

Molecular Basis for Nonanaphylactogenicity of a Monoclonal Anti-IgE Antibody¹

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IgE Abs mediate allergic responses by binding to specific high affinity receptors (FcεRI) on mast cells and basophils. Therefore, the IgE/FcεRI interaction is a target for clinical intervention in allergic disease. An anti-IgE mAb, termed BSW17, is nonanaphylactogenic, although recognizing IgE bound to FcεRI, and interferes with binding of IgE to FcεRI. Thus, BSW17 represents a candidate Ab for treatment of IgE-mediated disorders. By panning BSW17 against random peptide libraries displayed on phages, we defined mimotopes that mimic the conformational epitope recognized on human IgE. Two types of mimotopes, one within the Cε3 and one within the Cε4 domain, were identified, indicating that this mAb may recognize either a large conformational epitope or eventually two distinct epitopes on IgE. On the basis of alignments of the two mimotopes with the human IgE sequence, we postulate that binding of BSW17 to the Cε3 region predominantly blocks binding of IgE to FcεRI, leading to neutralization of IgE. Moreover, binding of BSW17 to the Cε4 region may explain how BSW17 recognizes FcεRI-bound IgE, and binding to this region may also interfere with degranulation of IgE sensitized cells (basophils and mast cells). As a practical application of these findings, mimotope peptides coupled to a carrier protein may be used for the development of a peptide-based anti-allergy vaccine by induction of anti-IgE Abs similar to the current approach of using humanized nonanaphylactogenic anti-IgE Abs as a passive vaccine. *The Journal of Immunology*, 2000, 165: 813–819.

Immunoglobulin E interacts with the α-chain of its high affinity receptor (FcεRIα) to form a complex with a K_d of $\sim 10^{-10}$ M. Cross-linking of the FcεRI/IgE complex by an Ag results in degranulation of mast cells and basophils and release of a variety of preformed inflammatory mediators, e.g., histamine, serotonin, and leukotrienes (for review, see Ref. 1). In the human system, high affinity binding of IgE to FcεRI is achieved by a complex protein-protein interaction, involving various parts of the third heavy chain constant region domain (Cε3) of IgE (2–4) and the membrane-proximal Ig-like domain α (2) of the FcεRIα subunit (Refs. 5–9; for review, see Ref. 10). However, the detailed mechanism of the binding process still remains to be characterized.

The monoclonal anti-IgE Ab BSW17 (11) is of particular interest because it is not capable of triggering histamine release from IgE-sensitized human basophils (12). Furthermore, BSW17 reduces in a time-dependent manner the amount of IgE bound to FcεRI (13) resulting in a de facto removal of cell-bound IgE by interfering with the association of IgE to FcεRI. Finally, BSW17 inhibits de novo synthesis of IgE in an in vitro culture system of human B cells (14). This unique capacity to recognize and to re-

move receptor-bound IgE without triggering effector cells renders BSW17 a candidate mAb for immunotherapy (15).

In this paper, we report that recently isolated mimotopes recognized by BSW17 (16) showed a structural homology (but no amino acid homology) to a part of the Cε4 domain of human IgE. Because this result was in contrast to our previous findings, mapping the epitope of BSW17 to Cε3 (17), we screened BSW17 again with other random peptide libraries. This selection procedure resulted in the isolation of peptides with sequence homology to Cε3. Inhibition and cross-inhibition experiments as well as molecular homology modeling clearly confirmed the presence of epitopes in both Cε3 and Cε4 of IgE. Our data indicate that this mAb may recognize either a large conformational epitope or eventually two distinct epitopes on IgE. Importantly, our findings provide the structural basis to explain the functional characteristics of BSW17.

Materials and Methods

Antibodies

Rat anti-mouse Ig conjugated to peroxidase (anti-mouse Ig-HRP) was purchased from Nordic Immunology (Tilburg, The Netherlands). Production of rabbit anti-phage Ab (RaP)⁶ was described earlier (18). Abs Le27 and BSW17 are described elsewhere (11). The anti-human FcεRIα mAb 5H5/F8 was generated at the Novartis Forschungsinstitut (Vienna, Austria), and is described elsewhere (19). The anti-IgE mAbs 11-3, 11-4, and 11-5 were obtained from Dr. R. Vasilov (NPO Biotechnologia, Moscow, Russia). 4F4 was a gift of Dr. M. P. Samoilovich and Dr. V. B. Klimovich (Hybridoma Technology Laboratory, Central Research Institute for Roentgeno-Radiology, St. Petersburg, Russia). Human myeloma IgE-PS was affinity purified from serum of a patient with an IgE myeloma (a gift of Dr. K. Ishizaka, La Jolla, CA) as described elsewhere (20).

Domain mapping experiments

Binding experiments of various truncated Cε1–3 proteins to mAbs 8E7/H8, 5H10, and BSW17 were performed as described (21). Briefly, linear DNA templates encoding various Cε fragments were in vitro expressed using

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⁶ Abbreviations used in this paper: RaP, rabbit anti-phage Ab; PBS-C, PBS containing 1.5 mg/ml casein; KLH, keyhole limpet hemocyanin.

