

Modulation of the allergic immune response in BALB/c mice by subcutaneous injection of high doses of the dominant T cell epitope from the major birch pollen allergen Bet v 1

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(Accepted for publication 31 October 1996)

SUMMARY

Several *in vitro* and *in vivo* studies indicate that application of high doses of dominant T cell epitopes can induce a state of antigen-specific non-responsiveness (anergy). In the present study, we developed a murine model of an allergic immune response to Bet v 1, the major birch pollen allergen. Mice were sensitized by injection of rBet v 1 and the allergic state was proven by the presence of allergen-specific IgE and positive immediate-type skin tests to Bet v 1. In epitope mapping experiments, an immunodominant T cell epitope of Bet v 1 in BALB/c mice was identified by the use of overlapping peptides. This peptide (BV139) was subsequently employed for treatment. Two tolerization protocols were used: in one approach, the peptide was administered to naive mice before immunization (group BV139-S), in the second, already sensitized mice were treated (S-BV139). The results demonstrated that administering high doses of the dominant T cell epitope of Bet v 1 profoundly diminished T cell proliferation to the peptide in the BV139-S group, and to the peptide as well as to the whole protein in the S-BV139 group. Skin test reactivity to Bet v 1 was reduced in the BV139-S group. However, no differences in terms of specific antibody production between treated and untreated mice could be observed. This study provides evidence that administration of dominant T cell epitopes can down-regulate the allergen-specific T cell response. Proceeding on the assumption that the T lymphocyte response to allergens is crucial for the induction and maintenance of the allergic disease, a modulation of the immune response to allergens by treatment with T cell epitope peptides could represent a promising concept for immunotherapy in the future.

Keywords type-I allergy tolerance immunotherapy T cell epitope

INTRODUCTION

Specific immunotherapy (SIT) has been successfully used in the treatment of type I allergies for more than 80 years [1]. The potential risk of severe immediate-type side-effects during this treatment (injection of allergen extract) is well established [2,3]. The precise mechanisms by which SIT achieves clinical improvement are poorly understood [4]. CD4⁺ T cells, which play a crucial role in the immune response of the atopic patient, appear to be an important target in SIT [5]. CD4⁺ Th2 cells provide B cell help, induce IgE class switch, and, in addition, contribute significantly to the allergic inflammatory response by secretion of proinflammatory cytokines [6]. Recent studies investigating patients who had undergone successful SIT, indicated a modulation of the immune response at the level of T lymphocytes. This is represented by a

shift from the type I allergy-associated Th2-like cytokine pattern to one resembling Th0/Th1 (decreased IL-4 production and increased interferon-gamma (IFN- γ) production) in allergen-specific T cells from treated patients [7–9].

Several *in vitro* and *in vivo* studies indicate that presentation of high doses of dominant T cell epitopes to T cells under appropriate conditions can induce antigen-specific non-responsiveness [10–13]. This is consistent with the 'two-signal model' of T cell activation [14]: T cells require both, T cell receptor signalling and a second signal via ligation of CD28 or CTLA-4 on T cells with CD80 or CD86 on antigen-presenting cells (APC) for efficient activation [15–18]. Anergy is induced under certain conditions provided that just one signal is delivered. Briner *et al.* as well as Hoyne *et al.* were able to demonstrate that administration of one immunodominant peptide in naive and primed mice reduced T cell reactivity as well as specific IgG antibody production [19,20]. Treatment with T cell peptides could therefore tolerize Th cells and at the same time make SIT safer, since it does not bear the potential risk of cross-linking

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IgE antibodies on mast cells [21]. In recent years, T cell epitopes of several major allergens have been identified [21–28], and first clinical trials of T cell peptide treatment in allergic individuals are now under way [29].

In the current study, we established a murine model of type I allergy to Bet v 1, the major birch pollen allergen. We present direct evidence by *in vitro* and *in vivo* analyses that a naive as well as an ongoing allergic immune response can be modulated by application of high doses of the dominant T cell epitope in experimental animals. This may also be a feasible way in the human system to ameliorate allergic symptoms in the atopic patient.

