Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

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BRIEF COMMUNICATION

An assay that may predict the development of IgG enhancing allergen-specific IgE binding during birch immunotherapy

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To cite this article: Selb R, Eckl-Dorna J, Vrtala S, Valenta R, Niederberger V. An assay that may predict the development of IgG enhancing allergen-specific IgE binding during birch immunotherapy. *Allergy* 2013; DOI: 10.1111/all.12204.

Keywords
Bet v 1; birch pollen allergy; enhancing IgG antibodies; immunotherapy; recombinant allergen.

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Accepted for publication 9 May 2013

DOI:10.1111/all.12204

Edited by: Michael Wechsler

Abstract

Background: It has been shown that birch pollen immunotherapy can induce IgG antibodies which enhance IgE binding to Bet v 1. We aimed to develop a serological assay to predict the development of antibodies which enhance IgE binding to Bet v 1 during immunotherapy.
Methods: In 18 patients treated by Bet v 1-fragment-specific immunotherapy, the effects of IgG antibodies specific for the fragments on the binding of IgE antibodies to Bet v 1 were measured by ELISA. Blocking and possible enhancing effects on IgE binding were compared with skin sensitivity to Bet v 1 after treatment.
Results: We found that fragment-specific IgG enhanced IgE binding to Bet v 1 in two patients who also showed an increase of skin sensitivity to Bet v 1.
Conclusion: Our results indicate that it may be possible to develop serological tests which predict the induction of unfavourable IgG antibodies enhancing the binding of IgE to Bet v 1 during immunotherapy.

Birch pollen allergy is a highly prevalent disease (1), which is due to IgE sensitization to the major allergen of birch, Bet v 1, a 17-kDa protein harbouring mainly conformational IgE epitopes (2). Birch pollen-specific immunotherapy (SIT) with birch pollen extract or purified recombinant Bet v 1 is a clinically effective treatment, and it has been shown that clinical improvement is associated with a reduction in birch pollen-induced immediate skin reactions and the development of Bet v 1-specific IgG antibodies (3). However, the molecular and immunological characterization of human Bet v 1-specific IgG antibodies has demonstrated that besides IgG antibodies, which inhibit allergic patients' IgE binding to Bet v 1 and therefore can block Bet v 1-induced allergic reactions, two other types of Bet v 1-specific antibodies can develop in patients (4, 5). One type of these Bet v 1-specific IgG antibodies recognizes other epitopes than patients' IgE, and hence, these antibodies do not inhibit IgE binding to Bet v 1. Another type of IgG antibodies was found to even enhance IgE binding to Bet v 1 and Bet v 1-induced immediate-type skin reactions (4, 5). The induction of the latter type of Bet v 1-specific IgG antibodies during SIT is therefore not desirable. Although it may be assumed that enhancing antibodies of the IgG isotype and potentially also of other isotypes may develop during immunotherapy against other allergen sources, to our knowledge, the presence of such undesirable antibodies has only been proven in birch pollen-allergic patients.

In this study, we investigated whether it is possible to develop an *in vitro* test that may predict the development of such nonbeneficial Bet v 1-specific IgG antibodies during injection immunotherapy.

Materials and methods

We used serum samples from 18 birch pollen-allergic patients obtained before and after SIT with recombinant Bet v 1

