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Inhibition of rBet v 1-induced basophil histamine release with specific immunotherapy –induced serum immunoglobulin G: no evidence that FcγRIIB signalling is important

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Summary

Background Human basophils and mast cells express the low-affinity immunoglobulin (Ig)G receptor FcγRIIB. It has previously been shown in artificial model systems that cross-linking of the high-affinity IgE receptor FcεRI and FcγRIIB leads to inhibition of FcεRI signalling.

Objective The aim of the present study was to investigate whether cross-linking of FcεRI and FcγRIIB contributes to IgG-mediated inhibition of histamine release in human basophils in a system using the sera from specific immunotherapy (SIT) patients and the major allergen from birch pollen, Bet v 1. As IgG4 furthermore has been proposed to have special blocking properties, we investigated the significance of IgG subclass specificity for this inhibition.

Methods Binding of recombinant Bet v 1–IgG complexes to FcγRII and IgG-binding activities in the sera from 25 birch pollen-allergic patients treated with SIT were measured using ¹²⁵I-rBet v 1. Inhibition of basophil histamine release was assessed by incubating washed leucocytes with complexes of rBet v 1–IgG with or without blocking of FcγRII.

Results We observed low binding of rBet v 1–IgG complexes to FcγRII, which was negatively correlated with the relative IgG-binding activities. Blocking of FcγRII did not reverse the SIT–IgG-induced inhibition of basophil histamine release. However, IgG-binding activities correlated significantly with the ability of the SIT sera to inhibit basophil histamine release.

Conclusion We suggest that at least in birch pollen SIT, the contribution of FcγRIIB-mediated inhibitory signalling to SIT–IgG-induced inhibition of human basophil histamine release is of minor importance. The main contributor to the inhibitory effect of SIT-induced IgG seems to be blocking of the allergen–IgE interaction.

Keywords basophils, blocking antibodies, FcγRII, IgE, IgG, IgG4, immunotherapy

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Introduction

Specific immunotherapy (SIT) is a potentially curative treatment of immunoglobulin (Ig)E-dependent allergy [1]. The clinical effect of SIT is well documented, and reductions in clinical parameters such as early- and late-phase reactions in the lungs, nose and skin have been shown. Furthermore, SIT has long-term efficacy [2, 3] and may prevent new sensitizations and inhibit progression from rhinitis to asthma [2, 4–6].

The immunological mechanisms behind the beneficial effect of SIT have not been fully clarified. An increase in allergen-specific IgG is one of the earliest described effects of SIT [7, 8]. Lichtenstein et al. [8] showed in 1968 that SIT-induced IgG blocked basophil histamine release; this observation has subsequently been confirmed by

many other investigators [9–11]. Furthermore, SIT-induced IgG blocks passive cutaneous anaphylaxis and serum-IgE facilitated antigen presentation to T cells [7, 12–15]. It has been hypothesized that ‘blocking’ antibodies represent an important aspect of the mechanism of SIT [16, 17].

It appears that competition for allergen binding between IgE and SIT-induced IgG is essential for the inhibitory effects of ‘blocking’ antibodies; however, studies in artificial model systems have shown that cross-linking of FcγRIIB and the high-affinity IgE receptor FcεRI leads to inhibition of IgE-mediated histamine release, Ca²⁺ signalling and cytokine production in human basophils, murine mast cells and rat basophilic leukaemia (RBL) cells [18–20]. From these experiments, in which different cells and cross-linking strategies were applied, it

is clear that activation of Fc γ RIIB in human basophils and mast cells represents an inhibitory pathway that may act on IgE-mediated Fc ϵ RI signalling. It remains to be established, however, whether this pathway is actually relevant *in vivo* in allergic patients undergoing SIT, or whether the inhibition is because of simple blocking of the allergen-IgE interaction.

Fc γ RII is the only Fc γ receptor expressed on human basophils, and of its three isoforms mainly, the Fc γ RIIB isoform has been observed [20–22]. Fc γ RII is a low-affinity receptor; K_d for monomeric IgG is 10^{-7} M, whereas IgG complexes bind with high avidity. However, monomeric IgG competes for binding depending on the relative concentrations of monomeric and complexed IgG [21]. The increase in allergen-specific IgG during long-term SIT occurs mainly in the IgG4 subclass [23, 24]. Some studies have indicated that IgG4 has special blocking properties, and inhibition of basophil histamine inhibition has been suggested to be primarily caused by IgG4 [25–28]. SIT-induced increases in allergen-specific IgG1, however, have also been demonstrated [10, 11, 29, 30], and inhibition of basophil histamine release has been observed using both monoclonal IgG1 and polyclonal IgG1 and IgG4 [31, 32].

The aim of the present study was to investigate the importance of Fc γ RIIB activation and IgG subclass specificity for the inhibitory effect of SIT-induced IgG on basophil histamine release in a system using the sera from SIT patients and the major allergen from birch pollen, Bet v 1.

Materials and methods

Patients, blood and serum samples

Sera from healthy controls and birch pollen-allergic patients treated with SIT using *Betula verrucosa* pollen extract (Alutard SQ, ALK-Abelló A/S, Hørsholm, Denmark) were obtained from the *in vitro* Diagnostics Department, ALK-Abelló. Sera from more than 10 healthy donors were pooled and used as negative serum control. All sera were blinded and clinical data were not available. The patients had been treated with SIT for 1–5 years at the time of blood sampling. All patients were RAST class 3–5 (specific IgE 31–778 SU/mL) before SIT. Blood samples, used for isolation of leucocytes, were obtained from healthy controls and birch pollen-allergic patients (specific IgE 1.3–8.8 SU/mL), who were not treated with SIT, after written informed consent. The study was approved by the local Ethical Committee.

