

High Affinity Human IgE Receptor (FcεRI)

ANALYSIS OF FUNCTIONAL DOMAINS OF THE α-SUBUNIT WITH MONOCLONAL ANTIBODIES *

(Received for publication, February 20, 1991)

Frank Riske†§, John Hakimi¶, Michael Mallamaci‡, Maureen Griffin‡, Bob Pilson¶, Nancy Tobkes||, Ping Lin¶, Waleed Danho**, Jarema Kochan‡, and Richard Chizzonite‡

From the Departments of ‡Molecular Genetics, ¶Immunopharmacology, **Peptide Research, and ||Protein Biochemistry, Roche Research Center, Hoffman-LaRoche, Inc., Nutley, New Jersey 07110

The binding of IgE to the high affinity Fcε receptor (FcεRI) on mast cells and basophils is mediated by the α-subunit of the tetrameric receptor complex. Based on sequence homologies, the 50-kDa α-subunit is a member of the immunoglobulin superfamily of proteins and has two predicted disulfide-bonded loops. Monoclonal antibodies specific for the human α-subunit have been identified and separated into two major classes: inhibitory and noninhibitory antibodies. Inhibitory antibodies (*i.e.* 15A5) block ¹²⁵I-IgE binding to a recombinant chimeric α-subunit (ch-α-protein) expressed on Chinese hamster ovary cells and immunoprecipitate ¹²⁵I-labeled purified ch-α-protein. Noninhibitory antibodies (*i.e.* 22E7) immunoprecipitate both ¹²⁵I-labeled ch-α-protein and the soluble complex of ¹²⁵I-IgE cross-linked to ch-α-protein but do not block ¹²⁵I-IgE binding to the ch-α-protein expressed on Chinese hamster ovary cells. Both classes of antibodies bind to natural FcεRI present on human basophils and induce histamine release from these cells.

Inhibitory antibody 15A5 specifically binds to a peptide corresponding to amino acids 125–140 of the putative second domain of the α-subunit sequence. All the inhibitory antibodies compete with ¹²⁵I-15A5 for binding to the ch-α-protein, indicating that these antibodies recognize inhibitory epitopes that are either identical or sterically overlapping. Noninhibitory antibodies (*i.e.* 22E7) do not block ¹²⁵I-15A5 binding to the ch-α-protein. These data suggest that antibodies binding to the predicted second domain of the α-subunit can inhibit IgE binding to the α-subunit, while antibodies binding at a distance from this site do not inhibit IgE binding. These inhibitory antibodies may block IgE binding to the ch-α-protein by direct overlap, steric inhibition, or induced conformational changes of the receptor contact points for IgE.

The binding of the Fc domain of immunoglobulin E to the high affinity IgE receptor (FcεRI) found on mast cells and basophils is mediated by the α-subunit of this four-subunit receptor complex (1). Cross-linking of this receptor by IgE and antigen induces cellular degranulation and release of preformed mediators such as histamine and serotonin (1).

The FcεRI on rat basophilic leukemia (RBL)¹ cells consists of a single ~50-kDa α-subunit (2, 3), one 33-kDa β-subunit (4–6), and two 7–9-kDa disulfide-linked γ-subunits (7, 8). Complementary DNA clones coding for the rat α (9, 10), β (11), and γ (12) subunits, the mouse α-, β-, and γ-subunits (13), and the human α (10, 14) and γ (15) subunits have been isolated.

The cDNAs coding for the α-subunits predict proteins with signal peptides of 23 (rat and mouse) or 25 (human) amino acids, extracellular domains of 180–181 amino acids, transmembrane domains of 20–21 amino acids, and cytoplasmic domains of 22 (rat), 25 (mouse), or 33 (human) amino acids. The three α-subunits are members of the immunoglobulin superfamily (9, 10, 13, 14), and the sequence for the human α-subunit predicts two disulfide-bonded loops which are composed of amino acids 51–93 and 132–176 of the full length α-protein sequence (10).

Transfection of the cDNA coding for either the human α and rat or human γ subunits (14, 16) or a chimeric human α-subunit alone (17) into heterologous mammalian cells results in cell lines capable of binding human IgE with high affinity. The chimeric human α-subunit (ch-α-protein) contains the extracellular domain of the human α-subunit and the transmembrane and cytoplasmic domains of the p55 subunit of the interleukin 2 receptor (17). The ch-α-protein has been solubilized and partially purified from a transfected Chinese hamster ovary cell line which expresses approximately 4–10 × 10⁵ ch-α-protein molecules per cell (CHO-3D10 cells). This paper describes the development and characterization of monoclonal anti-ch-α-protein antibodies. These antibodies bind to 1) the ch-α-protein expressed on CHO cells; 2) the native FcεRI expressed on human peripheral blood basophils; and 3) one of five epitopes on the α-subunit. The inhibitory and noninhibitory epitopes have been useful for localizing regions of the α-subunit which may participate in binding IgE.