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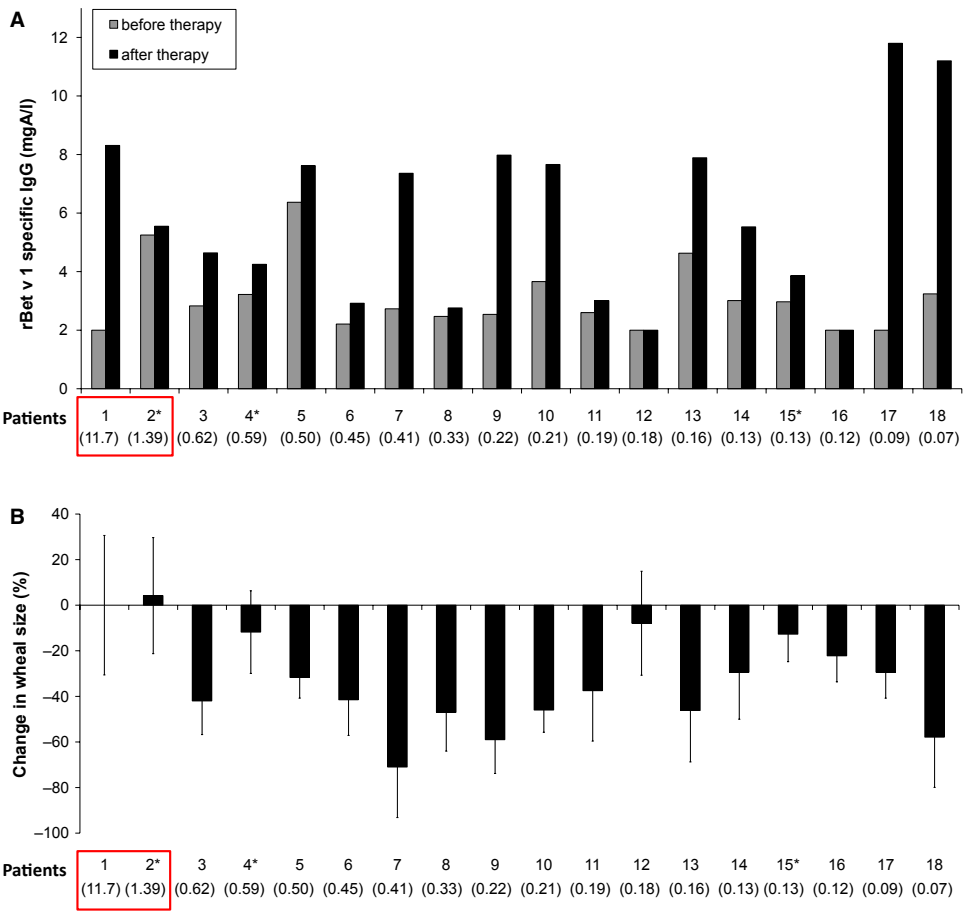


Figure 1 (A) Bet v 1-specific IgG (mgA/l – milligram antigen per litre) before and after immunotherapy (*y*-axis) for each patient (after immunotherapy: in May; patients 2*, 4* and 15* in October). (B) Change in immediate-type skin response after SIT in May (patient 2*, 4* and 15*: October) compared with values before immunotherapy. (A, B) The factor of (i.e. fold) change in IgE binding to Bet v 1 by fragment-specific IgG is shown for each of the patients (*x*-axes, in parentheses).

fragments (6). The presera of the patients were obtained before injection 1 of the treatment course. Bet v 1-specific IgG and IgE levels were measured by ImmunoCAP (Phadia, Uppsala, Sweden) (6).

In these serum samples, we determined changes (i.e. reduction or enhancement) in IgE binding to immune complexes consisting of Bet v 1 and IgG antibodies from rabbits that had been immunized with the same Bet v 1 fragments that had been injected into the patients (7). For this purpose, we performed an ELISA experiment with Bet v 1 on the solid phase and assessed to what extent the binding of IgE in patients' sera (obtained before treatment) to the allergen was altered by pre-incubation of plate-bound Bet v 1 with fragment-specific rabbit IgG. The ELISA experiments are described in the online repository. ELISA results represent mean values of duplicate determinations with a deviation of <5%. The factor of blocking/enhancing of IgE binding by Bet v 1 fragment-specific rabbit IgG was calculated according to the following formula: Factor of change of IgE binding by rabbit IgG = absorbance fragment-specific IgG/absorbance control rabbit IgG. Thus, values >1 express an enhancement, whereas values <1 express an inhibition of IgE binding by fragment-specific rabbit IgG, respectively.

