

Immunoreactivity of allergen (Bet v 1) conjugated to crystalline bacterial cell surface layers (S-layers)

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Abstract

Background: Crystalline cell surface layers (S-layers) from Gram-positive eubacteria had been demonstrated as carrier/adjuvants for chemically synthesized tumor-associated oligosaccharide haptens and capsular polysaccharide antigens of *Streptococcus pneumoniae* strains. **Objectives:** The applicability of S-layers as vaccine carrier for treatment of Type I allergy was investigated. **Study design:** Native or cross-linked S-layer self-assembly products and cell wall preparations from *Bacillus sphaericus* CCM 2177 and *Thermoanaerobacter thermohydrosulfuricus* L111-69 and L110-69 were used for immobilization of recombinant major birch pollen allergen Bet v 1. **Results and conclusions:** Depending on the carrier used, amounts of approximately 20–40 µg allergen per mg conjugate could be immobilized. By application of L-glutamic acid dimethyl ester as a spacer, this value could be increased approximately 10-fold. The functionality of the rBet v 1-conjugates was assessed in immunological systems. (i) The presence of intact B-cell epitopes was demonstrated in inhibition experiments using human Bet v 1-specific IgE. (ii) The rBet v 1-S-layer conjugates were immunogenic in mice. (iii) The proliferation of rBet v 1-specific T-cell clones suggested that the peptides created by processing of immobilized Bet v 1 were similar to those derived from natural allergen. (iv) Stimulation of human allergen-specific T_H2 lymphocytes with S-layer-conjugated Bet v 1 led to a modulation of the cytokine production pattern from T_H2 to T_H0/T_H1. This study indicates that S-layers may be suitable carriers for new immunotherapeutic vaccines for Type I hypersensitivity.

Keywords: Crystalline cell surface layers (S-layers); Vaccines; Immunotherapy; *Bacillus sphaericus*; Recombinant birch pollen allergen Bet v 1; Type I hypersensitivity

1. Introduction

The construction of vaccines for treatment of IgE-mediated allergies [1] has been suggested as a

means to modulate or redirect the T_H2-like immune responses of atopic patients towards a T_H0/T_H1-like cellular pattern which is typical for healthy individuals [2,3]. Crystalline bacterial cell surface layers (S-layers) composed of identical proteinaceous subunits (for reviews see [4,5]) have been used as carrier/adjuvant systems for the

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preparation of vaccine prototypes [6–9]. In previous studies, depending on the nature of the S-layer preparations (native or cross-linked with glutaraldehyde), the conjugates have induced immune responses either of a predominantly T_H1 -like cellular, or predominantly T_H2 -like, humoral nature [8,9].

In the present contribution, we report on studies in which Bet v 1, the major birch pollen allergen [10–13], was covalently coupled either to cell wall fragments, the protein S-layer self-assembly products or S-layer-depleted peptidoglycan sacculi of *Bacillus sphaericus* [14] or to the glycosylated S-layer proteins of *Thermoanaerobacter thermohydrosulfuricus* [15]. The different Bet v 1-conjugates were tested for authentic exposure of B-cell epitopes, for immunogenicity in mice, and for their ability to activate and modulate human allergen-specific T-cell responses.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacillus sphaericus CCM 2177 (henceforth abbreviated 2177) was grown aerobically at 32°C in continuous culture as described previously [14]. *Thermoanaerobacter* (formerly *Clostridium*) *thermohydrosulfuricus* strains L111-69 (henceforth abbreviated L111) and L110-69 (DSM 568; henceforth abbreviated L110) were grown anaerobically at 57°C in continuous culture as described previously [15].

2.2. Chemicals, antigens and antibodies

1-Ethyl-3,3'-(dimethylaminopropyl)carbodiimide (EDC) and ethylacetimidate were obtained from Sigma and glutaraldehyde and L-glutamic acid dimethyl ester.HCl (GDM) were from Fluka (Sigma-Aldrich-Fluka GmbH, Vienna). Recombinant Bet v 1 (rBet v 1) [11,13] was obtained from BIO-May GmbH (Linz, Austria). The ELISA chemicals goat anti-rabbit IgG alkaline phosphatase conjugate (A 8025) and *p*-nitrophenyl phosphate tablets (N 2765) were also obtained from Sigma.

2.3. Analytical techniques

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [16] was performed as described previously [17]. Electron microscopy of either freeze-dried, negatively stained or thin sectioned preparations was carried out on a Philips CM100 electron microscope as published previously [17,18].

