

Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens

Pål Johansen¹, Gabriela Senti¹, Julia Maria Martínez Gómez¹,
Brunello Wüthrich¹, Adrian Bot² and Thomas M. Kündig¹

¹ Unit for Experimental Immunotherapy, University Hospital of Zurich, Zurich, Switzerland

² MannKind Corporation, Valencia, USA

Allergen-specific immunotherapy (SIT) leads to a long-term amelioration of IgE- and Th2-mediated allergic diseases. However, SIT efficiency is low, with years of treatment along with frequent allergic side effects. The goal of this study was to reduce the side effects by destroying IgE-binding epitopes, *i.e.* by heat-denaturation, while preserving the therapeutic effect. Mice were immunised with bee venom, birch pollen, grass pollen or cat hair allergens, or with ovalbumin. Heat-denatured allergens bound less IgE but enhanced Th1-dependent IgG2a production as measured by ELISA. The strong IgG2a antibody responses also prevented allergic anaphylaxis in mice, as measured by body temperature drop after a challenge with a high allergen dose. We found that optimal heat-denaturation of allergens left a small proportion in the native conformation to sufficiently stimulate B cells, while non-B cell-mediated effects were probably amplified. The enhanced immunogenicity of heat-denatured allergens is likely explained by enhanced antigen presentation to T cells due to the particulate nature of heat-denatured proteins. This enables Th1 skewing of the immune response with strong production of IgG2a in mice. Therefore, heat-denaturation represents probably the simplest way to enhance the efficiency of SIT while reducing its side effects.

Received 25/5/05

Revised 19/7/05

Accepted 4/10/05

[DOI 10.1002/eji.200535076]

Key words:

Antibody isotypes

· Hymenoptera allergy

· Immunotherapy

· Th1/Th2

· Vaccination

Introduction

IgE-mediated type I hypersensitivity is characterised by prominent activity of mast cells, eosinophils, T helper type 2 (Th2) cells and cytokine actions. Allergen-specific immunotherapy (SIT) leads to long-term amelioration of allergic symptoms and is therefore recommended as a first-line therapy for allergies [1]. During SIT, gradually increasing allergen doses are subcutaneously administered until a maintenance dose

is reached, and then treatment continues for 3–5 years. Such lengthy treatment, requiring 30–80 allergen injections, is very costly [2]. Also, SIT frequently causes adverse events due to the interaction of specific IgE with the injected allergen. These adverse events frequently include a local reaction at the site of allergen injection, but also life-threatening systemic reactions such as asthma and anaphylaxis. This is why the administered allergen doses must be kept relatively low, which unfortunately favours Th2 immune responses in contrast to higher doses [3–5].

Several strategies have been developed to tackle the problems of SIT, *i.e.* to reduce its side effects and to enhance its immunogenicity. One strategy is to replace the currently used aluminium salts, which have Th2-polarizing properties, with novel adjuvants such as Toll-like receptor ligands [6, 7] that trigger Th1 responses. Another strategy is to increase safety and tolerability of SIT by injecting the allergen in a form that is not recognised by IgE, such as naked DNA vaccines

Correspondence: Pål Johansen, Unit for Experimental Immunotherapy, University Hospital of Zurich, Gloriastrasse 31, CH-8091 Zurich, Switzerland

Fax: +41-44-2554418

e-mail: pal.johansen@usz.ch

Abbreviations: **ABTS:** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt · **BV:** bee venom ·

HRP: horseradish peroxidase · **PLA₂:** phospholipase A₂ ·

SIT: allergen-specific immunotherapy

expressing the allergen [8]. Recombinant DNA technology has also allowed the production of mutated allergens with low IgE-binding capacity [9–14], such that higher therapeutic doses can be used [15, 16]. For the same reasons, short, synthetic peptide sequences corresponding to T cell epitopes from the allergen have been used for SIT. This approach was able to modify surrogate markers of allergy including cutaneous responses to allergen challenge and *ex vivo* parameters of T cell activation [17–19]. Finally, chemical cross-linking of allergens by use of bi-functional aldehydes to produce so-called allergoids [20] has been shown to reduce reactogenicity with IgE while yet improving antigen processing, Th1 cytokine secretion, and efficacy [21–23].

Heat-denaturation is well known to reduce the reactogenicity of allergens with IgE, as cooked foods are usually well or better tolerated by allergic individuals [24–26]. In this study, we tested whether this very simple strategy to reduce the reactogenicity of allergens might be useful for SIT, by investigating the effect of heat-denaturation on reactogenicity *versus* immunogenicity of several frequently used allergens. Heat-denatured allergen extracts of bee venom (BV), cat hair, grass pollen and birch pollen were studied in mice for their stimulation of specific immune responses. Expectedly, heat-denatured allergens showed reduced reactogenicity with IgE. Surprisingly, heat-denaturation of allergens markedly enhanced Th1-type immune responses characterised by antibody production of the IgG2a subclass. In allergic mice, the strong antibody responses induced by heat-denatured allergens were

protective against anaphylaxis upon challenge with the native allergen. Moreover, denatured allergens had little IgE-binding capacity but were recognised by human IgG4 equally well as native allergens, demonstrating a strong therapeutic potential of heat-denatured allergens also in humans.

