

Hypoallergenic and T cell reactive analogue peptides of bovine serum albumin, the major beef allergen

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Abstract

We recently identified IgE-binding epitopes of bovine serum albumin (BSA), the major beef allergen, and found that the major epitope structure contains an EXXV motif. Here we report hypoallergenic analogue peptides of BSA that preserve T cell reactivity to retain the capacity to induce immunotolerance. Substitution of the glutamic acid in the EXXV sequence to aspartic acid leads to a remarkable reduction of IgE-binding ability and induces potent T cell proliferation. Moreover, in response to the substitutions, the production of INF- γ is markedly increased compared with that elicited by the native peptides. Therefore, these analogue peptides represent potential candidates for specific immunotherapy.

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1. Introduction

Type I allergy, including food allergy, represents an immune disorder that affects almost 30% of the population in some countries (Hopkin, 1997). In this disease, allergens crosslink specific IgE antibodies bound to the surface of mast cells and basophils, to trigger the release of inflammatory mediators, such as histamine and leukotriene, which cause allergic symptoms. Although allergic symptoms can be ameliorated by pharmacological treatment, hyposensitizing therapy or allergen-specific immunotherapy (SIT) represents the only curative approach to type I allergy (Durham et al., 1999); (Rolland and O’Hehir, 2002). SIT protocols involve the repeated administration of the allergen itself over prolonged periods of time, which induces relative immunologic tolerance. However, the provocation of possibly life-threatening anaphylactic reactions occasionally occurs with this approach, due to the binding of the administered allergen to allergen-specific IgE antibodies. This can be avoided if allergen-derived synthetic peptides are used in-

stead of whole allergenic proteins (Rolland and O’Hehir, 2002). Until now, accumulating evidence implicates the usefulness of T cell reactive peptides in the clinical treatment of cat and bee venom allergy (Norman et al., 1996); (Muller et al., 1998).

Beef allergy is reported to have with an incidence between 3.3 and 6.5% among children with atopic dermatitis (Fiocchi et al., 2000). The major allergen in beef is bovine serum albumin (BSA) (Fiocchi et al., 2000); (Tanabe et al., 2002). Fiocchi et al. (2000) reported that all beef-allergic children tested reacted to BSA in a skin-prick test. Serum albumins are also known causes of cat- and dog-dander allergy (van Ree et al., 1999); (Pandjaitan et al., 2000). BSA and non-allergenic human serum albumins (HSA) display approximately 76% sequence homology and a repeating pattern of disulfides that is strictly conserved (Gelamo and Tabak, 2000). Recently, we found that BSA-specific IgE antibodies and T cells react primarily with sequential epitopes that differ greatly between BSA and HSA (Tanabe et al., 2002). Among eight IgE-binding epitopes identified by us (Tanabe et al., 2002), three were found to contain an EXXV motif (HPEYAVSVLL (p336–345), PVESKVT (p466–472), and VMENFVAF (p545–552)). The corresponding sequences in HSA are HPDYSVLLLL, PVSDRVT, and VMDDFAAF, respectively. Comparing two epitopic sequences (p336–345 and p545–552) in BSA

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HSA, human serum albumin; PBMC, peripheral blood mononuclear cells; SI, stimulation index; SIT, specific immunotherapy

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Table 1
List of BSA peptides and their analogues used in this study

Peptides	Sequence synthesized	Corresponding sequence in HSA
p336–345	HPEYAVSVLL	HPDYSVVLLL
E338D	HPDYAVSVLL	
p545–552	VMENFVAF	VMDDFAAF
E547D	VMDNFVAF	

Two epitopic peptides were synthesized (p336–345 and p545–552). Their analogue peptides were also synthesized; that is, E338D and E547D indicate that ³³⁸Glu and ⁵⁴⁷Glu are substituted to Asp. Synthesized peptides were dissolved in DMSO at a concentration of 5 mM for storage. Underlined residues represent amino acid differences between BSA and HSA.

with the corresponding sequences in HSA, it is likely that E residues (E338 and E547) are important for recognition by IgE-antibodies since the corresponding residues in HSA are D in both p336–345 and p545–552. Therefore, in this study, we synthesized two analogue peptides, E338D (HPDYAVSVLL) and E547D (VMDNFVAF), with amino acid substitutions from E to D (see Table 1), and characterized their IgE-binding abilities. The effects of the analogue peptides on T cell responses were also examined. Here, we report that the substitution of E to D leads to a remarkable reduction in IgE-binding ability and induces potent T cell proliferation. Cytokine secretion by PBMC following incubation with the analogue peptides was also investigated in this study, and it was revealed that the analogues alter the INF- γ :IL-4 ratio by selectively enhancing INF- γ secretion. The usefulness of these analogues for allergen-specific immunotherapy is discussed.