Table I. Domain mapping of anti-IgE mAbs using truncated Cε1–3 fragments

Cε1–3 Fragment ^a Truncated at Position ^c	Recognition by Anti-IgE ^c		
	BSW17	8E7/H8	5H10
Untruncated	+	+	+
445 ^d	–	+	+
422	–	–	–
403	–	–	–
379	–	–	–
375	–	–	–
365	–	–	–

^a Radioactively labeled, truncated Cε1–3 fragments were generated by coupled in vitro transcription/translation of PCR fragments in bacterial cell extracts (21).

^b Numeration of amino acids according to EMBL L00022.

^c Recognition of Cε1–3 fragments by anti-IgE mAbs was measured by RIA.

^d The complete Cε3 domain of human IgE is comprised of aa 357–464.

bacterial extracts and affinity purified by metal chelate chromatography. The radiolabeled proteins were tested for binding to the indicated Abs in a RIA.

Fcε constant domains displaying phage clones

Combinations of the following primers were used to clone different Cε fragments from cDNA Cε1–4 into *Xho*I and *Bam*HI sites of pComb8 (22): primer FcεH1 forward, GCGGGATCCGCTCCACACAGAGCC; primer FcεH2 forward, CGCGGATCCGCTCTGCTCCAGGG; primer FcεH2 reverse, GCGCTCGAGTGCACACTTCTTGCTGC; primer FcεH4 forward, GCGGGATCCGCGCCGCTGCTGCC; primer FcεH4 reverse, GCGC TCGAGTTTACCGGGATTACACACCG.

pComb8 vector and PCR fragments were digested with *Bam*HI and *Xho*I (Boehringer Mannheim, Rotkreuz, Switzerland) and purified on agarose gels followed by phenol extraction before ligation. Ligated DNA was electroporated into 200 μl electrocompetent *Escherichia coli* XL-I blue (Stratagene, La Jolla, CA) using a Bio-Rad Gene pulser (Bio-Rad, Richmond, CA) according to the manufacturers' instructions. After cloning, Cε-displaying phages were produced as described elsewhere (16).

Immunodot assays with phage particles

Monoclonal anti-IgE (1 μl; 500 μg/ml) was dotted onto nitrocellulose (Schleicher & Schüll, Riehen, Switzerland) and after drying was blocked with PBS containing 1.5 mg/ml casein (PBS-C, Fluka Chemie, Buchs, Switzerland) for 1 h. Strips were incubated overnight at room temperature with 10¹¹ CFU/ml phage particles diluted in PBS-C. After washing, bound phages were detected with RaP-HRP (1:1000 in PBS-C) after incubation for 4 h. Staining was performed as described earlier (23).

Phage libraries

Random peptide phage display libraries were obtained from different sources as outlined in Table I. Amplification and precipitation of phages were performed according to standard protocols (24).

Panning of phage particles

StarTubes (Nunc, Fakola, Basel, Switzerland) were coated with 20 μg/ml BSW17 in 0.1 M carbonate buffer at pH 9.6 for 16 h at 4°C, washed with water, and blocked with PBS-C for 1 h at 37°C. Amplified phage libraries were pooled (10¹² CFU of each individual library) in 2 ml PBS-C and incubated at 37°C for 2 h in coated/blocked tubes with constant agitation. Tubes were washed 10 times with PBS-C containing 0.1% Tween 20, and bound phage particles were eluted with 1 ml 0.1 M HCl, pH 2.2, containing 1% BSA (Fluka) for 10 min at 37°C with constant agitation. Eluted phage suspension was neutralized with 2 M Tris and amplified before use for a next round of panning.

Selection of mAb specific clones

After three rounds of panning, individual clones were incubated in single wells of 96-well EIA/RIA plates (Costar, Integra Bioscience, Walisellen, Switzerland) in 150 μl/well NZY medium (1% NZ amine A, 0.5% yeast extract, 0.5% NaCl) containing 100 μg/ml kanamycin and 20 μg/ml tetracycline and grown overnight at 37°C. Plates were centrifuged at 4000 × g for 20 min at 4°C, and supernatant was tested in an ELISA for the

presence of BSW17-specific phages. For this purpose, Tc96 flat-bottom ELISA plates were coated with 10 μg BSW17 in carbonate buffer (pH 9.6) overnight at 4°C, washed once with water, and blocked with PBS-C-Tween for 2 h at 37°C. Fifty microliters of supernatant from culture were transferred and incubated for 4 h at room temperature, washed three times with PBS, 0.5% Tween 20, and subsequently incubated as described below. Wells showing a staining intensity 3 times above the background (VCS-M13 in bacterial growth medium NZY) were considered positive. Positive phage clones were amplified, and phage DNA isolated and sequenced.

Peptide synthesis and coupling to keyhole limpet hemocyanin (KLH)

The following chemically synthesized peptides were used: Cε3 epitope (C3E), ³⁹⁷VNLTWSRASC⁴⁰⁶; Cε3 mimotope (C3M), VNLPSWFGLE; Cε4 mimotope linear (C4M-lin), INHRGYWV; Cε4 mimotope circular, (C4 M-circ), GEFCINHRGYWVCGDPA; Cε3 peptide Pro⁴⁵³-Arg⁴⁶⁷; ⁴⁵³PRALMRSTTKTSGPR⁴⁶⁷, Cε3 peptide Pro³⁷⁰-Leu^{375,370}PSPFTL³⁷⁵.

