

Fusarium solani major allergen peptide IV-1 binds IgE but does not release histamine

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Summary

Background *Fusarium solani* (FS) is an important allergen source afflicting 4% of the nasobronchial allergy patients. Fus s I₃₅₉₆*, a 65 kDa major glycoprotein allergen of FS reacts with 95% fungus sensitive patients.

Objectives To purify and characterize a potent peptide from Fus s I₃₅₉₆* which may be useful for therapeutic purposes.

Methods The 65 kDa protein was sequentially cleaved with trypsin and cyanogen bromide (CNBr). The cleaved products were purified on reverse phase high performance liquid chromatography (rpHPLC) column and functionally characterized by *in vitro* and *in vivo* methods for its IgE binding and histamine release.

Results The protein on cleavage showed 11 peaks (I to XI). Of these, peaks I, III, IV and V were highly allergenic as determined by IgE ELISA. These peaks were further purified and peptide IV-1 was most potent in comparison to other peptides by ELISA-inhibition. This peptide showed IgE binding but could not evoke intradermal response in *Fusarium*-sensitive patients. Heparinized blood challenged with peptide IV-1 does not release histamine. Preincubation of heparinized blood with peptide IV-1 and challenging with crude extract blocked histamine release in a dose dependent manner.

Conclusion Peptide IV-1 binds to IgE but does not release histamine, demonstrating its potential use in therapy of *Fusarium*-allergic patients.

Keywords: allergens, fungus, *Fusarium*, immunotherapy, peptide

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Introduction

Airborne fungal spores are important triggers of respiratory allergy and are present worldwide. *Fusarium solani* (FS) is an important fungal allergen for IgE-mediated allergic reaction in 4% of the nasobronchial allergy patients. In previous studies, we have standardized the growth parameters of FS and characterized FS allergens [1]. A 65 kDa protein was present in culture filtrate (CF) extract, spore and mycelium of FS and was recognized by sera of all FS-sensitive patients. This protein was purified to homogeneity and was designated as Fus s I₃₅₉₆* [2,3]. It is present in two other *Fusarium* species, *F. equiseti* and *F. moniliforme*, and

crossreacts with fungi responsible for allergic manifestations [4,5].

Immunotherapy is practiced as a preventive method for allergy disorders. However, the present regimen is associated with adverse reactions. Efforts are on the way to develop safer and tolerable preparations. There is an immediate rise in IgE with the therapy resulting in mediator release/anaphylaxis. To increase the safety of immunotherapy, allergen derivatives which have reduced IgE binding capacity but contain T cell stimulating sequences, or inhibit the IgE crosslinking on the effector cells, are possible candidates [6,7]. Small peptides have potential to bind IgE and inhibit its receptor crosslinking thereby reducing allergic manifestations. In addition, higher peptide concentration can be injected, with no adverse reactions, to raise protective antibodies. Recently, *Aspergillus* peptides

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have been shown to elicit humoral and cell mediated responses [8], and the peptide containing T cell epitopes of allergen are important for immunotherapy. *Asp f 1* peptide A5 (aa36–51) demonstrated reduced IgE binding but stimulated peripheral blood mononuclear cells (PBMC) from allergic bronchopulmonary aspergillosis patients [8]. Peptide from *Alt a 1*, a major allergen from *Alternaria*, induced both IgE and IgG antibodies in Balb/c mice but bound weakly with IgE [9].

In the present study, a peptide from *Fus s I₃₅₉₆** was isolated by rpHPLC after CNBr and trypsin cleavage. The allergenic peptide was purified to homogeneity for *in vitro* and *in vivo* studies and was characterized by its IgE binding and histamine releasing ability to evaluate its usefulness in immunotherapy.

Materials and methods

Preparation of extract

Eleven days culture of *F. solani* (strain 3596, Indian Agriculture Research Institute, Delhi, India) was grown in synthetic medium [10] and CF extract was prepared as described earlier [1].

Purification of a 65 kDa major allergen of *F. solani*

F. solani was sequentially fractionated using DEAE-Sephadex A-50, Sephadex G-200 and Mono Q column (FPLC) to isolate a 65 kDa major allergen designated as *Fus s I₃₅₉₆** [2].

