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Abbreviations: **GM**: Goat's Milk, **CM**: Cow's Milk, **GSM:** Goat's or sheep's milk, **βcap**: caprine β-casein, **βbov**: bovine β-casein, **mAb**: monoclonal antibody, **EAST**: Enzyme Allergo Sorbent Test, **RBL**: Rat Basophil Leukemia, **OIT**: oral immunotherapy.

Abstract

Background and objective: Goat's milk (GM) allergy associated with tolerance to cow's milk (CM) has been reported in patients without history of CM-allergy and in CM-allergic children successfully treated with oral immunotherapy. The IgE antibodies from GM-allergic/CM-tolerant patients recognize caprine β-casein (βcap) without cross-reacting with bovine β-casein (βbov) despite a sequence identity of 91%. In the present study, we investigated the non-cross-reactive IgE-binding epitopes of βcap.

Methods: Recombinant βcap was genetically modified by substituting caprine domains with the bovine counterparts and by performing site-directed mutagenesis. We then evaluated the recognition of modified βcap by IgE antibodies from 11 GM-allergic/CM-tolerant patients and 11 CM-allergic patients or by monoclonal antibodies (mAb) raised against caprine caseins. The allergenic potency of modified βcap was finally assessed by degranulation tests of humanized rat basophil leukemia (RBL)-SX38 cells.

Results: Non-cross-reactive epitopes of βcap were found in domains 44-88 and 130-178. The substitutions A55T/T63P/L75P and P148H/S152P induced the greatest decrease of IgE-reactivity of GM-allergic/CM-tolerant patients toward βcap. The pivotal role of threonine 63 was particularly revealed as its substitution also impaired the recognition of βcap by specific mAb which could discriminate between βcap and βbov. The modified βcap containing the five substitutions was then unable to trigger the degranulation of RBL-SX38 cells passively sensitized with IgE antibodies from GM-allergic/CM-tolerant patients.

Conclusions: Although IgE-binding epitopes are spread all over βcap, a non-cross-linking version of βcap was generated with only five amino acid substitutions and could thus provide new insight for the design of hypoallergenic variants.

INTRODUCTION

Cow's milk (CM) allergy is the most common food allergy in early childhood, affecting 2% to 3% of infants [1-3]. As goat's or sheep's milk (GSM) proteins are highly homologous to CM proteins, clinical cross-reactivity is expected and IgE sensitization to GSM proteins has been found to be as high as 92% to 98% in children with IgE-mediated CM allergy [4;5]. Conversely, CM-tolerant individuals should also tolerate GSM. However, several cases of GSM allergy without CM allergy have been regularly reported [6-13]. The development of this allergy is not dependent of an earlier sensitization to CM proteins since only 19% of the GSM-allergic/CM-tolerant patients had suffered from an outgrown CM allergy [14]. Moreover, a relatively high prevalence of allergy to GSM has been recently reported in a population of CM-allergic children successfully treated with oral immunotherapy [15-17].

With a mean age of 6 years, GM-allergic/CM-tolerant patients are generally older than those presenting CM allergy [10]. The symptoms are frequently severe with cases of angio-oedema or anaphylaxis induced by minimal amounts of GSM or cheese [10;14]. Allergic reactions are elicited by caprine caseins, as IgE antibodies of GM-allergic/CM-tolerant patients recognized principally αS1-, αS2- and β-caseins, and not the whey proteins [10]. Under physiological conditions, caseins are considered as natively unfolded or intrinsically unstructured proteins, with some elements of secondary structure but no defined tertiary structure [18-21]. As a consequence, immunogenicity and allergenicity of caseins are not affected by heat-treatment [22;23]. Accordingly, a recent study reported that heating does not decrease immunogenicity of GSM [24]. The levels of casein-specific IgE antibodies have also been found to be informative in predicting reactivity to baked CM [25]. In this regard, development of hypoallergenic caseins could provide an attractive alternative for immunotherapeutic treatment of patients reactive to baked milk.