Radio-labelling of rBet v 1

rBet v 1.2801 (EMBL accession number Z80104) was expressed and purified as described previously [33]. rBet

v 1.2801 (MW: 17 kD) was labelled with ^{125} I using chloramine-T and subsequently subjected to molecular size chromatography on Sephadex G-75 superfine columns (Pharmacia, Uppsala, Sweden) [34]. Fractions in which more than 95% of ^{125} I-rBet v 1 bound specific IgG and IgE from birch pollen-allergic patients were used. The specific activity was $2\text{--}3 \times 10^5$ cpm/ng.

Immunoglobulin E and immunoglobulin G depletion

Serum samples were heat inactivated at 56 °C for 30 min. Serum depletion of IgE was performed as described previously using supraoptimal amounts of anti-IgE monoclonal antibody (ALK-Abelló) covalently coupled to magnetic beads [34]. Residual activity of less than 4% was detected. In order to determine the non-IgE, non-IgG binding of rBet v 1 IgE-depleted serum was fractionated on protein G Sepharose columns, and binding of ^{125} I-rBet v 1 was tested as described previously [35].

rBet v 1 binding to immunoglobulin G

All dilutions were made in Pipes buffer (10 mM piperazine- N,N' -bis-2-ethanesulphonic acid, 140 mM Na-acetate, 5 mM K-acetate, 0.6 mM CaCl₂, 1.1 mM MgCl₂, pH 7.4, Bie & Berntsen, Copenhagen, Denmark) with 0.5% human serum albumin (HSA, Sigma, St Louis, MO, USA), and the binding assays were performed in duplicate.

Dilutions of heat-inactivated IgE-depleted serum were preincubated 18 h at 4 °C with ^{125} I-rBet v 1 and unlabelled rBet v 1 in a final total concentration of 5 ng/mL. In order to measure the binding activities under the same conditions as those used for histamine release experiments, samples were subsequently prewarmed 30 min at 37 °C before addition of Pipes buffer with 0.5% HSA to a final concentration of 2.5 ng/mL rBet v 1, and incubated 30 min at 37 °C before the binding of ^{125} I-rBet v 1 to IgG was determined by affinity separation at 4 °C applying 200 μ L on a 0.5 mL protein G column as described previously [35]. The protein G columns were reused following one extra washing sequence. The background binding was measured by applying ^{125} I-rBet in assay buffer to the columns. Unchanged background binding was found after four runs of positive sample, and likewise after the last run on the columns. Radioactivity (cpm) in fractions containing bound and nonbound ^{125} I-rBet v 1 was determined with errors below 2% in a gamma counter (Wizard 1470 automatic gamma counter, Wallac, OY, Finland) for estimation of bound and free rBet v 1. The background binding was below 2% of cpm added. The inter- and intraassay variation was below 15%. The rBet v 1-binding activities were calculated relative to an internal standard serum, for which the binding activity was assigned an arbitrary value of 100 U/mL.

rBet v 1 binding to immunoglobulin G4

The relative contribution of IgG4 to IgG-rBet v 1 complexes was estimated by simultaneous anti-IgG4 and protein G affinity separation of heat-inactivated IgE-depleted serum preincubated with ^{125}I -rBet v 1. The relative IgG4-binding activity was calculated as the ratio between absorption to anti-IgG4 and protein G columns, respectively.

Anti-IgG4 columns were prepared as described previously using anti-human IgG4 monoclonal antibody G4.T9 (ALK-Abelló) [35]. The specificity of the affinity column with respect to IgG4 was confirmed in ELISA for IgG1 and IgG4 using sheep anti-human IgG1 and IgG4 (The Binding Site, Birmingham, UK). Measurements were made at a concentration of IgE-depleted serum where 50% of the total ^{125}I -Bet v 1 (0.2 ng/mL) was bound to the IgG. All dilutions were made in Pipes buffer with 0.5% HSA. Simultaneously, 100–200 μL of the same sample of heat-inactivated IgE-depleted serum preincubated with ^{125}I -Bet v 1 was added to the anti-IgG4 (1 mL anti-IgG4 antibody-agarose beads) and the protein G column (0.5 mL protein G Sepharose CL-4B (Pharmacia)), respectively. Nonbound ^{125}I -Bet v 1 was eluted in 3 mL of PBS pH 7.4 containing 0.025% gelatin and 0.1% Triton X-100, and the bound material was eluted with 3 mL of 0.1 M glycine-HCl, 0.15% bovine serum albumin (Sigma), pH 2.5. The anti-IgG4 and the protein G columns were reused, and no changes in background binding were found. Radioactivity in both fractions was determined, and the ratio of bound and free cpm (B/F) was calculated for both columns. For all samples, the background binding on both the IgG4 and the protein G column was determined in the presence of 200 ng/mL rBet v 1 using the same columns. The background binding was below 1% for the anti-IgG4 column and below 2% for the protein G column; these values were subtracted from values measured without a competitor. The reproducibility of the measurements was between 88% and 94%. IgG4-binding activities were calculated as the IgG-binding activities (see the previous section) multiplied by the fraction of IgG4 binding measured in this assay.

