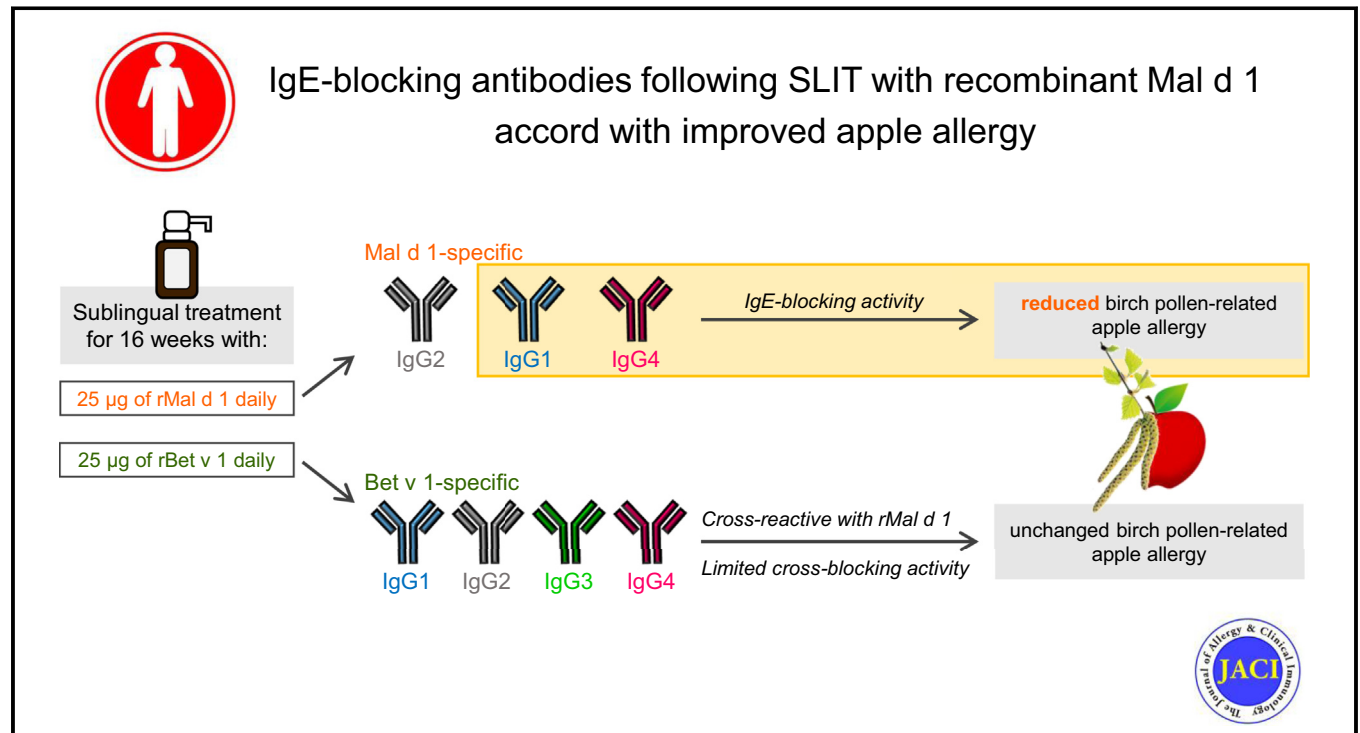


IgE-blocking antibodies following SLIT with recombinant Mal d 1 accord with improved apple allergy

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GRAPHICAL ABSTRACT



Background: We recently reported that 16 weeks of sublingual immunotherapy (SLIT) with recombinant (r) Mal d 1, but not rBet v 1, significantly improved birch pollen-related apple allergy. Allergen-specific IgE-blocking IgG antibodies have been associated with clinical efficacy.

Objective: We compared the quantity, quality, and IgE-blocking bioactivity of SLIT-induced Mal d 1-specific IgG antibodies in both treatment groups.

Methods: Pre- and post-SLIT sera were assessed for rMal d 1-specific IgG antibodies in ELISA and for their ability to inhibit apple allergen-induced upregulation of CD63 on basophils from

nontreated individuals with birch pollen-related apple allergy. Post-SLIT sera depleted of IgG1 or IgG4 were compared for their IgE-blocking activity. IgG1 binding to rMal d 1 was competed with rMal d 1 and rBet v 1 in ELISA.

Results: SLIT with rMal d 1 and rBet v 1 induced comparable levels of rMal d 1-specific IgG1, IgG2, IgG3, and IgG4 antibodies. Only post-rMal d 1 SLIT sera displayed IgE-blocking activity, which was significantly reduced by depletion of IgG1 and less so by IgG4 depletion. In competition ELISA, IgG1 binding to Mal d 1 in post-rMal d 1 SLIT sera was fully inhibited with rMal d 1 but not with rBet v 1. Correspondingly,

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submitted work. The rest of the authors declare that they have no relevant conflicts of interest.

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Bet v 1 was the more potent competitor for IgG1 binding to Mal d 1 in post-rBet v 1 SLIT sera.

