



Non-anaphylactic combination of partially deleted fragments of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy

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Abstract

Allergen-specific immunotherapy, in which repeated injections of allergens over prolonged periods are used to induce tolerance, has proven an effective treatment of allergy. A major side effect of allergen-specific immunotherapy is anaphylactic reaction. House dust mite allergens are major causative factors associated with various allergic diseases. Der f 2 is the major house dust mite allergen composed of 129 amino acid residues. Analysis using deletion mutants of Der f 2 suggested that T-cell epitopes of Der f 2 were multiple in mite-allergic patients. We found that some IgE epitopes were renatured by dialysis of a mixture of two denatured C- and N-terminal deletion mutants, 1–112 and 85–129 in 13 patients out of 14. On the other hand, IgE binding activity was negative in the separately dialyzed fragments and their mixture in each patient tested. Furthermore, we demonstrated that neither of the two separately prepared polypeptides induced *in vivo* skin prick test reactivity. These findings are important for improvement of T-cell targeting allergen-specific immunotherapy and development of monovalent IgE haptens. The use of combinations of overlapping non-anaphylactic fragments of allergen covering all of the T-cell epitopes achieves the removal of IgE reactivity, the cause of harmful anaphylactic reactions, without affecting the T-cell reactivity essential for immunotherapy, offering potentially safer and more effective treatment for allergic disease. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

IgE-mediated type I allergic diseases such as asthma, rhinitis and atopic dermatitis are worldwide problems from which more than 20% of the world's population suffers. Allergen-specific immunotherapy, in which repeated injections of allergens over prolonged periods are used to induce tolerance, has proven an effective

treatment of allergic disease since the first trials in the early 1900s (Noon, 1911). At present, induction of unresponsiveness of T cells or a change in the pattern of cytokines produced by CD4⁺ T cells is considered the major mechanisms of allergen-specific immunotherapy (Norman, 1993; Suko et al., 1995; O'Brien et al., 1997; Hoyne et al., 1995; Norman et al., 1996; van Neerven et al., 1996). Administration of an excess amount of allergen causes an anaphylactic reaction that follows the release of mediators from mast cells and basophils activated by crosslinking of the complex of allergen-specific IgE and high-affinity IgE receptor (FcεRI) on the cell surface with multivalent allergen

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(Ishizaka and Ishizaka, 1984; Metzger et al., 1986). A major problem with allergen-specific immunotherapy is the risk of anaphylactic reaction.

Recently, clinical trials of allergen-specific immunotherapy using short length peptides containing T-cell epitopes of allergens have been conducted (Hoyne et al., 1995; Norman et al., 1996; van Neerven et al., 1996). Peptide-based immunotherapy has the advantage of reduced risk of anaphylactic reaction over conventional allergen-specific immunotherapy because of an absence or reduction in the number of IgE epitopes (van't Hof et al., 1991). However, the peptide-based immunotherapy has important drawbacks i.e. the multiplicity of T-cell epitopes on individual allergens and the large diversity in T-cell epitopes due to the diversity of major histocompatibility complex (MHC) and T-cell receptor (TCR) among individual patients (van Neerven et al., 1996; WHO, 1995; Davis, 1990; O'Brien et al., 1995; Yssel et al., 1992). We chose the major house dust mite allergen Der f 2 as a typical target allergen for development of strategies of immunotherapy that avoid the problems while maintaining the advantages of peptide-based immunotherapy.

House dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*) allergens are major causative factors associated with various allergic diseases (Platts-Mills and Weck, 1989; Voohorst et al., 1967; Miyamoto et al., 1968), and the major allergens are classified as group 1 (Der f 1 and Der p 1, M_r 25,000) or group 2 (Der f 2 and Der p 2, M_r 14,000) (Platts-Mills and Chapman, 1987; Haida et al., 1985; Yamashita et al., 1989). The majority of sera from patients allergic to house dust mites have binding activities to both group 1 and 2 allergens. Genetic linkages between specific IgE responses to these allergens and genes on human chromosomes have been intensively investigated (Moffatt et al., 1994; Marsh et al., 1994). Analysis of B (van't Hof et al., 1991; Lombardero et al., 1990; Takai et al., 1997a,b; Nishiyama et al., 1995; Chua et al., 1991; Smith and Chapman, 1996) and T-cell epitopes (O'Brien et al., 1995; Yssel et al., 1992; van Neerven et al., 1993; O'Hehir et al., 1993; Tsitoura et al., 1996) of the major allergens also has been carried out to clarify their relationships to immunogenicity and to apply such epitope moieties to immunotherapy (Hoyne et al., 1995; van Neerven et al., 1996; Marshall and Francis, 1997; Valenta and Kraft, 1995).

The cDNA clones for Der f 2, the group 2 allergen of *D. farinae* (Yuuki et al., 1990, 1991), encode proteins consisting of 129 amino acid residues with marked homology to Der p 2, the group 2 allergen of *D. pteronyssinus* (Chua et al., 1990). It has been suggested that IgE epitopes of Der f 2 and Der p 2 are dependent on the integrity of the conformational structure of the allergens rather than the contiguous

sequence of amino acids (van't Hof et al., 1991; Takai et al., 1997a; Chua et al., 1991). We determined the location of three disulfide bonds of native Der f 2 (Cys8–Cys119, Cys21–Cys27, and Cys73–Cys78) (Nishiyama et al., 1993). We found that two disulfide bonds, Cys8–Cys119 and Cys73–Cys78, played critical roles in expressing IgE-binding capacity in Der f 2 (Takai et al., 1997b). Smith and Chapman (1996) reported that Cys73–Cys78 in Der p 2 was critical.

In this paper, we demonstrated the multiplicity of T-cell epitopes of Der f 2 in allergic patients, characterized the antigenic structure of Der f 2 recognized by polyclonal IgE and obtained insight into the refolding of IgE epitopes. Furthermore, we demonstrated that neither of the separately prepared, overlapping partially deleted Der f 2 polypeptides, 1–112 or 85–129, induced in vivo skin prick test reactivity. These results are important for improvement of T-cell targeting allergen-specific immunotherapy and development of monovalent IgE haptens. Combinations of overlapping non-anaphylactic recombinant fragments of allergen covering all of the multiple T-cell epitopes essential for immunotherapy are potentially useful for safer and more effective immunotherapy for allergic disease.

2. Materials and methods

2.1. Sera and cells

Sera and peripheral blood mononuclear cells were obtained from mite sensitive atopic patients with allergic rhinitis, bronchial asthma and/or atopic dermatitis who proved positive to a skin prick test.

2.2. Expression of fusion proteins of Der f 2 and its deletion mutants

The expression vectors of the fusion proteins of Der f 2 and its deletion mutants with the half-sized β -galactosidase were constructed as described (Takai et al., 1997a). *Escherichia coli* MC1061 transformed with these vectors were cultured and induced to express the fusion proteins (Yuuki et al., 1991).