CNBr-trypsin digestion

One hundred microgram of *Fus s I₃₅₉₆** in 200 µl of 50 mM ammonium bicarbonate buffer pH 7.8 containing 1 mM dithiothreitol (DTT) was treated with 200 µl CNBr solution (10 mg/mL in 0.2 M HCl) [2]. The sample was dialysed, lyophilized and further digested with 1 µg trypsin in 10 mM ammonium bicarbonate, pH 7.8 [11]. After digestion, the enzyme was inactivated and the sample lyophilized.

Fractionation of peptides on rpHPLC column

The CNBr-trypsin cleaved protein was purified on rpHPLC (Waters C₁₈ column). One ml fractions were collected and lyophilized. The IgE binding activity of each fraction was checked by ELISA using the sera of *Fusarium*-sensitive patients. The IgE binding peaks I, III, IV and V were further purified using a gradient programme. Peaks I and V eluted as homogeneous peaks in isocratic conditions with the solvents: 50% A and 50% B but peak III eluted into three subfractions (IIIa, IIIb and IIIc). Peak IV was

separated into IV-1, IV-2 and IV-3 using a linear gradient. The subfractions IIIb and IV-1 were homogeneous and highly allergenic in comparison to other subfractions.

Enzyme linked immunosorbent assay (ELISA) and ELISA-inhibition

IgE and IgG specific ELISA of the isolated peaks were performed [12]. Briefly, 100 ng of each *F. solani* extract, *Fus s I₃₅₉₆** and peptides in 100 µl of carbonate buffer (pH 9.6) were coated separately on the plate. *Imperata cylindrica* (grass pollen), *Salmelia* (tree pollen) and old paper dust extracts were coated similarly and used as negative controls. After washing, the plates were blocked (1% bovine serum albumin (BSA)) and incubated overnight at 4 °C with 1 : 10 v/v diluted allergic serum pool. The plates were washed and incubated for 4 h with 1 : 1000 v/v in PBS-Tween diluted anti-human IgE or anti-human IgG peroxidase (Sigma, St Louis, MO, USA). The colour was developed with o-phenylene diamine in sodium citrate buffer and absorbance was read at 490 nm.

Allergenic potency of peaks I, IIIb, IV-1 and V was determined by ELISA-inhibition against solid phase protein *Fus s I₃₅₉₆** (500 ng). Sera from *Fusarium*-sensitive patients (50 µl of 1 : 5 v/v diluted) was mixed with equal volume of buffer containing purified peptides in different concentrations (2–5000 ng/50 µl). This mixture was added to the plate and incubated overnight at 4 °C. The percent inhibition was calculated [2].

IgE specific ELISA with individual *Fusarium* positive patients sera was also done to check the IgE binding of peak IV-1. The peak IV-1 (100 ng) was coated on the plate and incubated with individual patient's serum. Other steps were similar to what is described above.

Tricine-SDS-PAGE/Immunoblot

Peak IV-1 was electrophoresed on 20% SDS-PAGE containing tricine and transferred to nitrocellulose membrane in tris-glycine buffer with 20% methanol as described by Towbin *et al.* [13]. After saturation with 1% BSA, membrane was incubated with 1 : 10 v/v diluted pooled patients sera at 4 °C. IgE binding was detected by anti-human IgE peroxidase and diaminobenzidine.

Intradermal skin testing

Intradermal (ID) tests were performed with crude extract, purified 65 kDa major allergen and peak IV-1 in *Fusarium*-sensitive patients at V. P. Chest Institute, Delhi, India. Briefly, for skin tests, lyophilized proteins (extracts) were reconstituted (1 : 500 w/v; 120 µg protein/mL) in PBS. About 0.02 mL of each extract was injected intradermally

