

A MONOCLONAL ANTI-IgE ANTIBODY AGAINST AN EPITOPE (AMINO ACIDS 367–376) IN THE CH3 DOMAIN INHIBITS IgE BINDING TO THE LOW AFFINITY IgE RECEPTOR (CD23)¹

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We have produced three different mAb specific for human IgE-Fc. Their binding pattern to either heat-denatured IgE or a family of overlapping IgE-derived recombinant peptides and their ability to affect interaction of IgE with its low affinity receptor FcεR2/CD23 demonstrate that they recognize distinct epitopes on the IgE molecule. All three mAb were able to induce basophil degranulation as measured by the induction of histamine release. mAb 173 recognizes a thermolabile epitope in the CH4 domain. It does not affect the binding of IgE to FcεR2/CD23. mAb 272 recognizes a thermostable epitope that maps to a sequence of 36 amino acids (AA) spanning part of the CH2 and CH3 domain and it does not affect the binding of IgE to FcεR2/CD23. mAb 27 recognizes a thermolabile epitope located on a 10 AA stretch (AA 367–376) in the CH3 domain. This area contains one N-linked oligosaccharide (Asn-371), but the antibody is not directed against carbohydrate because it binds to *Escherichia coli*-derived IgE peptides. mAb 27 inhibits the binding of IgE to FcεR2/CD23 but is still capable of reacting with IgE already bound to FcεR2/CD23. These data suggest that upon binding to FcεR2/CD23, the IgE molecule engages one of two equivalent-binding sites close to the glycosylated area of the CH3 domain.

Cell surface receptors for the Fc fragments of IgE (FcεR) have been identified on a variety of cell types from hematopoietic origin. A low affinity type of receptor has been identified on T and B lymphocytes, monocytes, platelets, and eosinophils (1, 2). It has been recently demonstrated that the low affinity receptor for IgE on B cells is identical to the CD23 Ag earlier described as a B cell-activation marker (3–5). In rodents, lymphocytes bearing low affinity receptors for IgE and their soluble products, collectively called IgE-binding factors, have been shown to play a major role in the regulation of IgE

production (6, 7).

Recently, IL-4 (8, 9) has been shown to induce enriched B cells preparations to produce IgE (10, 11), possibly through the induction of FcεR2/CD23³ or the release of a soluble form of FcεR2/CD23 (12, 13). Basophils and mast cells express FcεR1, which bind IgE with high affinity, and the reaction of allergen with cell-bound IgE induces the release of a variety of pharmacologically active mediators that are responsible for the clinical manifestations of allergic disorders (14, 15). The region of the IgE molecule that interacts with the high affinity IgE receptor (FcεR1) on basophil/mast cells has been recently mapped to span the junction between the CH2 and CH3 domains (residues 301–376) using the capacity of IgE fragments to inhibit passive cutaneous anaphylaxis (16–18). In contrast, the region of the IgE molecule that interacts with the FcεR2/CD23 on B cells has not yet been identified.

In this study, we report the characterization of three mAb specific for human IgE. One of these antibodies, mAb 27, is specific for an AA epitope in the CH3 domain of IgE (residues 367–376) and inhibits the binding of IgE to FcεR2/CD23, suggesting that a site close to or involving this epitope interacts with FcεR2/CD23.

MATERIALS AND METHODS

Generation of anti-human IgE mAb. BALB/c mice were immunized i.p. with 50 µg of IgE PS (a kind gift from Dr. Ishizaka) preincubated one night at 4°C with 5 µl of anti-human IgG mouse serum in CFA (GIBCO, Grand Island, NY). Two weeks later, the same preparation was injected s.c. in incomplete Freund's adjuvant. One month after the last boost, and for 3 consecutive days the mice received i.v. 50 µg of IgE PS in PBS. The following day, one mouse was bled and its spleen was removed for the fusion. Spleen cells were fused with NS1 mouse myeloma cells (ratio 5/1) in polyethylene glycol 4000. After the fusion, cells were incubated for one night at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Flow laboratories, Irvine, Scotland). The next day, cells were resuspended in RPMI 1640 complete medium containing 0.1 mM hypoxanthine, 6 × 10⁻³ mM azaserine, 10⁻³ U/ml insulin (all from Sigma, St Louis, MO) at 5 × 10⁵ cells/ml and distributed in 96-well plates with mouse peritoneal macrophages. Two weeks later, hybridoma supernatants were harvested and tested for their ability to bind IgE in a sandwich ELISA. As control, all supernatants were tested in the same manner for their cross-reaction on human IgG. Hybrids positive on IgE alone were cloned and expanded for supernatant production or for ascitic fluid production.

Preparation of IgE-derived peptides. The cloning and expression of gene fragments coding for the CH2-4 region of IgE (19) and smaller IgE-derived gene fragments have been described in detail previously

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³ Abbreviations used in this paper: FcεR2/CD23, low affinity receptor for IgE on lymphocytes; AA, amino acid; FcεR1, high affinity receptor for IgE on basophils.

(18, 20). The recombinant ϵ -chain polypeptides used in the present study were solubilized from cultures of *Escherichia coli* and partially purified by selective ammonium sulfate precipitation. Figure 4A shows the genetic constructs employed for antibody mapping; the names of the recombinant ϵ -peptides correspond to the names of the domains they contain; primed numbers indicate a truncated domain.

