

Bet v 1, the major birch pollen allergen, conjugated to crystalline bacterial cell surface proteins, expands allergen-specific T cells of the T_H1/T_H0 phenotype *in vitro* by induction of IL-12

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Abstract

Modulation of allergic immune responses by using adequate adjuvants is a promising concept for future immunotherapy of type I hypersensitivity. In the present study, recombinant Bet v 1 (rBet v 1, the major birch pollen allergen) was conjugated to cross-linked crystalline surface layer proteins (SL) derived from Gram-positive eubacteria. T cell lines (TCL) and clones (TCC) were established from peripheral blood of birch pollen-allergic patients. TCL and TCC were induced either using rBet v 1 alone or rBet v 1/SL conjugates (rBet v 1/SL) as initial antigen stimulus. Cytokine production after re-stimulation with rBet v 1 was investigated. TCL initiated with rBet v 1/SL showed significantly increased IFN- γ production as compared to rBet v 1-selected TCL. TCC were established from TCL of five patients. As expected, the majority of CD4⁺ TCC induced by rBet v 1 (55%) displayed a T_H2-like pattern of cytokine production. However, only 21% of Bet v 1-specific TCC isolated from TCL established with the Bet v 1/SL revealed this phenotype. The majority of SL-specific TCC (80%) belonged to the T_H1 phenotype. In cultures of peripheral blood mononuclear cells, both, SL and Bet v 1/SL (but not rBet v 1) stimulated the production of high levels of IL-12, a pivotal mediator of T_H1 responses. Moreover, stimulation of rBet v 1-induced TCC with rBet v 1/SL led to an increased IFN- γ production. This effect could be reversed by neutralizing anti-IL-12 mAb. Together these results indicate an adjuvant effect of SL mediated by IL-12. Our results indicate that bacterial components, such as SL, displaying adjuvant effects may be suitable for immunotherapeutic vaccines for type I allergy.

Introduction

In the last few years, evidence has accumulated that antagonizing activities of IL-4 and IFN- γ produced by different T cell subsets (T_H2 and T_H1) play a pivotal role in the allergic immune response in mice and humans (1–4). Allergic individuals demonstrate T_H2-like immune responses to allergenic molecules, leading to high antibody levels, including the production of IgE antibodies. Non-allergic

individuals, on the other hand, produce low levels of specific IgG to atopic allergens (5).

Specific immunotherapy (SIT), i.e. inducing tolerance to an allergen in an allergic patient by injecting increasing doses of specific allergen, was proved to be an effective treatment for patients suffering from type I allergy resulting from airborne allergens (6–9). Although SIT has been

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practised since 1911 (6), the underlying mechanism of its effectiveness is still unclear. Recent studies investigating patients which had undergone successful SIT indicated a modulation of the immune response at the level of T lymphocytes. Successful SIT was associated with a shift to a more T_H0/T_H1 -like cytokine pattern (decreased IL-4 production and/or increased IFN- γ production) in allergen-specific T cells from treated patients (10–14).

Thus, a redirection of regulatory T cells towards T_H0/T_H1 -like responses could represent a promising strategy in therapy and maybe even in prophylaxis of type I allergy (15). One possibility to influence the immune response to an antigen is the use of adjuvants and/or carriers inducing the desired type of immune response. It has long been known that bacterial components favor T_H1 -like cellular immune responses. Bacterial antigens result in immune responses with T_H1 characteristics (4,16–19). Recently an inverse association between tuberculin responses and atopic disorder became evident (20). Crystalline bacterial surface layers (SL) are common archeobacterial and eubacterial cell surface components. They form two-dimensional crystalline arrays (21) and can be used as carrier and adjuvants. In a mouse system, cross-linked carbohydrate/SL conjugates induced typical T_H1 -like immune responses (22,23).

