

Epitope-Specific Antibody Response to IgE by Mimotope Immunization¹

Michael P. Rudolf,^{2*} Monique Vogel,* Franz Kricek,[†] Christine Ruf,[†] Adrian W. Zürcher,* Roland Reuschel,[†] Manfred Auer,[†] Sylvia Miescher,* and Beda M. Stadler^{3*}

We have previously described a mouse monoclonal anti-human IgE antibody (BSW17) capable of recognizing receptor-bound IgE without inducing mediator release from human basophils or mast cells. Moreover, immune complexes of IgE and BSW17 are not able to bind to the IgE receptor. An initial attempt to map the precise epitope recognized by this mAb by using Fc ϵ -derived peptides of variable length was unsuccessful. However, by screening random peptide phage display libraries we isolated circular nona- and octapeptides specifically recognized by BSW17. These constrained peptides mimic at least a part of a conformational epitope and are thus called mimotopes. These mimotopes, either phage displayed or synthetically synthesized, did not react with any other anti-human IgE antibody tested, but efficiently inhibited the binding of human IgE to BSW17 only. The use of Rhodol-Green-labeled free cyclic peptide proved that these interactions were not carrier dependent. Immunization of rabbits with phage clones displaying the specific peptides on the surface induced an anti-human IgE response specific for the epitope of BSW17. Therefore, we conclude that such mimotopes or mimotope-derived peptides might be used for vaccination to induce in vivo a beneficial anti-IgE response as a novel immunotherapy. *The Journal of Immunology*, 1998, 160: 3315–3321.

Naturally occurring anti-IgE autoantibodies in human sera were reported in the early seventies (1) and were recently cloned by repertoire cloning (2). The physiologic role of such autoantibodies in the immune response or allergic reactions remains an enigma (3, 4). However, animal and in vitro experiments had previously indicated a role for anti-IgE Abs in down-regulation of IgE. So it was proposed to treat patients with atopic disease by passive immunization with humanized or reshaped mouse monoclonal anti-human IgE Abs (5–7), and such anti-IgE Abs are now in clinical trials (8–11).

Certain murine monoclonal anti-IgE Abs have been shown to inhibit IgE production in vitro (12–15) and the binding of IgE to the low affinity receptor CD23 (Fc ϵ RII) (16), or to prevent binding of IgE within immune complexes to the high affinity receptor Fc ϵ RI (17). Thus we intended to define the corresponding epitope of a murine monoclonal anti-human IgE Ab (BSW17) (18) that possesses all these activities. Peptides representing the recognized epitope of this Ab may be capable of inducing autoantibodies in vivo.

Peptide libraries displayed on filamentous phages have proven to be a powerful tool to define specific epitopes for mAbs, polyclonal sera, or receptor molecules on various cell types (19–22). Constrained peptide libraries exist that can be used for the definition of conformational or discontinuous epitopes. In these libraries the peptides are flanked by cysteine residues that form a disulfide bridge (23, 24), constraining the structure of the peptide. Phage

display-derived peptides isolated by affinity selection from linear or circular libraries are often called mimotopes, since they mimic the structure of the original epitope (25, 26). Upon immunization, mimotopes have been shown to induce an Ag-specific immune response directed against the epitope recognized by the mAb used for the affinity selection of phage clones (27, 28). Mimotope immunizations could therefore be a new way to induce epitope-specific Ab responses in vivo for cases where the complete Ag would be harmful (e.g., toxins) or induce undesired Ab specificities. Indeed, immunization with intact IgE does not necessarily induce exclusively Abs of the desired specificity (BSW17).

To identify the epitope specific for BSW17 we used two different approaches. First, we constructed and then screened a random ϵ peptide phage library, displaying Fc ϵ -derived peptides of varying length. Because this approach did not generate peptides binding to BSW17, we screened two random nonapeptide libraries (23, 29), displaying the nonapeptides in either a linear or a circular form. In this report we describe the identification and characterization of 13 clones from the circular nonapeptide library that were not recognized by other available anti-human IgE Abs. No clones were identified from the linear library. The clones from the circular library efficiently inhibited the binding of IgE to BSW17. This finding was also reproduced using synthetic peptides either coupled to a carrier protein or as free peptide. Rabbits immunized with phage particles displaying the peptides produced Abs directed against human IgE, inhibiting the binding of BSW17 to human IgE. Thus, such peptides might be used for vaccination to induce an anti-IgE response in humans, resulting eventually in the generation of human autoantibodies of the same specificity and therefore with the same beneficial properties as the mouse mAb BSW17.

Materials and Methods

Reagents

Goat anti-rabbit IgG (Fc and κ/λ)-conjugated peroxidase (POX)⁴ was purchased from Nordic Immunology (Tilburg, The Netherlands). Polyclonal

*Institute of Immunology and Allergology, University of Bern, Inselspital, Bern, Switzerland; and [†]Novartis Forschungsinstitut, Vienna, Austria

Received for publication May 5, 1997. Accepted for publication December 9, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported in part by Grants 32–36083.92 and 31–41915.94 from the Swiss National Science Foundation and by a grant from the Novartis Forschungsinstitut (Vienna, Austria).

² Current address: Loyola University Medical Center, Oncology Institute, 2160 South First Ave., Maywood, IL 60153.

³ Address correspondence and reprint requests to Prof. B. M. Stadler, Institute of Immunology and Allergology, University of Bern, Inselspital, CH-3010 Bern. E-mail address: beda.stadler@insel.ch

⁴ Abbreviations used in this paper: POX, peroxidase; SB, super broth; LB, Luria broth; TBS, Tris-buffered saline; KLH, keyhole limpet hemocyanin; RG, Rhodol Green; RP-HPLC, reverse phase high performance liquid chromatography.

sheep anti-human IgE-conjugated POX was purchased from The Binding Site Ltd. (Birmingham, U.K.). Mouse monoclonal anti-human IgE antibodies Le27 and BSW17 were described previously (17). Human myeloma IgE-PS (a gift from Drs. K. and T. Ishizaka, La Jolla, CA), IgE-Savazal (a gift from Dr. V. Savazal, Pilsen, Czech Republic), IgE-WT (a gift from Dr. Stanworth, Birmingham, U.K.) were affinity purified as described previously (30). Chimeric IgE-JW8 (31) was purified from supernatants of the productive cell line by affinity chromatography using anti-IgE mAb Le27 bound to affinity adsorbents (Boehringer Mannheim, Mannheim, Germany). IgE-SUS11 was purified from supernatant of an in-house produced hybridoma (32). The anti-IgE Abs 11-1, 11-3, 11-4, and 11-5 were obtained from Dr. R. Vasilov (NPO Biotechnologia, Moscow, Russia), and 4F4, 4F12, 5D4, 2B9, 5B9, 1E10, 4F1, and ICI were obtained from Drs. M. P. Samoilovich and V. B. Klimovich (Hybridoma Technology Laboratory, Central Research Institute for Roentgeno-Radiology, St. Petersburg, Russia). AbIT is a commercial Ab from Immunotech International (Marseille, France).

