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High-affinity IgG to a major *A. fumigatus* allergen, Asp f 2, retards allergic response

Elena V. Svirshchevskaya^{1ACDEF}, Natalya Viskova^{1BCF}, Marina Shevchenko^{1BCF},
Ludmila Alekseeva^{1E}, Alexei Marchenko², Sergei Benevolensky^{2BF},
Viswanath P. Kurup^{3AG}

¹ Department Immunology, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, Moscow, Russia

² State Research Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia

³ Medical College of Wisconsin, U.S.A.

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Summary

Background:

Allergic diseases represent a major health threat to humans. Allergen-specific immunotherapy (SIT) is one of the significant approaches to the treatment of IgE-mediated allergy and its control. The mechanisms involved in SIT-induced responses are complex and still speculative. Immunological events associated with successful SIT include an increase in allergen-specific "blocking" IgG, reduction in cytokine production, and induction of regulatory or suppressor cells. The aim of this study was to estimate the effect of SIT using a single major allergen of *A. fumigatus*, Asp f 2, or its dominant B-cell epitope, aa254-268, in a murine model of allergic aspergillosis. It is known that *A. fumigatus* (Af), a ubiquitous fungus, is implicated in the pathogenesis of a number of clinically different allergic diseases.

Material/Methods:

BALB/c and C57BL/6 mice were immunized with Asp f 2, its proteolytic fragments or the recombinant peptide aa254-268 to induce high-affinity IgG to Asp f 2. Allergy to Af was induced by subcutaneous and intranasal immunization of previously SIT-treated animals with an Af crude extract.

Results:

The results of immunological and lung histological studies demonstrate a simultaneous increase in Asp f 2-specific IgG and amelioration of allergic inflammatory symptoms in mice immunized with Asp f 2 or its peptides before exposure to Af crude allergen.

Conclusions:

Thus it was shown that the induction of IgG specific to major allergens or even to their B-cell epitopes induces protection from allergy provoked by natural allergens.

key words:

allergy • *Aspergillus fumigatus* • specific immunotherapy • virus-like particles • animal models

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Author's address:

Elena V. Svirshchevskaya, Ph.D, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya St, 16/10, Moscow 117997, Russia, e-mail: esvir@mail.ibch.ru

BACKGROUND

Allergic diseases are the result of chronic immune-system activation induced by proteins penetrating through mucosal tissues. These proteins, having diverse origins and properties, are in most cases presented by immune-competent cells to T-helper cells, which induce humoral immune response leading to IgE production. Subsequent cycles of immune-system activation lead to the recognition of additional new proteins from the complex allergens. *Aspergillus fumigatus* (Af), an ubiquitous fungus, possess such complex allergens, and on activation of the immune system with allergens the disease can progress from hyperreactivity to asthma, hypersensitivity pneumonitis, or more severe forms of allergy, such as allergic bronchopulmonary aspergillosis (ABPA) [1–4]. ABPA is characterized by the presence of specific IgE against a number of Af proteins, enhanced peripheral blood and lung eosinophilia, mild bronchospasm, and airway hyperreactivity to inhaled Af allergens [3–5]. The molecular characterization of the immunodominant allergens and antigens of Af presents complex proteins, polysaccharides, and glycoproteins. The allergens are enzymes such as elastase, protease, catalase, dismutase, ribonuclease, heat shock proteins, and unique proteins with no apparent identified functions or characteristics.

Successful allergy treatment includes specific immunotherapy (SIT) as well as more modern, peptide-based immunotherapy (PIT) [6–12]. SIT results in down-regulation of the specific immune response or enhancement of specific IgG production [6–8]. Down-regulation is primarily based on the mechanisms of peripheral tolerance induction in CD4⁺ T cells, while enhanced IgG production may reduce or even replace IgE expression, resulting in decreased allergic symptoms. On the other hand, peptide immunotherapy, such as with bee-venom peptides, induces late-phase reactions, demonstrating that active immunization precedes the induction of antigen-specific hyporesponsiveness in atopic subjects [9–10,12].

It is generally accepted that immunotherapy is efficient when applied to the treatment of patients sensitized to one or two allergens and with a mild form of hyperreactivity [13–15]. In some trials, immunotherapy was successful when 3 to 5 allergic extracts were used; however, multiple-allergen immunotherapy in allergic children with moderate-to-severe perennial asthma was ineffective [14,16]. The described effects observed after immunotherapy with natural allergen extracts resulted in improvement in the universal symptom-medication scores in the actively treated group. These included an increase in allergen-specific serum IgG, little or no change in the serum total and specific IgE levels, decrease in eosinophil counts, and Th2-associated lymphokine production [10–16]. Immunotherapy for allergic fungal diseases is not widely used because it is ineffective due to non-standardized mold extracts and the possible danger of anaphylaxis and sensitization to new allergens. The characterization and production of standardized recombinant allergens will greatly improve the diagnosis of ABPA and other forms of Af-induced hypersensitivity diseases. This may also help in developing specific immunotherapy for fungal allergy, including ABPA.

Adverse reactions resulting from SIT are the main concern when natural extracts are used [17]. The most severe side-effects

include anaphylaxis induced via IgE-mediated degranulation of mast cells leading to release of vasoactive mediators, and IgE-facilitated antigen uptake by dendritic cells [18]. IgE B-cell epitopes are mostly conformational, while IgG epitopes are both linear and conformational [19]. The disruption of IgE-binding epitopes in allergens used for SIT decreases allergenic properties, but retains most immunological responses [20–21]. Hence, deletion mutants or proteolytically modified Af proteins can be used for SIT if their IgE-binding B-cell epitopes are disrupted and non-functional [22–23]. Various studies carried out in recent years demonstrate that removal of IgE-binding B-cell epitopes reduces the risk of anaphylaxis [20–23]. Modified allergens can safely be used for rush SIT protocols for inducing stable remission faster.