The results of the ELISA were compared with the induction of Bet v 1-specific IgG and the alterations of cutaneous and nasal sensitivity to Bet v 1 in the patients in the course of SIT (Fig. 1, Table 1 right column). Methods for skin prick testing and nasal provocation testing are described in the online repository.

Results

In Figures 1A and B, patients were ordered from right to left according to the extent of inhibition of IgE binding (maximum inhibition, right – no inhibition and enhancement, left). In 16 of the 18 patients, a blocking of IgE binding to Bet v 1 (factor 0.62–0.07) by fragment-specific IgG was observed. Interestingly, in two patients, fragment-specific IgG enhanced the binding of IgE to Bet v 1 (Fig. 1, Table 1 and Table S1: Patient 1: factor 11.7; patient 2: factor 1.39).

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Table 1 Patient data: demographic data, Bet v 1-specific IgE levels (kUA/L: kilo units antigen per litre), factors of (i.e. folds) change in IgE binding to Bet v 1 by fragment-specific IgG, numbers of injections and cumulative injected dose (CID) are displayed. In the right column, changes in nasal sensitivity to birch pollen extract as determined by nasal provocation tests before therapy and 1 year thereafter are shown: '+': improvement, '−': deterioration, '=': no change in nasal sensitivity to birch pollen extract. n.d.: not done

Patient number	Sex	Age	Bet v 1-spec. IgE (kuA/L)	Factor of change of IgE binding by fragment-specific IgG	Immunizations		Nasal tolerance
					Number	CID (μg)	
1	m	36	2.22	11.7	9	245.00	=
2	f	23	5.46	1.39	3	4.00	–
3	m	28	2.33	0.62	9	167.00	–
4	f	49	67.7	0.59	6	9.00	n.d.
5	m	33	10.8	0.50	9	245.00	+
6	f	45	11.6	0.45	8	165.00	=
7	f	23	13.3	0.41	8	165.00	+
8	f	38	25.7	0.33	7	85.00	+
9	m	51	>100	0.22	7	85.00	+
10	f	45	24.7	0.21	9	103.00	+
11	f	48	26.2	0.19	9	185.00	=
12	m	31	7.35	0.18	3	5.00	=
13	m	35	58.1	0.16	9	205.00	–
14	f	58	41.1	0.13	9	88.00	=
15	m	34	8.4	0.13	7	28.00	=
16	m	33	39.70	0.12	5	25.00	+
17	f	25	24.4	0.09	9	245.00	+
18	f	41	56.4	0.07	9	197.00	=

We then assessed the levels of Bet v 1-specific IgG that was developed by the patients after SIT (Fig. 1). We found that 15 of the 18 patients showed increases in Bet v 1-specific IgG antibodies, and in two (i.e. patients 12 and 16), no relevant increase was detected. Next, we analysed the changes in immediate-type skin responses to Bet v 1 before and after the treatment. Two patients (i.e. patient 1 > patient 2) showed a development of Bet v 1-specific IgG during SIT, but showed no improvement in skin reactivity to Bet v 1. Furthermore, these two patients showed either no change or a deterioration of nasal sensitivity to birch pollen extract (Table 1, right column). Interestingly, for these two patients, we found that fragment-specific IgG enhanced IgE binding to Bet v 1 (Fig. 1). The development of IgG enhancing allergen-specific IgE in the patients may explain why they did not show a reduction in skin sensitivity after SIT and no improvement in nasal tolerance for birch pollen extract. In fact, patient 1 indeed failed to develop IgG antibodies against the major IgE-reactive area described by Gieras (2) (data not shown).

Discussion

Several research groups that develop allergy vaccines immunize animals with their vaccine candidates to study whether the vaccine can induce IgG antibodies upon immunization which block allergic patients' IgE binding to the allergen (8). Such tests have been used for the evaluation of various recombinant allergen-based vaccines and in particular for vaccines that contain recombinant modified allergen derivatives before these derivatives underwent clinical testing (9).