The objective of the present study was to characterize allergen-specific T cell populations induced by allergen/SL conjugates. In our model system Bet v 1, the major birch pollen allergen (24, 25), was used for several reasons. The immune response of allergic and non-allergic individuals to Bet v 1 has been extensively characterized in the recent past (5,26). In addition, it is available as standardized, recombinant protein in unlimited quantities. rBet v 1 has been shown to display identical qualities compared to natural Bet v 1 *in vitro* and *in vivo* (26–28).

We analyzed the cytokine production and epitope recognition of Bet v 1-specific TCL and TCC induced either with rBet v 1 or with rBet v 1/SL and investigated the mechanism of the observed differences in T_H subset distribution. Our investigation supports the idea of using bacterial carriers as adjuvants in vaccines for SIT of type I allergy in the future.

Methods

Patients

Peripheral blood was obtained from birch pollen-allergic patients. Type I allergy to birch pollen was proved by typical case history, positive skin tests and positive radioallergosorbent test. Furthermore, all patients investigated displayed IgE binding to rBet v 1 in immunoblot analyses (not shown).

Allergen and allergen/SL conjugates

Purified rBet v 1 was obtained from Biomay (Linz, Austria). SL protein was prepared from two Gram-positive bacterial strains, *Bacillus sphaericus* CCM 2177 (29) and *Thermoanaerobacter thermohydrosulfuricus* L111-69 (30). Cells were broken up by sonication to obtain cell walls. SL was then extracted using guanidine hydrochloride. Non-soluble peptidoglycan was removed by ultracentrifugation. During dialysis, the SL formed self assembly products. Glutaral-

dehyde fixation was performed as described (31), only the concentration of glutaraldehyde was reduced to 0.05%. rBet v 1 was coupled to the fixed SL self assembly products after activation of carboxyl groups of the SL by 1-ethyl-3, 3'-(dimethyl-aminopropyl)-carbodiimide as previously described (32). SL were controlled by SDS-PAGE and negative stain electron microscopy before and after fixation and conjugation. The rBet v 1/SL contained 30–50 μ g rBet v 1/mg SL (w/w), which corresponds to ~ 0.3 mol allergen/mol SL protomer. Their sizes ranged from ~ 1 to 5 μ m. Conjugation of the allergen to SL and the products have been previously described (33). rBet v 1/SL containing cross-linked SL of either strain were combined at equivalent amounts. This homogeneous mixture of conjugates was used throughout the study.

Immunological characterization of rBet v 1/SL

The reactivity of rBet v 1 and rBet v 1/SL was comparable on a molar basis. This was tested in ELISA inhibition experiments using human Bet v 1-specific IgE as described before (26). Moreover, epitope composition, proliferation inducing capacity and kinetics of cytokine production were studied using characterized Bet v 1-specific TCC (5,33).

Specificities of rBet v 1- and SL-specific TCL and TCC

TCL and TCC were obtained according to a technique described previously (5). Briefly, $1\text{--}1.5 \times 10^6$ peripheral blood mononuclear cells (PBMC) were either stimulated with 20 μ g rBet v 1 or with rBet v 1/SL containing also 20 μ g Bet v 1 bound to 300 μ g SL in 2 ml Ultra Culture medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM glutamine and 20 μ M 2-mercaptoethanol in 24-well flat-bottomed culture plates (Costar, Cambridge, MA). After 5 days of culture, human rIL-2 (10 U/ml; Boehringer Mannheim, Mannheim, Germany) was added and the culture continued for additional 7 days. Viable T cell blasts were then separated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). These cells were investigated for secondary proliferative responses and the pattern of cytokine production. In addition, TCL blasts of five patients were cloned by limiting dilution (0.5 cells/well) in 96-well U-bottom plates (Nuncclone; Nunc, Copenhagen, Denmark) in the presence of 2×10^5 irradiated (60 Gy) allogeneic PBMC as feeder cells, 1% phytohemagglutinin (PHA; Gibco, Grand Island, NY) (v/v) and rIL-2 (4 U/well) in the medium described above. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2. The specificity of the clones was assessed in proliferation assays as described (5). A stimulation index of >10 was considered positive.