Conclusion: rMal d 1 SLIT for 16 weeks induced functional, primarily Mal d 1-specific IgE-blocking antibodies, whereas rBet v 1 SLIT induced Bet v 1-specific, Mal d 1-cross-reactive IgG antibodies with limited cross-blocking activity. These results provide a possible explanation for the limited effectiveness of birch pollen immunotherapy in birch pollen-related food allergy and indicate a dominant protective role of functional IgE-blocking IgG1 antibodies in the early phase of allergy treatment. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: Food allergy, birch pollen allergy, birch pollen-related food allergy, IgE, allergen-specific immunotherapy, IgE-blocking antibodies, recombinant allergens, Bet v 1, Mal d 1

Birch pollen-related apple allergy (BPRAA) is the most prevalent food allergy in adolescents and adults in Northern Europe, and it affects up to 70% of individuals allergic to birch pollen.¹ Its main manifestation is the oral allergy syndrome, which refers to itching, swelling, and burning sensations in the oropharynx that develops within minutes after the consumption of fresh apples. Unlike seasonal birch pollinosis, BPRAA often persists perennially and significantly impairs the quality of life of the affected individuals.² BPRAA results from respiratory sensitization to the major birch pollen allergen, Bet v 1, and subsequent cross-reactivity of specific IgE antibodies with the homologous major allergen in apple, Mal d 1.^{3,4} Furthermore, Bet v 1-specific T cells cross-react with the apple allergen.^{5,6} Yet, the success of allergen-specific immunotherapy (AIT) with birch pollen in BPRAA has been debated.⁷⁻¹⁴ A limited effect of AIT with birch pollen has also been observed for birch pollen-related allergy to hazelnuts.¹⁵ In search of more effective treatment options, we recently performed a 16-week, double-blind placebo-controlled study of sublingual immunotherapy (SLIT) with recombinant (r) Mal d 1, rBet v 1, or placebo in 60 individuals with confirmed BPRAA.¹⁶ SLIT with rMal d 1 significantly reduced clinical reactions to oral challenges with the apple allergen, whereas this was not the case in either rBet v 1-treated or placebo-treated individuals.¹⁶

Successful SLIT has been associated with a modulation of allergen-specific cellular and humoral responses.^{17,18} Accordingly, we observed a downregulation of rMal d 1-specific T_H2 responses¹⁹ and significantly increased rMal d 1-specific IgG4/IgE ratios after SLIT with rMal d 1.¹⁶ Clinically effective AIT has been associated with the induction of allergen-specific blocking IgG antibodies that compete with IgE for allergen binding.^{20,21} In particular, allergen-specific IgG4 antibodies are thought to account for IgE blocking.²²⁻²⁴ However, the IgE-blocking bioactivity has been shown to be determined by the specificity and not by the subclass of allergen-specific IgG antibodies.^{25,26}

The aim of this study was to investigate whether 16 weeks of SLIT with rMal d 1 and rBet v 1 induced rMal d 1-specific IgE-blocking antibodies. In basophil inhibition tests we detected IgE-blocking bioactivity in post-SLIT sera from rMal d 1-treated individuals but not from rBet v 1-treated individuals. This finding tempted us to further characterize the Mal d 1-specific IgG response induced by either treatment. We compared the subclasses of Mal d 1-specific IgG antibodies and in particular, the

Abbreviations used

AIT: Allergen-specific immunotherapy
BAT: Basophil activation test
BPRAA: Birch pollen-related apple allergy
HRP: Horseradish peroxidase
SLIT: Sublingual immunotherapy

quantity, quality, and functional IgE-blocking activity of IgG1 and IgG4 antibodies.

METHODS

Serum samples

Ample volumes of serum samples collected at baseline and after 16 weeks of daily SLIT with 25 µg of either rMal d 1 or rBet v 1¹⁶ (ClinicalTrials.gov identifier NCT01449786 and EudraCT identifier 2011-001221-24) were available from 18 and 15 individuals, respectively. Ethical clearance for this study has been provided by the local ethics committee (EK228/2011).

Allergens

rMal d 1 (isoform rMal d 1.0108) and rBet v 1 (isoform Bet v 1.0101) were produced in-house in BL21 (DE3) *Escherichia coli* and purified by anion exchange chromatography and hydrophobic interaction chromatography followed by anion exchange chromatography, respectively. Both allergen preparations were controlled for purity, integrity, and IgE-binding activity as previously described,¹⁶ and both contained less than 40 endotoxin activity units of endotoxin per mg of protein as measured by Endozone II recombinant factor C assay (Hyglos, Bernied am Starnberger See, Germany). The apple extract was produced from Golden Delicious apples following a published protocol.²⁷ Briefly, 60 g of apple pulp was homogenized for 2 minutes in a blender in chilled 23 mM sodium phosphate buffer containing 0.8 mM EDTA disodium salt, 10 mM sodium diethyldithiocarbamate, 4% polyvinylpyrrolidone, 0.5 mM phenylmethanesulfonyl fluoride, and a protease inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation at 10,000 g for 30 minutes, the supernatant was filtered (0.45-µm filter, Millipore, Bedford, United Kingdom) and stored at -80°C. The protein content was measured by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Mass).