As the most often registered effect after SIT with crude extracts is the increase in specific IgG, we decided to investigate whether the induction of high-affinity IgG to major Af allergens such as Asp f 2 can protect from allergy induced by a crude extract. In this report we demonstrate that the induction of IgG antibodies to Asp f 2, its proteolytic digests, or dominant B-cell epitopes reduces allergic symptoms in mice.

MATERIALS AND METHODS

Subjects

Sera from patients with ABPA, diagnosed using approved clinical and laboratory diagnostics features, were studied for Asp f 2-specific IgE by ELISA. Of the 5 patients studied, Asp f 2-specific IgE was found in three sera. All these 3 sera were used for the antibody-inhibition assay.

Animals

Ten- to 12-week-old inbred BALB/c (H-2^d) or C57BL/6 (H-2^b) mice, bred at the Institute of Bioorganic Chemistry Animal Department, Moscow, were used in this study. Institutional Animal Use and Care Committee approved the experimental protocol followed in this study.

Antigens

Recombinant Asp f 2, a 37 kDa protein, from Af was cloned and expressed in *Escherichia coli* using a pET vector [23]. The C-terminal histidine tag was used to purify the allergen by Ni-affinity chromatography. Crude Af antigen extract was prepared as previously described [24]. A mixture of culture filtrate and mycelial extract of Af was diluted to 5 mg/ml and kept frozen at –20°C. Ten peptides from Asp f 2 predicted as possible T- and B-cell epitopes [25,26] were synthesized commercially (Alpha Diagnostic Int, San Antonio, TX). Peptide sequences are shown in Table 1.

SDS-PAGE

Antigen preparations (20 to 50 µg of protein) were boiled in 2.3% SDS/10% glycerol/63 mM Tris-HCl, 5% 2-mercaptoethanol buffer (pH 6.8) for 5 min and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system using a 5% stacking gel and a 15% resolving gel. The gel was

Table 1. IgG B-cell epitopes recognized by sera from BALB/c and C57BL/6 mice immunized with full-length Asp f 2 protein or its fragments obtained by proteolysis either with trypsin or duodenase. Data are shown as O.D._{492 nm}*

Position	Peptide Sequence	BALB/c			C57BL/6			
		Asp f 2	Asp f 2-Tr*	Asp f 2-Duo	Asp f 2	Asp f 2-Tr	Asp f 2-Duo	
1	3–24**	GAVTSFPIHSSCNATQRRQIEA						
2	21–29	QIEAGLNEA						
3	31–42	ELARHAKAHILR			1.2	2.28	2.14	SD
4	92–106	GWGGHWRGANATSET	1.1***	0.63	D#	1.14	1.07	SD
5	113–127	YTTRRWLVSMCSQGY						
6	172–186	KSNGTESTHDEAFE			1.87	2.14	1.72	D
7	193–207	YAFDIAAPGVGCAGE	0.55	0.76	SD			
8	212–226	DQGHDTGSASAPAST						
9	254–268	PSNCHTHEGGQLHCT	1.83	1.73	D	1.71	2.36	1.88
	Asp f 2	Full-length protein	2.72	2.82	2.85	2.83	2.76	2.74

* Asp f 2-Tr and Asp f 2-Duo are fragments of Asp f 2 obtained by proteolysis with trypsin or duodenase, respectively;
** synthetic peptides corresponding to the indicated position in the amino-acid sequence of Asp f 2 were used to coat ELISA plates;
*** net values of optical density are shown. Sera pooled from 5 mice in each group were diluted 1:100. Standard deviation was less than 10% in all cases. Three separate experiments were performed and data from a representative experiment are shown;
dominant IgG B-cell epitopes are marked as ‘D’ for dominant and ‘SD’ for subdominant epitopes. Dominant epitopes are shown in bold.

electrophoresed at 30 mA for 4 h in 0.025 M Tris/0.19 M glycine/0.1% SDS (pH 8.3) and stained with Coomassie blue R-250. Molecular weight (MW) standards (Helicon, Moscow) used were cellulase (94.6 kDa), bovine serum albumin (66.2), ovalbumin (46); carbonic anhydrase (31), trypsin inhibitor (21.5), and lysozyme (14.4).

Antigens for specific immunotherapy

Proteolytic peptides

Asp f 2 was degraded by either trypsin or duodenase [27] to produce a digest containing polypeptides with molecular masses of 10 kDa or less. The enzyme-to-protein ratio was selected based on the profile in 15% SDS-PAGE. These digests were used to immunize mice and in the ELISA for demonstrating specific antibodies to Af. Asp f 2, degraded with trypsin or duodenase, was designated as Asp f 2-Tr and Asp f 2-Duo, respectively.

Virus-like particles containing Asp f 2 peptide aa254-268

Recombinant peptide corresponding to dominant B-cell epitope of Asp f 2 PSNCHTHEGGQLHCT (aa254-268) was expressed in the yeast *Saccharomyces cerevisiae* as virus-like particles (VLPs) [28,29]. In brief, double-stranded DNA fragments encoding Asp f 2 peptide (aa254-268) were obtained after annealing the following synthetic oligonucleotides:

5'-gatctccatcaaaactgccatacacatgaaggtggtcattacattgtactg-3';
5'-gatccagtacaatgaattgaccacctcatgtgtatggcagtttgatgga 3'.