Results

Heat-denaturation reduces the sensitising potential of allergens

The effect of heat-denaturation on the sensitisation potential of an allergen was evaluated in CBA mice sensitised by intraperitoneal injections, a route that predominantly induces IgE. Heat-denatured BV extract was prepared by heating a 0.1 mg/mL aqueous solution on a thermoblock at 80°C for 2 h. As illustrated in Fig. 1A, heating reduced the capability of BV in aluminium hydroxide to stimulate IgE production ($p < 0.05$ for dilutions 1 : 20 through 1 : 80) but increased IgG production. Interestingly, while heat-denaturation of BV enhanced production of IgG1 ($p < 0.05$ for dilutions 1 : 80 through 1 : 640), a subclass that is dependent on Th2, serological conversion of the Th1-dependent IgG2a was only observed in mice treated with the heat-denatured BV; IgG2a is rarely secreted upon low-dose intraperitoneal immunisation. Blood samples drawn at earlier time points than the illustrated 10 wk showed similar results (data not shown).

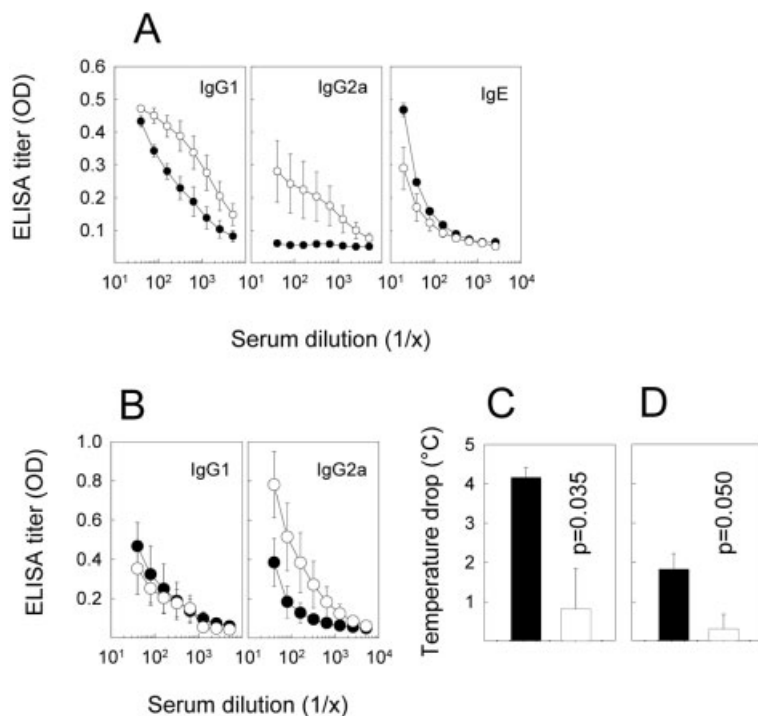


Figure 1. Heat-denaturation reduces the sensitising potential of allergens. Mice ($n = 3$) were sensitised by seven weekly intraperitoneal injections with 0.3 μ g native (filled symbols and bars) or heat-denatured (open symbols and bars) BV (A, C) or ovalbumin (B, D) in $Al(OH)_3$. Anti-PLA₂ antibody isotypes were measured by ELISA. Representative results from two experiments show titration curves of sera prepared 10 wk after the priming dose. At 12 wk after priming and 6 wk after the last injection, mice were challenged with 30 μ g PLA₂ (C) or 300 μ g ovalbumin (D) intraperitoneally, and anaphylaxis was measured as temperature drop after 30 min.

To analyse whether the effect of heat-denaturation on IgG production was a particular characteristic of BV, we treated CBA mice with weekly injections of 0.3 µg ovalbumin intraperitoneally. Heat-denaturation (80°C, 2 h) improved IgG2a production, although less significantly than for BV, while only non-significant changes in the IgG1 production were observed (Fig. 1B).

A common method for testing allergy in mice is to induce anaphylaxis by means of high doses of systemically administered allergens. We therefore injected the mice described in Fig. 1A, B with 30 or 300 µg phospholipase A₂ (PLA₂) or ovalbumin, respectively, 6 wk after the last sensitisation injection. After 30 min, we analysed anaphylaxis as changes in rectal temperature. Mice sensitised with native BV (Fig. 1C) or ovalbumin (Fig. 1D) had a profound drop in body temperature. In contrast, mice that were originally sensitised with the heat-denatured formulation had developed less allergy and, consequently, showed lesser changes in body temperature ($p = 0.035$ for BV and $p = 0.050$ for ovalbumin).

Heat-denaturation triggers Th1-associated IgG2a isotype production

To further test the IgG-stimulating capacity of heat-denatured allergens in a vaccination approach, we immunised CBA mice by four semi-weekly administrations of BV directly into the inguinal lymph nodes (Fig. 2). The BV was prepared by heating a 0.1 mg/mL aqueous solution at 60°C or 80°C for 1 or 2 h, respectively. In contrast to intraperitoneal injections (Fig. 1), native BV with aluminium hydroxide was only weakly immunogenic under the described experimental conditions and only IgG1 was induced. Heat-denaturation at 60°C for 1 h insignificantly improved IgG1 induction. However, more excessive denaturation at 80°C for 2 h had a highly significant effect on the immunogenicity ($p < 0.05$). High titres of IgG1 were obtained, and again most surprisingly, very high titres of the Th1-dependent isotype IgG2a were secreted. None of the preparations induced detectable IgE antibodies against PLA₂, as typically found for the intralymphatic route of administration (unpublished observations). Blood samples drawn at time points earlier than the illustrated 14 wk showed similar results (data not shown).