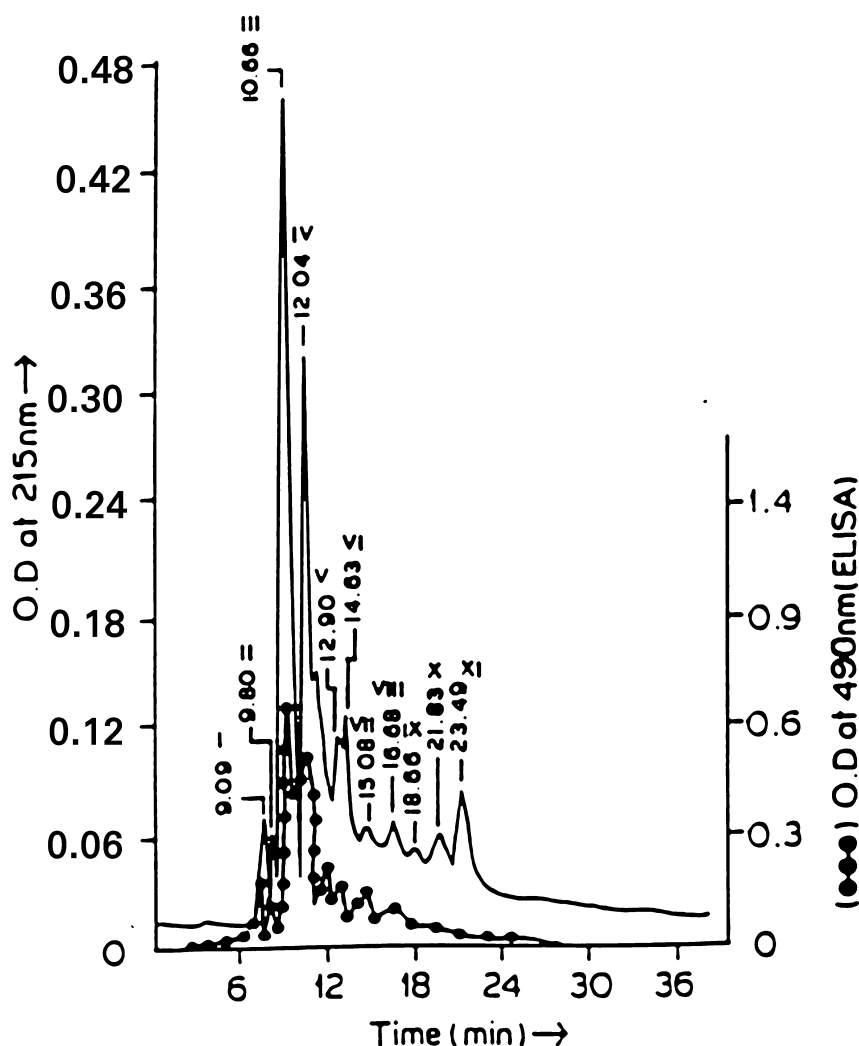


Fig. 1. Elution profile of CNBr-Trypsin treated *Fus s* I₃₅₉₆* on rpHPLC. Solvent A = 0.5% TFA in water, Solvent B = 0.5% TFA in acetonitrile. Programme: 70% A–30%B. Flow rate, 1.0 mL/min; Chart speed, 2.5 cm/min; Chart attenuation, 256. (.....) – IgE binding capacity of each eluted fraction as determined by ELISA.

in the forearm, raising a bleb of 2–3 mm at the injection sites. Phosphate buffer saline was taken as a negative control and histamine diphosphate as a positive control. After 20 min, wheal size more than three times the negative control (> 12 mm) was graded as markedly positive [14]. Blood was collected from these patients for histamine release assay. Blood from non-allergic volunteers was also taken as control. Consent of every patient and normals was obtained prior to skin testing.

Histamine release assay

This assay was performed according to the manufacturer's instructions using a kit (Immunotech, Marseille, France). Briefly, heparinized blood was challenged either with 10 ng each of crude extract, purified 65 kDa protein or peptide IV-1 for 30 min at 37 °C. (The assay was standardized with 5–500 ng of each extract). The histamine standards (0–100 nM) and released histamine in the super-

natant were acylated and assayed by ELISA. Spontaneous histamine release was measured in an unchallenged sample and total histamine in the blood samples was measured after cell lysis by freeze thawing.

Histamine release inhibition assay

Heparinized blood was mixed with varying concentrations of peptide IV-1 (2–25 ng/50 µl) and incubated at 37 °C for 30 min. *F. solani* CF extract (10 ng protein) was added to these cells and released histamine was measured as described above. The percent histamine release inhibition in each patient was determined.

Results

Characterization and purification of peptides

The CNBr and trypsin-treated *Fus s* I₃₅₉₆* allergen eluted into

Table 1. IgE and IgG binding activity of fractions obtained from rpHPLC column with *Fusarium* sensitive patients sera.