Expression of Fc γ RII

Expression of Fc γ RII on Raji cells and basophils from all donors was examined by flow cytometry using an anti-CD32 clone AT.10 (Serotec, Oxford, UK). In order to identify basophils in the suspension of purified leucocytes, samples were also stained with a-CD203c.

Flow cytometry assay for binding of rBet v 1-immunoglobulin G complexes

rBet v 1 was labelled with fluorescein-isothiocyanate (FITC) using standard protocols provided by the manufac-

turer (Sigma). Complexes of FITC-rBet v 1 and heat-inactivated IgE-depleted serum were prepared by incubating 10–65% serum with 1 ng/mL to 10 $\mu\text{g/mL}$ FITC-rBet v 1 for 18 h at 4 °C. Leucocytes were purified from fresh heparinized blood samples from healthy controls by lymphoprep separation. Basophils accounted for 0.5–2% of the purified cells, the remainder consisting of B- and T cells, monocytes and granulocytes. Washed leucocytes were added to the preincubated complexes and incubated for 30 min at 37 °C or for 18 h at 4 °C while gently shaking. Samples were stained with a-CD203c PE (Immunotech, Marseille, France) and analysed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Receptor assay for binding of rBet v 1-immunoglobulin G complexes to Raji cells

Raji cells, obtained from the American Type Culture Collection, were maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK) and 100 IU penicillin-streptomycin (Gibco), and grown at 37 °C in a humidified atmosphere of 5% CO₂. Complexes of rBet v 1 and heat-inactivated IgE-depleted serum were prepared by incubating 10% serum with ^{125}I -rBet v 1 and unlabelled rBet v 1 in combined final concentrations of 0.5, 5 and 50 ng/mL rBet v 1 for 18 h at 4 °C. The cell-binding assay was performed in duplicate samples of 240 μL by adding washed Raji cells from 48 h cultures (final concentration 6×10^6 cells/mL) to preincubated complexes of rBet v 1 and serum and incubating 18 h at 4 °C or 30 min at 37 °C while gently shaking. The final concentrations were 5% serum and 0.25, 2.5 and 25 ng/mL rBet v 1. For Fc γ RII blocking experiments, Raji cells were preincubated with a-CD32 Fab (clone 7.3, Ancell, MN, USA) for 30–40 min at 4 °C prior to the cell-binding assay. Tubes were put on ice and cell-bound rBet v 1 was separated by centrifugation of 200 μL of the cell suspension on dibutyl phthalate and bis(2-ethylhexyl) phthalate ratio 1 : 1 (Merck, Darmstadt, Germany). Cpm in cell pellet and supernatant were determined, and rBet v 1 binding to cells (B/T) was calculated as cpm in cell pellet/total cpm.

Inhibition of basophil histamine release

Dilutions of heat-inactivated IgE-depleted serum and rBet v 1 were incubated overnight at 4 °C. The concentration of rBet v 1 was 5 ng/mL and the serum concentration was kept constant at 10% by adding a pooled negative control serum.

Leucocytes were purified from heparinized fresh blood samples from birch pollen-allergic patients by lymphoprep separation, washed in Hepes buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1.2 mM K₂HPO₄, 5.5 mM glucose, pH 7.4, Bie & Berntsen) with 0.5% HSA, and resuspended in Pipes buffer with 0.1% HSA. Serum-rBet v 1 complexes and cell suspensions were prewarmed simultaneously for

30 min at 37 °C with or without addition of 5 µg/mL a-CD32 Fab (clone 7.3) to the cells, before 75 µL cell suspension was added to 75 µL serum-rBet v 1 complexes in triplicate and incubated 30 min at 37 °C. Plates were centrifuged, and the supernatants collected and stored at -20 °C. Furthermore, the total histamine content was measured in control wells on each plate. Histamine was measured using a commercial ELISA kit (Immunotech).

Statistical analyses

Student's *t*-test was performed and Pearson's correlation coefficient was calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). *P*-values below 0.05 were considered significant.

Results

Binding activities in the sera

In this study, we investigated sera from 25 birch pollen-allergic patients treated with SIT for 1–5 years. Preliminary experiments showed that some of the SIT sera contained complement activity. Complement activation could lead to binding of IgG complexes to complement receptors present on basophils. Furthermore, the complement fragments C3a and C5a can induce histamine release from basophils. Therefore, all experiments reported were performed using heat-inactivated serum.

To investigate the serum factors contributing to the rBet v 1-binding activity, we depleted sera of IgE and IgG and observed that less than 2% of the rBet v 1-binding activity remained (*n* = 5). All further experiments were performed with IgE-depleted serum, and the measured binding activities were ascribed to IgG.

To characterize the rBet v 1-binding activities in the sera, IgG binding to rBet v 1 was measured and expressed relative to an internal standard. The ratio of IgG4 and IgG binding was determined by simultaneous absorption to a-IgG4 and protein G columns. The results are shown in Table 1. The IgG-binding activities varied 15-fold between patients. The ratios of IgG4- and IgG-binding activities varied between 0.04 and 0.87, with 80% of the SIT sera displaying ratios above 0.6.

