

Responses of human birch pollen allergen-reactive T cells to chemically modified allergens (allergoids)

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Summary

Background Allergoids are widely used in specific immunotherapy for the treatment of IgE-mediated allergic diseases.

Objective The aim of this study was to analyse whether a modification of birch pollen allergens with formaldehyde affects the availability of T-cell epitopes.

Methods Efficient modification of the allergens was verified by determining IgE and IgG binding activity using ELISA inhibition tests. T-cell responses to birch pollen allergoids were analysed in polyclonal systems, using peripheral blood mononuclear cells (PBMC) of five birch pollen-allergic individuals, as well as birch pollen extract-reactive T-cell lines (TCL), established from the peripheral blood of 14 birch pollen-allergic donors. To determine whether the modification of natural (n)Bet v 1 with formaldehyde or maleic anhydride results in epitope-specific changes in T-cell reactivities, 22 Bet v 1-specific T-cell clones (TCC), established from nine additional birch pollen-allergic individuals, were tested for their reactivity with these products.

Results The majority of PBMC and TCL showed a reduced response to the birch pollen extract allergoid. Bet v 1-specific TCC could be divided into allergoid-reactive and -non-reactive TCC. No simple correlation between possible modification sites of formaldehyde in the respective T-cell epitopes and the stimulatory potential of the allergoid was observed. Mechanisms of suppression or of anergy induction were excluded as an explanation for the non-reactivity of representative TCC. All TCC could be stimulated by maleylated and unmodified nBet v 1 to a similar extent.

Conclusion These results demonstrate differences in the availability of T-cell epitopes between allergoids and unmodified allergens, which are most likely due to structural changes within the allergen molecule.

Keywords: allergoid, maleylated allergen, T-cell reactivity, T-cell epitope, birch pollen

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Introduction

The clinical efficacy of specific immunotherapy (SIT) for the treatment of IgE-mediated allergic disease using allergen or allergoid preparations is well known and has been verified by double-blind clinical trials [1]. Whether the mechanisms of action are the same in both cases is not

fully understood. In a murine model, allergoid obtained by glutaraldehyde polymerization of ovalbumin inhibited the induction of IgE responses [2] and abrogated ongoing IgE synthesis [3]. Furthermore, *in vivo* administration of glutaraldehyde-modified ovalbumin lead to preferential induction of TH₁-like responses [4]. In clinical studies ragweed formallergoids, representing formaldehyde-modified ragweed extracts, induced significant IgG antibody responses in ragweed-allergic patients [5,6]. IgG responses after formallergoid therapy were directed towards ragweed

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major allergens such as Amb a 1 and minor allergens such as Amb a 3, Amb a 5 and Amb a 6 [7].

The response of human allergen-specific T cells, which are the major target cell population in immunotherapy due to their ability to support antibody production by B cells, to modified allergens has not been analysed. We have used formaldehyde-modified birch pollen extract (FA-BP-extract) or nBet v 1 (FA-nBet v 1) in an *in vitro* model system. Formaldehyde reacts primarily with ϵ -amino groups of lysine residues, which can then interact with side chains of arginine and tyrosine, leading to intermolecular and intramolecular cross-linking of the allergen molecules [7–10]. In this study, we have compared the T-cell stimulating potency of formaldehyde-modified and native birch pollen allergens for peripheral blood mononuclear cells (PBMC), allergen-specific T-cell lines (TCL) and T-cell clones (TCC). To evaluate the effects on T-cell reactivity of pure lysine modification and of allergen cross-linking as a result of formaldehyde modification, a control maleylated nBet v 1 allergen (MA-nBet v 1), known to modify lysine residues without cross-linking, was used.

Materials and methods

Antigens

Birch pollen extract (BP-extract), natural (n)Bet v 1, purified by affinity chromatography and HPLC, formaldehyde-modified BP-extract (FA-BP-extract), formaldehyde-modified nBet v 1 (FA-nBet v 1) and nBet v 1 modified by treatment with maleic anhydride (MA-nBet v 1) [11] were kindly supplied by Dr H. Fiebig, Allergopharma, Reinbek, Germany. In FA-nBet v 1, 75% free ϵ -amino groups and in MA-nBet v 1, 50% free ϵ -amino groups were blocked as determined by TNBS [12]. Recombinant (r) Bet v 1a was prepared as described [13].

ELISA inhibition experiments

Microtitre wells (Maxisorp, Nunc, Roskilde, Denmark) were coated with rBet v 1a ($2 \mu\text{g/mL}$ in 0.1 mol/L sodium carbonate buffer, pH 9.6) for 6 h at room temperature. After washing twice with PBS/0.05% Tween 20, free binding sites were blocked with PBS/0.05% Tween 20/1% HSA overnight at 4°C . Thereafter, a serum pool of birch pollen-allergic individuals, which had been pre-incubated with serial dilutions of the modified allergens or the unmodified allergens as a control, was applied onto the rBet v 1a-coated wells and incubated for 4 h at room temperature. After washing five times, the wells were incubated with alkaline phosphatase-conjugated horse anti-human IgE antibodies (Allercoat EAST, Kallestad, Chaska, MN, USA), diluted 1:2 in PBS/0.05% Tween 20/0.5% HSA overnight at 4°C and developed with the substrate *para*-

nitrophenylphosphate, applied at a concentration of 1 mg/mL in diethanolamine buffer, pH 9.8. The reaction was stopped by adding 1 mol/L tribasic potassium phosphate. The optical density was measured at 405 nm, using the wavelength of 550 nm as reference value [14].