Basophil activation and inhibition tests

Basophil activation tests were performed as previously described.²⁸ Briefly, titrated amounts of recombinant allergens or apple extract (0.25-100 ng/mL) in HEPES calcium buffer (pH 7.4) supplemented with IL-3 (2 ng/mL) were added to heparinized whole blood from 4 untreated subjects with BPRAA who displayed rMal d 1-specific IgE levels of 1.1, 1.4, 2.6, and 5.8 kU_A/L, respectively. As a negative control, blood was stimulated with HEPES/IL-3 to confirm that basophils were not activated nonspecifically. After incubation for 15 minutes at 37°C the reaction was stopped with HEPES/EDTA (20 mmol/L) buffer and cells were stained with anti-CCR3 (allophycocyanin), anti-CD123 (peridinin-chlorophyll-protein), and anti-CD63 (phycoerythrin) (all from BioLegend, San Diego, Calif). After lysis of erythrocytes, basophils were defined as CD123⁺CCR3⁺ cells by using BD FACSCanto II and BD FACSDiva software (version 6.1.3) (BD Pharmingen, San Jose, Calif). The concentration of allergen that induced approximately 40% to 50% of CD63⁺ basophils in reference to untreated cells was incubated with the indicated pre- and post-SLIT serum samples for 1 hour at 37°C before addition to heparinized whole blood of the respective individual.

Detection of allergen-specific antibodies

Microplates (Nunc MaxiSorp, Thermo Scientific, Waltham, Mass) were coated overnight with rMal d 1 (2 µg/mL) and saturated for 6 hours with PBS/

0.05% Tween 20 supplemented with 1% human serum albumin. Samples were incubated overnight at 4°C. After the samples were washed 5 times with PBS/0.05% Tween 20, bound IgG1 was detected with a purified mouse anti-human IgG1 (Invitrogen, Waltham, Mass) followed by a horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG antibody (Amersham, GE Healthcare, Pittsburgh, Pa). Bound IgG2 and IgG3 antibodies were detected with biotinylated mouse anti-human IgG2 and IgG3 antibodies (Hybridoma Reagent Laboratory, Baltimore, Md) followed by streptavidin-HRP. Bound IgG4 antibodies were detected with alkaline phosphatase-conjugated anti-human IgG4 (BD Pharmingen).

For competition ELISA, sera from patients were incubated with titrated amounts of either rMal d 1 or rBet v 1 for 6 hours at room temperature before incubation with plate-bound rMal d 1.

Depletion of IgG subclasses of serum samples

Sera (2.5 mL) were incubated with CaptureSelect IgG1 (500 μ L) or CaptureSelect IgG4 (200 μ L) human affinity matrices (Thermo Fisher Scientific) for 30 minutes at room temperature. After centrifugation fresh CaptureSelect matrices were added to the supernatant. These cycles of depletion were repeated until complete removal of Mal d 1-specific IgG1 or IgG4 antibodies was detected by means of ELISA. IgG1 and IgG4 antibodies were eluted from the respective matrices by incubation with 0.1 M glycine (pH 2.5) for 5 minutes at room temperature. The pH of the eluted fractions was immediately neutralized by addition of 1 M Tris/HCl (pH 9). Thereafter, the pooled antibody fractions were dialysed against PBS. After adjustment to equal volume, serial dilutions of the antibody fractions were subjected to rMal d 1-specific ELISA as already described. Bound IgG antibodies were detected with an HRP-labeled goat anti-human IgG (Fc γ fragment-specific, Jackson ImmunoResearch Laboratories, Cambridgeshire, United Kingdom). All values with an OD value greater than the mean value plus 5 SDs of the buffer control were considered positive.

This study ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier NCT01449786 and EudraCT identifier 2011-001221-24) was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki and with approval of the local ethics committee (EK228/2011).

Statistics

Statistical analyses were performed by using IBM SPSS 20.0 software (IBM, Armonk, NY) with the Mann-Whitney *U* test used for intergroup analyses, the Wilcoxon signed rank test used for pairwise analyses, and the Spearman rho test used for correlations. All tests were 2 tailed, and differences were considered significant if *P* was less than or equal to .05.

RESULTS

Post-rMal d 1 SLIT sera display functional IgE-blocking bioactivity

Basophil inhibition assays were performed with pre- and post-SLIT sera from 18 and 15 subjects undergoing SLIT with rMal d 1 and rBet v 1, respectively. The study population's characteristics and clinical outcomes are provided in Table E1 (in this article's Online Repository at www.jacionline.org). In tests performed with rMal d 1 (ie, the isoform used for treatment), post-rMal d 1 SLIT sera displayed a significantly stronger inhibitory activity than post-rBet v 1 SLIT sera did (Fig 1). Similar results were obtained when basophils were activated with apple extract containing natural Mal d 1, which comprises several isoforms.²⁹⁻³¹ Despite their limited inhibition of apple allergen-induced basophil activation, post-rBet v 1 SLIT sera potentially blocked basophil activation by rBet v 1 (Fig 1). Post-rMal d 1 SLIT sera did not markedly inhibit basophil activation by rBet v 1.