2. Materials and methods

2.1. Patients

Peripheral blood samples (2–3 mL) were obtained from five 4-year-old patients (#1–5) with atopic dermatitis and beef allergy. Beef allergy was proved by clinical symptoms and a positive radioallergosorbent test (CAP-RAST score: 4–6 for beef). Informed consent was obtained from all donors (their parents). Serum from a non-allergic adult was also used as a negative control. Serum samples were used for ELISA after appropriate dilution with 0.1% HSA and 0.05% Tween 80 containing PBS. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density centrifugation in lymphocyte separation medium (ICN Biomedicals Inc., USA), and used in the lymphocyte proliferation assays.

2.2. Synthesis of BSA-derived peptides (Table 1)

As epitopic peptides of BSA that bind IgE, peptides corresponding to amino acid positions 336–345 (HPEYAVSVLL) and 545–552 (VMENFVAF) of BSA were synthesized es-

entially according to the solid phase method using a peptide synthesizer (PSSM-8, Shimadzu, Japan). Their analogue peptides, E338D (HPDYAVSVLL) and E547D (VMDNFVAF), were also synthesized. The synthesized peptides were applied to reversed-phase HPLC, and the main peak was collected. The purified peptides were lyophilized, dissolved in dimethylsulfoxide (DMSO) to a concentration of 5 mM, and stored at -80°C until use.

2.3. Inhibition ELISA

Inhibition ELISAs were performed by the usual method (Tanabe et al., 1996, 2002). Briefly, peptides (50 μM final concentration) were added to serum samples diluted fivefold in 0.05% Tween 80 containing PBS. After 1 h preincubation at ambient temperature, the peptide-serum mixture was applied to polystyrene microtiter plates (Nunc, Denmark) coated with BSA (5 μg per well) as an antigen and blocked with 1% HSA in PBS. Serum not treated with peptides was used as a control. The binding of IgE-antibody to BSA was determined by sequential incubations with biotinylated anti-human IgE, streptavidin-peroxidase conjugate, and o-phenylenediamine. Percent inhibition was calculated as $(1 - A/B) \times 100$, where A is the ELISA value of serum preincubated with peptide, and B is that of the control.

2.4. Lymphocyte proliferation assays

PBMCs from four patients (#1–4) were washed and resuspended in RPMI1640 medium containing 10% human AB serum, 3 mM glutamine, 50 μM 2-mercaptoethanol, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. (We could not perform this assay using a sample from patient #5 due to the difficulty in obtaining a sufficient volume of blood from the patient.) The PBMCs (1×10^5 per well) were cultured in a 96-well cell culture plate (Nunc) for 72 h at 37°C under a humidified 5% CO_2 atmosphere in the presence of 10–50 μM of synthesized peptides. Cultures of PBMCs alone were included as negative controls. Cultures of PBMCs in the presence of 10 $\mu\text{g}/\text{mL}$ phytohemagglutinin (PHA, Wako Pure Chemical Industries) were also assayed as positive controls. The cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for the final 24 h of culture. After culture, the plate was centrifuged at 300 g for 10 min, and the BrdU incorporated by the pelleted cells was measured with an ELISA kit (Roche, USA).

2.5. Determination of INF- γ and IL-4 concentrations

PBMCs from patient #3 were used for cytokine analyses. PBMCs (1×10^5 per well) were cultured in a 96-well cell culture plate for 24 h with or without the BSA-derived peptides (25 μM) as described above. After culture, the plate was centrifuged at 300 g for 10 min, and INF- γ and IL-4 concentrations of the culture supernatants were determined

by sandwich ELISAs (Biotrak, Amersham Biosciences, Japan). The cytokine secretion of control cultures (PBMCs alone) was subtracted from that of cultures containing BSA-derived peptides.

