

Anti-IgE autoantibodies mistaken for specific IgG

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During isolation of specific IgE by affinity chromatography, we obtained IgG acting as "specific IgG" in dot immunoassay and RAST. Further analysis of this fraction revealed that this specific IgG was indeed an immune complex consisting of IgG anti-IgE autoantibodies and specific IgE that falsely appeared as specific IgG in the immunoassays. After disruption of complexes, more IgE became accessible, resulting in increased counts per minute in the RAST. Thus, anti-IgE autoantibodies may hinder determination of specific IgE in in vitro immunoassays, since they hide specific IgE immunologically. To demonstrate the isotope specificity of such immune complex-derived IgG, the purified anti-IgE autoantibodies were radiolabeled and used as detection antibodies in immunoblotting. These autoantibodies recognized different IgE preparations, including a recombinant IgE peptide. (J ALLERGY CLIN IMMUNOL 1992;89:31-43.)

Key words: Specific IgE, specific IgG, allergen, anti-IgE autoantibody, RAST, immunoassay

Since the first description of blocking antibodies in serum of hay fever-allergic patients,¹ various attempts have been made to understand the basic mechanisms of immunotherapy. The increase of specific IgG antibodies during immunotherapy was later found to be restricted to IgG subclasses 1 and 4,^{2,3} and the predominant rise of IgG4 during immunotherapy was explained as being an effect of prolonged immunization with allergen.⁴ These findings initiated numerous studies in which levels of specific IgG were assessed in normal and atopic subjects,⁵ during hyposensitization with various allergens,⁶⁻¹¹ during therapy with β -lactam antibiotics.¹²

In a recent study with immunoblotting, we have also demonstrated that hyposensitization leads to significant increases of IgG1 and IgG4, but the same increase in specific IgG occurred also in patients who were not successfully treated.¹³ In contrast, we have also demonstrated that a large proportion of serum IgE occurs in complexes with IgG anti-IgE autoantibodies.¹⁴ This finding could mean that in our previous study on hyposensitization, in which we used im-

Abbreviations used

PBS:	Phosphate-buffered saline
PS-IgE:	Myeloma IgE
OD:	Optical density
TBS:	Tris-buffered saline
IC:	Immune complex
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
V _H :	Variable domain of heavy chain
MW:	Molecular weight

munoblots for measuring "specific" IgG, we might not have been able to distinguish whether the immunologically detected IgG was specific IgG or actually IgG anti-IgE autoantibodies in complexes with specific IgE.

To investigate whether specific IgE indeed occurs in such ICs of IgE with IgG anti-IgE autoantibodies, we intended to isolate specific IgE. We thereby demonstrated that IgG anti-IgE autoantibodies hide specific IgE and preclude its precise determination in immunoassays, such as immunodot and RAST. In contrast, in the same immunoassays, IgG anti-IgE autoantibodies, bound to specific IgE, were mistaken for "specific IgG," if detection was performed by an anti-IgG antibody. We also demonstrated that levels of IgG anti-IgE autoantibodies in serum of nonhyposensitized patients allergic to house dust mite or timothy-grass pollen were in the same order of magnitude as specific IgE.

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MATERIAL AND METHODS

Patients and sera

Patients allergic to either house dust mite or grass pollen were selected according to positive case history, positive skin prick test, and RAST class values, 4 (allergen disks for mite, d_1/d_2 ; for grass pollen, g_3/g_4 ; Pharmacia, Uppsala, Sweden). None of the patients had undergone hyposensitization therapy previously. Equal parts of four sera from mite-allergic patients (two male and two female patients; mean age, 26 years) were pooled (mite-serum pool). This mite-serum pool (57 ml) contained 1106 IU/ml of total IgE determined in PRIST (Pharmacia). The serum pool of grass pollen-allergic patients (grass serum pool, 2 ml) consisted of equal parts of 11 patients' sera (four female and seven male patients; mean age, 20.4 years) and had a total IgE content of 1062 IU/ml (evaluated by PRIST).

Affinity chromatography

Allergen extracts of house dust mite (*Dermatophagoides pteronyssinus*) and of timothy-grass pollen (*Phleum pratense*) were a kind gift from ALK (Allergologisk Laboratoriet Koebenhavn, Hoersholm, Denmark); 20 mg of each extract was bound to 2 gm of glutaraldehyde-activated affinity adsorbent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers' instructions. Mite- and grass-serum pools (1 ml per separation run) were diluted 1:1 in 0.9% NaCl before application to the respective column. Sera were recirculated for 1 hour at 4° C. Then, effluents were collected. After an extensive wash with 0.9% NaCl, bound material was eluted with 3 mol/L of NaSCN and immediately dialyzed against PBS at 4° C (dialysis membranes, Visking, Medicell Int., London, England; cutoff, 10 kd); 57 ml of mite-serum pool and 2 ml of grass-serum pool were applied consecutively in 1 ml portions to the respective allergen-affinity column. Samples eluted from the allergen columns were concentrated (Amicon filtration chambers, Amicon Corp., Lexington, The Netherlands) with ultrafiltration membranes with a cutoff of 10 kd (Diaflo, Amicon Corp.). The protein concentration of these samples was adjusted according to OD (at 280 nm) to 1 mg/1 ml of PBS. The material eluted from mite- and from timothy grass-pollen column was then individually loaded onto an anti-IgE column (1 gm of glutaraldehyde-activated affinity adsorbent coupled with 30 mg of Le 27, a mouse monoclonal antihuman IgE antibody,¹⁵ as described above). Loaded samples were recirculated for 1 hour at 4° C. The effluent was collected again, and after an extensive wash with 0.9% NaCl, the bound antibodies were eluted by 3 mol/L of potassium thiocyanate. Eluent and effluent were immediately dialyzed against PBS and concentrated as described above.

IgE preparations

As standard IgE, myeloma IgE (PS-IgE from a patient was a kind gift from Drs. T. and K. Ishizaka, La Jolla, Calif.) was used in dot immunoassays and immunoblots. PS-IgE was additionally purified to obtain monomeric IgE.¹⁶ In short, PS-IgE in 0.2 mol/L of Tris HCl, pH 8.6, was

reacted with dimethyl suberimidate. After the reaction was stopped with glycine, the reaction mixture was applied to a Sephadex G-200 connected with an Ultrogel AcA22 column (Bromma, Sweden) and eluted with *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid buffer. The eluted peak, which, according to MW, contained monomeric IgE, was concentrated, rerun on the same columns, and then frozen at -20° C.

