

MONOCLONAL ANTIBODIES DEFINING EPITOPES ON HUMAN IgE

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Abstract—Twelve monoclonal antibodies (mAb) were isolated that bound to six clusters of epitopes on the constant region of the epsilon chain of human IgE. Four of the mAb bound to the C₁ or early C₂ regions; three of these bound to the IgE myeloma protein PS and to serum IgE but not to the IgE myeloma protein ND. These mAb probably recognize an allotypic marker. Another mAb reacted with heat-denatured, but not native IgE. Four of the mAb failed to release histamine; the epitopes recognized by these mAb are in the C₁, C₂ and C₃₋₄ regions of IgE. Three of these non-histamine releasing mAb did not bind to IgE on the basophil surface. These mAb recognize epitopes in C₂ and C₃₋₄ that are not accessible when IgE is bound to its receptor. Four mAb inhibited IgE binding to basophils; two of these did not release histamine, and two others that bind to epitopes in the C₂₋₄ domain, released histamine and therefore blocked IgE binding by steric hindrance. Inhibition of IgE binding by different mAb suggest that the Fc_{RI} and Fc_{RII} bind to partly overlapping regions of the IgE molecule although the sites do not appear to be identical. A number of sites on C₁ and C₃₋₄ were accessible when IgE is bound to its basophil receptor. The data support the concept that only part of the Fc portion of IgE is hidden in the receptor and that portions of C₁₋₄ are accessible on the cell surface. These mAb should be useful in determining the domains of IgE that are critical for its biological activity.

INTRODUCTION

The binding of IgE to the high affinity receptors on mast cells or basophils is a critical event in initiating allergic reactions. The bridging of IgE by antigen or heterologous anti-IgE then results in the activation of the cells for the release of the vasoactive mediators of inflammation (Ishizaka and Ishizaka, 1984; Metzger *et al.*, 1986; Siraganian, 1988). The IgE binds to its receptor through the Fc portion of the molecule (Bennich and Johansson, 1971; Ishizaka *et al.*, 1970). Studies of heat denatured IgE suggested that the C₃ and C₄ domains are involved in the binding to the receptor (Dorrington and Bennich, 1978). However, the digestion of IgE bound to the receptor compared to IgE in solution suggested that a region close to the C₂:C₃ juncture might interact with the receptor (Perez-Montfort and Metzger, 1982). The C₄ por-

tion of the IgE is not hidden in the receptor and is available for binding by antibodies (Holowka *et al.*, 1985; Baird and Holowka, 1985). Their data also suggests that IgE binds via only one face and that the Fc region bends at the C₂:C₃ interface when IgE is in its receptor. However, there appear to be no significant changes in the flexibility of IgE following its binding to the receptor (Slattery *et al.*, 1985).

Baniyash and Eshhar (1984) produced 7 mAb to epitopes on the Fc region of mouse IgE; some of these inhibited the binding of IgE to rat basophilic leukemia cells (RBL-2H3). These mAb released serotonin and therefore bind to epitopes on the IgE molecules accessible on the basophil surface. However, there were differences in the binding of these mAb to IgE in solution compared to IgE bound to the RBL-2H3 cells (Baniyash *et al.*, 1986). Subsequently, another mAb was isolated (mAb 84.1C) that bound to mouse IgE in solution, inhibited ¹²⁵I-IgE binding, did not bind to IgE when it was on the RBL-2H3 cell surface and did not release serotonin (Baniyash *et al.*, 1988). Site-directed mutagenesis of the IgE heavy chain gene demonstrated that these mAb bind to the C₃ and C₄ domains and that C₃ is important in binding to the receptor on RBL-2H3 cells (Schwarzbaum *et al.*, 1989).

Synthetic peptides have also been used for studies of IgE binding to cells. Peptides 13 to 16 amino acids long from the C₃ and C₄ regions were found to inhibit the binding of ¹²⁵I-labeled rat IgE to mast cells (Burt and Stanworth, 1987b). Antibodies have been raised to synthetic peptides from different regions of

Abbreviations: BBS, 0.2 M borate-buffered saline pH 8.0; HR₅₀, concentration of anti-IgE_{hu} required for 50% histamine release; HSA, human serum albumin; IgE_{hu}, human IgE; IgE_{hu}(ND), purified human IgE myeloma protein derived from patient "ND"; IgE_{hu}(PS), purified human IgE myeloma protein derived from patient "PS"; mAb, monoclonal antibody/antibodies; PAAT, 0.02 M phosphate buffered 0.15 M NaCl saline solution containing 0.25% NaN₃, 0.2% BSA and 0.25% Tween 20, pH 7.4; PIPES, 1,4-piperazinediethanesulfonic acid; PIPES AC, buffered saline containing 119 mM NaCl, 5 mM NaCl, 5 mM KCl, 25 mM PIPES, 1.5 mM CaCl₂ and 0.03% human serum albumin; RIA, radioimmunoassay; Fc_{RI}, high affinity IgE receptor; Fc_{RII}, low affinity IgE receptor.

the IgE molecule. Antibodies to peptides from the C_ε3 and C_ε4 regions inhibited ¹²⁵I-IgE binding to rat mast cells although they also released histamine and therefore bind to the IgE on the cell surface (Burt *et al.*, 1987a). Similarly, Robertson and Liu (1988) prepared antisera to mouse IgE peptides from the C_ε2, C_ε3 and C_ε4 domains. One of these antisera towards a peptide from the C_ε3 region bound well to soluble IgE but not to IgE on RBL-2H3 cells.