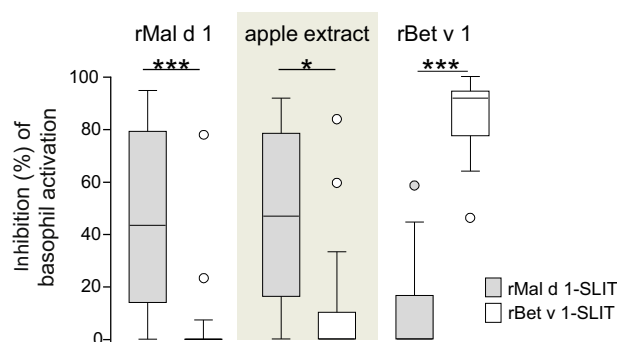


FIG 1. IgE-blocking activity after SLIT with rMal d 1 and rBet v 1. Allergens were incubated with pre- or post-SLIT sera from 18 rMal d 1- and 15 rBet v 1-treated individuals before addition to basophils from untreated individuals with BPAA. CD63⁺ basophils in the presence of pre-SLIT sera were normalized to 100%. The percentage of inhibition of basophil activation by post-SLIT sera is shown. Dots indicate outliers. **P* ≤ .05; ****P* ≤ .001; Wilcoxon signed rank test.

rMal d 1-specific IgG subclasses induced by SLIT with rMal d 1 or rBet v 1

We previously reported that SLIT with both rMal d 1 and rBet v 1 significantly enhanced rMal d 1-specific IgG4 antibodies.¹⁶ The levels of Mal d 1-specific IgG4 were comparable in the post-SLIT sera of both treatment groups (Fig 2, A [*P* = .843, Mann-Whitney *U* test]) and could not explain the observed discrepancy of functional blocking. Therefore, we assessed rMal d 1-specific IgG1, IgG2, and IgG3 responses to both treatments. SLIT with either allergen significantly enhanced rMal d 1-specific IgG1 and IgG2 levels (Fig 2, B and C). Interestingly, rMal d 1-specific IgG3 antibodies remained unchanged with rMal d 1 SLIT but increased significantly with rBet v 1 SLIT (Fig 2, D). Still, no significant differences in the levels of Mal d 1-specific IgG1, IgG2, and IgG3 antibodies in post-SLIT sera of both treatment groups were found (for IgG1, *P* = .274; for IgG2, *P* = .244; and for IgG3, *P* = .307 [Mann-Whitney *U* test]). Thus, SLIT with rMal d 1 and rBet v 1 induced a quantitatively similar rMal d 1-specific IgG response.

IgE-blocking activity of Mal d 1-specific IgG1 and IgG4 antibodies

To determine whether the inhibitory activity of post-SLIT sera resided within the IgG1 or IgG4 fraction, we depleted these subclasses from 7 post-rMal d 1 SLIT sera with an IgE-blocking bioactivity greater than 50%. Successful depletion and preservation of the respective rMal d 1-specific IgG subclass was confirmed individually by ELISA (see Fig E1 in this article's Online Repository at www.jacionline.org). Then, the IgE-blocking activities of depleted and nondepleted post-SLIT samples of each individual were assessed by using pre-SLIT sera as a reference. In 6 of 7 individuals the depletion of IgG1 restored the percentages of CD63⁺ basophils to those measured after activation with rMal d 1 in the pre-SLIT sera (Fig 3). This effect was clearly less pronounced after depletion of IgG4 for all individuals, with the exception of patient 2. Statistical analysis was performed between pre-SLIT and IgG1-depleted post-SLIT (*P* = .289) and IgG4-depleted post-SLIT (*P* = .018) sera with use of the Wilcoxon signed rank test.

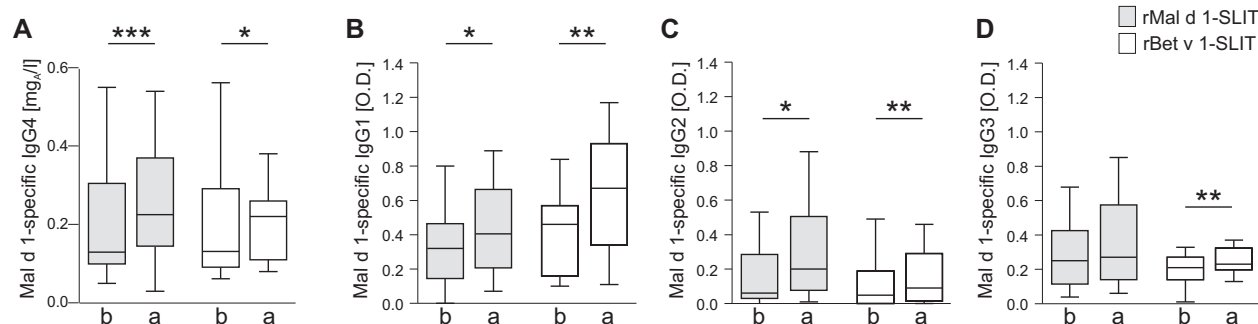


FIG 2. rMal d 1-specific IgG responses to SLIT with rMal d 1 and rBet v 1. Mal d 1-specific IgG4 levels were determined by ImmunoCAP in sera collected before (b) and after (a) SLIT with rMal d 1 (gray) and rBet v 1 (white) (A); IgG1 (B), IgG2 (C), and IgG3 (D) levels were analyzed by ELISA. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; Wilcoxon signed rank test. O.D., Optical density.