Binding of rBet v 1-immunoglobulin G complexes to FcγRII

To measure binding of rBet v 1-IgG complexes to FcγRII on basophils, we initially used a flow cytometric assay with FITC-labelled rBet v 1 and a basophil- and mast cell-specific antibody (a-CD203c). FITC-rBet v 1 in concentrations ranging from 1 ng/mL to 10 µg/mL was preincubated with between 10% and 65% serum-IgG, added to washed leucocytes from healthy nonallergic donors and incubated for 30 min at 37 °C. The experiments were performed

Table 1. Binding of rBet v 1 to IgG and IgG4 in SIT-sera

Serum	SIT (months)*	IgG binding (U/mL) [†]	IgG4 binding/IgG binding [‡]
1	42	14.4	0.78
2	24	36.1	0.81
3	36	6.6	0.66
4	36	15.2	0.62
5	36	22.8	0.70
6	60	27.9	0.75
7	52	26.8	0.43
8	39	33.8	0.81
9	48	17.5	0.87
10	48	23.1	0.74
11	48	9.7	0.51
12	36	25.4	0.64
13	36	8.5	0.75
14	12	11.2	0.60
15	29	13.2	0.62
16	48	14.1	0.69
17	36	24.9	0.64
18	36	15.0	0.25
19	18	10.4	0.054
20	42	13.9	0.71
21	60	13.6	0.81
22	60	16.3	0.68
23	30	43.0	0.036
24	30	100.0	0.69
25	30	42.0	0.70

*Months of SIT treatment at the time of blood sampling. [†]IgG binding was calculated relative to an internal standard (serum 24) where the binding activity was assigned the arbitrary value 100 U/mL. [‡]The ratio of IgG4 binding to IgG binding was measured by simultaneous a-IgG4 and prot G affinity separation.

SIT, specific immunotherapy; IgG, immunoglobulin G.

using several basophil donors, and expression of FcγRII was confirmed on all donors; however, under these conditions no binding of rBet v 1-IgG complexes could be detected. Binding of FITC-labelled rBet v 1 alone to specific IgE on the surface of basophils from birch pollen-allergic individuals was detectable by flow cytometry and the binding characteristics of FITC-labelled rBet v 1 were furthermore shown to be similar to unlabelled rBet v 1 in a fluid phase assay (data not shown). Attempts to use more potent detection systems with other fluorochromes in the flow cytometry assay did not result in detectable binding. Therefore, we next investigated the binding of rBet v 1-IgG complexes to FcγRII in a receptor assay system using ¹²⁵I-labelled rBet v 1 and FcγRII expressing Raji cells. Raji cells were used in this assay as it was not possible to obtain the large numbers of pure basophils needed (approximately 2.5 × 10⁶ cells per duplicate sample). The only FcγR expressed on Raji cells is FcγRII, and expression of the isoforms FcγRIIA, FcγRIIB1 and FcγRIIB2 has been shown by PCR and FACS [36]. The extracellular parts of the FcγRII isoforms are more

than 95% identical, and differences in binding of IgG complexes have not been demonstrated [37]. We found a similar expression level of Fc γ RII when the Raji cell line and basophils from the leucocyte donors in this study were compared using flow cytometry ($n=5$, data not shown). Raji cells are thus a useful model for studying binding to Fc γ RII and have been widely used to detect immune complexes [38].

Initial experiments with the receptor assay showed that binding of ^{125} I-rBet v 1-IgG complexes was detectable and furthermore enhanced by incubation at 4 °C for 18 h compared with 37 °C for 30 min (data not shown). rBet v 1-IgG complexes were prepared with rBet v 1 in varying concentrations and a constant concentration of serum-IgG, and binding was detected following incubation at 4 °C for 18 h to optimize binding. A representative example of rBet v 1-IgG binding to Raji cells is shown in Fig. 1. Binding of rBet v 1-IgG complexes increased with increasing rBet v 1 concentration in all the tested sera. Repeating the tests with the most active sera but using FITC-rBet v 1 and flow cytometry did not make binding of rBet v 1-IgG complexes to Raji cells detectable. Thus, the flow cytometry assay was less sensitive than the ^{125} I-rBet v 1-binding assay, and further experiments were performed with Raji cells using the ^{125} I-rBet v 1-binding assay and 18 h of incubation at 4 °C. The rBet v 1-IgG complexes used for the receptor assay were analysed by protein G absorption to investigate how much rBet v 1 was bound by IgG at the different concentrations of rBet v 1. At 0.25 ng/mL rBet v 1 87–97% of the rBet v 1 was bound, at 2.5 ng/mL 80–95% and at 25 ng/mL 35–83% depending on the serum. In addition, the rBet v 1-IgG complexes were stable; when a 200-fold excess of unlabelled ligand was added after preincubation of the complexes, less than