Extracts	IgE	IgG
<i>F. solani</i>	0.825	0.624
<i>Fus s</i> I ₃₅₉₆ *	0.678	0.425
I	0.181	0.128
II	0.169	0.317
III	0.188	0.239
IV	0.497	0.187
V	0.226	0.188
VI	0.138	0.318
VII	0.177	0.320
VIII	0.134	0.370
IX	0.141	0.428
X	0.129	0.349
XI	0.105	0.354
<i>Salmelia</i> tree pollen	0.000	0.027
Old paper dust	—	0.014
<i>Cynodon</i> grass pollen	0.026	0.022

11 peaks on rpHPLC column at the retention times (Rt) of 9.09, 9.80, 10.66, 12.04, 12.90, 14.63, 15.08, 16.68, 18.66, 21.83 and 23.49 (Fig. 1). The peaks were designated as peptide I to XI. All the peaks showed both IgE and IgG binding activity with *Fusarium*-sensitive patients sera (Table 1). Peak IV showed maximum IgE binding followed by peak V, peak III and peak I. These allergenic peaks were further purified on

rpHPLC. Peptide I and V eluted as a single peak in isocratic conditions while peak III and IV were not homogeneous and separated on rpHPLC at different elution conditions. Peak III eluted into three subfractions (IIIa, IIIb and IIIc) with an Rt of 9.75, 12.13 and 14.9 respectively, while peak IV eluted into IV-1, IV-2 and IV-3 subfractions at the Rt of 6.09, 7.16 and 7.62 (Fig. 2a), respectively. Peak IV-1 resolved as a homogeneous single allergenic peptide (Fig. 2b) of approx. 3.4 kDa on tricine-SDS-PAGE (Fig. 3).

ELISA-inhibition

The potency of the four purified allergenic peptides was determined quantitatively by ELISA-inhibition using *Fus s* I₃₅₉₆* as solid phase (Fig. 4). Purified major allergen, *Fus s* I₃₅₉₆*, required 8 ng protein for 50% inhibition of IgE binding to self protein. The peptide IV-1 required 31 ng while peptides I, IIIb and V required more than 750 ng for 50% inhibition demonstrating peptide IV-1 as the most potent peptide.

IgE binding and Intradermal test on *Fusarium* patients

To determine IgE binding ELISA was performed with *F. solani* extract, 65 kDa protein and peptide IV-1 (Table 2). Crude extract showed maximum IgE binding followed by 65 kDa protein and peptide IV-1 in all the patient's sera. Out of the 14 patients, 7 were intradermally tested with peptide IV-1. Six patients showed no reactivity with peptide IV-1 but

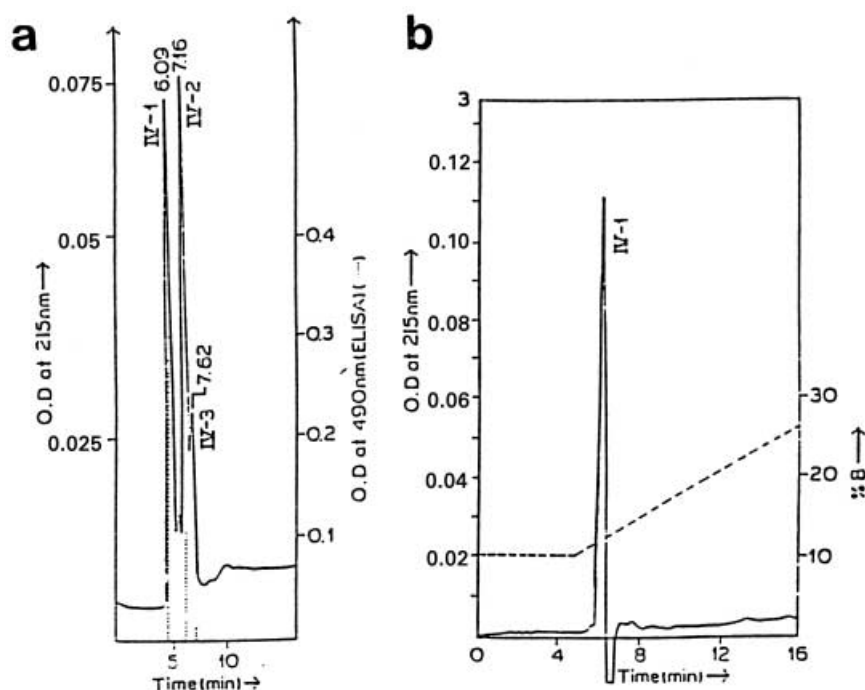


Fig. 2. Elution profile of (a) peptide IV and (b) peptide IV-1 on rpHPLC (Waters C18). Solvent A = 0.5% TFA in water, Solvent B = 0.5% TFA in acetonitrile. Programme: 50% A–50% B. Flow rate 1.0 mL/min, Chart speed, 2.5 cm/min; Chart attenuation, 256. (...) – IgE binding capacity of each eluted fraction (IIIa, IIIb and IIIc) of peak III as determined by ELISA.