Chimaeric IgE, consisting of a mouse V_H and human C_ϵ constant region, was purified from culture supernatants of the mouse hybridoma line, JW8/5/13,¹⁷ by affinity chromatography with a hapten-Sepharose column, as previously described.¹⁷

The DNA fragment coding for the CH3-CH4 peptide was obtained from the pC_ε-spl plasmid, kindly provided by Dr. Honjo (Osaka University Medical School, Osaka, Japan), as recently described in detail.¹⁸ The pC_ε-spl plasmid was cut with the restriction enzyme, Sac I, and a 1 kb DNA fragment, coding for the CH3-CH4 domains, including the last three adjacent amino acids of the CH2 domain, was isolated. The purified fragment was then ligated into the Sac I, digested expression vector pTrec 99A,¹⁹ which carries a tac promoter-operator and was kindly provided by Dr. E. Amann (Behringwerke AG, Hamburg, Germany). The recombinant DNA was used for transformation of *Escherichia coli* XL blue strain (Stratagene, San Diego, Calif.). Transformants were selected on Luria broth plates containing 100 µg of ampicillin per milliliter and then screened for CH3-CH4 peptide production. Screening was performed according to the procedure of Helfman et al.²⁰ with polyclonal and monoclonal anti-IgE antibodies (data not presented). Expression of CH3-CH4 peptide by one selected positive transformant was achieved as follows: 10 ml culture in Luria broth containing 50 µg of ampicillin per milliliter was grown for 2 hours at 37° C. When an OD of 0.5 at 600 nm was reached, 1 mmol/L of isopropyl-β-D-thiogalactoside was added, and cells were additionally incubated for another 2 hours. Then, from 1 ml of cell suspension, a whole cell extract was prepared in lysate buffer (62 mmol/L of Tris HCl, pH 6.8, 10% (vol/vol) of glycerol, 2% (wt/vol) of SDS, 700 mmol/L of 2-β-mercaptoethanol, and 0.001% (wt.vol) of bromphenol blue).

Immunodot assay

Antigens and antibodies were dotted onto nitrocellulose (Millipore Corp., Bedford, Mass.), as previously described.²¹ Briefly, the following reagents in TBS (2 mol/L of Tris, 1 mol/L of HCl, and 2 mol/L of NaCl, pH 7.4) were dotted (1 µl dots) in triplicate onto nitrocellulose strips: house dust-mite extract (10 mg/ml), timothy grass-pollen extract (10 mg/ml), monoclonal anti-IgE antibody, Le 27 (1 mg/ml), monoclonal anti-IgE antibody, BSW 17 (originally named BS¹⁵ but renamed because of commercially available anti-IgG BS 17; see below), PS-IgE (0.05 mg/ml), and myeloma IgG1, IgG2, IgG3, and IgG4 (World Health Organization references obtained from Dr. Skvaril, Bern, Switzerland) (1 mg/ml). Dots were air-dried and blocked with TBS containing 0.15% casein (Fluka Chemie AG, Buchs, Switzerland). Then, samples, adjusted to OD

0.15 at 280 nm (except serum pools and effluents from mite and timothy-grass column, which were diluted 1:10 in TBS/casein for detection of IgE and 1:100 for IgG), were applied onto the strips overnight at room temperature with constant shaking. On control strips only, TBS/casein was applied (blank control). After a wash with TBS (30 minutes; three changes), detection antibodies were applied for 2 hours at room temperature. Bound IgG (specific IgG binding to allergen dots; IgG anti-IgE autoantibodies on PS-IgE), IgG in complexes with IgE on dotted BSW 17 and Le 27, was detected by peroxidase-labeled anti-IgG BS 17 (Biotest AG, Frankfurt, Germany) (1:2000 in TBS/casein). Specific IgE on allergen dots was detected by peroxidase-labeled BSW 17 or, alternately, by Le 27 (1:500 in TBS/casein). Strips were then washed with TBS and developed for approximately 30 minutes with a solution of 16.6% (vol/vol) developing solution (stock: 4-chloro-1-naphthol and 3 mg/ml of methanol), 0.042% (vol/vol) of hydrogen peroxide in TBS. The reaction was stopped by washing with H₂O. Enzymatic reactions were analyzed with a hand-held densitometer (Gretag Ltd., Regensdorf, Switzerland). Absolute IgE or IgG values were then evaluated by comparison with IgE or IgG standard dots. For these standards, PS-IgE or human IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) were dotted in serial dilutions and developed with labeled anti-IgG BS 17, BSW 17, or Le 27 anti-IgE antibodies, respectively. IgE and IgG concentrations were calculated, based on logarithmic regression analysis with a computer program.

SDS-PAGE and electrotransfer

Six percent or 8% SDS-polyacrylamide separation and 5% stacking gels were prepared according to the method of Laemmli.²² Electrophoresis was carried out in a vertical electrophoresis unit (LKB, Bromma, Sweden). Samples were boiled in Laemmli sample buffer without mercaptoethanol for 5 minutes. Isolated antibodies (5 mg), recombinant IgE peptide CH3-CH4 (6 µg), PS-IgE (2.5 or 4 µg), chimaeric IgE (4 µg), and standard IgG (2.5 or 4 µg) were loaded per lane for immunoblotting and for Coomassie blue staining of the gels. For silver staining, 0.7 µg of antibody preparations was loaded per lane. Gels were stained with Coomassie or a silver-stain kit (Bio-Rad Laboratories, Richmond, Calif.). MWs were determined with prestained MW markers (Amersham Intl., Buckinghamshire, England). Proteins were transferred to nitrocellulose, 0.2 µm pore size (Schleicher & Schuell, Kassel, Germany), in a blotting tank (Bio-Rad Laboratories) for immunoblotting or staining with India ink.²³

Immunoblot

Peroxidase-labeled rabbit antihuman κ light chain and rabbit antihuman λ light chain antibodies (Dakopatts, Copenhagen, Denmark) were used. After saturation of blotted sheets, revealing antibodies were applied, 1:2000 in TBS/casein, overnight. Washing and developing was performed as described above (Immunodot assay).

BSW 17, BS 17, eluent, and effluent from the anti-IgE column were ¹²⁵I-labeled according to the method of Mc-

Conahay and Dixon²⁴; 50 µg of antibodies was labeled with 0.5 mCi ¹²⁵Iodine; 10⁶ cpm/50 cm² of nitrocellulose was applied overnight. After a wash with TBS, blots were dried and exposed to x-ray films (Fuji Photo Film Co., Ltd., Tokyo, Japan) for 24 hours or up to 18 days at -70°C.

Modified RAST

RAST allergen disks, standard disks, and standard serum were purchased from Pharmacia. For the experiments described, we used disks coupled with extracts from house dust mite (*D. pteronyssinus*, d.) and from timothy-grass pollen (*P. pratense*, g.). Samples (eluates from allergen columns and eluates and effluents from anti-IgE column) were adjusted to the same protein concentrations (OD 0.15 at 280 nm: 50 µl per disk). Effluents from allergen columns and serum pools were diluted 1:10 in PBS. An aliquot of each sample was heated for 30 minutes at 56°C just before application on RAST disks. Standard disks were tested with undiluted standard serum (Pharmacia) and developed with polyclonal anti-IgE (Pharmacia), as described by the supplier, except that 10⁵ cpm of labeled-revealing antibody was used. In the case of IgE and IgG detection with monoclonal antibodies, separate standards were prepared with the same commercial standard serum, but ¹²⁵I-labeled monoclonal anti-IgE, BSW 17, or monoclonal anti-IgG, BS 17, as developing antibodies; 50 µl of each sample (diluted serum, eluates, and effluents of allergen columns, and eluates and effluents of the anti-IgE column, and undiluted standard serum to the standard disks) was applied overnight. Disks were then washed with 0.9% NaCl containing 0.05% Tween 20 (30 minutes; three changes) and incubated with ¹²⁵I-labeled polyclonal rabbit antihuman IgE (Pharmacia), BSW 17, or BS 17. For each antibody, 10⁵ cpm in 100 µl of PBS was applied overnight. Disks were washed, and bound radioactivity was measured in a γ-counter (Gammamatic; Kontron Instruments AG, Zurich, Switzerland).

