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Molecular patterns in the isotype-specific antibody responses to the major cedar aeroallergen Jun a 1

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Abstract

Most studies of the immune responses in allergic rhinitis have focused on IgE antibodies to mixtures of allergenic proteins. Based on our previous studies of the major mountain cedar allergen Jun a 1, we sought to describe a broader assessment of the humoral immune responses to a single, dominant allergen, in three groups of allergic subjects, all of whom had similarly exposures to the whole cedar pollen. The major outcomes of this study was that, with the onset of allergic rhinitis symptoms, and after treatment with immunotherapy, serum IgE and IgG (but not IgA) antibodies to Jun a 1 increased. Interestingly, both IgE and IgG4 antibodies to the Jun a 1 allergen were strongly focused on its conformation epitopes. These IgG antibodies to conformational structures may be a useful marker of the therapeutic response to immunotherapy. (136 words)

Keywords

Allergen; cedar pollen hypersensitivity; Jun a 1; epitopes; IgG; immunoglobulin isotypes

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1. Introduction

Seasonal allergic rhinitis due to cedar pollens are a common problem in multiple areas in the world (Goldblum *et al.*, 2016; Pichler *et al.*, 2015; Midoro-Horiuti, 1992; Midoro-Horiuti *et al.*, 1992). For instance, about 30–40% of the populations of central US, Southern Europe and Japan, suffer from cedar pollen hypersensitivities to highly cross-reactive allergens of the mountain cedar (*Juniperus ashei*, Cupressaceae), Italian cypress (*Cupressus sempervirens*, Cupressaceae) and Japanese cedar (*Cryptomeria japonica*, Taxodiaceae). We have previously isolated, cloned, sequenced and elucidated the crystal or model structure and IgE epitopes of two of these allergens, Jun a 1 and Jun a 3, from mountain cedar pollen (Midoro-Horiuti *et al.*, 1999; Midoro-Horiuti *et al.*, 2000; Soman *et al.*, 2000; Midoro-Horiuti *et al.*, 2003; Liu *et al.*, 2003; Czerwinski *et al.*, 2005; Varshney *et al.*, 2007). We also reported that the majority of human IgE antibodies from the sera of mountain cedar allergy sufferers are directed against several conformational epitopes on the surface of the dominant allergen, Jun a 1 (Goldblum *et al.*, 2014). There are reports about the analyses of IgE and IgG epitopes on the other pollen allergens. The conformational IgE epitopes are reported on Bet v 1 (Mirza *et al.*, 2000; Gieras *et al.*, 2011; Subbarayal *et al.*, 2013), Amb a 4 (Pablos *et al.*, 2018), Amb a 8 (Offermann *et al.*, 2016), Cry j 1 (Aoki *et al.*, 2009), Phl p 2 (Padavattan *et al.*, 2009), Phl p 3 (Devanaboyina *et al.*, 2014) and Phl p 5 (Focke-Tejkl *et al.*, 2014). Also, conformational IgG epitopes are reported on Bet v 1 (Subbarayal *et al.*, 2013).

We describe here a population-based study comparing three groups of atopic subjects, all of whom were naturally exposed yearly to mountain cedar pollen, in the same region. The first group did not display allergic symptoms or skin prick test (SPT)¹ responses to mountain cedar extracts (SPT⁻). The second group had seasonal, clinical manifestations of cedar pollinosis and positive SPT, but had not been treated with specific immunotherapy (SPT⁺SIT⁻). The third group had seasonal symptoms, was SPT⁺ and had been treated with subcutaneous immunotherapy with mountain cedar pollen extracts (SPT⁺SIT⁺). Our goal for this epidemiologic study was to identify the similarities and differences in the serum antibody responses to Jun a 1, in these three clinical groups.

2. Methods

2.1 Patient selection and serum collection

Each subject provided serum samples under a University of Texas Medical Branch IRB Protocol (06–050) and had a repeat SPT with a commercial extract of mountain cedar pollen (Hollister-Stier, Spokane, WA), if they had not been tested in the previous year. Sera from the three groups of atopic adults, all of whom were seasonally exposed to mountain cedar pollen in the same region and were recruited from Dr. van Bavel's clinic at Austin, Texas. We distributed these subjects into three clinical groups. The first group did not display allergic symptoms or SPT responses to mountain cedar extract (SPT⁻, n=10). The second group had seasonal clinical manifestations of cedar pollinosis and positive skin prick tests, but were not treated with specific immunotherapy (SPT⁺ SIT⁻, n=12). The third group had

Abbreviations: Jun a 1; *Juniperus ashei* allergen 1, SIP; specific immunotherapy, SPT; skin prick test

seasonal symptoms, positive SPT and were treated with allergen specific immunotherapy (SPT⁺SIT⁺, n=12).

2.2 Purification of Jun a 1

Jun a 1 was purified from mountain cedar pollen, as we previously described (Midoro-Horiuti *et al.*, 1999).