The effect of heat-denaturation was further demonstrated with other allergens, namely cat hair, grass pollen and birch pollen allergen extracts (Fig. 3). After heat-denaturation at 80°C for 2 h, also these allergens gained immunogenicity in terms of IgG2a secretion. With the exception of birch pollen, which showed increased IgG1 immunogenicity after heating, the grass pollen and cat dander gained no IgG1 benefit by heat-denaturation.

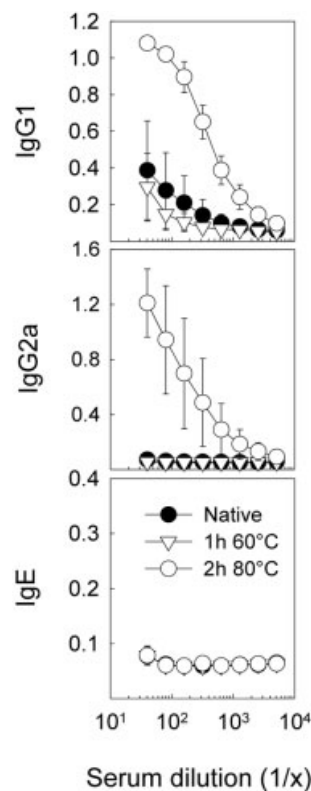


Figure 2. Heat-denaturation triggers Th1-associated IgG2a isotype production. Mice were immunised four times semi-weekly with 0.2 µg native or heat-denatured BV in Al(OH)₃ by intralymphatic injections. Anti-PLA₂ IgG1, IgG2a and IgE antibodies were measured by ELISA. Representative results from three experiments ($n = 3$ –4 mice) show titration curves of sera prepared 14 wk after the first dose.

A low amount of native allergen is required to achieve strong and specific IgG2a immune responses and prevents allergy in mice

To test whether an optimum condition for heat-denaturation existed or whether the IgG2a responses continuously increased as a function of temperature and time, we immunised mice intralymphatically as described above with 0.3 µg BV untreated or heated at 60, 80 or 99°C for 1, 2 and 20 h, respectively. The results again showed that heating at 60°C and especially 80°C increased the BV immunogenicity for IgG2a, but not notably for IgG1 (Fig. 4). However, treatment at 99°C and for 20 h fully abolished BV immunogenicity. This could be partly (IgG1) or fully (IgG2a) restored by the addition of a minute amount of native BV (0.03 µg). Most interestingly, when mice were first sensitised with native BV and then de-sensitised with differentially heat-denatured BV, a subsequent challenge with a high but not lethal dose of PLA₂ caused anaphylaxis in mice treated with the fully destroyed BV. In contrast, the addition of a minute amount of native BV prevented anaphylaxis (Fig. 5). The addition of 0.03 µg native BV

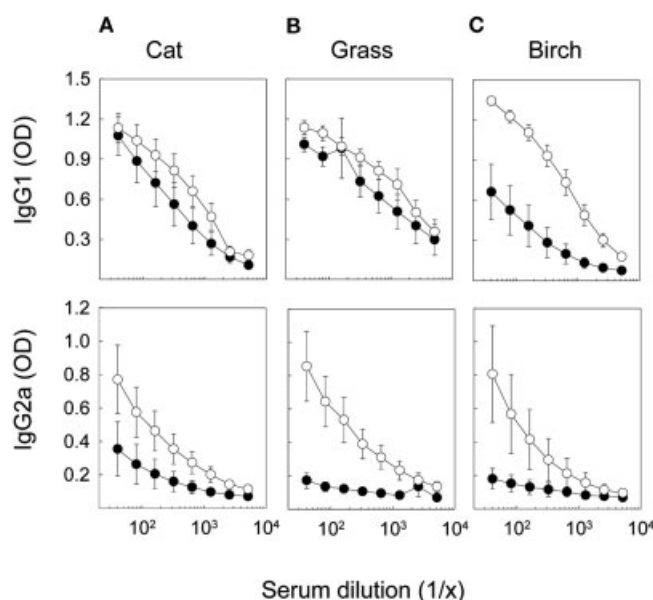


Figure 3. The vaccination potential of heat-denatured allergens is applicable to a wide range of allergens. Mice were immunised four times semi-weekly by intralymphatic injections of native (filled symbols) or heat-denatured (2 h, 80°C; open symbols) cat hair extract (A), grass pollen allergen extract (B) or birch pollen allergen extract (C) in $\text{Al}(\text{OH})_3$. IgG1 and IgG2a antibodies were measured by ELISA 12 wk after the priming dose using the allergen extract as coating allergen. Comparable results were obtained at earlier time points, and only non-significant levels of IgE were detected (not shown).