A panel of 50 dodecapeptides according to the Bet v 1 sequence and overlapping for nine amino acids (Cambridge Research Biochemicals, Cambridge, UK) was used to define the T cell epitopes of rBet v 1-specific clones. Peptides were used at 5 μ g/ml for the proliferation assays.

Analysis of phenotype and cytokine production

The phenotype of the TCC was analyzed by flow cytometry as described (5) on a FACScan (Becton Dickinson, Mountain View, CA) using FITC-labeled antibodies: anti-Leu4/CD3, anti-Leu2a/CD8, anti-Leu3a/CD4, anti-TCR-1 $\alpha\beta$ WT31 and anti-TCR $\gamma\delta$ I (Becton Dickinson).

For cytokine production of TCL and TCC induced with either rBet v 1 or rBet v 1/SL, 10^6 T cells were incubated with 5×10^5 irradiated (60 Gy) autologous PBMC in 1 ml of culture medium in the presence of 10 μ g rBet v 1. SL-specific TCC were stimulated with 150 μ g SL. Cultures containing PBMC plus antigen and T cells plus PBMC without antigen served as negative controls. Supernatants were collected after 24 h. IL-4 and IFN- γ levels in the supernatants were measured using commercial ELISA systems (Medgenix Diagnostics, Fleurus, The Netherlands). Detection limits were 1.5 pg/ml for IFN- γ and 2 pg/ml for IL-4. For the classification of TCC, exclusive IFN- γ production or high IFN- γ /IL-4 ratio (10 or higher) was considered a T_H1 -like and exclusive IL-4 production or low IFN- γ /IL-4 ratio (0.1 or less) a T_H2 -like cytokine pattern. TCC which produced considerable amounts of IFN- γ and IL-4 were labeled T_H0 -like TCC. In some TCC no cytokines could be detected in spite of high proliferative responses. These TCC were also attributed to the T_H0 subset.

To assay IL-12 production, 2×10^6 PBMC/ml were cultured for 24 h with medium, rBet v 1 (10 μ g/ml), rBet v 1/SL (10 respectively 150 μ g/ml) or SL (150 μ g/ml) alone. The concentration of IL-12 in the supernatants of these cultures was tested in ELISA assay systems specific for total IL-12p40 (p40/p40 and p40/p35 dimers; Medgenix) or the IL-12p70 heterodimer (Quantikine HS; R&D Systems, Minneapolis, MN). *Staphylococcus aureus* Cowan strain I (SAC), a strong IL-12 inducing agent (34), was used as positive control (Pansorbin 1:10000; Calbiochem, La Jolla, CA).

Stimulation of established Bet v 1-specific TCC with Bet v 1/SL
TCC cells (10^6) established with rBet v 1 (5) were stimulated with autologous PBMC and rBet v 1 (5 μ g/ml) or rBet v 1/SL respectively. Thereafter supernatants were collected as described above and analyzed for IFN- γ and IL-4.

IL-12 inhibition experiments

20c2, a strongly neutralizing mAb (35), kindly provided by R. Chizzonite (Hoffmann-LaRoche, Nutley, NJ), was used at a concentration of 50 μ g/ml. In pilot experiments, this concentration was shown to inhibit $74 \pm 6\%$ of the effect of 500 pg huIL-12p70 (R&D Systems) in a PHA-blast proliferation assay (36). Clone cells (10^6) were incubated with either rBet v 1/SL or rBet v 1/SL plus anti IL-12 or an isotype-matched control antibody (rat IgG1 50 μ g/ml; PharMingen). The antibodies were added to the cultures 30 min prior to the antigens. Non-irradiated PBMC were used as antigen-presenting cells (APC). Supernatants were recovered and subjected to ELISA as mentioned above. The cytokine levels produced within the control cultures (PBMC plus respective antigen) were subtracted from the results obtained with the TCC.

Statistics

Cytokine production was analyzed by Wilcoxon's signed rank test and univariate analysis of variance subsequent to square root transformation.