2.3. Purification and refolding of fusion proteins of Der f 2 and its deletion mutants

The method that has been previously described (Yuuki et al., 1991; Nishiyama et al., 1994) was used with some modifications. Briefly, the fusion proteins of Der f 2 and its deletion mutants were produced as inclusion bodies in *E. coli* MC1061. Cells were harvested by centrifugation, suspended with buffer and disrupted by a sonication. The cell lysate was centrifuged and the precipitate of inclusion bodies recovered, washed

with buffer and solubilized with buffer containing urea. The fusion proteins denatured with urea were refolded by dialysis against PBS. After centrifugation, the supernatant was recovered and the protein concentration was determined with Protein Assay Kit (Bio-Rad, Richmond, CA). Purity was checked by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) (8% acrylamide).

2.4. T-cell proliferation assays

T cells from peripheral blood mononuclear cells from mite-allergic volunteers were cultured in 96-well culture plates with the fusion proteins of Der f 2 and its deletion mutants (100 µg/ml) and [³H]thymidine incorporation was measured as described (Mori et al., 1997).

2.5. Immunoblot analysis

The method used has been described previously (Takai et al., 1997a). Briefly, total cellular proteins expressed in *E. coli* MC1061 transformed with expression vectors of the fusion proteins of Der f 2 and its deletion mutants were subjected to SDS-PAGE and electroblotted. The membranes were stained with Coomassie Brilliant Blue, or probed with a combination of a mite-allergic patient serum and horse radish peroxidase-conjugated anti-human IgE (ε-specific) antibodies (Cappel, Malvern, PA).

2.6. RAST-EIA

IgE reactivities of the fusion proteins of Der f 2 and its deletion mutants were measured by RAST-EIA using a RAST-FEIA kit (Pharmacia, Uppsala, Sweden) as described (Takai et al., 1997b) with some modifications. Briefly, allergic patient serum of 1/4 dilution was incubated overnight at room temperature with paper discs coupled with each of the fusion proteins of Der f 2 and its deletion mutants. Then, IgE that bound to the disc was detected with β-galactosidase-conjugated anti-human IgE antibodies by measuring the fluorescence at 355 nm for excitation, and 460 nm for emission on a fluorometer, Fluoroskan II (Flow Laboratories, Lugano, Switzerland).

2.7. Skin prick test

Skin prick test was performed as described (Dreborg, 1985). Solutions of the recombinant Der f 2 of non-fused form, the Der f 2 fusion protein with half-sized β-galactosidase, its deletion mutants (22–129, 1–112, and 85–129), and half-sized β-galactosidase (20 ng/20 µl) were applied.

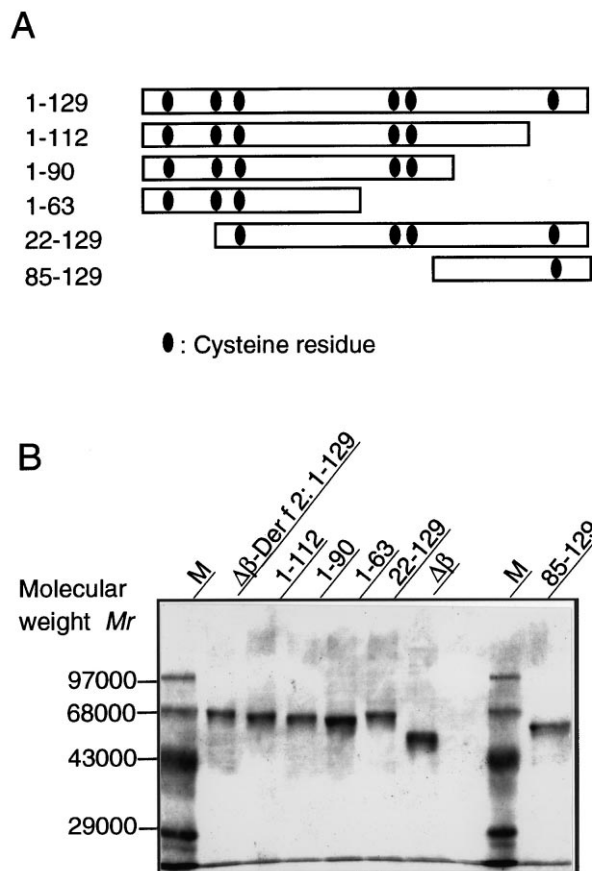


Fig. 1. (A) Schematic representation of Der f 2 and its deletion mutants expressed as fusion proteins in *E. coli*. (B) Purification of fusion proteins of Der f 2 and its deletion mutants. Purified inclusion bodies expressed in *E. coli* MC1061 transformed with the expression vector of Der f 2 fusion protein with half-sized β-galactosidase (Δβ-Der f 2: 1–129), its deletion mutants (1–112, 1–90, 1–63, 22–129, and 85–129), and a control vector encoding half-sized β-galactosidase (Δβ) were subjected to SDS-PAGE (8% acrylamide). The gel was stained with Coomassie Brilliant Blue. M: molecular weight marker.

3. Results

3.1. Preparation of fusion proteins of Der f 2 and its deletion mutants

Der f 2 and its five deletion mutants, that dispersed within various sizes among those constructed by the use of restriction sites in Der f 2 cDNA or by digestion of the cDNA by exonuclease III (Takai et al., 1997a), were expressed in *E. coli* as fusion proteins with half-sized β-galactosidase. Insoluble inclusion bodies containing the fusion proteins were recovered and solubilized. The fusion proteins refolded by dialysis against buffer were purified as shown in Fig. 1. These samples were used in all following experiments except immunoblot analysis.