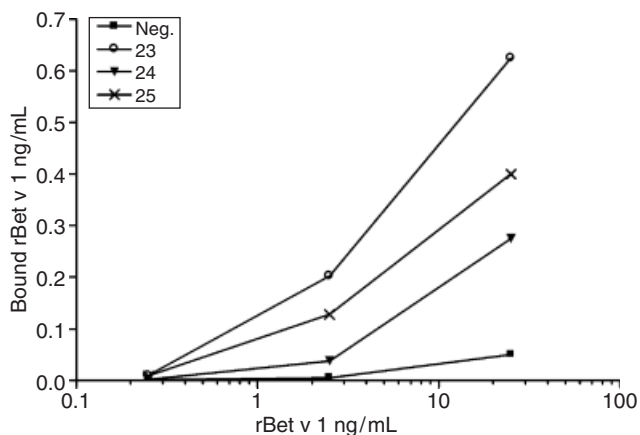


Fig. 1. Binding of complexes of rBet v 1 and immunoglobulin (Ig)E-depleted specific immunotherapy (SIT)serum to Raji cells. IgE-depleted SIT sera and negative control serum were preincubated with ^{125} I-rBet v 1, added to Raji cells and incubated for 18 h at 4 °C. The final concentrations were 5% serum and 0.25, 2.5 and 25 ng/mL rBet v 1. Data are shown as mean values of duplicate experiments.

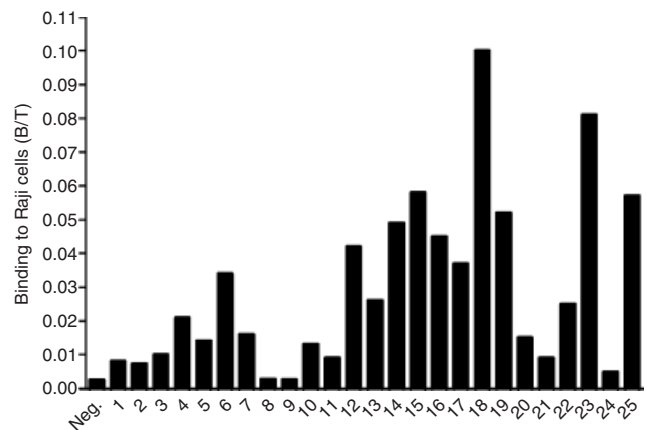


Fig. 2. Binding of complexes of rBet v 1 and immunoglobulin (Ig)E-depleted specific immunotherapy (SIT) serum to Raji cells. IgE-depleted SIT sera and negative control serum were preincubated with ^{125} I-rBet v 1, added to Raji cells and incubated for 18 h at 4 °C. Final concentrations were 5% serum and 2.5 ng/mL rBet v 1. Data are shown as mean values of duplicate experiments. Binding was calculated as B/T: cpm bound/total cpm.

20% of the ^{125} I-rBet v 1 was displaced after 30 min at 37 °C. Based on these results, we decided to perform further analysis of rBet v 1-IgG complex binding with a final rBet v 1 concentration of 2.5 ng/mL. At this concentration, more than 80% of the rBet v 1 was bound by IgG and the concentration was relevant for performing histamine release experiments in human basophils.

The binding of rBet v 1-IgG complexes to Raji cells was measured in all 25 SIT sera. As shown in Fig. 2, a large heterogeneity in Fc γ RII binding was observed. The maximum binding of ^{125} I-rBet v 1-IgG complexes to Raji cells was 10% of the total rBet v 1 concentration, equivalent to approximately 1500 molecules of rBet v 1 bound per cell. However, in 22 of the 25 tested sera binding was above two times the background. When the same experiment was repeated with incubation of complexes and cells for 30 min at 37 °C, binding was reduced more than fourfold but the same pattern of relative binding was observed (data not shown).

Binding of rBet v 1-IgG complexes to Raji cells was blocked in a dose-dependent manner with Fab fragments of an Fc γ RII-specific antibody (clone 7.3). Figure 3 shows blocking of ^{125} I-rBet v 1-IgG complex binding to Raji cells with two different sera demonstrating high-binding activity to Raji cells. Binding to Raji cells was reduced to the level of negative control serum by preincubation with 1 μ g/mL of α -Fc γ RII Fab. Similar blocking by the α -Fc γ RII Fab fragments was found using six other SIT sera with high Raji-binding activities (data not shown), indicating that the binding of rBet v 1-IgG complexes to Raji cells was mediated by Fc γ RII and confirming that the α -Fc γ RII Fab was blocking as shown earlier [39].

There was no correlation between the IgG-binding activities in the sera and their capacity to bind to Fc γ RII

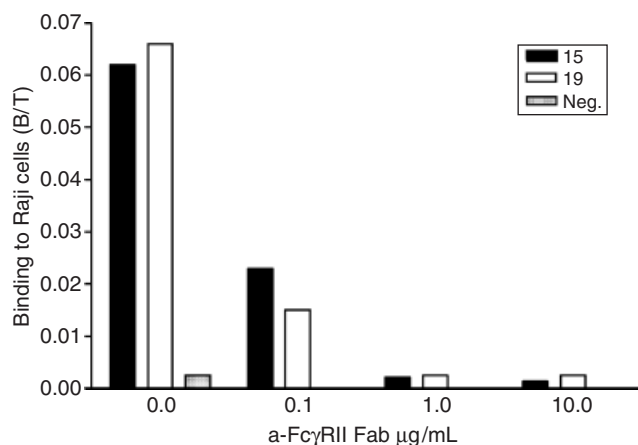


Fig. 3. Raji cell binding of complexes of rBet v 1 and IgE-depleted specific immunotherapy (SIT) sera is blocked by a-Fc γ RII Fab fragments. Immunoglobulin E-depleted SIT sera and negative control serum were preincubated with ^{125} I-rBet v 1 and added to Raji cells with or without a-Fc γ RII Fab in the concentrations indicated. A representative example of a blocking experiment is shown. The final concentrations were 5% serum and 2.5 ng/mL rBet v 1. Data are shown as mean values of duplicate experiments. Binding was calculated as B/T: cpm bound/total cpm.

at this concentration of rBet v 1; however, a significant negative correlation between IgG4/IgG-binding activities and binding to Fc γ RII was observed ($r = -0.68$, $P = 0.0002$, Fig. 4).