For birch pollen allergy, it was observed that SIT may induce an unfavourable IgG antibody response, which enhances allergic patient's IgE binding to Bet v 1 (4, 5). Therefore, we were interested whether it may be possible to use IgG antibodies induced in an experimental animal system with Bet v 1-based vaccines to identify patients whose IgE binding to Bet v 1 may be enhanced by Bet v 1-specific SIT. Our finding that exactly those patients whose IgE binding to Bet v 1 was enhanced by Bet v 1-specific IgG showed deterioration or no improvement in skin responses to Bet v 1, and no improvement in nasal tolerance of birch pollen extract after SIT indicates that it may indeed be possible to develop serological tests that may predict the development of unwanted IgG in the course of SIT.

Our results also indicate that not only the mere increase in allergen-specific IgG levels after SIT but also the quality (i.e. affinity, avidity, epitope specificity) of therapy-induced IgG is important for success of SIT (10). In conclusion, we provide evidence that it may be possible to develop tests that can predict the quality of allergen-specific IgG responses during SIT. The test system developed by us may depend on the animals used for immunization and the vaccine formulation, but outbred rabbits seem to be a quite good model system. Our results suggest that this system allows the prediction of the development of unfavourable IgG in the course of Bet v 1-specific SIT and may be useful to identify patients who will not benefit from Bet v 1-specific SIT. A similar approach could potentially also be developed to evaluate vaccines against other allergen sources. It may also be used to evaluate new forms of recombinant allergen-based allergy vaccines, which can

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focus IgG responses towards IgE-binding sites on allergens and may improve the efficacy of birch SIT in the future (11, 12).

Acknowledgments

We thank Irene Steiner, MSc, Center of Medical Statistics, Informatics and Intelligent Systems, Medical University of Vienna, for her help regarding the statistical analysis of our data.

Funding

Supported by grants F4602, F4604 and F4613 of the Austrian Science Fund (FWF).

Conflict of interest

R.V. has received grant support from Phadia/Thermofisher, Uppsala, Sweden, and BIOMAY AG, Vienna, Austria, and serves as consultant for both companies. V.N. serves as a consultant for BIOMAY AG.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Online repository; Material and methods.

Table S1. Blocking/enhancing of IgE binding by rabbit IgG.

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Online Repository

An assay which may predict the development of IgG enhancing allergen-specific IgE binding during birch immunotherapy

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Methods:

ELISA experiments

ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with rBet v 1 (Biomay, Vienna, Austria) at a concentration of 1 µg/ml diluted in coating buffer (100 mM sodium carbonate-bicarbonate) and incubated overnight at 4°C. Plates were washed with washing buffer (PBS, 0.02% v/v Tween 20) and blocked with buffer A (PBS, 0.02% v/v Tween 20, 1% w/v BSA (PAA, Pasching, Austria)) at room temperature for 2h. Sera of two rabbits immunized with fragment 1 or fragment 2 of Bet v 1 respectively were mixed at a 1:1 ratio, diluted 1:50 in buffer A, added to the plates and incubated overnight at 4°C. As control, 1:50-diluted serum from a non-immunized rabbit was used. After a washing step, patient's pre-immune sera (obtained before immunotherapy) were applied at a dilution of 1:5 in buffer A and again incubated at 4°C overnight. Patient's IgE was detected with a HRP-labelled anti-human IgE antibody (KPL, Maryland, USA) at a dilution of 1:2500 in buffer A and incubated at room temperature for 2h. After a further washing step, ABTS detection was performed. ABTS (2,2'-Azino-bis(3-ethylbenzothialzoline-6-sulfonic acid) di-ammonium salt – Sigma-Aldrich, Saint Louis, Missouri, USA) was used at a concentration of 1 mg/ml in phosphate buffered citrate (70 mM) and 0.1 µl/ml H_2O_2 (30%, Sigma-Aldrich) was added. Plates were read at 405 nm (Victor3, PerkinElmer, Waltham, Massachusetts, USA). Mean values of duplicate determinations were calculated.