Results

TCL induced by rBet v 1 and rBet v 1/SL

TCL were established from peripheral blood of birch pollen-allergic donors using either rBet v 1 or rBet v 1/SL as initial

Table 1. Comparison of cytokine production in TCL induced with either rBet v 1 or rBet v 1/SL

Patient	IFN- γ /IL-4-ratio	
	TCL-rBet v 1	TCL-rBet v 1/SL
1	3.5	203.5
2	1.8	95.6
3	58.7	111.1
4	1.2	1.0
5	149.0	567.5
6	1.7	6.8
7	1600	1600
8	8.6	114.0
9	5.8	17.3
10	21.5	43.8
11	6.1	7.6
Median	6.1	95.6 ^a

PBMC (1.5×10^6) were stimulated with 20 μ g/2 ml rBet v 1 alone or rBet v 1/SL for 5 days and 20 U huIL-2 were added. At day 12 the TCL were re-stimulated with rBet v 1 and autologous irradiated APC. Supernatants were collected after 24 h and the concentrations of IFN- γ and IL-4 were determined by ELISA.

^a $P = 0.003$ (Wilcoxon's signed rank test).

stimulant. As shown in Table 1, re-stimulation of these TCL with rBet v 1 revealed a significantly increased IFN- γ /IL-4 ratio ($P = 0.003$) in TCL that had originally been induced with the allergen conjugate.

TCC induced by rBet v 1 and rBet v 1/SL

A total of 53 clones were isolated from the TCL of five selected donors. No significant differences were observed between the cloning efficiencies of the cloning experiments using T cell blasts originating from rBet v 1- and rBet v 1/SL-initiated TCL. 23/53 Bet v 1-specific TCC were derived from TCL initially stimulated with rBet v 1. Cloning of TCL induced with rBet v 1/SL resulted in 30 TCC, 19 allergen-specific TCC and 11 TCC with specificity for SL. Allergen-reactive T_H cell clones are shown in Table 2.

Epitope mapping

Testing allergen-specific TCC with 50 overlapping epitopes spanning the whole Bet v 1 sequence revealed 13 different T cell epitopes (Table 2). The epitopes were scattered over the whole molecule. No difference was observed in the distribution of epitopes recognized by the TCC derived from either rBet v 1 or rBet v 1/SL-induced TCL. No epitope was associated with a particular T_H subset.

Phenotype and cytokine production

FACS analyses revealed that all TCC expressed TCR $\alpha\beta$. Two CD4⁺CD3⁺CD8⁺ clones were detected. One was derived from a rBet v 1-stimulated TCL and the other one was SL specific (induced by rBet v 1/SL). The remaining 51 TCC belonged to the T_H phenotype (CD3⁺CD4⁺CD8⁻).

After stimulation of TCC with rBet v 1, supernatants were analyzed for their content of IFN- γ and IL-4 (Table 3). SL-specific TCC were stimulated with SL protein alone (data not shown). The CD4⁺ clones were then divided into T_H subsets

Table 2. Proliferative responses and epitope specificities of rBet v 1-specific CD4⁺ T cell clones