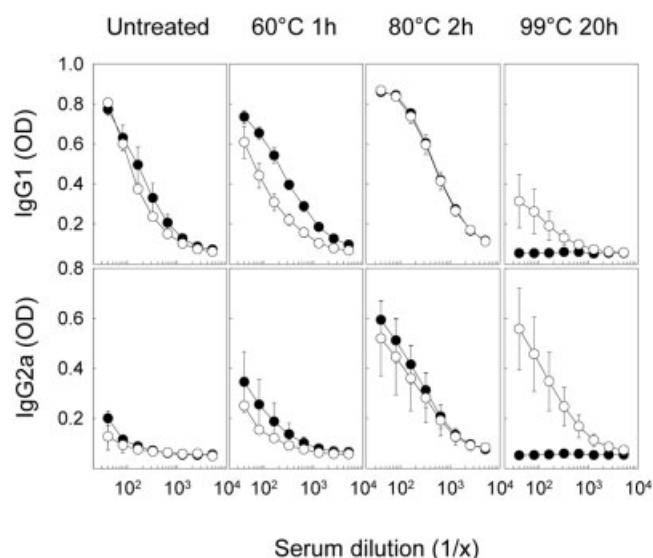


Figure 4. A low amount of native allergen is required to achieve strong and specific immune responses. Mice were immunised with 0.3 μg native or differentially heat-denatured BV in $\text{Al}(\text{OH})_3$ four times semi-weekly by intralymphatic injections (filled symbols). Alternatively, to the same vaccine modifications were added another 0.03 μg native BV prior to injection (open symbols). Anti-PLA₂ IgG1 and IgG2a antibodies were measured by ELISA 10 wk after the priming dose.

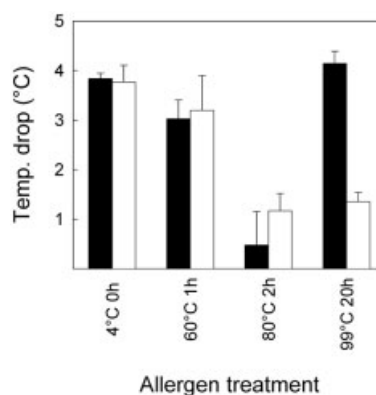


Figure 5. Heat-denatured BV can be used therapeutically to prevent allergic anaphylaxis. Mice ($n = 3$) were sensitised by seven weekly intraperitoneal injections with 0.3 μg native BV in $\text{Al}(\text{OH})_3$. At 4 wk thereafter, they were immunised as described in Fig. 4 with 0.3 μg native or differentially heat-denatured BV in $\text{Al}(\text{OH})_3$ (filled bars) or with the same vaccines with the addition of 0.03 μg native BV in $\text{Al}(\text{OH})_3$ prior to injection (open bars). For induction of anaphylactic responses, mice were challenged intraperitoneally with 30 μg PLA₂. The change in rectal temperature was measured after 30 min.

to 0.3 μg untreated (4°C) or partly treated (60°C and 80°C) BV did not further improve protection (Fig. 4, 5). Taken together, these experiments demonstrate that heat-denaturation provides strong immunogenicity, which is, however, dependent on residual amounts of native allergen, and more importantly, heat-denatured BV can be used therapeutically to prevent allergy.

Heat-denatured allergens have therapeutic potentials in humans

Having demonstrated the therapeutic potential of heat-denatured BV in mice, we tested whether native and heat-denatured PLA₂ or BV could be recognised by human immunoglobulins. This was analysed by a competitive inhibition assay using human antibodies specific for IgG4 and IgE. Both native and denatured allergens were recognised by human IgG4 (Fig. 6A, B), results which were verified in Western blot analysis (data not shown). However, the IgE-binding capacity was virtually abolished upon heat-denaturation of the allergens (Fig. 6C, D). Hence, while maintaining a therapeutic potential, the risk of an adverse reaction is reduced.

Discussion

Type I hypersensitivity is mediated through IgE bound to the surfaces of mast cells and basophils. Subsequent exposure to the allergen can cross-link the IgE and cause degranulation of the cells with the release of histamine

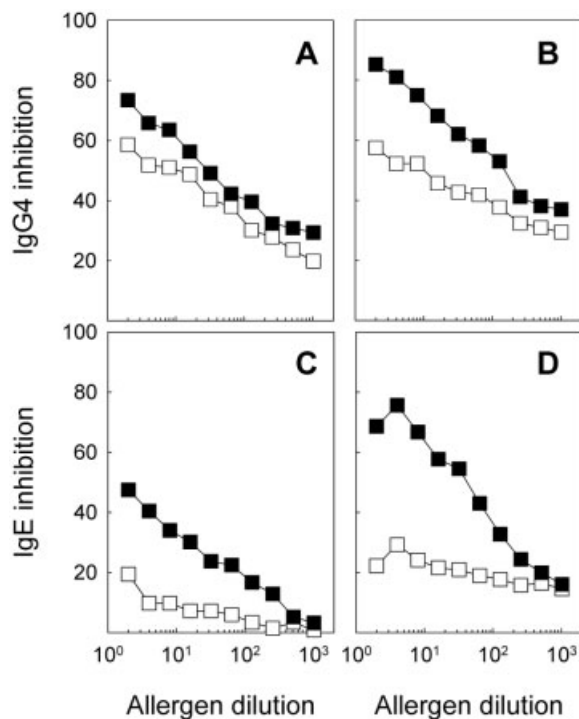


Figure 6. Heat-denatured allergens have impaired IgE-binding capacity but unaffected IgG-binding capacity in human anti-serum. *In vitro* inhibition of human IgG4 (A, B) and IgE (C, D) by native (filled symbols) and heat-denatured (open symbols) PLA₂ (A, C) and BV (B, D). Inhibition plates were prepared by incubating titrations of the native or the heat-denatured allergens with a seropositive human serum against BV. The ELISA plates were coated with PLA₂ and the inhibition mixtures were subsequently added. The results are representative of three experiments.

