

A Novel Approach to Specific Allergy Treatment: The Recombinant Fusion Protein of a Bacterial Cell Surface (S-Layer) Protein and the Major Birch Pollen Allergen Bet v 1 (rSbsC-Bet v 1) Combines Reduced Allergenicity with Immunomodulating Capacity¹

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Counterregulating the disease-eliciting Th2-like immune response of allergen-specific Th lymphocytes by fostering an allergen-specific Th1-like response is a promising concept for future immunotherapy of type I allergy. The use of recombinant allergens combined with more functional adjuvants has been proposed. In this respect, we present a novel approach. The gene sequence encoding the major birch pollen allergen, Bet v 1, was fused with the gene encoding the bacterial cell surface (S-layer) protein of *Geobacillus stearothermophilus*, resulting in the recombinant protein, rSbsC-Bet v 1. rSbsC-Bet v 1 contained all relevant Bet v 1-specific B and T cell epitopes, but was significantly less efficient to release histamine than rBet v 1. In cells of birch pollen-allergic individuals, rSbsC-Bet v 1 induced IFN- γ along with IL-10, but no Th2-like response, as observed after stimulation with Bet v 1. Intracellular cytokine staining revealed that rSbsC-Bet v 1 promoted IFN- γ -producing Th cells. Moreover, rSbsC-Bet v 1 induced IFN- γ synthesis in Bet v 1-specific Th2 cell clones, and importantly, increased IL-10 production in these cells. In conclusion, genetic fusion of an allergen to S-layer proteins combined reduced allergenicity with immunomodulatory capacity. The strategy described in this work may be generally applied to design vaccines for specific immunotherapy of type I allergy with improved efficacy and safety. *The Journal of Immunology*, 2004, 172: 6642–6648.

It is well established that Th2 cells, i.e., CD4⁺ T lymphocytes producing high levels of IL-4, IL-5, and IL-13 in response to particular Ag, play a central role in type I allergy and up-regulate the production of allergen-specific IgE Ab (1, 2). At the moment, specific immunotherapy (SIT)³ represents the only causative treatment for type I allergy (3). The treatment consists of the consecutive injections of increasing doses of specific allergen to induce specific tolerance in the allergic patient. Different immunological mechanisms have been discussed to contribute to a successful treatment. During SIT, a rise of allergen-specific IgG, especially IgG4, was observed and interpreted as the formation of blocking Ab (4–6). At the level of Th cells, a down-regulation of the allergen-specific Th2 response became evident. Diminished lymphoproliferative responses to the allergen measured in vitro indicated the induction of peripheral tolerance to the injected allergen (7, 8). In this context, a crucial role of IL-10 was suggested (9). For the remaining T cell reactivity, an immunodeviation was

observed, i.e., the shift to a more Th1/0-like immune response, characterized by the production of either higher amounts of IFN- γ or reduced levels of IL-4 and IL-5 (7, 8, 10, 11).

However, traditional injection-SIT has a limited scope of efficacy, which may in part be due to the current nature of the vaccines used. These consist in general of crude extracts of the allergen source, e.g., tree or grass pollen, adsorbed to aluminum hydroxide. The extracts represent complex mixtures of allergenic and nonallergenic molecules that are difficult to standardize. Aluminum hydroxide was shown to support Th2- rather than Th1-type immune responses (12). Thus, attempts to improve vaccines for SIT focused on the allergenic molecules as well as on adjuvants that support the shift of the Th2-biased immune response toward a more balanced phenotype. In the recent past, many relevant allergens have been produced as recombinant proteins facilitating the standardization of the allergen content in vaccines (13). Simultaneously, different biological substances were characterized with respect to their Th1-fostering properties and potential use as adjuvants for SIT (14–19). A conjugate consisting of an allergen chemically linked to oligodeoxynucleotides with immunomodulatory capacity represented a particularly promising strategy to improve vaccines for future SIT (20, 21).

Crystalline bacterial cell surface proteins (S-layers) are well-characterized cell envelope components of many bacteria and archaea that form regularly structured crystalline arrays (22–24). These S-layer lattices were successfully exploited for the immobilization of foreign molecules, e.g., haptens, Ag, or enzymes, to develop diagnostic test systems (25–27). Possibly due to their natural assembly, S-layers activated the immune system of vertebrates, implying their application as carrier/adjuvants for vaccines

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Received for publication October 8, 2003. Accepted for publication March 31, 2004.

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¹ This work was supported by Grant SFB F018-07 and P14639-M013 from the Fonds zur Förderung der Wissenschaftlichen Forschung (Vienna, Austria), and by Biomay.

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³ Abbreviations used in this paper: SIT, specific immunotherapy; RT, room temperature; SI, stimulation index; SN, supernatant; TCC, T cell clone; TCL, T cell line.