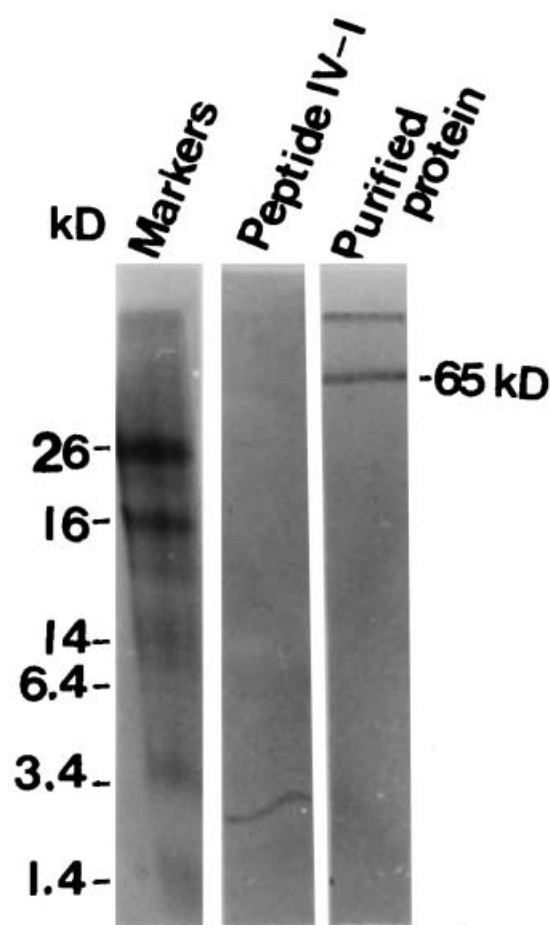


Fig. 3. Tricine SDS-PAGE (20% gel) profile. (A)-Polypeptide markers (B)-Peptide IV-1 (C)-*Fus s* I₃₅₉₆* (65 kDa major allergen).

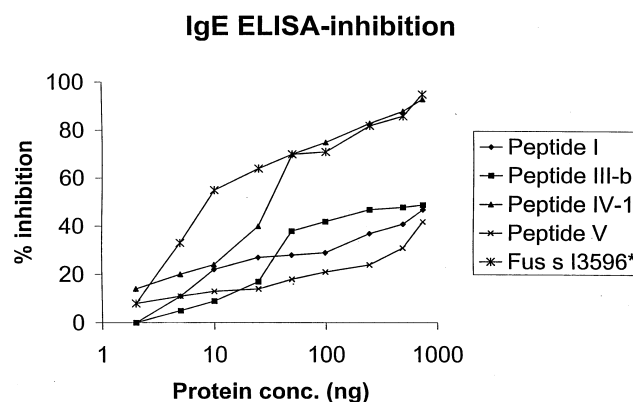


Fig. 4. ELISA-inhibition dose response curves of peptides isolated from *Fus s* I₃₅₉₆* by sequential cleavages with CNBr and trypsin.

one patient showed mild reactivity. All the patients tested showed IgE binding with peptide IV-1 by ELISA indicating that the peptide may act as a monomer and thus does not bring the two IgE receptors together for mediator release (Table 2).

Histamine release assay

Histamine release was performed with heparinized blood obtained from seven *Fusarium* allergic patients who were ELISA-positive to *F. solani*, purified 65 kDa protein and peptide IV-1 (Table 2). Histamine release was observed on challenge with crude extract and purified major allergen but peptide IV-1 did not release any histamine on challenge (Fig. 5). Histamine release > 10% of the total histamine

Table 2. Intradermal test and IgE binding (ELISA) of *F. solani* extract, *Fus s* I₃₅₉₆* and peptide IV-1