Patient	TCC	Thymidine uptake ($\times 10^{-3}$ c.p.m.)			
		Neg Co ^a	r Bet v 1	Epitope	Peptides (amino acids)
1	4 ^b	0.2	49.6	58.8	142–156
	161	0.1	26.8	63.0	76–90
	9 ^c	0.2	8.4	25.1	142–153
	16	0.2	1.3	14.7	28–42
	48	0.1	1.3	1.5	139–150
	62	0.2	48.7	76.6	34–51
2	94	4.0	47.4	ND	ND
	97	0.1	41.0	75.2	112–123
	98	0.1	16.6	77.9	112–123
	101	0.1	19.1	15.6	10–24
	104	3.5	33.1	ND	ND
	106	0.2	15.6	67.6	100–111
	110	0.1	12.5	113.3	10–27
	112	0.1	43.6	91.8	112–123
	121	0.1	18.2	83.6	10–27
	124	7.2	67.1	ND	ND
	2	0.2	22.4	31.5	142–153
	9	0.4	70.8	143.8	106–120
	19	3.1	30.9	27.0	76–90
	37	6.7	38.1	50.2	55–69
	47	0.3	25.8	28.8	112–123
	49	0.5	14.1	52.3	85–96
3	72	3.2	18.4	ND	ND
	75	1.7	3.5	9.0	22–33
	83	0.4	4.2	ND	ND
	86	1.5	10.4	18.8	13–24
	88	7.5	36.7	ND	ND
	16	1.6	70.1	16.2	13–24
	27	1.3	13.5	ND	ND
4	25	0.3	2.2	18.5	142–156
	7	3.9	33.0	24.3	109–120
	36	0.1	8.4	90.0	4–18
	37	0.2	34.7	91.1	7–18
5	12	2.6	46.7	31.1	4–15
	16	0.5	5.6	ND	ND
	18	0.2	20.3	56.3	4–15
	20	0.2	67.1	ND	ND
	31	7.3	139.7	108.9	64–75
	33	4.8	141.2	116.5	64–75
	34	0.1	7.6	28.9	88–102
	110	1.8	128.5	125.2	64–75

^aAPC and T cell clone in the absence of antigen.^bPlain figures: clones induced by rBet v 1.^cItalic figures: clones induced by rBet v 1/SL conjugates.

according to the IFN- γ /IL-4 ratio. The results are summarized in Fig. 1. As expected, the majority of TCC (12 of 22, 55%) derived from allergen-stimulated TCL displayed a T_H2-like pattern of cytokine production. Within TCC isolated from rBet v 1/SL activated TCL, a lower percentage of T_H2-like TCC (4 of 19, 21%) was observed. Concomitantly, the number of TCC producing T_H0 and T_H1-like patterns of cytokines increased (15 of 19, 79%).

This increase in T_H0/T_H1 subsets appeared to be due to the increased IFN- γ production as indicated by the mean values of IFN- γ (399 $\pm$ 935 versus 893 $\pm$ 1223 pg/ml), rather

Table 3. Specific cytokine production by Bet v 1-specific CD4⁺ T cell clones

Patients	TCC	IFN- γ (pg/ml)	IL-4 (pg/ml)	T _H subset
1	4 ^a	270	570	0
	161	0	136	2
	9 ^b	0	334	2
	16	3080	164	1
	48	100	826	0
	62	2590	76	1
2	94	0	34	2
	97	3250	2568	0
	98	860	204	0
	101	0	604	2
	104	3150	1880	0
	106	100	10	1
	110	0	4	2
	112	700	2118	0
	121	200	184	0
	124	0	138	2
	2	120	630	0
	9	510	298	0
	19	790	2486	0
	37	3020	2480	0
	47	2840	934	0
	49	30	0	1
3	72	0	172	2
	75	0	0	0
	83	0	2	2
	86	0	2182	2
	88	0	2332	2
	16	280	1762	0
	27	0	210	2
4	25	180	2000	2
	7	2730	338	0
	36	0	0	0
5	37	0	0	0
	12	85	0	1
	16	0	31	2
	18	0	0	0
	20	0	796	2
	31	65	2402	2
	33	150	2515	2
	34	160	400	0
	110	520	1862	0

^aPlain figures: T cell clones induced with r Bet v 1.^bItalic figures: T cell clones induced with rBet v 1/SL conjugates.

than to changes in IL-4 (801 $\pm$ 961 versus 932 $\pm$ 971 pg/ml). Using square root transformation and variance analysis, the differences in IFN- γ production observed with both stimuli were statistically significant ($P = 0.009$). SL-specific CD4⁺ TCC produced high concentrations of IFN- γ and no or only low concentrations of IL-4 (1202 $\pm$ 1475 and 75 $\pm$ 220 pg/ml). Thus, eight of 10 (80%) TCC belonged to the T_H1-like T_H subset and two of 10 TCC to the T_H0-like subset (Fig. 1).