(25). In previous studies, we showed that natural S-layers as allergen carriers represented a promising tool to promote the desired Th1-like response in SIT. rBet v 1, the major birch pollen allergen, chemically linked to S-layers purified from Gram⁺ bacterial strains, displayed strong Th1-promoting capacities and expanded allergen-specific Th0/Th1-like cells from birch pollen-allergic individuals in vitro (28–30). As the gene encoding the S-layer of *Geobacillus stearothermophilus* was recently cloned and sequenced (31), the concept emerged to create a fusion construct consisting of the recombinant S-layer protein and an allergen. The gene sequence encoding Bet v 1 was added to the C terminus of the truncated S-layer gene sequence (32). The resulting recombinant fusion protein, rSbsC-Bet v 1, maintained its ability to self-assemble and contained Bet v 1 in a correctly folded conformation located at the outer surface of the monomolecular S-layer lattice (33). In addition, rSbsC-Bet v 1 overcame major disadvantages of chemical Bet v 1-S-layer conjugates, i.e., low resuspendibility and the use of potentially toxic chemicals during the coupling procedure. The purpose of the present study was to characterize rSbsC-Bet v 1 with respect to immunological functionality, i.e., authentic exposure of Bet v 1-specific B and T cell epitopes, and its ability to modulate human allergen-specific T cell responses.

Materials and Methods

Allergic individuals

In total, 18 individuals (12 female, 6 male; age, 25–43 years; median, 30) were included in this study. All individuals had a history of hayfever during early spring, a positive skin prick reaction (wheal >5 mm) to birch pollen (Soluprick; ALK, Hørsholm, Denmark), and a radioallergosorbent test to birch pollen >3 (median, 24.9 kU/ml; RAST/CAP; Pharmacia, Uppsala, Sweden). None had received immunotherapy before.

Allergens

rBet v 1 was purchased from Biomay (Vienna, Austria). rSbsC-Bet v 1 was produced, as previously described (33). Bacterial endotoxin was detected using the LAL ENDOTOXIN test (Charles River Endosafe, Wilmington, MA). Endotoxin levels in rBet v 1, rSbsC-Bet v 1, and rSbsC were below 1 EU/mg. In all experiments, the amount of rSbsC-Bet v 1 was adjusted to the amount of rBet v 1, i.e., 5.9 μ g of rSbsC-Bet v 1 contained 1 μ g of Bet v 1. Recombinant bacterial S-layer (rSbsC) served as a control and was used equivalent in amount to the fusion protein (31).

IgE inhibition ELISA

Sera of 11 individual birch pollen-allergic patients were preincubated overnight at room temperature (RT) with various concentrations (0.04–5 μ g/ml) of either rBet v 1 or rSbsC-Bet v 1 containing 0.04–5 μ g/ml Bet v 1. As controls, each serum was preincubated with either 20 μ g/ml BSA or adjusted amounts of rSbsC. Maxisorp 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at RT with rBet v 1 (1 μ g/ml in carbonate buffer, pH 9.6). After washing, the plates were saturated with PBS/0.05% Tween 20/1% human serum albumin for 6 h at RT. Subsequently, preincubated sera and controls were incubated overnight at 4°C. Bound IgE Ab were detected using an alkaline phosphatase-conjugated horse anti-human IgE Ab (Kallestad, Chaska, MN), which was incubated for 2 h at RT.

Basophil histamine release assay

Histamine release from basophil granulocytes was performed with whole blood samples using a histamine release kit, followed by a histamine ELISA (IBL, Hamburg, Germany), according to the manufacturer's instructions.

Stimulation of PBMC

PBMC were obtained from peripheral blood of allergic patients by Ficoll-Hypaque density gradient centrifugation (Pharmacia). Cells were cultivated in serum-free Ultra Culture Medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine and 2×10^{-5} M 2-ME. To detect Th2 cytokines, the protocol described by Marshall et al. was modified (21). PBMC (2×10^5 /200 μ l) were cultured in triplicates in 96-well plates (Nunc; Nunc) and stimulated with rBet v 1 in the concentration range

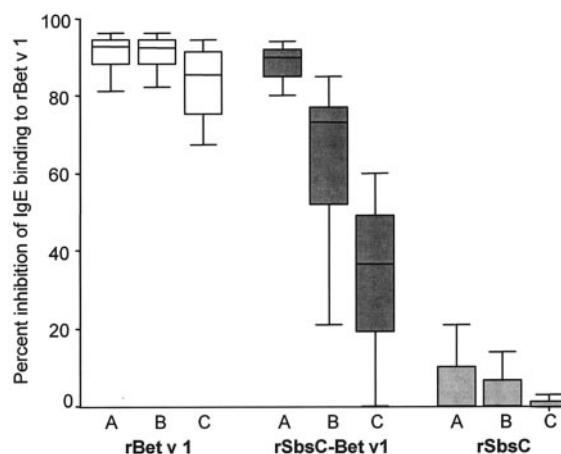


FIGURE 1. IgE inhibition ELISA. IgE binding to immobilized rBet v 1 was inhibited by preincubation of sera from 11 birch pollen-allergic individuals with 1 μ g/ml (A), 0.2 μ g/ml (B), and 0.04 μ g/ml (C) rBet v 1 or rSbsC-Bet v 1 adjusted to equal amounts of allergen. rSbsC had no effect on IgE binding. Box plots are shown: each box represents the interquartile range containing the 50% values. The line across the box indicates the median. The whiskers extend from the box to the highest and lowest values.