2.4. Cell wall preparations and S-layer self-assembly products

To obtain large cell wall fragments of 2177 the S-layer isolation procedure [17,19] was modified. In previous experiments, harsher sonication conditions were used to increase the yield of cell walls and less attention was paid to their dimensions. Sonication of intact cells was performed for 2 min with a Branson model 450 sonifier at maximum output and a duty cycle of 25%. The specimen was cooled in an ice bath. Intact and broken cells were recovered by centrifugation at $11\,300 \times g$ (Beckman J2-21 centrifuge, 4°C, 15 min). The pellet was treated with 0.5% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.2 as described previously [19,20] and centrifuged at $11\,300 \times g$. A two-layer pellet was formed which contained in the upper layer cell wall fragments of approximately half the size of an intact bacterial cell. This layer was collected by gentle vortexing and treated again with Triton X-100. After centrifugation, the cell walls were washed twice with 50 mM Tris-HCl buffer, pH 7.2 and distilled water, respectively. Upon lyophilization this material was stored at –20°C for the haptentation experiments. For the remaining intact cells, the sonication and isolation procedure was repeated. S-Layer-depleted cell wall fragments which represent the peptidoglycan-containing layer were prepared by repeated extraction with 5 M guanidine hydrochloride (GHC), as described before. All cell wall preparations and the S-layer-free, peptidoglycan-containing sacculi were analysed by SDS-PAGE, negative staining and thin sectioning electron microscopy.

Self-assembly products of 2177 in a size range of approximately 1–5 μm were prepared by a con-

trolled self-assembly process, using the GHCl extract [21]. After sonication of the biomass (2 min, output 50%, duty cycle 50%), cell wall fragments of variable size were obtained. Purification of the latter followed essentially the previously described methods. The S-layer protein was extracted with GHCl (5 M in distilled water; 1 ml/g frozen biomass pellet) for 2 h at room temperature. The peptidoglycan-containing layer was removed from the extract by ultracentrifugation (Beckman SW-41 rotor, 30 000 rev./min, 30 min). The supernatant was dialysed against distilled water (2× for 1 h, room temperature). In order to obtain S-layer self-assembly products in a narrow size range of a few μm , precipitated protein-containing S-layer self-assemblies of various size were removed by centrifugation at 23 000 $\times g$ (Beckman J2-21 centrifuge, 4°C, 30 min). The resulting supernatant was dialysed against 2 mM calcium chloride (2× for 1 h, room temperature and overnight, 4°C) and the self-assembly products obtained were lyophilised. This material was analysed by SDS-PAGE and negative-stain electron microscopy.

Cell wall fragments of *T. thermohydrosulfuricus* strains (L111 and L110) were obtained according to the previously published methods [17,19]. Self-assembly products of L110 were prepared by extraction of cell walls with 5 M GHCl and subsequent dialysis against 5 mM potassium chloride at 60°C [22]. The washed product was lyophilised and stored at -20°C.

2.5. Immobilization of antigens on S-layers

Recombinant Bet v 1 was conjugated to either native or cross-linked S-layer preparations of both strains. Primary amino groups in the S-layer protein were blocked by amidination of non-crosslinked cell wall fragments or S-layer self-assembly products with ethylacetimidate prior to EDC activation [23,24]. Cross-linking with glutaraldehyde of native cell wall fragments or S-layer self-assembly products was performed according to Sára and Sleytr [25] and Messner et al. [26] with the modification that the final glutaraldehyde concentration was 0.05% instead of 0.5%.

In a typical EDC activation experiment 20 mg of lyophilised cell wall or 30 mg of S-layer preparations were resuspended in distilled water and the pH was adjusted to 4.7. The following sample/EDC ratios (per weight) were determined in a concentration series: native and cross-linked cell walls, 2.9:1.0; native and cross-linked S-layer self-assembly products, 0.7:1.0. After incubation with EDC for 1 h at room temperature the suspensions were centrifuged (Hettich Microliter centrifuge, 11 650 $\times g$, 15 min) and 1 ml of a Bet v 1 solution (in distilled water, containing either 0.3 mg antigen for cell wall preparations or 0.6 mg for S-layer self-assemblies) was added. The reaction mixtures were stirred for 1 h at room temperature and then overnight at 4°C. Subsequently, the suspensions were centrifuged in the microcentrifuge and the pellets were washed 1× with 0.3 M sodium chloride solution, 3× with 0.1 M phosphate buffer, pH 7.0, and 3× with distilled water. All supernatants were investigated by SDS-PAGE and the antigen concentrations were determined using the Bradford protein assay [27] and an antigen-specific ELISA.

Coupling of antigen to S-layer self-assembly products and cell wall fragments of 2177 was also carried out after introduction of L-glutamic acid dimethylester (GDM) as a spacer molecule [28]. The coupling procedure was essentially the same as for EDC activation but GDM was added to the reaction mixture at the same time. The following sample/GDM ratios (per weight) were applied: cell walls, 1:1; S-layer self-assembly products, 1:2.