Induction of IL-12

IL-12 is a pivotal inducer of T_H1 responses (37). rBet v 1/SL and SL alone induced the synthesis of high levels of IL-12 in PBMC. Both IL-12p40 (total dimers) and also the bioactive IL-12p70 heterodimer could be demonstrated (Table 4).

Table 4. SL-induced IL-12 production by PBMC derived from allergic individuals

PBMC donor	IL-12 specificity	Stimulant				
		Control	Bet v 1	Bet v 1/SL	SL	SAC
1	p40	78 ^a	75	1860	898	7870
	p70	<0.6	<0.6	20.5	8.5	>40
2	p40	24	28	1254	NT	8290
	p70	<0.6	<0.6	6.2	NT	>40
3	p40	5	5	773	390	4180
	p70	<0.6	<0.6	1.8	<0.6	>40
4	p40	<1.5	2	579	167	3210
	p70	<0.6	<0.6	1.4	<0.6	>40
5	p40	8	6	362	279	2940
	p70	<0.6	<0.6	2.5	1.1	>40

^aIn units of pg/ml measured by IL-12 p40/p70-specific ELISA. Supernatants of 2×10^6 PBMC were collected after 24 h stimulation with rBet v 1 (10 µg/ml), SL (150 µg/ml), rBet v 1/SL conjugate or SAC (1:10,000).

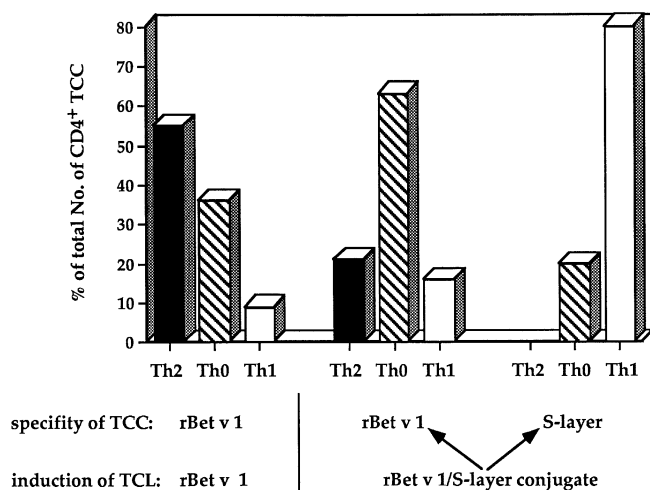


Fig. 1. Distribution of T_H subsets within $CD4^+$ TCC induced by rBet v 1 or rBet v 1/SL conjugates and stimulated by rBet v 1. T_H subsets were classified according to IFN- γ /IL-4 ratio (see Methods).

Induction of IFN- γ in established Bet v 1-specific TCC by rBet v 1/SL

IL-12 not only primes for stable IFN- γ production during T cell differentiation, but has also been shown to induce transient IFN- γ production in already established T_H2 TCC (37). To study the effect of rBet v 1/SL in this respect, additional rBet v 1-derived TCC were investigated. As shown in Fig. 2, stimulation with rBet v 1/SL induced an elevated IFN- γ /IL-4 ratio in culture supernatants when compared with rBet v 1-stimulated TCC. These changes were due to a significant increase of IFN- γ ($P = 0.003$, Wilcoxon's signed rank test), while the reduction of concentration of IL-4 was not significant.

IL-12 inhibition experiments

To evaluate the role of IL-12 in mediating the effects of rBet v 1/SL on T cell cytokine production, neutralizing mAb to huIL-12 were added to cultures of rBet v 1-specific TCC stimulated by rBet v 1/SL. The increase of the IFN- γ /IL-4 ratio promoted by the SL conjugates could be reversed by anti-

IL-12 mAb, but not by isotype-matched control antibodies (Fig. 2).