and other mediators of allergic symptoms. While current treatment is generally symptomatic, the only treatment with a long-lasting effect that can also stop progression of the allergy is SIT. However, SIT is time consuming, costly and bears the risk of allergic side effects. The latter can be reduced by pretreatment with anti-histamines [27, 28], or by the use of allergen-derived peptides [17–19], chemically modified allergens [29, 30] or recombinant allergens [11–13, 31] with a reduced capacity to bind IgE. For instance, by producing fusion proteins of major allergens, one aim was to reduce IgE binding and therefore the allergic side effects, while yet retaining the immunotherapeutic potential due to preserved T cell epitopes, changes in protein folding and relative exposure of B cell epitopes [9]. As an alternative and easier method to achieve the same result, we tested whether the disruption of the secondary and tertiary structure of protein allergens by a simple physical treatment such as heat-denaturation would reduce IgE-binding capacity, probably due to damaging of crucial B cell epitopes, while promoting Th1 skewing of the immune response by exposure of other epitopes with new immunodominance.

As expected, we found that heat-denaturation of allergen extracts or purified proteins reduced IgE-binding capacity. This was previously reported for other allergens [25, 26, 32] and fits well with the clinical observations that allergic individuals usually tolerate cooked food better than raw food [25]. We also found that heat-denatured allergens showed a lower potential to sensitise mice. Animals immunised intraperitoneally with heat-denatured BV allergen extract or ovalbumin produced less IgE than their littermates that had received the native allergen. The significance of this difference in IgE induction was attested by the fact that challenge with the native allergen induced anaphylaxis only in mice immunised with the native allergen, but not in mice immunised with heat-denatured allergens (Fig. 1). Since heat-denatured allergens bound and induced less IgE, they would be safer for use in SIT. In addition, the fact that allergic mice could be treated with heat-denatured allergens, which also were recognised by human IgG, would make such vaccine preparations ideally suited for SIT.

However, not only must the vaccine be safe, but it must also be immunogenic. In case of SIT, the desired immune response is a cytokine environment favourable for the protection against a subsequent natural exposure to the allergen with the production of IgG [1]. Rather strikingly, heat-denaturation of allergens markedly enhanced IgG2a production in mice (Fig. 1–3). In contrast, mice immunised with a native preparation of BV extract, BV PLA₂, birch pollen extract, grass pollen extract or cat dander extract produced high titres of IgG1 but not IgG2a, indicating a predominant Th2-type response for native proteins. This capacity of heat-denatured allergens to enhance the immunogenicity of allergens and to shift the cellular response towards Th1 would ideally fulfil the consensus requirements for successful SIT [1]. Although the frequencies of regulatory CD4-positive T cells and the levels of secreted IL-10 have been shown to increase in PBMC from desensitised patients [33–36], the fact that clinical symptoms develop within seconds of a bee sting suggests that neutralising antibodies play an important role in protection, unless there exist regulatory T cells that exert a more direct effect on mast cells and basophils. The importance of antibodies is further underlined by the fact that short immunotherapy with heat-denatured allergens, but not with native allergens, protected allergic mice against naturally induced anaphylaxis (Fig. 5). This superior immunotherapeutic effect of heat-denatured allergens correlated with enhanced IgG2a production, but less with the presence of IgG1 (Fig. 4). In mice, antibodies of the IgG2a subclass have high neutralising capacity and are, in contrast to IgG1 and IgE antibodies, compatible with strong T cell responses and the production of Th1-type cytokines [37, 38].

One possible explanation for enhanced IgG2a production by heat-denatured allergens could be the exposure of a different set of epitopes. It is clear that heat-denaturation generates new B cell epitopes by formation of novel conformational epitopes and by exposure of linear epitopes that are hidden inside the native allergen. However, it is difficult to envision why the newly generated or exposed epitopes would preferentially induce a different subclass of antibodies, such as IgG2a.

Our hypothesis is that heating only partially denatured the allergens and that residual amounts of native allergen were sufficient to stimulate specific B cells. However, after heating, most of the allergen will be denatured and aggregated. Such particulate proteins are typically more immunogenic and more Th1-stimulating than soluble proteins [39]. The improved immunogenicity could not only be a result of heat-induced aggregation of BV proteins, since such microscopic aggregation increased with the applied temperature and time (not shown). In fact, allergen treated with excessive heating at 99°C for over 20 h no longer induced detectable IgG, unless a small amount of native allergen was added. The latter preparation fully restored the IgG2a response and protection against anaphylaxis, whereas neither components alone, *i.e.* the excessively heat-denatured allergen nor the small amount of native allergen, were immunogenic. This may indirectly implicate non-antibody-mediated mechanisms in which the heat denaturation results in a mixture of fully denatured antigen (T cell inducer) and minute amounts of native-like antigen (B and T cell inducers).