POX (horseradish POX; Sigma Chemical Co., St. Louis, MO)-coupled Abs (Le27-POX, BSW17-POX) were prepared by the periodate method as described previously (33). Anti-IgE-POX was used at a dilution of 1/1000 in PBS containing 0.15% casein (Fluka Chemie AG, Buchs, Switzerland).

Construction of a random ϵ peptide library

Four micrograms of cDNA encoding Fc ϵ constant domains 1 to 4 was ligated with 10 U of T4 DNA ligase (Boehringer) at 16°C overnight, phenol extracted, and digested with 1 mU of DNase I (Boehringer) in 50 mM Tris and 10 mM MnCl₂, pH 7.4, at 16°C for 40 min. After extraction twice with phenol, samples were heated to 65°C for 10 min and cooled on ice; then 0.05 mM dNTP (Boehringer, Germany) was added, and the mixture was incubated with 5 U of T4 DNA polymerase (Boehringer) and 5 U of *Escherichia coli* DNA polymerase (Boehringer) in 5 mM MgCl₂ for 15 min. After phenol extraction, 1 μ g of DNA was ligated into the *EcoRV* site of pComb8 vector (34) in ligase buffer (Boehringer) containing 1.2% polyethylene glycol 8000 (Fluka) and 2 U of T4 DNA ligase (Boehringer) at 16°C overnight. After phenol extraction, DNA was introduced by electroporation into 200 μ l of electrocompetent *E. coli* XL-I blue (Stratagene, La Jolla, CA) using a Bio-Rad Gene pulser (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. Transfected bacteria were grown in SB medium (35) containing 10 μ g/ml tetracycline (Boehringer) and 20 μ g/ml carbenicillin (Fluka) at 37°C for 2 h with vigorous shaking. One hundred milliliters of SB containing 10 μ g/ml tetracycline and 50 μ g/ml carbenicillin was added, and incubation was continued for 1 h. VCS M13 helper phage (10¹² CFU; Stratagene) were added; after 2 h at 37°C, kanamycin (Boehringer) was added to a final concentration of 70 μ g/ml, and incubation was continued overnight. Phage particles were precipitated by centrifugation at 4,000 $\times g$ at 4°C for 20 min, supernatant was mixed with 38 ml of an ice-cold, sterile-filtered solution of 12% NaCl/16% polyethylene glycol 8000, cooled on ice for 30 min, and centrifuged for 30 min at 10,000 $\times g$ at 4°C. The phage pellet was resuspended in 2 ml of PBS/0.15% casein and stored at 4°C.

Phage amplification and biopanning

For amplification of phage libraries, 2 ml of a liquid culture of *E. coli* XL-I blue grown in SB to OD₆₀₀ = 1.0 was incubated with eluted phages from biopanning or 10¹⁰ CFU from the amplified library was incubated for 15 min at room temperature. Ten milliliters of SB containing 10 μ g/ml tetracycline and 20 μ g/ml carbenicillin was added, and 10, 1, and 0.1 μ l were plated on LB plates (36) containing 100 μ g/ml carbenicillin. The culture was incubated at 37°C for 1 h, then 100 ml of SB medium containing 10 μ g/ml tetracycline and 50 μ g/ml carbenicillin was added, and incubation was continued for 1 h. VCS M13 helper phage (10¹² CFU) were added, and after 2 h at 37°C, kanamycin was added to a final concentration of 70 μ g/ml, and incubation continued overnight. Phage precipitation was performed as described above.

For biopanning, Costar RIA plates A/2 (Costar, Cambridge, MA) were coated overnight at 4°C with 20 μ g/ml BSW17 in 0.1 M carbonate buffer, pH 9.6, and later blocked with PBS/0.15% casein. Phage (2 $\times$ 10¹¹ CFU) were added and incubated at 37°C for 2 h, then washed 10 times with PBS/0.1% Tween-20. The wells were rinsed with water, and the bound phage were eluted with a total of 200 μ l of 0.1 M HCl, pH 2.2, for 10 min. Eluted phages were neutralized with 2 M Tris base and amplified as described.

Selection of positive clones

Fifty microliters of an *E. coli* XL-I blue liquid culture grown in SB to OD₆₀₀ = 1.0 was incubated with 1 μ l of a 10⁻⁸ dilution of amplified phages after the third round of biopanning for 15 min at room temperature

and afterward plated on LB plates containing 100 μ g/ml carbenicillin and grown overnight. Colonies were randomly picked and plated on LB plates containing 100 μ g/ml carbenicillin. After 4 h at 37°C, nitrocellulose filters soaked with 10 mM isopropyl- β -D-thiogalactopyranoside (Biofinex Wallisellen, Switzerland) were placed on top of the plates, and incubation was continued overnight at 32°C. Filters were removed and incubated at 37°C for 30 min in a chloroform atmosphere. Bacterial debris was removed by incubating filters in 50 mM Tris (pH 8), 150 mM NaCl, 5 mM MgCl₂, 3% BSA, 100 U of DNase I, and 40 mg of lysozyme (Boehringer) per 100 ml for 1 h, blocked in TBS/1.5% casein, and incubated overnight with BSW17-POX in PBS/0.15% casein. Filters were sequentially washed with TBS, TBS/0.05% Tween-20, and TBS for 10 min each. Filters were incubated in TBS containing 600 μ g/ml 4-chloro-1-naphthol (Merck, Darmstadt, Germany) and 0.042% hydrogen peroxide for staining.

IgE binding to solid phase anti-IgE mAb BSW17 for RIA

Soft microtiter plates (Dynatech, Chantilly, VA) were coated with 20 μ g/ml BSW17 in 0.1 M carbonate buffer, pH 9.6, overnight at 4°C and subsequently blocked with PBS/0.15% casein. IgE-SUS11 was ¹²⁵I labeled using the chloramine-T method (37), and 100,000 cpm/well was used. All experiments were performed at room temperature; plates were washed three times with 0.9% NaCl/0.05% Tween-20 and cut in pieces, then each well was measured individually in a gamma counter (LKB, Zürich, Switzerland) for 1 min.