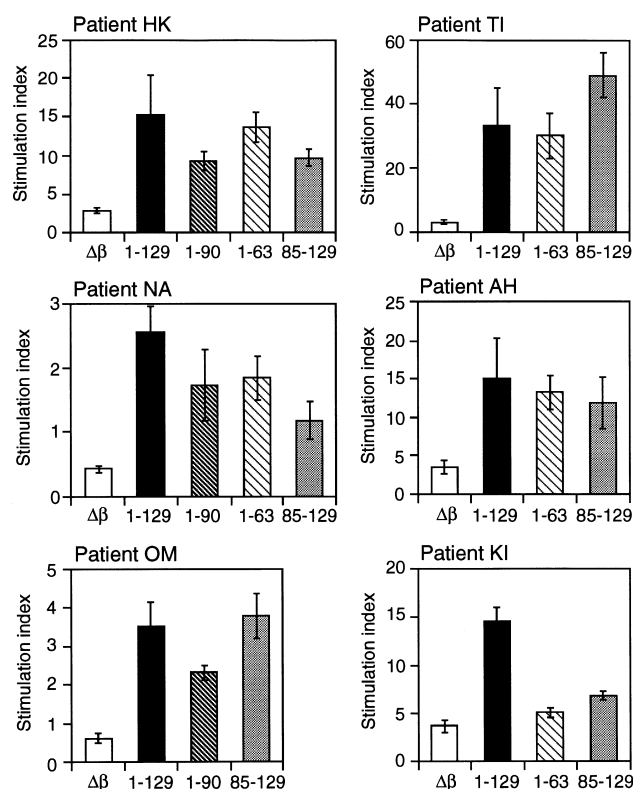


Fig. 2. Proliferative response of T cells stimulated with purified fusion proteins of Der f 2 and its deletion mutants. Peripheral blood mononuclear cells were obtained from six mite-allergic patients. The Der f 2 fusion protein with half-sized β -galactosidase (1–129), its deletion mutants (1–90, 1–63, and 85–129), and half-sized β -galactosidase ($\Delta\beta$) as a negative control were used as antigens to stimulate polyclonal T cells in the peripheral blood mononuclear cells. Initials of the patients' names are indicated (HK, NA, OM, TI, AH, and KI). Results are presented as stimulation indices that were calculated as the ratio of the counts/min of culture wells containing each antigen with that of wells containing medium alone. The mean and SEM of four points are shown.

3.2. T-cell proliferative responses against deleted Der f 2 molecules

T-cell reactivities against the deletion mutants of Der f 2 were examined in six mite-allergic patients. T-cell reactivities against the whole Der f 2 fusion 1–129, a deletion mutant 85–129, and half-sized β -galactosidase were examined in six patients (HK, NA, OM, TI, AH, and KI), those against 1–90 in three patients (HK, NA, and OM), and those against 1–63 in five patients (HK, NA, TI, AH, and KI). The proliferation of polyclonal T cells in peripheral blood mononuclear cells was significantly higher in patients stimulated with 1–129, 1–90, 1–63, or 85–129 than with half-sized β -galactosidase ($\Delta\beta$) (Fig. 2). In a patient, NA, however, the stimulation index against 85–129 was not significantly higher than that against medium alone.

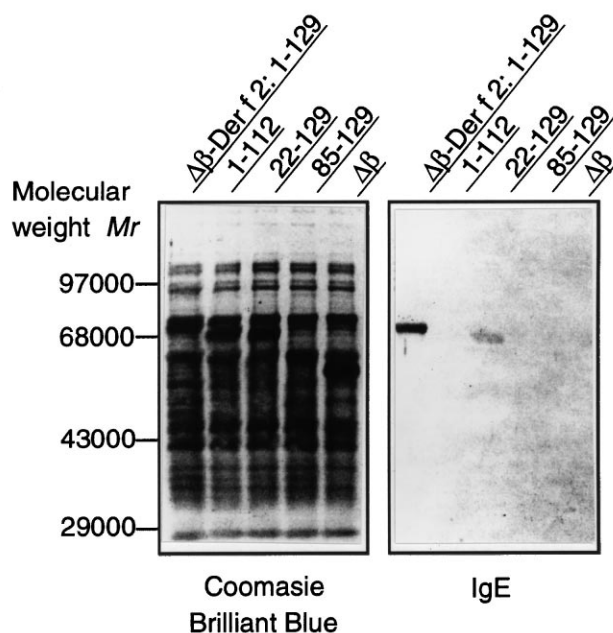


Fig. 3. Immunoblot analysis of fusion proteins of Der f 2 and its deletion mutants expressed in *E. coli*. Total cellular proteins of *E. coli* MC1061 transformed with the expression vector of the Der f 2 fusion protein with half-sized β -galactosidase ($\Delta\beta$ -Der f 2: 1–129), its deletion mutants (1–112, 22–129, and 85–129), and pFL500, a control vector encoding half-sized β -galactosidase ($\Delta\beta$) were subjected to SDS-PAGE (8% acrylamide) and electroblotted. The membranes were stained with Coomassie Brilliant Blue or probed with human atopic serum and horse radish peroxidase-conjugated anti-human IgE antibodies.

3.3. Reactivities of deleted Der f 2 molecules to IgE in allergic patient sera

Immunoblot analysis of whole lysate of *E. coli* cells expressing three deletion mutants (1–112, 22–129, and 85–129) was performed with mite-allergic patient sera. The same result that two deletion mutants, 1–112 and 85–129, had no detectable IgE reactivity while a deleted Der f 2, 22–129, had weak IgE reactivity compared with the whole Der f 2 fusion, 1–129, was obtained with each of the tested ten patient sera. A typical result is shown in Fig. 3.

The IgE-binding capacities of five deletion mutants (1–112, 1–90, 1–63, 22–129, and 85–129) which were recovered from inclusion bodies and not treated with SDS were determined by RAST-EIA. All 14 patients tested showed similar profiles of IgE-binding capacity: no or markedly reduced reactivity in four deletion mutants (1–112, 1–90, 1–63, and 85–129), and weak reactivity in a mutant 22–129 compared with the whole Der f 2 fusion, 1–129 (Fig. 4A and B). In two patients, approximately 5 to 10-fold increase in the concentration of 22–129 to achieve the levels of binding observed in the whole Der f 2 fusion (Fig. 4B).