Numeration of amino acid position according to the putative human IgE protein is as described under EMBL accession numbers L00022 and V00555.

The mimotope and epitope peptides were synthesized by piCHEM Research and Development (Graz, Austria) using Fmoc chemistry on a Bolyx 4170 automated peptide synthesizer as described in detail in Ref. 16. All peptides were >80% pure (HPLC) and characterized by mass spectroscopy. Amino-terminal coupling of 2.2 mg mimotope or epitope peptides (in 0.1 M NaHCO₃/Na₂CO₃, 0.5 M NaCl, pH 8.0) and 2.1 mg KLH (dialyzed into the same buffer) was performed in PIERCE Reacti-Vials (Pierce, Rockford, IL) with 3.5 mM bis(sulfosuccinimidyl) suberate for 90 min at room temperature. The mixture was loaded onto a Pharmacia PD-10 desalting column (10 ml Sephadex G-25, equilibrated with 40 ml 38 mM phosphate buffer, 0.9 M NaCl, pH 7.2), and eluted with 3.5 ml of the same buffer. Purified protein was stored at –20°C. Coupling ratio was estimated to 200 mol peptide per mol KLH.

ELISA experiments

Chemically synthesized peptides (100 ng/ml) were covalently coupled to DNA-BIND microtiter plates (Costar, Cambridge, MA). The remaining reactive surface of the wells was blocked by incubation for 2 h at 37°C with 5% BSA (Sigma, St. Louis, MO) (200 μl/well). Subsequently, BSW17 in increasing amounts specified in Results was incubated for 2 h at 37°C in 100 μl PBS (pH 7.5) containing 0.05% Tween 20 (Serva, Heidelberg, Germany), and 2% FCS (BioWhittaker, Verviers, Belgium). After washing (twice with 300 μl PBS, pH 7.5, 0.05% Tween 20), bound BSW17 was determined by incubation for 2 h at 37°C with HRP-conjugated anti-mouse Ig Ab (1:1000 dilution in the same buffer). After removal of the incubation mixture and two washing steps, color reaction was induced using the HRP Substrate Kit purchased from Bio-Rad (Hercules, CA), and optical density at 405 nm was measured in an ELISA plate spectrophotometer (EasyReader, SLT Labinstruments, Vienna, Austria). The readout shown in the figures was corrected for background binding to microtiter plates coated with the indicated control and represents mean values of duplicates.

Inhibition and cross-inhibition experiments

For inhibition of BSW17 binding to mimotopes/epitopes with IgE-PS, EIA/RIA plates (Costar) were coated with KLH-coupled mimotopes/epitopes (10 μg/ml) or IgE-PS (5 μg/ml) overnight at 4°C in carbonate buffer, pH 9.6, and thereafter blocked with PBS containing 5% BSA. Immune complexes of BSW17 (5 μg/ml) with IgE-PS (20 μg/ml) (both in PBS, 2% FCS, 0.05% Tween 20) were formed overnight at room temperature. Immune complexes were added for 4 h at room temperature and bound BSW17 detected by incubation with anti-mouse IgG-HRP. Wells were washed and incubated with 100 μl 0.5 mM tetramethylbenzidine in 30 mM potassium citrate buffer, pH 4.1, containing 4 mM H₂O₂. Reaction was stopped by the addition of an equal amount of 1 M H₂SO₄ and OD_{450 nm} was determined on an automated ELISA reader.

For inhibition of BSW17 binding to KLH-coupled mimotopes/epitopes with phage-displayed mimotopes, plates were coated and blocked as described above with KLH-coupled mimotope/epitope. Immune complexes of BSW17 (1 μg/ml) with mimotope-displaying phages (10¹³ CFU/ml) were formed for 4 h at room temperature and incubated for 2 h on the ELISA plates. Bound BSW17 was detected and revealed as described above.

Amino acid sequence alignment

Alignment of the postulated putative BSW17 epitopes within the three-dimensional homology based model of Fcε (25), the atomic coordinates of

Table II. Alignment of mimotope peptides with Cε4 by pattern similarity

Origin ^a		Sequence										Frequency
PhBSW.6-9	C ^b	R	R	H	N	Y	G	F	W	V	C	<i>n</i> = 7
PhBSW.29-8	C	<i>I</i>	N	H	R		G	Y	W	V	C	<i>n</i> = 3
PhBSW.C€4a	C	T	R	<i>L</i>	H	T	G	Y	W	V	C	<i>n</i> = 2
PhBSW.C€4b	C	T	<i>L</i>	S	<i>V</i>	<i>F</i>	G	Y	W	V	C	<i>n</i> = 1
C€4 (524–532)		K	T	K	G	S	G	F	F	V		

^a Origin of amino acid sequences: PhBSW.6-9, PhBSW.29-8, PhBSW.Cε4a, and PhBSW.Cε4b are different phage clone-derived sequences isolated after panning described in Ref. 16. Cε4 (524–532) represents the sequence of aa 524–532 of human IgE.