Immunodot assays with phage particles

Anti-IgE mAbs were diluted in PBS to a concentration of 500 μ g/ml, and 1 μ l was dotted onto nitrocellulose (HWG 304 FO, Schleicher and Schuell, Riehen, Switzerland). Nitrocellulose was blocked in PBS/0.15% casein for 1 h. Strips were incubated overnight at room temperature with phage supernatants diluted 1/10 in PBS/0.15% casein. Washing was performed sequentially in PBS, PBS/0.05% Tween-20, and PBS for 10 min each. Developing rabbit anti-phage-POX was diluted 1/1000 in PBS/0.15% casein and incubated for 4 h. Washing and staining were performed as described and read with a hand-held reflection densitometer (Gretag Ltd., Regensburg, Switzerland).

Immunodot assays with rabbit serum

IgE mAbs were diluted in PBS to a concentration of 500 μ g/ml, and 1 μ l was dotted onto nitrocellulose. Nitrocellulose was blocked in PBS/0.15% casein for 1 h. Rabbit serum was diluted 1/200 in PBS/0.15% casein and incubated overnight. Washing was performed sequentially in PBS, PBS/0.05% Tween-20, and PBS for 10 min each. Goat anti-rabbit IgG (Fc and κ/λ)-conjugated POX was diluted 1/1000 in PBS/0.15% casein and incubated for 4 h. Washing and staining were performed as described, and staining was read with a hand-held reflection densitometer.

Inhibition of binding of BSW17 to IgE

Strips were prepared as described above and incubated with rabbit serum at a dilution of 1/50 in PBS/0.15% casein overnight. After washing, mAb BSW17-POX was added in a 1/50,000 dilution in PBS/0.15% casein for 4 h. Subsequent washing and staining were performed as described.

Inhibition of binding of BSW17 to IgE on sensitized CHO-Fc ϵ RI transfectants

CHO cells (2 $\times$ 10⁵) transfected with human Fc ϵ RI α -chain (17) were sensitized with 20 U of IgE-SUS11-IgE for 15 min. After washing, FITC-labeled BSW17 that had been complexed with different amounts of synthetically synthesized KLH-coupled BSW17 mimotope (pepBSW.29-8-KLH; for synthesis details see next paragraph) for 30 min was incubated with the cells for 15 min. After washing, cells were analyzed by FACS (EPICS Profile II, Coulter Electronics, Hialeah, FL).

Synthesis of N-Rhodol Green (RG)-labeled pepBSW.29-8

The peptide corresponding to PhBSW.29-8 was prepared on a Biolynx 4170 automated peptide synthesizer (LKB Pharmacia, Uppsala, Sweden). All acylation reactions were conducted using a fivefold excess of F-moc amino acids activated with 1 equivalent of benzotriazole-1-yl-oxy-rs-pyrrolidino-phosphonium in the presence of 1 equivalent of hydroxybenzotriazole and 2 equivalents of di-isopropylethylamine. All reactions were conducted in dimethylformamide. A coupling time of 45 min was used throughout. The cleavage of the peptide from the resin and side chain deprotection were conducted by treatment of the peptide resin with trifluoroacetic acid/ethanedithiol/water (94/5/1) for 2.5 h. After precipitation of

the crude peptide with ether, lyophilization was performed in a water/t-butanol (1:1) mixture. The peptide was checked for purity by HPLC and mass spectrometry (m.w. = 1924.15).

The cyclization of the freshly reduced peptide was conducted on Eka-thiox Resin (Peptides International, Inc., Louisville, KY, catalogue no. RSH-1299-PI). The reaction was checked with Ellman's reagent. After separation from the resin, the peptide was purified by RP-HPLC. The cyclic peptide was checked by capillary electrophoresis and mass spectrometry (MW = 1922.10 C₈₅ H₁₁₆ N₂₄ O₂₄ S₂).

The peptide was labeled with RG carboxylic acid succinimidyl ester (R-6108, Molecular Probes, Eugene, OR) and purified by RP-HPLC to a purity >98%. Pooled fractions were lyophilized and stored at -80°C.

Binding of RG-labeled mimotope (pepBSW.29-8-RG) to BSW17

Steady state fluorescence measurements were performed on a SLM 8000C spectrofluorometer equipped with JD-490 photomultipliers and a 450-watt xenon Arclamp (SLM Instruments, Urbana, IL). Spectral band widths were set at 8 and 16 nm for excitation and emission, respectively. Measurements were performed at 25°C in a 750Tl quartz cuvette (10-mm optical path length). PepBSW.29-8-RG at a concentration of 30 nM in 150 mM sodium phosphate, pH 6.8, was incubated with increasing concentrations of BSW17 from a 15.33-μM stock solution in the same buffer. Titrations were performed with the excitation wavelength set at 500 nm, and the emission wavelength set at 533 nm. The spectra were corrected for Raman scattering from the buffer solution, for dilution, and for inner filter effects due to BSW17 additions. The signal was adjusted for lamp intensity changes by ratio mode detection with a metal scatter in the reference channel. An OG 515 emission cut-off filter was used for stray light reduction. For each addition of BSW17, 10 intensity measurements were taken with an integration time of 10 s each. For data analysis the two points with the highest and lowest values each were dropped, and the average was calculated automatically by a hand-written program, ANIS3, which also provides for all possible individual corrections to judge the quality of the titration.

For data analysis, the percent enhancement of the integrated fluorescence intensity of the pepBSW.29-8-RG/BSW17 complex was plotted as function of the total concentration of BSW17 and fit to the equation

$$F = F_{\min} + (F_{\max} - F_{\min}) * (1/[\text{pepBSW.29-8-RG}]) * (([\text{pepBSW.29-8-RG}] + n*[\text{BSW17}] + K_d)/2 - ([\text{pepBSW.29-8-RG}] + n*[\text{BSW17}] + K_d)^2/4 - (4n*[\text{pepBSW.29-8-RG}] * [\text{BSW17}])^{1/2}).$$

The Marquardt algorithm in the program GraFit 3.0 Erithacus Software Ltd, Staines, U.K. was used to optimize the maximal fluorescence intensity, $F_{\max}$, and K_d , the intrinsic dissociation constants for the pepBSW.29-8-RG interaction with BSW17, assuming a 1:2 stoichiometry with two independent and equal binding sites.

Immunization

Freshly prepared phage particles (10^{12} CFU) were dialyzed against PBS at 4°C. One milliliter was emulsified with CFA for the first immunization or with 1 ml of IFA for boosting. Immunization was repeated s.c. every 14 days. After the third boost, 12 ml of blood was taken and clotted for 4 h at room temperature in glass vials, centrifuged for 10 min at $2000 \times g$. The supernatant was frozen at -20°C.