MATERIALS AND METHODS

Mice

Female BALB/c, 6 weeks of age, were obtained from Charles River (Sulzfeld, Germany). All experimental animals used in this study were covered by a protocol approved by the local ethics committee of the University of Vienna, Austria.

Epitope mapping

A panel of 50 peptides was synthesized according to the Bet v 1 sequence by Cambridge Research Biochemicals Ltd (Cambridge, UK). The dodecapeptides were overlapping for three amino acids (neighbours shared nine residues). To examine cellular reactivity to the peptides, spleens were removed from the animals 10 days after immunization. Cells were forced through a stainless steel mesh, mononuclear cells (MNC) were purified by density gradient centrifugation (Lympholyte, Cedarlane, Ontario, Canada), washed, and cultured at $1\text{--}2 \times 10^5$ cells/well in 96-well round-bottomed plates (Nuncclone; Nunc, Roskilde, Denmark). Peptides were added at a concentration of 5 µg/ml. Cells were cultured at 37°C in 5% CO₂ in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 0.1 mg/ml gentamycin, and 50 µM 2-mercaptoethanol for 5 days. In the last 16 h of culture ³H-TdR (Amersham, Aylesbury, UK; 0.5 µCi/well) was added and incorporated radioactivity was measured by scintillation counting.

Antigens

Purified rBet v 1 was obtained from Biomay/Biotechnik (Linz,

Austria) [30,31]. The peptide BV139 of a purity in excess of 95%—as determined by high performance liquid chromatography (HPLC; pi-Chem, Graz, Austria)—was used for treatment.

Treatment protocol

Twenty-five mice were investigated.

Group S. Five mice were injected on four occasions, 12 days apart with 1 µg rBet v 1 mixed with 50 µl PBS and 100 µl Al(OH)₃ (Serva, Heidelberg, Germany; 2 mg/animal) intraperitoneally.

Group BV139-S. For induction of tolerance, eight mice were injected subcutaneously at three dorsal sites with 25 µg of the Bet v 1 peptide MGETLLRAVESYLL (representing the Bet v 1-aa 139–152, from now on referred to as BV139) in 150 µl PBS on 5 consecutive days 7 days before sensitization, following the schedule described above.

Group S-BV139. Eight mice were immunized according to the protocol described. Two weeks after sensitization, mice were treated with peptide BV139. One week after peptide application, mice were subcutaneously rechallenged with rBet v 1 at the dose described for immunization.

Group N. A control group was neither sensitized nor treated.

Blood was taken from the tail vein before the first injection with rBet v 1 or peptide BV139 and thereafter four times (BV139-S) and five times (S-BV139), respectively. The last collection was performed 1 day before skin testing and cellular analyses. Immunization and tolerance induction protocols are summarized in Fig. 1.

Proliferation assays

Spleens were removed from the animals 10 days after the last immunization. Culture conditions were as described for epitope mapping. Antigen concentrations used in proliferation assays were 10 µg/ml for Bet v 1 and 5 µg/ml for the peptides.

Intradermal skin testing

Skin testing was performed according to a protocol described [32], 10 days after the last immunization. For each skin test, 30 µl of test solution were injected intradermally. The concentrations of rBet v 1 in PBS solutions were 1.67 µg/ml and 167 ng/ml, respectively. The mast cell degranulation compound 48/80 (Sigma Chemical Co., St Louis, MO) at a concentration of 1 µg/ml served as positive