of 1–10 μ g/ml. After 6 days, cells were washed and stimulated with PMA (10 ng/ml) plus ionomycin (1 μ M; Sigma-Aldrich, Munich, Germany) for 24 h. In most cultures, no IL-4 was detectable at any of the concentrations. A concentration of 3 μ g/ml rBet v 1 was determined to induce optimum IL-5 synthesis. Maximum levels of IFN- γ and IL-10 were detected when PBMC (1×10^6 /200 μ l) were stimulated for 48 h.

Allergen-specific T cell lines (TCL)

To generate allergen-specific TCL, 1.5×10^6 PBMC/1 ml were stimulated with 10 μ g/ml purified rBet v 1 or the respective amount of rSbsC-Bet v 1 in 24-well flat-bottom culture plates (3524; Costar, Cambridge, MA). After 5 days, 10 U/ml human rIL-2 (Boehringer Mannheim, Mannheim, Germany) was added, and at day 7, T cell blasts were enriched by density gradient centrifugation and cultures were expanded at weekly intervals with irradiated PBMC and rIL-2 (34, 35). To test allergen specificity, the TCL were stimulated with rBet v 1 (5 μ g/ml) and corresponding amounts of rSbsC-Bet v 1, rSbsC, or birch pollen (25 μ g/ml) in the presence of 5×10^4 irradiated autologous PBMC for 48 h in duplicates. After a 16-h pulse with 0.5 μ Ci of [^{3} H]TdR (Amersham, Little Chalfont, U.K.), cultures were harvested and radionuclide uptake was measured by scintillation counting. The stimulation index (SI) was calculated as ratio between cpm of TCL plus PBMC plus Ag and cpm of TCL and PBMC only. TCL were considered as specific when the SI was >5. Epitope mapping was performed according to previously described protocols (34, 35). Peptides inducing a SI >5 were considered positive.

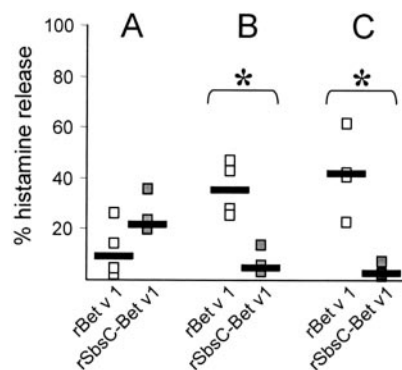


FIGURE 2. Histamine release by rSbsC-Bet v 1. Whole blood samples from four patients were incubated with 1 μ g/ml (A), 0.1 μ g/ml (B), and 0.01 μ g/ml (C) free rBet v 1 or rSbsC-Bet v 1 containing equivalent amounts of Bet v 1. The percentage of total histamine release is expressed on the y-axis, and the horizontal lines indicate the median (Wilcoxon signed ranks; *, $p < 0.05$).

Table 1. Proliferative responses of allergen-specific TCC and TCL to rBet v 1, rSbsC-Bet v 1, rSbsC, and birch pollen extract^a

TCC	Epitope (aa)	Stimulated with			TCL	Stimulated with			
		rBet v 1	rSbsC-Bet v 1	rSbsC		rBet v 1	rSbsC-Bet v 1	rSbsC	Birch Pollen
RR19/5	4–21	8.6*	13	n.d.	WIP	42	27	1.1	63
MAK3	4–18	16.6	10.9	0.7	FIM	352	376	26	322
WS9	49–63	51.6	255	0.9	MAK	11	12	1.5	18
WIP56	55–66	18	17	0.6	SAL	285	247	39	242
SD218	76–90	21.7	38.6	n.d.	KLU	135	131	9	155
MAK39	94–108	21.2	27	1.0	OLP	17	16	1.4	18
CMF118	109–123	17.4	7	0.9	GRU	71	49	1.2	32.4
CMF103	145–159	46.8	189	1.0	MAH	259	217	41	215
SUG1	145–159	7.1	43.8	1.2					

^a SI are shown.

Allergen-specific T cell clones (TCC)

Bet v 1-specific TCC established from PBMC of birch pollen-allergic individuals were stimulated in triplicates with either rBet v 1 (5 µg/ml), adjusted amounts of rSbsC-Bet v 1, or rSbsC in the presence of irradiated autologous PBMC (5 × 10⁴/ml) as APC and tested for proliferation after 48 h. Appropriate controls with APC alone, APC and clones, and APC plus each stimulus without clones were performed. To analyze the cytokine patterns in response to the different stimuli, TCC were stimulated in the presence of PBMC (5 × 10⁴/ml), and supernatants (SN) were harvested after 24 h. Cytokine levels measured in cultures containing APC plus stimulus without clones were subtracted from the levels obtained from TCC plus APC plus stimulus.