Molecular biological techniques have been used to produce fragments of human IgE in bacteria. Molecules that contain the majority of the Fc portion of IgE produced by bacteria are unglycosylated but bind to human basophils with high affinity (Liu *et al.*, 1984; Kenten *et al.*, 1984). A recombinant polypeptide synthesized in bacteria contained about two-thirds of the C_ε2 and all of the C_ε3 and C_ε4 domains. This polypeptide bound to cultured human basophils (Kenten *et al.*, 1984) and inhibited the binding of IgE both to cultured basophils and to skin mast cells (Kenten *et al.*, 1984; Ishizaka *et al.*, 1986; Geha *et al.*, 1985). A smaller IgE fragment of 76 amino acids from the junction of the C_ε2 and C_ε3 domains (Gln 301 to Arg 376) inhibited passive sensitization of skin mast cells and basophils for histamine release (Helm *et al.*, 1988; Helm *et al.*, 1989). It is likely that residues 363 to 376 are not required for binding (Helm *et al.*, 1989).

The present experiments define regions of the IgE molecule that are critical for binding to receptors using monoclonal antibodies (mAb). If a mAb reacts with IgE in solution, but does not when it is in its receptor, this indicates that the epitope is unavailable when the IgE is on the cell surface. These epitopes may correspond to sites that are actually hidden in the receptor, or to allosteric conformational changes occurring when IgE binds to the receptor.

MATERIALS AND METHODS

Production of hybridomas

Five BALB/c mice were injected subcutaneously at four-week intervals with 50 µg of purified IgE_{hu}(PS), human myeloma protein from patient PS (kindly supplied by Dr Henry Metzger, NIAMSD, NIH) emulsified in complete Freund's adjuvant (Ra 37). Following adoptive transfer into x-irradiated BALB/c mice, the spleen cells were fused with the non-immunoglobulin producing plasmacytoma cell line X63-Ag8.653 (Kearney *et al.*, 1979; Fox *et al.*, 1981) and cultured as described previously (Siraganian *et al.*, 1983). Culture supernatants were screened after two or three weeks by radioimmunoassay (RIA) for binding to ¹²⁵I-IgE_{hu}. Positive wells were expanded, retested and cloned by a limiting dilution method (Siraganian *et al.*, 1983). Hybridoma supernatants that bound IgE_{hu} (either to PS or ND), and whose binding was not blocked by 170 µg ml⁻¹ of human IgG, were injected i.p. into BALB/c mice

(8 × 10⁶ cells), previously primed with 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, WI).

Purification of monoclonal antibodies

Each mAb was purified from ascitic fluid by ammonium sulfate precipitation followed by DEAE chromatography (DE52, Whatman Inc., Clifton, NJ). Protein concentration was determined by absorbance at 280 nm with the OD value for 1 mg ml⁻¹ taken as 1.5. The isotype of the mAb was determined by radial immunodiffusion of the purified preparations reacted against subclass-specific antisera (Litton Bionetics, Inc., Charleston, S.C.).

Enzymatic digestion of IgE

IgE_{hu}(PS) was incubated with either pepsin or with papain to produce F(ab')₂ and Fc respectively under conditions as described previously (Bennich and Johansson, 1971; Ishizaka *et al.*, 1970). To prepare the F(ab')₂ fragment, 100 µg of IgE_{hu}(PS) was treated with 2% w/w of pepsin for 2 or 18 hr at 37°C in 0.1 M pH 4.5 acetate buffer. The Fc fragment was prepared by digestion of 100 µg of IgE_{hu}(PS) with 2% w/w of papain for 18 hr at 37°C in 0.2 M pH 5.6 acetate buffer containing 4 mM EDTA and 4 mM cysteine. The fragments were purified by HPLC using 7.5 × 300 mm Spherogel TSK-2000 and TSK-3000 gel filtration columns in series (Beckman-Altex, Berkeley, CA). The F(ab')₂ had a M_r of 150 k and the Fc 85 k. The purity of the fragments was demonstrated by SDS-PAGE followed by silver staining.

SDS-polyacrylamide gel electrophoresis and immunoblots

Electrophoresis was performed on 12.5% polyacrylamide slab gels (Laemmli, 1970). For immunoblots the proteins were transferred to 0.45 µm nitrocellulose membranes (Towbin *et al.*, 1979), blocked with 3% bovine serum albumin (BSA) and reacted with 5 µg ml⁻¹ mAb. After washing, ¹²⁵I-rabbit anti-mouse immunoglobulin (0.6 µg ml⁻¹, 6.7 × 10⁵ cpm µg⁻¹ protein) was added and after further washing the strips were dried for autoradiography on XAS-2 film (Eastman Kodak, Rochester, NY).

¹²⁵I-radiolabeling of proteins

All proteins were iodinated using Iodo-BeadsTM (Pierce Chemical Co., Rockford, IL) as described previously (Basciano *et al.*, 1986). Specific activity was 5 × 10⁵ to 5 × 10⁶ cpm µg⁻¹ protein. ¹²⁵I-labeled IgE_{hu}(ND) with a specific activity of 25 µCi µg⁻¹ and ¹²⁵I-labeled rabbit anti-human IgE (specific activity of 6.8 µCi mg⁻¹) were purchased from Pharmacia Diagnostics (Piscataway, NJ).

