000 (vol/vol) dilutions.

Peripheral blood eosinophilia. The eosinophils were counted on a hemocytometer, using 1 µl heparinized blood stained with 9 µl of Dunger’s reagent (an aqueous solution containing 0.1% wt/vol eosin, 10% vol/vol acetic acid, and 0.1% wt/vol Na₂CO₃).

Preparation of single-cell suspension from lungs. Lungs were isolated from mice and homogenized in RPMI-1640 medium containing 10% (vol/vol) bovine serum albumin and 0.1% wt/vol analysis of purified preparations of SP-D under reducing as well as nonreducing conditions (Coomassie staining). A recombinant, homotrimeric fragment composed of the eight Gly-Xaa-Yaa repeats, α-helical coiled-coil neck region, and CRD of human SP-D was expressed in E. coli as the inclusion bodies and purified. The recombinant protein behaved as a homotrimer of about 60 kDa when examined by gel filtration chromatography and chemical cross-linking (data not shown). Under reducing conditions (lane 2), it ran as a monomer of about 18 kDa. No higher oligomers were seen when rSP-D was run under nonreducing conditions (lane 3), confirming that the trimerization was not a result of aberrant disulfide bridges between CRD regions. The rSP-D was also assessed for correct folding using disulfide mapping, and its crystallographic structure complexed with maltose in the carbohydrate-binding pockets (A.K. Shrive et al., unpublished data). (b) SDS-PAGE (10% wt/vol) analysis of purified preparations of SP-D and SP-A under reducing conditions (Coomassie staining). The majority of SP-D is composed of a 43-kDa polypeptide chain (lane 1) with faint bands corresponding to dimers and trimers of the 43-kDa chain (confirmed by immunoblotting). Two bands are seen, a major band corresponding to the 32-kDa polypeptide chain of SP-A (lane 2), together with a proportion of nonreducible dimers (64 kDa). Traces of higher oligomers and some aggregates (confirmed by immunoblotting) can also been seen. The nonreduced preparations of SP-D and SP-A behaved on SDS-PAGE as described previously (13).

Figure 1

(a) SDS-PAGE (15% wt/vol) analysis of purified preparations of rSP-D under reducing as well as nonreducing conditions (Coomassie staining). A recombinant, homotrimeric fragment composed of the eight Gly-Xaa-Yaa repeats, α-helical coiled-coil neck region, and CRD of human SP-D was expressed in E. coli as the inclusion bodies and purified. The recombinant protein behaved as a homotrimer of about 60 kDa when examined by gel filtration chromatography and chemical cross-linking (data not shown). Under reducing conditions (lane 2), it ran as a monomer of about 18 kDa. No higher oligomers were seen when rSP-D was run under nonreducing conditions (lane 3), confirming that the trimerization was not a result of aberrant disulfide bridges between CRD regions. The rSP-D was also assessed for correct folding using disulfide mapping, and its crystallographic structure complexed with maltose in the carbohydrate-binding pockets (A.K. Shrive et al., unpublished data). (b) SDS-PAGE (10% wt/vol) analysis of purified preparations of SP-D and SP-A under reducing conditions (Coomassie staining). The majority of SP-D is composed of a 43-kDa polypeptide chain (lane 1) with faint bands corresponding to dimers and trimers of the 43-kDa chain (confirmed by immunoblotting). Two bands are seen, a major band corresponding to the 32-kDa polypeptide chain of SP-A (lane 2), together with a proportion of nonreducible dimers (64 kDa). Traces of higher oligomers and some aggregates (confirmed by immunoblotting) can also been seen. The nonreduced preparations of SP-D and SP-A behaved on SDS-PAGE as described previously (13).
pared using unpaired two-tailed Mann-Whitney (non-parametric) test. The P values were considered statistically significant if they were less than 0.05.