In the case of the IgG inhibition experiments, mouse anti-human IgG antibodies (Pharmingen, San Diego, CA, USA) diluted 1:1000 in PBS/0.05% Tween 20/0.5% HSA were used overnight at 4°C . Thereafter the wells were washed five times and incubated with peroxidase-conjugated sheep anti-mouse IgG (Amersham-Buchler) diluted 1:2000 in PBS/0.05% Tween 20/0.5% HSA for 2 h at 37°C and developed with the substrate ABTS. The reaction was stopped by adding 0.32% (w/v) NaF. The optical density was measured at 405 nm (with 490 nm as reference wavelength).

Generation of T-cell lines and T-cell clones

BP-extract-reactive T-cell lines were established from PBMC obtained from birch pollen allergic individuals, following the method described by Parronchi *et al.* [15] with slight modifications. PBMC were separated by density gradient centrifugation of blood samples on ficoll (density 1.077, Biochrom, Berlin, Germany) and resuspended in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 100 IU penicillin, $100 \mu\text{g/mL}$ streptomycin, $5 \times 10^{-5} \text{ M}$ 2-mercaptoethanol and 5% pooled, heat-inactivated AB⁺ serum (Blood Transfusion Service, Mainz and Bad Kreuznach, Germany) (complete AB-medium). PBMC ($5 \times 10^5/\text{mL/well}$) were stimulated with $20 \mu\text{g/mL}$ BP-extract in complete medium in 24-well culture plates (Costar, Cambridge, MA, USA) for 5 days. Subsequently, human rIL-2 (20 U/mL , a kind gift of Cetus, Emeryville, CA, USA) was added, and the cells were cultured for an additional 8–10 days.

Bet v 1-reactive TCC were established and characterized according to the methods described [14].

Proliferation assays

TCL ($2 \times 10^4/\text{well}$) were cultured in triplicates in 96-well, flat-bottom microtiter plates (Costar) and TCC ($2\text{--}4 \times 10^4/\text{well}$) were cultured in duplicates in 96-well, round-bottom microtitre plates (Nunc, Roskilde, Denmark) in the presence of irradiated (50 gray) autologous PBMC ($10^5/\text{well}$) and various allergen preparations as indicated for 48 h at 37°C in a 5% CO_2 humidified incubator. After pulsing for 18 h with $0.5 \mu\text{Ci/well}$ [^3H]thymidine (Amersham-Buchler, Amersham, Germany), cells were harvested and the radioactivity incorporated into cellular DNA was measured by liquid scintillation counting. PBMC ($1 \times 10^5/\text{well}$) were cultivated with the allergen preparations indicated in round-bottom microtitre plates for 6 days. [^3H]thymidine

(1 μ Ci/well) was present during the last 8 h of culture. The results are expressed as stimulation index (SI, ratio of cpm obtained in cultures with allergen and cpm obtained in cultures without allergen).

Test for cytokine production

TCC ($2-4 \times 10^4$) were cultivated with 1×10^5 irradiated (50 gray) autologous PBMC and with 5 μ g/mL nBet v 1 or FA-nBet v 1 or without allergen for 48 h. Cell-free

supernatants were analysed at a 1:2.5 dilution by ELISA for content of IL-5 (R & D Systems, Wiesbaden, Germany) and IFN γ (CLB, Amsterdam, The Netherlands) according to the manufacturer's protocol. The limit of detection was 39.06 pg/mL for IL-5 and 6.25 pg/mL for IFN γ .

Results

Verification of the efficiency of the formaldehyde modification procedure in reducing allergenicity of the BP-extract and nBet v 1

ELISA inhibition experiments revealed a drastic reduction in binding of Bet v 1a-reactive IgE and IgG antibodies to FA-BP-extract and FA-nBet v 1 (Fig. 1). In contrast, modification of nBet v 1 with maleic anhydride did not affect the IgE and IgG binding properties.

Polyclonal T-cell reactivity with FA-BP-extract

Heterogeneity in the reactivity of PBMC from birch pollen-allergic donors to FA-BP-extract as compared with BP-extract was observed (Fig. 2). While PBMC from donor DF were stimulated by FA-BP-extract more efficiently than by BP-extract, PBMC from donor DA did not respond to FA-BP-extract. PBMC from other donors tested reacted to FA-BP-extract to a variable extent. In further experiments, T-cell lines established in the presence of the unmodified BP-extract were tested for their capacity to respond to the FA-BP-extract (Fig. 3). The majority of 14 TCL tested proliferated more strongly in response to the BP-extract as compared with identical concentrations of the FA-BP-extract. Again, extensive heterogeneity in the reactivity of individual TCL to the FA-BP-extract was observed. Thus, TCL SC30.8 showed little or no reactivity to the FA-BP-extract, whereas TCL AA12.2 showed comparable reactivities to the unmodified and the modified BP-extract. To exclude the possibility that these differences were due to reduced or lacking B-cell presentation of the FA-BP-extract, CD19-depleted PBMC were used as antigen presenting cells (APC) for several of the TCL. In these experiments the stimulating capacity of the FA-BP-extract was comparable when unseparated or CD19-depleted PBMC were used as APC (data not shown).