For the presentation of heterologous peptide from Asp f 2 as the C-terminal domain of *Saccharomyces cerevisiae* p1 pro-

tein, the *Bgl*II/*Bam*HI double-stranded DNA fragment was cloned in frame with the *TyA* gene into the *Bam*HI site of plasmid pPDX-TyA [28]. As a result, the construct pPDX-TyA-Cp₂₅₄₋₂₆₈ was obtained which encoded peptide 254-268 expressed at the C-terminus (Cp₂₅₄₋₂₆₈). VLPs without heterologous inserts were used as a control preparation. The VLP was purified by stepwise sucrose-density gradient centrifugation. For the *in vivo* experiments, solutions containing VLP were dialyzed against PBS and filter sterilized before injecting into animals.

Experimental protocol for specific immunotherapy (SIT)

Six groups of BALB/c and C57BL/6 mice (5 mice per group) were used in the experiments. Mice from groups 1-6 underwent specific immunotherapy using the following antigens or vehicles: 1) Asp f 2, 2) Asp f 2-Tr, 3) Asp f 2-Duo, 4) VLP-Cp₂₅₄₋₂₆₈, 5) VLP vehicle, and 6) PBS.

To induce IgG antibodies, the antigens were dissolved in PBS at a concentration of 1 mg/ml and injected intraperitoneally. One hundred micrograms of the different preparations were injected five times, with 2- to 3-day intervals between injections. A week after the last immunization, sera were collected and tested for specific IgG1 and IgE. Three weeks after the last injection, all mice were subjected to Af crude antigen exposure to induce allergy as described below.

Induction of allergic response in mice

To induce allergy, all treated mice from groups 1-6 were primed subcutaneously (s.c.) with 100 µg of Af in Freund's complete adjuvant (FCA) (1:1 ratio of aqueous/mineral oil) twice at monthly intervals. Two days after the last s.c.

immunization, the mice were exposed intranasally (i.n.) 5 times every week to 50 µg of Af in PBS (25 µl/mouse) for two weeks. Two days after the last i.n. instillation, the mice were sacrificed and the lungs were fixed and histopathologically studied.

B-cell epitope recognition analysis by ELISA

Cross-reactivity experiments were carried out using sera from mice which underwent SIT as described above. The sera collected from these immunized mice were tested for specific IgG antibodies to Asp f 2 using enzyme-linked immunosorbent assay (ELISA). B-cell epitope analysis was studied using synthetic peptides in ELISA.

Enzyme-linked immunosorbent assay (ELISA)

Aspergillus-specific IgG and IgE were determined according to the instructions of the manufacturer. In brief, 5 µg/ml of Asp f 2 or its digests in 100 µl/well of PBS were coated on ELISA plates (Costar). Af crude antigen was used at a concentration of 100 µg/ml for coating the plates. All dilutions of the reagents were made in 1% of BSA in PBS (BSA-PBS) and incubations were made at room temperature. Plates were washed three times between incubations with 0.05% Tween 20 in PBS (PBS-T). Biotinylated IgE and IgG1-specific anti-mouse conjugates (Pharmingen) and extravidin-HRP conjugate (Sigma Chemicals) were then added and incubated as before. The color was developed using orthophenylene diamine (OPD) as the substrate, and the optical density (O.D.) was measured at 492 nm in an ELISA reader. The data are presented as net O.D. values after subtracting the blanks. B-cell epitopes of Asp f 2 were determined by peptide ELISA. Plates were coated overnight with 5 µg/ml of Asp f 2 synthetic peptides dissolved in carbonate buffer, pH 9.0, in 100 µl/well. For peptide ELISA, all dilutions of the reagents were made in PBS-T. The remaining steps of the ELISA were performed as described above.

Competitive ELISA

For determining Asp f 2-specific IgE in ABPA sera, biotinylated anti-human IgE and IgG conjugates (Vector Laboratories, Burlingame, CA) were used at 2 µg/ml. For the competitive ELISA, Asp f 2-specific IgE-positive sera were diluted to give an O.D. of 1.0 after prior estimation by ELISA as described above. IgE and IgG binding of recombinant Asp f 2 proteins were studied by competitive ELISA, where a full-length protein was used to coat the solid phase. The proteolytic digests of Asp f 2, i.e. Asp f 2-Tr or Asp f 2-Duo, as well as the full-length Asp f 2 protein were used in fluid phase for competitive binding. The procedure used for competitive binding was briefly as follows. The plates were coated with 5 µg/ml full-length Asp f 2 in 100 µl/well in PBS overnight. The plates were then washed with PBS-T and blocked with BSA-PBS for 1 h at room temperature (RT). After washing, Asp f 2, Asp f 2-Tr, or Asp f 2-Duo were added at 10, 1, 0.1, and 0.01 µM in BSA-PBS. Control wells contained only BSA-PBS. Plates were incubated after adding ABPA serum appropriately diluted overnight at 4°C to reach the equilibrium state between immobilized and fluid-phase antigens. After washing, biotinylated anti-human IgE or IgG conjugates were added and incubated for 1 h at room temperature. ExtrAvidin-HRP conjugate (Sigma Chem. Co) was