^b The conserved carboxyl-terminal part is boxed. The characteristic features of the more degenerated amino-terminal part are displayed as: bold face, positively charged; light face, uncharged polar; italic, nonpolar; n, number of randomly selected clones with identical sequence.

which were obtained from the Brookhaven Protein Data Bank, was performed with Weblab Viewer version 2.01 (Molecular Simulations, San Diego, CA).

Results

BSW17 recognizes peptide sequences with homology to Cε3 and Cε4

Earlier attempts to map the epitope of the monoclonal anti-human IgE Ab BSW17 with recombinant heavy chain constant region domains of IgE (Cε) produced in *E. coli* indicated an epitope within the third constant Fcε domain (Cε3) (13, 17). The specificity of BSW17 for Cε3 was further confirmed in a domain-mapping experiment by binding of truncated, recombinant in vitro expressed Cε fragments to BSW17 and other anti-Cε3 mAbs (Table I). The binding pattern demonstrates that BSW17 indeed recognizes intact Cε3 and that removal of the carboxyl-terminal 20 amino acids abrogates binding. Because the other anti-Cε3 mAbs still bound to Cε3 truncated at Cys⁴⁴⁵, we conclude that the intact carboxy terminus of Cε3 is essential for binding of BSW17. Nevertheless, whether the deleted part itself contains the BSW17 epitope or is of importance for a stable structure of the epitope within Cε3 cannot be deduced from the presented data.

However, by screening a constrained random nonapeptide library (26), nona- and octapeptide mimotope sequences have been identified that were specifically recognized by BSW17 (16). None of these peptides (Table II) showed amino acid homology to the primary sequence of human IgE (EMBL accession numbers. V00555 and L00022). Nevertheless, it is possible to deduce structural homology between these peptides by pattern alignment based on the properties of the amino acids: 1) the carboxyl-terminal region of the central glycine is highly conserved and contains aromatic side chains; and 2) the region amino terminal to the central

glycine is less conserved but must contain positively charged amino acids spaced by uncharged polar and nonpolar residues to maintain strong BSW17 binding if displayed on the surface of the bacteriophages. Surprisingly, screening of the Cε sequence for the presence of a region with similar organization revealed a structural homology to a region within Cε4 rather than within Cε3 (Table II), stretching from IgE Cε4 aa 524–532. Thus, to confirm the eventual existence of an epitope for BSW17 within Cε4, we tested the binding of BSW17 and a panel of other monoclonal anti-IgE Abs to different Cε domains displayed on the surface of phages. For this purpose, anti-human IgE mAbs were immobilized on nitrocellulose strips and incubated with 10¹¹ CFU phage particles displaying different Cε domains. As shown in Table III, phage particles displaying Cε4 (PhCε1–4, PhCε4) specifically bound to both anti-Cε4 Abs (Le27 and 11-4) but not the anti-Cε3 mAb 4F4. Similarly, PhCε2 reacted only with anti-Cε2 Abs but not with anti-Cε3 or anti-Cε4 Abs. Importantly, phage particles displaying Cε4 (PhCε1–4, PhCε4) also bound to the anti-Cε3 mAb BSW17. In contrast, no reaction between BSW17 and PhCε2 was observed. Taken together, these results suggested the presence of an epitope for BSW17 within the Cε4 domain of IgE.

Screening of BSW17 with random peptide phage display libraries

Our previous domain mapping of BSW17 suggested that the actual epitope most likely resides within Cε3 (13, 17). Thus, the data presented above prompted us to screen for mimotopes in other types of peptide phage display libraries than used previously (16) to gain a more detailed information on the structure of the epitope recognized by BSW17 on human IgE. Several phage display peptide libraries of varying complexity were pooled and screened against immobilized BSW17 (Table IV). After three rounds of

Table III. Binding of phage-displayed single or multiple Fcε constant domains to monoclonal anti-IgE Abs

Dotted Anti-IgE mAb	Cε Domain Specificity of mAb	Incubation with					
		PhCε2 ^a	PhCε1–4	PhCε4	PhBSW29.8 ^b	IgE-PS	M13 control
11-4	Cε4	– ^c	+	+	–	+	–
Le27	Cε4	–	+	+	–	+	–
4F4	Cε3	–	–	–	–	+	–
BSW17	Cε3	–	+	+	+	+	–
11-3	Cε2	+	–	–	–	+	–
11-5	Cε2	+	–	–	–	+	–

^a Single or multiple constant Fc domains of IgE expressed on the surface of M13 as described in *Materials and Methods*.

^b Octapeptide isolated by screening a random peptide library against BSW17 (16).

^c Binding of phage-displayed Fcε constant domains to monoclonal anti-IgE was measured by immunodot and quantified by densitometry. +, binding; –, no binding. A cutoff level of a relative OD of 0.05 was chosen.

Table IV. Phage display libraries used for panning

Library	Insert Size ^a	Library Size ^b	Ref.
6-mer Amp EP Lib	6-mer	2 × 10 ⁸	42
15-mer Amp EP lib	15-mer	Unknown	42
am 3–4	10-mer	4 × 10 ⁸	43
fAFF1 6-mer Lib	6-mer	2 × 10 ⁷	44

^a Number of amino acids of a random sequence displayed on the phage surface.