3. Results

3.1. Effects of amino acid substitution on IgE-binding properties

In order to clarify whether E residues in BSA epitopic peptides (HPEYAVSVLL (p336–345) and VMENFVAF (p545–552)) are essential for IgE-binding, we first synthesized two analogue peptides, E338D (HPDYAVSVLL) and E547D (VMDNFVAF), with amino acid substitutions from E to D residues, as well as the native peptides (Table 1), and used them for inhibition ELISA. As demonstrated in Fig. 1, the native p336–345 peptide was recognized by IgE antibodies from all five patients, while the native p545–552 was recognized by IgE antibodies from two (#2 and #4) of the five patients. The substitution at E338D led to a marked reduction of IgE-binding in all five patients (p336–345 ver-

sus E338D). Especially, in patients #1 and #3, the ELISA values for E338D dropped below the limit of detection, and, in patient #2, the ELISA value was much less than that for the native p336–345. In addition, the analogue peptide carrying an amino acid substitution at ⁵⁴⁷E, E547D, abrogated IgE-binding in both two patients (#2 and #4). Thus, it is likely that ³³⁸E in p336–345 and ⁵⁴⁷E in p545–552 are important for recognition by patient IgE antibodies. IgE antibodies from patients #1, #3 and #5 recognized neither p545–552 nor E547D.

3.2. Analogue peptides induced potent T cell proliferation

Cultured PBMCs from patients #1–4 were stimulated with the BSA peptides and their analogues, and the results of the lymphocyte proliferation assays are shown in Fig. 2. PBMCs from all patients tested showed very low (stimulation index (SI) <1.5) lympho-proliferative responses to the native peptides, p336–345 and p545–552. Stimulation by E338D induced slightly more potent proliferation in patients #1–3 compared to the native p336–345. Of interest was that the substitution of ⁵⁴⁷E to D resulted in outstanding proliferation of PBMCs from all patients (indicated by black bars in Fig. 2). In fact, the SI values for E547D rose above 2 for the PBMCs from patients #1 and #4. Moreover, stimulation by

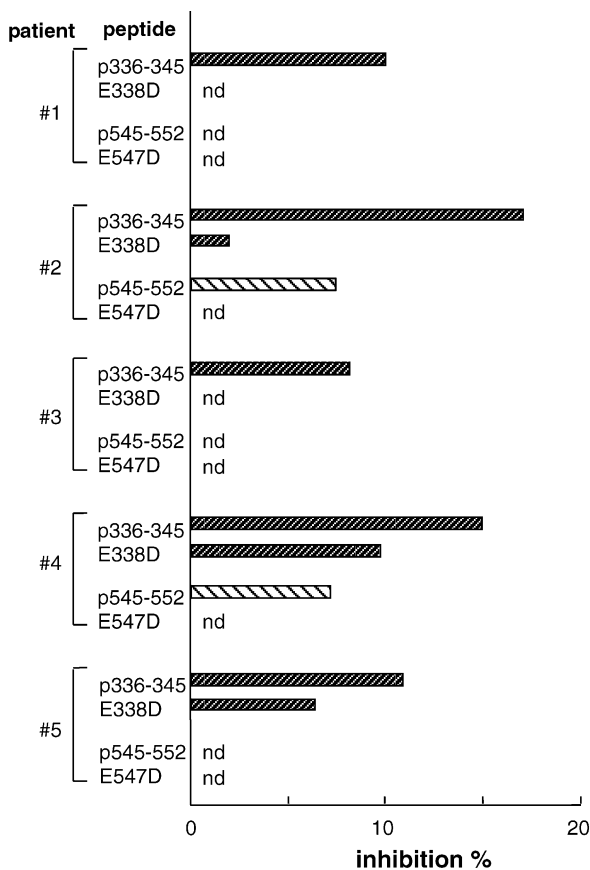


Fig. 1. Inhibition ELISA of BSA peptides and their analogues. The importance of E residues in p336–345 and p545–552 on IgE-binding was evaluated. The substitutions at E338D and E547D led to remarkable reductions in IgE-binding in all five patients, which suggests that E338 and E547 are important for recognition by patient IgE antibodies.