Effect of Fc γ RII blocking on specific immunotherapy-immunoglobulin G-mediated inhibition of basophil histamine release

To determine the contribution of Fc γ RII activation to the inhibitory effect of SIT sera on basophil histamine release, we investigated whether blocking of Fc γ RII on basophils

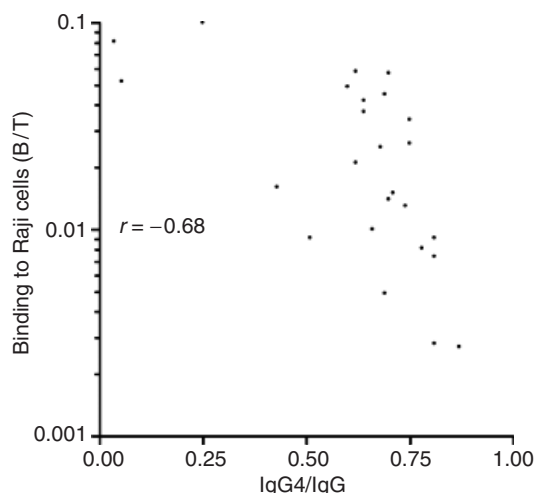


Fig. 4. Relationship between the ratio of immunoglobulin (Ig)G4- and IgG-binding activities and binding of complexes of IgE-depleted specific immunotherapy serum and rBet v 1 to Raji cells.

would reverse inhibition of histamine release. First, we investigated blocking of Fc γ RII using the same concentrations of complexed serum-IgG and rBet v 1 used for detection of rBet v 1-IgG complex binding to Raji cells; Fig. 5a shows a representative example of a blocking experiment. Blocking Fc γ RII on basophils from different donors ($n = 5$) did not reverse the inhibition of histamine release in seven sera, all of which bound to Raji cells with high activity compared with the rest of the sera. We did observe reversal of inhibition during blocking of Fc γ RII in one SIT-serum; however, these results could not be

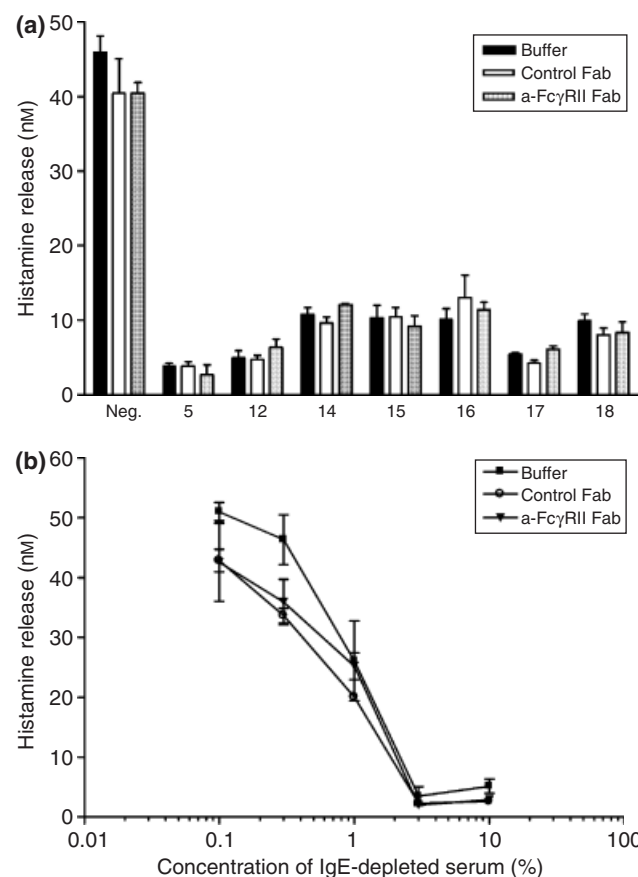


Fig. 5. Blocking of Fc γ RII does not reverse the specific immunotherapy (SIT)-immunoglobulin (Ig)G-mediated inhibition of basophil histamine release. In (a) and (b), representative examples of blocking experiments are shown. (a) IgE-depleted serum from seven birch pollen SIT patients and negative control serum were preincubated with rBet v 1 and added to washed leucocytes from a birch pollen-allergic patient preincubated with buffer, control Fab (5 μ g/mL) or a-Fc γ RII Fab (5 μ g/mL). The final concentration of rBet v 1 was 2.5 ng/mL rBet v 1 and the serum concentration was 5%. Data are shown as mean values of triplicates; error bars represent SEM. (b) Dilution series of IgE-depleted serum from patient 23 were preincubated with rBet v 1 and added to washed leucocytes from a birch pollen-allergic patient preincubated as in (a). The final concentration of rBet v 1 was 2.5 ng/mL rBet v 1 and the serum concentration was maintained at 5% by addition of negative control serum. Data are shown as mean values of triplicates; error bars represent SEM.

reproduced using other basophil cell donors. The lack of effect of blocking Fc γ RII under these conditions could be because of a close to maximal inhibition of Fc ϵ RI signalling, resulting in only a minor activation signal left to be inhibited by Fc γ RII activation. Thus, we investigated blocking of Fc γ RII with serial dilutions of IgE-depleted SIT serum. However, as shown in Fig. 5b, blocking Fc γ RII under these conditions also did not reverse inhibition; similar results were obtained with three other sera using leucocytes from different donors ($n = 5$). On the contrary, we did, in some experiments, observe that preincubation with the a-Fc γ RII antibody had additional inhibitory effects on histamine release (data not shown).