^b Number of clones present in the original library as stated in the original publication.

panning, highly specific clones were isolated, which were exclusively recognized by BSW17 (data not shown). Twenty independent clones were sequenced, and their inserts were aligned with the Fcε chain sequence. Eighteen of twenty clones analyzed had an insert length of 10 amino acids and showed high sequence homology to amino acids 397–402 of IgE, a region within Cε3, (Table V). All clones showed a conserved VxxxWx motif, which is also found in the Cε3 domain (aa 397 and 401). This putative epitope recognized by BSW17 within the Cε3 domain overlaps with the putative binding site of IgE to the α-chain of FcεRI as reported by Presta et al. (2) and Henry et al. (4). The remaining 2 binding clones had an insert length of 15 amino acids with no apparent homology to the primary IgE sequence (data not shown); therefore, they were not included into further analysis.

Chemically synthesized mimotopes and Cε3-derived peptide PRALMRSTTKTSGPR are recognized by BSW17

To exclude any contribution of phage proteins to the interaction of the isolated peptides with BSW17 and, most importantly, to demonstrate that BSW17 recognizes both (Cε3 and Cε4) regions, BSW17 was tested for binding to chemically synthesized Cε3 and Cε4 mimotopes as well as to the linear sequences identical with the putative epitopes on IgE as deduced by sequence homology analysis (Fig. 1A). Because previous experiments revealed that BSW17 was not able to recognize these mimotope peptides when immobilized to microtiter plate wells using a standard alkaline coating procedure (data not shown), mimotope peptides were covalently coupled to the activated polystyrene surface of DNA-BIND plates as described in *Materials and Methods* and incubated with increasing amounts of BSW17. The linear Cε3 epitope (C3E) was only weakly recognized by BSW17, and no binding to the linear Cε3 mimotope (C3M) as compared with the nonspecific glycine background was observed. In contrast, Cε4 mimotopes and epitopes (C4E, C4M-lin, C4M-circ) all were bound by BSW17. The C3E shows some weak binding at a high concentration of BSW17 Ab (20 μg) in one experiment but not in the second (Fig. 1). Similar results have been obtained in identical experiments, and C3E binding was always slightly above background but with variable in-

tensity at high concentrations of BSW17 Ab. Currently, we do not have an explanation for this phenomenon.

The domain mapping experiments shown in Table I indicated that amino acids belonging to the carboxyl-terminal region of the Cε3 domain might also contribute to BSW17 binding. Therefore, a peptide comprising this region of Cε3 was chemically synthesized (⁴⁵³PRALMSTTKTSGPR⁴⁶⁷) and tested for recognition by BSW17 (Fig. 1B). As a negative control, a Cε3-derived peptide (³⁷⁰PSPFTL³⁷⁵) located at the amino-terminal region of Cε3 was included in testing. As shown in Fig. 1B, specific binding was demonstrated only for C4 M-lin and for peptide ⁴⁵³PRALMSTTKTSGPR⁴⁶⁷, indicating that the ⁴⁵³PRALMSTTKTSGPR⁴⁶⁷ sequence within Cε3 also contributes to the BSW17 epitope.

IgE or phage-displayed mimotopes inhibit binding of BSW17 to mimotopes/epitopes

To confirm the specificity of the interaction of BSW17 with the mimotopes/epitopes, inhibition assays using IgE-PS or phage-displayed mimotopes as inhibitors were performed. As shown in Fig. 2A, the Cε4 epitope and the Cε4 mimotope were bound by BSW17. Similarly, the Cε3 mimotope was also bound by BSW17. This finding is in contrast to Fig. 1A, where the directly coupled Cε3 mimotope was not bound by BSW17 and indicates that the Cε3 mimotope as well as the Cε3 epitope, when covalently coupled to DNA-BIND plates, do not present a conformation which allows binding of BSW17. Importantly, preincubation of BSW17 with 20 μg/ml IgE-PS efficiently inhibited the binding of BSW17 to all three KLH-coupled peptides as well as to IgE-PS itself.

Subsequently, Cε3 mimotopes and Cε4 mimotopes were used in a cross-inhibition assay. For this purpose, immune complexes of BSW17 with a 10-fold excess of phage particles displaying either the Cε4 or the Cε3 mimotope were performed before incubation with KLH-coupled mimotope coated to ELISA plates (Fig. 2B). An almost complete inhibition of binding to both KLH-coupled mimotopes was observed in the presence of either the Cε4 or the Cε3 mimotope displaying phage particles. Some inhibition of BSW17 binding to Cε4 was observed in the presence of a control phage displaying a peptide not recognized by BSW17 (irrelevant mimotope, a linear 9-mer mimotope recognized by mAb Le27 (16)) probably due to unspecific adsorption of phage particles to the coated proteins. The cross-inhibition clearly demonstrates that each of the mimotopes inhibits binding of BSW17 to the other mimotope, although the mimotopes have no obvious homology to each other and mimic two different structures in the Fcε of IgE.