Results
Afu-IgG and Afu-IgE Ab’s in mouse sera. A significant increase in Afu-IgG and Afu-IgE levels was observed in the groups of mice immunized for 4 weeks with 3wcf (the ABPA mice groups before any treatment), in comparison with those of control mice (the control groups before any treatment) immunized with PBS alone (Figures 2 and 3; significantly different at \( P < 0.05 \)). On intranasal administration of SP-A (3 \( \mu \)g/mouse), SP-D (1 \( \mu \)g/mouse), and rSP-D (1 \( \mu \)g/mouse) for 3 consecutive days to the groups of SP-A–treated ABPA mice, SP-D–treated ABPA mice, and rSP-D–treated ABPA mice, respectively, the Afu-IgG levels decreased in comparison with the untreated ABPA mice and BSA-treated ABPA mice groups and continued to do so until the day 16 of the treatment study (Figure 2). The SP-A–treated ABPA mice showed the lowest Afu-IgG levels on day 16 of the treatment study with a ratio of 1.512 ± 0.075 to 1 for the SP-A–treated control mice (decreased from the 0-day level of 2.752 ± 0.132 to 1). The SP-D–treated and rSP-D–treated ABPA mice groups also showed significant decreases in the Afu-IgG levels in the serum in comparison with their respective controls. The Afu-IgG levels in the groups of control mice were not affected by administration of SP-A, SP-D, rSP-D, and BSA, and were comparable to those of the untreated control groups. The ratios of Afu-IgG levels on day 16 of the treatment study in various groups — untreated ABPA mice, SP-A–treated ABPA mice, SP-D–treated ABPA mice, rSP-D–treated ABPA mice, and BSA-treated ABPA mice — to their respective control groups were 3.137 ± 0.098, 1.964 ± 0.089, 1.792 ± 0.082, 1.745 ± 0.076, and 3.227 ± 0.126, respectively (Figure 3). The BSA-treated ABPA mice and control mice showed an increase in Afu-IgE levels (Afu-IgE absorbance for BSA-
The levels of eosinophil peroxidase (EPO) activity in the lung suspensions of all the ABPA mice were raised quite significantly in comparison with the control mice on day 0 of the treatment study (Figure 6), although a significant increase in peripheral eosinophilia seen on day 4 in the untreated and BSA-treated ABPA mice was not observed with the EPO activity of these groups. The ratio of EPO activity in the groups of untreated ABPA mice versus the untreated control mice on day 0 was found to be 1.752 ± 0.128, which gradually declined to 1.220 ± 0.068 on day 16 of the treatment study. Administration of SP-D and rSP-D reduced the EPO activity in the ABPA mice on day 4 after therapy. The ratio of SP-D–treated ABPA mice to SP-D–treated control mice was 0.589 ± 0.032 and that of the rSP-D–treated ABPA mice to the rSP-D–treated control mice was 0.941 ± 0.047. Administration of SP-A, SP-D, rSP-D, and BSA did not affect the EPO activity in the control mice. Furthermore, the BSA-treated ABPA mice did not show a significant lowering in EPO activity when compared with the untreated ABPA mice. Although SP-A treatment resulted in a decrease in the EPO activity initially (ratio of the SP-A–treated ABPA mice to the SP-A–treated control mice on day 4 of the treatment study was 0.968 ± 0.048), the levels became comparable to those in the untreated and BSA-treated ABPA mice groups on day 16 of the treatment study.
Cytokines in splenic supernatants. Administration of SP-A, SP-D, and rSP-D in the ABPA mice significantly changed the levels of IL-2, IL-4, IL-5, and IFN-γ on day 10 of the treatment study in comparison to their respective controls (Figure 7). Ratios of IL-2, IL-4, IL-5, and IFN-γ levels in splenic supernatants of the untreated ABPA mice to their respective controls were found to be 2.744 ± 0.137, 5.469 ± 0.226, 1.750 ± 0.078, and 0.663 ± 0.039, respectively (Figure 7). The levels of IL-2, IL-4, and IL-5 decreased while IFN-γ showed higher concentrations in the splenic supernatants of those ABPA mice that were treated with SP-A, SP-D, and rSP-D. The percentage increase in IFN-γ levels of SP-A–, SP-D–, and rSP-D–treated ABPA mice in comparison with the untreated ABPA mice was 136.25%, 93.82%, and 156.14%, respectively. The cytokine levels in the BSA-treated ABPA mice were not significantly different from the untreated ABPA mice. Administration of SP-A, SP-D, rSP-D, and BSA did not affect the cytokine levels in the control mice.