Results

Construction of a random ϵ peptide library and selection of mAb-specific epitopes by biopanning of phage display libraries

In a first attempt to characterize the epitope of the anti-IgE mAb BSW17, we chose an M13-based bacteriophage system as described previously (34, 35). Pilot experiments using the vector pComb8 showed that we were able to express single as well as multiple ϵ domains along the surface of the phage body that were recognized by different anti-IgE mAbs (data not shown).

To obtain smaller parts of the Fc ϵ constant domains, possibly containing only the epitope of BSW17, cDNA for the Fc ϵ part was digested with DNase I, resulting in random fragments of varying lengths (data not shown). After ligation into the phagemid vector pComb8, the phage display library resulted in 3×10^6 transformants. This represents 4 times the theoretical number of fragments longer than 12 bp, assuming that 8×10^5 12-mer or longer frag-

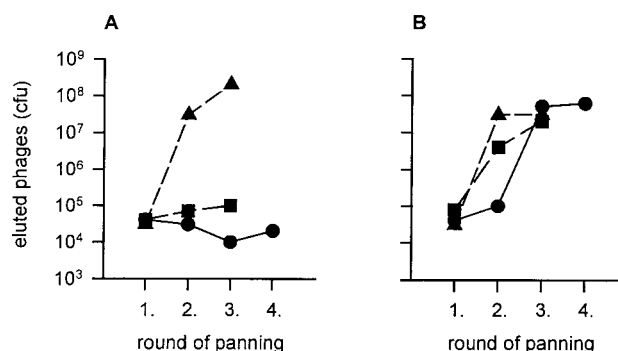


FIGURE 1. Enrichment of peptide-displaying phages by serial rounds of biopanning. Three different peptide libraries displayed on filamentous phages were selected for binding to mouse monoclonal anti-human IgE antibodies by several rounds of biopanning. The values are given in colony-forming units after each round of biopanning, representing the amount of infectious phage particles eluted from the Ab-coated plates. The closed circles represent the biopanning of a random ϵ peptide library derived from a random digest of the cDNA encoding the constant ϵ part; the squares indicate a linear, and the triangles indicate a circular random nonapeptide library. The various libraries were panned either on mAb BSW17 (A) or on Le27 (B).

ments can be generated from the full-length cDNA of 1284 bp. The transformed bacterial cells produced phage particles displaying this random ϵ library as fusion proteins with the major coat protein pVIII on the phage surface. Affinity selection of the phage display library with BSW17 was performed by biopanning as described previously. For a control we used another mouse monoclonal anti-human IgE Ab, termed Le27, recognizing a heat-resistant epitope within the fourth constant ϵ domain. However, our attempt to select epitope displaying phages specific for mAb BSW17 after four rounds of biopanning was clearly negative (Fig. 1A).

Using the control Ab Le27, we achieved a high titer of phages binding to Le27 within three rounds of biopanning (Fig. 1B). After the third round the library was analyzed, and clones were isolated displaying peptides shorter than 35 amino acids, as judged by DNA analysis on agarose gels (data not shown). In contrast, BSW17 recognized none of these clones, but some of the longer peptides (>50 amino acids in length) were recognized by a polyclonal sheep anti-human IgE Ab (data not shown). Thus, we assumed that no short linear primary amino acid sequence had been displayed by the phages in a conformation that could be recognized by the mAb BSW17.

Selection of BSW17-specific mimotopes and sequence analysis

To search for a possible conformational epitope, we screened two random peptide libraries (23, 29). One of the libraries displays linear peptides of nine amino acids in length, of a random sequence, and fused to the major coat protein pVIII. The second, circular library is similar to the first, but has additionally two cysteines flanking the random nonapeptide sequence, giving a constraint to the peptide by allowing the formation of a disulfide bridge. After three rounds of biopanning with the two libraries using BSW17 as a target, we observed an approximately 6000-fold increase in the number of eluted phages, but only in the case of the circular library (Fig. 1A).

From the third round of biopanning on the mAb BSW17, we randomly picked 82 clones and tested them for binding of POX-labeled BSW17. By means of this assay we arbitrarily selected 13 clones (16% of the screened clones) reacting strongly with BSW17. These clones were negative on other available mouse

Table I. Interaction of different phage clones with monoclonal anti-human IgE antibodies

mAb	Isotype ^a	Domain Specificity	Phage Clone ^b														Co ^c
			1	4	5	6	12	15	16	18	20	24	25	29	36		
Le27	IgG1	CεH4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
11-4	ND	CεH4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
BSW17	IgG1	CεH3	++	++	++	++	++	++	+	+++	(+)	+	+	+++	+++	—	
	11-1	ND	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
4F4	IgG2a	CεH3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
4F12	IgG1	CεH3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
IT	IgG2a	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
11-3	ND	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
11-5	ND	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
5D4	IgG2a	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
2B9	IgG1	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
5B9	IgG1	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
IE10	IgG2a	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
4F1	IgG2a	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
ICI	IgG2a	CεH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

^a Isotype and domain specificity as determined earlier (17).^b Recognition of clones by the anti-IgE antibody in a dot assay. +++, relative OD >100; ++, between 100 and 60; +, between 60 and 30; (+), between 30 and 10, and —, less than 10 relative units as determined by a hand held reflection densitometer.^c Negative control: VCS M13 helper phage.

mAb anti-human IgE as shown in Table I. This result indicated that the peptides were recognized by the binding site of BSW17. DNA sequence analysis of the 13 binding phage clones revealed that the displayed peptides can be grouped into four classes according to the deduced amino acid sequence (Table II). None of the peptide sequences showed homology to the primary IgE amino acid sequence (EMBL accession no. V00555; L00022). Surprisingly three clones contained a peptide of eight amino acids instead of nine. Moreover, the phage clones displaying the octapeptide bound stronger to BSW17 compared with the other nonapeptide clones and determined by immunodot assays (Tables I and II, clones 18, 29, and 36). The postulated structure common to all the clones consists of a core of seven amino acids, where the two amino acids toward the amino terminus have a polar charge, followed by three polar, but uncharged, and two nonpolar amino acids (Table II). All clones contained a tryptophan and a valine at the carboxyl terminus of the nonapeptide and a glycine in the middle.