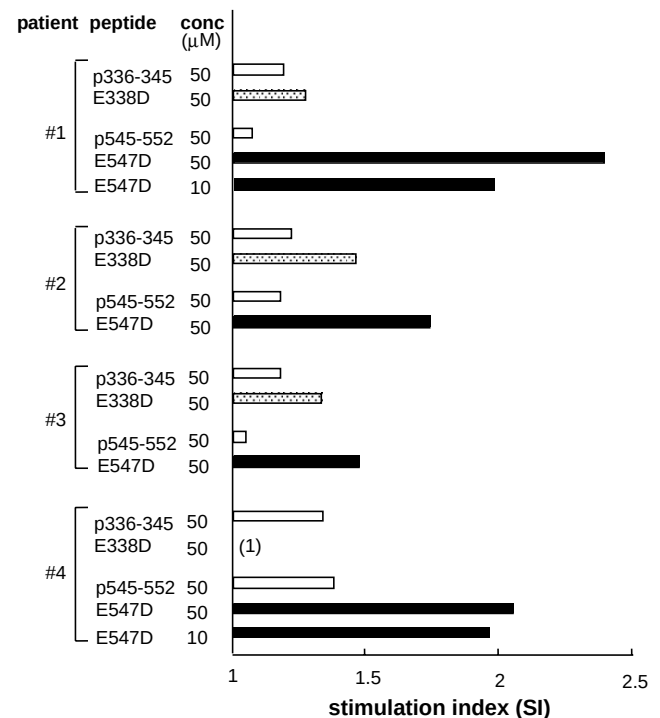


Fig. 2. Lymphocyte proliferation assay of BSA peptides and their analogues. PBMCs (patients #1–4) were stimulated with BSA peptides (50 μM) or their analogues (10 or 50 μM) for 72 h, and BrdU incorporation was measured. The substitutions of E to D induced potent T cell proliferation.

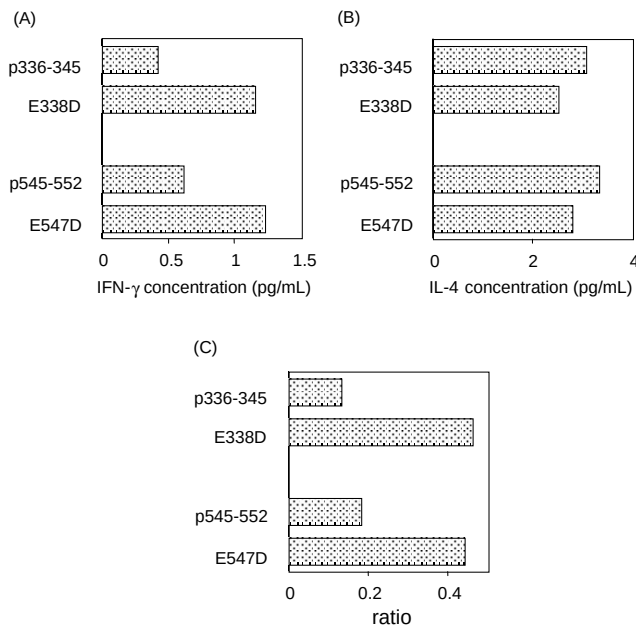


Fig. 3. Cytokine secretion by PBMCs following incubation with BSA peptides and their analogues. PBMCs (patient #3) were stimulated with BSA peptides or their analogues. Supernatants were harvested 24 h later for the determination of IFN- γ and IL-4 concentrations (A and B). IFN- γ /IL-4 ratios induced by BSA peptides and their analogues were calculated (C). The production of IFN- γ induced by the analogues (E338D and E547D) was markedly higher than that elicited by the native peptides (p336–345 and p545–552) (A). In the meantime, the analogues induced almost the same level of IL-4 secretion as the native peptides (B).

a one-fifth concentration of E547D (10 μ M) induced more potent proliferation in patients #1 and #4 compared to the native p545–552 (50 μ M). Thus, fairly low concentrations of E547D can induce a stronger proliferative response than at least a 5 times greater concentration of native p545–552.