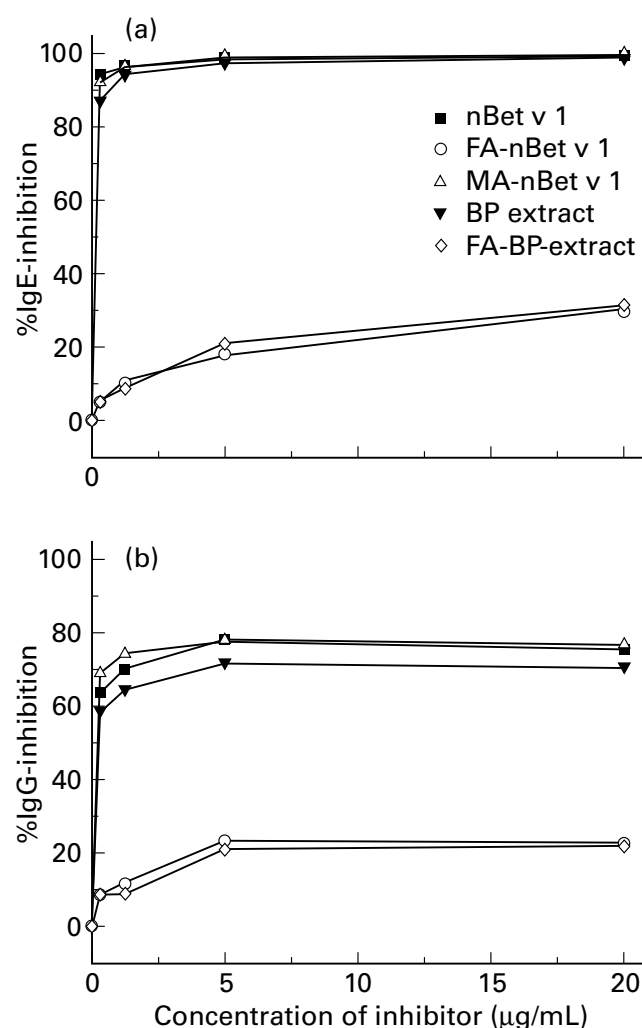


Fig. 1. Inhibition of IgE (a) and IgG (b) binding to purified rBet v 1a by chemically modified allergens. A 1:4 (a) or 1:50 (b) diluted serum pool from birch pollen-allergic patients was absorbed with serial dilutions of nBet v 1, FA-nBet v 1, MA-nBet v 1, BP extract or FA-BP-extract and subsequently tested in an ELISA assay for reactivity with rBet v 1a. The values of percentage of inhibition were calculated as following: $(1 - [\text{OD} \text{ in the presence of inhibitor}] / \text{OD in the absence of inhibitor}) \times 100$.

Responsiveness of Bet v 1-specific TCC to chemically modified allergens

To analyse whether distinct epitopes have been destroyed or altered by the chemical modification procedure, a panel of 22 epitope-mapped, Bet v 1-specific TCC, established from PBMC of nine birch pollen-allergic individuals, were tested

with respect to their proliferative response to the modified allergens. Eight out of 22 TCC proliferated in the presence of FA-nBet v 1 ($SI > 5$) while 14 TCC were unresponsive to FA-nBet v 1 stimulation (Figs 4 & 5). Not all FA-nBet v 1-non-reactive TCC have constraining lysin-residues in their T-cell epitopes, as seen for the TCC HPR77, HPR111 or RR4R which recognize Bet v 1a(142–153) (TLLRAVESYLLA), although potential secondary modification sites are present. Nevertheless, potential primary or secondary modification sites do not necessarily result in a loss of T-cell reactivity to FA-nBet v 1 as can be seen in the case of TCC SAZ53/III which recognizes Bet v 1a(27–42) (DNLFPKVAPQAISSE), SD25 which recognizes Bet v 1a(49–66) (GPGTIKKISFPEGFPFKY), MAH3 recognizing Bet v 1a(76–90) (HTNFKYNYSVIEGGP) or GZ24R recognizing Bet v 1a(118–129) (NKYHTKGDHEVK). Furthermore, TCC RR9 m and WD36/III which both recognize Bet v 1a(4–18) (NYETETTSVIPAARL) react differently to FA-nBet v 1. While TCC WD36/III shows a good response to FA-nBet v 1, no FA-nBet v 1 reactivity was observed for TCC RR9 m.

The TCC used express a TH_2/TH_0 phenotype. It was interesting to analyse whether stimulation with FA-nBet v 1 alters the pattern of cytokines produced by the TCC. All TCC tested produced lower levels of both IL-5 and IFN- γ when cultured in the presence of FA-nBet v 1 as compared with the nBet v 1.