Inhibition of IgE binding to BSW17 with mimotope displaying phage clones

For further studies we selected two phage clones: one displaying the nonapeptide (PhBSW.6-9), and one displaying the octapeptide (PhBSW.29-8) as shown in Table II. Both phage clones were used at a concentration of 10^{11} CFU/ml and inhibited the binding of ^{125} I-labeled IgE to BSW17 (Fig. 2). To quantitate this inhibition, we determined the amount of cold IgE necessary for the same reduction in binding of [^{125} I]IgE-SUS11. The inhibition achieved

with 10^{11} CFU/ml of phage clone PhBSW.6-9 corresponded to a concentration of 1 μ g/ml IgE-SUS11 (corresponding to 6×10^{12} epitopes that can theoretically be recognized by BSW17 in the IgE molecule, based on a 188 kDa molecular mass for human IgE), and that for clone PhBSW.29-8 corresponded to a concentration of 1.7 μ g/ml IgE-SUS11 (corresponding to 1×10^{13} epitopes). Assuming that both peptides have comparable affinities for BSW17, we estimated that 64 or 109 binding sites are blocked per PhBSW.6-9 or PhBSW.29-8 phage particle, respectively.

Inhibition of BSW17 binding to IgE on sensitized CHO-FcεRI transfectants

To further elucidate the specificity of the selected peptides, the mimotope of phage clone PhBSW.29-8 was synthesized chemically and coupled to KLH (pepBSW.29-8-KLH). We used this construct to inhibit the interaction of BSW17 with IgE bound to FcεRI-transfected CHO cells. As shown in Figure 3, preincubation of BSW17 with KLH-coupled peptide (0.05, 0.5, 5, and 50 μ g) inhibited binding to CHO-bound IgE in a dose-dependent manner (87, 86, 40, and 48% inhibition, respectively), whereas KLH alone did not inhibit this interaction. No binding to the untransfected parental cell line was observed.

Binding of RG-labeled pepBSW.29-8 to BSW17

To prove that the cyclic mimotopes not only bind to BSW17 when associated with phage particles or a carrier protein, the PhBSW.29-8 mimotope was synthetically synthesized and labeled

Table II. Sequence alignment of mimotopes specific for anti-IgE mAb BSW17

Sequence ^a															Recognition by BSW17 ^b		Clones with Identical Sequence	
•	E	F	C	R	R	H	N	Y	G	F	W	V	C	G	D	•	++	7/13
•	E	F	C	I	N	H	R	—	G	Y	W	V	C	G	D	•	+++	3/13
•	E	F	C	T	R	L	H	T	G	Y	W	V	C	G	D	•	+	2/13
•	E	F	C	T	L	S	V	F	G	Y	W	V	C	G	D	•	(+)	1/13

^a Sequence of 13 clones from the pVIII-9aa.Cys library after the third biopanning as shown in Figure 1. Only the random nonapeptide inserts plus flanking amino acids are shown. The two cysteines forming the disulfide bridge are typed in italics. Amino acids with a polar charge are underlined in black, nonpolar amino acids are boxed, and polar/not charged amino acids are shaded.^b Recognition of clones by BSW17 in a dot assay. +++, a relative OD >100; ++, between 100 and 40; +, between 40 and 10; and (+), less than 10 relative units as determined by a hand held reflection densitometer.

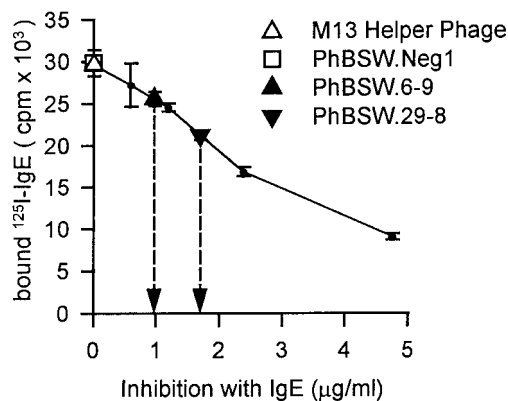


FIGURE 2. Binding of ^{125}I -labeled IgE-SUS11 to anti-IgE mAb BSW17 in the presence of epitope-displaying phage clones. Each point represents the mean $\pm$ SD (triplicates) of bound ^{125}I -labeled IgE. Closed symbols represent the two epitope-bearing phage clones, while open symbols are controls, as explained in the figure legend. Small closed circles show a standard inhibition curve by unlabeled IgE-SUS11 for estimation of inhibition of binding of labeled IgE in the presence of 10^{11} CFU/ml of phage particles.

with the rhodamine derivative RG (pepBSW.29-8-RG) at the N-terminus. A titration of this fluorescent derivative at a concentration of 30 nM with BSW17 resulted in an approximately 20% increase in RG fluorescence intensity (Fig. 4) at saturation. The affinity of this construct to BSW17 was determined by a nonlinear, least squares fit to the quadratic equation describing the relative increase in RG fluorescence emission intensity enhancement as a function of the total BSW17 concentration. Two equivalent, independent binding sites for pepBSW.29-8-RG on BSW17 were assumed. The resulting intrinsic dissociation constant was 78.1 ± 25.1 nM.

The interaction of this synthetic peptide with BSW17 could only be reproducibly detected with a freshly prepared solution of pepBSW.29-8-RG. Storage of the stock solution at concentrations between 100 and 200 μM at 4°C resulted in deactivation within 5 days. Mass spectroscopic and HPLC analysis of the stored pepBSW.29-8-RG solution showed unaltered homogeneity and retention time, and confirmed the oxidized cystine bridge. We therefore assume that the peptide can undergo a conformational change from an active to an inactive stage.

FIGURE 3. Synthetic BSW17 mimotopes can inhibit the binding of BSW17 to IgE on sensitized CHO-Fc ϵ RI transfectants. Flow cytometry was used to analyze the binding of FITC-labeled BSW17 to IgE-sensitized CHO transfectants in either the absence (100% binding) or the presence of preformed immune complexes of BSW17-FITC with different amounts of synthetic KLH-coupled BSW17 mimotope. Results are shown as the percent binding of BSW17-FITC to IgE-sensitized CHO in the presence of pepBSW29-8-KLH. The inset shows fluorescence profiles of IgE-sensitized CHO (straight line) and BSW17-FITC-stained CHO (dotted line), and inhibition of this interaction with 50 μg of pepBSW.29-8-KLH (bold line).