RESULTS

Purification of mite-specific antibodies

For purification of specific IgE and specific IgG antibodies, we fractionated a serum pool of patients allergic to house dust mite by a house dust mite-affinity column. The material eluted from this column was further passed over an affinity column coupled with monoclonal anti-IgE antibody (Le 27). This antibody and the monoclonal anti-IgE antibody, BSW 17, recognized PS-IgE but demonstrated no reaction with myeloma IgG₁, IgG₂, IgG₃, or IgG₄ in dot immunoassay. Their reactivity was directly compared with the monoclonal anti-IgG BS 17 and is presented in Table I. Antibodies in the serum pool, eluates, and effluents of the mite and anti-IgE columns were then measured by the immunodot assay.

As illustrated in Table II, specific IgE was detected by both monoclonal anti-IgE antibodies, BSW 17 and Le 27, in the serum pool. In the fraction eluted from the mite column and, additionally, from the anti-IgE

TABLE I. Reactivity of monoclonal antihuman immunoglobulin antibodies with immunoglobulin myelomas

Immunoglobulin†	% Binding of monoclonal antibodies*		
	Anti-IgE (Le 27)	Anti-IgE (BSW 17)	Anti-IgG (BS 17)
IgE	100	100	0
IgG1	0	0	96
IgG2	0	0	20
IgG3	0	0	100
IgG4	0	0	8

*Dot strips were developed with peroxidase-labeled monoclonal anti-IgE and anti-IgG antibodies. ODs were set equal 100% for the values obtained by the antibody specific for the respective immunoglobulin isotype.

†Purified immunoglobulin myeloma proteins (World Health Organization reference standards obtained from Dr. Skvaril, Bern, Switzerland) were dotted onto nitrocellulose strips at 1 µg per dot. PS-IgE (obtained from Dr. Ishizaka, La Jolla Calif.) was used at concentration of 50 ng per dot.

TABLE II. Immunodot analysis of antibody fractions isolated on house dust-mite and anti-IgE-affinity column

Detection method*	Immunoglobulin corresponding to 1 ml of serum (µg/ml)				
	Serum†	Mite column		Anti-IgE column	
		Effluent	Eluent	Effluent	Eluent
Specific IgE					
By monoclonal anti-IgE Le 27	0.78	0.00	0.00	0.00	0.00
By monoclonal anti-IgE BSW 17	1.42	0.00	3.32	0.72	2.30
Specific IgG					
By monoclonal anti-IgG BS 17	5.87	4.18	1.07	0.36	0.51
Anti-IgE					
By PS-IgE and BS 17	3.12	3.05	0.83	0.16	0.65
By Le 27 and BS 17	7.09	3.25	5.87	0.30	2.97
By BSW 17 and BS 17	5.51	4.18	1.77	0.23	1.02

*Unfractionated and fractionated samples were tested in immunodot assays. House dust mite-specific IgE and specific IgG reacting with mite extract (*D. pteronyssinus*) were dotted on nitrocellulose and detected by peroxidase-labeled monoclonal anti-IgE antibodies (BSW 17 or Le 27) or monoclonal anti-IgG (BS 17). IgG anti-IgE autoantibodies reacting with dots of IgE (PS-IgE) were detected by anti-IgG (BS 17). Complexes of IgE and anti-IgE autoantibodies were determined in a sandwich assay with Le 27 as IgE-catching antibody. Anti-IgG (BS 17) was used for developing complex-bound IgG anti-IgE autoantibodies. Data are corrected for volume and dilution with PS-IgE standards or normal human IgG standards and are expressed as the amount corresponding to 1 ml of the unfractionated serum pool. Detection limit of the sandwich immunodot assay is 1 to 10 ng of immunoglobulin per milliliter of incubation fluid.

†Serum pool of mite-allergic patients (1106 IU/ml total IgE in PRIST) and effluent from the mite column were diluted 1:10 for IgE determinations and 1:100 for IgG determinations. Eluent from the mite column, as well as eluent and effluent from the anti-IgE column, was tested at an equal protein concentration according to OD at 280 nm (see MATERIAL AND METHODS).

column, in which specific IgE was expected, only BSW 17 detected specific IgE (2.30 µg/ml of fractionated serum). Unexpectedly, specific IgG (0.51 µg/ml of serum) was also detected in the same sample. This finding prompted us to examine the sample for the presence of anti-IgE autoantibodies. By two different measuring principles, such anti-IgE autoantibodies were detected, namely, IgG anti-IgE either directly binding to PS-IgE (0.65 µg/ml of serum) or

IgG anti-IgE autoantibody in complexes with IgE (1.02 to 2.97 µg/ml of serum), thus retained by a monoclonal anti-IgE antibody. This finding suggested that IgE was present in the sample but hidden within ICs. The amounts of specific IgE and anti-IgE antibodies determined by these sandwich assays were similar and suggested a 1:1 ratio of IgE to IgG in the ICs.

Furthermore, that allergen-specific IgG was not as

TABLE III. Specific immunoglobulins in house dust-mite RAST

Detection method*	Treatment†	Serum pool‡	CPM (RAST class)	
			Mite-eluent after anti-IgE column	
			Effluent	Eluent
Specific IgE				
By polyclonal anti-IgE	Nonheated	15119 (III)	999 (0)	1296 (0)
	Heated	10801 (III)	1279 (0)	1501 (I)
By monoclonal anti-IgE BSW 17	Nonheated	2893 (III)	195 (I)	759 (II)
	Heated	3848 (III)	348 (II)	1018 (II)
Specific IgG				
By monoclonal anti-IgG BS 17	Nonheated	1256	34	234
	Heated	820	78	205

*Unfractionated and fractionated samples were incubated with commercial RAST disks (*D. pteronyssinus*, d₁; Pharmacia) and developed with either the supplied polyclonal anti-IgE (background, 208 cpm), monoclonal anti-IgE (BSW 17) (background, 481 cpm), or with monoclonal anti-IgG (BS 17) (background, 421 cpm). All three antibodies were applied at 100,000 cpm per disk. Data are expressed as average counts per minute of duplicate determinations minus indicated background.

†Heated samples were incubated for 30 minutes at 56° C in a waterbath.