We extended our findings to humans by demonstrating that the binding capacity of heat-denatured BV extract to human IgG (by Western blot; not shown) and human IgG4 (by competitive inhibition assay; Fig. 6) was comparable to that of native BV, whereas the IgE-binding capacity of heat-denatured BV was decreased. This further illustrates the dichotomy between epitopes bound by different isotypes, and suggests that heat-denatured allergens could also be useful in SIT in humans. Moreover, it allows the use of higher therapeutic doses, which *per se* is known to promote Th1 immune responses [40, 41] and to be favourable in combating allergic immunopathology [15, 16].

In conclusion, heat-denatured allergen extracts show reduced IgE binding and therefore would increase the safety and tolerability of SIT. Yet surprisingly, heat denaturation of allergen extracts enhanced production of the Th1-dependent IgG2a subclass, which worked therapeutically in allergic mice. We also present evidence that these findings can be extended to humans. As extracts are easily available and produced, and because heat-denaturation is simple, our findings may represent a socio-economically very viable strategy to improve the efficacy and safety of SIT.

Materials and methods

Immunisation protocols

Young female CBA mice from Harlan (Horst, The Netherlands) were immunised with native or heat-denatured extracts of BV and grass pollen (ALK-Abelló, Denmark) and cat hair (Stallergenes, France) purchased from Trimedial Ltd. (Bruttisellen, Switzerland). Ovalbumin was purchased from Sigma-Aldrich (Buchs, Switzerland). Denaturation was performed using concentrated allergen solutions in saline containing human serum albumin under agitation at 60, 80 and 99°C using a Compact Thermomixer from Eppendorf. Allergens were injected with 30% v/v of a 2% suspension of aluminium hydroxide (Alu-Gel-S; Serva, Heidelberg, Germany) in phosphate-buffered saline. For intraperitoneal and intralymphatic injections, 50 and 10 µL, respectively, of the vaccine was injected; intralymphatic injections were performed under anaesthesia with Ketamin and Xylazin as described [42]. Serum was prepared from clotted blood collected from the tail vein and was frozen at -20°C until analysed by ELISA.

For induction of anaphylactic responses, sensitised mice were challenged intraperitoneally with 30 µg PLA₂ purified from BV (purchased from Sigma Aldrich) or mice were challenged with 300 µg ovalbumin. Rectal temperature was measured with a calibrated digital thermometer after 30 min. All animal experiments were performed according to guidelines formulated by the Federal Swiss Veterinary Service.

Antibody determination

For detection of antibodies, microtitre 96-well plates (Nunc Maxisorb) were coated at 4°C overnight with 100 µL of 5 µg/mL PLA₂, 1 µg/mL whole birch pollen extract, 1 µg/mL whole cat hair allergen extract, or 2 µg/mL whole grass pollen extract, respectively, in buffered carbonate (pH 9.4). Plates were washed with PBS-0.05% Tween-20 (PBST) and saturated with 150 µL 2.5% non-fat dry milk (PBSTM) at room temperature (RT) for 1 h. After washing, serial dilutions of individual sera in 100 µL PBSTM were added to the plates, which were then incubated at RT for 2 h. Plates were washed and then incubated with 1 µg/mL goat anti-mouse IgG1 or IgG2a conjugated to biotin (BD Biosciences Pharmingen, San Diego, CA) in 100 µL PBSTM at RT and for 2 h. After further washing and incubation with 100 µL of a 1 : 1000 dilution of streptavidin-conjugated horseradish peroxidase (HRP) (BD Pharmingen) at RT for 1 h, the plates were washed again and 100 µL of the enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS; Sigma) in 1 M sodium dihydrogen phosphate was added. The absorption was read at 405 nm after 20 min of substrate incubation.

For detection of PLA₂-specific IgE, plates were coated with 5 µg/mL of a mouse anti-IgE antibody (6HD5) kindly provided to us by Rolf M. Zinkernagel. As secondary reagent to bound mouse serum, biotinylated PLA₂ (in-house preparation) was used at 3 µg/mL.

Inhibition assay

To evaluate the recognition of native and heat-denatured allergens by human antibodies, the allergens were tested in an ELISA inhibition assay. This was performed by coating 96-well plates at 4°C overnight with 100 µL of a 5 µg/mL PLA₂ solution in buffered carbonate (pH 9.4). Subsequently, the plates were blocked with PBSTM. In a second series of plates, twofold dilutions of the native and heat-denatured allergens were prepared at 60 µL. These were incubated at 37°C and for 2 h with 60 µL of 1 : 400 dilutions of a seropositive human serum against BV. Subsequently, 100 µL of the antiserum-allergen solutions were transferred to the washed and emptied PLA₂-coated and blocked plates for further incubation at 37°C for 2 h. After discarding the solution and washing the plates, 100 µL of biotinylated anti-human IgG4 or IgE antibodies were added for 1.5 h, as recommended by the provider (BD Pharmingen). The plates were finally developed with streptavidin-HRP (1 : 1000) and ABTS as described above.

Acknowledgements: This work was supported in part by MannKind Corporation, Valencia, USA. We thank Susi Pena-Rodriguez for excellent technical assistance and Diana Mittag for helpful discussions.