Correlation between immunoglobulin G-binding activities and inhibition of basophil histamine release

In order to compare the capacity of the sera to inhibit basophil histamine release with the IgG-binding activities, we quantified the abilities of the SIT sera to inhibit basophil histamine release. rBet v 1 was preincubated with varying concentrations of IgE-depleted serum and added to washed leucocytes from birch pollen-allergic patients. Histamine release was measured and inhibition titres were calculated from inhibition curves as the concentration of serum that induced 50% inhibition of histamine release compared with controls with negative serum. The experiments were performed with leucocytes from two different birch pollen-allergic patients. Overall, the levels of inhibition titres were different for the two patients, reflecting a difference in basophil sensitivity to rBet v 1-induced histamine release, but when the sera were ranked according to inhibitory capacity, a similar pattern was found in the two patients (data not shown). To investigate whether IgG4 is particularly important for the inhibitory effect of SIT-induced IgG, we compared the inhibition titres of the two patients with the IgG4-binding activities in the SIT sera. A significant correlation was found for one of the patients ($r = -0.47$, $P = 0.021$); however, for the other patient, no correlation was found ($r = -0.45$, $P = 0.052$). Comparing the inhibition titres with the total IgG-binding activities, however, significant correlations were found for both patients ($r = -0.47$, $P = 0.018$ and $r = -0.54$, $P = 0.0068$, respectively, Figs 6a and b).

Discussion

It is well established that SIT-induced serum IgG is capable of inhibiting basophil histamine release [8–11, 40]. As cross-linking of the inhibitory Fc γ RIIB and the high-affinity IgE receptor Fc ϵ RI in artificial model systems has been shown to inhibit mediator release from RBL cells, mouse mast cells and human basophils, it has been hypothesized that activation of Fc γ RII by SIT-induced IgG

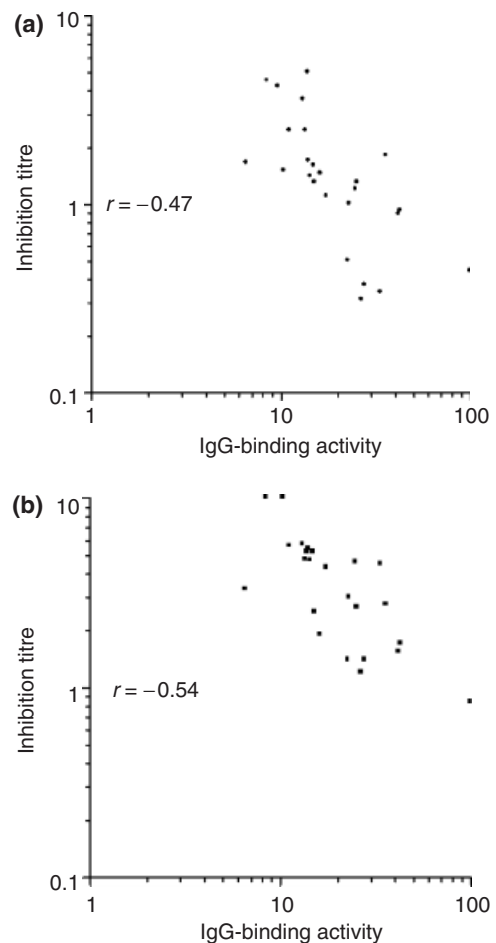


Fig. 6. Relationship between immunoglobulin G-binding activities in the specific immunotherapy sera and their ability to inhibit histamine release in washed leucocytes from two different birch pollen-allergic patients A and B.

could be a mechanism of SIT [18–20]. All the studies on Fc γ RII-induced inhibition of Fc ϵ RI activation, however, were performed under experimental conditions where the receptors were cross-linked by receptor antibodies, or cells were sensitized with mono-specific IgE and IgG.

The aim of the present study was to investigate whether Fc γ RIIB activation plays a role in the inhibitory effect of SIT-induced IgG on basophil histamine release under more physiological conditions using SIT sera and the major allergen from Birch pollen; Bet v 1. The increase in allergen-specific IgG was demonstrated to be mainly in the IgG4 subclass and it has been suggested that IgG4 has particular blocking properties [23–28]. Thus, we also investigated the role of SIT-induced rBet v 1-specific IgG4 in the inhibition of basophil histamine release.

To investigate a possible contribution of Fc γ RII signalling to SIT-IgG-mediated inhibition, we established an experimental model where rBet v 1 and IgE-depleted SIT-serum was preincubated to ensure optimal formation of complexes. This was done to create optimal conditions for

binding of complexes to Fc γ RII, which binds complexed IgG with high avidity. We hypothesized that binding of rBet v 1-IgG complexes to specific IgE on the Fc ϵ RI would enable cooperative binding of IgG to nearby Fc γ RIIs. The *in vivo* situation may be quite different; one study in cat-allergic patients indicated the presence of circulating immune complexes containing allergen, IgE and IgG [41].