‡Serum pool (class IV positive in house dust-mite RAST) and effluent from mite-affinity column were diluted 1:10; eluent from mite-affinity column, as well as eluent and effluent from anti-IgE column, was tested at protein concentrations of 3.5 µg per disk as described in MATERIAL AND METHODS. RAST classes (in parentheses) were calculated according to the supplier's instructions.

effectively retained by the allergen-affinity column as IgE is presented in Table II; but in the effluent from the anti-IgE column, specific IgG was in the same order of magnitude as the specific IgE, which had not bound to the column coupled with monoclonal anti-IgE Le 27. Also, anti-IgE autoantibodies were detected, but in lower amounts than in the sample eluted from the anti-IgE column.

Analysis of specific IgE and IgG by house dust-mite RAST

Results of the dot immunoassay implied that most measurable specific IgE bound to the mite-affinity column. In contrast, more IgG than that eluted from the mite column was found in the effluent. This finding could be due to different affinities of specific antibodies, depending on their isotype or caused by the quality of the allergen extract on the affinity column. To evaluate the latter possibility, we tested our samples with allergen extract obtained from a different supplier. We chose the commercial RAST and tested the mite-serum pool as well as the effluent obtained after the anti-IgE column at a 1:10 dilution.

In all samples, relatively more specific IgE was detected by the polyclonal than by the monoclonal anti-IgE antibody, as presented in Table III. However, with the corresponding standards, similar RAST classes were obtained in the serum pool. In the more diluted, purified samples, also lower RAST classes were detected. As expected, the monoclonal anti-IgG antibody detected specific IgG in the effluent material

from the anti-IgE column. But, most interestingly, even more "specific IgG" was observed in the eluent from the same column, supposedly containing the specific IgE only. These results suggested that the "specific IgG" antibodies in this fraction might actually be anti-IgE antibodies. Consecutively, we heated the samples to disrupt ICs, as described by Ingenäs et al.²⁵ Indeed, more specific IgE was detected by polyclonal and monoclonal anti-IgE in the heated samples. By use of the polyclonal anti-IgE (Pharmacia), the observed increase even resulted in an increase in RAST class in the material eluted from the anti-IgE column.

Analysis of mite-specific antibodies in immunoblot

Thus far, our experiments indicated that specific IgE was complexed with anti-IgE autoantibodies and was thereby hidden. Based on the estimation by the dot assay, these complexes appeared to contain IgE and IgG anti-IgE in a ratio of approximately 1:1. Therefore, we examined the composition of ICs by SDS-PAGE and immunoblotting (Fig. 1).

For comparison with the eluted material, we used normal human IgG that resulted in a broad protein band lower than the protein band of PS-IgE. Effluent and eluent from anti-IgE column contained bands of an MW corresponding to IgG. The eluted material demonstrated, in addition, a protein band at 98 kd. No band at the expected MW of IgE was revealed at the protein concentrations used.

In immunoblot, monoclonal anti-IgE (BSW 17) de-

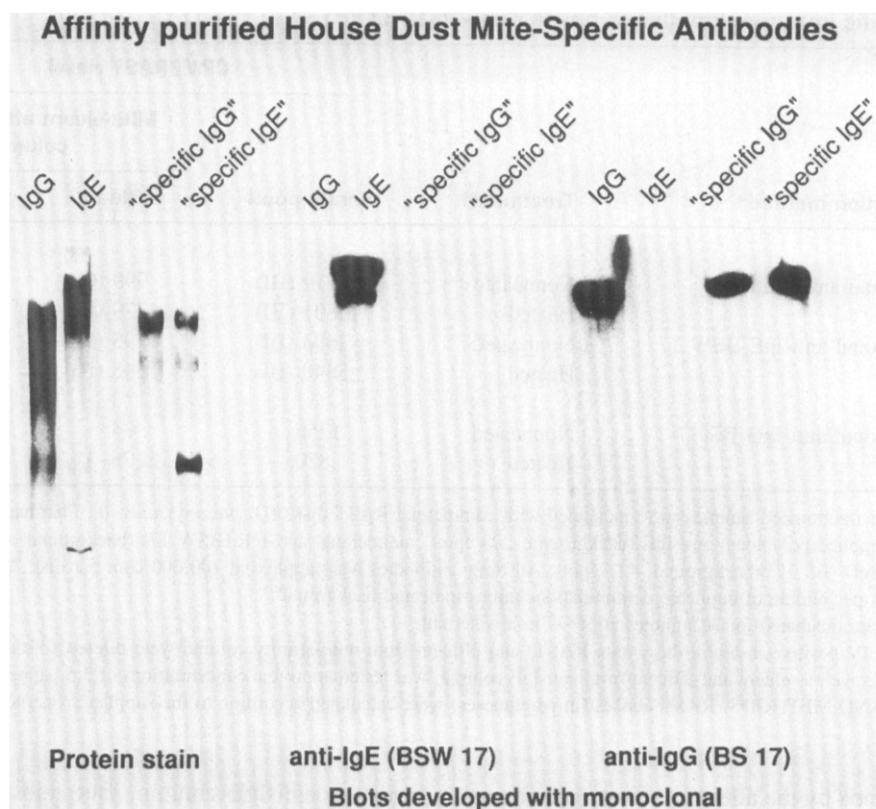


FIG. 1. Affinity-purified house dust mite-specific antibodies analyzed by SDS-PAGE and immunoblotting. Human normal IgG, PS-IgE, and samples eluted from a mite-affinity column and additionally separated on an anti-IgE column (0.7 μ g each per lane) are illustrated in silver-stained 6% SDS-PAGE (protein stain). Effluent from the anti-IgE column is termed "specific IgG"; the eluent, "specific IgE." Same samples were also transferred to nitrocellulose (IgG, 2.5 μ g; PS-IgE, 2.5 μ g; eluent and effluent, 5 μ g each per lane) and developed with ^{125} I-labeled monoclonal anti-IgE (BSW 17) or monoclonal anti-IgG (BS 17) antibody; 10^6 cpm of each antibody was used per membrane. Blots were exposed 24 hours at -70° C.

tected only standard IgE (2.5 mg per slot) but did not bind to any of the protein bands as detected by the protein stain in eluent and effluent sample (5 mg per slot) at an exposure time of 48 hours. Furthermore, another monoclonal anti-IgE (Le 27) and polyclonal anti-IgE antibody (Pharmacia) failed to detect IgE in any of the column samples (data not presented). On the contrary, the anti-IgG antibody (BS 17) detected strong IgG bands in the eluent as well as in the effluent from the anti-IgE column with the same MWs as standard IgG. The 98 kd component was not recognized by anti-IgE or anti-IgG antibodies at the indicated exposure time of the autoradiographs.