The GM-allergic/CM-tolerant patients display no or very weak IgE cross-reactivity between bovine and caprine caseins despite sequence identities ranging from 87% to 91% [10]. The caprine β-casein (βcap) can constitute up to 60% of caprine whole casein and is 91% and 99.5% identical to the bovine and ovine counterparts, respectively [26;27]. In a previous study, we identified the βcap peptide (f29-107) as the most immunoreactive fragment among different plasmin-derived peptides [28]. In the present work, we used an approach based on the production of recombinant fusion proteins between different domains of βcap and the bovine β-casein (βbov) in order to further characterize the non-cross-reactive epitopes. Substitutions occurring between βbov and βcap were then introduced in IgE-binding domains of rβcap. Their impact on rβcap immunoreactivity was evaluated with IgE antibodies from GM-allergic/CM-tolerant patients or with murine monoclonal antibodies (mAb). Substitution of only five amino acids was then shown to abolish the capacity of rβcap to trigger the degranulation of rat basophil leukemia (RBL)-SX38 cells loaded with IgE antibodies from GM-allergic/CM-tolerant patients.

MATERIALS and METHODS

Human sera

Sera for this retrospective study were collected from 11 CM-allergic children and 11 GM-allergic/CM-tolerant children patients recruited in two different paediatric allergy units, after informed consent from patient's parents (Table 1 and [10;28]). The IgE-mediated allergy to GM was confirmed by clinical manifestations observed in less than one hour after ingestion of GM proteins. The absence of CM allergy was established by routine ingestion of CM or derived products without any adverse reaction.

Recombinant and native allergens

Genes encoding βbov and βcap (Swiss-Prot accession number P02666 and P33048, Fig. S1) were synthesized by using codons optimized for bacterial expression (Genscript USA Inc., Piscataway, NJ, USA). The SacI, PstI, AatII, KpnI and BamHI restriction sites were silently inserted into the *βbov-csn2* and *βcap-csn2* open reading frames in order to generate fusion constructs between 5' end of *βbov-csn2* and complementary 3' end of *βcap-csn2* (Fig. S2). Site-directed mutagenesis was generated by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Expression and purification of recombinant proteins were performed (Fig. S3), as previously described [29;30]. Circular dichroism analysis of the recombinant proteins confirmed the unfolded nature of β-caseins with the presence of regions of little ordered structure (Fig. S4), as previously described [20;21]. For more information, see the supplemental Materials and Methods section.

Production of mAb specific to caprine β-casein

Monoclonal antibodies were produced by conventional techniques as previously described [31]. Before being sensitized to caprine caseins, eight BALB/c mice received 5 mg of bovine whole casein by intragastric gavages on days 1, 2, 3, 7, 8 and 9 in order to induce oral tolerance toward bovine caseins. After three intraperitoneal injections on days 13, 36 and 50 of caprine whole casein (50µg) emulsified in incomplete Freund's adjuvant, the mouse displaying the highest IgG1 response to caprine caseins was selected for preparation of monoclonal antibodies. Spleen cells were fused with NS1 mouse myeloma cells and specific antibodies were measured in myeloma culture supernatants. Positive hybridoma cells were cloned and expanded as ascitic fluids in BALB/c mice. All experiments were performed in compliance with the French and European regulations on care and protection of Laboratory Animals (EC Directive 86/609, French Law 2001-486, June 6, 2001) with permission 91-493 of French Veterinary Services in a laboratory animal facility care-approved by the French Veterinary Services and CEA agreement D-91-272-106 from the Veterinary Inspection Department of Essonne (France).

IgE quantification by direct Enzyme Allergo Sorbent Test (EAST)

IgE responses to the different recombinant β-caseins were quantified using a direct EAST in which purified antigens (5µg/mL) were passively adsorbed on microtiter plates as previously described [5]. After incubation with adequate dilutions of sera (50 µL/well), IgE-binding was revealed by the addition of labelled anti-human IgE monoclonal antibody BS17. Tracer was prepared by covalent linkage of the purified proteins to the tetrameric form of acetylcholinesterase according to Bernard *et al.* [5]. After washing, Ellman's reagent was used as the enzyme substrate and absorbance was measured at 414 nm.

IgE-binding capacity as determined by reverse EAST inhibition

Plates were coated with anti-human IgE monoclonal antibody LE27 [32]. Fifty µL/well of serum from each patient at adequate dilutions were incubated overnight at 4°C. After washing, 50 µL of inhibitors (*i.e.* increasing concentrations of recombinant β-casein) and 50 µL of native βbov or βcap, labelled as previously described [28], were mixed and incubated for 4 h at room temperature. Results were expressed as B/B0, where B0 and B represent the amount of labelled β-casein bound to immobilised IgE antibodies in the absence or presence of a known concentration of inhibitor. The IgE reactivity of competitors was calculated with the concentration inhibiting 50% of the IgE binding to labelled β-casein (IC50) by using GraphPad Prism 5.01 software.