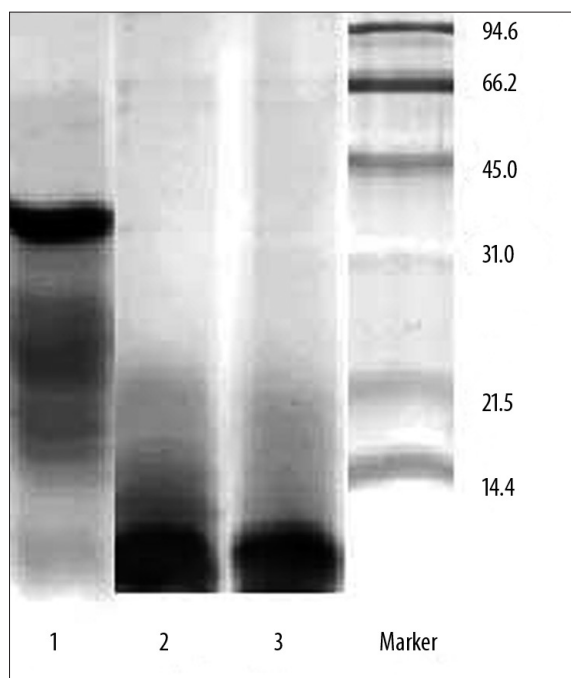


Figure 1. Gel-electrophoresis of Asp f 2 (Lane 1), Asp f 2-Tr (Lane 2), and Asp f 3-Duo (Lane 3) in 15% PAAG. MW of Asp f 2 corresponds to 37 kDa, while MW of polypeptides are less than 10 kDa. MW of markers are shown in kDa.

added after the washing and the color developed by OPD was measured as described above.

Lung Histology

Lungs from the controls and Af antigen-treated mice were inflated with one ml of 10% (vol/vol) neutral buffered formaldehyde and fixed in 10% neutral buffered formaldehyde. Tissues were processed, sectioned, and stained with hematoxylin and eosin (Sigma Chem. Co). Sections were analyzed by two independent pathologists who were blinded to treatment information.

Statistics

The means and standard deviations were calculated for each group, and the groups were compared with paired t-test analysis using the Excel Statistics Program.

RESULTS

IgE B-cell epitopes are mostly conformational, while IgG epitopes are linear

Allergenicity is characterized by the ability of an allergen to bind to specific IgE in the sera of patients. Human sera from ABPA patients with specific IgE to Asp f 2 were used to determine the effect of protein degradation on IgE binding. To compare the allergenicity, Asp f 2 was degraded to polypeptides by digestion with trypsin or duodenase [26]. The degraded pools contained polypeptides with molecular weights frequently less than 10 kDa (Figure 1). These pools as well as the full-length Asp f 2 protein were used in a fluid phase to inhibit ABPA serum