Discussion

SIT is an efficient form of treatment of type I allergy (6–9). The mechanism of its effectiveness is still unclear, although increasing evidence evolves indicating a working mechanism on the level of regulating T cells (10–14). The molecular characterization of allergens, the availability of the major allergens as recombinant proteins together with the knowledge of epitopes open possibilities to develop new allergy vaccines (15,38,39). One possibility to modulate the typical allergic T_H2 -like immune response and to direct it towards T_H0 or T_H1 might be the administration of allergens together with appropriate adjuvants. Bacterial products have been shown to induce T_H1 -like immune responses (4,17–20). In this respect, cross-linked SL of Gram-positive eubacteria have been shown to be effective (23). Therefore, in the present study bacterial SL were investigated for their usefulness as carrier/adjuvants for allergens.

TCL and TCC derived from the peripheral blood of birch pollen-allergic individuals were established using rBet v 1/SL. T lymphocyte cultures were characterized and compared to cultures which had been produced in parallel using rBet v 1 alone. Oligoclonal Bet v 1-specific TCL induced with rBet v 1/SL revealed a significantly increased IFN- γ production when re-stimulated with rBet v 1 alone, as compared to TCL initiated with rBet v 1 and re-stimulated with rBet v 1 (Table 1). The presence of IFN- γ in the TCL-induction phase has been described to lead to a preferential development of TCC with a T_H1 -like phenotype (40). To investigate this mechanism we cloned TCL that had been induced with rBet v 1 or rBet v 1/SL respectively. Indeed, the majority of TCC derived from the TCL which had been initiated with rBet v 1 (55%) displayed a typical T_H2 -like cytokine production pattern. Due to a significant increase of IFN- γ levels, less T_H2 -like (21%) and increased numbers of IFN- γ -producing TCC were obtained from TCL initially stimulated with rBet v 1/SL. Most of the TCC specific for SL protein (80%) expressed a T_H1 -like cytokine production pattern (Fig. 1).