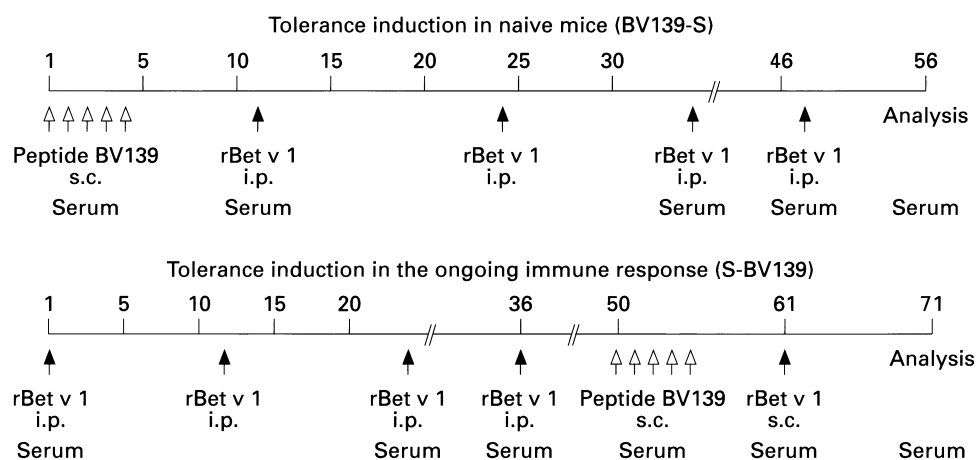


Fig. 1. Two different tolerization protocols of BALB/c mice with administration of high doses (5×25 µg) of the dominant Bet v 1 epitope BV139 before or after immunization with recombinant (r)Bet v 1. A non-treated and a non-sensitized, non-treated group of mice with immunizations at the same time points are not shown. i.p., Intraperitoneal; s.c., subcutaneous.

control, PBS being used as negative control. Before intradermal testing, Evans blue (Sigma) solution of a concentration of 0.5% (w/v) was injected intravenously to visualize positive reactions. Mice were killed 15 min after testing. Reactions were read on the reversed skin and compared with controls. A reaction as strong as or stronger than the positive control was scored as double-positive (++), single-positive (+) referred to positive reactions weaker than positive control, but larger than negative control.

Measurement of serum antibodies

An ELISA system was used to investigate Bet v 1-specific IgE, IgG1, IgG2a, IgG2b, IgG3, and IgM antibody levels in sera. All experiments were performed in one assay, and a reference serum pool was included as positive control. Microtitre plates (Nunc) were coated with rBet v 1 (2 µg/ml carbonate buffer, pH 9.6) overnight at 4°C. After washing twice with PBS containing 0.05% Tween 20 (PBS-T), unspecific binding sites were saturated with PBS-T-1% bovine serum albumin (BSA) at room temperature for 2 h. Thereafter, sera diluted in PBS-T-0.5% BSA were incubated overnight at 4°C (IgE 1:20, IgG1 1:20 000, IgG2a 1:1000, IgG2b 1:400, IgM 1:500). After five washings rat anti-mouse IgE, IgG subclass, and IgM antibodies (Pharmingen, San Diego, CA) diluted 1:1000 in PBS-T-0.5% BSA were added overnight at 4°C. After five washings bound anti-mouse antibodies were detected using a horseradish peroxidase (HRP)-conjugated mouse anti-rat IgG antibody (diluted 1:1000; Dianova, Hamburg, Germany). Colour development was performed using 2,2 azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma) as substrate. OD was measured at 405/490 nm using a Dynatech microplate reader. Results are expressed in ELISA OD units.

Cytokine assays

In order to measure murine IL-4 and IFN-γ levels in primary cell culture supernatants (SN), MNC were cultured at 4×10^5 cells/well in 96-well round-bottomed plates without stimulation or with rBet v 1 for 48 h. Ninety-six-well plates (Maxisorp; Nunc) were coated with rat anti-murine IL-4 (Pharmingen) or rat anti-murine IFN-γ (Pharmingen) antibodies (1 µg/ml in carbonate buffer pH 9.6) overnight at 4°C. Undiluted culture SN and standard solutions were incubated overnight at 4°C. Bound murine IL-4 and IFN-γ were detected by incubation with biotinylated rat anti-murine IL-4 (Pharmingen) or rat anti-murine IFN-γ (Pharmingen) antibodies at 1 µg/ml in PBS-T-1.5% BSA. Bound antibodies were detected using an HRP-conjugated avidin (Sigma) and ABTS (Sigma) as substrate. Values were calculated according to standard curves obtained with mIL-4 (Pharmingen) and mIFN-γ (Pharmingen); sensitivity thresholds were 5 pg/ml for IL-4 and 25 pg/ml for IFN-γ, respectively.