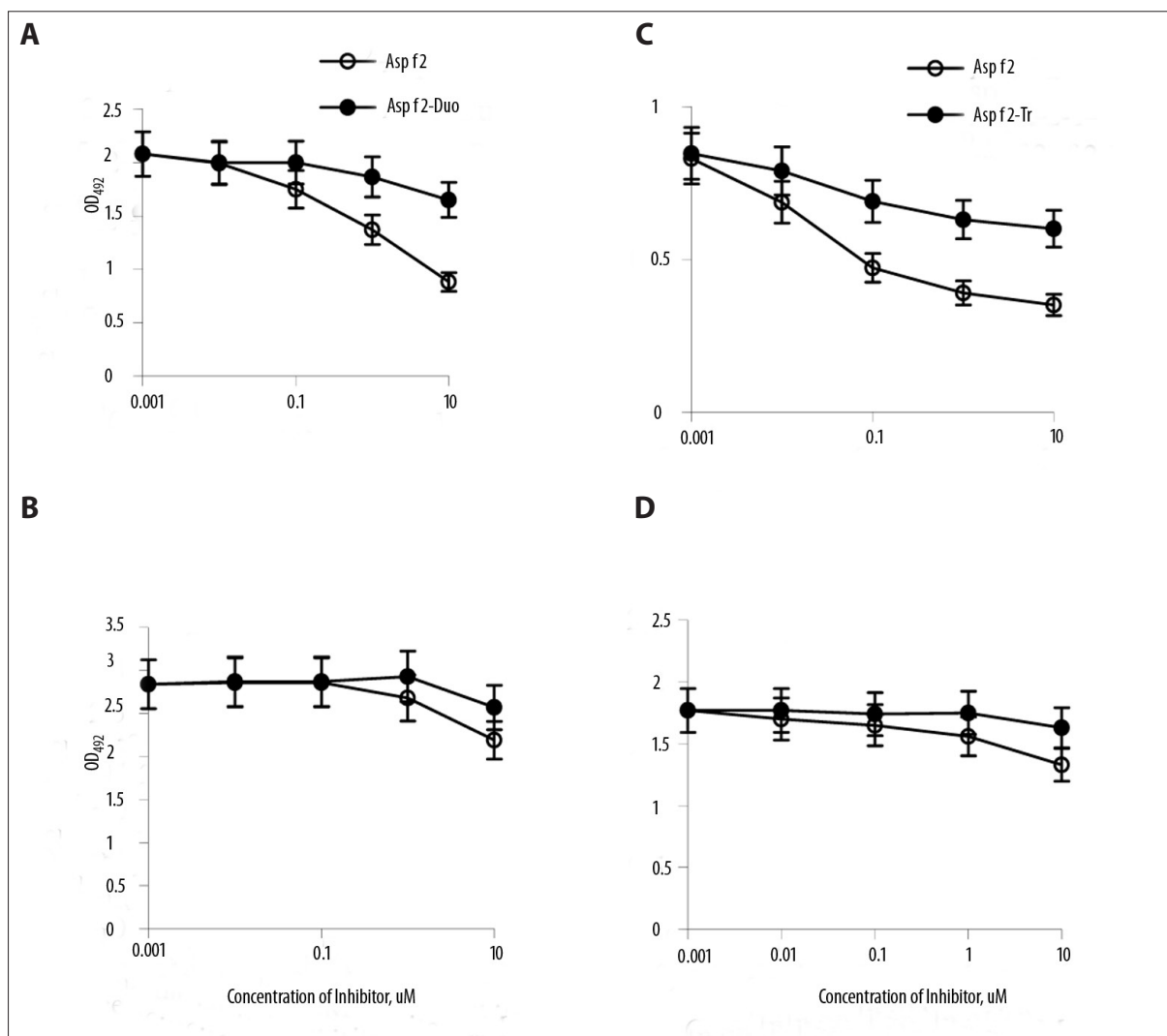


Figure 2. Competitive inhibition of IgE (A,C) and IgG (B,D) binding to immobilized Asp f 2 by Asp f 2 or its proteolytic fragments Asp f 2-Tr and Asp f 2-Duo in fluid phase. Analogous data were obtained for three different sera. (A) and (B) represents the results with serum #1, (C) and (D) with serum #2.

IgG and IgE binding using a competitive binding assay. The full-length protein in the system was able to inhibit 50% to 80% of IgE binding (Figure 2A,C), while only 20% to 30% inhibition of IgG binding (Figure 2B,D). This indicates that IgE binds mostly to conformational B-cell epitopes preserved in a fluid phase. In contrast, IgG binds to linear B-cell epitopes, and only 20% of the antibodies recognize conformational epitopes. Proteolytic degradation of proteins induces conformational changes leading to a 10^3 – 10^4 times less effective inhibition of IgE binding (Figure 2A,C) and inability to compete with IgG binding at all concentrations used (Figure 2 B,D). These results demonstrate that proteolytic digestion results in loss of allergenicity and hence the polypeptides can be used at a higher concentration in SIT.

Immunogenic properties of polypeptides

To compare the immunogenic properties of the proteolytic peptides, we injected mice every 2-3 days for 5 times with antigens reconstituted in PBS. Immunization of mice with

polypeptides was sufficient to induce antigen-specific IgG antibodies (Table 2).

Cross-recognition of proteins by sera from mice immunized with full-length protein was studied by ELISA employing the polypeptides immobilized on a solid phase. Similarly, the ability of sera from mice immunized with digested peptides to bind the corresponding full-length protein was studied using ELISA plates coated with full-length Asp f 2. These experiments were carried out in both BALB/c and C57BL/6 mice, using the two different enzyme-digested peptides (Table 2). The IgG in all the sera was able to cross-recognize both properly folded full-length Asp f 2 or its proteolytic peptides, which contained mostly linear B-cell epitopes. However, up to 40% of antigenic activity was lost due to the disruption of conformational epitopes induced by proteolysis. The remaining activity of the degraded polypeptides was sufficient to use them for SIT. No IgE response was found when mice were immunized with Asp f 2 or peptides (data not shown).

Table 2. Cross-recognition of Asp f 2 and its proteolytic polypeptides by sera from BALB/c and C57BL/6 mice immunized with full-length protein or its fragments obtained by proteolysis either with trypsin (Tr) or duodenase (Duo).

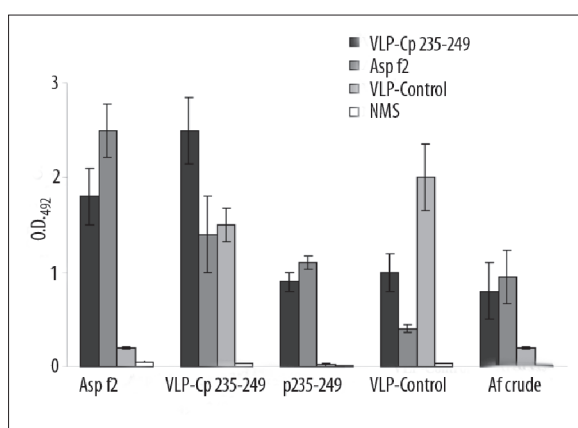
BALB/c						
Immunogen	Asp f 2*		Asp f 2-Tr		Asp f 2-Duo	
Antigens coated on plates	O.D.	%	O.D.	%	O.D.	%
Asp f 2**	1.54***	100#	0.93	60	0.81	53
Asp f 2-Tr	1.25	81	0.94	61	0.92	60
Asp f 2-Duo	1.2	78	0.94	61	0.93	60
C57BL/6						
Asp f 2	1.3	100	1.07	82	0.86	66
Asp f 2-Tr	1.18	91	1.06	82	0.97	75
Asp f 2-Duo	1.17	90	1.13	87	1.1	85

* Asp f 2-Tr and Asp f 2-Duo are fragments of Asp f 2 obtained by proteolysis with trypsin or duodenase, respectively;

** immunogens used for *in vivo* immunization are shown in the upper row, while antigens used to coat plates for ELISA are shown in the left column;