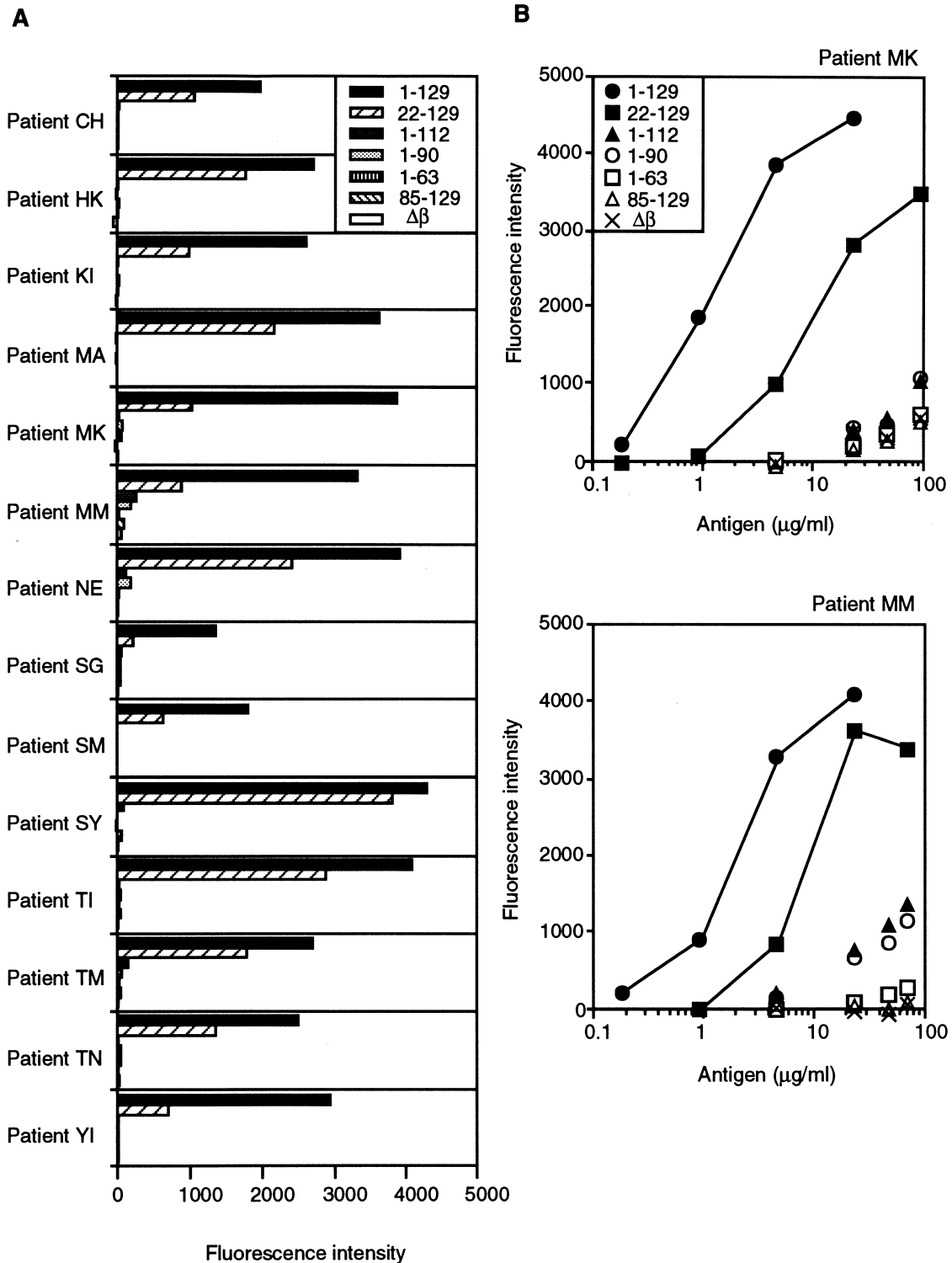


Fig. 4. RAST-EIA using purified fusion proteins of Der f 2 and its deletion mutants. (A) IgE-binding profiles of 14 patients allergic to house dust mite. Initials of the patients' names are indicated. The concentration of antigens in the immobilization step was 5 $\mu\text{g/ml}$. (B) IgE-binding profiles of two patients, MK and MM.

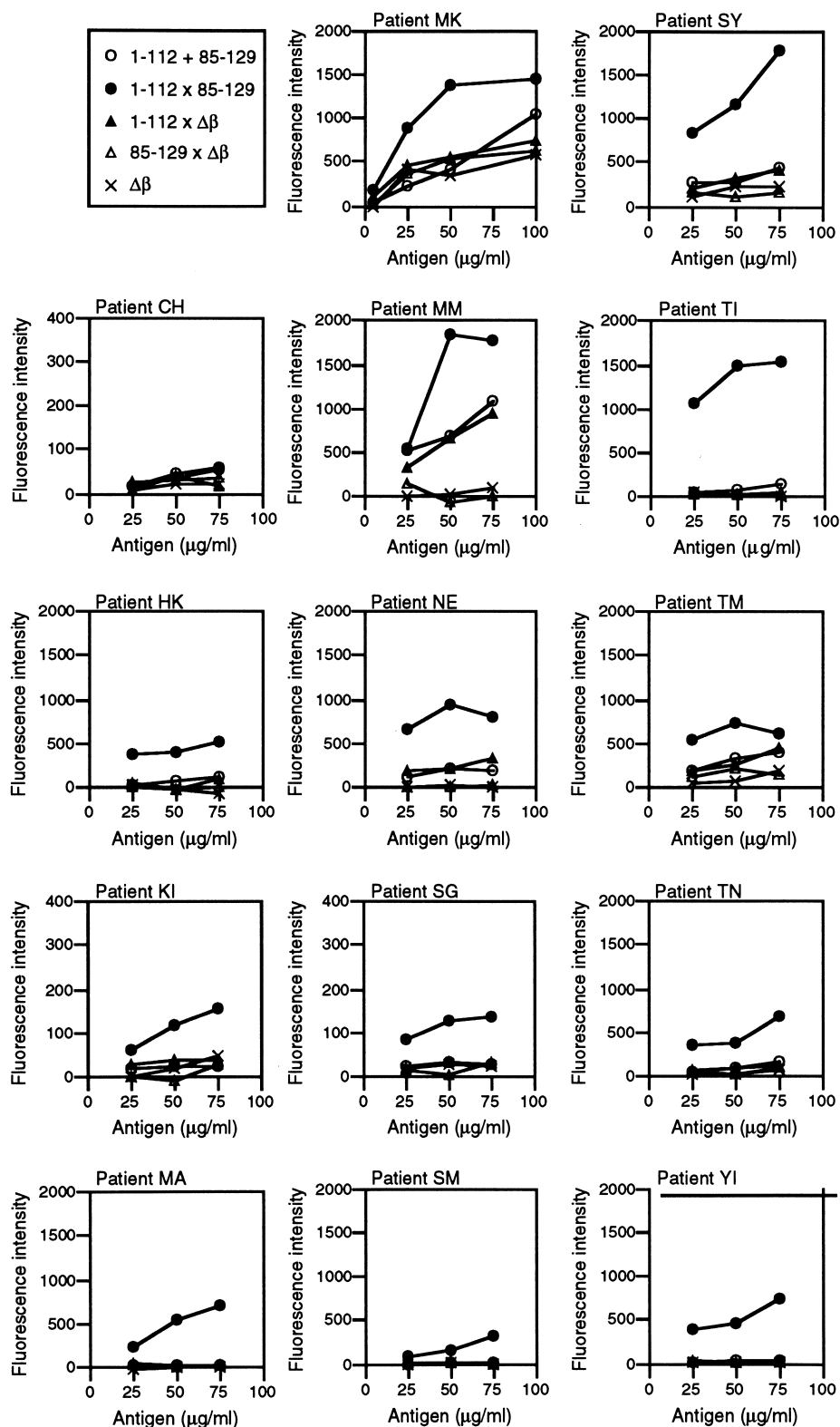


Fig. 5. Refolding of IgE epitopes by interaction of C- and N-terminal deletion mutants, 1-112 and 85-129. 1-112 + 85-129: the mixture of equal amounts of dialyzed 1-112 and dialyzed 85-129. 1-112 x 85-129: the sample generated by dialysis of the mixture of equal amounts of urea-containing 1-112 and urea-containing 85-129. 1-112 x $\Delta\beta$: the sample generated by dialysis of the mixture of equal amounts of urea-containing 1-112 and urea-containing $\Delta\beta$ as a negative control. 85-129 x $\Delta\beta$: the sample generated by dialysis of the mixture of equal amounts of urea-containing 85-129 and urea-containing $\Delta\beta$ as a negative control.