2.6. Bet v 1-specific ELISA

To test for authentic exposure of Bet v 1-specific epitopes, conjugates were examined by Bet v 1-specific ELISA. Microtiter plates (FluoroNunc™ Plates, MaxiSorp) were coated overnight at 4°C with 0.25 $\mu\text{g}/\text{ml}$ of the monoclonal antibody BIP1 [12] as capture antibody for rBet v 1. The remaining binding sites were saturated by incubation with 1% bovine serum albumine, in buffer A (phosphate buffered saline with 0.05% Tween 20) for 2 h at room temperature. After washing three times with buffer A the plates were incubated with decreasing concentrations of rBet v 1 (200 $\mu\text{g}/\text{ml}$,

100 µg/ml, 50 µg/ml, 25 µg/ml,...) or diluted sample. For optimal binding conditions, the plates were first incubated for 30 min at 37°C and then for 30 min at 4°C. After washing three times with buffer A the plates were incubated with rabbit-anti-Bet v 1 serum, kindly provided by Dr Karin Hoffmann-Sommergruber, (diluted 1:20 000 with 1% bovine serum albumine in buffer A). The plates were incubated 30 min at 37°C and 30 min at 4°C. After washing, incubation was repeated using identical conditions with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies and developed with the substrate *p*-nitrophenyl phosphate (one tablet in 20 ml 0.1 M diethanolamine buffer, pH 9.8 containing 0.5 mM magnesium chloride). The optical density was measured at 405 nm (with 492 nm as reference wavelength), using an ELISA reader (Easy Reader EAR 400 AT, SLT-Labinstruments).

2.7. Inhibition of human IgE binding

To ascertain whether the Bet v 1 epitopes exposed in the conjugates correspond to those involved in the binding to IgE, conjugates were analyzed in a human IgE binding assay. Inhibition experiments were performed with minor alterations as previously described [13]. A Bet v 1-specific serum pool was obtained from eight allergic patients and characterized by immunoblotting. Aliquots were diluted 1:6 in PBS and preincubated overnight at 4°C with different concentrations of rBet v 1 (0.015–20 µg/ml), rBet v 1-conjugates (corresponding to Bet v 1 concentration of 0.3–20 µg/ml) or carrier material alone, and no inhibitor as a control. ELISA plates were coated overnight with 2 µg/ml rBet v 1. Unspecific binding sites were saturated with 1% human serum albumine, 0.05% Tween 20 in PBS. On these plates, the remaining IgE-binding of the preincubated samples was measured using alkaline phosphatase-conjugated horse anti-human IgE antibodies (Kallestad, Chaska) and *p*-nitrophenyl phosphate as substrate.

2.8. Proliferation of Bet v 1-specific T-cell clones (TCC)

The ability of conjugate-bound rBet v 1 epitopes

to stimulate proliferation of Bet v 1-specific TCC was examined in a T-cell proliferation assay. Epitope-mapped, Bet v 1-specific TCC that had been established from birch pollen allergic individuals as previously described [29], were used for proliferation assays. Cell cultures were performed in Ultra Culture Medium (Bio Whittaker, Walkersville, MD) supplemented with 2 mM glutamine and 2×10^{-5} M 2-mercaptoethanol in 96 U-bottom microtiter plates.

Triplicate cultures of 2×10^4 resting T cells which had not been stimulated for at least 5 days were incubated in the presence of 10^5 autologous, irradiated (60 Gy) peripheral blood mononuclear cells (PBMC) and different concentrations (≤ 10 µg/ml) of rBet v 1, S-layer-Bet v 1-conjugates or carrier controls. After an incubation of 48 h at 37°C in 5% CO₂, 0.5 µCi [³H]thymidine was added. After a further 16 h, cells were harvested and incorporated radioactivity was measured by scintillation counting.

2.9. Production of cytokines

For cytokine production, 10^6 T cells were incubated with 5×10^5 autologous PBMC (60 Gy) in 1 ml of culture medium in the presence of 10 µg rBet v 1 or rBet v 1-S-layer conjugates. Cultures containing PBMC and antigen, or TCC and PBMC without antigen served as controls. Supernatants were collected after 24 h and levels of interleukin (IL)-4 and interferon (IFN)-γ were measured using commercial ELISA systems (Medgenix Diagnostics, Fleurus, The Netherlands). Statistical significance was determined by the Mann-Whitney *U*-test.

2.10. Immune response in mice

Seven-week-old female BALB/c mice (Charles River, Sulzfeld, Germany) were immunized every 10–12 days. Non-cross-linked rBet v 1-S-layer conjugates, rBet v 1 mixed with corresponding amounts of S-layers, or rBet v 1 alone were resuspended in PBS and 150 µl were injected intraperitoneally. For the first three immunizations, L111 preparations were used, and the three subsequent immunizations were performed with S-layers of 2177. Serum was obtained by bleeding the tail veins.