References

- Bousquet, J., Lockey, R., Mallin, H. J., Alvarez-Cuesta, E., Canonica, G. W., Chapman, M. D., Creticos, P. J. *et al.*, Allergen immunotherapy: Therapeutic vaccines for allergic diseases. World Health Organization. American Academy of Allergy, Asthma and Immunology. *Ann. Allergy Asthma Immunol.* 1998. **81**: 401–405.
- Durham, S. R., Walker, S. M., Varga, E. M., Jacobson, M. R., O'Brien, F., Noble, W., Till, S. J., Hamid, Q. A. and Nouri-Aria, K. T., Long-term clinical efficacy of grass-pollen immunotherapy. *N. Engl. J. Med.* 1999. **341**: 468–475.
- Kundig, T. M., Bachmann, M. F., Ohashi, P. S., Pircher, H., Hengartner, H. and Zinkernagel, R. M., On T cell memory: Arguments for antigen dependence. *Immunol. Rev.* 1996. **150**: 63–90.
- Maruyama, M., Lam, K. P. and Rajewsky, K., Memory B-cell persistence is independent of persisting immunizing antigen. *Nature* 2000. **407**: 636–642.
- Gray, D. and Skarvall, H., B-cell memory is short-lived in the absence of antigen. *Nature* 1988. **336**: 70–73.
- Gangloff, S. C. and Guenounou, M., Toll-like receptors and immune response in allergic disease. *Clin. Rev. Allergy Immunol.* 2004. **26**: 115–125.
- Chisholm, D., Libet, L., Hayashi, T. and Horner, A. A., Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities. *J. Allergy Clin. Immunol.* 2004. **113**: 448–454.
- Jilek, S., Barbey, C., Spertini, F. and Cortes, B., Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J. Immunol.* 2001. **166**: 3612–3621.
- Kussebi, F., Karamloo, F., Rhyner, C., Schmid-Grendelmeier, P., Salagianni, M., Mannhart, C., Akdis, M. *et al.*, A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy. *J. Allergy Clin. Immunol.* 2005. **115**: 323–329.
- Westritschnig, K., Focke, M., Verdino, P., Goessler, W., Keller, W., Twardosz, A., Mari, A. *et al.*, Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. *J. Immunol.* 2004. **172**: 5684–5692.
- Valenta, R. and Kraft, D., From allergen structure to new forms of allergen-specific immunotherapy. *Curr. Opin. Immunol.* 2002. **14**: 718–727.
- Schmid-Grendelmeier, P., Holzmann, D., Himly, M., Weichel, M., Tresch, S., Ruckert, B., Menz, G. *et al.*, Native Art v 1 and recombinant Art v 1 are able to induce humoral and T cell-mediated *in vitro* and *in vivo* responses in mugwort allergy. *J. Allergy Clin. Immunol.* 2003. **111**: 1328–1336.
- Tresch, S., Holzmann, D., Baumann, S., Blaser, K., Wuthrich, B., Cramer, R. and Schmid-Grendelmeier, P., *In vitro* and *in vivo* allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract. *Clin. Exp. Allergy* 2003. **33**: 1153–1158.
- Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A. J., Grimm, R. *et al.*, Modulation of IgE reactivity of allergens by site-directed mutagenesis: Potential use of hypoallergenic variants for immunotherapy. *Faseb J.* 1998. **12**: 231–242.
- Andre, C., Perrin-Fayolle, M., Grosclaude, M., Couturier, P., Basset, D., Cornillon, J., Piperno, D. *et al.*, A double-blind placebo-controlled evaluation of sublingual immunotherapy with a standardized ragweed extract in patients with seasonal rhinitis. Evidence for a dose-response relationship. *Int. Arch. Allergy Immunol.* 2003. **131**: 111–118.
- Ewbank, P. A., Murray, J., Sanders, K., Curran-Everett, D., Dreskin, S. and Nelson, H. S., A double-blind, placebo-controlled immunotherapy dose-response study with standardized cat extract. *J. Allergy Clin. Immunol.* 2003. **111**: 155–161.
- Kay, A. B. and Larche, M., Allergen immunotherapy with cat allergen peptides. *Springer Semin. Immunopathol.* 2004. **25**: 391–399.
- Fellrath, J. M., Kettner, A., Dufour, N., Frigerio, C., Schneeberger, D., Leimgruber, A., Corradin, G. and Spertini, F., Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: Results of a phase I trial. *J. Allergy Clin. Immunol.* 2003. **111**: 854–861.
- von Garnier, C., Astori, M., Kettner, A., Dufour, N., Heusser, C., Corradin, G. and Spertini, F., Allergen-derived long peptide immunotherapy down-regulates specific IgE response and protects from anaphylaxis. *Eur. J. Immunol.* 2000. **30**: 1638–1645.
- Patterson, R., Suszko, I. M., Pruzansky, J. J., Zeiss, C. R., Metzger, W. J. and Roberts, M., Polymerization of mixtures of grass allergens. *J. Allergy Clin. Immunol.* 1977. **59**: 314–319.
- Grammer, L. C., Shaughnessy, M. A. and Patterson, R., Standardization of modified allergens using polymerized ragweed as a model system. *Clin. Rev. Allergy* 1987. **5**: 107–116.
- Gieni, R. S., Yang, X., Kelso, A. and Hayglass, K. T., Limiting dilution analysis of CD4 T-cell cytokine production in mice administered native versus polymerized ovalbumin: Directed induction of T-helper type-1-like activation. *Immunology* 1996. **87**: 119–126.
- Bousquet, J., Hejjaoui, A., Soussana, M. and Michel, F. B., Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. IV. Comparison of the safety and efficacy of two dosages of a high-molecular-weight allergoid. *J. Allergy Clin. Immunol.* 1990. **85**: 490–497.
- Kim, M. J., Lee, J. W., Yook, H. S., Lee, S. Y., Kim, M. C. and Byun, M. W., Changes in the antigenic and immunoglobulin E-binding properties of hen's egg albumin with the combination of heat and gamma irradiation treatment. *J. Food Prot.* 2002. **65**: 1192–1195.
- Sanchez-Monge, R., Blanco, C., Perales, A. D., Collada, C., Carrillo, T., Aragoncillo, C. and Salcedo, G., Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J. Allergy Clin. Immunol.* 2000. **106**: 190–195.
- Koppelman, S. J., van Koningsveld, G. A., Knulst, A. C., Gruppen, H., Pigman, I. G. and de Jongh, H. H., Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *J. Agric. Food Chem.* 2002. **50**: 1562–1568.
- Halmerbauer, G., Hauk, P., Forster, J., Urbanek, R., Kaufmehl, K. and Koller, D. Y., *In vivo* histamine release during the first minutes after deliberate sting challenges correlates with the severity of allergic symptoms. *Pediatr. Allergy Immunol.* 1999. **10**: 53–57.
- Muller, U., Hari, Y. and Berchtold, E., Premedication with antihistamines may enhance efficacy of specific-allergen immunotherapy. *J. Allergy Clin. Immunol.* 2001. **107**: 81–86.
- Ibarrola, I., Sanz, M. L., Gamboa, P. M., Mir, A., Benahmed, D., Ferrer, A., Arilla, M. C., Martinez, A. and Asturias, J. A., Biological characterization of glutaraldehyde-modified *Parietaria judaica* pollen extracts. *Clin. Exp. Allergy* 2004. **34**: 303–309.