Discussion

We have used phage display peptide libraries to identify putative regions on human IgE recognized by the anti-human IgE mAb BSW17. Based on alignment of the mimotope peptide sequences

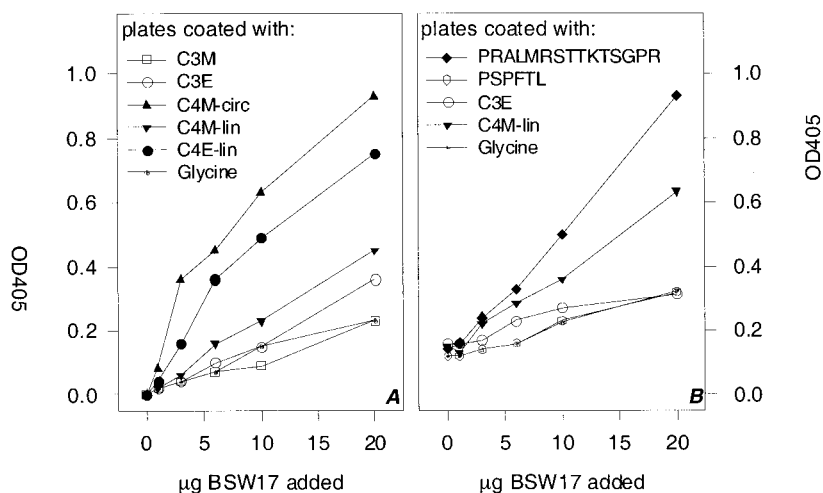
Table V. Sequence of phage clones with homology to IgE-Cε3

Origin ^a	Sequence ^b										Frequency
PhBSW.Cε3a	V	N	L	P	W	S	F	G	L	E	12/18
PhBSW.Cε3b	V	N	R	P	W	S	F	G	L	E	5/18
PhBSW.Cε3c	V	K	L	P	W	R	F	Y	O	V	1/18
Cε3 (397–406)	V	N	L	T	W	S	R	A	S	G	

^a Origin of the amino acid sequences: PhBSW.Cε3a–c are different phage clone-derived sequences after panning, Cε3 (397–406) represents the sequence of aa 397–406 of human IgE.

^b Amino acid sequence of peptides displayed on phage surface (region with a high degree of homology to Cε3 is boxed).

FIGURE 1. A, Recognition of chemically synthesized mimotope peptides by BSW17. A 100-ng sample of each peptide was chemically coupled to wells of DNA-BIND plates, incubated (in duplicates) with increasing amounts of BSW17, and developed with anti-mouse IgG-HRP. Variations of duplicates were generally $\leq \pm 0.05$. B, Recognition of chemically synthesized C ϵ 3-derived peptide ^{453} PRALMRSTTKTSGPR ^{467} by BSW17. Binding was compared with C ϵ 3 epitope (C3E), to linear C ϵ 4 mimotope (C4 M-lin) and to the C ϵ 3-derived control peptide ^{370} PSTFTL ^{375} located at the amino-terminal part of C ϵ 3. The experimental protocol was the same as for A. Variations of duplicates were generally $\leq \pm 0.07$.



with the sequence of human IgE, our results indicate that BSW17 recognizes regions in C ϵ 3 as well as in C ϵ 4. Our findings may explain the experimentally observed characteristics of BSW17.

The parts involved within IgE C ϵ 3 and C ϵ 4 are incorporated in the homology-based model of IgE Fc shown in Fig. 3A. Both regions of the putative BSW17 epitope as defined by homology anal-

ysis of peptides isolated from peptide libraries (Fig. 3A, red, blue) are surface exposed and accessible from the same direction in this model. They are separated from each other by a distance of 20–30 Å, allowing formation of a complex epitope covering an area of 400–900 Å², which is in accordance with published crystallographic data obtained for discontinuous epitopes (27–30). Furthermore, according to this three-dimensional model, the peptide ^{453} PRALMRSTTKTSGPR ^{467} within the C ϵ 3 domain (drawn in light green in Fig. 3A), which is also recognized by BSW17, is surface exposed and resides within the “steric environment” of the postulated conformational BSW17 epitope. Importantly, the mimotope sequences mapping to a region within C ϵ 3 (C ϵ 3 epitopes) (Fig. 3A, red), covering the region Val³⁹⁷-Gly⁴⁰⁶, and the ^{453} PRALMRSTTKTSGPR ^{467} peptide (Fig. 3A, light green) contain four (Arg⁴⁰³, Ser⁴⁰⁵, Arg⁴⁵⁴, Met⁴⁵⁷) of nine amino acids which by alanine scanning mutagenesis have been identified to be directly involved in binding of IgE to Fc ϵ RI. All nine amino acids contributing to this putative IgE binding site as described in Refs. 2 and 4 are shown in Fig. 3B in dark green.