Radioimmunoassay for anti-IgE antibody

Anti-IgE activity was measured by RIA using ¹²⁵I-labeled IgE_{hu} myeloma proteins. The diluent for binding assays was PBS containing 0.25% NaN₃,

0.2% BSA and 0.25% Tween 20 (PAAT). To each tube was added 2 to 10 ng (0.1 ml) of labeled IgE_{hu} and 0.1 ml of the diluted hybridoma supernatant or 1, 3, 10, 30 and 100 µg ml⁻¹ of the purified mAb. After 30 min on a rotary mixer at room temperature, 0.1 ml affinity-purified rabbit anti-mouse immunoglobulin (120–250 µg ml⁻¹) was added and the mixtures again incubated for 30 min. Finally, 0.5 ml (10 mg cells ml⁻¹) of washed, formalin-fixed *Staphylococcus aureus* (Immuno-Precipitin, BRL, Gaithersburg, MD) were added and tubes incubated with mixing for 2.5 hr. The pellets were washed three times and their radioactivity determined.

Competition radioimmunoassay with the mAb

The different mAb at 3 µg ml⁻¹ were coupled to CNBr activated discs (Ceska *et al.*, 1972). Each mAb (3 µg) was incubated with 0.05 µg of ¹²⁵I-IgE_{hu} (PS or ND) for 90 min at 25°C on a rotary shaker. After 90 min, mAb-coupled discs were added and the incubation continued for another 90 min. The discs were washed with PAAT and the bound counts determined. Percent inhibition was calculated from the determination of binding in the absence of a competing mAb.

Binding of ¹²⁵I-mAb to IgE on basophils

Leukocytes were enriched for basophils using (Beckman Instruments Inc., Palo Alto, CA) counter-current centrifugal elutriation (Meyer *et al.*, 1983) and the basophils stained with Alcian blue (Gilbert and Ornstein, 1975). Elutriated cells (typically 5×10^7 , 7–33% basophils) were incubated for 18 hr at 35°C CO₂ in 15 ml Eagle's minimal essential medium containing 15% fetal calf serum and 10% pooled serum containing 2000 ng ml⁻¹ IgE_{hu}. After washing with PBS containing 0.03% human serum albumin (HSA) and 0.02% NaN₃, cells (0.1 ml) were incubated first for 10 min at 4°C with 0.1 ml normal rabbit IgE (4 mg ml⁻¹) to block IgG Fc receptor binding, then with ¹²⁵I-labeled mAb (7 to 57 nM) for 2 hr in an ice bath with occasional agitation. The cells were then washed three times using the same PBS-HSA-NaN₃ medium, and 0.1 ml aliquots in duplicate were layered over 0.2 ml of an oil mixture containing 40% bis(2-ethylhexyl) phthalate and 60% dibutylphthalate (Eastman, Rochester, NY). Tubes were centrifuged at 4°C for 2 min in a Microfuge (Beckman Instruments Inc., Palo Alto, CA). The tips of the tubes containing the pelleted cells were cut off and counted for ¹²⁵I. The reaction mixtures contained $3.6 \pm 0.9 \times 10^6$ ($\bar{x} \pm \text{SEM}$) cells (21% $\pm$ 2.6% basophils). Non-specific binding was determined by adding unlabeled hybridoma at 20- to 200-fold excess to some tubes 10 min before the ¹²⁵I-labeled mAb. This non-specific binding in the presence of excess unlabeled mAb was 1 to 3 times the background count and specific binding with the positive mAb was 10- to 100-fold above background. A control for each experiment measured the capacity of the ¹²⁵I-labeled

mAb to bind IgE_{hu} in a solid-phase assay: paper discs coated with sheep anti-human IgE were incubated with pooled human serum (containing 24 ng of IgE) washed and reacted with the ¹²⁵I-labeled mAb.

Inhibition by mAb of ¹²⁵I-IgE binding to basophils and lymphocytes

Elutriated cells were incubated for 18 hr under the conditions described above but in the absence of human serum IgE. The cells were then washed with ice-cold 0.15 M NaCl and the cell surface IgE stripped by a 5 min incubation in a glycine pH 2.9 buffer (Isersky *et al.*, 1979). The cells were washed and added to tubes containing 0.5–3.0 µg of ¹²⁵I-IgE_{hu} (PS) which had been preincubated for 90 min with varying concentrations of the different mAb in culture medium containing 2% fetal calf serum and 1.25 mg of rabbit IgG. After 90 min on ice, an aliquot of each sample was centrifuged through oil and the cell-bound radioactivity determined. Excess unlabeled IgE_{hu} (PS) was added to the basophils in some tubes to determine non-specific binding. The reaction mixtures contained $1.4 \pm 0.24 \times 10^6$ ($\bar{x} \pm \text{SEM}$, $n = 22$) basophils per tube. In the absence of the mAb there was 90 ± 17 ng ($\bar{x} \pm \text{SEM}$, $n = 22$) IgE bound 10^{-7} basophils. Inhibition by the mAb of IgE binding to lymphoblastoid RPMI 8866 cells having the low affinity Fc RII was done in a manner similar to the methods described above.

Histamine release from human leukocytes

Dilutions of anti-IgE_{hu} (0.25 ml) were incubated for 45 min at 37°C with an equal volume of washed leukocytes from non-allergic donors as previously described (Siraganian and Hook, 1986). Histamine was assayed by an automated fluorometric technique (Siraganian, 1974; Siraganian and Hook, 1986). Spontaneous release in all experiments was less than 5%. Results are expressed as the percentage of maximum release obtained with an optimal concentration of goat anti-IgE_{hu} (from Miles-Yeda, Elkhart, IN).

RESULTS

Production of monoclonal anti-IgE_{hu}

Spleen cells from mice immunized with IgE_{hu} (PS) were hybridized with a non-secreting myeloma cell line. Hybridomas whose supernatants bound to native or heated IgE_{hu} (PS and ND) and did not bind to pooled human IgG were selected. The purified immunoglobulin from ascites was used for all the present studies. The mAb described in this report were obtained from five different hybridizations. Of the original 216 wells plated with cells, 23 gave positive results and were expanded and cloned.