2.3 Enzyme linked immuno assay (ELISA) assays

To quantify the IgE, IgA, IgG and IgG4 serum antibodies to Jun a 1, 96 well microtiter plates were coated by incubating with native, purified Jun a 1 (3 µg/mL) at 37°C overnight (Midoro-Horiuti *et al.*, 1999). Then various dilutions of the sera (1:10 to 1:10⁹ in 0.01% Tween 20-Tris buffered saline, TTBS) from each subject were incubated in duplicate wells for 4 hours. After washing the microtiter plates, the quantity of IgE bound to the wells was detected, using biotinylated goat anti-human IgE (Vector, Burlingame, CA), followed by horseradish peroxidase (HRP)-streptavidin conjugates. The binding of IgA, IgG and IgG4 were detected by incubation with anti-human IgA, IgG or IgG4 enzyme conjugates (Zymed, San Francisco, CA). After washing the microtiter plates, the patient's immunoglobulins of each isotype, that bound to the wells was quantified using 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma). The results from the colorimetric enzyme assays were then plotted, to establish the highest dilution that had color significantly above the buffer control. The number of dilutions was considered the titer for that isotype.

2.4 ELISAs to distinguish between antibodies that bind to native and denatured Jun a 1.

To determine the extent to which individual patient's IgE, IgA, IgG and IgG4 antibodies react to conformational and linear epitopes of Jun a 1, we used similar ELISA assays, which we described previously (Varshney *et al.*, 2007;Goldblum *et al.*, 2014). Some of these coated wells were incubated with 6 mol/L guanidine-HCl for 30 minutes, to denature the Jun a 1. The sera from the members of each of the three clinical groups were then serially diluted and incubated in either the guanidine-treated and untreated wells. The binding of each isotype, to native and denatured Jun a 1, was detected and the results plotted as described above.

3. Results and Discussion

3.1 Patient demographics

There were no significant differences in the gender or age distribution between these three groups. The mean male: female ratios were (3:7, 7:5 and 4:6), and ages $\pm$ SD were 31.9 $\pm$ 11.7, 37.9 $\pm$ 12.1 and 44.2 $\pm$ 8.9) for SPT⁻, SPT⁺SIT⁻ and SPT⁺SIT⁺, respectively. (Table I)

3.2 Titer of Jun a 1 specific immunoglobulins

We first assessed each subject's titers of serum IgE, IgA, IgG and IgG4 antibodies to Jun a 1, using ELISA assays, as described above (Fig 1) (Goldblum *et al.*, 2014). We could not distinguish any differences in the concentration of serum IgA anti-Jun a 1 antibodies

between these three groups (Fig 1). However, we found a significantly higher concentration of IgG anti-Jun a 1 antibodies in the sera of the SPT⁺SIT⁻, than in the other two groups. The IgG4 anti-Jun a 1 concentrations were too low to be accurately quantified in most of the 1:10 diluted sera. The lines above the graph indicate the significant differences between the groups for IgG and IgE isotypes.

3.3 Recognition of conformational vs. linear epitopes of Jun a 1 by each antibody isotype

We next examined the types of molecular structures on Jun a 1 that were recognized by each isotype, by testing the same sera in ELISA wells, in which the conformational (discontinuous) epitopes were disrupted by pretreating Jun a 1 coated wells with guanidine (Fig 2), as we previously described. The results of these assays showed that IgE antibody are strongly directed toward conformational epitopes, while IgA and total IgG are less selective for these structures. This is indicated in the SPT⁺SIT⁻ group (Fig 2B), by the loss (80–100%) of their IgE reactivity when assayed on the denatured Jun a 1 (mean $\pm$ SD = 95 $\pm$ 3). However, binding of their IgA, IgG and IgG4 was less affected by this denaturation (43 $\pm$ 24, 27 $\pm$ 22 and 58 $\pm$ 27%, $p < 0.00005$, 0.0005 and 0.06), respectively. These findings strongly suggest that pathologic IgE antibodies to Jun a 1 predominantly bind to conformational epitopes, while the IgA and total IgG antibodies recognize both linear and conformational epitopes on Jun a 1, in the SIT⁻ groups. IgG4 antibody binding, generally favored denatured Jun a 1.

3.4 Potential mechanism of allergen specific immunoglobulin production

Together these findings suggest that IgE and most of the IgG4 antibodies to cedar allergen were directed against native Jun a 1 in the local mucosal tissues, prior to inhalation or ingestion, during which the pollen proteins would be denatured, as reported by others (Rondon *et al.*, 2007; Rondon *et al.*, 2012; Wise *et al.*, 2009; Yoshida *et al.*, 2005). However, IgG and IgA antibodies, produced in response to the same exposures, are most likely produced against allergens that are transported through the nasal mucosa or swallowed, and then denatured in the gastrointestinal tract, or upon systemic absorption. None, or very few, IgE antibodies were detected in the sera from the SPT⁻ subjects. However, these subjects did produce Jun a 1-specific IgA, IgG and IgG4 antibodies. The proportion of antibodies that were directed against conformational epitope was IgA: 56 $\pm$ 19, IgG: 51 $\pm$ 24 and IgG4: 73 $\pm$ 37 in the SPT⁻ group. After SIT (i.e. in the SPT⁺SIT⁺ group, Fig 2C), the reactivity to conformational epitopes of IgE, IgA, total IgG and IgG4 were (mean $\pm$ SD = 86 $\pm$ 17, 47 $\pm$ 19, 68 $\pm$ 19 and 77 $\pm$ 27%), respectively. Significant differences between SPT⁺SIT⁻ and SPT⁺SIT⁺ groups were detected in IgG reactivity, with 27 $\pm$ 22% in SPT⁺SIT⁻ and 68 $\pm$ 19% in SPT⁺SIT⁺ group (Fig 2B and C), suggesting that SIT enhanced the production of IgG antibodies to conformational epitopes of Jun a 1. These IgG antibodies induced by SIT are likely to reduce the IgE binding to Jun a 1, thereby preventing symptoms. Interestingly, the patterns of Jun a 1 recognition by total IgG and IgG4 in SPT⁺SIT⁺ group are similar to those of the SPT⁻ group.