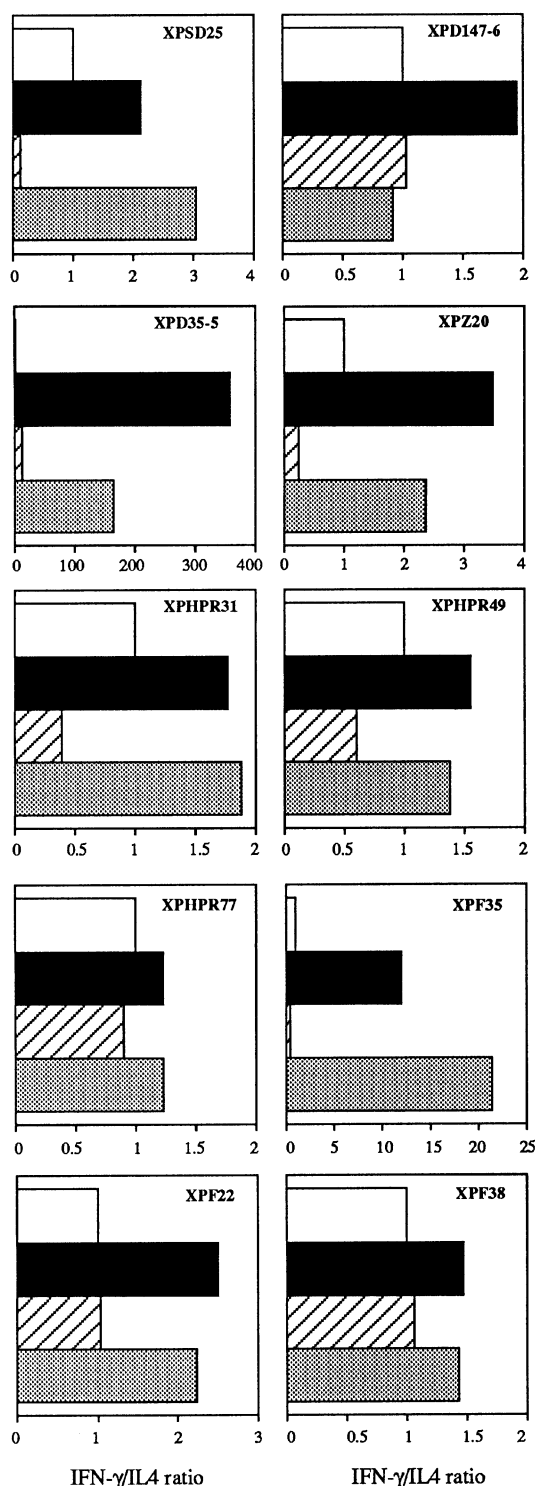


Fig. 2. Stimulation of Bet v 1-specific TCC with rBet v 1/SL leads to increased IFN- γ /IL-4 ratios (black bars) as compared to stimulation with rBet v 1 alone (white bars). Addition of neutralizing anti-IL-12 mAb inhibits the effects of rBet v 1/SL (hatched bars), whereas isotype-matched control antibodies are not effective in this respect (grey bars). The concentrations of IFN- γ and IL-4 in the supernatants were analyzed by ELISA. The relative IFN- γ /IL-4 ratios (stimulation with rBet v 1 = 1) are indicated.

The induction of particular cytokines in responding T lymphocytes depends on the expression of certain co-stimulatory surface molecules on APC and the presence of certain mediators in the microenvironment. Due to their size of 1–5 μ m, a targeting of the rBet v 1/SL to macrophages, which are the most efficient phagocytic APC (41), may be assumed. Macrophages apparently are specialized APC in the promotion of T_H1 immune responses. This can be explained by the production of large amounts of IL-12, a heteromeric cytokine produced by activated macrophages. IL-12 elicits IFN- γ production and thereby T_H1 responses, and is induced by intracellular parasites and bacterial products (34). The presence of IL-12 in the initial cultures may be responsible for the observed effects in our study. Indeed, we could show that in PBMC high levels of IL-12 (including the bioactive IL-12p70 heterodimer) were induced by SL or rBet v 1/SL, while allergen alone had no effect on the basal level of this cytokine. IL-12 has been described to elicit IFN- γ production in TCC of the T_H2 type (37). To test whether IL-12 is an important factor contributing to the effects observed with our bacterial SL conjugates, we stimulated rBet v 1-induced allergen-specific TCC with rBet v 1/SL and compared IFN- γ /IL-4 ratios in response to rBet v 1 or rBet v 1/SL. As expected, stimulation with rBet v 1 associated with bacterial SL led to increased levels of IFN- γ production by TCC. This phenomenon could be reversed by the addition of neutralizing anti-IL-12 mAb, underlining the importance of IL-12 for the deviation induced by SL conjugates (Fig. 2). In addition other factors may be involved, e.g. we cannot rule out that the observed modulation of the T cell response are partly due to the particularization *per se* (42), also an increased antigen dose and peptide load per APC might contribute to the skewing of Th subsets (43,44).

The T cell epitopes of rBet v 1- and rBet v 1/SL-induced TCC were scattered over the whole Bet v 1 molecule (5). No association between particular T_H subsets and certain epitopes on the allergen could be observed. Therefore, no evidence for the selection of T_H1-associated specificities within the allergen molecule (which would have been of special therapeutic interest) could be observed. In our experiments SL had no influence on the induction of CD8⁺ TCC. Only two CD8⁺ TCC were isolated. One of each was derived from rBet v 1 respectively rBet v 1/SL-induced TCL, and both produced IFN- γ and IL-4 (45).

In conclusion, the results of our study indicate that bacterial components such as SL may be suitable carriers for allergens used as immunotherapeutic vaccines for SIT of type I allergy in the future.

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Abbreviations

APC	antigen-presenting cell
Bet v 1	the major pollen allergen of birch (<i>Betula verrucosa</i>)

Bet v 1/SL	Bet v 1/SL conjugate
PBMC	peripheral blood mononuclear cell
PHA	phytohemagglutinin
SAC	<i>Staphylococcus aureus</i> Cowan strain I
SIT	specific immunotherapy
SL	crystalline bacterial surface layer protein
TCC	T cell clone
TCL	T cell line

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