Measurement of cytokines in SN

SN were analyzed for cytokine content using ELISA with endogen matched Ab pairs (Endogen, Woburn, MA), according to the manufacturer's instructions (sensitivity limits: IL-4, 9 pg/ml; IL-5, 7.4 pg/ml; IFN-γ, 9.5 pg/ml; IL-10, 4.7 pg/ml).

Intracytofluorometric analysis of cytokine production

Allergen-specific TCL were stimulated with PMA (10 ng/ml) plus ionomycin (1 µM) plus 1 µg/ml brefeldin A (Sigma-Aldrich) for 4 h. Cells were harvested and washed with PBS, and unspecific binding sites were saturated by incubation with PBS containing 20% human AB serum for 20 min on ice. As the expression of CD4 was down-regulated after stimulation with PMA/ionomycin, T lymphocytes were stained with PerCP-conjugated anti-CD3 mAbs and PE-conjugated anti-CD8 (BD Biosciences, San Jose, CA) in PBS/0.1% AB serum for 30 min on ice. After washing with PBS, cells were fixed with 2% formaldehyde for 20 min at RT. Cells were washed with PBS/0.1% saponin and stained with 0.5 µg/ml FITC-labeled anti-human IFN-γ or anti-human IL-4 mAb (BD Pharmingen, San Diego, CA) in PBS/0.1% saponin for 30 min at RT. After washing with PBS/0.1% saponin and PBS, cells were analyzed on a FACStar^{Plus} (BD Biosciences).

Statistical analysis

Statistical significance of differences was determined by the Wilcoxon signed ranks test. Differences were considered statistically significant for $p < 0.05$.

Results

rSbsC-Bet v 1 contains the major Bet v 1-specific B cell epitopes

By means of immunoblotting, it was previously demonstrated that IgE Ab of birch pollen-allergic patients recognized rSbsC-Bet v 1 (33). To test the authentic exposure of Bet v 1 epitopes in the self-assembled fusion protein, we performed IgE inhibition ELISA with sera from 11 birch pollen-allergic patients (Fig. 1). At a concentration equivalent to 1 µg/ml rBet v 1, rSbsC-Bet v 1 inhibited IgE binding to the major birch pollen allergen to the same extent as free rBet v 1. At lower concentrations, the fusion protein displayed a reduced inhibition capacity compared with free rBet v 1. Preincubation of the sera with rSbsC had in general no effect on Bet v 1-specific IgE binding (Fig. 1). These data indicate that the

self-assembled fusion protein contains the major IgE-binding epitopes of Bet v 1, although some epitopes seem less accessible for IgE Ab.

rSbsC-Bet v 1 displays a reduced histamine-releasing capability

The histamine release induced by rSbsC-Bet v 1 was measured in the concentration range equivalent to 1–0.01 µg/ml rBet v 1 (Fig. 2). As observed in previous investigations, rBet v 1 displayed optimum histamine-releasing capability at 0.1 and 0.01 µg/ml, respectively. At both concentrations, rSbsC-Bet v 1 released significantly less histamine than free rBet v 1 ($p = 0.043$ and 0.046). rSbsC did not induce detectable histamine release (data not shown).

rSbsC-Bet v 1 contains all Bet v 1-specific T cell epitopes

Nine TCC from seven different donors covering relevant T cell epitopes spreading the entire Bet v 1 molecule were incubated with rBet v 1, rSbsC-Bet v 1, and rSbsC (Table I). All TCC proliferated in response to the fusion protein; none of the TCC responded to rSbsC. Next, we established short-term TCL with rSbsC-Bet v 1 from eight different patients. These TCL were subsequently restimulated with either rBet v 1, rSbsC-Bet v 1, rSbsC, or birch pollen extract containing natural Bet v 1 (Table I). Comparable proliferative responses to either variant of Bet v 1 were observed. In some TCL, a weak reactivity with rSbsC was observed (Table I). In addition, rSbsC-Bet v 1-initiated TCL were epitope mapped using a panel of 50 synthetic peptides representing the entire Bet v 1 molecule (34, 35). A TCL initiated with rBet v 1 from the same individual served as control (Fig. 3). In both TCL of all patients, a comparable T cell recognition pattern was detected. Hence, Ag processing of the self-assembled fusion protein does not influence the presentation of the major Bet v 1-specific T cell epitopes.