Exclusion of mechanisms of suppression or anergy for the non-reactivity of representative TCC to FA-nBet v 1

To exclude mechanisms of suppression, T cells of clone WF24R or HPR111 were stimulated with rBet v 1a or with rBet v 1a in combination with FA-nBet v 1 in proliferation assays. As depicted in Fig. 6, the presence of FA-nBet v 1 did not result in the suppression of the proliferative response of these TCC to rBet v 1a.

To test whether the non-reactivity of TCC to FA-nBet v 1 was due to anergy induction, T cells of clone HPR77, which do not proliferate in response to FA-nBet v 1, were pre-treated with FA-nBet v 1 or medium in the presence of PBMC as antigen-presenting cells. Subsequently, the cells

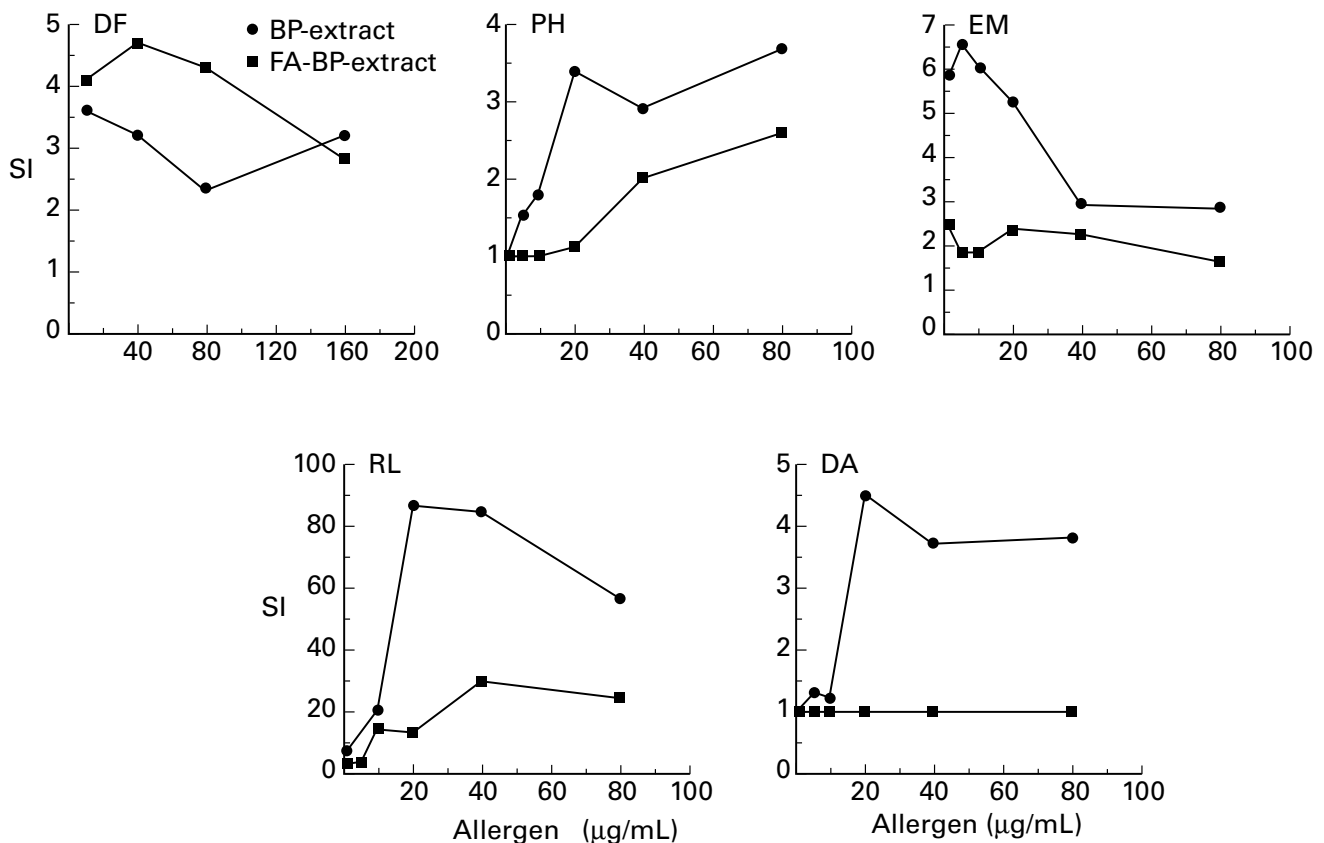


Fig. 2. Reactivity of PBMC to BP-extract and FA-BP-extract. PBMC (1×10^5) were cultivated in the presence of BP-extract or FA-BP-extract at the indicated concentrations or in the absence of allergen for 6 days. [^3H]thymidine ($1 \mu\text{Ci}$) was present during the last 8 h of culture.

were washed and stimulated with nBet v 1 or medium together with PBMC. Proliferation in response to nBet v 1 was not inhibited by pre-treatment of the T cells with FA-nBet v 1, indicating that anergy induction had not taken place (Fig. 7). Rechallenge with phytohaemagglutinin/IL-2 served as a control.