IgE-binding to βcap presented by mAb SCB1D

In this assay, the βcap-specific mAb SCB1D was used as a solid-phase coupled allergen-catching reagent as previously described [32;33]. After washing of the plates directly coated with mAb SCB1D, 100 µL of βcap (5 µg/mL) were dispensed in each well. After 4h incubation, plates were washed and adequate dilutions of serum from each patient were added. After 24h incubation at 4°C, specific IgE-binding was revealed by the addition of labelled anti-human IgE monoclonal antibody BS17.

Mediator release assay

Degranulation assay was performed with RBL SX -38 cells [34]. Cells were passively sensitized with IgE antibodies immunopurified from a pool of five sera from GM-allergic/CM-tolerant patients (serum 178, 183, 193, 86 and 24) or a pool of two sera from CM-allergic patients (serum 76 and 100). Mediator release induced by incubation with

different concentrations of caseins was determined by measuring the enzymatic activity of β-hexosaminidase. Results were expressed as percentage of the reference release induced with anti-human IgE (LE27 clone; 100 ng/mL).

Statistical analysis

Data were analyzed using the non-parametric Wilcoxon matched pairs signed rank test. Statistical analyses were performed with GraphPad Prism 5.01 software and a $P<0.05$ was considered significant (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

RESULTS

IgE responses to native and recombinant β-caseins

The recombinant β-caseins, rβbov and rβcap, were produced and purified near to homogeneity (Fig. S3). As shown in Fig. 1, rβbov and rβcap displayed IgE-binding capacities comparable to those of the native allergens. While IgE antibodies of CM-allergic patients recognized similarly rβbov and rβcap, IgE responses of GM-allergic/CM-tolerant patients to rβcap were always much higher than those to rβbov (rβcap *vs* rβbov, p=0.001, Fig. 1).

Localization of the non-cross-reactive immunodominant epitopes in caprine β-casein

Four fusion proteins were generated by substituting caprine domains with the corresponding bovine domains (Fig. 2A). These fusion proteins displayed an IgE-binding capacity similar to those of rβcap and rβbov when testing sera from CM-allergic patients (Fig. S5). Conversely, with sera from GM-allergic/CM-tolerant patients, IgE-binding capacity of fusion proteins was decreasing as the caprine sequence was progressively replaced by that of rβbov (Fig. 2B). Two immunodominant non-cross-reactive domains were thus identified. For eight out of eleven patients, 50% to 100% of rβcap IgE-binding capacity was sustained by the caprine domain 44-88. For three patients, the highest decrease of IgE response occurred with the substitution of the caprine domain 130-178 (Fig. 2B).

Identification of the substitutions supporting the non-cross-reactivity of rβcap

Compared to rβbov, domains 44-88 and 130-178 of rβcap contain three and five substitutions, respectively (Fig. S1). Site-directed mutagenesis of the gene encoding rβcap was then undertaken to identify the critical residues for IgE-binding. Considering the domain 44-88, the eight tested sera displayed a reduced IgE-reactivity toward the allergen containing

the T63P substitution (Fig. 3A). In comparison, substitutions L75P or A55T had less influence on IgE-binding capacity of rβcap. However, IgE-reactivity of the triple mutant was much lower than that of the T63P mutant, thus showing a cumulative impact of these three substitutions. Considering the domain 130-178, the double mutation P148H/S152P affected the IgE-binding capacity of rβcap with sera of patients 24, 86 and, to a lesser extent, 53 (Fig. 3B and 3C). The IgE response of patient 53 to the domain 130-178 also involved the K132N substitution (Fig. 3C). The IgE-binding capacity of rβcap was never affected by the substitution P168S.

Production of murine mAb distinguishing rβcap from rβbov

In order to produce monoclonal antibodies which could recognize βcap without cross-reacting with βbov, BALB/c mice were fed six tolerogenic doses of whole bovine casein before being immunized to whole caprine casein (see Materials and Methods). Among the 16 positive hybridoma selected for expansion as ascitic fluids, 14 recognized αS1- or κ-caseins and two mAbs were directed against βcap. These two mAbs, SCB1D and SCB4C, did not recognize βbov and the substitution T63P was shown to drastically impair the binding of both mAbs to rβcap (Fig. 4).