Binding of the mAb to IgE_{hu}

The mAb chosen for study are listed in Table 1. All were of the IgG₁ or IgG_{2a} isotype. The mAb 2, mAb 3 and mAb 4 bound to IgE_{hu} (PS), but not to the

Table 1. IgE binding properties of the anti-IgE_{hu} mAb*

mAb [†]		Ig isotype	IgE _{hu} (ND)		IgE _{hu} (PS)		Concentration for 50% binding§
Code	Cell line		Native	Heated‡	Native	Heated	
Percent of maximum binding‡							
							nM
2	E11AC2HC3	IgG _{2a}	3	1	98	49	4.0 ± 1.5
3	E11BA11D1	IgG _{2a}	2	1	70	27	7.4 ± 31.0
4	E11BC11B6	IgG _{2a}	9	0	82	53	261.0 ± 105.0
6	E11AC21D6	IgG _{2a}	6	67	5	74	20.0 ± 11.0
8	E11BA31D4	IgG _{2a}	64	20	97	46	7.0 ± 4.5
9	E9AD21A5	IgG _{2a}	89	10	89	12	3.6 ± 0.8
10	E11BB51A6	IgG _{2a}	81	7	109	10	3.2 ± 0.7
11	E5AA11A6	IgG ₁	43	9	72	26	34.0 ± 19.0
12	E5BB31A4	IgG _{2a}	75	3	100	27	9.8 ± 6.4
13	E14C51B1	IgG _{2a}	91	11	88	18	3.7 ± 1.1
14	E8BD11A6	IgG _{2a}	43	3	43	4	81.0 ± 34.0
15	E11AC31B4	IgG ₁	78	3	88	2	12.0 ± 2.0

*¹²⁵I-IgE_{hu} myeloma proteins (ND) or (PS) were incubated with concentrations of 1.0 to 100 µg of the different mAb followed by rabbit anti-mouse IgG and *Staphylococcus aureus* cells.

†The mAb purified from ascites were used in all assays.

‡Binding assays were with ¹²⁵I-IgE_{hu} heated at 56 °C for 4 hr.

§The monoclonal anti-IgE_{hu} concentration (nM ± SEM) giving 50% of the maximum binding of ¹²⁵I-IgE_{hu}(PS).

||The binding by each hybridoma is expressed as a mean percentage of the maximum ¹²⁵I-IgE_{hu} binding by an optimal concentration of mouse anti-IgE_{hu} serum [62% ± 3.5% with IgE(PS) and 70% ± 2.3% with IgE(ND)]. Results are the mean from 3 to 12 experiments and SEM values were less than 10%.

myeloma IgE_{hu}(ND). Because the mice were originally immunized with IgE_{hu}(PS), it was possible that these mAb recognized idiotypic determinants. However, this possibility is unlikely: mAb 2, 3 and 4 reacted well with serum IgE from 22 different individuals when their IgE was immobilized on paper discs coated with polyclonal anti-IgE_{hu} (data not shown). The mAb 6 bound better to heated than to native IgE_{hu}, and bound similarly to heated (PS) and (ND). Because this mAb did not bind to native IgE it was omitted from further studies. The remaining mAb (mAb 8–15) bound well to both IgE_{hu}(PS) and (ND). The concentration of mAb that gave 50% maximum binding of IgE_{hu}(PS) is shown in Table 1. The reciprocal of this value can be used as an approximation of the apparent K_a for IgE_{hu} binding. Most of these mAb have an apparent K_a of approximately 10^8 M^{-1} (median of $1 \times 10^8 \text{ M}^{-1}$, range 3.8×10^6 to $3.1 \times 10^8 \text{ M}^{-1}$).

The mAb were tested by immunoblotting for their reactivity to both reduced and non-reduced IgE_{hu}. All bound to non-reduced IgE_{hu}(PS); following reduction, the mAb reacted predominantly with the ϵ chain; mAb 13, 14 and 15 bound less to the ϵ chain than to the intact molecule suggesting that a number of determinants were lost following reduction of the IgE molecule (data not shown).

Binding of the mAb to different fragments of ¹²⁵I-IgE

Human IgE was digested with either pepsin or papain, and the respective F(ab')₂ and Fc fragments purified and used to test for the binding of the different mAb (Table 2). The F(ab')₂ fragment contains the C₁ and C₂ domains, and the Fc fragment the C₂ to C₄ regions. Therefore, we have defined the epitopes recognized by these mAb on the following basis: those that bind to F(ab')₂ but not Fc, recognize epitopes in C₁; mAb that react with both F(ab')₂ and Fc apparently bind to C₂ and those

reacting with only the Fc fragment probably identify epitopes in C₂ or C₄. In general, the mAb bound better to either one or the other of the fragments. For example, mAb 2, 3, 4 and 8 bound well to the F(ab')₂ but poorly, if at all, to the Fc fragment of the molecule and therefore the epitopes must be in the C₁ domain. Another group of mAb (mAb 9, 10, 12, 13 and 14) bound well to the Fc but not the F(ab')₂ fragments and therefore probably recognize epitopes in the C₃ or C₄ domains. The remaining 2 mAb (mAb 11 and 15) bound to both the F(ab')₂ and the Fc fragments. Of these, mAb 15 bound better to the F(ab')₂, therefore the epitope recognized by this mAb is in the C₂ region. In contrast, mAb 11 binds better to the Fc fragment and probably has an epitope on both C₂ and C₃. With 3 mAb the binding to the F(ab')₂ was greater than to the intact IgE. This could