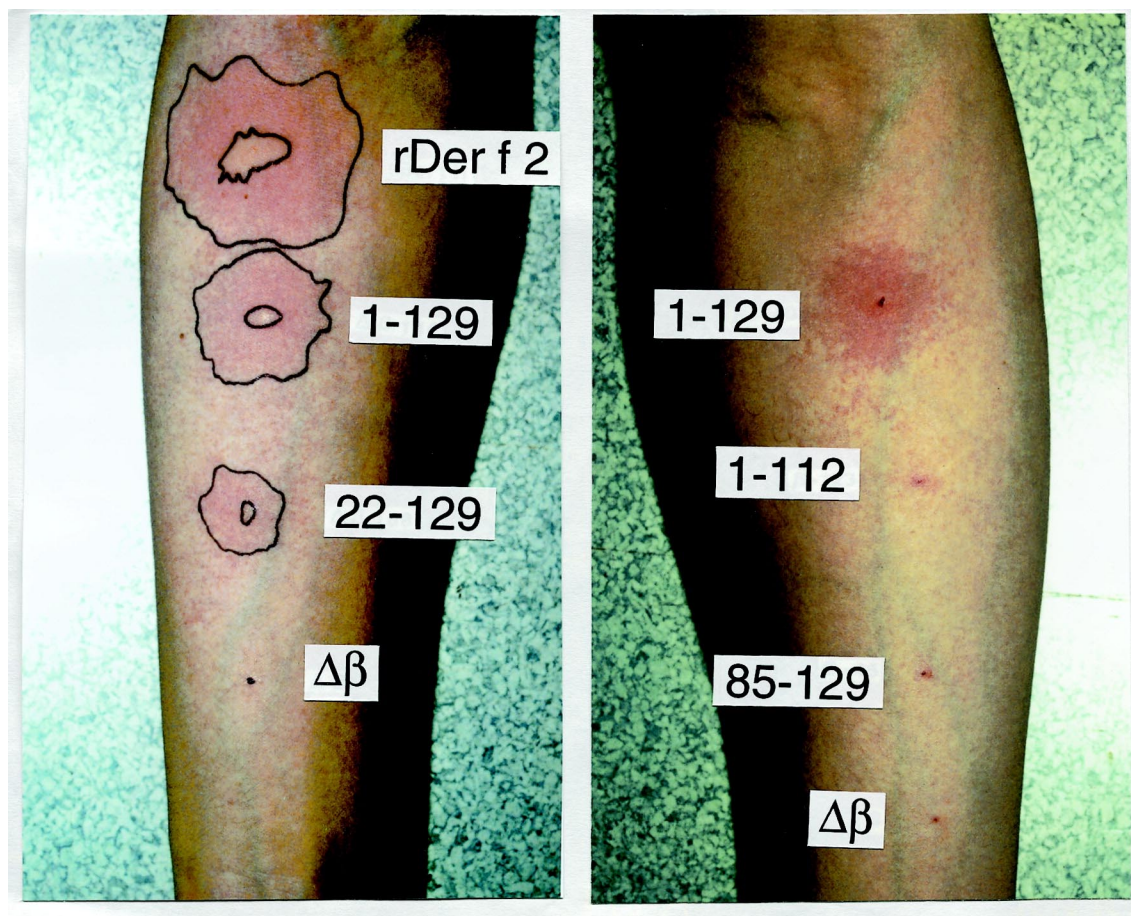


Fig. 6. Skin prick test with purified fusion proteins of Der f 2 and its deletion mutants. The Der f 2 fusion protein with half-sized β -galactosidase ($\Delta\beta$ -Der f 2: 1–129), its deletion mutants (1–112, 22–129, 1–112, and 85–129), the recombinant Der f 2 of non-fused form (rDer f 2), and half-sized β -galactosidase ($\Delta\beta$) as a negative control were used.

3.4. Renature of IgE epitopes by the presence of both N- and C-terminal sequences of Der f 2 in the refolding process

A C-terminal deletion mutant 1–112 and an N-terminal deletion mutant 85–129, overlapping each other and both negative in IgE reactivity, were selected for further study of IgE epitopes dependent on the whole sequence of Der f 2 by RAST-EIA. Results are shown in Fig. 5. RAST-EIA was performed with 14 mite-allergic patient sera. Antigens were coupled to paper discs at higher concentrations in this assay. Interestingly, the material generated by dialysis of the urea-containing mixture of denatured 1–112 and denatured 85–129 had significantly higher IgE reactivity than the mixture of the separately dialyzed two deletion mutants which had no or very low reactivity in 13 patients out of 14 except one patient, CH. The material generated by dialysis of the urea-containing mixture of denatured 1–112 and denatured $\Delta\beta$ and that of 85–129 and $\Delta\beta$ were both negative.

3.5. Activities of deleted Der f 2 molecules to induce urticarial type I reactions in an allergic patient

The capacity of the recombinant fusion proteins of Der f 2 and its deletion mutants to elicit cutaneous reactions was evaluated in a volunteer allergic to house dust mites by in vivo skin prick test. The volunteer showed a typical type I reaction using the recombinant Der f 2 of non-fused form (rDer f 2) (Iwamoto et al., 1996) and the fusion of the whole Der f 2 (1–129), a relatively weak but significantly positive reaction using an N-terminal deletion mutant 22–129, and a negative reaction using two deletion mutants, 1–112 and 85–129, and $\Delta\beta$ (Fig. 6).

4. Discussion

IgE antibodies against specific allergens are directly correlated with symptoms of type I allergy in allergic patients (Ishizaka and Ishizaka, 1984; Metzger et al.,

1986; Moffatt et al., 1994; Marsh et al., 1994). Differentiation and activation of B cells to secrete the allergen-specific IgE is regulated by T cells that recognize the complexes of the allergen-derived peptide and MHC on the surface of antigen presenting cells via TCR (van Neerven et al., 1996). Although a patient, NA, did not show a positive T-cell response to a deletion mutant, 85–129, the other patients showed a positive response to each of tested deletion mutants (Fig. 2). The results of proliferation assay suggests that T-cell epitopes of Der f 2 in patients allergic to house dust mites are multiple and disperse throughout the entire amino acid sequence of Der f 2 in each patient.