Skin prick tests

Skin prick tests were performed before therapy, after the birch pollen season following treatment with the Bet v 1 fragments and one year after the first skin test. Tests were done in triplicates with birch pollen extract containing 16 µg/ml of Bet v 1, 0.9 saline (negative control) and histamine dihydrochloride (positive control). After 15 minutes, reactions were recorded by transferring a line drawn round the perimeter of the wheal with adhesive tape to paper. Computerized digital planimetry was used for measuring the size of the reactions.

Nasal provocation experiments

Nasal provocation was performed before the beginning of immunotherapy with the Bet v 1 fragments and one year thereafter. First, basal levels of nasal flow and resistance without any intervention and 10 minutes after spraying the nose with 0.9% sodium chloride solution were determined by active anterior rhinomanometry (Allergopharma Rhinomanometer; Allergopharma, Reinbek, Germany). Nasal provocation was performed with natural birch pollen extract which was prepared from a standardized lyophilized birch pollen extract and then used for a maximum of 48 hours. Approximately 0.05 ml of birch pollen extract solutions containing standardized amounts of Bet v 1 (0.0064, 0.064, 0.64, 6.4, 64 µg/ml) were applied to both nostrils using a metered dose pump. Changes in nasal flow and resistance were measured 15 and 20 minutes after allergen application. Subjective symptoms were recorded 15 minutes after provocation (mild nasal secretion = 1 score, intensive nasal secretion = 2 scores; 3-5 sneezes = 1 score, >5 sneezes = 2 scores; tear flow and/or itching of throat and/or ears: 1 score, conjunctivitis and/or chemosis and/or urticaria and/or cough and/or dyspnea = 2 scores). The test was stopped if a 40% or more flow reduction was measured by rhinomanometry or if the symptoms score was at least 4

scores. The maximum allergen concentration tolerated by the subject was recorded. Concentrations tolerated before and after immunotherapy with the fragments were compared.

Supplementary Table 1: Change of IgE binding to Bet v 1 by fragment-specific IgG measured by ELISA (A405nm)

Binding of patients´IgE to Bet v 1 after pre-absorption with either fragment-specific rabbit IgG or control sera is displayed. In the right column, calculated fold of change of IgE binding is shown. SD: standard deviation of duplicate ELISA measurements.

Supplementary Table: Blocking/Enhancing of IgE binding by rabbit IgG

	A 405 nm				
patient	with fragment specific rabbit IgG	SD	with control sera	SD	change of IgE binding
1	**1.52**	0.03	**0.13**	0.02	**11.7**
2	**2.52**	0.01	**1.82**	0.02	**1.39**
3	**0.53**	0.03	**0.85**	0.07	**0.62**
4	**1.59**	0.04	**2.68**	0.07	**0.59**
5	**1.42**	0.02	**2.83**	0.01	**0.50**
6	**0.29**	0.05	**0.65**	0.01	**0.45**
7	**0.84**	0.01	**2.07**	0.03	**0.41**
8	**0.23**	0.05	**0.72**	0.06	**0.33**
9	**0.64**	0.02	**2.84**	0.06	**0.22**
10	**0.48**	0.02	**2.24**	0.15	**0.21**
11	**0.56**	0.02	**2.99**	0.05	**0.19**
12	**0.19**	0.02	**1.05**	0.06	**0.18**
13	**0.43**	0.03	**2.70**	0.10	**0.16**
14	**0.38**	0.03	**2.94**	0.02	**0.13**
15	**0.13**	0.02	**1.03**	0.08	**0.13**
16	**0.34**	0.02	**2.82**	0.05	**0.12**
17	**0.25**	0.00	**2.90**	0.02	**0.09**
18	**0.21**	0.00	**2.96**	0.00	**0.07**