The mice were killed 7 days after the last immunization and the spleens were removed. Single cell suspensions were prepared by a passage through stainless steel sieves and mononuclear cells were prepared using a Lympholyte M (Cedarlane, Hornby, Canada) density gradient. Antigen-specific proliferative T-cell responses were measured as described above. Triplicates of 10^5 cells per well were stimulated for 4 days with 10 $\mu\text{g/ml}$ rBet v 1 or S-layer proteins and pulsed with [^3H]thymidine for the last 16 h.

3. Results

3.1. Preparation of allergen-S-layer conjugates

3.1.1. Preparation of the carriers

In order to standardize and optimize the uptake of S-layer conjugates by antigen-presenting cells

(APC) S-layer self-assembly products, cell wall fragments and S-layer depleted peptidoglycan-containing sacculi of 2177 were prepared in a size distribution resembling intact bacterial cells. The S-layer of 2177 exhibits a square lattice, is non-glycosylated, and the protomer has a molecular mass of approximately 122 kDa by SDS-PAGE [14]. The controlled self-assembly process of isolated S-layer subunits led to 1–5 μm large, flat, assembly products with the two layers associated in a mirror-symmetric fashion (Fig. 1a). L111 and L110 possess glycosylated S-layer proteins with a molecular mass of the protomers of approximately 130 kDa and an identical glycan structure [15]. Upon dialysis of GHCl extracts of L110, vesicle-like self-assembly products in dimensions comparable to 2177 were obtained (Fig. 1b). For unknown reasons no self-assembly products were obtained from GHCl extracts of L111. The applied

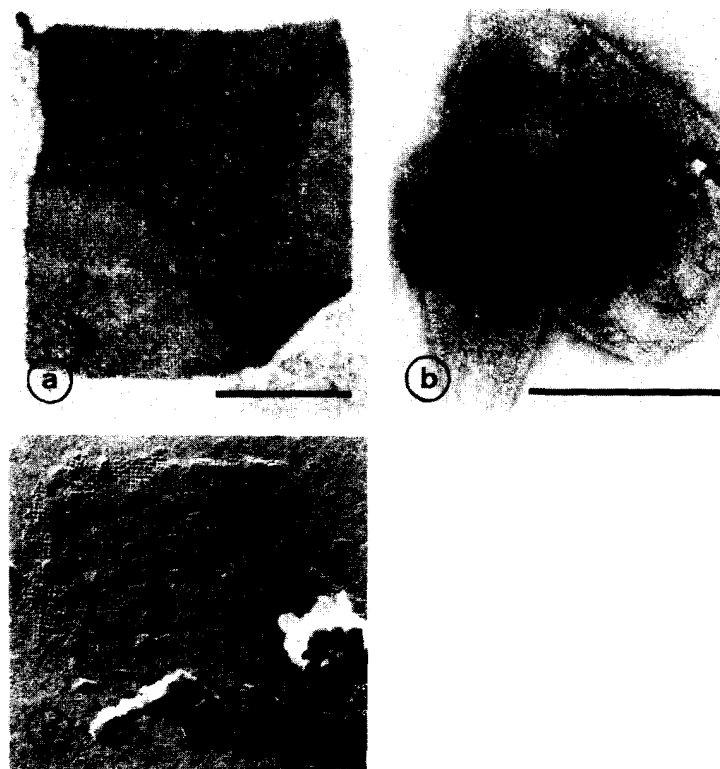


Fig. 1. Electron micrographs of (a,b) negatively-stained untreated S-layer self-assembly products of (a) *B. sphaericus* CCM 2177, showing a square S-layer lattice and (b) *T. thermohydrosulfuricus* L110-69 showing a hexagonal S-layer lattice. After freeze-drying (c) of the rBet v 1-2177 S-layer conjugate the S-layer lattice is still visible; bar, 0.5 μm .

sonication protocol for disrupting intact cells of 2177 led to cup-shaped cell wall fragments with a length of approximately 3 μm and a diameter of approximately 1 μm (not shown) closely resembling the overall shape of the bacterial rods. Thin-sectioned cell wall preparations indicated that the peptidoglycan-containing layer was covered on both sides with an S-layer [30,31]. Repeated extraction of cell wall preparations of 2177 with GHCl completely removed the S-layers from the peptidoglycan-containing sacculi.