rSbsC-Bet v 1 induces IFN-γ and IL-10, but no IL-5 in PBMC of allergic donors

PBMC from birch pollen-allergic patients were stimulated with 3 µg/ml rBet v 1 and corresponding amounts of rSbsC-Bet v 1 and rSbsC. To measure IFN-γ and IL-10, SN were harvested after 48 h. rSbsC-Bet v 1 induced a substantial production of both IFN-γ and IL-10, as compared with the medium control and rBet v 1 (Fig. 4). Increased amounts of both cytokines were also detected in cultures stimulated with rSbsC, but were significantly lower than levels induced by rSbsC-Bet v 1 (IFN-γ, $p = 0.038$; IL-10, $p = 0.008$). To detect Th2 cytokines, PBMC were stimulated, as described in *Materials and Methods*. As IL-4 levels were constantly below the sensitivity limit, we alternatively detected IL-5 as readout for Th2 cytokine production. Compared with cultures incubated with medium alone or rSbsC, stimulation with rBet v 1 resulted in a

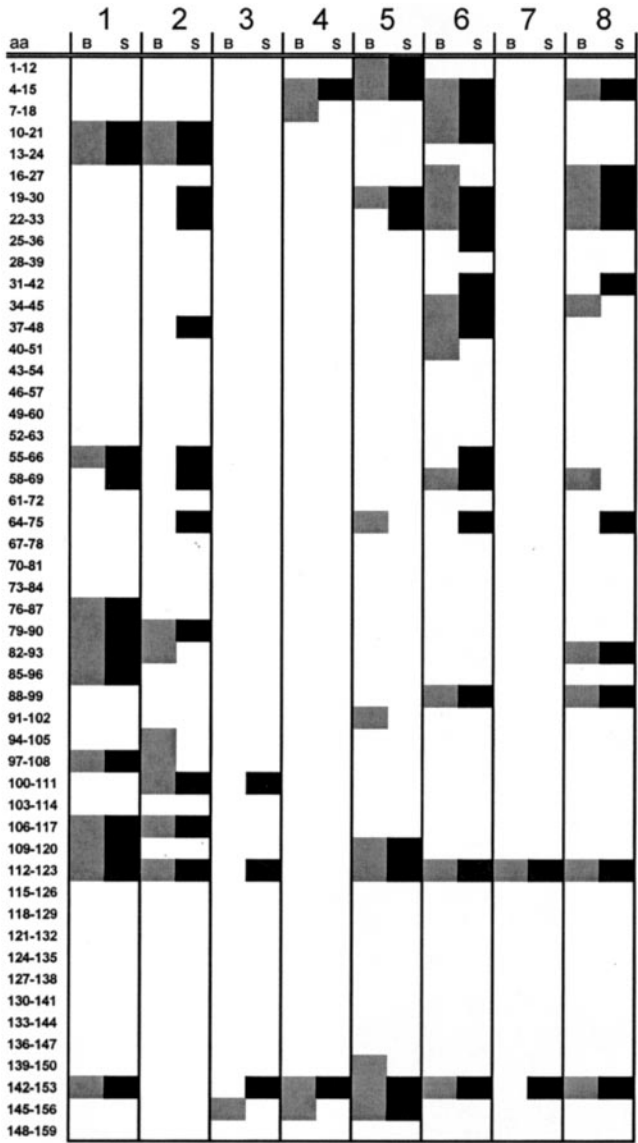


FIGURE 3. Bet v 1-specific T cell epitopes present in TCL established with rSbsC-Bet v 1. TCL from eight patients were established with rBet v 1 (B) or rSbsC-Bet v 1 (S). Proliferation was tested with overlapping peptides spanning the entire Bet v 1 sequence. Proliferative responses ($SI > 5$) are depicted as gray boxes in Bet v 1-initiated TCL, and as filled boxes in rSbsC-Bet v 1-initiated TCL.

marked increase of IL-5 levels ($p = 0.028$ and 0.006). However, allergen-induced synthesis of IL-5 was not observed in cultures stimulated with rSbsC-Bet v 1 ($p = 0.008$).

rSbsC-Bet v 1 favors the development of allergen-specific T cells into Th1/0-like effectors

To investigate the effect of rSbsC-Bet v 1 on the in vitro development of allergen-specific T lymphocytes, two TCL using either rBet v 1 or rSbsC-Bet v 1 were generated from the same patient. After proof of allergen specificity, both TCL were analyzed by flow cytometry for intracellular synthesis of IL-4 and IFN- γ (Fig. 5). A significant increase in the percentage of IFN- γ -producing CD4 $^{+}$ T cells was detected in TCL initiated with rSbsC-Bet v 1 as compared with rBet v 1-initiated TCL ($p = 0.023$). Although in a few cultures a reduction of IL-4-secreting T cells was observed, overall the percentage of IL-4 $^{+}$ CD3 $^{+}$ CD8 $^{-}$ cells did not differ significantly (Fig. 5).

rSbsC-Bet v 1 induces IFN- γ synthesis in Bet v 1-specific Th2-like TCC

To investigate whether rSbsC-Bet v 1 influenced an already established allergen-specific Th2-like immune response in vitro, five different Bet v 1-specific TCC belonging to the Th2 subset were stimulated with either rBet v 1, rSbsC-Bet v 1, or rSbsC plus autologous PBMC as APC. The levels of IL-4, IFN- γ , and IL-10 in the SN were determined by ELISA. The results are summarized in Fig. 6. When stimulated with rSbsC-Bet v 1, TCC synthesized significantly higher amounts of IFN- γ and IL-10 as compared with stimulation with rBet v 1. However, IL-4 levels remained unchanged. In all experiments, proliferation did not differ between the two different stimuli. RSbsC did not induce cytokines in Bet v 1-specific TCC, confirming allergen specificity. Cytokine levels measured in TCC incubated in medium plus APC were below the sensitivity limit (data not shown).