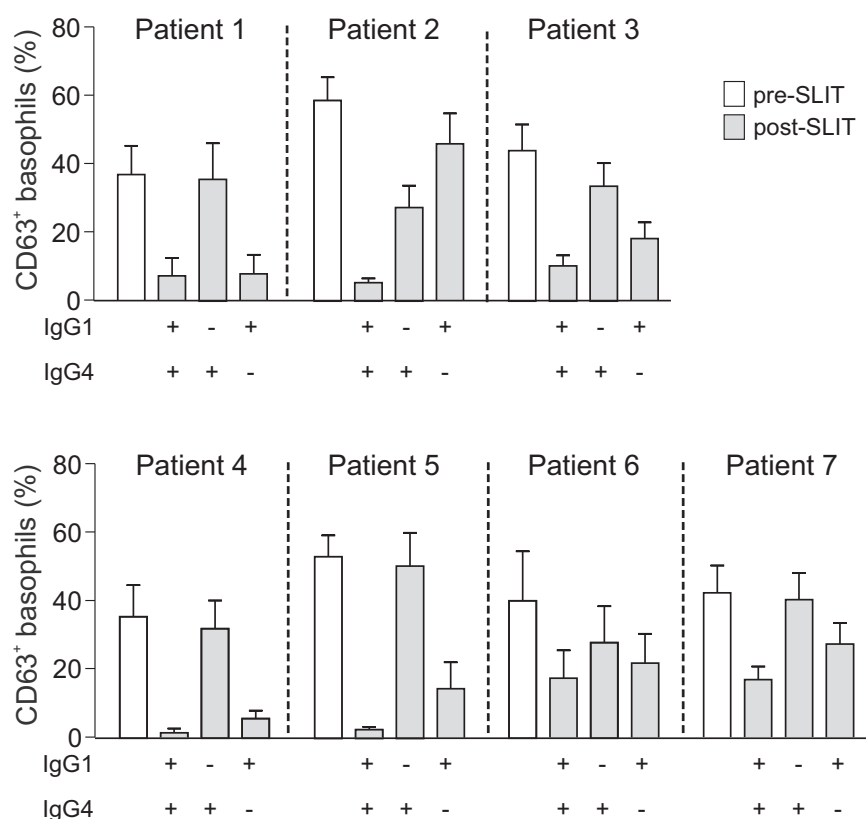


FIG 3. IgE-blocking activity of post-rMal d 1 SLIT sera after depletion of IgG1 or IgG4. Percentages of CD63⁺ basophils after activation with rMal d 1 in the presence of pre-SLIT (white bars) or post-SLIT sera (gray) with and without IgG1 and IgG4, respectively. Data are shown as means $\pm$ SDs of 2 to 4 experiments per SLIT-treated individual. Statistical analysis was performed between pre-SLIT and IgG1-depleted post-SLIT ($P = .289$) and IgG4-depleted post-SLIT ($P = .018$) sera with use of the Wilcoxon signed rank test.

To compare the quantity of Mal d 1-specific IgG1 and IgG4 antibodies, matrix-bound IgG1 and IgG4 antibodies were eluted and adjusted to the same volume. Subsequently, the titers of Mal d 1-specific antibodies were determined by means of ELISA (see Fig E1). IgG1 titers were significantly higher than IgG4 titers in the post-SLIT sera from all individuals (Table I). The ratios of IgG1 to IgG4 ranged from 4 to 64 (median = 8 [Table I]).

Binding quality of Mal d 1-specific IgG1 antibodies induced by SLIT with rMal d 1 and rBet v 1

To elucidate possible qualitative differences of Mal d 1-specific IgG1 antibodies induced by SLIT with rMal d 1 or rBet v 1, we compared their binding behavior with that of rMal d 1 in competition ELISA. Preincubation of post-SLIT sera from rMal d 1-treated individuals with increasing amounts of rMal d 1 resulted in complete inhibition of IgG1 binding to the plate-bound

TABLE I. Mal d 1–specific IgG1 and IgG4 titers in post-SLIT sera

Patient	Titer of rMal d 1–specific		Ratio of IgG1 to IgG4
	IgG1	IgG4	
1	128	2	64
2	64	8	8
3	128	8	16
4	8	1	8
5	64	4	16
6	4	1	4
7	64	8	8

apple allergen (Fig 4, A). Notably, equal doses of rBet v 1 were significantly less competitive, with a maximum inhibition of around 60%. The latter suggests that the rMal d 1 SLIT–induced IgG1 antibodies recognized epitopes not present on the pollen allergen. Otherwise, rBet v 1 was the more potent competitor for IgG1 binding to rMal d 1 in post–rBet v 1 SLIT sera (Fig 4, B). This finding indicates that these IgG1 antibodies bear a higher affinity to the pollen allergen than to the apple allergen.