Thus, while allergen-specific IgE antibodies from mountain cedar allergic patients recognize predominantly conformational epitopes, less than half of their IgA and IgG antibodies, like those from non-cedar allergic donors, recognize conformational epitopes. This pattern of

epitope recognition suggests a local (mucosal) production of allergen-specific IgE. However, after systemic immunotherapy, the proportion of allergen specific IgG, and particularly IgG4, that recognize conformational epitopes is increased. Taken together these findings may indicate that successful subcutaneous immunotherapy for cedar pollen may depend, at least in part, on the ability of IgG antibodies to recognize conformational epitopes.

Conclusion

Detailed examination of the isotype-specific patterns of human antibody responses to structurally distinct features on allergens, and their responses to therapeutic interventions, may allow us to identify new approaches to allergic diseases and/or help to predict which patient's will respond to these interventions.

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Highlights:

- Development of the symptoms of mountain cedar pollen hypersensitivity is associated with an increase in specific IgE and IgG antibodies, relative to the non-sensitive, but atopic group.
- Allergen specific IgG produced after immunotherapy, predominantly recognizes conformational epitopes.
- The epitope-specificity of IgE and IgG may provide clues to the anatomical sites and the mechanisms of production of these immunoglobulins.

Fig 1.

Titer of Jun a 1-specific IgE, IgG and IgA antibodies are shown with their mean $\pm$ SD (open bars). IgG4 antibodies were not shown in this figure because of their very low titers.

Significance differences in the titer of different isotypes are indicated by * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Fig 2. Decrease in the binding of each immunoglobulin isotype after Jun a 1 was denatured with guanidine is shown as the percent decrease. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$, as indicated.

Table 1

SPT+SIT ⁻ group		J.		
#	sex	age	ashei	positive SPT to other allergens
1	M	26	+	tree, ragweed, dust mite, Alternaria
2	F	43	+	no other
3	M	27	+	ragweed, Alternaria
4	M	26	+	tree, ragweed, Alternaria
5	F	39	+	tree, ragweed, dust mite tree, ragweed, dust mite, Alternaria,
6	M	30	+	penicillium
7	M	55	+	tree, grass, ragweed, cat, Alternaria
8	M	45	+	tree, grass, ragweed, dust mite, cat tree, grass, ragweed, dust mite, cat,
9	F	42	+	Alternaria
10	F	56	+	tree, ragweed, cat
11	F	47	+	tree, grass, ragweed
12	M	19	+	grass, dust mite, ragweed
mean		37.9	12.1	
SPT+SIT ⁺ group		J.		
#	sex	age	ashei	positive SPT to other allergens
				tree, grass, ragweed, dust mite, dog,
1	M	43	+	cat
2	M	35	+	tree, grass, ragweed
3	F	42	+	tree, grass, ragweed, dog, cat
4	M	44	+	tree, grass, ragweed, dust mite
5	M	47	+	grass, dust mite
6	F	65	+	ragweed
7	F	34	+	tree, ragweed, penicillium, dog tree, grass, ragweed, dust mite, dog,
8	F	50	+	cat, cockroach
9	F	44	+	tree, dust mite, cat tree, grass, ragweed, dust mite,
10	F	38	+	Alternaria, dog, cat, cockroach
mean		44.2	8.9	
SPT ⁻ group		J.		
#	sex	age	ashei	positive SPT to other allergens
1	F	39	-	tree, grass, dog, cat, Alternaria tree, ragweed, dog, dust mite,
2	F	31	-	Alternaria
3	M	20	-	tree, grass, dog, dust mite tree, grass, ragweed, dust mite, cat,
4	F	11	-	Alternaria
5	F	47	-	tree, dust mite, dog
6	F	32	-	tree, Alternaria, dog, cat, Alternaria
7	M	24	-	tree, cat, Alternaria
8	F	30	-	tree, grass, dust mite, Alternaria
9	F	36	-	tree, grass, dust mite, dog, Alternaria

SPT+SIT ⁺ group		<i>J.</i>		
#	sex	age	<i>ashei</i>	positive SPT to other allergens
10	M	49	-	dust mite, Alternaria
mean		31.9	11.7	

SPT: skin pick test, SIT: specific immunotherapy