3.1.2. Covalent coupling of rBet v 1

In either instance, the allergen was coupled by standard carbodiimide methodology to activated carboxyl groups of the respective preparations. The results of the immobilization experiments are summarized in Table 1. Coupling of antigen to the double-layered assembly products of 2177 did not disturb the S-layer lattice (Fig. 1c). Between 30 and 50 μg rBet v 1/mg S-layer (wt/wt) could be immobilized both on native or cross-linked S-layer preparations of either 2177 or L111/L110. On a molar basis, this value represents approximately 0.3 mol allergen/mol S-layer protomer. Introduction of GDM as a spacer molecule increased the amount of immobilized allergen up to approx-

imately 220 μg allergen/mg S-layer. S-Layer self-assembly products of 2177 and L110, cell wall preparations and peptidoglycan-containing sacculi of 2177 were in the desired size range for optimal uptake by APC and represented a pool of carriers in a narrow size distribution. The lyophilised S-layer protein of L111 formed aggregates of unpredictable dimensions. In comparison to self-assembly products much lower amounts of allergen were bound per mg of dry weight to cell wall fragments and peptidoglycan-containing sacculi of 2177 (Table 1). No significant differences in the binding capacity were observed between native and cross-linked preparations of either strain.

3.2. Immunological functionality of allergen-S-layer conjugates

3.2.1. Inhibition of human IgE binding

The immunological properties of rBet v 1 after chemical conjugation to the respective S-layer carriers were characterized in different assays. In order to investigate whether different B-cell epitopes were retained, all types of conjugates were tested for their capacity to bind human Bet v 1-specific IgE. After preincubation of the serum pool with the rBet v 1-conjugates the remaining

Table 1
Concentration of rBet v 1 immobilized on S-layer self-assembly products, cell wall preparations and S-layer-free peptidoglycan-containing sacculi

Conjugate	Antigen concentration		
	<i>n</i>	$\mu\text{g}/\text{mg}$	mol/mol
Native S-layer self-assembly products, 2177	3	32 ± 8	0.23 ± 0.05
Cross-linked S-layer self-assembly products, 2177	5	41 ± 9	0.28 ± 0.06
Native S-layer material, L111/L110	4	25 ± 4	0.19 ± 0.03
Cross-linked S-layer material, L111/L110	4	31 ± 8	0.23 ± 0.06
Native S-layer self-assembly products with GDM, 2177 ^a	1	221	1.54
Cross-linked S-layer self-assembly products with GDM, 2177 ^a	1	222	1.55
Native cell wall preparations, 2177	3	7 ± 4	0.07 ± 0.04^b
Cross-linked cell wall preparations, 2177	4	15 ± 9	0.15 ± 0.09^b
Native cell wall preparations with spacer, 2177 ^a	1	18	0.19^b
Cross-linked cell wall preparations with spacer, 2177 ^a	1	28	0.27^b
Untreated peptidoglycan, 2177	1	1	

Average values and standard deviation; *n*, number of repeats.

^aL-glutamic acid dimethylester (GDM) as a spacer molecule.

^bBased on an S-layer content of 70% in the cell walls.

IgE binding to rBet v 1 was evaluated with ELISA assays. An inhibitory effect was found with all rBet v 1-conjugates. S-Layer carrier without Bet v 1 as controls did not substantially decrease the IgE