Table 2. Binding of the anti-IgE mAb to fragments of IgE_{hu}(PS)*

mAb code	F(ab') ₂	Fc
Percent of binding to undigested IgE (x ± SEM)		
2	96 ± 3	14 ± 12
3	94 ± 3	5 ± 5
4	124 ± 6	25 ± 11
8	117 ± 2	4 ± 1
9	3 ± 0	99 ± 5
10	4 ± 0	102 ± 2
11	43 ± 1	91 ± 8
12	3 ± 0	103 ± 6
13	4 ± 0	95 ± 13
14	2 ± 0	96 ± 9
15	185 ± 6	94 ± 1
Polyclonal†	90 ± 6	115 ± 2

*IgE_{hu}(PS) was digested and purified by HPLC as described in Methods. The different IgE fragments were incubated 18 hr with the various anti-IgE mAb immobilized on paper discs. These were then washed and incubated with ¹²⁵I-rabbit anti-IgE_{hu}. After washing, discs were counted for radioactivity. The F(ab')₂ prepared by 2 hr or 18 hr pepsin digestion gave similar results.

†Goat anti-IgE_{hu}.

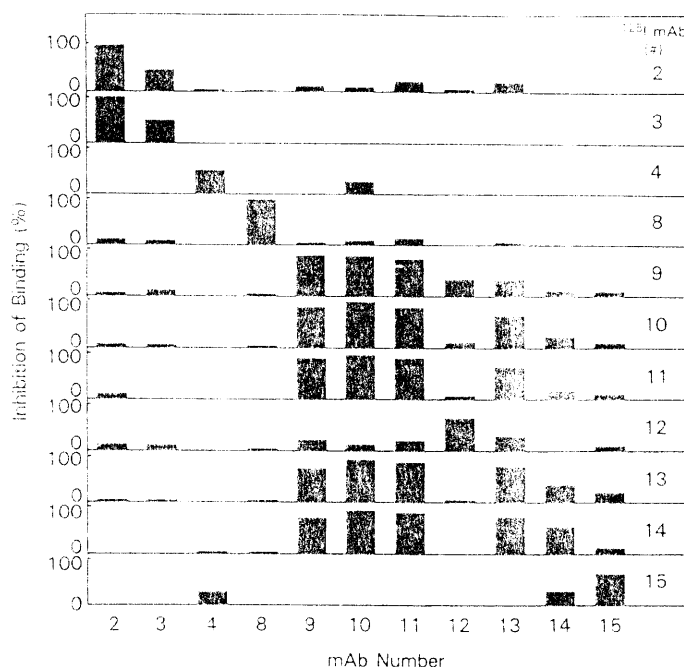


Fig. 1. Recognition of epitopes on IgE_{hu} by the mAb. Each mAb was incubated with ^{125}I -IgE_{hu}(PS or ND). The different mAb coupled to paper discs were then added and the incubation continued for 90 min. The discs were washed and the bound radioactivity determined. Percentage inhibition was calculated from the binding in the absence of a competing mAb. Results are the means of three experiments.

be due to the increased accessibility of epitopes to mAb following digestion. Therefore, the mAb bind to epitopes in different regions of the IgE molecule.

Binding cross-inhibition studies with the different mAb

The capacity of one mAb to inhibit the binding of a different mAb to radiolabeled IgE was used to determine the relationship of the different epitopes recognized by these antibodies (Fig. 1). The mAb detect 6 different epitopes on IgE as determined by cross-inhibition studies. Four of these epitopes, those recognized by mAb 4, 8, 12 and 15 are unique with little or no cross-inhibition by other mAb. Another epitope is recognized by two different antibodies, mAb 2 and 3. The final epitope is recognized by a group of mAb (mAb 9, 10, 11, 13 and 14) all of which have similar cross-inhibition patterns and probably bind to the same region of the IgE molecule.

Inhibition by the mAb of ^{125}I -IgE_{hu}(PS) binding to basophils

The different mAb were tested for their capacity to inhibit the binding of ^{125}I -IgE to Fcε RI on basophils. In leukocyte preparations enriched by basophils, IgE on the cell surface was stripped by low pH and the cells used for binding experiments with ^{125}I -IgE. Only four of the mAb inhibited the binding of IgE to basophils (Table 3). There was 50% inhibition with mAb 10 and 11 at concentrations of 3 to 5 $\mu\text{g ml}^{-1}$, whereas mAb 13 and 15 inhibited at higher concentrations. Although mAb 9, 10, 11, 13 and 14 belong to one epitope cluster, mAb 9 and 14 did not inhibit ^{125}I -IgE binding at concentrations as high as

100 $\mu\text{g ml}^{-1}$. Therefore, 4 mAb (mAb 10, 11, 13 and 15) inhibited binding of IgE to the Fcε RI either by steric hindrance or by binding to sites involved in binding to the receptor.

Inhibition by the mAb of ^{125}I -IgE_{hu}(PS) binding to the RPMI 8866 lymphoblastoid cell line

The different mAb were also tested for their capacity to inhibit binding of ^{125}I -IgE to the low affinity IgE receptor (Fcε RII) on human lymphoblastoid RPMI 8866 cells (Table 3). Several of the mAb that inhibited IgE binding to Fcε RI on basophils also suppressed binding to RPMI 8866 cells. Thus, mAb

Table 3. Blocking by the mAb of ^{125}I -IgE_{hu}(PS) binding to human basophils or lymphoblastoid cell line RPMI 8866*

mAb†	Concentration for 50% inhibition of ^{125}I -IgE _{hu} binding to:‡ ($\mu\text{g ml}^{-1}$)	
	Basophils	RPMI 8866 cells
8	>100	11 ± 5.5
9	>100	1.4 ± 0.50
10	4.8 ± 0.4	0.27 ± 0.04
11	3.0 ± 0.1	16 ± 12
13	34.0 ± 12.0	0.29 ± 0.05
14	>100	1.2 ± 0.62
15	76.0 ± 36.0	5.7 ± 1.5

* ^{125}I -IgE_{hu}(PS) at 4 $\mu\text{g ml}^{-1}$ was incubated 90 min at room temperature with different concentrations of each mAb (4–120 $\mu\text{g ml}^{-1}$). Human basophils (1.4×10^5) or RPMI 8866 cells (2×10^6) were added and tubes incubated 90 min in an ice bath, followed by centrifugation through oil and counting for ^{125}I .