Statistical analysis

Immunoglobulin levels (OD values) and stimulation indices of T cell proliferation are expressed as the mean $\pm$ s.e.m. Student's two-tailed unpaired *t*-test matrix was used to determine the level of difference between the four groups of mice investigated in the study. In the case of skin test reactivity, Fisher's exact test was used to determine the level of difference between groups.

RESULTS

Choice of appropriate mouse strains

In preliminary experiments, five different strains of mice (BALB/c,

SJL/J, DBA/1 J, CBA/J, C57Bl/6) were investigated. Six mice of each strain were immunized four times with 1 µg of rBet v 1 mixed with 50 µl PBS and 100 µl Al(OH)₃ (Serva; 2 mg/animal) intraperitoneally. A significant IgE antibody response could be induced in all mouse strains. IgE and IgG1 antibodies dominated in BALB/c (data not shown), therefore this strain was used for all further experiments.

BV 139 is the dominant T cell epitope of Bet v 1 in BALB/c mice

In pilot experiments, 30 BALB/c mice were immunized with rBet v 1 according to a protocol described for group S. Ten days after the last injection, splenic MNC were cultured with 50 overlapping peptides in proliferation assays. Lymphocytes of each mouse proliferated (SI of 4 or more) upon stimulation with rBet v 1 and at least with one of the peptides. Twenty-five of 30 (83%) recognized peptide 47 of the 50 mapping peptides (MGETLLRA-VESY will be referred to as BV139), which clearly appeared to be the immunodominant T cell epitope on Bet v 1 in BALB/c mice. Therefore, peptide BV139 (Bet v 1-aa 139–152) was used for tolerance induction in all further experiments. The frequency of recognition of overlapping peptides representing the whole Bet v 1 protein is summarized in Fig. 2. In five mice immunized according to a protocol described for group S, skin tests were performed using BV 139 and rBet v 1. Immediate-type skin reactions could be observed for rBet v 1 (1.67 µg/ml, 167 ng/ml) in all mice; in comparison, BV 139 in the same concentration ($\approx$ 10-fold molar excess) was negative (not shown).

No differences in terms of antibody production between sensitized (S) and treated (BV139-S, S-BV139) mice

Specific IgE was induced only after two injections of rBet v 1 in Al(OH)₃ (Fig. 3). Although the IgE antibody increase was generally lower in group BV139-S, no marked differences could be found between the non-treated, sensitized (S) and peptide-treated groups (BV139-S, S-BV139; Fig. 3). The same was true for other isotypes and subclasses of antibodies investigated (IgG2a, IgG2b, IgG3, IgM; Fig. 4). A trend to higher IgG1 levels in the sensitized group could be observed. No specific antibody production was seen in group N.

Effect of peptide treatment on skin test reactivity

Skin test reactivity to rBet v 1 was reduced in pretreated, sensitized mice (group BV139-S) compared with sensitized, but untreated animals (group S), but the difference did not reach the level of statistical significance ($P < 0.12$). No differences in skin test reactivity to rBet v 1 were observed between group S-BV139

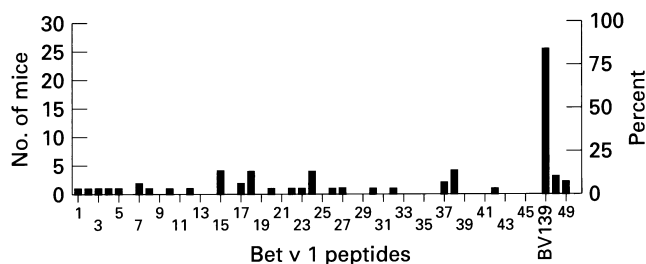


Fig. 2. BV139 is the dominant T cell epitope of Bet v 1 in BALB/c mice. Splenic mononuclear cells (MNC) of 30 allergic BALB/c mice were tested in proliferation assays with 50 overlapping peptides spanning the whole Bet v 1 amino acid sequence (stimulation indices >4 were considered positive).