Sandwich ELISA. Rabbit polyclonal antibodies anti-human IgE (Dakopatts, Glostrup, Denmark) or anti-human IgG (Behring, Marburg, Germany) diluted 1/2000 in carbonate-bicarbonate buffer were coated on ELISA plates (Immunoplates, Nunc, Denmark) for one night at 4°C. Plates were then incubated with PBS containing 1% BSA for 1 h at room temperature. After washes with PBS 0.05% Tween (all washes were performed with this buffer), polyclonal human IgE (Behring), monoclonal human IgE PS (λ, ϵ), monoclonal human IgE Lyon (κ, ϵ) (21), rIgE-derived fragments, polyclonal human IgG (a gift from Dr. Rigal, Blood Bank, Lyon, France) or medium were incubated for 2 h at room temperature. After washes, hybrid supernatants were added for 2 h at room temperature. The binding of the mAb was monitored by using an anti-mouse Ig conjugated to alkaline phosphatase (Sigma). Finally, enzyme substrate *p*-nitrophenylphosphate (sigma) in 1 M diethanolamine buffer was added. Plates were incubated at 37°C and optical density was measured at 405 nm using a Dynatech MR 580 Autoreader.

Indirect ELISA. In this assay, human IgE, IgG, IgM, or IgA purified as described earlier (22) were directly coated at 200, 100, and 50 ng/well. The mAb and the conjugates were added as described above. Positive controls were mAb against IgG clone 8a4 (Immunotech, Luminy, France) anti-IgM clone 145-8 (Becton Dickinson, Mountain View, CA) and anti-IgA (Serotec, Oxon, England).

mAb purification: Fab fragment preparation. Isotype determination of the mAb was carried out using a Meloy radial immunodiffusion kit (Meloy, Springfield, VA). All mAb were purified by using protein A affinity chromatography (Bio-Rad, Richmond, CA) either from ammonium sulfate precipitates of hybridoma supernatants or from ascitic fluids. For the Fab preparation, purified mAb diluted in PBS containing 0.01 M cysteine and 2 mM EDTA, pH 7, was incubated for 1 h at 37°C with papain (24 U for 100 mg of Ig). The pH was then adjusted to 8.5 and after dialysis the mixture was passed over a protein A column. The Fab fragment was recovered in the effluent, digestion and purity were checked on SDS-PAGE followed by silver staining (BioRad Kit).

Western blot analysis and dot immunoassay. SDS polyacrylamide gel electrophoresis of 10 μ g of IgE or partially purified rIgE peptides in sample buffer containing 0.1 M 2-ME was carried out according to Laemmli (23) on a 15% acrylamide gel. After electrophoresis, gels were transferred onto nitrocellulose paper (Bio-rad) as described by Towbin et al. (24) in an electroblot apparatus (Transblot cell, Bio-Rad) at 100 mA overnight. For the dot immunoassay, 10 and 50 ng of IgG, IgA, IgM, or IgE in PBS were dotted on nitrocellulose. After drying, strips were incubated for 30 min in PBS 0.1% Tween at room temperature. Hybridoma supernatants were added overnight at 4°C. After three washes in PBS with 0.05% Tween, binding of the mAb was monitored by addition of ^{125} I-labeled rabbit anti-mouse IgG or horseradish peroxidase linked to rabbit anti-mouse IgG (diluted in PBS 1% BSA 0.05% Tween for 2 h at room temperature). After three washes, sheets were dried and exposed for autoradiography or, in cases in which horseradish peroxidase linked antibody was used, developed by the method of Adams (25).

Immunoprecipitation. A total of 30 ng (about 100,000 cpm) of IgE or IgG ^{125} I-labeled by the chloramine T method were incubated with 100 μ l of hybrid supernatant for 30 min at room temperature. Precipitation was achieved by adding 10 μ l of a rabbit anti-mouse Ig diluted with 10% human serum. After incubation for 1 h at room temperature and overnight at 4°C, tubes were centrifuged 15 min at 10,000 $\times$ g. Pellets were washed twice and counted in a gamma counter (LKB, Bromma, Sweden).

Competition experiment between anti-human IgE mAb. RIA tubes were coated overnight at 4°C with 400 μ l of polyclonal anti-human λ -chain (Behring) dilution 1/2000 in carbonate-bicarbonate buffer. After saturation for 1 h at room temperature with PBS containing 1% BSA and three washes in PBS with 0.05% Tween (all washes were performed with this buffer), 400 μ l of IgE PS (12.5 ng/ml in PBS with 1% BSA) were added and incubated for 2 h at room temperature. After three washes, ^{125} I-labeled (chloramine T method) purified anti-IgE mAb alone or in the presence of unlabeled purified mAb (or 200 μ l of clone supernatant) were added and incubated 2 h at room temperature. After three washes, radioactivity of the tubes was determined with a gamma counter.