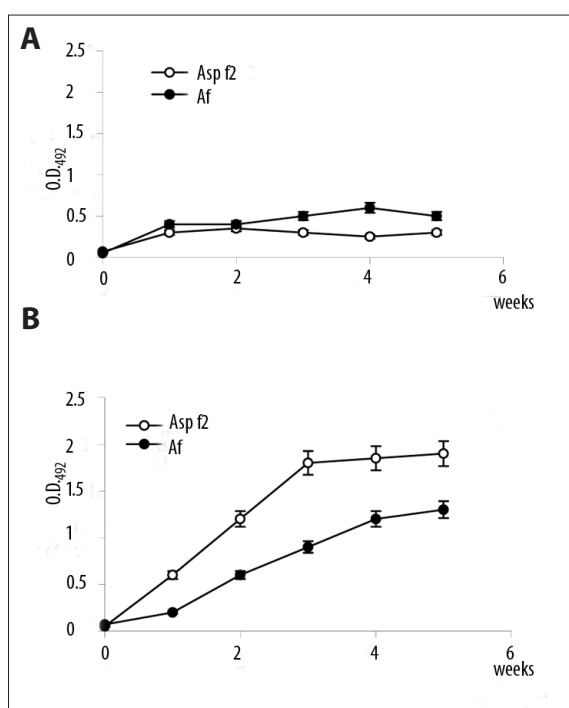
*** net values of optical density are shown. Sera pooled from 5 mice were diluted 1:100. Standard deviation was less than 10%. Three separate experiments were carried out and the data from a representative experiment are shown;

immune responses in mice immunized with full-length Asp f 2 and analyzed against Asp f 2 were taken as 100% and are shown in bold.

**Figure 3.** Cross-recognition of different antigens by sera from mice immunized with VLP-Cp254-268 (see legends in the insert), Asp f 2, VLP-Control, and from normal mice (NMS). Antigens used to coat ELISA plates are shown along the X-axis. All sera were tested at 1:100 dilution.

Fine specificity of B-cell response

To compare the specificity of B-cell epitopes recognized by sera from mice immunized with polypeptides, we analyzed the sera using a peptide ELISA, where synthetic peptides from Asp f 2 were coated on the ELISA plates (Table 1). Asp f 2-dominant B-cell epitopes recognized by BALB/c mice were demonstrated in p4 and p9 peptides, while C57BL/6 mice recognized p6 and p9 peptides. One dominant and some subdominant epitopes were preserved when mice were immunized with degraded peptides. Peptide p9 has a common dominant epitope recognized both by BALB/c and C57BL/6 mice. It was selected for producing a recombinant peptide.

**Figure 4.** Induction of IgG to Af (black circles) and Asp f 2 (white circles) in mice immunized with Af (A) or Asp f 2 (B). Mice were immunized with 100 µg/mouse of Asp f 2 or 500 µg Af, both antigens dissolved in FCA. Sera were collected and tested in dynamics.

These results demonstrate that the polypeptides obtained after proteolytic degradation were not only immunogenic, but also induced a humoral immune response similar to that induced by the full-length protein.

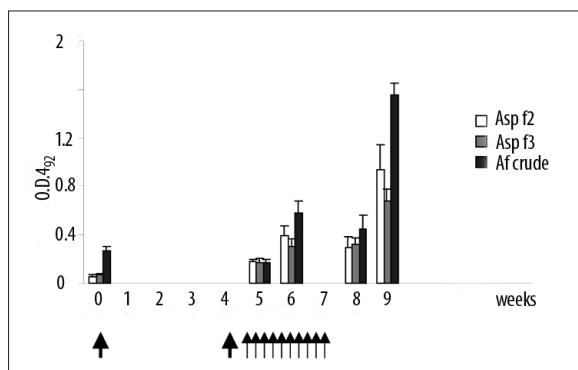


Figure 5. IgG1 production in BALB/c mice induced by Af crude extract. Thick arrows show immunizations with Af in FCA, thin arrows show i.n. immunizations. Sera pooled from 5 mice and diluted 1:50 were tested against Af crude (black bars), Asp f 2 (white bars) and Asp f 3 (gray bars).

Immunogenic properties of VLP-Cp₂₅₄₋₂₆₈

VLP-Cp₂₅₄₋₂₆₈ is a recombinant Asp f 2 peptide representing a B-cell epitope comprising residues aa254-268 and recognized by both BALB/c and C57BL/6 mice. This peptide was expressed previously in the form of virus-like particles in the yeast *S. cerevisiae* [28]. To analyze the immunogenic properties of VLP-Cp₂₅₄₋₂₆₈, sera from mice immunized with this peptide were tested against full-length Asp f 2, synthetic peptide p9, and VLP-vehicle. Peptide-specific serum recognized Asp f 2, VLP-Cp₂₅₄₋₂₆₈, VLP-vehicle, p9, and Af crude extract (Figure 3). The recognition of Asp f 2 in Af crude extract is an indication that recombinant VLP-based peptide can be used for SIT.

Immune response to Af crude allergens

Immune response to crude allergens differs from the response to pure recombinant proteins originating from the same source. This difference is clearly evidenced in the IgG production after exposure to crude extract in mice. Mice immunized with two injections of Af in FCA produce only low levels of Af- or Asp f 2-specific IgG (Figure 4A), while similar immunization with Asp f 2 induces high antibody production (Figure 4B). Additional s.c. and a series of i.n. immunizations with Af led to the induction of IgG (Figure 5). To estimate the recognition of individual antigens by the sera of mice immunized by two s.c. and 10 i.n. injections of Af, we tested it against two major Af proteins, namely Asp f 2 and Asp f 3. Recognition of these antigens by antibody correlated well with the Af response in mice (Figure 5). Thus it is likely that if Af-specific IgGs are induced, they recognize not only Asp f 2 and Asp f 3, but also many other different proteins from the Af extract. No Af-specific IgE was detected in the sera, although a significant increase in the total IgE was found (data not shown). The inability of Af to induce IgG antibody response after two injections of Af in FCA is probably the consequence of the presence of the low concentration of individual proteins in the extract. Besides, the presence of a number of allergens may also modify the immune response to the protein components.