Discussion

In this study we have investigated the reactivity of PBMC, TCL and TCC derived from birch pollen-allergic patients to chemically modified birch pollen allergens. To verify that modification of birch pollen allergens with formaldehyde resulted in an altered protein structure, we first analysed the binding of specific antibodies to the modified allergens. As

expected, we observed a drastic reduction in IgE and IgG binding to formaldehyde-modified BP-extract and to formaldehyde-modified nBet v 1. In contrast, modification of nBet v 1 by treatment with maleic anhydride did not alter IgE and IgG binding. In a murine system, maleylation of diphtheria toxoid has been shown to disrupt native B-cell epitopes of diphtheria toxoid and to create new epitopes [16]. This finding suggests that maleylation has divergent effects on the structural properties of individual proteins and thus on the availability of specific B-cell epitopes.

With one exception, PBMC isolated from birch pollen-allergic donors proliferated to a lesser extent to the FA-BP-extract than to the BP-extract. Furthermore, T-cell lines, established in the presence of BP-extract, exhibited in most cases a reduced proliferative response to the FA-BP-extract,

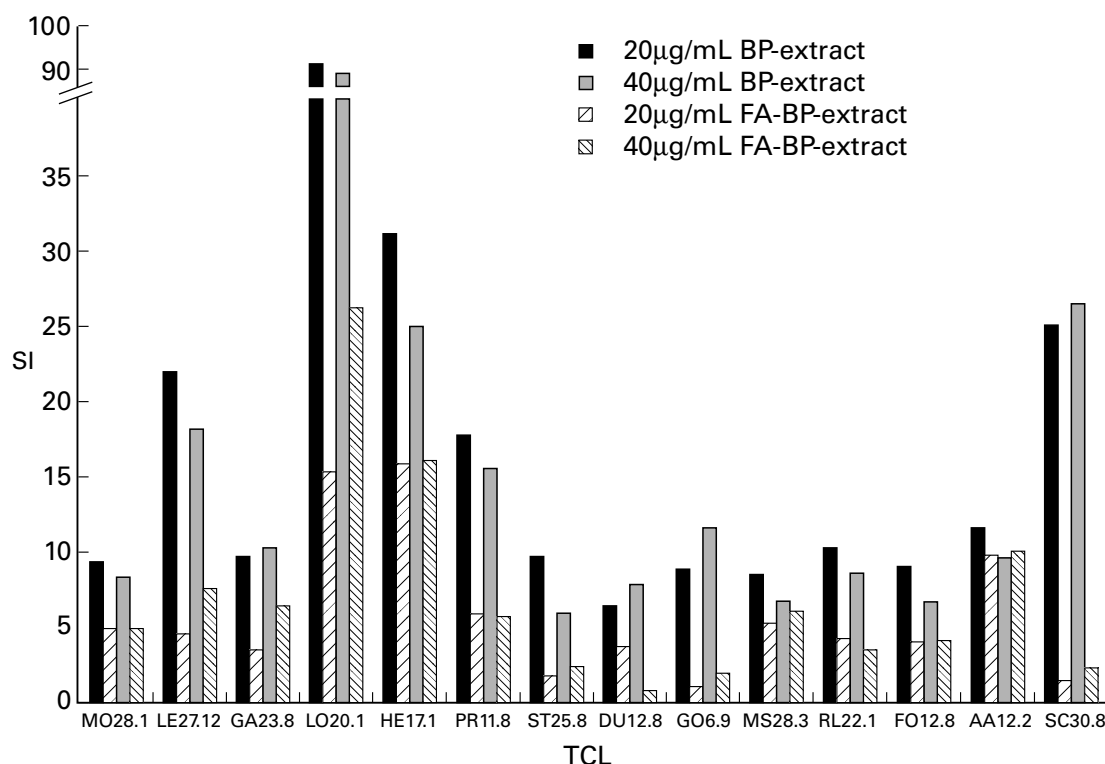


Fig. 3. Comparison of the proliferative response of BP-extract-reactive TCL to BP-extract and FA-BP-extract. TCL (2×10^4) co-cultured with 1×10^5 irradiated (50 gray) autologous PBMC and BP-extract (20 µg/mL or 40 µg/mL) or FA-BP-extract (20 µg/mL or 40 µg/mL) or medium as control for 66 h. [^3H]thymidine 90.5 µCi) was present during the last 16–18 h of culture.

Fig. 4. Proliferative responses of Bet v 1-specific T-cell clones to nBet v 1, FA-nBet v 1 and MA-nBet v 1. TCC ($2\text{--}4 \times 10^4$) were cocultured for 66 h with 1×10^5 irradiated (50 gray) autologous PBMC and with medium or with nBet v 1 (—▲—) FA-nBet v 1 (—□—) or MA-nBet v 1 (—○—) at the concentrations indicated. [^3H]thymidine was present during the last 18 h of culture.