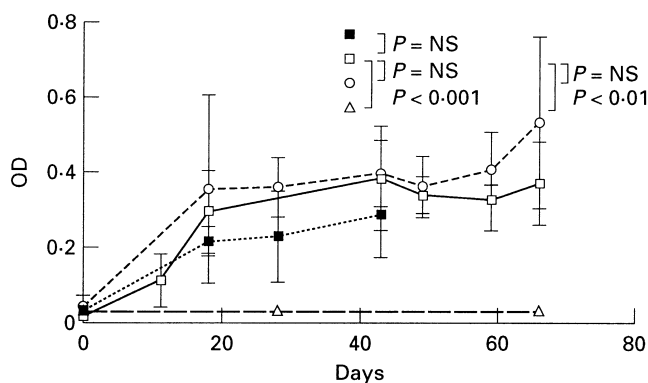


Fig. 3. No significant difference between the sensitized and treated mouse groups in terms of IgE antibody production. Bet v 1-specific/IgE-ELISA: comparison of IgE antibody levels (values represent the arithmetic mean $\pm$ s.e.m. of one group) of four groups of mice (S (□), BV139-S (■), S-BV139 (○), N (Δ)). OD, Optical density.

and group S. No positive skin test reactivity to rBet v 1 was seen in group N. Twenty-three of 25 mice reacted to the mast cell degranulating compound 48/80; in two mice positive controls were negative, and in these cases results were not interpretable. No reactivity was observed to PBS, the negative control. Results from intradermal skin testing are summarized in Table 1.

Proliferation of splenic MNC from sensitized and treated (BV139-S, S-BV139) mice

Ten days after the last immunization, splenic MNC were collected and investigated with rBet v 1, BV139, and a control peptide of Bet v 1 KIVATPDGGSIL (referred to as BV103) in proliferation assays. Proliferation to the dominant epitope (BV139) was reduced to levels of the irrelevant peptides, i.e. responses to BV139 were markedly reduced (Fig. 5). In addition, in group S-BV139, proliferation to the whole Bet v 1 molecule was decreased to the same degree. This trend was also found in group BV139-S. No proliferation was detected in group N (Fig. 5).

Effect of peptide treatment on IL-4 and IFN- γ production

Ten days after the last immunization, splenic MNC were prepared,

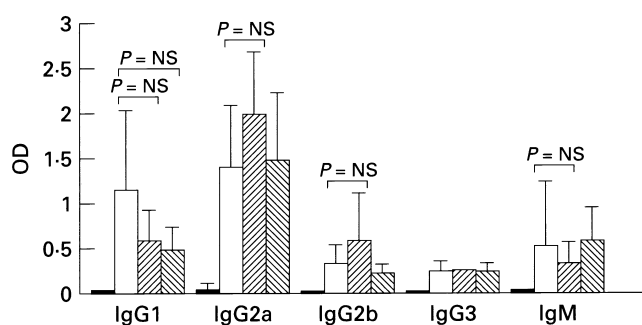


Fig. 4. No difference between sensitized and treated mouse groups in terms of IgG subclass and IgM antibody production. Bet v 1-specific/IgG subclass, IgM-ELISA: serum samples were taken 10 days after the last immunization. Comparison of IgG subclass and IgM antibodies. Each bar represents the arithmetic mean $\pm$ s.e.m. of the OD values of three groups of mice (S (□), BV139-S (▨), S-BV139 (▩)). ■, Control. OD, Optical density.

Table 1. Effects of peptide treatment as determined by intradermal skin testing

Mice	PBS			5 ng rBet v 1			50 ng rBet v 1		
	–	+	++	–	+	++	–	+	++
S	5	0	0	0	2	3	0	1	4
BV139-S	7	0	0	4	2	1	2	4	1
S-BV139	7	0	0	0	4	3	0	3	4
N	4	0	0	4	0	0	4	0	0

Skin testing was performed 10 days after the last immunizations. Test solutions were injected intradermally. PBS, Negative control. ++, Positive reactions as strong as or stronger than positive control; +, positive reactions which are weaker than positive control; –, negative skin reaction. In two mice, positive controls (compound 48/80) were negative and these mice are not listed.

incubated with rBet v 1 for 48 h. Thereafter, the concentration of IL-4 and IFN- γ in the culture SN was measured. IFN- γ was detectable in the sensitized (group S) and treated groups (BV139-S, S-BV139) of mice, whereas IL-4 production was found in group S, exclusively (Table 2). According to their cytokine secretion pattern, the immune response of the mice in group S could be classified as Th0-like, whereas the administration of high doses of peptide BV139 (groups BV139-S and S-BV139) induced a Th1-like cytokine pattern. Neither IL-4 nor IFN- γ could be measured in the culture SN of mice in group N. Results are presented in Table 2.