†The 50% inhibitory concentration was determined graphically from the inhibition curve. Each value is the $\bar{x} \pm \text{SEM}$ of 4–9 experiments.

‡The unlisted mAb (mAb 2, 3, 4 and 12) did not inhibit binding (<20%).

10, 11, 13 and 15 inhibited IgE binding for both the high and low affinity IgE receptors; among these mAb 13 had relatively less activity for suppressing IgE binding to basophils whereas mAb 11 was less active in inhibiting binding to RPMI 8866 cells. In contrast, mAb 8, 9 and 14 inhibited IgE binding to RPMI 8866 cells but were inactive in suppressing binding to the basophils. The mAb that inhibited binding to both Fc_ε receptors required lower concentrations for suppressing binding to Fc_εRII on lymphocytes than to Fc_εRI on basophils. The rank order of the activity of different mAb for inhibition of binding IgE to basophils and lymphocytes was also distinct, further suggesting that the sites for binding to the two receptors are not identical.

Histamine release by mAb

The different mAb were tested for their capacity to release histamine from the washed leukocytes of 11 different donors (Table 4). The mAb 2, 3 and 8–12 released histamine from the cells of all the donors tested, and two of the mAb (mAb 9 and 10) frequently released more histamine than did polyclonal goat anti-IgE_{hu} (Table 4). There was no relationship between the serum IgE level of the donors and the sensitivity of their cells to release histamine. Maximum release was generally induced with 10 $\mu\text{g ml}^{-1}$ of the mAb, and antibody concentrations of 100 $\mu\text{g ml}^{-1}$ or greater usually resulted in decreased histamine release (data not shown). The concentration of mAb required for 50% release (HR₅₀) was generally less than 1 $\mu\text{g ml}^{-1}$ (approximately 6 nM). For most of the mAb, the concentration for 50% maximum histamine release was similar to the concentration for 50% of maximal binding in the radioimmunoassay (Table 1). There were two exceptions: with mAb 2 the 50% binding was at 4.0 nM whereas

the HR₅₀ was at 78 nM; and with mAb 3, the 50% binding value was 76 nM and HR₅₀ was 5.7 nM $\pm$ 1.9 nM (Tables 1 and 4). This suggests differences in the affinity of binding of these mAb to IgE in solution compared to IgE on the basophil surface.

Five of the mAb (mAb 4, 6, 13, 14 and 15) failed to induce histamine release from the cells of all individuals tested (Table 4). Antibody concentrations of 100 $\mu\text{g ml}^{-1}$ or greater were also incapable of releasing histamine. The addition of a second antibody (rabbit anti-mouse immunoglobulin) was also ineffective in inducing release with these mAb. Pretreatment of leukocytes with a non-releasing mAb did not inhibit the release induced by a histamine releasing mAb (data not shown). The range of concentrations for 50% binding of IgE_{hu} in the RIA with mAb 8 to 11 is similar to that of the histamine releasing mAb 13 to 15. Therefore, the lack of histamine releasing capacity cannot be attributed to a low binding affinity. The data that follow suggest that the non-histamine releasing mAb were also incapable of binding to IgE_{hu} on the basophil surface.

Binding of ¹²⁵I-labeled mAb to IgE on human basophils

Three histamine releasing mAb (mAb 8, 9 and 12) and 3 mAb that did not release histamine (mAb 13, 14 and 15) were tested for their capacity to bind to IgE-sensitized basophils (Fig. 2). The 3 histamine releasing mAb bound well (243 ± 38 fmoles 10^{-6} basophils, $\bar{x} \pm \text{SEM}$, $n = 17$). The curves of these 3 mAb were similar for binding to IgE both on basophils and a solid matrix. There were $1.3 \times 10^5 \pm 0.2 \times 10^5$ ($\bar{x} \pm \text{SEM}$) mAb molecules basophil⁻¹ range 5×10^4 to 4.2×10^5 . In contrast, the 3 mAb that did not release histamine (mAb 13, 14 and 15) bound poorly to basophils ($13.8 \times 10^3 \pm 1.6 \times 10^3$; $\bar{x} \pm \text{SEM}$ mAb molecules basophil⁻¹) despite binding well to IgE_{hu} on a solid matrix. The mAb 13, which had an apparent binding constant similar to mAb 8 or mAb 9 (Table 1) did not bind at all to sensitized basophils. However, mAb 14 and mAb 15 appeared to bind somewhat at the highest mAb concentrations tested. Therefore, compared to IgE on a solid surface, the binding to basophils of the non-histamine releasing mAb was either absent or greatly reduced.