We were not able to detect binding of complexes of rBet v 1 and IgE-depleted serum to basophils using a flow cytometric assay. However, using a more sensitive receptor assay we could demonstrate binding of ^{125} I-rBet v 1-IgG complexes to Raji cells. Blocking Fc γ RII binding with α -Fc γ RII (clone 7.3 Fab) fragments showed that the binding of rBet v 1-IgG complexes was mediated by Fc γ RII, and that the monoclonal antibody had blocking properties, in agreement with earlier observations [39]. The expression level of Fc γ RII on Raji cells was similar to that found on basophils. When the binding of rBet v 1-IgG complexes was tested in all 25 SIT sera following incubation at 4 °C for 18 h, a highly heterogeneous result was observed. Under the present experimental conditions, maximally 10% of the rBet v 1 was bound, corresponding to approximately 1500 molecules of rBet v 1 per cell. However, when binding was tested after incubation at 37 °C for 30 min binding was still detectable but more than fourfold lower. When considering SIT-induced IgG and its ability to activate Fc γ RIIB, it should be noted that allergen-specific IgG only accounts for a very small fraction of the total IgG. Although monomeric IgG binds with low affinity to Fc γ RII, monomeric IgG is in far excess over allergen-IgG complexes and it will compete significantly for binding to the receptor [21].

Interestingly, an increased IgG4/IgG ratio was significantly correlated with lower binding to Raji cells, indicating that the subclass composition of the specific antibody response is important for binding to Fc γ RII. It has previously been shown that the affinity of Fc γ RIIB for complexed IgG4 is lower than for other IgG subclasses [42]. IgG4 is functionally monovalent, leading to formation of small nonprecipitating complexes [43, 44]; this could be a reason for the reduced affinity. It has been shown by several investigators, and was also observed in this study, that allergen-specific IgG responses of most SIT-treated patients eventually become dominated by IgG4 during treatment, thus making activation of Fc γ RII by SIT-induced IgG progressively difficult [23, 24].

In our experiments, blocking Fc γ RII on human basophils did not reverse the SIT-IgG-induced inhibition of histamine release. The concentration of Fc γ RII-specific Fab fragments used for blocking Fc γ RII on basophils was chosen from experiments using Raji cells. We first investigated blocking using the same concentrations of rBet v 1 and IgG as those used for measuring binding of complexes to Raji cells and found no effect of blocking. This could, however, be because of a close to maximal inhibition of

Fc ϵ RI activation caused by allergen binding to IgG, and thus, it was possible that under these conditions activation of Fc γ RIIB would not be detectable. We therefore investigated blocking of Fc γ RII with serial dilutions of serum inducing varying degrees of inhibition; this also did not influence inhibition of histamine release. Our results indicate that activation of Fc γ RII does not play an important role in SIT-IgG-mediated inhibition of basophil histamine release under these experimental conditions.

Fab fragments were used to block Fc γ RII in order to avoid cross-linking and possible activation of Fc γ RII. Earlier studies have shown that aggregation of Fc γ RII alone had no effect on Fc ϵ RI signalling, and that activation of Fc γ RII depended on phosphorylation by Fc ϵ RI-associated Lyn; however, contrary to this, another study showed that independent aggregation of Fc γ RII and Fc ϵ RI inhibited Fc ϵ RI-mediated release [19, 20, 45].

Investigating the relationship between IgG-binding activities in the SIT sera and their ability to inhibit basophil histamine release in washed leucocytes from two birch pollen-allergic patients, we could show significant correlations, confirming the results of Sobotka et al. [40], who found a significant correlation between levels of IgG binding of Amb a 1 and inhibition of basophil histamine release in 33 SIT-treated ragweed-allergic patients. Furthermore, this result indicates a simple relationship between allergen-specific IgG and blocking activities, according to which the total amount of allergen-specific antibodies contributes to inhibition and not specific subclasses.

Theoretically, it could be argued that the correlation between IgG-binding activities and histamine release inhibition titres is conferred by Fc γ RII, as activation of this receptor could be proportional to the antibody concentration. This does not seem to be the case, however, as sera with similar binding activities showed large differences in the ability to form complexes with rBet v 1 and binding to Raji cells.

Cross-linking of Fc γ RII and Fc ϵ RI was theoretically proposed to represent an inhibitory pathway in human basophils and mast cells during SIT. Our results, however, show that birch pollen SIT-induced IgG does not activate this pathway to any significant degree. It is, however, a pathway that has therapeutic potential. Studies have shown that a fusion protein of the Fc part of IgE and IgG can inhibit IgE-mediated release from human basophils and mast cells [46, 47]. Recently, another study has shown inhibitory properties of a fusion protein consisting of Fab parts of an α -IgE and an α -Fc γ RIIB antibody [48].

In conclusion, we suggest that at least in birch pollen SIT, the contribution of Fc γ RIIB-mediated inhibitory signalling to SIT-IgG-induced inhibition of basophil histamine release in human basophils is of minor importance compared with the direct blocking of the interaction between IgE and rBet v 1 by SIT-induced allergen-specific IgG.

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