Recognition of IgE bound to RPMI 8866 by anti-IgE mAb. A total of 50 μ l of RPMI 8866 cell suspension (10^7 cells/ml) was incubated with 40 μ l of monomeric IgE PS (20 μ g/ml) in 96-well, V-bottom plates in PBS containing 0.1% sodium azide and 1% BSA (all dilutions and all washing steps were carried out using this buffer) for 30 min

at 4°C. Plates were washed twice and cells were resuspended with 50 μ l of clone supernatant or a dilution of purified mAb for 30 min at 4°C. After two washes, binding of the mAb to IgE was detected by fluoresceinated F(ab')₂ fragments of rabbit anti-mouse IgG (Sigma). After three washes, cells were analyzed with a FACS 440 (Becton Dickinson, Sunnyvale, CA) equipped with a 5-W argon laser running at 488 nm, 0.5 W. Fluorescence parameters were collected by using a built-in logarithmic amplifier after gating on the combination of forward light scatter and perpendicular light scatter, which was used to discriminate viable from nonviable cells.

Inhibition of IgE binding to RPMI 8866 cells. Several dilutions of mAb in 50 μ l were incubated for 45 min at room temperature with 40 μ l IgE PS (20 μ g/ml). A total of 5×10^5 RPMI 8866 cells in 100 μ l was then added for 30 min at 4°C. After two washes, the IgE anti-IgE complex binding was monitored by incubation with i) an affinity-purified rabbit anti-human IgE (prepared in the laboratory) followed by incubation with a goat anti-rabbit Ig coupled to fluorescent microspheres as described earlier (22) or ii) a fluoresceinated rabbit anti-mouse Ig.

Degranulation of basophils and histamine assay. Histamine levels were measured by using a specific RIA (Immunotech). Basophil degranulation was performed on whole blood from normal donors. Fifty microliters of mAb dilution were added to 100 μ l of blood previously diluted 1/4 with manufacturer's buffer and incubated 30 min at 37°C. After centrifugation, supernatants were harvested and histamine content was measured following manufacturer's instruction. Total histamine content was determined after dilution of blood in distilled water and freeze thawing.

RESULTS

Generation of mAb Specific for Human IgE

In order to generate mAb specific for human IgE, mice were immunized with purified IgE that had previously been preincubated with a mouse antiserum specific for human IgG. This procedure was used to mask epitopes common to IgG and IgE. Spleen cells were fused with NS-1 myeloma cells and the supernatants from the resulting hybridomas were tested for their ability to recognize IgE bound to microtiter plates via an adsorbed rabbit polyclonal antibody specific for IgE. In order to eliminate mAb that would recognize epitopes common to both IgE and IgG (e.g., anti-L-chain antibodies), supernatants were also tested for their ability to bind to IgG bound to microtiter plates via an adsorbed rabbit polyclonal antibody specific for IgG. According to these criteria, eight hybridomas specific for IgE were selected, cloned, and subcloned. mAb originating from these hybridomas were obtained in purified form from ascites or cell culture supernatants. They were able to bind to IgE but not to IgG, IgM, or IgA directly coated to microtiter plates (Table I). These mAb were able to immunoprecipitate soluble ^{125} I IgE but not ^{125} I IgG (not shown). In addition, these mAb could bind to IgE adsorbed on nitrocellulose filters. All mAb were found to recognize two different myelomatous IgE (PS and Lyon) and a polyclonal IgE preparation either in dot immunoassay or in ELISA (data not shown) show-

TABLE I
Binding specificity of monoclonal anti-IgE antibodies (OD $\times 10^3$)^a

	IgG ^a	IgA	IgM	IgE
Anti-IFN- γ mAb	77	83	96	93
Anti-IgG mAb	818	95	96	164
Anti-IgA mAb	128	271	98	98
Anti-IgM mAb	112	94	>1500	106
mAb 27	102	115	110	>1500
mAb 272	166	157	144	>1500
mAb 173	166	141	130	>1500

^a A total of 100 ng purified IgG, IgA, IgM, and IgE was coated into microtiter plates wells; 200 μ l mAb anti-Ig were added for 2 h. Then, alkaline phosphatase-conjugated anti-mouse Ig was added for 2 h. Paranitrophenylphosphate in diethanolamine buffer was added and OD were measured after 1 h at 405 nm. Each value expressed in thousands of OD is the mean of quadruplicates (SD < 5% of the mean).

ing that their reactivity was not limited to the IgE used as the immunogen. Competition studies (not shown here) performed with these eight mAb allowed us to demonstrate that they were specific for three distinct antigenic sites on the IgE molecules. mAb 173 was studied in detail as a representative of a group of six mAb whereas mAb 27 and mAb 272 were unique. These three mAb were of the IgG1 isotype. As determined in an ELISA indirect assay mAb 272 could bind to heat-denatured IgE whereas mAb 27 and mAb 173 failed to do so (Fig. 1) suggesting that the latter were recognizing an epitope localized within the CH3 and CH4 domains.

Effects of the Anti-IgE mAb on the Interaction of IgE with Its High and Low Affinity Receptors

Induction of basophil degranulation. We have investigated whether the three anti-IgE mAbs were able to induce basophil degranulation and histamine release. Whole blood was incubated with increasing concentrations of anti-IgE mAb and the histamine released was measured with a RIA. Data in Table II show that the three tested mAb were able to induce basophil histamine release, demonstrating that these antibodies bind to epitopes on the IgE molecule that are not masked when IgE is bound to the high affinity IgE receptor (see Discussion).