Thus, by immunoblotting, IgG was revealed with relative ease in the effluent and in the eluent. The IgG in the effluent might therefore represent the specific IgG. However, we failed to detect IgE in the eluent

from the anti-IgE column at the protein concentrations used, even though our specific immunoassays suggested it is present but partially hidden by IgG anti-IgE autoantibodies. The ratio of specific IgE to anti-IgE autoantibodies might therefore be even greater than estimated by immunologic assays.

To examine further whether a longer exposure time might reveal IgE with the monoclonal anti-IgE (BSW 17), the same membrane as illustrated in Fig. 1 was exposed for 18 days (Fig. 2). Interestingly, a clear signal from the 98 kd band was obtained by the monoclonal anti-IgE (BSW 17).

If corresponding membranes were tested with anti- κ and anti- λ light chain-specific antibodies, both detected light chains in standard IgG as well as in the isolated IgG antibody fractions. Standard PS-IgE contained exclusively λ chains that were detected in the

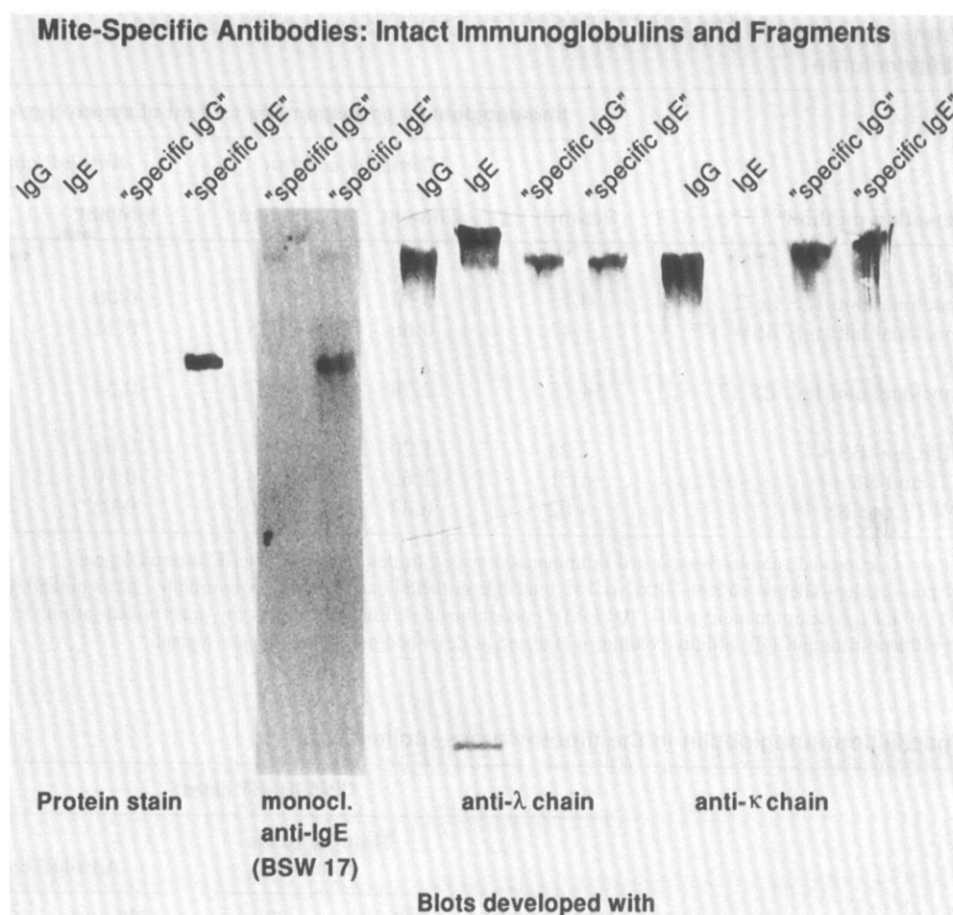


FIG. 2. Mite-specific antibodies: intact immunoglobulins and fragments; isolated house dust mite-specific antibodies analyzed by immunoblot. Similar membranes as illustrated in Fig. 1 were India ink stained (protein stain) or immunoblotted; the membrane tested with ¹²⁵I-labeled monoclonal anti-IgE (BSW 17), as illustrated in Fig. 1, is illustrated after exposure for 18 days. In addition, membranes were tested with peroxidase-labeled polyclonal anti-λ and anti-κ light chain antibodies.

intact molecule as well as in a sharp band of 25 kd. However, none of the column samples tested were positive for light chains in bands other than the IgG.

Affinity chromatography and specificity of antibodies from timothy grass-pollen serum pool

To reexamine our above findings by use of another allergen, we fractionated a serum pool of patients allergic to grass pollen on a timothy grass pollen-affinity column followed by anti-IgE column (Le 27). As in the fractionated mite-serum pool, only little specific IgE was detected by the dot immunoassay in material eluted from the anti-IgE column (Table IV). Again, "specific IgG" was detected in the sample ex-

pected to contain only timothy grass pollen-specific IgE, and anti-IgE autoantibodies were detected in the same sample. Values for specific IgE and "specific IgG" were in a similar range in the sample derived from the grass pollen-allergic patients' serum, suggesting again a 1:1 ratio of IgE to IgG in the ICs.

To demonstrate that timothy grass pollen-specific IgE also might be hidden by anti-IgE autoantibodies, samples were tested by the commercial RAST assay (Table V). The supplied polyclonal anti-IgE antibody (Pharmacia) or our monoclonal anti-IgE (BSW 17) detected specific IgE in the order of RAST class 3 in the timothy grass pollen-serum pool. Specific IgE was again detectable in the eluted fractions. The monoclonal anti-IgG antibody (BS 17) again detected

TABLE IV. Immunodot analysis of antibody fractions isolated on timothy grass-pollen- and anti-IgE-affinity column

Detection method*	Immunoglobulin corresponding to 1 ml of serum ($\mu\text{g/ml}$)				
	Serum†	Timothy column		Anti-IgE column	
		Effluent	Eluent	Effluent	Eluent
Specific IgE					
By monoclonal anti-IgE Le 27	0.75	0.32	0.12	0.00	0.16
By monoclonal anti-IgE BSW 17	1.47	0.66	0.25	0.22	0.00
Specific IgG					
By monoclonal anti-IgG BS 17	14.18	2.78	0.57	0.54	0.64
Anti-IgE					
By PS-IgE and BS 17	2.28	1.20	0.05	0.04	0.06
By Le 27 and BS 17	5.51	12.62	0.14	0.10	0.37
By BSW 17 and BS 17	4.02	6.47	0.10	0.08	0.22

*Unfractionated and fractionated samples were tested in immunodot assay, as described in Table II, *asterisk* legend.

†Serum pool of grass pollen-allergic patients (1062 IU/ml total IgE in PRIST) and effluent from timothy, grass pollen-affinity column were diluted 1:10 for IgE determinations and 1:100 for IgG determinations. Eluent from timothy grass pollen-affinity column, as well as eluent and effluent from anti-IgE-affinity column, was tested as described in Table I, *dagger* legend.