Epitopes of group 2 allergens of house dust mites, Der f 2 from the dust mite species *D. farinae* and Der p 2 from *D. pteronyssinus*, for polyclonal IgE antibodies in atopic patient sera do not appear to consist of simply sequential polypeptides. Chua et al. (1991) constructed and analyzed four deletion mutants of Der p 2 fused to glutathione transferase, 1–69, 42–117, 69–117, and 69–129. They reported that the deletion mutants did not bind IgE in the majority of adult patients, or weakly bound in only a few of them. Previously, we demonstrated that the sequences of 1–24, 25–29 and 121–123 are the minimum N- and C-terminal sequences required for IgE binding by immunoblot analysis of numbers of more precisely serially deleted Der f 2 fused to maltose binding protein (Takai et al., 1997a). In the present study, RAST-EIA of the half-sized β -galactosidase fusions was performed with 14 sera (Fig. 4) and immunoblot analysis with 10 sera (Fig. 3). In all tested sera, no or markedly reduced reactivity in four deletion mutants (1–112, 1–90, 1–63, and 85–129), and weak reactivity in a mutant 22–129 compared with the whole Der f 2 fusion. The RAST-EIA revealed that 22–129 had IgE epitopes common to all tested patients although the entire amino acid sequence was essential for full IgE reactivity.

On the basis of the results of the RAST-EIA, we assumed that interaction between N- and C-terminal sequences of Der f 2 protein was critical for binding to IgE. To test this hypothesis directly, we analyzed gain of IgE reactivity in mixed deleted fragments. We performed a refolding experiment using a C-terminal deletion mutant, 1–112, an N-terminal deletion mutant, 85–129, and combinations of the two. Interestingly, the refolded material generated by the dialysis of the mixture of denatured 1–112, and denatured 85–129 had significant IgE reactivity (Fig. 5) in 13 patients out of 14. We considered this to be a result of the renature of structures for some IgE epitopes directly composed of amino acid residues present in the N- and C-terminal deleted sequences of 1–84 and 113–129, those not in the sequences but indirectly maintained by both of the sequences, and/or those efficiently refolded by the help of the interaction mediated by the sequences.

Recently we reported that disruption of a disulfide bond that bridged N- and C-terminal sequences of Der f 2, Cys8–Cys119, caused marked reduction of reactivity to IgE (Takai et al., 1997b). The disulfide bond formation of Cys8–Cys119 or amino acid residues surrounding the disulfide bond might be involved in the interaction of the two fragments of Der f 2, 1–112 and 85–129 (Nishiyama et al., 1993, 1995; Takai et al., 1997b).

The results of the refolding experiment give useful insights for the development of IgE haptens of Der f 2. IgE haptens contain only one IgE epitope structure and are not able to crosslink the complex of IgE and Fc ϵ RI on mast cells or basophils (Valenta and Kraft, 1995; Ball et al., 1994). IgE haptens have therapeutical potential in treatment for allergy in a different context from that of T-cell targeting allergen-specific immunotherapy described in the following paragraphs. Identification of the combinations of sequences critical for the reconstitution of each IgE binding structure in the Der f 2 molecule would provide useful information to design IgE haptens mimicking local tertiary structures of Der f 2. The structures renatured reactive to IgE in the material generated by dialysis of the urea-containing mixture of denatured 1–112 and denatured 85–129 might be candidates for IgE haptens composed of peptides that were located separately in the primary amino acid sequence of the allergen.

Allergen-specific immunotherapy involves the stepwise subcutaneous injection of increasing concentrations of specific allergens to which a patient is sensitive to achieve a state of immunological tolerance (Norman, 1993; Suko et al., 1995; O'Brien et al., 1997). It seems that ideally, allergen should be administered at a higher dose to change the Th1/Th2 bias (Secrist et al., 1995). However, this may cause an anaphylactic reaction. Neither the C-terminal deletion mutant of Der f 2, 1–112 nor the N-terminal deletion mutant, 85–129, as we described above, had significant reactivity to IgE in patient sera. Furthermore, in vivo skin prick test showed that skin mast cells did not degranulate in the presence of 1–112 or 85–129 (Fig. 6). Therefore, allergen-specific immunotherapy with the deletion mutants would not cause anaphylaxis even at relatively high doses. That the mixture of the separately dialyzed two deletion mutants was negative in RAST-EIA suggests that significant reconstitution of IgE epitope structures is unable to occur once the detergent is removed from the solution by dialysis (Fig. 5). Preparing the overlapping two fragments separately is preferable to avoid occurrence of such IgE epitope structures that were refolded in the material generated by dialysis of the mixture of the two denatured fragments.

Recently, clinical trials of allergen-specific immunotherapy using chemically synthesized short-lengthed

peptides containing T-cell epitopes of allergens have been carried out (Hoyne et al., 1995; Norman et al., 1996; van Neerven et al., 1996). As with immunotherapy using non-anaphylactic combinations of the deletion mutants of allergens such as 1–112 and 85–129 of Der f 2 fragments, the peptide-based immunotherapy has the advantage of less IgE binding (van't Hof et al., 1991). However, a problem associated with peptide-based immunotherapy is a multiplicity of T-cell epitopes of the individual allergen in each patient and a diversity of them due to the diversity of MHC class II molecules and TCRs in human. Indeed, T-cell epitopes of Der f 2 proved to be often multiple in allergic patients (Fig. 2) and those of many allergens including Der p 2 from the dust mite species *D. pteronyssinus* have been reported not only to be multiple in each patient but also to have diversity among patients (van Neerven et al., 1996). Litwin et al. (1991) demonstrated clinical effectiveness of enzymatically digested fragments of ragweed allergen. However, in the case that cleavage sites are present within T-cell epitopes, condition of digestion might have to be carefully optimized to avoid excessive degradation that caused loss of peptides containing T-cell epitopes. Immunotherapy with a combination of overlapping non-anaphylactic fragments of allergen such as that of the two deleted Der f 2 molecules, 1–112 and 85–129, would solve these problems. Vrtala et al. (1997) reported that both of the two recombinant half fragments of the major birch pollen allergen Bet v 1 were non-anaphylactic because of the disruption of the three dimensional structure that was considered occurring in the case of deletion mutants of Der f 2. The boundary between such non-overlapping half fragments should be designed not to be bestridden by T-cell epitopes. Peptides that are presented as complex with the class II molecules of MHC have been reported to range in length from 10 to 34 amino acid residues and almost all are shorter than 28 residues long (Chiz et al., 1993). As 1–112 and 85–129 overlap for 28 amino acid residues, combined they cover all of the T-cell epitopes in the whole Der f 2 sequence theoretically.