Patients	ID testing			IgE binding by ELISA (O.D.)		
	<i>F. solani</i>	<i>Fus s</i> I ₃₅₉₆ *	Peptide IV-1	<i>F. solani</i>	<i>Fus s</i> I ₃₅₉₆ *	Peptide IV-1
1	++	ND	ND	3.30	2.93	1.13
2	+++	ND	ND	3.12	1.39	0.95
3	+++	ND	ND	3.09	2.00	1.43
4	++	ND	ND	2.87	1.57	0.66
5	+++	ND	ND	3.27	2.52	1.53
6	+++	ND	ND	2.15	1.90	0.33
7	++	ND	ND	2.87	1.13	1.53
8*	+++	++	- ve	2.15	1.79	0.23
9*	++	+++	- ve	1.98	1.71	0.86
10*	++	++	- ve	1.25	0.75	0.38
11*	++	++	- ve	2.00	1.69	0.66
12*	++	±	- ve	1.53	1.43	0.72
13*	++	++	+ ve	1.70	1.31	0.54
14*	++	±	- ve	1.39	1.52	0.92

ND, not done; *Blood from these patients was used for histamine assay.

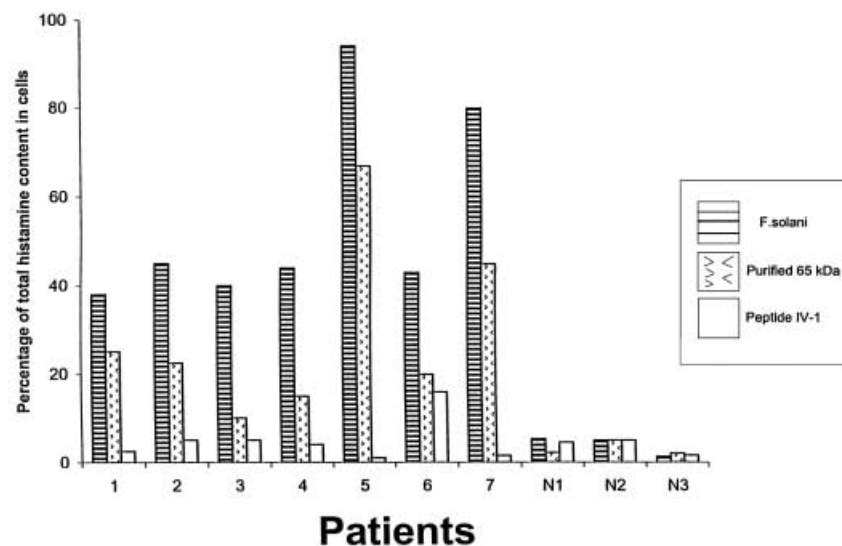
Histamine release from the blood cells of *Fusarium* positive patients in response to allergens

Fig. 5. Histamine release of peptide IV-1, purified major allergen *Fus s* I₃₅₉₆* and *F. solani* extract from the heparinized blood of seven individual subjects allergic to *Fusarium*. Histamine release was calculated as percentage (%) of the total histamine in cells.

content was considered as positive. Five patients showed 38–45% of histamine release when challenged with crude extract while in two patients 80–90% release of the total histamine content was observed on challenge with the same extract. Challenge with purified allergen resulted in nearly half the release compared to the crude extract, while the peptide released < 10% of the total histamine in most of the patients. One patient (no. = 6), showed 16% histamine release on challenge with peptide IV-1. None of the control subjects released histamine on challenge.

Histamine release inhibition assay

Peptide IV-1 was used in different concentrations to inhibit histamine release from heparinized blood on challenge with

F. solani extract. Peptide IV-1 inhibits histamine release in a dose-dependent manner in all the patients (Fig. 6). Each patient had different responses to peptide ranging from 92–5 ng for 50% histamine release. It indicates that peptide IV-1 had blocked the IgE receptor and further action of histamine release was inhibited.