3.3. Analogue peptides alter the IFN- γ :IL-4 ratio by selectively enhancing IFN- γ secretion

Using PBMCs from patient #3, we evaluated cytokine secretion by PBMCs following incubation with BSA peptides and their analogues. In response to substitutions with D at positions 338 and 547, the production of IFN- γ (Th-1 type cytokine) was markedly increased compared with that elicited by the native peptides p336–345 and p545–552 (Fig. 3A). The ability of the BSA-derived peptides to stimulate IL-4 (Th-2 type cytokine) production by PBMCs was also investigated. The analogue peptides induced almost the same level of IL-4 secretion as the native peptides (Fig. 3B). Consequently, these divergences in cytokine profiles between the native and analogue peptides are clearly reflected by the higher ratio of IFN- γ : IL-4 secretion induced by the two analogue peptides, E338D and E547D (Fig. 3C).

We got the almost same results from other patients and showed here the results from patient #3 as representative ones. Except for patient #3, we could not perform this assay sufficiently and repeatedly due to the difficulty in obtaining

a plenty volume of fresh whole blood from other patients, all of whom were very young children.

4. Discussion

Serum albumin is one of the most widely studied and applied proteins in biochemistry. It is also known as the major beef allergen (Fiocchi et al., 2000; Tanabe et al., 2002). The importance of bovine serum albumin (BSA) in beef allergy was substantiated first by Fiocchi et al. (1995), who showed that all beef-allergic children tested reacted to BSA in a skin-prick test. Japanese groups, including ours, confirmed this fact afterwards (Han et al., 2000). However, there had been limited information on the IgE-binding and T cell epitope of BSA until we carried out a break-through study (Tanabe et al., 2002). Some human IgE-binding epitopes were identified from the regions in which the amino acid sequences of BSA and human serum albumin (HSA) differ greatly (Tanabe et al., 2002).

We also predicted that the EXXV sequence was minimally required for IgE-binding (Tanabe et al., 2002). Thus, the importance of E residues in p336–345 and p545–552 for IgE-binding was first evaluated in this study (Fig. 1). We found that substitutions of ³³⁸E and ⁵⁴⁷E to D lead to a remarkable reduction in IgE binding. This indicates that these E residues are important for recognition by IgE antibodies in patients. Since the sequences corresponding to HPEYAVSVLL (p336–345) and VMENFVAF (p545–552) in non-allergenic HSA are HPDYSVLL and VMDDFAAF, respectively (Table 1), it is highly likely that the difference between D (human type) and E (bovine type) at positions 338 and 547 is a major cause for IgE-recognition by patients. According to the three-dimensional structure of HSA, these two D residues are located at the surface of the molecule (Fig. 4). Although the three-dimensional structure of BSA is not available, the corresponding E residues at positions 338 and 547 in BSA are assumed to be located similarly to the D residues in HSA since the tertiary structures of BSA and HSA are very similar to one another (Peters, 1985). Therefore, it is possible that IgE-antibodies of patients easily recognize E residues at positions 338 and 547 on the surface of BSA, with the subsequent allergic reaction taking place.

Clinically, insight into the IgE-binding epitopes of BSA is quite useful, since modifying these epitopes might lead to better designs for beef-allergy-specific immunotherapy. In this respect, E338D and E547D might be useful for safer allergen-specific immunotherapy (SIT). Since, however, SIT is a T cell-targeted strategy explored with the awareness of the pivotal role of allergen-specific CD4⁺ T cells in regulating the immune response (Rolland and O'Hehir, 2002), the T cell responses against E338D and E547D should be evaluated. Lymphocyte proliferation assays of the analogue peptides, E338D and E547D, were thus performed (Fig. 2). As a result, the analogue peptides, especially E547D, induced