Discussion

Future strategies for type I allergy treatment favor the use of recombinant allergens and adjuvants supporting Th1 responses. Allergen/Adjuvant conjugates were shown to have a stronger immunomodulatory capacity than allergen/adjuvant mixtures, which might be attributed to the simultaneous delivery of allergen and immunomodulatory agent to the same APC creating the Th1-supporting cytokine environment directly at the site of APC/T cell interaction. In addition, chemical conjugation reduced the IgE-binding capacity, and thus, allergenicity of allergen conjugates (20, 21, 28). In the present study, we introduce a recombinant construct designed by genetical fusion of an allergen to an adjuvant, which has several advantages over a conjugate consisting of an allergen chemically linked to an adjuvant. The coupling position within the amino acid sequence of the allergen is precisely defined. The ratio between allergen and adjuvant is adjustable and remains constant. Most importantly, the fusion protein can be produced in recombinant form, which facilitates standardization and guarantees invariable immunological characteristics. RSbsC-Bet v 1 contained the major B cell epitopes, but Bet v 1-specific IgE molecules seemed sterically hindered from binding to the allergen within the self-assembled fusion protein (Fig. 1). This partial inaccessibility of intact Bet v 1 epitopes significantly reduced the histamine-releasing capacity of rSbsC-Bet v 1 in comparison with rBet v 1 (Fig. 2). Nine Bet v 1-specific TCC reactive with distinct epitopes on the entire birch pollen molecule responded to stimulation with rSbsC-Bet v 1 (Table I). Vice versa, TCL initiated with rSbsC-Bet v 1 proliferated after stimulation with recombinant as well as natural Bet v 1 and contained the same T cell specificities as the TCL initiated with rBet v 1 from the same individual (Table I; Fig. 3). Thus, rSbsC-Bet v 1 can be regarded as a recombinant hypoallergen that contains all relevant T cell epitopes.

The ability of rSbsC-Bet v 1 to alter the phenotype of the allergic immune response was tested in poly-, oligo-, and monoclonal T cell cultures. RSbsC-Bet v 1 as well as rSbsC induced the synthesis of IFN- γ in PBMC, confirming that the Th1-enhancing properties are clearly attributable to the S-layer protein (Fig. 4). This S-layer-induced IFN- γ production was previously shown to be triggered via IL-12 synthesized by phagocytotic cells of the innate immune system (30). The observation that rSbsC-Bet v 1-induced IFN- γ levels were significantly higher than levels induced by the S-layer protein alone indicated that allergen-specific cells were involved in IFN- γ production. Single cell staining of TCL confirmed that the fusion protein promoted the development of allergen-specific IFN- γ -secreting CD3 $^{+}$ CD8 $^{-}$ T cells (Fig. 5). Otherwise, stimulation of PBMC with rBet v 1 led to a substantial