DISCUSSION

We have isolated 12 mAb that bind to different portions of the human IgE molecule as demonstrated by binding studies with intact IgE or F(ab')₂ and Fc fragments (Fig. 3). By cross-inhibition studies, it was established that these mAb recognize six distinct epitopes. There were two epitopes that appeared to be on the C₁ domain of IgE: one epitope to which mAb 2 and 3 bound and a second epitope for mAb 8. A third epitope for mAb 4 appears to be at the C₁/C₂ junction. A fourth epitope, that identified by mAb 15,

Table 4. Histamine release by anti-IgE mAb from basophils of 11 donors

mAb code	Histamine release*	Concentration for 50% maximum release†
	(% maximum)	nM
2	68 $\pm$ 8.5	78.0 $\pm$ 18.0
3	98 $\pm$ 9.2	5.7 $\pm$ 1.9
4	0	—
6	0	—
8	79 $\pm$ 4.5	12.0 $\pm$ 5.2
9	132 $\pm$ 17.0	7.8 $\pm$ 2.5
10	120 $\pm$ 18.0	5.2 $\pm$ 1.4
11	69 $\pm$ 5.7	32.0 $\pm$ 10.0
12	90 $\pm$ 9.7	15.0 $\pm$ 4.4
13	0	—
14	0	—
15	0	—

*Washed leukocytes, from 11 different donors, were each incubated with 0.1, 1 and 10 μg of the different mAb, or with one of five dilutions of goat anti-IgE_{hu} antibody. Histamine release is expressed as the $\bar{x} \pm \text{SEM}$ of maximum release achieved by the polyclonal (goat) anti-IgE (56% $\pm$ 7.9%). Zero indicates no histamine release at any concentration of mAb.

†The mAb concentration (nM) giving 50% of the maximum histamine release achieved by the goat anti-IgE. Data are the $\bar{x} \pm \text{SEM}$ from 15 to 27 titrations of mAb. The minus sign (—) indicates no histamine release observed at any mAb concentration.

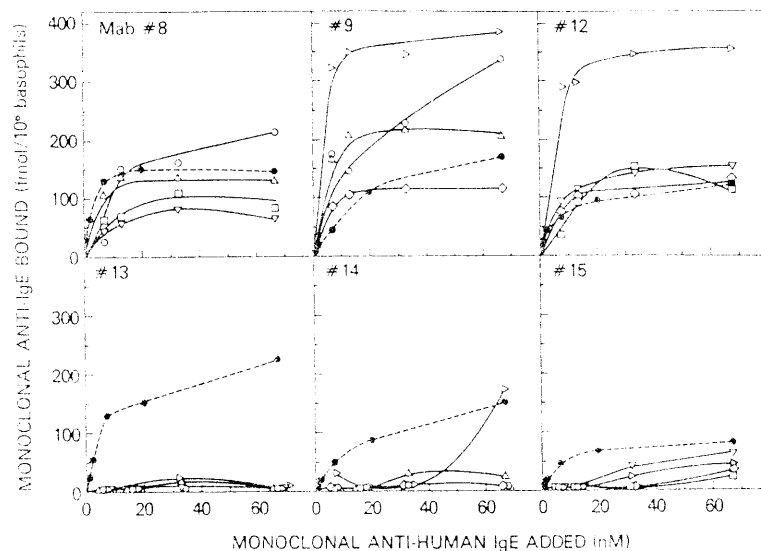


Fig. 2. Binding of ^{125}I -labeled mAb to IgE_{hu} on human basophils or on paper discs. Basophils from seven individual donors are designated by the different open symbols. Reaction mixtures contained $3.6 \pm 0.9 \times 10^6$ cells (21 ± 2.6 basophils), 1 mg ml^{-1} of rabbit IgG, and 7 to 57 nM of ^{125}I -mAb. After 2 hr in an ice bath, cells were washed and the cell-associated counts determined as described in Methods. The specifically-bound counts are expressed as fmoles 10^{-6} basophils. Binding of the ^{125}I -labeled mAb to IgE_{hu} bound to anti-human IgE fixed to paper discs is shown by closed symbols (●—●).

was in the C₂ region. The last two epitopes were in the C₃₋₄ domains and were the binding sites for mAb 12 and of the cluster recognized by mAb 9, 10, 11, 13 and 14. The binding studies with IgE fragments suggest that mAb 11 bind to the C_{2/C3} junction. Although mAb 11 is in the same epitope group as mAb 9 it appears to be closer than the others to C₂. Therefore, the epitopes defined by these mAb are on different domains of the IgE molecule.

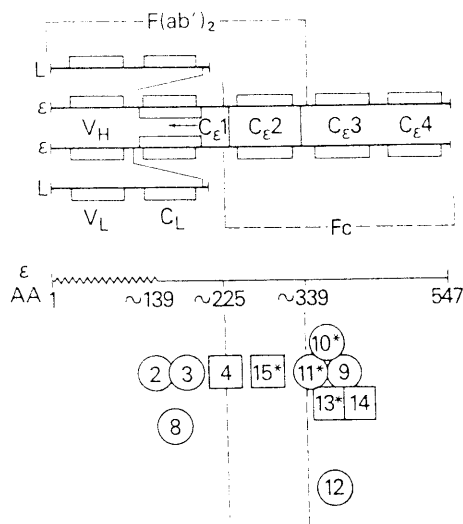


Fig. 3. Summary of binding, inhibition and histamine release results. Beneath the schematic of IgE is shown where the various mAb bind to enzyme-fragmented IgE_{hu} (PS). The mAb with asterisks block the binding of labeled IgE_{hu} (PS) to basophils. Cross-inhibiting epitopes are designated by contiguous symbols. The histamine-releasing mAb are designated by circles and the non-releasing by squares.