Protection of mice with SIT

To analyze the effect of SIT in allergy, BALB/c and C57BL/6 mice were immunized initially with Asp f 2, the proteolytic

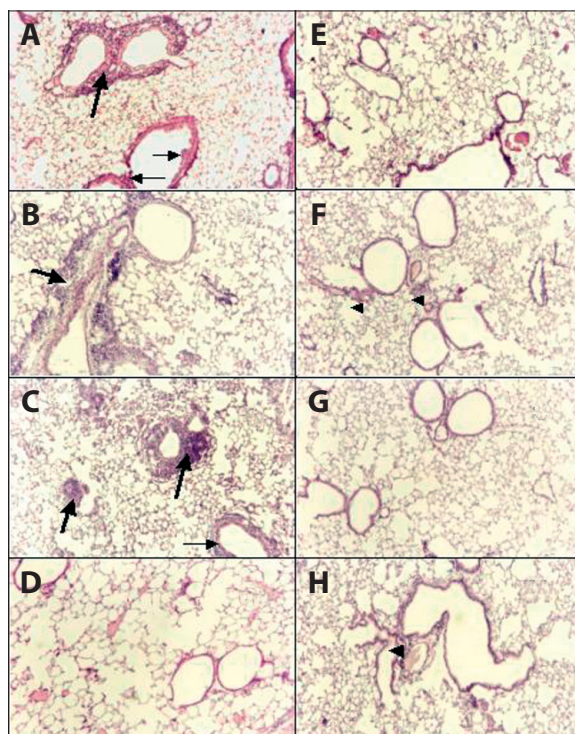


Figure 6. Lung histology of SIT treated mice. (A,B). Thick arrows show peribronchiolar and perivascular infiltrates with lymphocytes, neutrophils, and macrophages in the lungs of BALB/c (A) and C57BL/6 (B) mice immunized with PBS and then subjected to allergy induction with Af crude extract. Hyperplasia of epithelial cells is also present (thin arrows). (C). Lung section of VLP-vehicle immunized BALB/c mouse challenged with Af crude extract, showing peribronchiolar and perivascular infiltrates with lymphocytes, neutrophils, and macrophages (thick arrows). (D). Section of lung from intact mouse; the lung is normal. E-H. Sections of lungs from mice which underwent SIT with Asp f 2 (E), Asp f 2-Tr (F), Asp f 2-Duo (G), or recombinant Asp f 2 peptide VLP-Cp₂₅₄₋₂₆₈ (H). The lungs are practically normal, with small foci of perivascular infiltration (arrowheads) (H & E, $\times 160$).

digests Asp f 2-Tr, Asp f 2-Duo, or the Asp f 2 recombinant peptide VLP-Cp₂₅₄₋₂₆₈. Control mice were immunized with VLP-vehicle or PBS. To induce allergic inflammation in the lungs, mice were primed with Af extract i.p. twice and then exposed to Af by the i.n. route according to the scheme depicted in Figure 5. Antibody response in SIT-treated mice before and after Af crude extract sensitization is shown in Table 3. The lung histology of BALB/c mice is shown in Figure 6. Control mice treated previously with VLP-vehicle or PBS alone developed lung inflammation when exposed to the antigens. Numerous zones of inflammation characterized by perivascular and peribronchial infiltration with lymphocytes, neutrophils, macrophages, and occasional eosinophils were found in all lobes of lungs studied (Figure 6 A–C). Preventive immunization with Asp f 2, Asp f 2-Tr, Asp f 2-Duo, and VLP-Cp₂₅₄₋₂₆₈ ameliorated the allergic reaction in the lungs of all treated mice almost completely, with rare small foci of perivascular infiltration with lymphocytes (Figure 6 E,H). There were no major differences in the allergic response induced in BALB/c and C57BL/

Table 3. IgG response to Af in BALB/c mice during SIT and allergy induction by Af. Data are shown as O.D._{492 nm}*

Antigens used for SIT	Before SIT	After SIT and before allergy induction	After allergy induction, week 7	After allergy induction, week 9, histology
Asp f 2	0.05±0.01*	1.21±0.30**	1.55±0.35	1.23±0.37
Asp f 2-Tr	0.05±0.01	1.05±0.25	1.25±0.14	1.15±0.28
Asp f 2-Duo	0.06±0.02	1.09±0.15	1.32±0.22	1.06±0.12
VLP-Cp ₂₅₄₋₂₆₈	0.05±0.01	0.92±0.15	1.21±0.30	1.12±0.15
VLP-vehicle	0.04±0.01	0.22±0.05	0.53±0.03	1.54±0.42
PBS	0.05±0.01	0.25±0.03	0.44±0.03	1.64±0.36

* sera from 5 individual mice were collected, tested in ELISA, and the average is calculated. Representative data from three different experiments are shown;

** high titers of IgG are shown in bold.

6 mice (Figure 6 A,B). All preparations used for SIT were also protective in C57BL/6 mice.