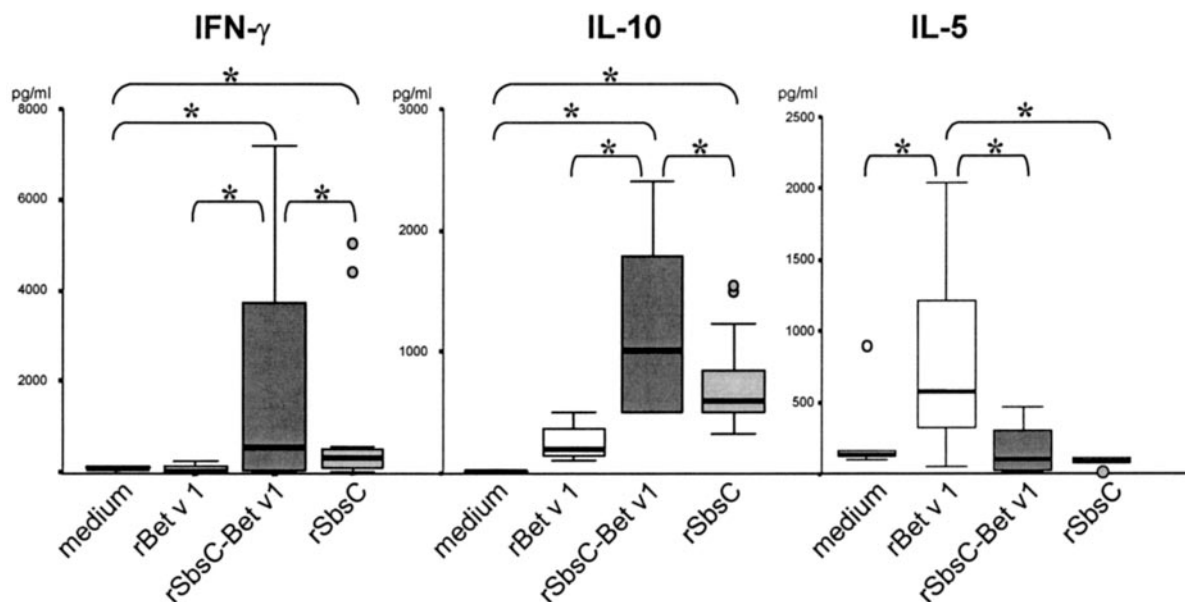


FIGURE 4. RSbsC-Bet v 1 induces a Th1-like response in PBMC. PBMC from nine birch pollen-allergic patients were incubated with medium, 3 μ g/ml rBet v 1, corresponding amounts of rSbsC-Bet v 1, and rSbsC. SN were assessed by ELISA for IFN- γ and IL-10 after 48 h; levels of IL-5 were analyzed after 6 days of culture, as described in *Materials and Methods* (Wilcoxon signed ranks test; *, $p < 0.05$). Box plots are shown, and the circles indicate outliers.

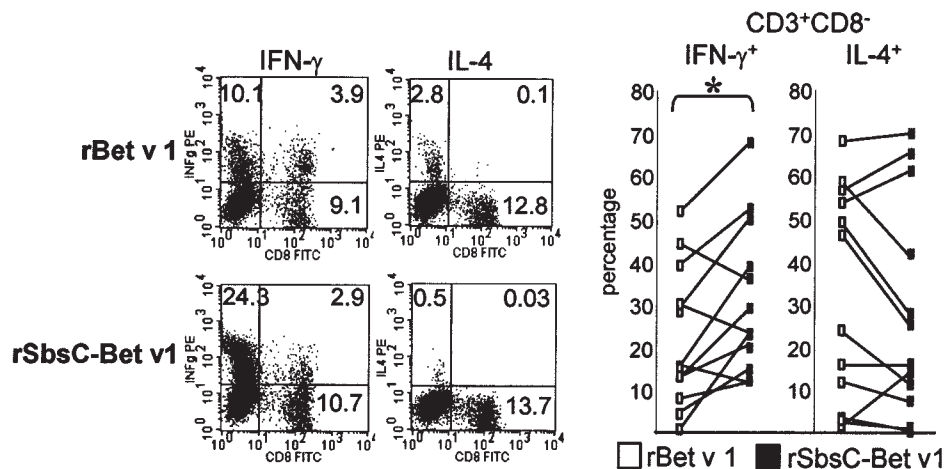
production of IL-5 that was not observed after stimulation with the fusion protein. In TCL initiated with rSbsC-Bet v 1, the proportion of IL-4⁺CD3⁺CD8[−] cells was solely reduced in some cultures as compared with rBet v 1-induced TCL (Fig. 5). We speculated that the Th1-dominated microenvironment created by rSbsC-Bet v 1 prevented the polarization of either naive or allergen-specific central memory T cells into Th2 effector cells, but did not modulate Th2 effector cells in these cultures. Bet v 1-specific TCC displaying a typical Th2 phenotype, i.e., high levels of IL-4 vs low/no IFN- γ , synthesized IFN- γ after stimulation with rSbsC-Bet v 1, whereas IL-4 levels remained unchanged (Fig. 6). Hence, the fusion protein altered the established Th2-dominated phenotype as well as the de novo cytokine secretion profile toward a Th1/Th0-like phenotype.

In addition to IFN- γ , rSbsC-Bet v 1 as well as rSbsC induced a substantial synthesis of the suppressive cytokine IL-10 in PBMC of allergic donors. More importantly, rSbsC-Bet v 1 significantly enhanced the allergen-specific IL-10 production in Bet v 1-specific TCC (Fig. 6). IL-10 was reported to be crucial for both the induction of T cell anergy, i.e., a state of Ag-specific hyporesponsive-

ness characterized by suppressed proliferation and cytokine synthesis, as well as the differentiation of regulatory CD4⁺CD25⁺ cells, which themselves suppress Th1 as well as Th2 responses through secretion of IL-10 (36–39). In successfully desensitized grass pollen-allergic patients, increased numbers of circulating allergen-induced IL-10-secreting CD4⁺CD25⁺ cells were reported (40). CD4⁺CD25⁺ cells were involved in the allergen-specific IL-10-dependent T cell suppression observed after SIT (41). In our opinion, rSbsC-Bet v 1 could support the induction of IL-10-producing T lymphocytes during SIT. Whether this might lead to suppression and/or anergy needs to be further evaluated. At present, we can only speculate that the shifted cytokine pattern of Bet v 1-specific Th2 cells toward enhanced IL-10 and IFN- γ synthesis in response to rSbsC-Bet v 1 supports the production of specific IgG4 Ab, which requires IL-4, IFN- γ , and is additionally favored by IL-10 (42–44). Addition of a neutralizing anti-IL-10 Ab to the cultures enhanced cytokine levels in the SN, indicating the immunosuppressive effect of this cytokine (data not shown).