Discussion

Different therapeutic approaches are in practice for the treatment of allergic diseases [15–17]. Various available drugs; anti-histamines, anti-leukotrienes, anti-inflammatory agents, immunosuppressants, corticosteroids, etc. are effective for atopic diseases but they only provide symptomatic relief and the allergic inflammation is likely to recur after stopping the treatment. Blocking of adhesion molecules may be an alternative new approach but immune response inhibition may lead to increased infection rates. A humanized murine monoclonal antibody (rhuMAb-E25) directed against FcεRI binding domain of human IgE decreases allergen specific IgE [18–20]. This therapy may be useful in the treatment of asthmatic and allergy patients with high IgE levels but repeated injections may not be feasible as a long-term treatment because of inherent problems associated with foreign proteins. Peptide therapy is a promising method for the treatment of allergic diseases as it can modulate immune response [21–23]. Small peptides derived from the whole allergen may not bind to IgE on effector cells [24]. In high doses, they induce tolerance in allergen specific Th2-like cells and indirectly shift the cytokine profile to Th1 [21]. Peptides containing major T cell epitopes have been proposed for the treatment

Histamine release inhibition

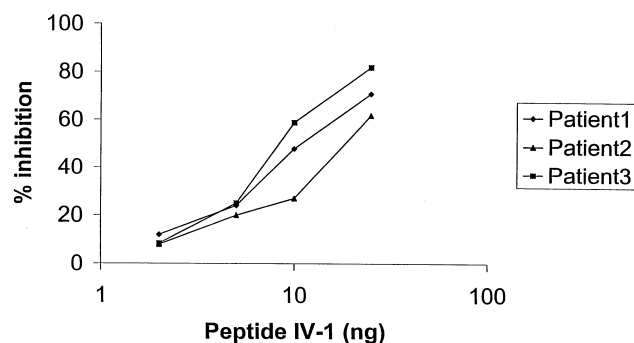


Fig. 6. Dose-dependent inhibition of histamine release. Heparinized blood from *Fusarium*-allergic patients was pretreated with 2, 10 and 50 ng of peptide IV-1 and then challenged with culture filtrate extract of *Fusarium solani*.

of allergic diseases because they induce T cell anergy [25]. Allergen-derived long peptide down-regulates specific IgE response and also protects presensitized mice from anaphylaxis upon challenge with native PLA₂ [26].

In the present study, major allergen of *F.solani* is sequentially cleaved with CNBr and trypsin to functionally characterize allergenically important peptides. We have shown that a 65 kDa major allergen (Fus s I₃₅₉₆*) contained at least three allergenic determinants [2]. Presently, out of 11 peaks obtained after chemical and enzymatic treatments, four (I, III, IV, and V) are highly allergenic by ELISA using *Fusarium* sensitive pooled patient's sera. Peptide IV-1, a 3.4 kDa fragment, is the most potent and caters to Fus s I₃₅₉₆* allergenicity. Kurup *et al.* (1998) demonstrated that decapeptides from Asp f 1 have distinct IgE binding activity with allergic bronchopulmonary aspergillosis (ABPA) patients sera and induce different proliferative responses [8]. Miller *et al.* (1999) have also used overlapping synthetic and tryptic peptides for the identification of IgE and IgG binding regions using brown Norway rat model [27].

In order to evaluate the potency of *F. solani* peptide IV-1, both *in vivo* and *in vitro* studies were performed. The results indicate that allergen specific immune response can be altered. Peptide IV-1 shows high IgE binding with the sera of most *Fusarium*-positive patients and inhibits up to 75% binding of *F. solani* specific antibodies to crude extract. However, *in vivo* (intradermal) testing with peptide elicited no reactivity in *Fusarium*-allergic patients. Earlier studies with cat allergens, have also emphasized the importance of screening the epitopes on the peptides for IgE binding to overcome the mediator release by receptor crosslinking [28].

Significant level of histamine is released after challenge with crude and purified allergen in all the patients. Peptide IV-1 did not show any histamine release in most of the patients. One patient showed histamine release and +1 reactivity on ID test. Probably the antibodies of this patient recognized minor epitopes on the peptide or the patient is hyper-reactive. Pretreatment of heparinized blood cells with peptide IV-1 was effective in blocking the allergen binding sites and decreased the histamine release on challenge with crude extract. Similarly, investigators have shown that naive mice pretreated with the dominant peptide of Fel d 1 or Der p 1 did not develop an immune response when challenged with native Der p 1 or Fel d 1 [29]. Thus, the allergic response could be substantially down-regulated by peptide treatment.

Until now, *F. solani* crude extract has been used for allergy diagnosis and immunotherapy. With the identification and characterization of a potent allergenic peptide from *F. solani* extract, it will be possible to develop new therapeutic preparations. Peptide IV-1 shows high IgE binding but is not functionally active. On the basis of these

results, it appears that peptide IV-1 is a monovalent peptide and seems to be a prospective candidate for immunotherapy.

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