DISCUSSION

Innate immunity plays a major role in the defense against Af by the recognition and clearance of the organism in immunocompetent hosts [30]. The concentration of Af proteins penetrating through the epithelium is probably very low due to the activity of innate immunity mechanisms of lung clearance. Nevertheless, the specific immune system of both patients with allergy and healthy individuals recognize Af proteins during chronic exposure to Af [31–32]. It has been shown that about 100 proteins and glycoproteins from Af can bind to human IgG [33]. However, IgE from the sera of asthmatic and cystic fibrosis patients sensitized to Af, but without ABPA, binds only to a limited number of proteins, such as Asp f 1 and Asp f 3. On the other hand, ABPA sera bind to Asp f 2, Asp f 4, Asp f 6, and Asp f 16 in addition to Asp f 1 and Asp f 3 [34,35]. Analogous data were obtained for grass pollen allergy [36]. These findings demonstrate that progression of the disease correlates with the increased number of allergens recognized by IgE. It is possible that the allergy results from recognition of many proteins from the allergens by the immune system. Total allergic response is likely to be the sum of these different responses. In contrast, a normal protective immune response to any pathogen is usually focused on a small number of major proteins from the pathogen [37,38]. It is possible that immunization with major allergens can limit the recognition of new proteins of the allergens present in small quantities.

We have evaluated the effect of specific immunotherapy based on active immunization of mice with a single major allergen from Af, Asp f 2, or its dominant B-cell epitope on allergic responses in a murine model of allergic aspergillosis. The main features of ABPA are the presence of high levels of total and specific IgE, lung, blood and bone marrow eosinophilia, hyperplasia of goblet cells, and mucus production in the lungs. In mice immunized with pure Af proteins, no marked increase in IgE and eosinophil counts were noted nor did these animals show presence of goblet cell hyperplasia and lung fibrosis [39]. In contrast, immunization with Af crude extract induced an increased level of total serum IgE,

but not IgE specific to Af. Moreover, immunization of mice with Af crude extract failed to induce significant IgG production (Figures 4,5, Table 3), but effectively induced allergic responses, including lung inflammation and infiltration with lymphocytes, neutrophils and eosinophils, goblet cell hyperplasia, and lung fibrosis in mice (Figure 5A–C). A significant level of IgG production in response to Af crude extract was achieved only after two s.c. immunizations and 10 i.n. administrations (Figure 5, Table 3). At that moment, not only Asp f 2, but also Asp f 3 and, likely, other Af proteins were recognized by IgGs (Figure 5). In contrast, a single s.c. immunization with pure Asp f 2 induced a high level of Asp f 2-specific IgG (Figure 4, Table 3). Sera of mice immunized with Asp f 2 or its B-cell epitope readily recognized this protein in Af crude extract (Figure 3). This difference can be a consequence of poor recognition of individual antigens when mice were immunized with a mixture containing many proteins. This can lead to the induction of low-affinity IgGs, which were not detected by ELISA technique. Immunization with pure antigens is more efficient as only the protein is recognized by cooperating T and B cells. Induction of high-affinity IgG to major proteins can permit focusing immune response on these proteins and by this means limiting the recognition of other, minor antigens. In fact, preventive immunization of mice with Asp f 2 protected them from induction of allergy (Figure 5E). This effect can be attributed to different mechanisms: induction of neutralizing, high-affinity antibodies to Asp f 2, activation of regulatory T cells, or the formation of anergic T cells which are refractory to i.n. stimulation with Af extract containing high concentrations of Asp f 2.

Nevertheless, usage of full-length Af proteins for SIT cannot be safe in patients having even a low concentration of IgE specific to these proteins. Disruption of IgE-binding epitopes decreases the allergenic properties of the full-length proteins while retaining their immunogenicity (Figure 2, Tables 1,2). Mice immunized with degraded allergen produced IgG antibodies which recognized both the full-length protein and its dominant epitope peptides (Tables 1,2). Thus, proteins degraded to polypeptides by proteolysis lost some of the conformational IgE-binding B-cell epitopes, while they preserved a number of linear IgG-binding epitopes. Immunization of mice with degraded peptides retarded allergic response as effectively as immunization with Asp f 2 (Figure 6F,G).

Immunization of mice with a pure protein induces IgG antibodies directed to a limited number of B-cell epitopes. Two dominant B-cell epitopes were recognized by BALB/c and C57BL/6 mice immunized with Asp f 2 (Table 2). We have tried to induce protection in mice using a single B-cell epitope of Asp f 2 produced as a VLP for SIT. Preventive immunization of mice with this construct protected mice from allergy induced later with Af extract (Figure 6H).

It is also known that IgG can be responsible for complement activation or immune complex disease induction in allergic patients. It is not known why IgGs specific to Af are pathogenic in some and harmless in others. We hypothesize that the IgGs involved in allergic reaction have low affinity. It is possible that in predisposed subjects these low-affinity IgGs form immune complexes with the antigens which are not cleared by FcγR⁺ cells, but in turn activate complement. It is possible that a balance between immune complex clearance and complement activation results in anergy to allergen in healthy people or type III allergic reaction in predisposed subjects. Chronic activation of B cells by the allergen via the BCR can induce Ig isotype switching, leading to IgE production. If this is true, one way to bias this process to immune complex clearance is the induction of high-affinity allergen-specific IgGs to major allergens at an early stage of the disease.

CONCLUSIONS

It was demonstrated that immunization with a high dose of a pure major allergen from *A. fumigatus*, Asp f 2, protects mice from lung inflammation induced by a crude extract of the fungus. The same effect was found when mice were immunized either with a proteolytic digest of Asp f 2 or its dominant B-cell epitope encompassed in residues 254-268 aa and expressed as a yeast virus-like particle. The use of proteolytic or recombinant peptides of Asp f 2 decreases IgE binding, providing a less allergenic preparation for SIT. We hypothesize that protection is a consequence of immune response focusing on major antigens presented in high quantity in crude allergen. Immunization with high doses of pure proteins or peptides induces IgG and, likely, IgA with higher affinity, able to compete with IgE and form immune complexes with fungus allergens which are thus better cleared from the lungs.

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