Discussion
ABPA, an allergic disorder induced by Afu, is clinically characterized by episodic bronchial obstruction, positive immediate skin reactivity (positive wheal and erythema reaction), presence of precipitins in serum to Afu antigens, elevated total IgE in serum, elevated Afu-IgG and Afu-IgE Ab’s in serum, peripheral and pulmonary eosinophilia, central bronchiectasis, and history of expectorating brown plugs or flecks (Rosenberg’s diagnostic parameters for ABPA). To assess the protective effects of SP-A and SP-D against Afu antigens and allergens, we have generated a murine model of fungal hypersensitivity using Afu antigens and allergens described previously. This murine model may not truly represent human ABPA, where the actively growing fungus sheds antigens and allergens continuously, it has helped our understanding of the role of eosinophilia, Afu-IgG, Afu-IgE, and Th2 response in its pathogenesis. For convenience, we have referred to this animal model as murine ABPA throughout the text.

In ABPA, Afu antigens and allergens cross-link mast cell–bound IgE with subsequent release of mediators such as histamine, leukotrienes, and platelet-activating factor (PAF), leading to bronchial smooth muscle contraction and vascular permeability. The leukotrienes B4 and PAF are known chemoattractants and stimulants for eosinophils. The Afu-IgG and Afu-
IgE are also considered to bind Fc receptors present on eosinophils, leading to secretion of inflammatory mediators such as major basic protein and eosinophil-derived neurotoxins (2). In the present study, exposure to Afu antigens and allergens appeared to affect eosinophil differentiation, as inferred by the EPO activity (15). The observed correlation between eosinophilia and the Afu-IgE levels appeared to suggest a possible interaction between eosinophils and IgE in the pathogenesis of ABPA. We also observed an increase in the levels of IL-2, IL-4, and IL-5 and decrease in the level of IFN-γ in the splenic supernatants, in addition to elevated Afu-IgG levels, suggesting predominance of a Th2 response (characterized by secretion of IL-4, IL-5, IL-10, and IL-13 and generation of humoral immune responses) in the ABPA mice (20). IL-5 is a differentiation factor for eosinophils (21, 22). IL-4 is an important immunoglobulin switch factor for B cells, leading to the secretion of IgG1 (human IgG4) and IgE (23). The observation that IL-4 levels in splenic supernatants appeared to correlate with serum Afu-IgE levels, implicates IL-4 as a modulator of IgE production in the ABPA mice. IL-2 and its receptor are central to the growth and differentiation of T and B lymphocytes, natural killer (NK) cells, macrophages,
and monocytes. An increase in the IL-2 levels in the ABPA mice probably causes clonal expansion of Afu-specific Th2 cells. Lower levels of IFN-γ in the splenic cultures of the ABPA mice, as also reported previously (20), is quite significant since IFN-γ, a Th1-type cytokine, promotes cellular immunity.