The C ϵ 4 mimotope sequences can be aligned with region Lys⁵²⁴-Val⁵³² (shown in blue in Fig. 3A) which forms a surface-exposed loop. This concurs with the identification of mimotopes only from a C-C constrained circular library but not from an analogous library displaying linear peptides. A peptide mapping to exactly the same C ϵ 4 region (drawn in Fig. 3B in orange) has already been described and characterized earlier (31, 32). The authors proposed that this region within C ϵ 4 might be involved in mast cell triggering (33). This hypothesis gained some support by that fact, that a serum generated against this decapeptide of IgE-C ϵ 4 inhibited histamine release from rat mast cells (34). These findings have remained controversial. However, others showed that disturbance of C ϵ 4 can interfere with binding of IgE to Fc ϵ RI even though C ϵ 4 is not directly involved in receptor binding (35).

In summary, data compiled in this paper provide the molecular basis to explain the observed biological characteristics of BSW17 (12–15, 17). Binding to C ϵ 3 (as shown in Ref. 17 and this study) may explain how BSW17 can remove IgE from Fc ϵ RI (13) and prevent resensitization of the receptor (13, 17). Furthermore binding to the C ϵ 4 part of the epitope (as shown in Ref. 16 and this study) may explain why BSW17, although nonanaphylactogenic (13), recognizes receptor-bound IgE. This feature is unique to BSW17 compared with other nonanaphylactogenic anti-IgE mAbs (36, 37). Binding to C ϵ 4 might as well contribute to the inability of BSW17 to provoke mediator release (12, 31).

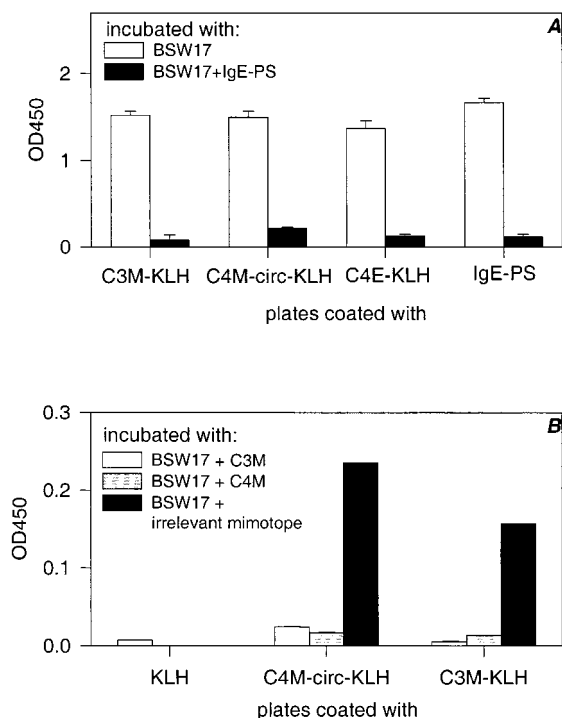


FIGURE 2. A, Inhibition by IgE-PS of the interaction of BSW17 with KLH-coupled mimotopes/epitopes and IgE-PS. KLH-coupled mimotopes/epitope (10 µg/ml) or IgE-PS (5 µg/ml) were coated onto ELISA plates and incubated with BSW17 (5 µg/ml) complexed with IgE-PS (20 µg/ml). Bound BSW17 was detected with HRP-labeled anti-mouse Ab. Background values (no BSW17 added) are subtracted. B, Inhibition by phage-displayed mimotopes of the interaction of BSW17 with KLH-coupled mimotopes. BSW17 was incubated with phage particles for formation of immune complexes and subsequently used in an ELISA assay on plates coated with either KLH or KLH-coupled mimotopes for 2 h. As a control, BSW17 was incubated with a phage clone displaying a peptide not recognized by BSW17 (irrelevant). Bound BSW17 was detected with HRP-labeled anti-mouse Ab.