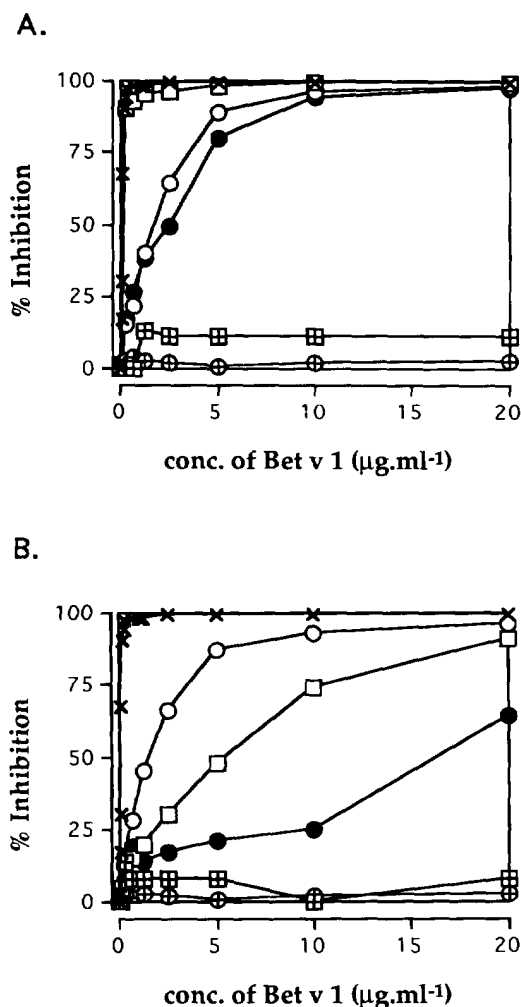


Fig. 2. Inhibition of IgE binding to Bet v 1. A serum pool from birch pollen allergic patients was absorbed with serial concentrations of Bet v 1 (—x—) and Bet v 1-S-layer conjugates: A. Native preparations of L111 S-layers (—□—, lot MG2-45) and 2177 S-layer self-assembly products (—○—, lot MG3-29 and —●—, lot MG3-26). B. Cross-linked preparations of L111 S-layers (—□—, lot MG2-62) and 2177 S-layer self-assembly products (—○—, lot MG2-34 and —●—, lot MG3-23). Lots MG3-23 and MG3-26 contain GDM as a spacer between antigen and carrier. The respective negative controls, S-layers alone, are indicated as —○— and —□—. The absorption value at 405 nm of the non-inhibited sample was 1.07 ± 0.08 ($n = 14$).

binding, indicating that the major epitopes are present and accessible. Free rBet v 1 showed the strongest IgE binding with 50% inhibition at a concentration (IC_{50}) of $0.05 \mu\text{g/ml}$. To observe equivalent IgE antibody binding with the conjugates, rBet v 1 concentrations of <0.3 – $15 \mu\text{g/ml}$ were necessary. As representative examples, results obtained with various S-layer self-assembly preparations are shown in Fig. 2. In general, native preparations and/or low amounts of immobilized rBet v 1 bound relatively more IgE than those preparations that had been cross-linked and/or contained high concentrations of rBet v 1. For example, rBet v 1-cell wall conjugates and rBet v 1-peptidoglycan conjugates were the most potent inhibitors (IC_{50} : <0.3 – $1.3 \mu\text{g/ml}$), whereas the GDM-linked S-layer conjugates with the highest rBet v 1 concentrations ($>200 \mu\text{g/mg}$ S-layer conjugate; Table 1) were the weakest inhibitors (IC_{50} : 2.5 and $15 \mu\text{g/ml}$; Fig. 2). In these cases the weak IgE binding may also be due to steric hindrance, i.e. decreased accessibility of Bet v 1 epitopes.

3.2.2. Immune response in mice

Due to the low amounts of allergen immobilized on conjugates with cell walls and peptidoglycan-containing sacculi (Table 1), *in vivo* immunization experiments were only performed with rBet v 1-S-layer conjugates. Initial experiments had demonstrated that such conjugates were capable of eliciting humoral as well as cellular, allergen-specific immune responses. No significant differences were observed between native and cross-linked S-layer preparations. Mice were primed and boosted with 1 or $10 \mu\text{g}$ of rBet v 1 conjugates (Table 2). Admixtures of allergen to S-layers or only rBet v 1 were injected into control mice. The mice immunized with $10 \mu\text{g}$ rBet v 1-conjugates, but not those with admixtures of rBet v 1 or rBet v 1 alone, became seropositive for Bet v 1 after three immunizations. The production of IgG antibodies of different subclasses (IgG1 and IgG2a) was further increased after the following booster injections (data not shown).

Bet v 1-specific T-cell responses in these mice were tested by assaying the proliferation of spleen cells. As shown in Table 2, only the spleen cells from those mice that had been immunized with

Table 2

T-cell response of mice immunized with rBet v 1, rBet v 1 admixed to S-layer self-assembly products and rBet v 1-S-layer conjugates

Immunization agent	Amount of rBet v 1 per dose (μg)	Stimulation ^a of T-cell proliferation with		
		rBet v 1	L111-S-layer	2177-S-layer
rBet v 1	1	1.2	n.t.	n.t.
	10	1.3	n.t.	n.t.
rBet v 1 admixed to S-layers	1	0.7	3.0	14.3
	10	1.1	3.7	20.8
rBet v 1-S-layer conjugate	1	1.4	3.4	4.4
	10	4.5	14.6	15.2

Mice were immunized three times with L111 preparations and three times with 2177 preparations every 10–12 days. Spleens were removed at day 7 after the last immunization and allergen- and S-layer-specific proliferation of 10^5 mononuclear cells were measured in triplicate; n.t.; not tested.

^aStimulation index, mean proliferation with antigen (counts/min)/medium control mean value (counts/min).

rBet v 1-conjugates or admixtures of rBet v 1 to S-layers showed T-cell responses to S-layer protein. A significant, allergen-specific proliferation, however, was only observed in mice which had been immunized with 10 μg rBet v 1-conjugates. Spleen cells derived from mice which had been treated with rBet v 1 alone, or with a mixture containing rBet v 1 and S-layers, did not show rBet v 1-specific proliferation.