- 30 Maasch, H. J. and Marsh, D. G., Standardized extracts modified allergens – allergoids. *Clin. Rev. Allergy* 1987. **5**: 89–106.
- 31 Ferreira, F., Wallner, M., Breiteneder, H., Hartl, A., Thalhamer, J. and Ebner, C., Genetic engineering of allergens: Future therapeutic products. *Int. Arch. Allergy Immunol.* 2002. **128**: 171–178.
- 32 Magi, M., Garcia, L., Vandenbranden, M., Palmantier, R. and Jacquet, A., Heat denaturation affects the ProDer p 1 IgE reactivity and downregulates the development of the specific allergic response. *J. Allergy Clin. Immunol.* 2004. **114**: 545–552.
- 33 Schmidt-Weber, C. B., Kunzmann, S. and Blaser, K., TGF-beta-mediated control of allergen-specific T-cell responses. *Curr. Allergy Asthma Rep.* 2002. **2**: 259–262.
- 34 Nakao, A., Is TGF-beta1 the key to suppression of human asthma? *Trends Immunol.* 2001. **22**: 115–118.
- 35 Levings, M. K., Bacchetta, R., Schulz, U. and Roncarolo, M. G., The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int. Arch. Allergy Immunol.* 2002. **129**: 263–276.
- 36 Akdis, C. A., Joss, A., Akdis, M. and Blaser, K., Mechanism of IL-10-induced T cell inactivation in allergic inflammation and normal response to allergens. *Int. Arch. Allergy Immunol.* 2001. **124**: 180–182.
- 37 Klaus, G. G., Pepys, M. B., Kitajima, K. and Askonas, B. A., Activation of mouse complement by different classes of mouse antibody. *Immunology* 1979. **38**: 687–695.
- 38 Baldridge, J. R. and Buchmeier, M. J., Mechanisms of antibody-mediated protection against lymphocytic choriomeningitis virus infection: Mother-to-baby transfer of humoral protection. *J. Virol.* 1992. **66**: 4252–4257.
- 39 Storni, T., Kundig, T. M., Senti, G. and Johansen, P., Immunity in response to particulate antigen-delivery systems. *Adv. Drug Deliv. Rev.* 2005. **57**: 333–355.
- 40 Boonstra, A., Asselin-Paturel, C., Gilliet, M., Crain, C., Trinchieri, G., Liu, Y. J. and O'Garra, A., Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: Dependency on antigen dose and differential Toll-like receptor ligation. *J. Exp. Med.* 2003. **197**: 101–109.
- 41 Von Garnier, C., Astori, M., Kettner, A., Dufour, N., Corradin, G. and Spertini, F., *In vivo* kinetics of the immunoglobulin E response to allergen: Bystander effect of coimmunization and relationship with anaphylaxis. *Clin. Exp. Allergy* 2002. **32**: 401–410.
- 42 Johansen, P., Haffner, A. C., Koch, F., Zepter, K., Erdmann, I., Maloy, K., Simard, J. J. et al., Direct intralymphatic injection of peptide vaccines enhances immunogenicity. *Eur. J. Immunol.* 2005. **35**: 568–574.