DISCUSSION

We have recently reported that equal doses of the homologous and highly cross-reactive allergens Mal d 1 and Bet v 1 administered directly at the site of food-induced allergic symptoms showed surprisingly different clinical effects on BPRAA.¹⁶ Daily sublingual administration of 25 µg of rMal d 1 for 16 weeks reduced BPRAA, whereas SLIT with rBet v 1 for the same time period failed to significantly reduce mucosal allergic reactions to the apple allergen. In search of immune mechanisms that may explain this divergent clinical outcome, we compared the quantity and quality of the Mal d 1–specific IgG response in both treatment groups. Although sera collected after either treatment contained comparable levels of Mal d 1–specific IgG1, IgG2, IgG3, and IgG4 antibodies, only rMal d 1 SLIT–induced antibodies were able to prevent Mal d 1–induced effector cell activation. Hence, clinical improvement was correlated with the inhibitory activity but not with the concentration of Mal d 1–specific antibodies in post-SLIT sera. This study confirms that functional assays of inhibitory IgG are more useful surrogates of treatment efficacy than the quantification of allergen-specific IgG levels.³²

To assess functional IgE blocking, we performed basophil inhibition tests because this assay measures not only the inhibition of IgE allergen-binding but also the prevention of IgE-mediated effector cell activation *in vitro*. As we obtained comparable inhibition results with individual post-SLIT sera and basophils from 2 to 4 different individuals with BPRAA, we can exclude the possibility that the IgE-inhibitory activity depended on the basophil donor. In addition, the inhibitory activities of post–rMal d 1 SLIT sera in basophil inhibition tests performed with rMal d 1 and apple extract were well correlated ($R = 0.903$; $P < .001$ [$n = 18$]). These results also indicated that SLIT with a single isoform of Mal d 1 induced antibodies that prevent IgE-mediated degranulation of effector cells induced by natural Mal d 1, which contains various Mal d 1 isoforms.^{29–31}

Indeed, all of the Mal d 1–treated individuals who tolerated the highest dose of rMal d 1 in sublingual challenge tests also tolerated challenge with 20 g of fresh apple.¹⁶ In summary, these findings support the idea that the isoform rMal d 1.0108 is well suited for sublingual treatment of BPRAA. In contrast, SLIT with rBet v 1 induced Mal d 1–reactive IgG antibodies with limited activity to prevent Mal d 1–induced IgE-mediated effector cell activation. Still, these antibodies efficiently inhibited Bet v 1–induced basophil activation. Thus, Bet v 1 SLIT induced functional Bet v 1–blocking antibodies that cross-react with Mal d 1 but lack sufficient specificity/affinity for effective cross-blocking. In line with this finding, Mal d 1 binding of Bet v 1 SLIT–induced IgG1 antibodies was more potently inhibited by Bet v 1 than by the apple allergen itself.

Several studies have shown that SLIT with allergen extracts enhances the levels of allergen-specific IgG1 and IgG4 antibodies.^{33,34} We found that SLIT with rMal d 1 gave rise to specific IgG1, IgG2, and IgG4 antibodies. In general, AIT-induced IgE-blocking bioactivity has been mainly attributed to specific IgG4, as this subclass displays very high binding affinity.^{35,36} However, the development of specific IgG4 antibodies with high affinity requires repetitive stimulation with allergen for several weeks, whereas the early antibody response in AIT is predominantly in the form of IgG1.³⁷ Our depletion experiments revealed that after 16 weeks of SLIT, allergen-specific IgG1 antibodies were the predominant blocking antibodies, with higher titers than the titers of IgG4 antibodies. The close monitoring of antibody levels during SLIT with birch pollen has revealed that allergen-specific IgG4 antibodies continuously rise until 24 weeks of treatment.^{38,39} Therefore, it is tempting to speculate that sublingual administration of rMal d 1 for more than 16 weeks may further increase the levels and affinity of Mal d 1–specific IgG4 antibodies and, correspondingly, their relevance for functional IgE blocking. In fact, the post-SLIT serum of patient 2 already displayed an IgG4-dominated protective response that was paralleled by higher levels of IgG4 compared with those in most of the other individuals. We hypothesize a biphasic response of IgE-blocking antibodies with an initially dominant protective role of allergen-specific IgG1 followed by IgG4. Nevertheless, the depletion of IgG4 from sera of patients with allergy that were collected after 24 months of AIT has revealed that allergen-specific IgG4 antibodies do not entirely account for the inhibitory bioactivity.^{21,40} Thus, allergen-specific IgG1 antibodies may still also contribute to effective IgE blocking in a later phase of AIT.