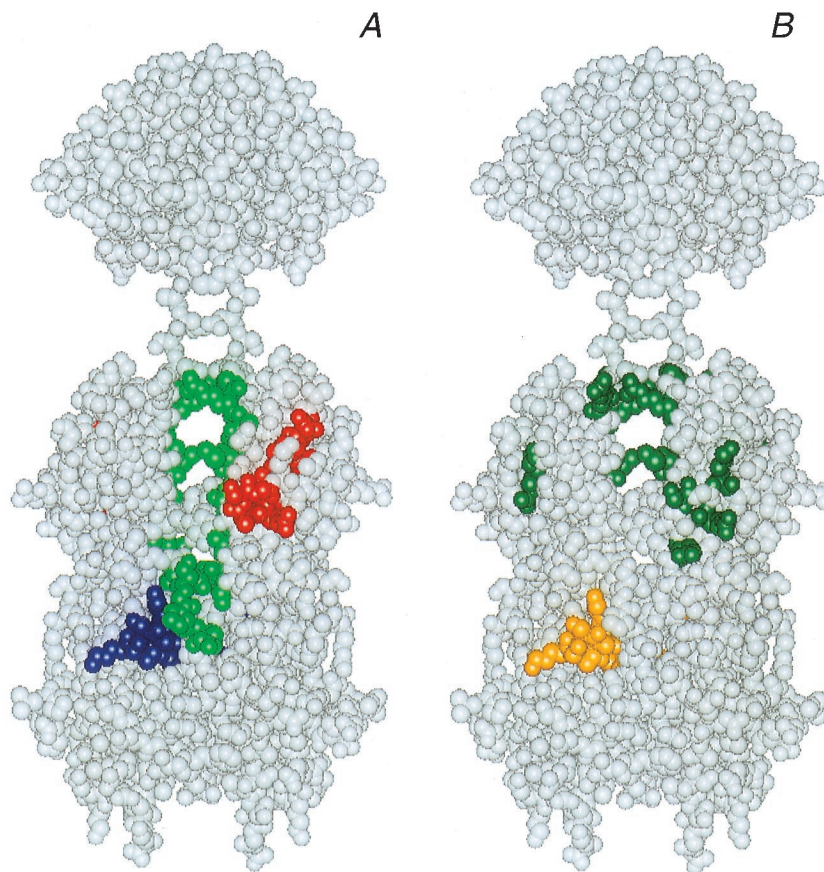


FIGURE 3. Three dimensional structural model of the putative BSW17 epitope. The model is based on computer-generated atomic coordinates for Fcε retrieved from the Brookhaven database and visualized for BSW17 epitope alignment using Weblab Viewer software. *A*, Regions identified on human IgE that are recognized by BSW17. The Cε3 epitope part, Val³⁹⁷-Gly⁴⁰⁶, is red, the Cε4 epitope part, Lys⁵²⁴-Val⁵³², is blue. The peptide Pro⁴⁵³-Arg⁴⁶⁷ (light green) is also recognized by BSW17. *B*, Regions on IgE responsible for binding to FcεRI α and for mediator release. The amino acid residues Arg⁴⁰³, Ser⁴⁰⁵, Lys⁴⁰⁷, Arg⁴²⁰, Glu⁴⁴¹, Arg⁴⁵⁴, Met⁴⁵⁷ (2), Pro³⁶⁰ and Arg³⁶¹ (4) have been demonstrated to be involved in FcεRI and are shown in dark green. The peptide Lys⁵²⁴-Val⁵³² postulated to be involved in IgE-mediated mast cell degranulation (31) is orange.

Nevertheless, the identification of BSW17-binding regions within IgE Cε3 and Cε4 allows two interpretations: either BSW17 recognizes a large conformational epitope spanning over parts of the Cε3 and the Cε4 domain as outlined above; or BSW17 recognizes two distinct epitopes on the same molecule, i.e., IgE. This phenomenon, referred to as intramolecular cross-reactivity, has been reported for T cells (38) as well as for Abs directed against diphtheria toxin (39) or *Plasmodium falciparum* (40). The principle of intramolecular cross-reactivity may also help to explain how nonrepetitive Ags are able to cross-link B cell receptors, a mandatory event for B cell activation (38–40). Structural organization of the Cε3 epitope (Val³⁹⁷-Gly⁴⁰⁶) and the Cε4 epitope (Lys⁵²⁴-Val⁵³²) allows the interpretation that both epitopes could be simultaneously bound by one molecule of BSW17. However, to bind to the two epitopes simultaneously on the same CH chain of IgE, the Fab part of BSW17 would have to bind at a very narrow angle. Alternatively, to bind one epitope on each of the two CH chains of the same IgE molecule, the Fab part of BSW17 would have to wrap itself around the IgE molecule. Nevertheless, our cross-inhibition experiments showing that the Cε3 mimotope is able to inhibit the binding of BSW17 to Cε4 mimotope (Fig. 2B) and vice versa may indicate the presence of two independent epitopes. However, we do not have enough evidence for either hypothesis; therefore, we cannot draw a final conclusion about which of the two hypotheses is correct.

As shown in Table III, PhCε1–4 containing the Cε3 domain was not recognized by the anti-Cε3 mAb 4F4. Similarly, we have previously observed that Cε3 recombinantly produced in *E. coli* was not recognized by 4F4 and by BSW17 (Ref. 17 and unpublished observation). However, an anti-Cε3 mAb (termed 8E7/H8) raised against recombinant Cε3 was shown to also recognize heat-denatured IgE (41). Thus, it may be speculated that recombinant Cε3 as

well as phage-displayed Cε3 were not properly folded, thereby adopting a structure similar to heat-denatured IgE. This might also explain why PhCε1–4 was not recognized by anti-Cε2 Abs 11-2 and 11-5 (Table III). From these observations, we conclude that the positive signals obtained for the reaction of BSW17 with PhCε1–4 (and with PhCε4) were due to recognition of Cε4 rather than Cε3.

Because binding of BSW17 to chemically synthesized Cε3 and Cε4 mimotope peptides coupled to KLH can be univocally demonstrated (Fig. 2), these conjugates might function as immunogens. Our own experimental data (16) have shown that immunization of rabbits with phage particles displaying the Cε4 mimotope induces Abs directed against human IgE. Mimotopes or mimotope/carrier conjugates are anticipated to represent structures that are “foreign” enough to the human immune system to elicit a strong immune response by immunization, but structurally similar enough to human IgE to specifically induce BSW17-like anti-IgE (auto) Abs. Induction of nonanaphylactogenic anti-IgE Abs should inhibit IgE-mediated responses and subsequently the occurrence of allergic reactions.

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