TABLE V. Specific immunoglobulin in the timothy grass-pollen RAST

Detection method*	Treatment†	Serum pool‡	CPM (RAST class)			
			Timothy grass column		Anti-IgE column	
			Effluent	Eluent	Effluent	Eluent
Specific IgE						
By polyclonal	Nonheated	21728 (III)	137 (0)	948 (0)	57 (0)	1025 (0)
Anti-IgE	Heated	20465 (III)	170 (0)	865 (0)	60 (0)	1697 (I)
By monoclonal	Nonheated	5535 (III)	55 (0)	275 (I)	22 (0)	165 (0)
Anti-IgE (BSW 17)	Heated	6312 (III)	67 (0)	205 (I)	23 (0)	348 (I)
Specific IgG						
By monoclonal	Nonheated	2387	85	122	33	135
Anti-IgG (BS 17)	Heated	2235	85	161	– 108	119

*Unfractionated and fractionated samples were incubated with commercial RAST disks (*P. pratense*) and developed with either the supplied polyclonal anti-IgE (background, 224 cpm), monoclonal anti-IgE (BSW 17) (background, 457 cpm), or with monoclonal anti-IgG (BS 17) (background, 300), as described in Table II, *asterisk* legend.

†Heated samples were incubated for 30 minutes at 56° C in a waterbath.

‡Serum pool (class IV positive in grass-pollen RAST) and effluent from timothy grass pollen-affinity column were diluted 1:10; eluent from timothy-grass column, as well as eluent and effluent from anti-IgE column, was tested at protein concentrations of 3.5 μg per disk, as described in MATERIAL AND METHODS. RAST classes (in parentheses) were calculated according to the supplier's instructions.

“specific IgG” in the eluent from the anti-IgE-affinity column, supposedly representing anti-IgE autoantibodies. The relative amount of this “specific IgG” in the eluent was once more higher than the real specific IgG in the effluent fraction. As in the case of the mite-specific material, heating of the samples led to an increase of measurable specific IgE in the eluent from

the anti-IgE-affinity column, resulting in an increase of RAST class from 0 to 1.

As illustrated in Fig. 3, again, no protein of an MW corresponding to IgE was revealed in the fraction eluted from the anti-IgE column, supposed to contain the timothy grass-specific IgE only. Also, by immunoblotting with monoclonal anti-IgE (BSW 17) (or

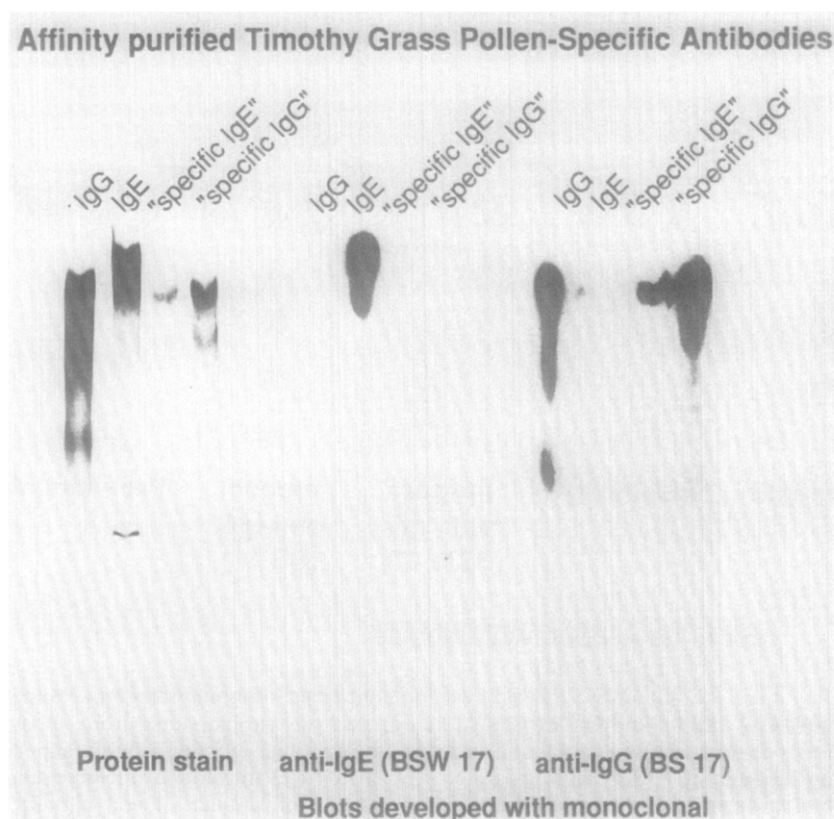


FIG. 3. Affinity-purified timothy grass pollen-specific antibodies analyzed by SDS-PAGE and immunoblotting. Human normal IgG, PS-IgE and, samples eluted from a house dust mite-affinity column and additionally separated on an anti-IgE column (0.7 μ g each per lane) are illustrated in silver-stained 6% SDS-PAGE (protein stain). Effluent from the anti-IgE column is termed "specific IgG"; the eluent, "specific IgE." Same samples were also transferred to nitrocellulose (IgG, 2.5 μ g; PS-IgE, 2.5 μ g; eluent and effluent, 5 μ g each per lane) and developed with ^{125} I-labeled monoclonal anti-IgE (BSW 17) or monoclonal anti-IgG (BS 17) antibody; 10⁶ cpm of each antibody was used per membrane. Blots were exposed 24 hours at -70° C.

monoclonal anti-IgE, Le 27, as well as polyclonal anti-IgE from Pharmacia; data not presented) as revealing antibody, no IgE was detected at the protein concentrations tested. However, the monoclonal anti-IgG antibody (BS 17) again clearly revealed a band corresponding to IgG in the sample, supposedly containing "specific IgE." Thus, similar results were obtained by use of timothy-grass pollen as allergen as obtained by house dust-mite extract in immunoassays and Western blot.

Use of isolated antibodies as developing antibodies

Until this stage of experimentation, the immunoassays (either in-house or commercial) clearly documented allergen-specific IgE in the material eluted from the anti-IgE column. However, based on the immunoblots, it appeared that most of the sample load was recognized by the anti-IgG antibody only. Instead

of loading more material onto the gels for eventually detecting a faint band corresponding to IgE, we preferred to study whether the presumed IgG anti-IgE autoantibodies, which represented most antibodies in the specific-IgE fraction, were indeed capable of recognizing IgE. The eluent and effluent from the anti-IgE column (obtained from house dust mite experiments) were therefore labeled with ^{125} iodine and used as revealing antibodies for immunoblotting (Fig. 4). As positive control antibodies, we used monoclonal anti-IgE (BSW 17) and monoclonal anti-IgG (BS 17). As already illustrated in Fig. 1, the monoclonal anti-IgE reacted exclusively with PS-IgE. The monoclonal anti-IgG antibody detected IgG in the normal human IgG preparation and in the sample eluted from the anti-IgE-affinity column.