Inhibition of IgE-binding to βcap presented by mAb SCB1D

Taking advantage of the specificity of mAb SCB1D, the IgE-binding to βcap directly coated to the solid phase was compared to that to βcap presented by mAb SCB1D. With patients whose IgE antibodies recognized principally the caprine domain 44-88, IgE-binding to βcap presented by SCB1D was markedly inhibited, up to 95% (Fig. 5). The immunodominant role of the area encompassing threonine 63 was thus confirmed. In contrast,

presentation of βcap by SCB1D did not significantly affect the IgE binding with sera from patients 86, 53 and 24 since their specific IgE antibodies were predominantly directed toward the caprine domain 130-178.

Abrogation of rβcap potency for GM-allergic/CM-tolerant patients

We further investigated the residual allergenicity of a modified βcap, rβcap-5, which contained the five critical substitutions A55T, T63P, L75P, P148H and S152P. As expected, rβcap, rβbov and rβcap-5 displayed similar inhibition capacities with sera from CM-allergic patients (Table 2). In contrast, rβbov was unable to inhibit the binding to βcap of IgE antibodies from GM-allergic/CM-tolerant patients. Nine out of 11 GM-allergic/CM-tolerant patients showed no IgE reactivity toward rβcap-5 while patients 86 and 178 exhibited greatly reduced IgE-reactivities, with IC50 about 80- and 130-fold higher than those determined with rβcap.

Finally, the capacity of rβcap-5 to trigger the degranulation of RBL SX-38 cells was evaluated. RBL cells were loaded with a pool of IgE antibodies immunopurified from patients 178, 183, 193, 86 and 24 in order to be representative of the different profiles of IgE-reactivity observed in this study. As expected, nβcap and rβcap exhibited similar capacities to induce cell degranulation whereas nβov and rβov were not potent (Fig. 6A). The five substitutions introduced in rβcap-5 were sufficient to fully suppress the allergenic activity of rβcap (Fig. 5A). It is noteworthy that rβcap-5 remained fully potent when RBL SX-38 cells were passively sensitized with IgE antibodies from CM-allergic-patients (Fig. 6B).

DISCUSSION

According to the WHO/FAO/EFSA/codex criterion for the assessment of the allergenic risk of novel proteins, cross-reactivity between two proteins needs to be considered when their sequences are more than 35% identical over a window of 80 amino acid residues. However, it has been suggested that this criterion should be revised as recent studies reported cross-reactive binding epitopes between proteins exhibiting very low sequence identity [35;36]. On the other hand, strong IgE cross-reactivity is expected between highly homologous proteins. In this regard, the absence of cross-reactivity between βcap and βbov in GM-allergic/CM-tolerant patients was intriguing in view of their high sequence identity (91%). We thus sought to identify the structural determinants supporting the restricted IgE specifity to βcap.

The IgE-binding epitopes of βbov and βcap have been detected all over the molecules [28;37;38]. The pattern of amino acids critical for IgE binding to βbov has been then shown to be heterogenous, in line with the diversity of IgE responses observed in CM-allergic patients [37;38]. In the present work, we delineated the location of immunodominant non-cross-reactive epitopes of βcap in only two domains: 44-88 and 130-178 and we showed that few epitopes were relevant when considering the allergenic activity of βcap. Mutation of only five residues was indeed sufficient to abolish βcap capacity to trigger the degranulation of RBL cells sensitized with IgE antibodies from GM-allergic/CM-tolerant patients. The approach to produce chimeric proteins between caprine and bovine allergens could certainly be extended to the identification of immunodominant IgE-binding domains of other caprine caseins. Considering cow's milk allergens, Cocco *et al.* reported that the mutation of at least eleven IgE-binding epitopes on βbov could be necessary to engineer mutated milk proteins potentially useful for allergy vaccination [38;39]. However, the present work suggests that the deletion of all IgE-binding epitopes may not be required to generate non-cross-linking variants. In this regard, the epitopes recognized by IgE antibodies with high affinity should be

targeted in priority since higher levels of cell degranulation has been correlated with high-affinity IgE-binding [40;41].