Effects of the anti-IgE mAb in the IgE-FcεR2/CD23 interaction. The preceding experiment has shown that the three anti-IgE mAb were able to bind to IgE occupied basophils. It was therefore investigated whether the purified anti-IgE mAb would bind to RPMI 8866 cells saturated with IgE, the binding being monitored by subsequent incubation with FITC-conjugated goat anti-mouse Ig F(ab')₂ fragments. Figure 2 demonstrates that the three anti-IgE mAb bind to IgE-saturated cells. Binding was shown to be specific since anti-IgE mAb did not bind to RPMI 8866 cells before incubation with IgE. These data indicate that sites on FcεR2/CD23-complexed IgE continue to be available to anti-IgE mAb. Next, we investigated if preincubation of IgE with three anti-IgE mAb could interfere with the binding of IgE to FcεR2/CD23. As shown in Figure 3, mAb 173 (Fig. 3D) and mAb 272 (fig. 3E) did not prevent binding of IgE as visualized by rabbit anti-IgE/fluorescent bead-conjugated anti-rabbit IgG. It could be demonstrated that bound IgE was actually an IgE/anti-IgE complex since cell staining could be observed with FITC-conjugated anti-mouse Ig (data not shown). In contrast, preincubation of IgE with mAb 27

totally abrogated the binding of IgE to FcεR2/CD23 as monitored by rabbit anti-IgE/fluorescent bead-conjugated anti-rabbit IgG (Fig. 3B). Fab fragments of mAb 27 also inhibited the binding of IgE to FcεR2/CD23, thus partially ruling out a steric hindrance effect (Fig. 3C).

Binding of Anti-IgE mAb to IgE-Derived Recombinant Peptides

In order to locate epitopes recognized by the three mAb, purified IgE was digested either with papain, pepsin, or V8 protease. IgE peptides were obtained which were still recognized by the mAb in Western blot analysis (data not shown). This allowed us to exclude that the mAb recognized conformational epitopes and therefore allowed immunoblotting studies with *E. coli*-derived recombinant peptides encompassing different sequences of the IgE molecule spanning the whole Fcε region. Figure 4 summarizes the expression products of cloned ε-chain cDNA fragments used in the present study and the binding pattern of mAb 27, mAb 173, and mAb 272 to these products. The ε-chain sequence common to all the recombinant peptides recognized by mAb 272 comprises residues 301 to 336, which are located at the junction between CH2 and CH3.

mAb 173, as well as five other members of this group binds to peptides rE 2-4, rE 2'-4, rE 3-4, and rE 4. It does not bind to rE 2. There is a weak cross-reactivity with rE 2'-3' and rE 2-3. Excluding this cross-reaction, this pattern indicates that mAb 173 (as well as five other members of this group) binds to an epitope in the CH4 domain (residues 440 to 547).

Of particular interest is mAb 27, which inhibits the binding of IgE to cells bearing the FcεR2/CD23. mAb 27 binds to peptides rE 2-4, rE 2'-4, rE 2-3, rE 2'-3', rE 2-3', rE 3-4 but not to peptides rE 2, rE 4, rE 2-3'A and rE 2-3'B (Fig. 5). The reactivity of this antibody shows that it is directed against a heat labile, nonglycosylated epitope in the N-terminal region of the CH3 domain (AA 367-376).

DISCUSSION

The aim of this work was to produce mAb specific for human IgE-Fc in order to map the sites on IgE interacting with receptors on target cells. Eight mAb specific for the ε-chain were obtained. They were able to recognize soluble IgE as well as partially denatured IgE obtained after