The labeled antibodies of the *eluent* fraction from the anti-IgE column reacted as expected with PS-IgE, and somewhat weaker with standard normal IgG prep-

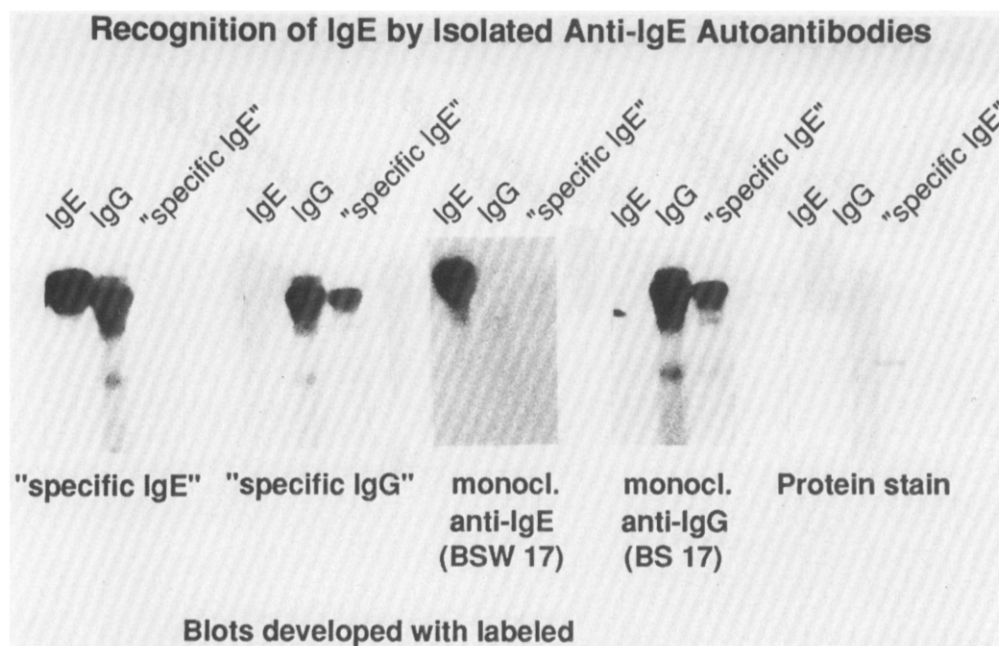


FIG. 4. Use of anti-IgE autoantibodies as revealing antibodies in immunoblotting; PS-IgE (*IgE*) (2.5 μ g per lane), normal human IgG (*IgG*) (2.5 μ g), and the sample eluted from house dust mite-affinity column and also additionally eluted from anti-IgE-affinity column, supposedly containing "specific IgE" (5 μ g), were separated in 8% SDS-PAGE and blotted onto nitrocellulose. Blots were revealed with ^{125} I-labeled house dust-mite eluent/anti-IgE eluent, termed "specific IgE," which contained anti-IgE autoantibodies, and with ^{125} I-labeled house dust-mite eluent/anti-IgE effluent, termed "specific IgG." Immunoblots were also developed with ^{125} I-labeled monoclonal anti-IgE (BSW 17) and monoclonal anti-IgG (BS 17); 10^6 cpm was applied onto one blot sheet. Blots were exposed for 24 hours. On the far right a protein stain of the membrane is illustrated (India ink stain).

aration, but did not react with themselves. Interestingly, antibodies of the *effluent* from the anti-IgE column, supposedly containing allergen-specific IgG, bound to standard normal IgG and to self-IgG eluted from the anti-IgE column, but did not recognize PS-IgE. This result suggested that only the antibodies *eluted* from the anti-IgE-affinity column are anti-IgE autoantibodies.

Thus far, we have demonstrated the anti-IgE binding of purified autoantibodies only by use of the PS-IgE. To study further the specificity of the isolated anti-IgE autoantibodies, we used additional IgE preparations (Fig. 5). A recombinant IgE peptide, consisting only of the CH3 to CH4 domains, was recognized as a single band by the isolated anti-IgE autoantibodies. No cross-reaction was observed with the numerous bacterial proteins in the unpurified sample. In addition, anti-IgE autoantibodies and monoclonal anti-IgE also recognized chimaeric IgE, consisting of mouse V_H combined with human constant region.¹⁷

Most interestingly, the latter experiments demonstrate that isolated anti-IgE autoantibodies recognize, very specifically, a recombinant nonglycosylated IgE

peptide in a crude preparation. This finding and the fact that they also detected a chimaeric IgE suggest that a large proportion of the isolated anti-IgE autoantibodies is strictly isotype specific. At the present time, it has not been evaluated how much of the anti-IgE activity (as observed in immunoassays) was due to the recognition of variable regions or other epitopes shared by IgE with other immunoglobulin isotypes.

DISCUSSION

The present study was initially intended to purify allergen-specific IgE. However, it became clear that it may be difficult to purify specific IgE without simultaneously obtaining IgG anti-IgE autoantibodies. Based on immunoblotting, anti-IgE autoantibodies appeared to be even in excess of measurable specific IgE. The presence of anti-IgE autoantibodies may also be the reason it is difficult, if not impossible, to precisely measure specific IgE in assays with a small amount of allergen on a solid phase. After dissociation of ICs of IgE and IgG by heating,²⁵ we detected more IgE in the heated samples, suggesting that specific IgE was liberated from ICs. The increased counts per