Another important finding in the present work was that the production of antibodies able to distinguish βcap from βbov could be induced *in vivo* in mice tolerized to bovine caseins before being sensitized to caprine caseins. The critical role of residue 63 for the generation of non-cross-reactive epitopes was thus confirmed by the specificity of mAb SCB1D and SCB4C and by their ability to interfere with IgE antibody binding. It is also noteworthy that administration of tolerogenic doses of bovine caseins did not prevent the development of antibody responses against caprine caseins. In this regard, the induction of oral tolerance to CM proteins in some CM-allergic children through oral immunotherapy (OIT) has been shown to be ineffective against GM allergy [15-17]. A prevalence of 26% of allergy to GSM has been reported in a population of children successfully treated by CM-OIT with 47% of positive oral food challenges to GSM leading to anaphylactic reactions [17]. In the present study, no CM allergy was reported in the medical history of the GM-allergic/CM-tolerant patients. It would be then interesting to determine whether the non-cross-reactive epitopes recognized by IgE antibodies from CM-OIT-treated patients match those identified in the present study. In addition, the IgE-reactivity to GSM proteins should be determined in the patients before and after the treatment in order to determine whether changes in epitope diversity and affinity, possibly leading to a higher reactivity toward GSM proteins, could occur over time as a result of the therapeutic intervention [41;42]. This could be of critical interest in order to limit the potential side effects induced during CM-specific immunotherapy, for example by mixing CM and GSM proteins during OIT.

To conclude, despite the wide distribution of non-cross-reactive IgE-binding epitopes in βcap, the allergenic activity of this allergen for GM-allergic/CM-tolerant patients could be abolished with only five amino acid substitutions located in two domains of the allergen. This

approach may provide new insights in the design of hypoallergenic variants. Future immunotherapeutic interventions on CM-allergic patients may also consider preventive measure in relation to the allergenic risk of GSM.

Conflict of interest

None

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Figure legends

Fig. 1. Specific IgE concentrations (IU/mL) to native and recombinant β-caseins quantified by direct EAST in CM-allergic patients (n=11) and in GM-allergic/CM-tolerant patients (n=11). Statistical analysis was performed with the non-parametric Wilcoxon matched pairs signed rank test.

Fig. 2. (A) Fusion proteins between rβcap and rβbov were generated by substituting progressively caprine domains with the bovine counterparts. (B) IgE-binding capacity of fusion proteins determined with sera from GM-allergic/CM-tolerant patients and quantified by EAST (expressed in percent of IgE response to rβcap). The fusion proteins displayed similar IgE-binding capacity when testing sera from CM-allergic patients (see supplemental Fig. S5).

Fig. 3. IgE-binding capacity of modified rβcap containing different amino acid substitutions in domain 44-88 (A), in domain 130-178 (B) or in both domains (C) with sera from GM-allergic/CM-tolerant patients and quantified by EAST (expressed in percent of IgE response to rβcap).

Fig. 4. Impact of the substitution T63P on the recognition of rβcap by mAb SCB1D and SCB4C.

Fig. 5. Inhibition of IgE-binding to βcap presented by mAb SCB1D (empty columns), compared to IgE-binding to βcap directly coated to the solid phase (black columns).

Fig. 6. Mediator release assay with RBL SX-38 cells passively sensitized with immunopurified human IgE antibodies from a pool of sera from five GM-allergic/CM-tolerant patients (A) or from a pool of two sera from CM-allergic patients (B) in response to increasing concentrations of native and recombinant βbov and βcap (see Methods). The construct rβcap-5 contains five substitutions: A55T, T63P, L75P, P148H and S152P.

Fig 1

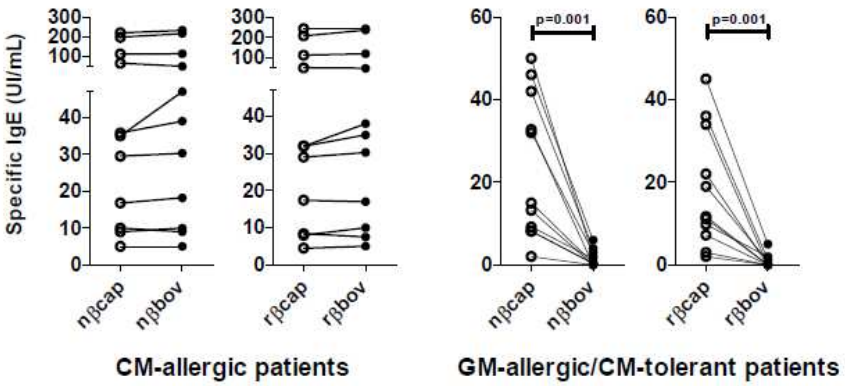

Specific IgE (UI/mL)
300
200
100
40
30
20
10
0
nβcap
nβbov
rβcap
rβbov
CM-allergic patients
60
40
20
0
p=0.001
GM-allergic/CM-tolerant patients