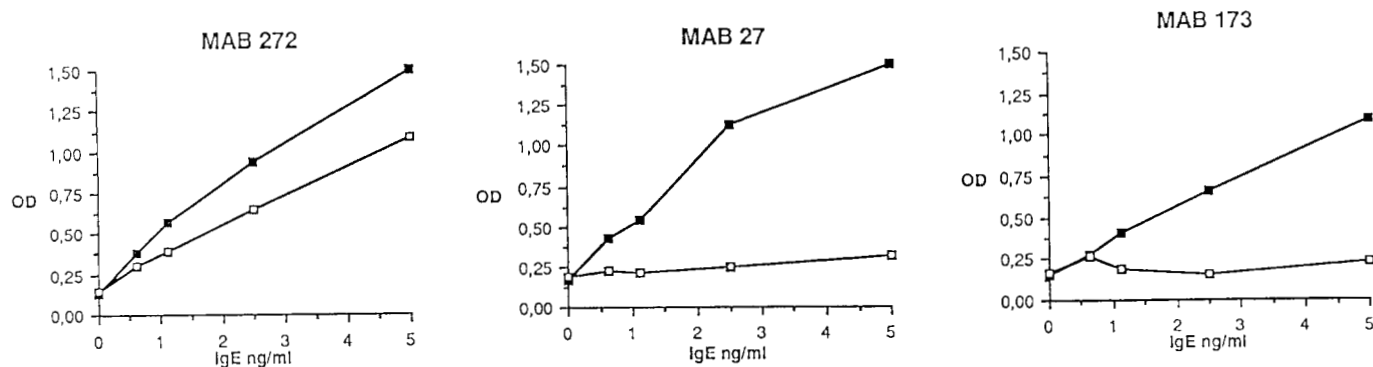


Figure 1. Binding of anti-IgE mAb to IgE and heat-denatured IgE in a sandwich ELISA. Plates were coated with a polyclonal rabbit anti-IgE antibody. Increasing concentration of heat-denatured (2 h at 56°C) IgE (open symbol) or nontreated IgE (closed symbol) were added. The anti-IgE mAb to assay was then added, followed by an alkaline phosphatase-conjugated goat anti-mouse Ig. Finally substrate was added and OD determined at 405 nm. Each point is a mean of duplicate determination. Similar results were obtained in four other experiments.

TABLE II

Induction of basophils degranulation by anti-IgE mAb (histamine nM)

mAb Concentration (μ g/ml)	mAb Control	mAb 27	mAb 173	mAb 272
0	3	3	3	3
0.5	ND	60	24	17
4	ND	30	48	48
8	2.5	7.8	36	54

^a A total of 100 μ l (1/4 diluted) blood was incubated for 30 min with increasing concentrations of the mAb. After centrifugation, histamine content of the supernatant was estimated by a specific RIA. The total histamine content released by addition of distilled water and freeze thawing was 250 nM. Each value is the mean of quadruplicates (SD <5% of the mean).

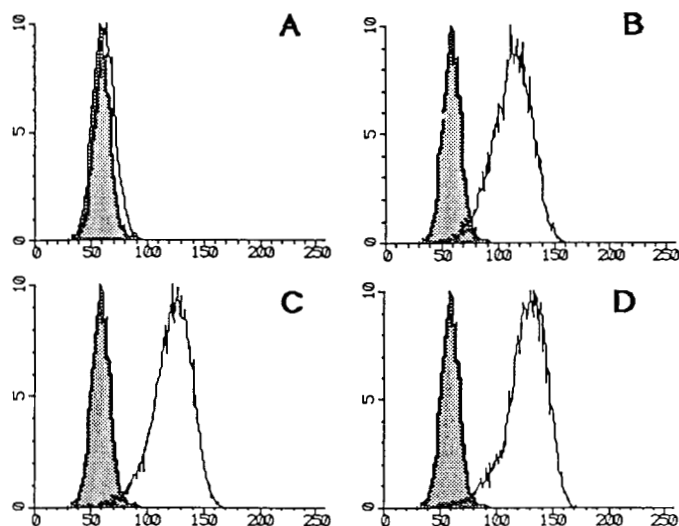


Figure 2. Fixation of anti-IgE mAb to IgE bound to Fc ϵ R2/CD23 on RPMI 8866 cells. Cells were first incubated with IgE then with the different anti-IgE mAb or a control mAb of the same isotype. Binding of the mAb was monitored by FITC-conjugated F(ab')₂ fragments of goat anti-mouse Ig. Samples were analyzed by flow cytometry. Histograms in dark, controls obtained by omission of IgE; abscissa, log fluorescence intensity; ordinate, relative number of cells; A, anti-IFN γ -mAb; B, mAb 27; C, mAb 173; D, mAb 272.

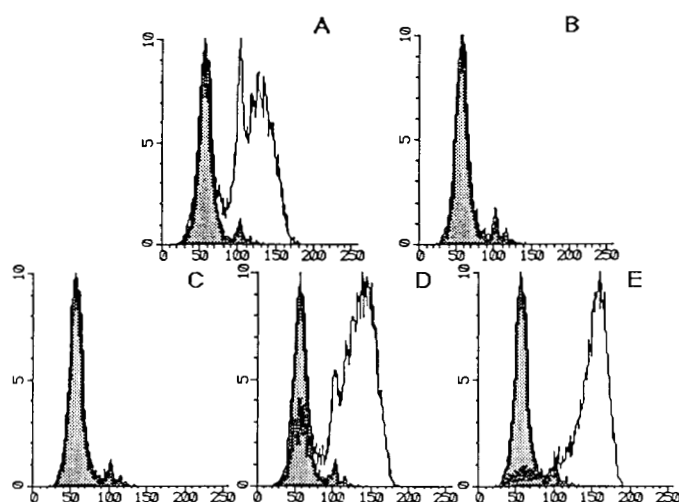


Figure 3. Fixation of IgE/anti-IgE mAb complexes to Fc ϵ R2/CD23 on RPMI 8866 cells. IgE (1 μ g) in 40 μ l were incubated with 1 μ g of anti-IgE mAb in 50 μ l for 45 min at room temperature. Then 5×10^5 RPMI 8866 cells in 100 μ l were added. A rabbit anti-IgE antiserum was added followed by goat anti-rabbit Ig coupled to fluorescent microspheres. Samples were analyzed by flow cytometry. Histograms in dark, controls obtained by omission of IgE; abscissa, log fluorescence intensity; ordinate, relative number of cells; A, anti-IFN γ -mAb; B, mAb 27; C, Fab fragments of mAb 27; D, mAb 173; E, mAb 272.