In summary, this study provides an immunologic explanation of why secondary food allergy cannot be effectively treated by administration of the sensitizing pollen allergen. We found that treatment with Bet v 1 promotes specific IgG antibodies that cross-react with Mal d 1 but lack sufficient affinity to induce cross-protection. The latter finding may be the result of very few differing amino acid residues in the epitope of cross-reactive allergens, as previously demonstrated for an IgE-blocking IgG4 mAb isolated from an AIT-treated individual.⁴¹ On the other hand, treatment with the secondary food allergen induced a food allergen-specific *de novo* antibody response that is characterized by IgG1 antibodies with IgE-blocking bioactivity and specific for epitopes exclusive of the apple allergen. Finally, we have demonstrated for the first time that allergen-specific IgG1 antibodies are the dominant protective blocking subclass in the early phase of allergy treatment.

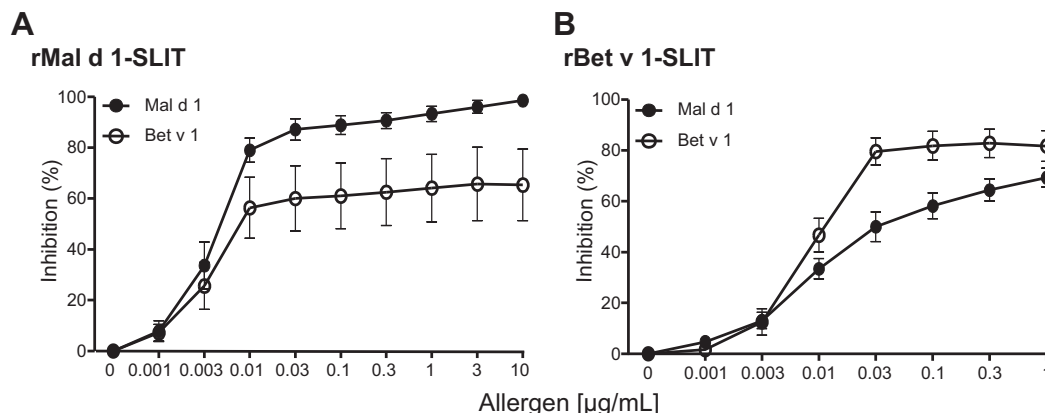


FIG 4. IgG1 competition ELISA. Post-SLIT sera of individuals treated with rMal d 1 ($n = 6$) (A) or rBet v 1 ($n = 8$) (B) were incubated with increasing doses of rMal d 1 (solid circles) or rBet v 1 (open circles). The inhibition of IgG1 binding to plate-bound Mal d 1 (2 $\mu\text{g/mL}$) is shown as mean $\pm$ SD.

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Clinical implications: The clinical success of SLIT with rMal d 1 is mediated by IgE-blocking IgG antibodies.

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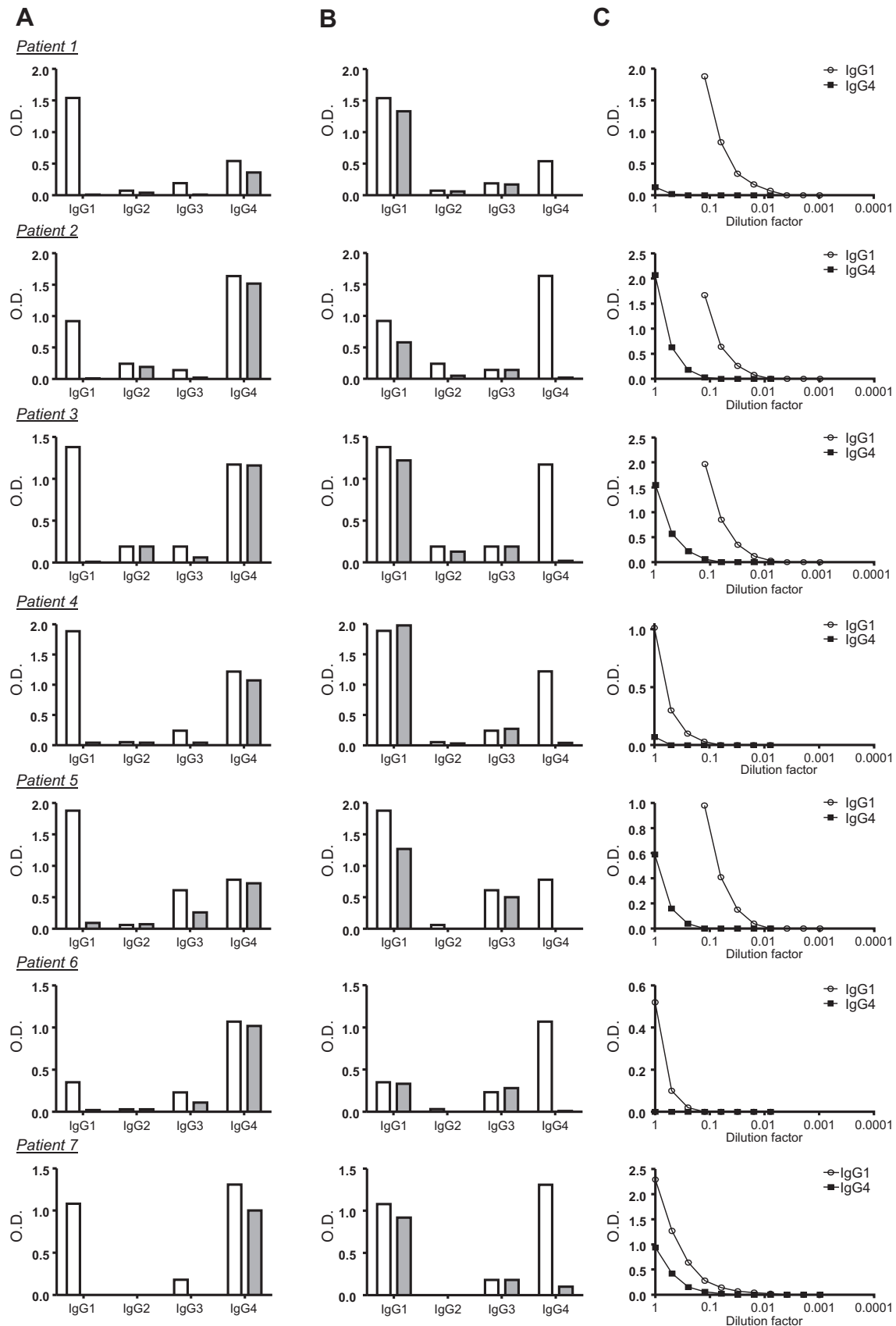