Fig 2

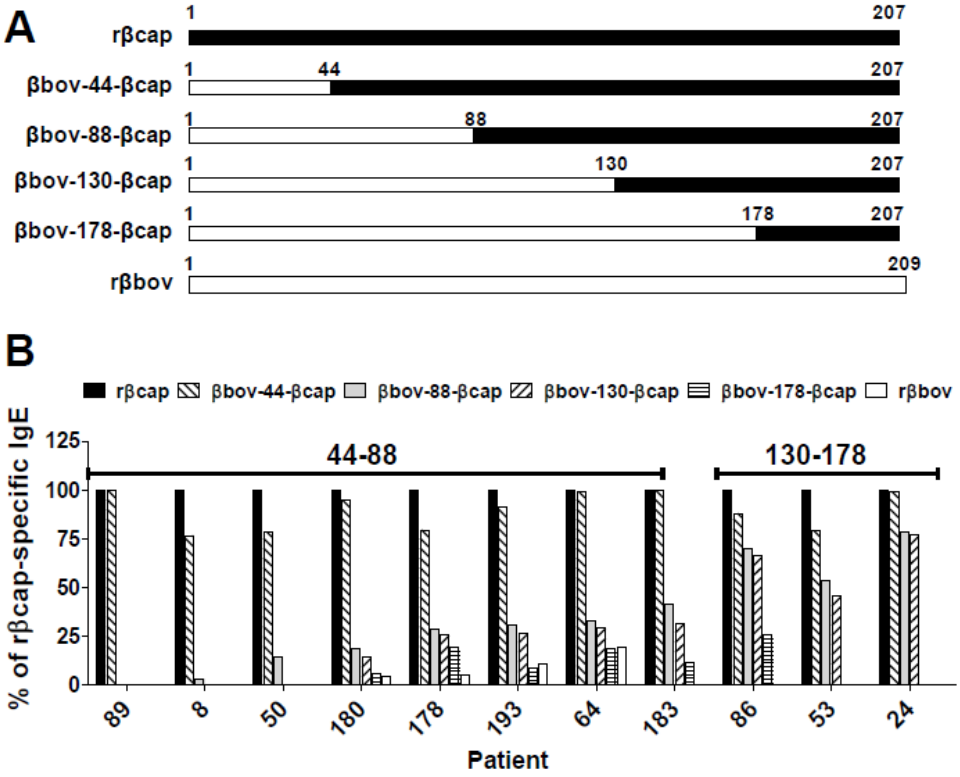

A
rβcap
βbov-44-βcap
βbov-88-βcap
βbov-130-βcap
βbov-178-βcap
rβbov
1
207
44
88
130
178
209
B
rβcap
βbov-44-βcap
βbov-88-βcap
βbov-130-βcap
βbov-178-βcap
rβbov
% of rβcap-specific IgE
125
100
75
50
25
0
44-88
130-178
89
8
50
180
178
193
64
183
86
53
24
Patient