3.2.3. Proliferation of rBet v 1-specific human TCC

The different rBet v 1-conjugates were compared to rBet v 1 alone with respect to their capability to induce antigen-specific proliferation of Bet v 1-specific TCC, which had been established and characterized previously [29]. In order to induce a Bet v 1-specific proliferation of the clones, the conjugates need to be taken up, processed and presented by autologous APC among

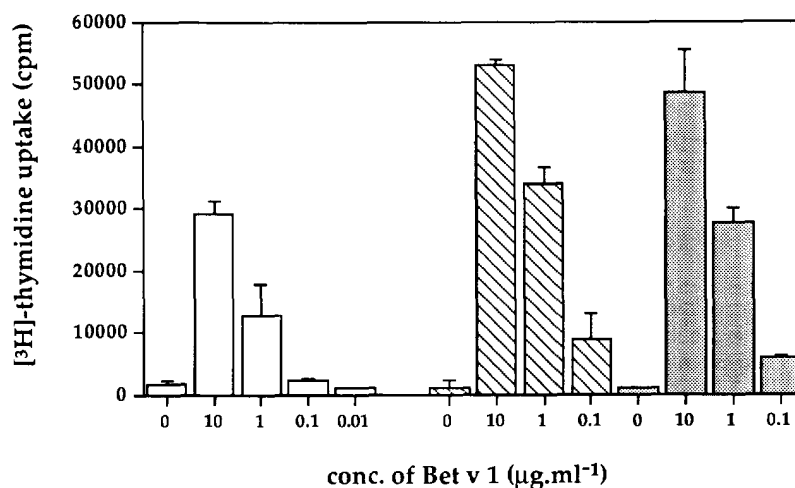


Fig. 3. Stimulation of a Bet v 1-specific T-cell clone with rBet v 1 and rBet v 1-S-layer conjugates; 2×10^4 T cells were incubated with 10^5 irradiated autologous peripheral blood mononuclear cells and different concentrations of Bet v 1 (open bars), non-fixed Bet v 1-S-layer conjugates (hatched bars; preparation MG2-26), or glutaraldehyde-cross-linked conjugates (grey bars; preparation MG2-23). After 48 h, proliferation was measured by [^3H]thymidine incorporation within 16 h.

Table 3

Stimulation of Bet v 1-specific human T cell clones demonstrating different epitope specificities with rBet v 1-S-layer conjugates

TCC	Epitope (aa)	[³ H]thymidine uptake ($\times 10^{-3}$ counts/min)			
		Neg control ^a	rBet v 1	rBet v 1-SL _N ^b	rBet v 1-SL _C ^c
1	75–90	1.4	97.9	n.t.	84.1
2	94–105	1.7	29.2	33.6	24.5
3	118–129	2.7	71.5	30.6	48.6
4	142–156	0.2	41.6	15.1	22.7

Values are mean of triplicates; n.t., not tested.

^aNegative control: TCC and APC alone.^bPool of native rBet v 1-S-layer conjugates.^cPool of cross-linked rBet v 1-S-layer conjugates.

the peripheral blood mononuclear cells which were used as feeder cells. Appropriate controls with feeder cells alone, feeder cells and clones, and feeder cells with rBet v 1 were performed.

All tested conjugates of rBet v 1 immobilized on S-layer self-assembly products induced concentration-dependent T-cell responses similar to rBet v 1 alone. This observation was irrespective of the relative amount of rBet v 1 bound to the carrier. Two representative S-layer conjugates are shown in Fig. 3. Interestingly, these two preparations had been relatively weak in binding human IgE (Fig. 2).

These experiments demonstrate that the rBet v 1-conjugates are taken up and processed by APC. By using TCC with different epitope specificity, it was demonstrated that four important rBet v 1-epitopes [3] were presented by APC (Table 3). However, conjugates containing only small amounts of rBet v 1, i.e. cell wall and peptidoglycan preparations, were not suitable for T-cell proliferation assays due to interference of excess carrier material. Therefore, only small amounts of these conjugates, containing much less than 10 μ g rBet v 1, could be tested. These samples still induced Bet v 1-specific T-cell responses (data not shown).

3.2.4. Induction of IFN- γ in human allergen-specific TCC of the T_H2 phenotype

Seven human Bet v 1-specific TCC displaying the typical T_H2-like phenotype (high IL-4 vs. low/no IFN- γ production) were stimulated with

rBet v 1 and rBet v 1-conjugates, respectively. The mean of the IL-4 production in response to rBet v 1 was 917 ± 955 pg/ml ($n = 7$), stimulation with rBet v 1-conjugates led to a mean of 674 ± 727 pg/ml ($n = 7$; no significant difference). However, when stimulated with rBet v 1-conjugates, TCC produced significantly more IFN- γ (mean 604 ± 1150 pg/ml; $n = 7$; $P \leq 0.001$) in comparison to stimulation with rBet v 1 (14 ± 37 pg/ml; $n = 7$), indicating a shift towards the T_H0/T_H1 phenotype (Fig. 4). In all experiments, measured proliferation did not differ between the two different stimuli.

