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 Capacity

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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 Capacity

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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.
(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 contain 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 ENDOCHROME test (Charles River Endosafe, Wilmington, MA). Endotoxin levels in rBet v 1, rSbsC-Bet v 1, and rSbsC were below 1 EU/ng. In all experiments, the amount of rSbsC-Bet v 1 was adjusted to the amount of rBet v 1 i.e., 5.9 μg of rSbsC-Bet v 1 contained 1 μg of Bet v 1. Recombinant bacterial S-layer (rSbsC) served as a control and was used equivalently 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 μg/ml) of either rBet v 1 or rSbsC-Bet v 1 containing 0.04–5 μg/ml Bet v 1. As controls, each serum was preincubated with either 20 μg/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 μg/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 × 10⁷/200 μl) were cultured in triplicates in 96-well plates (Nunclon; Nunc) and stimulated with rBet v 1 in the concentration range of 1–10 μg/ml. After 6 days, cells were washed and stimulated with PMA (10 ng/ml) plus ionomycin (1 μM; Sigma-Aldrich, Munich, Germany) for 24 h. In most cultures, no IL-4 was detectable at any of the concentrations. A concentration of 3 μg/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 × 10⁶/200 μl) were stimulated for 48 h.

**Allergen-specific T cell lines (TCL)**

To generate allergen-specific TCL, 1.5 × 10⁶ PBMC/1 ml were stimulated with 10 μg/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 μg/ml) and corresponding amounts of rSbsC-Bet v 1, rSbsC, or birch pollen (25 μg/ml) in the presence of 5 × 10⁴ irradiated autologous PBMC for 48 h in duplicates. After a 16-h pulse with 0.5 μCi of [3H]TdR (Amersham, Little Chalfont, U.K.), cultures were harvested and radioactivity 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 1.** IgE inhibition ELISA. IgE binding to immobilized Bet v 1 was inhibited by preincubation of sera from 11 birch pollen-allergic individuals with 1 μg/ml (A), 0.2 μg/ml (B), and 0.04 μg/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.

**FIGURE 2.** Histamine release by rSbsC-Bet v 1. Whole blood samples from four patients were incubated with 1 μg/ml (A), 0.1 μg/ml (B), and 0.01 μg/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).
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 × 10⁶/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 × 10⁶/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).

Intracyttofluorometric analysis of cytokine production

Allergen-specific TCC 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/10% 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 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 0.01–0.01 μg/ml rBet v 1 (Fig. 2). As observed in previous investigations, rBet v 1 displayed optimum histamine-releasing capability at 0.01 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 TCC with rSbsC-Bet v 1 from eight different patients. These TCC were subsequently restimulated with either rBet v 1, rSbsC-Bet v 1, or 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 TCC, a weak reactivity with rSbsC was observed (Table I). In addition, rSbsC-Bet v 1-initiated TCC were epotope mapped using a panel of 50 synthetic peptides representing the entire Bet v 1 molecule (34, 35). A TCC initiated with rBet v 1 from the same individual served as control (Fig. 3). In both TCC 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
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 genetic 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 immunological characteristics. RShsC-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 RShsC-Bet v 1 in comparison with rBet v 1 (Fig. 2).

The ability of RShsC-Bet v 1 to alter the phenotype of the allergic immune response was tested in poly-, oligo-, and monoclonal T cell cultures. RShsC-Bet v 1 as well as RShsC 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 phagocytic cells of the innate immune system (30). The observation that RShsC-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 reduction of IFN-γ production, which was shown to be mediated by IFN-γ-secreting CD4⁺ T cells (Fig. 6). When stimulated with RShsC-Bet v 1, TCL 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. RShsC did not induce cytokines in Bet v 1-specific TCL, confirming allergen specificity. Cytokine levels measured in TCL incubated in medium plus APC were below the sensitivity limit (data not shown).

FIGURE 3. Bet v 1-specific T cell epitopes present in TCL established with RShsC-Bet v 1. TCL from eight patients were established with rBet v 1 (B) or RShsC-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 RShsC-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 RShsC-Bet v 1 (p = 0.008).

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

To investigate the effect of RShsC-Bet v 1 on the in vitro development of allergen-specific T lymphocytes, two TCL using either RBet v 1 or RShsC-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 RShsC-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).
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 hyporesponsiveness 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
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.

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References