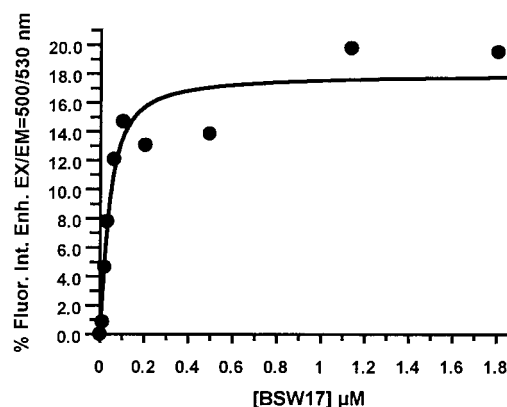
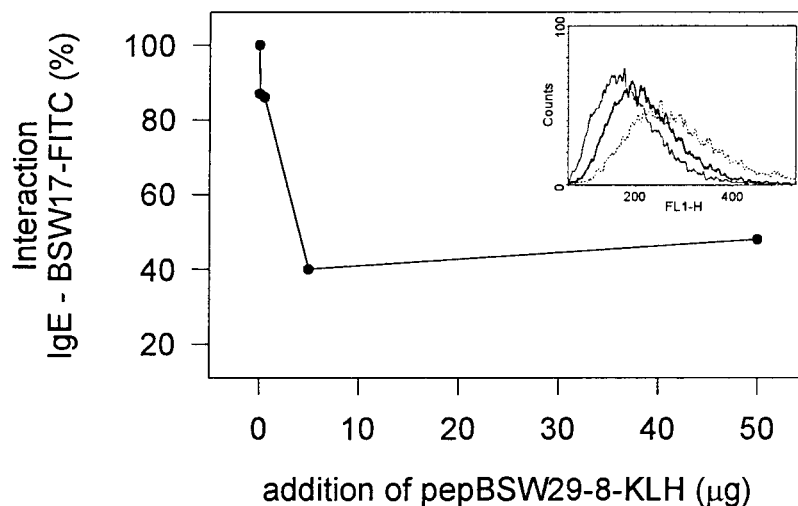


FIGURE 4. Binding of pepBSW29.8-RG to BSW17 determined by steady state fluorescence titration. A plot of the relative fluorescence intensity enhancement of RG-labeled peptide at 530 nm as a function of the total BSW17 concentration using a model equation for two independent and equivalent binding sites on BSW17 resulted in an intrinsic dissociation constant of 78.1 ± 25.1 nM.

Induction of an epitope-specific immune reaction by immunization of rabbits with mimotope-displaying phage clones

To investigate whether the mimotopes represent an immunogenic structure corresponding to the natural epitope on the IgE molecule, rabbits were immunized with phage particles, either with phage clone PhBSW.6-9, clone PhBSW.29-8, or with the helper phage VCS M13. After immunization and two boosts, sera from the rabbits were collected and tested for anti-human IgE Abs (Table III). The sera showed an anti-IgE response; meanwhile, the control rabbit immunized with M13 phage alone showed no anti-IgE reactivity.

Finally, we investigated the specificity of the rabbit anti-human IgE Abs by inhibiting the binding of POX-labeled BSW17 to human IgE in the presence of rabbit sera. As shown in Table IV, the serum of the control rabbit, immunized with M13 helper phage, did not inhibit binding of BSW17 to IgE. In contrast, sera from the two rabbits immunized with BSW17 mimotope reduced the signal up to 65%. In an additional control experiment we used Le27 at a concentration of 10 $\mu\text{g}/\text{ml}$ instead of serum. This anti-IgE Ab did not influence the binding of BSW17 to IgE (data not shown). These experiments clearly demonstrate the presence of an elicited

Table III. Anti-IgE response in rabbits immunized with mimotope displaying phages

Rabbit immunized with ^b	Recognition of Different IgE (relative OD) ^a		
	SUS11-IgE	PS-IgE	WT-IgE
VCS M13 helper phage	0.0 ± 0.6	0.0 ± 1	1.0 ± 0.5
PhBSW.6-9	3.0 ± 0.2	6.0 ± 0.6	19.0 ± 1
PhBSW.29-8	14.5 ± 0.7	7.0 ± 0.4	23.0 ± 1

^a IgE proteins are either from human myelomas or a human hybridoma (SUS11). Values represent relative OD and background values from six nonimmunized rabbit sera are subtracted.

^b Rabbits were immunized with 10¹² CFU of phages as described in *Materials and Methods*. Serum was taken 14 days after the third boost and used at a dilution of 1/200.

specific anti-IgE response directed against the epitope of BSW17 in the rabbit.

Discussion

IgE plays a central role in the manifestation of the allergic reaction. The basic idea to control the IgE response with anti-IgE Abs either at the level of IgE synthesis or at the effector phase by preventing the binding of IgE to FcεRI α-chain emerged several years ago (5–9, 13, 15). At present, two humanized anti-IgE Abs are in clinical trials (8–11). Both Abs are nonanaphylactogenic, since they recognize an epitope in the vicinity of or in the binding site for FcεRI and as a consequence are unable to recognize receptor-bound IgE.

In contrast, the nonanaphylactogenic mouse monoclonal anti-human IgE Ab BSW17 recognized receptor-bound IgE (17). Moreover, free immune complexes of IgE and BSW17 did not bind to FcεRI, probably due to a conformational change induced by BSW17 within the Fcε part. Therefore, BSW17 was able to form immune complexes with receptor-bound IgE, thereby shifting the thermodynamic balance of bound vs unbound immune complexes toward the unbound by preventing the reassociation with the receptor (17). These findings indicated that the part of the IgE molecule containing the epitope for BSW17 may be crucial for the biologic activity.

In the case of rat IgE, synthetic peptides corresponding to the sequence of the native Ag induced Abs in vivo (38). Furthermore, immunization with mimotopes also induced an epitope-specific immune response in vivo (28, 39). More importantly, even protective immune reactions elicited by mimotope immunizations have been shown for several infectious agents (27, 40, 41). Therefore, peptide mimotopes might be a valuable approach to develop vaccines for the induction of a defined Ab response to complex Ags.

In our case, using the mouse monoclonal anti-human IgE Ab BSW17, we identified phage clones displaying peptides mimicking a conformational epitope of the IgE molecule. The isolation of

these peptides for BSW17 was only possible using a constrained random nonapeptide library, in which peptides are displayed as circular loops held together by a disulfide bridge. The amino acid sequences of the BSW17-specific mimotopes were not present in the primary amino acid sequence of the IgE molecule, and degradation of the disulfide bond destroyed the mimotope for BSW17 (data not shown). Therefore, we assume that the epitope for BSW17 is of a discontinuous nature.