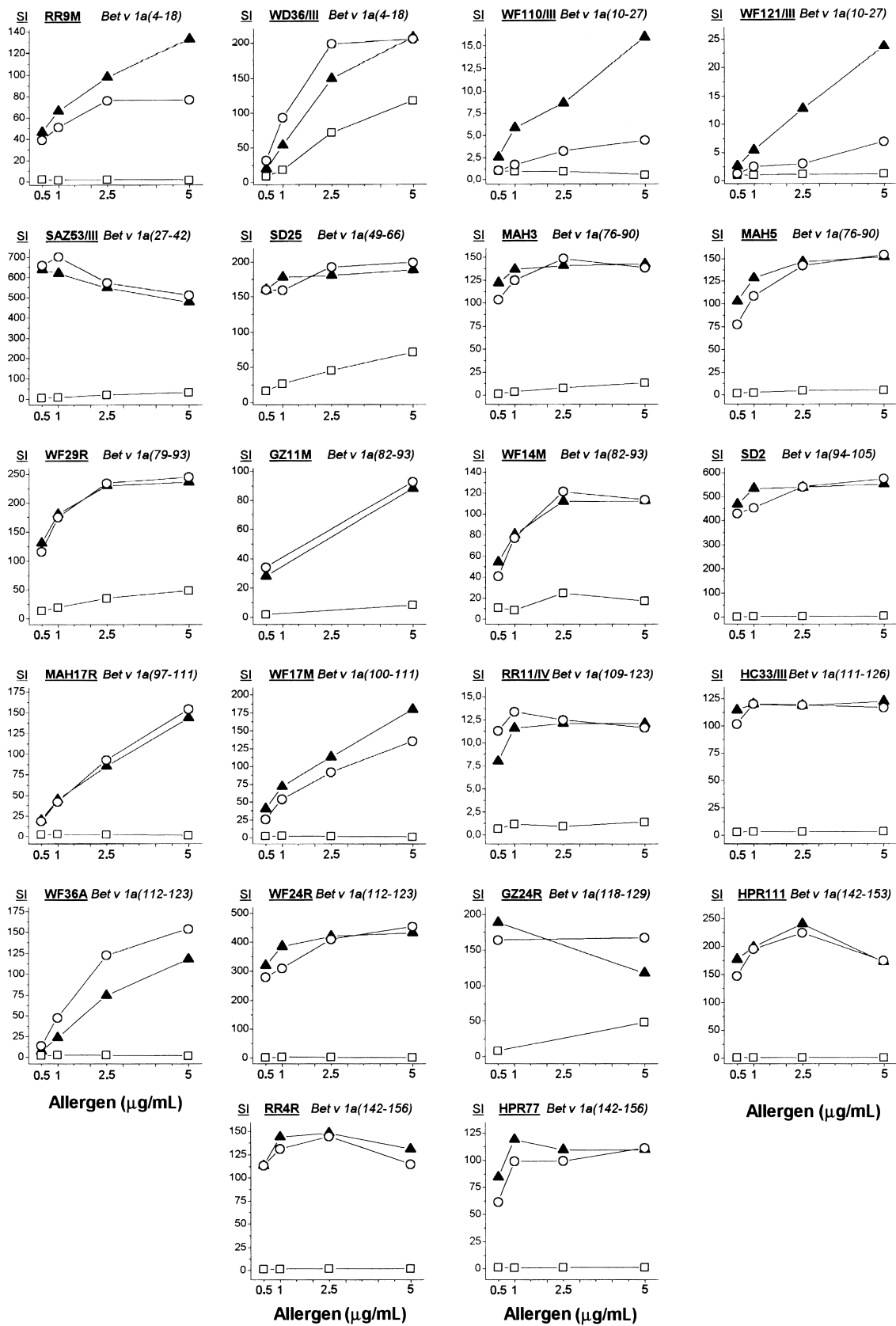




Fig. 5. Epitope specificity of the FA-nBet v 1-reactive and -non-reactive T-cell clones used. The positions of the epitopes, recognized by individual TCC, are indicated by bars underneath the Bet v 1a amino acid sequence. Potential primary modification sites (lysine residues) are indicated by an asterisk and potential secondary modification sites (arginine and tyrosine residues) are indicated by an arrow. ■, epitopes of TCC reactive solely with native nBet v1; ▨, epitopes of TCC reactive with both nBet v 1 and FA-nBet v1.