After intranasal administration of physiological concentrations of SP-A, SP-D, and rSP-D in the ABPA mice, we observed a sharp decline in the Afu-IgE and Afu-IgG levels and peripheral blood eosinophilia and pulmonary infiltration. Suppression of blood eosinophilia and specific Ab levels persisted up to 4 days in the SP-A–treated ABPA mice and up to 16 days in the SP-D– or rSP-D–treated ABPA mice. Cellular infiltration consisting of lymphocytes and eosinophils, seen in the lung sections, was markedly reduced in the ABPA mice treated with SP-A, SP-D, and rSP-D. The levels of IL-2, IL-4, and IL-5 were decreased, while IFN-γ levels were raised in the splenic supernatants of treated mice, indicating a shift from predominant Th2 type to the Th1 type. It is considered that a Th1 response is protective against Afu, whereas a Th2 immune response leads to the ABPA pathogenesis (2).

We have demonstrated previously the antifungal activity of SP-A and SP-D against Afu conidia (24). Both collectins can bind glycoprotein allergens present in the Afu 3wcf (and two immunodominant antigens and allergens, gp45 and gp55), compete with Afu-IgE to bind these allergens, and also block subsequent histamine release from the sensitized basophils isolated from ABPA patients (10). Since IgE-dependent mechanisms are important in the induction of a Th2 immune response and the subsequent pulmonary infiltration of leukocytes (25), it appears that by inhibiting binding of specific IgE to glycoprotein allergens, SP-A and SP-D could be modulating the allergic reactions. Both SP-A and SP-D have been shown to suppress PHA- and anti-CD3–stimulated proliferation of PBMCs isolated from normal individuals and inhibit IL-2 production (12, 26). They can also inhibit allergen-induced proliferation of PBMCs of asthmatic children sensitive to mite allergens (11). The ability of SP-A and SP-D to suppress proliferation of specific B-lymphocytes may account for the lowering of Afu-IgG and Afu-IgE levels in the ABPA mice following treatment with SP-A, SP-D, and rSP-D; this effect may well be amplified by a decrease in the IL-2 levels since IL-2 is central to lymphocyte growth and differentiation. Since histamine release and lymphocyte proliferation are two essential steps in the development of asthmatic symptoms, the possibility of using exogenous SP-A and SP-D (and rSP-D) as therapy for allergic disorders induced by Afu and other allergens is worth exploring. However, it is worthwhile to mention that the beneficial effects of treatment with SP-A, SP-D, and rSP-D observed in our study were obtained using BALB/c mice exposed to 3wcf antigens and allergens that originated from a clinical isolate of Afu. These effects may show variability when different strains of mice or of fungal pathogen are used.

The therapeutic effect of rSP-D observed in the present study is consistent with our recent observations on the anti-Aspergillus activity of this truncated form of SP-D. TherSP-D binds to Afu conidia and the 3wcf in a calcium-, dose-, and carbohydrate-dependent manner. It can also inhibit specific IgE binding to the 3wcf in a dose-dependent manner and subsequent release of histamine from basophils isolated from ABPA patients, as well as murine ABPA. It has suppressive effect on the lymphoproliferation of Afu-sensitized mouse splenic cells. When splenocytes from the ABPA mice were treated with rSP-D in vitro, there was a decrease in the levels of IL-4 and IL-5 and an increase in the level of IFN-γ in the splenic supernatants (T. Madan et al., unpublished data), suggesting a shift from Th2 to Th1 immune response. TherSP-D has been shown recently to inhibit RSV infectivity in cell culture, giving 100% inhibition of replication. Intrapulmonary administration of rSP-D to RSV-infected mice appeared to inhibit viral replication in the lungs, reducing viral load to 80% (27). This is quite significant since RSV is known to exacerbate asthma in children.

The experiments carried out using the transgenic mouse deficient in SP-A and SP-D emphasize a key role played by these surfactant proteins in pulmonary immunological response. The SP-A gene-deficient mice are less effective in clearing lung pathogens (28). Mice deficient in SP-D show chronic inflammation, foamy alveolar macrophages secreting tenfold higher levels of hydrogen peroxide, increased activity of metalloproteinases, emphysema, and fibrosis in the lungs (29). We are currently investigating whether SP-A and SP-D gene knockout mice are more susceptible to allergic challenge using 3wcf of Afu.

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