Fig 3

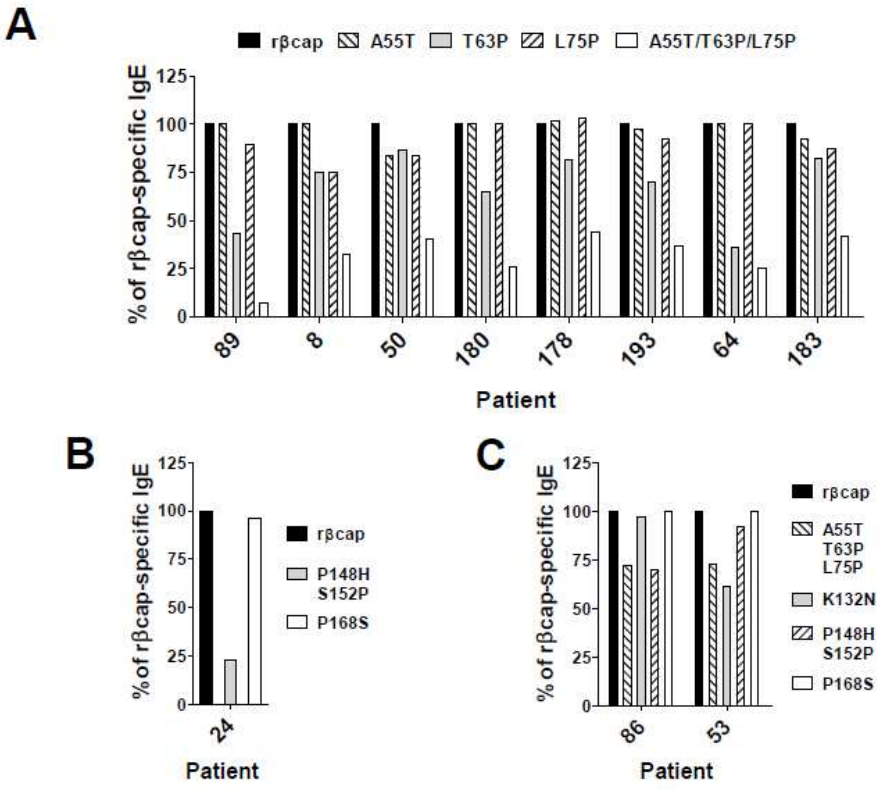

A
rβcap
A55T
T63P
L75P
A55T/T63P/L75P
% of rβcap-specific IgE
125
100
75
50
25
0
89
8
50
180
178
193
64
183
Patient
B
% of rβcap-specific IgE
rβcap
P148H
S152P
P168S
24
Patient
C
% of rβcap-specific IgE
rβcap
A55T
T63P
L75P
K132N
P148H
S152P
P168S
86
53
Patient

Fig 4

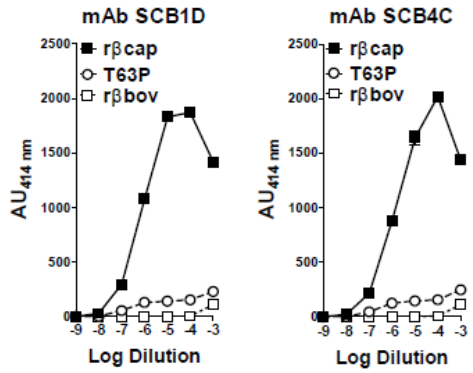

mAb SCB1D
mAb SCB4C
rβcap
T63P
rβbov
AU414 nm
2500
2000
1500
1000
500
0
-9
-8
-7
-6
-5
-4
-3
Log Dilution

Fig 5

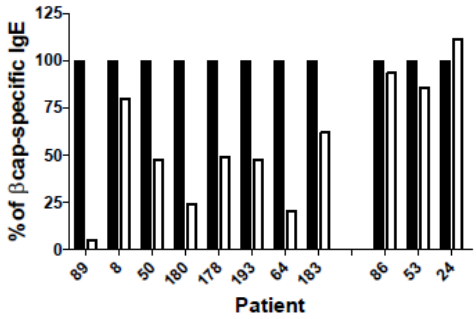
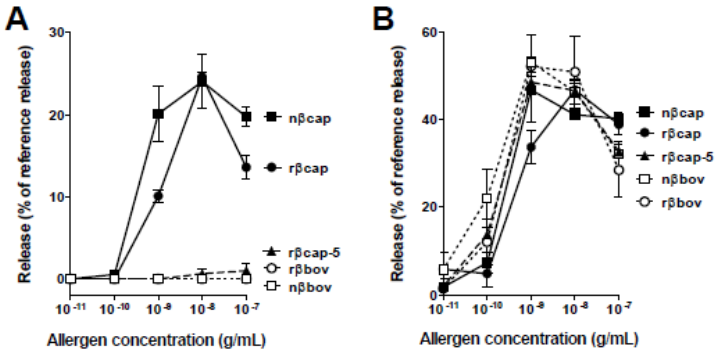

% of βcap-specific IgE
125
100
75
50
25
0
89
8
50
180
178
193
64
183
86
53
24
Patient

Fig 6


A
Release (% of reference release)
30
20
10
0
nβcap
rβcap
rβcap-5
rβbov
nβbov
Allergen concentration (g/mL)
B
Release (% of reference release)
60
40
20
0
nβcap
rβcap
rβcap-5
nβbov
rβbov
Allergen concentration (g/mL)