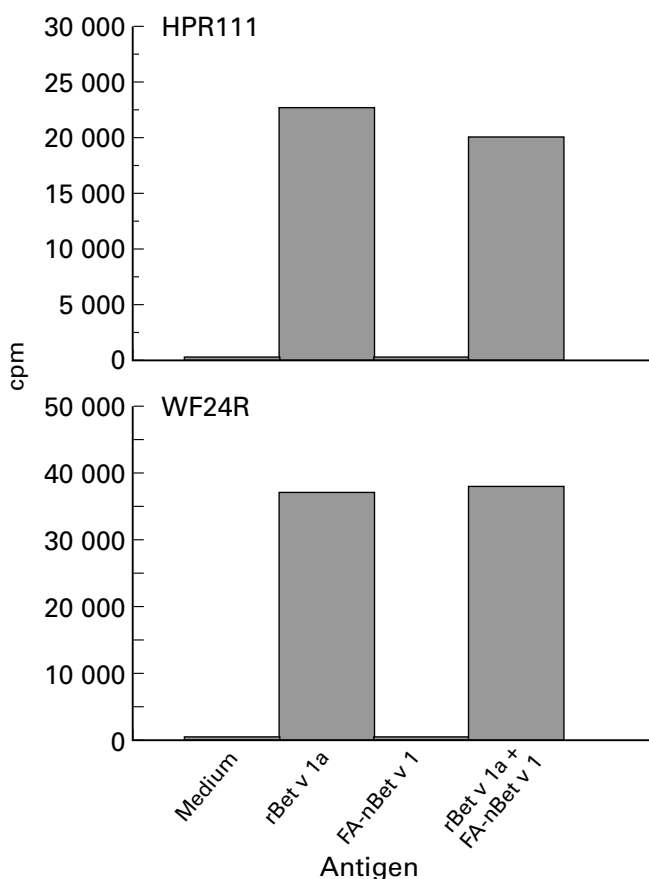


Fig. 6. FA-nBet v 1 does not suppress rBet v 1a-induced proliferation of FA-nBet v 1-non-reactive TCC. FA-nBet v 1-non-reactive cells of TCC HPR111 (3×10^4) or WF24R (4×10^4) were cultured in the presence of 1×10^5 irradiated (50 gray) autologous PBMC and antigen as indicated ($5 \mu\text{g/mL}$) for 66 h. [^3H]thymidine 0.5 (μCi) was present during the last 18 h of culture.

although TCL exist (TCL AA12.2, Fig. 4) which showed comparable responses to the FA-BP-extract and to the unmodified BP-extract. The inter-individual differences in reactivity of the PBMC and TCL to the FA-BP-extract are probably due to the different HLA-background of the donors and/or to different epitope specificities of the T cells.

In accordance with the reduced proliferative responses of PBMC and TCL towards FA-BP-extract, only eight out of 22 Bet v 1-specific TCC tested (36%) proliferated in the presence of FA-nBet v 1 ($\text{SI} > 5$). These responses were considerably weaker compared with responses to nBet v 1. The remaining 14 TCC were not stimulated by FA-nBet v 1. In contrast, the stimulatory capacity of MA-nBet v 1 was similar to that of nBet v 1, indicating that modification of ϵ -amino groups of lysine residues *per se* is not responsible for the loss of reactivity of TCC to FA-nBet v 1. The lack of reactivity of the representative TCC tested to FA-nBet v 1 was not due to anergy induction or suppressive effects by the modified allergen. We cannot discriminate whether formaldehyde modification of nBet v 1 results in a reduced number of available epitopes or in intrinsic changes in epitopes, which might alter signalling via the T-cell receptor. In both cases a reduced T-cell response would ensue. Only 75% free ϵ -amino groups had been blocked by formaldehyde treatment in the preparation of FA-nBet v 1 used. One would thus expect an efficient stimulatory potential of FA-nBet v 1 for TCC at a higher concentration. However, this was not the case for some of the TCC tested. One explanation for this finding is, that peptide generation for loading of MHC class II molecules might be affected by formaldehyde modification of nBet v 1. Possibly the unfolding of the antigen during the generation of class II-presentable peptides is not as effective when structural changes of the antigen, induced by inter- or intramolecular cross-linking, have occurred. Cross-linking of nBet v 1 may also

affect enzyme action during the intracellular degradation processes or the further trimming of extruding parts of large degradation fragments when bound to MHC class II molecules. In addition, the binding to class II molecules may be influenced by structural constraints on the unfolded,