Thus, the combination of the two non-anaphylactic deleted Der f 2 molecules, 1–112 and 85–129, is potentially useful for immunotherapy specific for house dust mites in that not only does it reduce the risk of anaphylaxis, but also all of the native multiple T-cell epitopes are theoretically covered. We have reported that a variant of Der f 2 with slight differences from the native sequence, designated C8/119 S, maintained full reactivity to proliferate T cells in patients allergic to house dust mite in spite of the low reactivities of IgE binding (Takai et al., 1997b). We emphasize that the strategies for the use of the overlapping non-anaphylactic combination of two partially deleted fragments

of allergen covering all the native amino acid sequence such as in the present study and of mutant allergen such as C8/119 S are based on a similar concept i.e. separation of IgE reactivity that depends largely on the tertiary protein structure from T-cell reactivity that depends on the primary amino acid sequence of T-cell epitope peptides (Marshall and Francis, 1997). These strategies have an advantage of maintenance of native sequences for T-cell epitopes over the use of hypoallergenic isoforms, for example, those proposed in Bet v 1 (Ferreira et al., 1996). The concept of separating of the B-cell epitopes of antigen from the T-cell epitopes or that of the latter from the former might be useful for not only allergen specific immunotherapy but immunotherapy for cancer or autoimmune diseases and safer DNA vaccination (Hsu et al., 1996; Raz et al., 1996).

References

- Ball, T., Vrtala, S., Sperr, W.R., Valent, P., Susani, M., Kraft, D., Valenta, R., 1994. Isolation of an immunodominant IgE hapten from an epitope expression cDNA library: dissection of the allergic effector reaction. *J. Biol. Chem.* 269, 28323–28328.
- Chiz, R.M., Urban, R.G., Gorga, J.C., Vignali, D.A.A., Lane, W.S., Strominger, J.L., 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* 178, 27–47.
- Chua, K.Y., Greene, W.K., Kehal, P., Thomas, W.R., 1991. IgE binding studies with large peptides expressed from *Der p* II cDNA constructs. *Clin. Exp. Allergy* 21, 161–166.
- Chua, K.Y., Doyle, C.R., Simpson, R.J., Turner, K.J., Stewart, G.A., Thomas, W.R., 1990. Isolation of cDNA coding for the major mite allergen *Der p* II by IgE plaque immunoassay. *Int. Arch. Allergy Appl. Immunol.* 91, 118–123.
- Davis, M.M., 1990. T cell receptor gene diversity and selection. *Annu. Rev. Biochem.* 59, 475–496.
- Dreborg, S., 1985. Skin prick test. *Allergy* 40, 55–59.
- Ferreira, F., Hirtenlehner, K., Jilek, A., Godnik-Cvar, J., Breiteneder, H., Grimm, R., Hoffmann-Sommergruber, K., Scheiner, O., Kraft, D., Breitenbach, M., Rheinberger, H.-J., Ebner, C., 1996. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Exp. Med.* 183, 599–609.
- Haida, M., Okudaira, H., Ogita, T., Ito, K., Miyamoto, T., Nakajima, T., Hongo, I., 1985. Allergens of the house dust mite *Dermatophagoides farinae* — immunochemical studies of four allergic fractions. *J. Allergy Clin. Immunol.* 75, 686–692.
- Hoyne, G.F., Kristensen, N.M., Yssel, H., Lamb, J.R., 1995. Peptide modulation of allergen-specific immune response. *Curr. Opin. Immunol.* 7, 757–761.
- Hsu, C.-H., Chua, K.-Y., Tao, M.-H., Lai, Y.-L., Wu, H.-D., Huang, S.-K., Hisieh, K.-H., 1996. Immunoanaphylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nature Medicine* 2, 540–544.
- Ishizaka, T., Ishizaka, K., 1984. Activation of mast cells for mediator release through IgE. *Prog. Allergy* 34, 188–235.
- Iwamoto, N., Nishiyama, C., Yasuhara, T., Saito, A., Yuuki, T., Okumura, Y., Okudaira, H., 1996. Direct expression of Der f 2, a