Inhibition and immunization experiments documented the specificity of the mimotopes for the mAb BSW17, excluding a non-specific binding via framework sequences or the Fcγ constant part of BSW17. Additionally, the mimotopes were specifically recognized by BSW17 in the context of another carrier protein (Fig. 3) and also without carrier (Fig. 4), indicating that the mimotope alone is responsible for the interaction with BSW17 without involvement of structures originating from the phage particle. A computer-generated model of the three-dimensional structure of our BSW17 mimotopes as displayed on the phage protein pVIII showed the heptapeptide motif pointing away from the phage pVIII protein, rendering it accessible for an Ab (data not shown). The described charge motif might be enough to mimic the structure of the natural epitope, as can be speculated from the sequence alignment of the mimotopes. A detailed analysis of the three-dimensional structure of the mimotope will be published elsewhere.

Despite the great number of independent clones in the libraries used, they cover only a small percentage of all the theoretically possible nonapeptides (5×10^{11}). It is possible that the linear or constrained nonapeptide library does not contain peptides mimicking the most optimal structure of the native epitope. Moreover, it has been shown that Ab-specific mimotopes derived from different combinatorial libraries varied among the different libraries used for screening (42). Finally, the intrinsic dissociation constant measured for pepBSW.29-8-RG binding to BSW17 (78.1 ± 25.1 nM) was more than 1 log higher than the dissociation constant known for the BSW17-IgE interaction (1.2 nM) (our unpublished observation). This further indicated that the mimotope may not represent the optimally fitting structure. Therefore, we are presently screening several phage libraries, displaying peptides of different lengths, to study whether additional mimotopes for BSW17 can be found.

Since our mimotopes are displayed as fusion proteins with the major coat protein (up to 60–100 copies along the filamentous phage particle), it might be possible that avidity rather than affinity played a role in selecting the mimotopes. Nevertheless, it was reported for streptavidin that screening of conformationally constrained peptide libraries revealed clones of much higher affinities than those of linear peptide libraries (43).

In summary, this report describes the isolation of mimotopes corresponding to the epitope of the monoclonal anti-IgE Ab BSW17. Binding and inhibition studies either with mimotope-displaying phage particles or with synthetic peptides proved the specificity of the isolated mimotopes. Finally, immunization of rabbits with mimotope induced anti-IgE Abs whose binding to IgE was

Table IV. Inhibition of BSW17/IgE interaction with rabbit sera

Rabbit immunized with	Inhibition of IgE Recognition, Relative OD ± SD (% inhibition) ^a			
	SUS11-IgE	WT-IgE	PS-IgE	Zavasal-IgE
VCS M13 helper phage	30.5 ± 0.5 (0%)	13 ± 1 (0%)	13.5 ± 0.5 (0%)	22.5 ± 0.5 (0%)
PhBSW.6-9	20 ± 0.5 (34%)	4.5 ± 0.5 (65%)	5 ± 1 (63%)	11 ± 0.5 (51%)
PhBSW.29-8	14.5 ± 0.5 (52%)	4.5 ± 0.5 (65%)	7 ± 1.5 (48%)	12 ± 1 (47%)

^a Values are given in relative OD at 450 nm. The percentage of inhibition was calculated based on the value of serum from rabbits immunized with the helper phage VCS M13.

inhibited by BSW17. Similar products may serve as agents for the prevention of allergic disease.

Acknowledgments

We thank S. Blaser, V. Ramseyer, M. Maus, M. Bouzas, C. Graf, W. Mayer-Granitzer, A. Neubauer, and Ch. Biaggi-Rudolf for their excellent technical assistance; S. Miescher, M. Stämpfli and I. Aebischer for critical reviews and discussions; and especially Dr. Franco Felici from the Istituto Ricerche di Biologia Molecolare (Rome, Italy) for the kind gift of the nonapeptide phage display libraries.