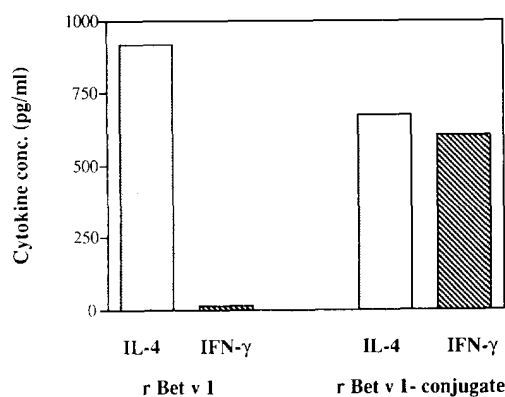


Fig. 4. Cytokine production of typical human Bet v 1-specific T_H2 T-cell clones; 10^6 T cells in the presence of autologous irradiated PBMC (60 Gy) were stimulated for 24 h with 10 μ g rBet v 1 or rBet v 1-S-layer conjugates in 1 ml medium. Cytokine concentrations in supernatants were measured by ELISA (values are the mean of seven clones).

4. Discussion

According to current concepts of immune regulation, IgE production in Type I-allergic patients results from T_H2 -like immune responses to allergens, whereas healthy individuals react to allergens with T_H0 - or T_H1 -like immune responses and restricted IgG production. One strategy for immunoprophylaxis or immunotherapy of allergies aims at modulation of the T_H2 response of atopic patients towards T_H1 -like responses [1–3].

Previous immunization experiments with carbohydrate-S-layer conjugates have indicated that immune responses in animals can be modulated towards a T_H1 - or a T_H2 -pattern through the choice and construction of the respective S-layer conjugates [8]. In the present study Bet v 1 was chosen for several reasons: (i) It is an important inhalant allergen with multiple cross-reactivities to other plant allergens [32]. (ii) Large amounts of purified rBet v 1 are available [11,13]. (iii) The immune response to Bet v 1 in allergic and healthy individuals has been extensively characterized [29,33].

Recombinant Bet v 1 was covalently coupled to different cell envelope components of two Gram-positive eubacteria by standard carbodiimide methodology (Table 1) and the immunological properties of the conjugates were characterized. In comparison to previous experiments, in which carbohydrate haptens were bound to S-layers [7–9], using rBet v 1 did not result in significant differences in the immunological reactivity between native and cross-linked S-layer preparations. The conservation of major B-cell epitopes on all preparations tested was demonstrated in inhibition assays using sera from birch pollen-allergic patients. This is of importance, since until now it has not been possible to map B-cell epitopes of Bet v 1 on the basis of linear peptides. In general, the more rBet v 1 was bound to S-layer carriers, the less inhibition was observed (Fig. 2). The decreased IgE binding results most likely from steric hindrance, i.e. decreased accessibility of the intact Bet v 1 epitopes.

Preliminary immunization experiments in mice indicated that S-layers serve not only as carriers

but also as adjuvants for the allergen (Table 2). At equivalent concentrations, rBet v 1 alone did not induce immune responses nor did rBet v 1 admixed to S-layers. Only rBet v 1-S-layer conjugates elicited significant allergen-specific T-cell responses. Considering that the antigen was applied intraperitoneally, this finding indicates that a close and enduring proximity of allergen and carrier is required for the adjuvant effect which is probably mediated by the antigen-presenting cells in the induction phase of the immune reaction.

Presumably, T-cell epitopes do not correspond to B-cell epitopes of Bet v 1 [34]. Experiments with Bet v 1-specific, epitope-mapped TCC (Table 3) indicate that conjugates were taken up and processed by APC. In view of the size of the conjugates, it is likely that monocytes acted as APC. The T-cell response correlated to the total amount of Bet v 1, was not dependent on the relative amount of Bet v 1 bound to the carrier, and displayed dose-dependency (Fig. 3).

These experiments have demonstrated the usefulness of S-layers as carrier/adjuvants for allergens. The value of these conjugates for the treatment of Type I allergy was further examined. We could show that activation of allergen-specific TCC with Bet v 1-S-layer conjugates led to a significant increase of IFN- γ production by these cells. In contrast, when stimulated with free allergen TCC displayed a typical T_H2 pattern of cytokine secretion (high levels of IL-4, low/no IFN- γ ; Fig. 4).

In conclusion, S-layers as allergen carriers demonstrated a promising effect on the induction of the desired T helper response for the treatment of Type I hypersensitivity. The underlying mechanisms, however, have still to be elucidated.

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