FIG E1. Mal d 1-specific IgG1, IgG2, IgG3, and IgG4 antibodies in post-SLIT sera before (white bars) and after depletion (gray bars) of IgG1 (A) and IgG4 (B). C, Equal volumes of eluted IgG1 and IgG4 antibodies were serially diluted and binding to rMal d 1 was detected by an HRP-labeled anti-human IgG antibody in ELISA. O.D., Optical density.

TABLE E1. Demographics and baseline characteristics of the study population

Subject	Age (y)	Sex	IgE (kU _A /L) specific for			SCT dose (μg)	
			Birch pollen	rBet v 1	rMal d 1	Before SLIT	After SLIT
M1	28	F	28.0	29.0	6.4	3.1	3.1
M2	64	F	29.1	29.7	5.4	6.3	Neg
M3	54	M	59.8	45.7	45.2	6.3	Neg
M4	48	M	25.9	45.9	27.5	1.6	25.0
M5	33	F	20.9	19.2	7.2	3.1	12.5
M6	31	M	20.5	21.7	7.2	12.5	12.5
M7	28	F	5.2	4.3	0.9	3.1	Neg
M8	22	M	2.0	3.8	3.5	3.2	12.5
M9	53	F	12.7	9.9	1.4	6.3	Neg
M10	45	M	12.9	13.1	9.3	1.6	6.3
M11	32	M	20.8	15.9	13.3	6.3	12.5
M12	52	F	19.1	18.4	13.8	12.5	3.1
M13	49	M	27.0	23.8	12.4	1.6	1.6
M14	33	M	34.1	30.3	7.3	6.3	25.0
M15	21	M	59.9	45.2	17.3	1.6	25.0
M16	35	F	11.6	10.8	2.4	12.5	6.3
M17	26	M	27.6	29.5	16.6	3.1	25.0
M18	28	M	8.1	16.2	6.7	12.5	Neg
B1	31	F	15.1	16.7	14.8	3.1	12.5
B2	43	F	10.8	11.2	6.7	25.0	25.0
B3	52	M	11.3	10.1	1.9	25.0	6.3
B4	37	F	9.8	9.6	7.7	12.5	25.0
B5	33	F	15.9	18.1	10.2	3.1	25.0
B6	54	F	27.5	20.3	8.7	12.5	25.0
B7	44	M	6.2	9.2	4.2	6.3	6.3
B8	27	F	21.4	14.5	2.8	6.3	12.5
B9	27	M	38.3	31.7	10.4	1.6	3.1
B10	49	F	2.3	2.3	1.1	6.3	6.3
B11	39	M	11.5	11.7	2.6	25	12.0
B12	34	F	17.8	16.7	7.8	6.3	50.0
B13	50	F	8.5	6.25	2.5	3.1	1.6
B14	20	F	10.3	8.0	5.3	25.0	12.5
B15	24	M	9.4	6.5	4.5	12.5	Neg

F, Female; M, male; Neg, negative; SCT, sublingual challenge test.

Subjects M1 to M18 received a daily sublingual dose of 25 μg of rMal d 1, and subjects B1 to B15 received a daily sublingual dose of 25 μg rBet v 1 for 16 weeks. The dose of rMal d 1 that induced allergic symptoms was recorded. Negative means that these subjects tolerated the highest dose of rMal d 1 and subsequent challenge with 20 g of fresh apple. From Kinacyan T, Nagl B, Faustmann S, Frommlet F, Kopp S, Wolkersdorfer M, et al. Efficacy and safety of 4 months of sublingual immunotherapy with recombinant Mal d 1 and Bet v 1 in patients with birch pollen-related apple allergy. J Allergy Clin Immunol 2018;141:1002-8.