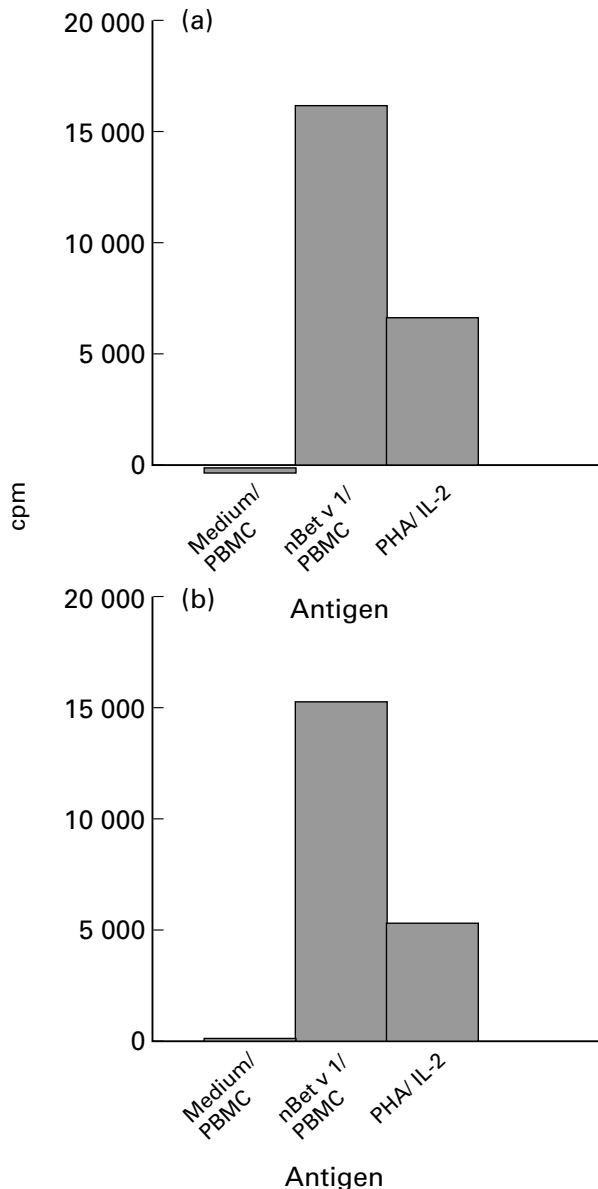


Fig. 7. Pre-incubation of T cells of the FA-n Bet v 1-unresponsive clone HPR77 with FA-nBet v 1 in the presence of APC does not induce anergy. T cells ($1 \times 10^5/\text{mL}$) were incubated with irradiated (50 gray) PBMC ($5 \times 10^5/\text{mL}$) as APC in the presence of (a) medium as a control or (b) $5 \mu\text{g}/\text{mL}$ FA-nBet v 1 for 72 h in 96-well round-bottom plates. After pre-treatment, cells were washed and challenged in a proliferation assay with $5 \mu\text{g}/\text{mL}$ nBet v 1 and PBMC, medium and PBMC, or PHA (0.1% v/v) and rhIL-2 (20 U/mL).

Table 1. Cytokine production by TCC^a

TCC	IL-5 (pg/mL)		IFN γ (pg/mL)	
	nBet v 1	FA-nBet v 1	nBet v 1	FA-nBet v 1
WD36/III	12770	2418	36	ND
SD25	85980	30920	24	ND
WF29R	9190	1528	56	ND
GZ11M	56240	1105	14	ND
WF14M	5640	880	60	ND
WF36A	1107	ND	130	ND
GZ24R	81060	7274	334	19
HPR111	4493	ND	447	ND
RR4R	26500	ND	157	ND

^a TCC ($2-4 \times 10^4$) were co-cultured with 1×10^5 irradiated autologous PBMC and with $5 \mu\text{g}/\text{mL}$ nBet v 1 or FA-nBet v 1 or without allergen for 48 h. Cell-free supernatants were analysed for content of cytokines by ELISA. In supernatants of control cultures without allergen added IL-5 and IFN γ were below the limit of detection. ND, not detectable.

partially degraded protein fragments [17] which may differ between modified and unmodified allergens.

The HLA background of individual patients may influence T-cell reactivity to allergoids. TCC RR9 M and WD36/III which both recognize Bet v 1a(4–18) show differences in reactivity to FA-nBet v 1. In this context we cannot exclude slight differences in the minimal epitopes recognized by TCC RR9 M or WD36/III which may also be responsible for this conflicting finding.

The clinical efficacy of SIT using allergoid preparations has been established by double-blind, placebo-controlled clinical trials [1]. The mechanism of action underlying clinical improvement has not been analysed in detail. Evidence is accumulating that SIT employing unmodified allergen preparations results in a shift in the ratio of TH₁- to TH₂-type cytokine production towards production of TH₁ cytokines [18–21]. This may be achieved by shifting cytokine secretion of established allergen-specific TH₂/TH₀-type cells towards increased production of IFN γ , by inducing anergy in established TH₂-type cells, or by stimulating the differentiation of naive T cells into TH₁-type or TC₁-type T cells. A large proportion of the Bet v 1-specific TCC tested were inefficiently stimulated by FA-nBet v 1 or were not activated at all. Furthermore, levels of IFN γ production were not augmented upon stimulation of reactive TCC with allergoid (FA-BP-extract) as compared with unmodified allergen. These findings suggest that SIT employing formaldehyde-modified allergen does not act on the level of the allergen-specific TH₂/TH₀-type cells. It seems more likely that allergoids act by inducing

differentiation of allergoid-specific TH₁-type or TC₁-type cells. Nevertheless, divergent reactivities of the TCC and TCL indicate that some common T-cell epitopes exist within allergen and allergoid as well as additional epitopes unique to unmodified allergen. B cells, which take up the allergen and process it to peptides, are expected to present both types of epitope. Along this reasoning they should therefore interact with the allergen-induced TH₂-like cells as well as with the allergoid-induced TH₁ like cells. Using *in vitro* systems, the ratio of IL-4 to IFN γ was shown to determine the relative production of IgE and IgG₄ antibodies [22]. IL-4 is required for the switch to both IgE and IgG₄, while IFN γ inhibits the switch to IgE and promotes switching to IgG₄ [23–27]. The allergen-specific TH₂-like cells are envisaged to produce the IL-4 necessary for a switch of the B cell towards IgG₄ production. The allergoid-induced TH₁-like cells would produce IFN γ as the second mediator essential for switching of the B cell to production of IgG₄.

The strong T-cell stimulating capacity of MA-nBet v 1 in combination with the knowledge that maleylation of various proteins converts them into specific ligands for macrophage scavenger receptors [28,29] might be the basis of alternative approaches towards immunotherapies for type I allergic disorders. Nevertheless, further investigations should be carried out in order to address the question whether cytokine production by T cells can be modulated by these chemically modified allergens.

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