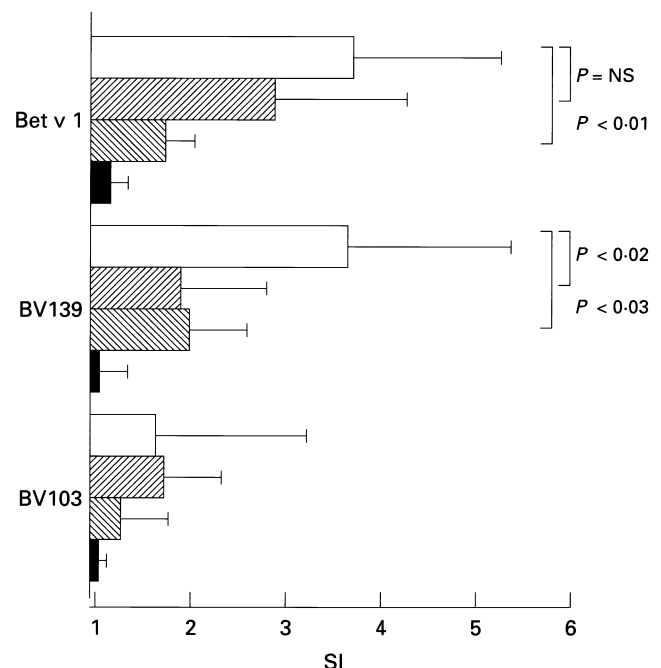


Fig. 5. Effect of peptide treatment on T cell proliferation to peptides BV103 and BV139, and Bet v 1 of four groups of mice (S (□), BV139-S (▨), S-BV139 (▩), C (■)). Each bar represents the arithmetic mean $\pm$ s.e.m. of SI of one group of mice.

Table 2. Effect of peptide treatment on the production of IL-4 and IFN- γ in four groups of mice (S, BV139-S, S-BV139, N)

Group	IL-4		IFN- γ	
	IL-4-containing supernatants	pg/ml	IFN- γ -containing supernatants	pg/ml
S	2/5	8–91	3/5	25–389
BV139-S*	0/3	<5	1/3	30
S-BV139	0/8	<5	6/8	25–72
N	0/4	<5	0/4	<25

Splenic cells were cultured with rBet v 1. The supernatants were harvested after 48 h and cytokine levels determined by ELISA. *Spleen mononuclear cells (MNC) from eight BV139-S treated mice were collected in three pools (3–3–2).

DISCUSSION

Several *in vitro* and *in vivo* models of tolerance induction using T cell epitopes have been established in antigen-specific systems [19,20,33,34]. In this study, we were able to tolerize T cells and reduce skin test reactivity in a mouse model of an IgE-mediated allergic immune response. We identified the peptide BV139 as the immunodominant epitope of Bet v 1 in BALB/c mice (Fig. 2). Interestingly, this peptide was also described as an important T cell epitope in patients suffering from birch pollen allergy [21,27]. By using two different regimens, we were able to modulate (i) a naive and (ii) an ongoing immune response to rBet v 1 in BALB/c mice by administration of high doses of the dominant T cell epitope BV139. In one approach, the peptide was administered in naive mice before immunization, in the second, already sensitized mice were treated (Fig. 1).