transfer to nitrocellulose filter. They could be classified into three categories by competition experiments and study of their binding to heat-denatured IgE and IgE-derived recombinant fragments. The properties of these mAb are summarized on Table III.

mAb 173 (as well as five other mAb of the same group) bound to a heat-sensitive determinant on the CH4 domain. It induces basophil degranulation and is unable to inhibit the binding of IgE to Fc ϵ R2/CD23. This indicates that none of the IgE epitopes recognized by mAb 173 is masked when IgE is bound to Fc ϵ R2 or Fc ϵ R1.

mAb 272 was found to recognize an epitope within a stretch of 36 AA (AA 301–336) spanning the junction between the C-terminal CH2 domain and N-terminal CH3 domain, which is not affected by heat denaturation. mAb 272 induces degranulation of IgE-occupied basophils and is unable to prevent the binding of IgE to Fc ϵ R2/CD23.

The third antibody, mAb 27, recognizes a heat-labile epitope involving a stretch of 10 AA (367–376) in the N-terminal CH3 domain. This antibody induces degranulation of IgE-sensitized basophils and inhibits the binding of IgE to Fc ϵ R2/CD23. Furthermore, we found that when IgE is preincubated with RPMI 8866 cells, mAb 27 can still engage receptor-bound IgE.

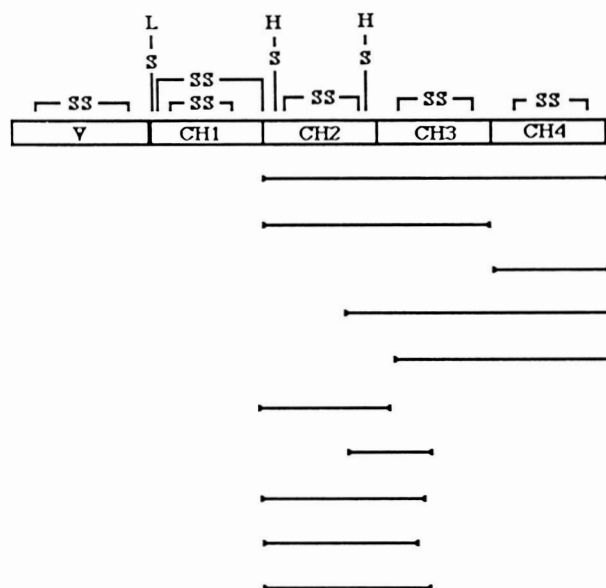
Although Baniyash et al. have described anti-IgE mAb that bind to several conformational determinants on rat IgE (26, 27), our mapping studies exclude this possibility. The most plausible explanation for our observation would be that IgE binds to a single Fc ϵ R2/CD23 molecule by involving epitopes from only one of the two ϵ -chains, leaving the other epitopes localized on the second ϵ -chain free to interact with the anti-IgE mAb.

It has been shown previously that an apparently monomeric recombinant peptide containing a sequence of 76 AA at the CH2/CH3 junction contains the Fc ϵ R1-binding site on human IgE (18). The fact that our three mAb triggered IgE-occupied peripheral blood basophils does not mean that epitopes recognized by these mAb are not involved in Fc ϵ R1 binding but only that these sites are still available when IgE is bound to Fc ϵ R1. In order to determine whether the IgE epitopes recognized by mAb 27 and mAb 272 are involved in binding to Fc ϵ R1, it would be of interest to test whether these mAb are able to induce degranulation of basophils sensitized with monomeric rE 2-3 or rE 2'-3'. Because a fraction of Fc ϵ R1 sites on peripheral basophils used in the present study is already occupied by endogenous IgE these experiments will have to await the outcome of unsensitized target cells.

Examination of a structural model of the human IgE-Fc region (28, 29) shows that accessible residues in the last two β -strands of CH2 involve residues 307 to 314, and 324 to 335, whereas the first two β -strands in CH3 face away from solvent and, by homology with the known structure of human IgG-Fc, would be mainly covered with carbohydrate (28, 29). Thus, the third β -strand (AA 362–370) in CH3 may contribute residues for both low and high affinity recognition sites on IgE. The inhibition of IgE binding by mAb 27 to Fc ϵ R2/CD23 suggests that an epitope involving AA 367–376 comprises, or is close to the binding site of IgE to Fc ϵ R2/CD23 and the model predicts that an epitope involving Pro-367, Ser-368, and Lys-369 has antigenic potential.

However, we and others, (D. Vercelli, B. Helm, manu-

A



B

	<u>MAB</u>	<u>MAB</u>	<u>MAB</u>
	<u>27</u>	<u>173</u>	<u>272</u>
r E 2-4 (218-547)	+	+	+
r E 2-3 (218-439)	+	±	+
r E 4 (440-547)	Ø	+	Ø
r E 2'-4 (301-547)	+	+	+
r E 3-4 (340-547)	+	+	Ø
r E 2 (218-336)	Ø	Ø	+
r E 2'-3' (301-376)	+	±	+
r E 2-3'A (218-367)	Ø	N.A	N.A
r E 2-3'B (218-361)	Ø	N.A	N.A
r E 2-3' (218-376)	+	Ø	+

EPIOTOPE DELINEATION

367-376 440-547 301-336

Figure 4. Reactivity of anti-IgE mAb with rIgE-derived peptides. A, genetic constructs used in mapping of the human ϵ -chain by anti-IgE mAb; B, binding pattern of mAb as analyzed by immunoblotting (see *Materials and Methods*); +, strong binding; $\pm$, weak binding; ϕ , no binding; N.A., not applicable.