The 3 mAb (mAb 2, 3 and 4) that bound to the C₁ domain of IgE reacted with myeloma protein PS but not with the second myeloma protein ND. These mAb also bound to IgE from 22 individual sera and mAb 2 and 3 released histamine from leukocytes of all tested donors. Therefore, these mAb recognize a determinant on C₁ that is present on IgE_{hu} (PS) but not on IgE_{hu} (ND). These mAb could be reacting with an allotypic site which is present at high frequency in the population that we studied. This could be similar to the rare IgE allotype that has been described by (van Loghem *et al.*, 1984). Alternatively, they might bind an epitope in C₁ that has been altered in IgE_{hu} (ND).

The heating of IgE causes prominent changes in the structure of the C₃ and C₄ regions (Dorrington and Bennich, 1978). In the present experiments, the heating of the IgE_{hu} resulted in alteration of the sites available for binding by the different mAb. One of the monoclonal antibodies reacted with heat-denatured, but not native IgE. In contrast, the binding of all the other mAb was markedly decreased when IgE was heated for 1 hr at 56°C. This suggests that the tertiary structure of IgE is important for recognition of epitopes in not only the C₃₋₄ domains, but in all the constant regions of the IgE molecule.

The mAb can be divided into two groups depending on whether or not they released histamine from human basophils (Table 4). Five of the mAb failed to release histamine with cells of all donors tested. The epitopes recognized by these mAb are in the C₁, C₂ and C₃₋₄ regions of IgE. The capacity to release histamine did not appear to be related to the binding constant of the mAb with IgE; e.g. the two non-histamine releasing mAb 13 and 15 had binding

constants similar to the histamine releasing mAb 9 and 12. Furthermore, the addition of the histamine-non-releasing mAb followed by anti-mouse Ig was ineffective in causing histamine release. Therefore, the lack of histamine release by these mAb is not likely due to the mAb binding univalently to IgE on the cell surface as has been suggested in the murine system (Baniyash *et al.*, 1986). The concentration for 50% maximal histamine release was generally similar to the amount for 50% maximal binding to IgE. There were, however, two exceptions; both were with mAb that bind to the C₁ region. Perhaps these epitopes are modified when the IgE is bound to the receptor resulting in an increase or decrease in accessibility of their sites. There have been suggestions of conformational bending of the IgE molecule when it binds to its receptor on the cell surface (Baird and Holowka, 1985; Holowka and Baird, 1983).

Four of the mAb define epitopes on IgE that are not accessible when it is bound to Fc_εRI on the cell surface. Such areas of IgE could be important for binding to the basophil. The mAb 13 and mAb 15 did not release histamine, inhibited ¹²⁵I-IgE binding and did not bind to IgE on the basophil cell surface. These mAb bind to epitopes in the C₂ and C₃₋₄ regions of IgE. There were two other mAb (mAb 4 and 14) that did not release histamine, but failed to inhibit the binding of labeled IgE to basophils. Both of these antibodies appear to have a lower binding affinity than other mAb that inhibited binding. Alternatively, the binding of IgE to the receptor could cause a conformational change in the epitopes preventing the binding of these mAb.

Antibodies could also bind to sites distant from the receptor binding sites but still block binding to Fc_εRI. The mAb 10 and 11 released histamine indicating the accessibility of their epitopes when IgE is on the basophil surface however, they also blocked the binding of ¹²⁵I-IgE to cells. There are other mAb with similar affinities that do not inhibit ¹²⁵I-IgE binding to basophils, e.g. mAb 2, 8, 9 and 12. The epitopes recognized by these mAb must therefore be more distant from the receptor binding site to explain their lack of interference with the binding of IgE. Although mAb 9 and 10 have similar affinities, cross-inhibition patterns, and both release histamine; only mAb 10 inhibited IgE binding. Therefore, mAb 9 and 10 must bind to slightly different portions of the same epitope cluster in the C₃₋₄ domain.

There were differences in the capacity of the mAb to inhibit the binding of ¹²⁵I-IgE to the high and low affinity IgE receptors (Table 3). In general, more mAb inhibited binding to Fc_εRII than to Fc_εRI. Several mAb that did not inhibit binding to the high affinity receptor were very active in blocking binding to Fc_εRII (i.e. mAb 9 and 14). The mAb that inhibited binding to both receptors had different rank orders for their capacity to block binding to one or other receptor. The most active mAb in blocking binding to the Fc_εRII were mAb that react with sites

in the C₃ and C₄ domains. However, the mAb that react with the C₂ domain were also active in inhibiting binding to the Fc_εRII. These results are similar to the published reports that mAb that bind to the C₄ domain did not inhibit binding to the Fc_εRII; whereas antibodies that bind to epitopes in the C₂ and C₃ domains inhibited binding (Chrétien *et al.*, 1988; Vercelli *et al.*, 1989). Our experiments demonstrate that the Fc_εRI and Fc_εRII bind partly overlapping regions of the IgE molecule. However, the sites do not appear to be identical.

These experiments demonstrate that the epitopes reactive with mAb 15 and 13 are sites on IgE that are in the C₂ and C₃₋₄ domains and are not accessible when IgE is bound to its receptor. The results do not distinguish whether the sites recognized by mAb 4 and mAb 14 on C₁ and C₃₋₄ are actually hidden on a part of the IgE within the receptor or are conformationally changed on binding to the cell. The epitope recognized by mAb 11 at the junction of C₂:C₃ and that of mAb 10 sterically hinders binding to the receptor. Furthermore, it is clear that a number of sites on C₁ and C₃₋₄ are accessible when IgE is bound to its basophil receptor. The data support the concept that only part of the Fc portion of IgE is in the receptor and that portions of C₄ are accessible on the cell surface. Further studies with these mAb could define better the sites on IgE important in binding to its receptors. These mAb could also be used to modulate the synthesis of IgE by B cells (Sherr *et al.*, 1989; Haba and Nisonoff, 1990).

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