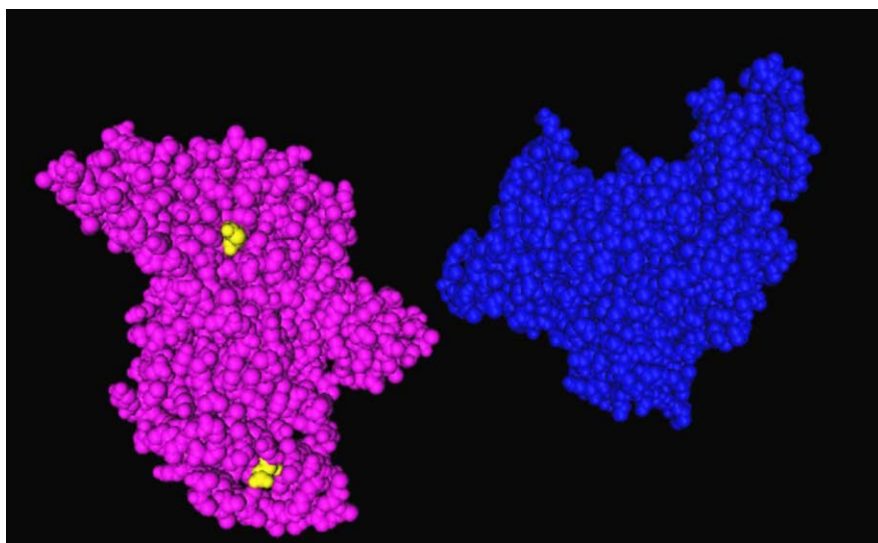


Fig. 4. Molecular structure of HSA. The molecular structure of HSA is shown in gray, and the two aspartic acid residues (upper, D338; lower, D547) are highlighted in white. The drawing was generated with software Cn3D ver. 4.1 (www.ncbi.nlm.nih.gov).

far more potent proliferation than intact BSA peptides in patient PBMCs. Therefore, these analogue peptides, especially E547D, represent potential candidates for SIT since it is the most hypoallergenic and T cell reactive one.

Recently, a shift in the balance between Th-1 and Th-2 cell responses has been stressed as the major underlying cause for atopic diseases, in which the Th-2 response producing IL-4 predominates over the Th-1 response producing INF- γ resulting in the production of excess IgE antibodies (Romagnani, 1997; Rolland and O'Hehir, 2002). In this context, SIT is now attracting clinical attention, since it is possible to improve the Th-1/Th-2 balance. SITs using T-cell reactive peptides (peptide immunotherapy) have already been successfully tried clinically for the major cat allergen *Feld 1* (Norman et al., 1996) and the major bee venom allergen phospholipase A2 (Muller et al., 1998). In addition, strategies for the generation of hypoallergenic recombinant allergens by site-directed mutagenesis have been developed for birch pollen (Vrtala et al., 2000), timothy grass pollen (Schramm et al., 1999) and house dust mite allergens (Takai et al., 1997); (Korematsu et al., 2000). Recombinant mite allergen, C8/119S, has been reported to induce potent and almost exclusive differentiation of Th-1 cells from the peripheral blood of atopic patients in vitro (Korematsu et al., 2000). Based on this background, we next investigated the production of two representative cytokines, INF- γ and IL-4.

INF- γ and IL-4 secretion by PBMCs from patient #3 following incubation with BSA peptides and their analogues was evaluated (Fig. 3). The analogue peptides were found to alter the INF- γ : IL-4 ratio by selectively enhancing INF- γ secretion. From the data shown in Figs. 2 and 3, the substitutions of ³³⁸E and ⁵⁴⁷E to D, especially ⁵⁴⁷E, were found to alter T-cell recognition significantly. The results shown in Fig. 3 coincide well with the studies on house dust mite

(Tsitoura et al., 1996) and Japanese cedar pollen (Ikagawa et al., 1996) allergens in which T cell epitope analogue peptides have been shown to enhance the production of INF- γ by T cells compared with the native peptides. Since it is recognized that an increase in INF- γ production brings about a reduction in allergen-specific IgE antibodies, BSA analogue peptides, especially E547D, with the ability to enhance INF- γ secretion, would be applicable for SIT. Hypoallergenic and short T cell reactive peptides such as E547D also offer considerable advantages for SIT because they cannot crosslink mast-cell-bound IgE antibodies and, thus, can be given safely at high doses, with consequent improved efficacy. Additional studies are planned to clarify the mechanisms by which the analogue peptides selectively enhance Th-1 responses.

In conclusion, we have shown that E338D and E547D are hypoallergenic analogue peptides of BSA that preserve T cell reactivity to retain the capacity to induce immunotolerance. The present results further imply that E547D, which completely abrogates IgE-binding, may provide a useful approach to developing safer and effective hyposensitizing tools for beef allergy. These analogue peptides are also applicable to immunological studies as an excellent tool for elucidating beef allergy.

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