Figure 5. Immunoblots of the anti-IgE mAb 27 with IgE-derived peptides. Peptides were run on 15% acrylamide SDS gels. Upper panel, gels were stained with Coomassie blue; lower panel, duplicate gels were transferred onto nitrocellulose then reacted with mAb 27 followed by anti-mouse IgG coupled to peroxidase (see *Materials and Methods*). 1, Prestained standards (Bethesda Research Laboratories, Bethesda, MD). From top to bottom, arrows show M_r in kDa 68, 43, 25.7, 18.4, 14.3; 2, IgE (ND); 3, rE2-4; 4, rE2; 5, rE2'-4; 6, rE2'-3'; 7, rE2-3; 8, rE3-4; 9, rE4; 10, rE2-3'; 11, rE2-3'A and rE2-3'B

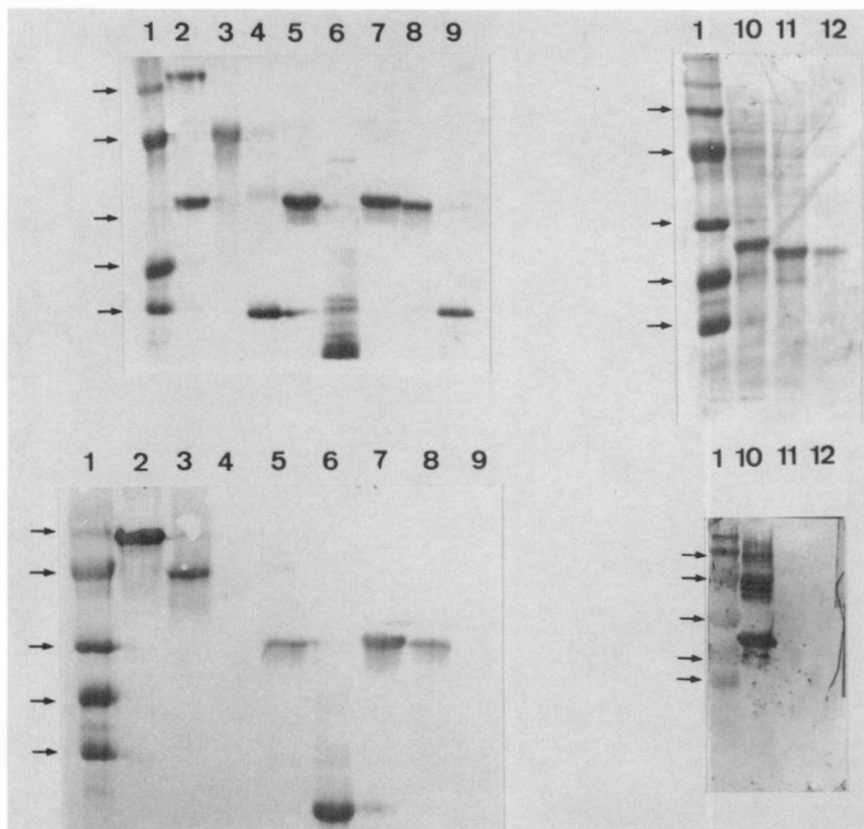


TABLE III
Summary of the properties of three distinct anti-IgE mAb

Antibody	Binding to IgE	Binding to Heat-Denatured IgE	Binding to IgE on FcεR2/CD23	Inhibition of IgE Binding to FcεR2/CD23	Basophil Degranulation
mAb 27	+	—	+	+	+
mAb 173	+	—	+	—	+
mAb 272	+	+	+	—	+

script in preparation), have tested another mAb, mAb P3 (a kind gift of Dr R. Pumphries of Manchester Infirmary), which inhibits the binding of IgE to FcεR2/CD23, and preliminary studies suggest that this antibody maps to an epitope comprising AA 307–314 in the CH2 domain. These data do not rule out the possibility that mAb P3 or mAb 27 prevent the binding of IgE FcεR2/CD23 by steric hindrance, furthermore, it cannot formally be excluded that 27 can alter the conformation of the IgE molecule so as to abolish its capacity to bind to FcεR2/CD23. If this were the mechanisms of action of this antibody, one would expect that mAb 27 would induce IgE to dissociate from FcεR2/CD23 since the affinity of mAb 27 for IgE (10^{-10} M) is about 1000 times higher than the affinity of IgE for FcεR2/CD23. The observation that mAb 27 Fab fragments also inhibit the binding of IgE to FcεR2/CD23, supports our claim that mAb 27 binding site on IgE comprises or is spatially very near the site where IgE engages FcεR2/CD23.

An attractive hypothesis presents itself when we consider possible sites on IgE involved in FcεR2/CD23 binding in the context of a 3D model structure of human IgE-Fc (Fig. 6). An inspection of this model clearly demonstrates the close proximity of one of the β-strands in CH2 (AA 307–314) recognized by mAb P3 to the area defined by mAb 27 on IgE (AA 367–376) thus suggesting that IgE

makes contact with its low affinity receptor in this region.