- major house dust mite allergen, in *Escherichia coli*. Int. Arch. Allergy Immunol. 109, 356–361.
- Litwin, A., Pesce, A.J., Fischer, T., Michael, M., Michael, J.G., 1991. Regulation of the human immune response to ragweed pollen by immunotherapy: a controlled trial comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. Clin. Exp. Allergy 21, 457–465.
- Lombardero, M., Heymann, P.W., Platts-Mills, T.A.E., Fox, J.W., Chapman, M.D., 1990. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens: effect of thermal and chemical denaturation on the binding of mouse IgG and human IgE antibodies. J. Immunol. 144, 1353–1360.
- Marsh, D.G., Neely, J.D., Breazeale, D.R., Ghosh, B., Freidhoff, L.R., Ehrlich-Kautzky, E., Schou, C., Krishnaswamy, G., Beaty, T.H., 1994. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science 264, 1152–1156.
- Marshall Jr, G.D., Francis, D., 1997. Dusting off recombinant allergens. Nature Biotechnology 15, 718–719.
- Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.-P., Pribluda, V., Quarto, R., 1986. The receptor with high affinity for immunoglobulin E. Annu. Rev. Immunol. 4, 419–470.
- Miyamoto, T., Oshima, S., Ishizaki, T., Sano, S., 1968. Allergic identity between the common floor mite (*Dermatophagoides farinae* Hughes 1961) and house dust as a causative agent in bronchial asthma. J. Allergy 42, 14–28.
- Moffatt, M.F., Hill, M.R., Cornélis, F., Schou, C., Faux, J.A., Young, R.P., James, A.L., Ryan, G., le Souef, P., Musk, A.W., Hopkin, J.M., Cookson, W.O.C.M., 1994. Genetic linkage of T-cell receptor alpha/delta complex to specific IgE responses. Lancet 343, 1597–1600.
- Mori, A., Kaminuma, O., Suko, M., Mikami, T., Nishizaki, Y., Ohmura, T., Hoshino, A., Asakura, Y., Miyazawa, K., Ando, T., Okumura, Y., Okudaira, H., 1997. Cellular and molecular mechanisms of interleukin-5 synthesis in atopic diseases: a study using allergen-specific human helper T cells. J. Allergy Clin. Immunol. 100, S56–S64.
- Nishiyama, C., Yuuki, T., Takai, T., Okumura, Y., Okudaira, H., 1993. Determination of three disulfide bonds in a major house dust mite allergen, *Der f* II. Int. Arch. Allergy Immunol. 101, 159–166.
- Nishiyama, C., Yuuki, T., Usui, Y., Iwamoto, N., Okumura, Y., Okudaira, H., 1994. Effects of amino acid variations in recombinant *Der f* II on its human IgE and mouse IgG recognition. Int. Arch. Allergy Immunol. 105, 62–69.
- Nishiyama, C., Fukada, M., Usui, Y., Iwamoto, N., Yuuki, T., Okumura, Y., Okudaira, H., 1995. Analysis of the IgE-epitope of *Der f* 2, a major mite allergen, by in vitro mutagenesis. Mol. Immunol. 32, 1021–1029.
- Noon, L., 1911. Prophylactic inoculation against hay fever. Lancet i, 1572–1573.
- Norman, P.S., 1993. Modern concepts of immunotherapy. Curr. Opin. Immunol. 5, 968–973.
- Norman, P.S., Ohman Jr, J.L., Long, A.A., Creticos, P.S., Geftter, M.A., Shaked, Z., Wood, R.A., Eggleston, P.A., Hafner, K.B., Rao, P., Lichtenstein, L.M., Jones, N.H., Nicodemus, C.F., 1996. Treatment of cat allergy with T-cell reactive peptides. Am. J. Respir. Crit. Care. Med. 154, 1623–1628.
- O'Brien, R.M., Byron, K.A., Varigos, G.A., Thomas, W.R., 1997. House dust mite immunotherapy results in decrease in *Der p* 2-specific IFN- γ and IL-4 expression by circulating T lymphocytes. Clin. Exp. Allergy 27, 46–51.
- O'Brien, R.M., Thomas, W.R., Nicholson, I., Lamb, J.R., Tait, B.D., 1995. An immunogenic analysis of the T-cell recognition of the major house dust mite allergen *Der p* 2: identification of high and low-responder HLA-DQ alleles and localization of T-cell epitopes. Immunology 86, 176–182.
- O'Hehir, R.E., Verhoef, A., Panagiotopoulou, E., Keswani, S., Hayball, J.D., Thomas, W.R., Lamb, J.R., 1993. Analysis of human T cell responses to the group II allergen of *Dermatophagoides* species: localization of major antigenic sites. J. Allergy Clin. Immunol. 92, 105–113.
- Platts-Mills, T.A.E., Chapman, M.D., 1987. Dust mites: immunology, allergic disease, and environmental control. J. Allergy Clin. Immunol. 80, 755–775.
- Platts-Mills, T.A.E., Weck, A.L., 1989. Dust mite allergens and asthma — a worldwide problem. J. Allergy Clin. Immunol. 83, 416–427.
- Raz, E., Tighe, H., Sato, Y., Corr, M., Dudler, J.A., Roman, M., Swain, S.L., Spiegelberg, H.L., Carson, D.A., 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. Proc. Nat. Acad. Sci. USA 93, 5141–5145.
- Secrist, H., DeKruyff, R.H., Umets, D.T., 1995. Interleukin 4 production by CD4⁺ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. J. Exp. Med. 181, 1081–1089.
- Smith, A.M., Chapman, M.D., 1996. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen *Der p* 2. Mol. Immunol. 33, 399–405.
- Suko, M., Mori, A., Ito, K., Okudaira, H., 1995. Oral immunotherapy may induce T cell anergy. Int. Arch. Allergy Immunol. 107, 278–281.
- Takai, T., Yuuki, T., Okumura, Y., Mori, A., Okudaira, H., 1997a. Determination of the N- and C-terminal sequences required to bind human IgE of the major house dust mite allergen *Der f* 2 and epitope mapping for monoclonal antibodies. Mol. Immunol. 34, 255–261.
- Takai, T., Yokota, T., Yasue, M., Nishiyama, C., Yuuki, T., Mori, A., Okudaira, H., Okumura, Y., 1997b. Engineering of the major house dust mite allergen *Der f* 2 for allergen-specific immunotherapy. Nature Biotechnology 15, 754–758.
- Tsitoura, D.C., Verhoef, A., Gelder, C.M., O'Hehir, R.E., Lamb, J.R., 1996. Altered T cell ligands derived from a major house dust mite allergen enhance IFN γ but not IL-4 production by human CD4⁺ T cells. J. Immunol. 157, 2160–2165.
- Valenta, R., Kraft, D., 1995. Recombinant allergens for diagnosis and therapy of allergic diseases. Curr. Opin. Immunol. 7, 751–756.
- van Neerven, R.J.J., Ebner, C., Yssel, H., Kapsenberg, M.L., Lamb, J.R., 1996. T-cell responses to allergens: epitope-specificity and clinical relevance. Immunology Today 17, 526–532.
- van Neerven, R.J.J., van't Hof, W., Ringrose, J.H., Jansen, H.M., Aalberse, R.C., Wierenga, E.A., Kapsenberg, M.L., 1993. T cell epitopes of house dust mite allergen *Der p* II. J. Immunol. 151, 2326–2335.
- van't Hof, W., Dridijk, P.C., van den Berg, M., Beck-Sickinger, A.G., Jung, G., Aalberse, R.C., 1991. Epitope mapping of the *Dermatophagoides pteronyssinus* house dust mite major allergen *Der p* II using overlapping synthetic peptides. Mol. Immunol. 28, 1225–1232.
- Voohorst, R., Spieksma-Boezeman, M.I.A., Spieksma, F.T.M., Varekamp, H., Leupen, M.J., Lyklema, A.W., 1967. The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces: identity with the house dust allergen. J. Allergy 39, 325–339.
- Vrtala, S., Hirtlenlehner, K., Vangelista, L., Pastore, A., Eichler, H.G., Sperr, W.R., Valent, P., Ebner, C., Kraft, D., Valenta, R., 1997. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candi-

- dates for a novel form of specific immunotherapy. J. Clin. Invest. 99, 1673–1681.
- WHO, Nomenclature Committee for Factors of the HLA System, 1995. Nomenclature for factors of the HLA system 1995. Vox Sang 69, 359–372.
- Yamashita, N., Haida, M., Suko, M., Okudaira, H., Miyamoto, T., 1989. Allergens of house dust mite *Dermatophagoides farinae*. II: immunological characterization of four allergic molecules. Int. Arch. Allergy Appl. Immunol. 88, 173–175.
- Yssel, H., Johnson, K.E., Schneider, P.V., Wideman, J., Terr, A., Kastelein, R., de Vries, J.E., 1992. T cell activation-inducing epitopes of the house dust mite allergen Der p. I: proliferation and lymphokine production patterns by Der p I-Specific CD4+ T cell clones. J. Immunol. 148, 738–745.
- Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M., Okudaira, H., 1990. Cloning and sequencing of cDNAs corresponding to mite major allergen *Der f* II. Jpn. J. Allergol. 39, 557–561.
- Yuuki, T., Okumura, Y., Ando, T., Yamakawa, H., Suko, M., Haida, M., Okudaira, H., 1991. Cloning and expression of cDNA coding for the major house dust mite allergen *Der f* II in *Escherichia coli*. Agric. Biol. Chem. 55, 1233–1238.