Treatment of naive mice with high doses of peptide BV139 followed by immunization (group BV139-S), markedly impaired T cell proliferation to the administered peptide (Fig. 5). This is consistent with the 'two-signal model' of T cell activation, suggesting that in addition to ligation of the T cell receptor (TCR) by antigenic peptide MHC complexes, costimulatory signals, such as CD28 or CTLA-4 on T cells and CD80 or CD86 on APC, are a prerequisite for T cell activation and prevent anergy induction [15–18]. It can be assumed that peptides, when administered in high doses, also target non-professional APC, which lack costimulatory signals, and can thereby induce T cell anergy. Administration of the immunodominant peptide in naive mice followed by immunization strongly reduced skin test reactivity to the whole rBet v 1 molecule (Table 1). However, IgE levels were not significantly affected (Fig. 3). Therefore, the reduction in skin test reactivity might be explained by the lack of priming, growth, and activating signals (histamine-releasing factors (HRF), IL-3, IL-4, IL-9) delivered from T cells, i.e. a reduced histamine releasability rather than by the lack of mast cell-bound IgE [35,36].

Treatment with the dominant peptide in mice with an ongoing immune response (group S-BV139), markedly reduced T cell reactivity to *in vitro* challenge with the peptide as well as with the whole rBet v 1 molecule (Fig. 5). This remarkable finding of tolerance to the whole Bet v 1 molecule induced by a single peptide confirms similar observations in experimental autoimmune encephalomyelitis, house dust mite or cat allergen models [11,19,20]. It

has been postulated that tolerized CD4⁺ T cells or an induced subset of regulatory T cells mediate cross-regulation of T cells directed to other epitopes through inhibitory cytokines [37]. In this group of mice, peptide application did not influence skin test reactivity to Bet v 1 or specific IgE production (Table 1, Fig. 3). Presumably, sufficient numbers of already primed mast cells were present at the time of peptide treatment.

Taken together, two major differences could be observed after peptide treatment between the immune reactivity of naive and specifically preimmunized mice: (i) skin test reactivity to rBet v 1 was only reduced in the pretreated animals (group BV139-S). Although T cell reactivity was impaired in the preimmunized mice (group S-BV139), one series of peptide administration might not be enough to abrogate skin test reactivity in mice with an ongoing immune response which possess sufficient numbers of primed mast cells. However, it can be speculated that—as in patients under SIT—it takes several injections over a period of many weeks until the first reductions in skin test reactivity can be seen; (ii) the group of mice with an ongoing immune response, which received just one allergen challenge after peptide treatment, developed a state of cellular non-responsiveness both to the peptide and to the whole Bet v 1 molecule. In contrast, tolerance to Bet v 1 could be overcome by several cycles of immunization in the pretreated group of mice. Therefore, there is a need for studies as to whether this cross-regulatory effect of tolerance induction in T cells would be sustained by several series of peptide treatments.

Interestingly, none of our treatment regimens led to substantial changes in antibody levels of any isotype (Fig. 4). Only small differences could be observed between the treated and non-treated animals in IgG1 antibody levels. These findings depart from recently published results [19,20], which display a concomitant decrease of specific IgG antibodies with a significantly impaired specific T cell reactivity. Several lines of evidence for an altered cytokine profile in patients under SIT with a predominance of IFN- γ (or reduction of IL-4) have been provided by a number of groups [7–9]. O'Hehir *et al.* demonstrated that exposure of T cell clones to supraoptimal concentrations of specific peptide followed by rechallenge led to undetectable levels of IL-4 release, while IFN- γ production was not inhibited under this condition [10]. In this study, the trend of deviation from a Th0-like to a Th1-like cytokine secretion pattern was observed in both groups of mice which received peptide treatment.

The ability to influence T cell and skin test reactivity to Bet v 1 in an animal model of type I allergy by subcutaneous injections of the immunodominant epitope was shown. Therapeutical approaches based on these findings could lead to a treatment of type I hypersensitivities which would target allergen-specific T lymphocytes, without the potential risk of cross-linking IgE antibodies on mast cells.

ACKNOWLEDGMENTS

This work was supported by Grant S 06704-MED of the 'Fonds zur Förderung der Wissenschaftlichen Forschung', Austria. H.R. is supported by the 'Deutsche Forschungsgemeinschaft (Re 737/4–3)' and A.D. by the Frauenförderung of the Freie Universität Berlin.

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