It is worth noting that although both mAb 272 and mAb P3 recognize an epitope in the C-terminal CH2 domain, they obviously bind to different determinants, because mAb 272 does not inhibit the binding of IgE to FcεR2/CD23. It indicates that the sequence comprising AA 301–336 possesses at least two antigenic determinants and that the epitope defined by mAb P3, but not by mAb 272 is sufficiently close to or involves the site on IgE interacting with FcεR2/CD23. Indeed, the 3D model structure shows that the putative epitope for mAb P3 (AA 307–314) is remote from the second site (AA 324–336) computed to contain antigenic determinants. It is tempting to speculate that an epitope in this sequence might be recognized by mAb 272, although this remains to be demonstrated.

Although the sequence defined by mAb 27 carries one of the two oligosaccharide moieties (linked to Asn-371) in CH3, the antibody is not directed against carbohydrate. In view of the fact that FcεR2/CD23 shares considerable homology with the asialoglycoprotein receptor (30–32) and that the carbohydrate moieties of IgE-binding factors seem to play an important role in the regulation of IgE synthesis (6, 7, 33, 34), one might invoke a role of carbohydrate in the interaction of IgE with its low affinity receptor. Whether such an interaction of IgE with FcεR2/CD23 plays a role in the regulation of IgE production still remains to be established.

In conclusion, our anti-IgE mAb allowed us to establish that occupation of epitopes on IgE involving AA 307–314 or 367–376 by Fab fragments of mAb P3 or mAb 27 will inhibit the binding to FcεR2/CD23. These observations are entirely consistent when considered in the context of current structural models (28, 29) for IgE-Fc, which show

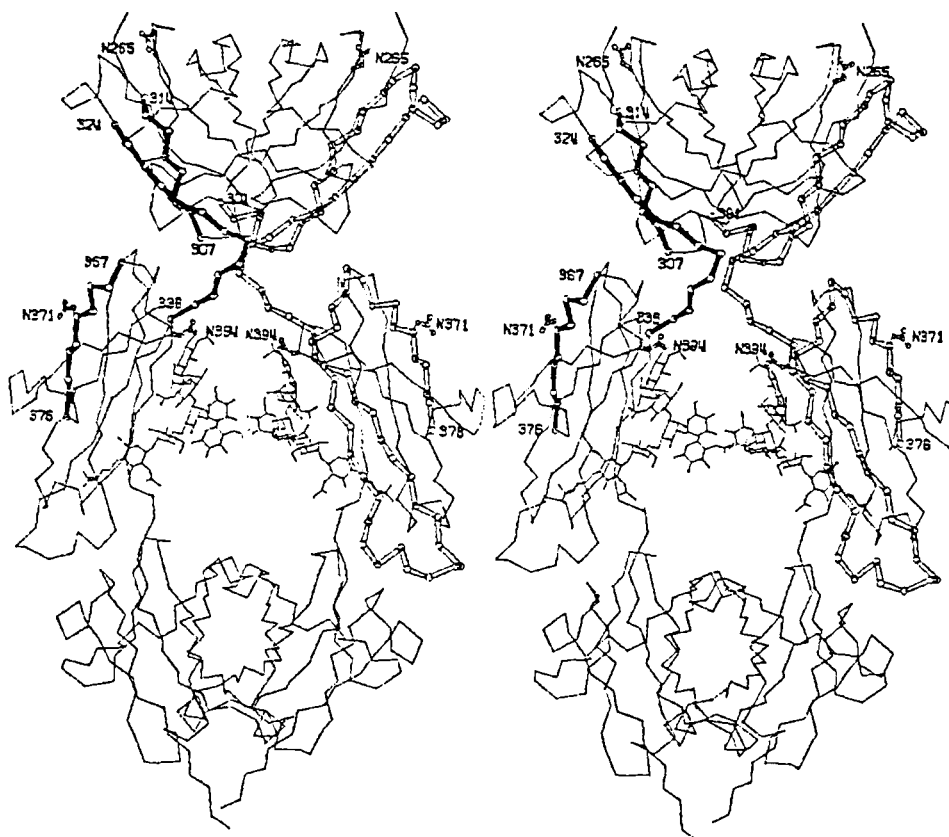


Figure 6. Three-dimensional model of human IgE Fc portion (stereo-pair) (28) showing as thick filled bonds on the left chain the putative location of peptide 367–376 (mAb 27). Within the 301–336 peptide (mAb 272), the epitope recognized by mAb P3 (307–324) (see Discussion) and a potential binding site for mAb 272 (324–336) (see Discussion) are also shown as thick filled bonds on the left chain. The thick open bonds on the right chain show the putative location of peptide 301–376 involved in binding to FcεR1 (18).

the close proximity of these epitopes in the cleft made up by residues of the C terminal CH2 and N-terminal CH3 domain that also comprise the sites where IgE engages FcεR1. In addition, the data suggest that only epitopes carried by one of the two epsilon chains are involved in the interaction of IgE with FcεR2/CD23.

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