References

- Williams, R. C., Jr., J. Griffiths, D. Emmons, and R. C. Field. 1972. Naturally occurring human antiglobulins with specificity for γ E. *J. Clin. Invest.* 51:955.
- Vogel, M., S. Miescher, C. Biaggi, and B. M. Stadler. 1994. Human anti-IgE antibodies by repertoire cloning. *Eur. J. Immunol.* 24:1200.
- Inganäs, M., S. G. O. Johansson, and H. Bennich. 1981. Anti-IgE antibodies in human serum: occurrence and specificity. *Int. Arch. Allergy Appl. Immunol.* 65: 51.
- Carini, C., and J. Brostoff. 1983. An antiglobulin: IgG anti-IgE: occurrence and specificity. *Ann. Allergy* 51:251.
- Davis, F. M., L. A. Gossett, K. L. Pinnston, R. S. Liou, L. K. Sun, Y. W. Kim, N. T. Chang, T. W. Chang, K. Wagner, J. Bews, V. Brinkmann, H. Towbin, N. Subramanian, and C. Heusser. 1993. Can anti-IgE be used to treat allergy? *Springer Semin. Immunopathol.* 15:1.
- Jardieu, P. 1995. Anti-IgE therapy. *Curr. Opin. Immunol.* 7:779.
- Kolbinger, F., J. Saldanha, N. Hardman, and M. M. Bending. 1993. Humanization of a mouse anti-human IgE antibody: a potential therapeutic for IgE-mediated allergies. *Protein Eng.* 6:971.
- Presta, L. G., S. Lahr, R. L. Shields, J. P. Porter, C. M. Gorman, B. M. Fendly, and P. M. Jardieu. 1993. Humanization of an antibody directed against IgE. *J. Immunol.* 151:2623.
- Chang, T. W., F. M. Davis, N. Sun, C. R. Y. Sun, D. W. MacGlashan, and R. G. Hamilton. 1990. Monoclonal antibodies specific for human IgE-producing B cells: a potential therapeutic for IgE-mediated allergic diseases. *BioTechnology* 8:122.
- Coyle, A. J., K. Wagner, C. Bertrand, S. Tsuyuki, J. Bews, and C. Heusser. 1996. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-Ig-antibody. *J. Exp. Med.* 183:1303.
- Corne, J., R. Djukanovic, L. Thomas, J. Warner, L. Botta, B. Grandordy, D. Gygax, C. Heusser, F. Patalano, W. Richardson, E. Kilcherr, T. Staehlin, F. Davis, W. Gordon, R. Liou, G. Wang, T. Chang, and S. Holgate. 1997. The effect of intravenous administration of chimeric anti-IgE antibody on serum IgE levels in atopic subjects: efficacy, safety and pharmacokinetics. *J. Clin. Invest.* 99:879.
- Haba, S., and A. Nisonoff. 1994. Effects of syngeneic anti-IgE antibodies on the development of IgE memory and on the secondary IgE response. *J. Immunol.* 152:51.
- Sherr, E. H., and A. Saxon. 1987. A mechanism for the suppression of ongoing IgE synthesis. *Int. Arch. Allergy Appl. Immunol.* 82:414.
- Benhamou, L. E., P. A. Cazenave, and P. Sarthou. 1990. Anti-immunoglobulins induce death by apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol.* 20:1405.
- Stämpfli, M. R., S. Miescher, I. Aebischer, A. W. Zürcher, and B. M. Stadler. 1994. Inhibition of human IgE synthesis by anti-IgE antibodies requires divalent recognition. *Eur. J. Immunol.* 24:2161.
- Miescher, S., M. Vogel, M. R. Stämpfli, E. Wasserbauer, F. Kricek, S. Vorburger, and B. M. Stadler. 1994. Domain-specific anti-IgE antibodies interfere with IgE binding to FcεRII. *Int. Arch. Allergy Immunol.* 105:75.
- Rudolf, M. P., K. Furukawa, S. Miescher, M. Vogel, F. Kricek, and B. M. Stadler. 1996. Effect of anti-IgE antibodies on FcεRI bound IgE. *J. Immunol.* 151:5646.
- Knutti-Müller, J. M., B. M. Stadler, C. M. Magnusson, and A. L. de Weck. 1986. Human IgE synthesis in vitro. *Allergy* 41:457.
- Cortese, R., F. Felici, G. Galfré, A. Luzzago, P. Monaci, and A. Nicosia. 1994. Epitope discovery using peptide libraries displayed on phage. *Tibtech* 12:262.
- Pasqualini, R., and E. Ruolahi. 1996. Organ targeting in vivo using phage display peptide libraries. *Nature* 380:364.
- Dybwad, A., B. Bogen, J. B. Natvig, O. Forre, and M. Sioud. 1995. Peptide phage libraries can be an efficient tool for identifying antibody ligands for polyclonal antisera. *Clin. Exp. Immunol.* 102:438.
- Yayon, A., D. Aviezer, M. Safran, J. L. Gross, Y. Heldman, S. Cabilly, D. Givol, and E. Katchalski-Katzir. 1993. Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage-epitope library. *Proc. Natl. Acad. Sci. USA* 90:10643.
- Luzzago, A., F. Felici, A. Tramontano, A. Pessi, and R. Cortese. 1993. Mimicking of discontinuous epitopes by phage-displayed peptides. I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene* 128:51.
- Koivunen, E., B. Wang, and E. Ruolahi. 1994. Isolation of a highly specific ligand for the $\alpha_5\beta_1$ integrin from a phage display library. *J. Biol. Chem.* 124:373.
- Hoess, R. H., A. J. Mack, H. Walton, and T. M. Reilly. 1994. Identification of a structural epitope by using a peptide library displayed on filamentous bacteriophage. *J. Immunol.* 153:724.
- Zhong, G., G. P. Smith, J. Berry, and R. C. Brunham. 1994. Conformational mimicry of a chlamydial neutralization epitope on filamentous phage. *J. Biol. Chem.* 269:24183.
- Stoute, J. A., W. R. Ballou, N. Kolodny, C. D. Deal, R. A. Wirtz, and L. E. Lindler. 1995. Induction of humoral immune response against *Plasmodium falciparum* sporozoites by immunization with a synthetic peptide mimotope whose sequence was derived from screening a filamentous phage epitope library. *Infect. Immun.* 63:934.
- Meola, A., P. Delmastro, P. Monaci, A. Luzzago, A. Nicosia, F. Felici, R. Cortese, and G. Galfré. 1995. Derivation of vaccines from mimotopes. *J. Immunol.* 154:3162.
- Felici, F., L. Castagnoli, A. Musacchio, R. Jappelli, and G. Cesareni. 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* 222:301.
- Hong, C. S., B. M. Stadler, M. Wälti, and A. L. de Weck. 1986. Dot-immuno-binding assay with monoclonal anti-IgE antibodies for the detection and quantitation of human IgE. *J. Immunol. Methods* 95:195.
- Neuberger, M. S., G. T. Williams, E. B. Mitchell, S. S. Jouhal, J. G. Flanagan, and T. H. Rabbitts. 1985. A hapten-specific chimeric IgE antibody with human physiological effector function. *Nature* 314:268.
- Zürcher, A. W., A. B. Lang, I. Aebischer, S. Miescher, and B. M. Stadler. 1995. IgE producing hybridomas established after B cell culture in the CD40 system. *Immunol. Lett.* 46:49.
- Vassella, C. C., A. L. de Weck, and B. M. Stadler. 1990. Influence of IgE fragments on IgE determination. *Int. Arch. Allergy Appl. Immunol.* 92:272.
- Kang, A. S., C. F. Barbas, K. D. Janda, S. J. Benkovic, and R. A. Lerner. 1988. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc. Natl. Acad. Sci. USA* 88:4363.
- Barbas, C. F., and R. A. Lerner. 1991. Combinatorial immunoglobulin libraries on the surface of phage (phabs): rapid selection of antigen-specific phabs. *Companion Methods Enzymol.* 2:119.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Bacterial media, antibiotics, and bacterial strains. In *Molecular Cloning: A Laboratory Manual*, 2nd Ed. C. Nolan, ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. A.1.
- Hunter, W. M. 1978. Radioimmunoassay. In *Handbook of Experimental Immunology*, Vol. 1. D. M. Weir, ed. Blackwell Scientific Publications, Oxford, p. 14.1.
- Hastings, G. Z., D. S. Burt, and D. R. Stanworth. 1988. A monoclonal antibody raised against a synthetic peptide representative of part of the amino acid sequence of rat immunoglobulin E detects thermally induced changes in that region of the IgE molecule. *Immunology* 65:149.
- Willis, A. E., R. N. Perham, and D. Wraith. 1993. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene* 128: 79.
- Motti, C., M. Nuzzo, A. Meola, G. Galfré, F. Felici, R. Cortese, A. Nicosia, and P. Monaci. 1994. Recognition by human sera and immunogenicity of HBsAg mimotopes selected from an M13 phage display library. *Gene* 146:191.
- Prezzi, C., M. Nuzzo, A. Meola, P. Delmastro, G. Galfré, R. Cortese, A. Nicosia, and P. Monaci. 1996. Selection of antigenic and immunogenic mimics of hepatitis C virus using sera from patients. *J. Immunol.* 156:4504.
- Adey, N. B., and B. K. Kay. 1996. Identification of calmodulin-binding peptide consensus sequences from a phage-displayed random peptide library. *Gene* 169: 133.
- Giebel, L. B., R. T. Cass, D. L. Milligan, D. C. Young, R. Arze, and C. R. Johnson. 1995. Screening of cyclic peptide libraries identifies ligands that bind streptavidin with high affinities. *Biochemistry* 34:15430.