In conclusion, genetic fusion of a major allergen to a bacterial S-layer protein resulted in a hypoallergen capable of modulating

FIGURE 5. Intracellular analysis of allergen-specific TCL initiated with rSbsC-Bet v 1. PBMC from birch pollen-allergic donors were stimulated with rBet v 1 or equivalent amounts of rSbsC-Bet v 1. After expansion and proof of Ag specificity, both TCL of 12 donors were assessed by flow cytometry for intracellular synthesis of IL-4 and IFN- γ (y-axis) after stimulation with PMA plus ionomycin. One representative example is shown. The x-axis of each dot blot depicts cells stained for CD8 in a gate set on CD3⁺ cells. The graph shows the percentages of IL-4⁺ and IFN- γ ⁺ cells of CD3⁺CD8[−] cells (Wilcoxon signed ranks test; *, $p < 0.05$).



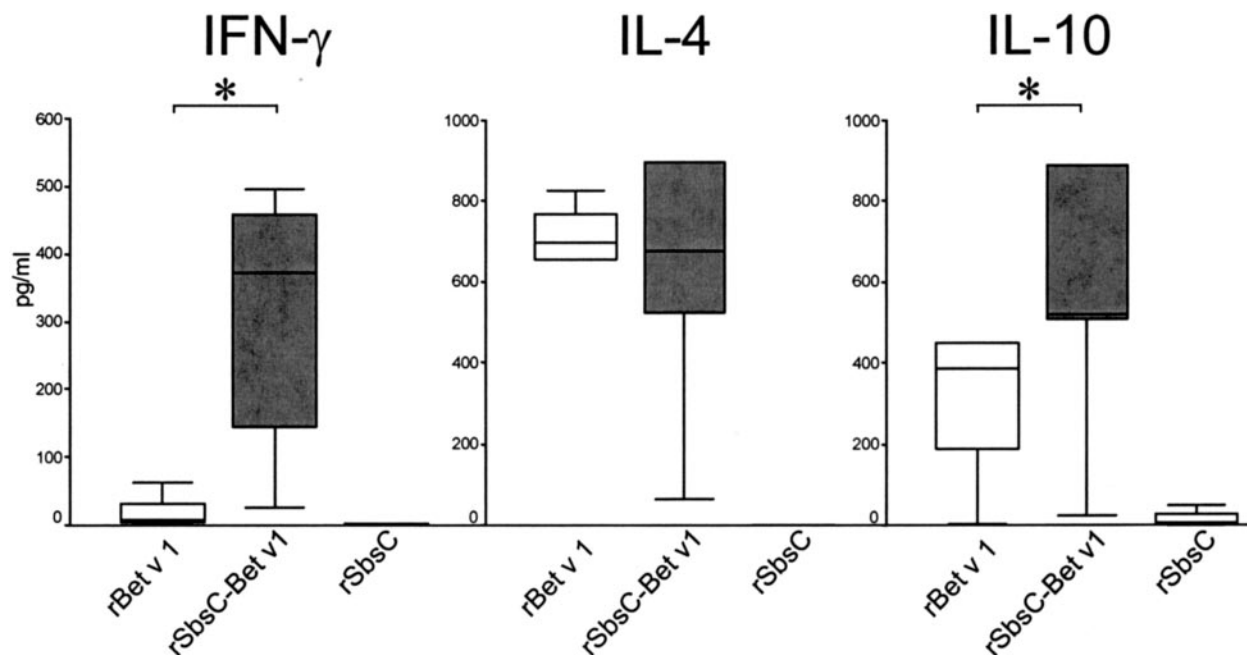


FIGURE 6. Cytokine production of Bet v 1-specific TCC in response to rSbsC-Bet v 1. Five different TCC from four different birch pollen-allergic patients were stimulated for 24 h with rBet v 1 (5 μ g/ml), corresponding amounts of rSbsC-Bet v 1, or rSbsC. Cytokine concentrations were measured in SN by ELISA. Cytokine levels in the medium controls were below the sensitivity limits (data not shown).

the allergen-specific Th2-dominated cellular response into a more balanced phenotype accompanied by enhanced production of IL-10. Our first proof of a novel concept for vaccine design can be applied to any allergen with known DNA sequence. Fusion constructs with modified allergen variants lacking IgE-binding capacity, but retained T cell-activating properties, can be considered to additionally improve the safety of the vaccine. At present, first studies to test the effects of rSbsC-Bet v 1 in vivo are underway.

Acknowledgment

We thank Renate Steiner for excellent technical assistance.

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