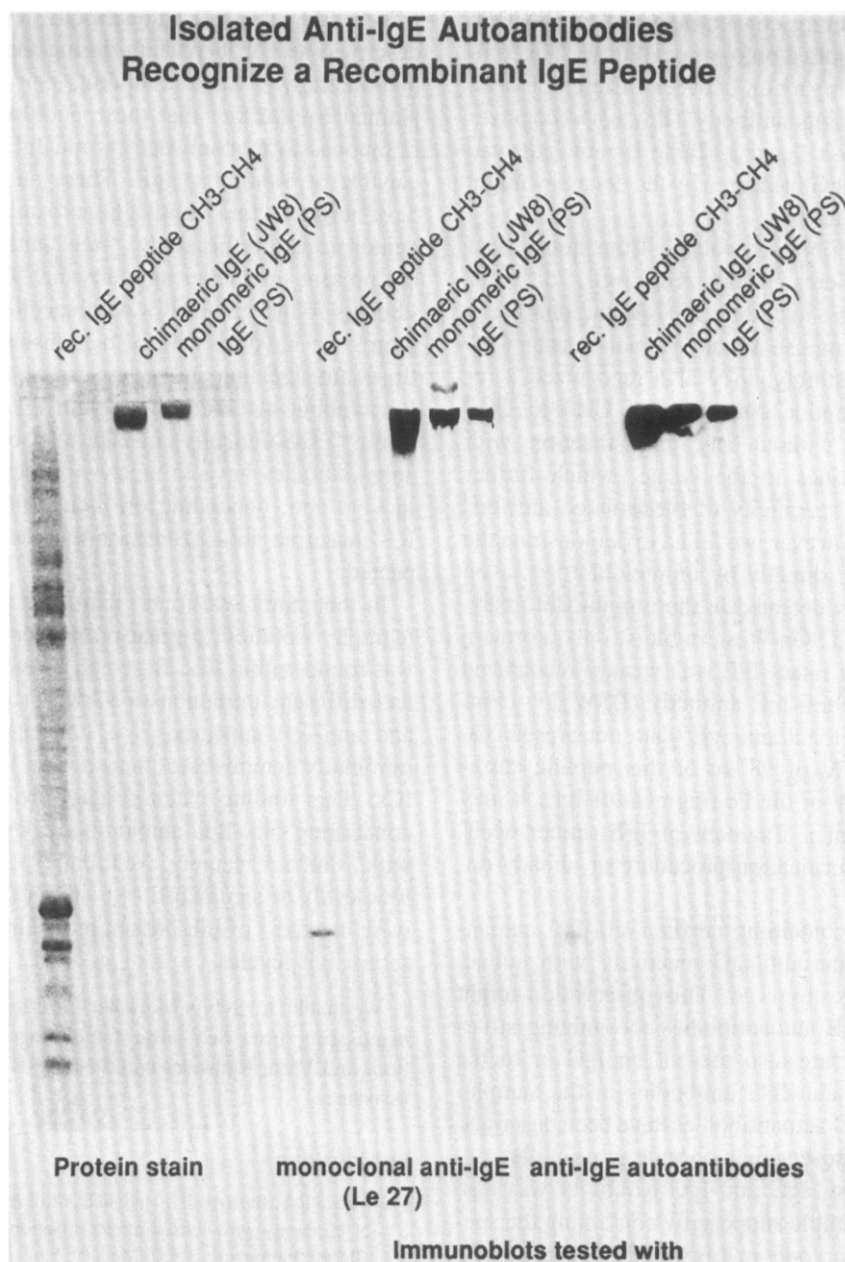


FIG. 5. Isolated anti-IgE autoantibodies recognize a recombinant IgE peptide. Recombinant IgE peptide CH3-CH4 (6 μ g), chimaeric IgE (JW8) (4 μ g), and monomeric (4 μ g), as well as unfractionated PS-IgE (4 μ g) were compared in protein-stained 8% SDS-PAGE (Coomassie). Same samples at same protein concentrations were also immunoblotted and incubated with peroxidase-labeled monoclonal anti-IgE (Le 27) or with ^{125} I-labeled anti-IgE autoantibodies (10^6 cpm). The radiolabeled blot was exposed for 4 days.

minute in the RAST may be, in part, due to anti-IgE autoantibodies no longer recognizing heat-labile epitopes on IgE, which now became accessible for revealing antibodies, or reassociation of anti-IgE and IgE was not yet completed and labeled antibodies were capable of detecting IgE instead.

We measured anti-IgE autoantibodies by two types

of assays: on the one hand, complexes of IgE and anti-IgE autoantibodies were measured by a sandwich technique with specific monoclonal antibodies. In contrast, IgG anti-IgE autoantibodies, which bound to dotted PS-IgE, were termed "free" anti-IgE autoantibodies because they had *free* valences to bind. The standardization of both types of assays is based

on measuring different concentration of purified human IgG directly dotted onto nitrocellulose. This procedure allowed comparison between different assays. Since our developing antibody (BS 17) recognizes mostly IgG subclass 1 and 3 (Table I), our indicated concentrations of IgG measured by the dot assay might even be underestimated.

Anti-IgE autoantibodies isolated from house dust mite-allergic patients recognized, well, different preparations of IgE, including PS-IgE and chimaeric IgE consisting of mouse V_H and human constant region. Most interestingly, they also recognized a recombinant IgE peptide consisting of CH3 to CH4 domains of Fc ϵ without any cross-reaction with the bacterial peptides in the lysate, which further demonstrated the specificity of the isolated anti-IgE autoantibodies. However, we failed to demonstrate the isolated intact IgE directly by immunoblotting, even when it was easily detected in the immunodot assay, as well as in RAST. On Western blots, only a protein band smaller than intact IgE was weakly recognized by a monoclonal anti-IgE antibody (BSW 17) (Fig. 2) and by a polyclonal anti-IgE (data not presented). The observed MW of 98 kd of this peptide corresponds to the MW of the Fc fragment of IgE. It may therefore represent a fragment of IgE, either accumulated by the purification procedure or already degraded *in vivo*.^{26, 27}

In the sample containing purified anti-IgE autoantibodies, also some anti-IgG reactivity with human standard IgG was observed. This observation might be due to anti-IgE autoantibodies recognizing structures of IgE that are also present on IgG or to the presence of IgG anti-IgG antibodies in the sample. Such IgG anti-IgG autoantibodies have been described to rise during hyposensitization with grass-pollen extract.²⁸ In contrast, IgG anti-IgG reactivity was also detected in the sample supposedly containing the specific IgG. However, only in this sample, IgG reactivity directed against the isolated IgG anti-IgE autoantibodies was observed.

Since the first description of anti-IgE autoantibodies,²⁹ substantial evidence has accumulated that IgG anti-IgE autoantibodies might play a role in different diseases.³⁰⁻³³ As demonstrated in this study, IgG anti-IgE autoantibodies can easily be mistaken for "specific IgG" because of their (indirect via IgE) binding to the allergen on the solid phase. Also, our RAST data suggested that IgG, interpreted as allergen specific, comprises more anti-IgE autoantibodies than true specific IgG. Thus, results obtained by measuring "specific IgG" by similar immunoassays may also reflect a certain contamination with anti-IgE autoantibodies, and some will probably have to be reexamined under this new aspect.⁵⁻¹²

Recently, anti-IgE autoantibody levels have been described as not increasing during the course of immunotherapy.³⁴ Such a finding may easily be explained by the fact that these authors have used an immunoassay that only detects anti-IgE autoantibodies capable of binding to IgE. These autoantibodies we have termed "free" anti-IgE autoantibodies. But as demonstrated in this study, "free" anti-IgE antibodies are present in smaller amounts than the anti-IgE antibodies found within ICs with specific IgE. Furthermore, we have previously demonstrated that sometimes anti-IgE autoantibodies can be assessed within ICs without detecting "free" anti-IgE autoantibodies at all.¹⁴ Theoretically, it can also be assumed that autoantibodies have to react with their antigen (IgE) *in vivo* and, depending on their affinity, even more ICs than free anti-IgE autoantibodies have to be expected.

In this study, we found that after affinity-chromatography isolation, a great proportion of specific IgE was found within ICs. Based on Western blot analysis and different immunoassays, we estimated that IgE and anti-IgE appeared in a 1:1 ratio. This finding minimally demonstrates that specific IgE exists within ICs. This finding extends our previous observation concerning anti-IgE autoantibody levels in children with asthma.³⁵ Namely, we found that in children with low levels of serum IgE (<5 IU/ml), anti-IgE levels were in excess of IgE but not in children with elevated serum IgE levels.

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