MATERIALS AND METHODS

Purification and Labeling of Human and Rat IgE with ¹²⁵I and Biotin—Human myeloma IgE(PS) was purified as previously described (17, 18), and rat monoclonal IgE was purified from the ascites fluid of nu/nu mice bearing Immunocytoma IR 162 (19). Human IgE was biotinylated with biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Calbiochem) (20). Human and rat IgE were radiolabeled with ¹²⁵I to a specific activity of 1–2 × 10⁶ cpm/μg of protein using chloramine T (21).

Purification of Soluble Chimeric α-Protein—CHO cells expressing

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

§ Present address: Bioprocess Development Dept., Hoffmann-LaRoche Inc., Nutley, NJ 07110.

¹ The abbreviations used are: RBL, rat basophilic leukemia; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EIA, enzyme immunoassay.

4–10 × 10⁵ ch-α-protein molecules per cell (CHO-3D10 cells) were maintained in Iscove's modified Dulbecco's medium containing 10% fetal calf serum as previously described (17). Cells were harvested, washed with cold phosphate-buffered saline (PBS, pH 7.4), and solubilized (10⁶ cells per ml) in cold PBS containing 10 mM CHAPS, 1 mM EDTA, 40 μg/ml phenylmethylsulfonyl fluoride, and 0.02% NaN₃ (10 mM CHAPS/PBS buffer). The cell extract was centrifuged at 100,000 × g for 70 min to remove cell debris and solubilized ch-α-protein was partially purified by IgE affinity chromatography.

Human or rat IgE (5 mg) was coupled to Affi-Gel 10 (1 ml) according to the manufacturer's protocol (Bio-Rad). Either rat or human IgE could be used interchangeably for the affinity purifications. Solubilized preparations of CHO-3D10 cells (1–6 × 10⁹ cell-equivalents) were applied to the column, and the column was washed with 10 mM CHAPS/PBS buffer and 2 mM CHAPS/PBS buffer (2 mM CHAPS, 1 mM EDTA, 40 μg/ml phenylmethylsulfonyl fluoride, 0.02% NaN₃ in PBS, pH 7.4). Proteins bound to the IgE column were eluted with 0.2 M acetic acid, 0.2 M NaCl, 2 mM CHAPS, pH 2.8. Each fraction was immediately neutralized with 3 M Tris, pH 9, and fractions containing active ch-α-protein were pooled and dialyzed against 10 mM CHAPS/PBS buffer. Soluble extracts of CHO-3D10 cells and affinity-purified ch-α-protein were assayed for active ch-α-protein by a modified slot blot assay (22). Briefly, cell extracts and purified ch-α-protein were bound to nitrocellulose and reacted with biotinylated human IgE. The biotinylated human IgE bound to the ch-α-protein was detected with streptavidin-peroxidase (Amersham) and visualized with 4-chloro-1-naphthol.

The extracellular domain of the α-subunit was expressed and secreted by stably transfected CHO cells and purified to affinity chromatography on a matrix of antibody 22E7 coupled by agarose.²

Labeling of Affinity-purified ch-α-protein with ¹²⁵I and N-Glycanase Treatment of ¹²⁵I-ch-α-protein—Affinity-purified ch-α-protein was labeled for 30 min with 0.2 mCi of Na¹²⁵I using Enzymobead reagent (Bio-Rad) according to the manufacturer's protocol. Labeled protein was separated from free ¹²⁵I on a Bio-Gel P10DG column (Bio-Rad) equilibrated with 2 mM CHAPS/PBS, diluted with an equal volume of 2 mM CHAPS/PBS with 1 mg/ml bovine serum albumin, and stored at 4 °C.

Radiolabeled ch-α-protein was dried under vacuum and resuspended in 0.2 M sodium phosphate, 10 mM EDTA, pH 7.0 (50 μl). Digestion with N-glycanase (3 units; Genzyme) proceeded for 2 days at 37 °C on a shaker, and the reaction was stopped with 0.1 M citrate buffer, pH 4.5 (50 μl).

Receptor Binding Assays and Affinity Cross-linking—Binding assays were performed essentially as described previously (17, 23). Briefly, CHO-3D10 or rat basophilic leukemia (RBL) cells were resuspended to 4 × 10⁶ cells per ml in RPMI 1640, 5% fetal bovine serum, 5 mM HEPES, pH 7.4 (binding buffer) and incubated at room temperature for 1 h with ¹²⁵I-labeled IgE (10 ng/ml to 1 μg/ml). Nonspecific binding was determined in the presence of a 50–100-fold molar excess of unlabeled IgE. Cell-bound radioactivity was separated from unbound label by centrifugation through silicone oil (24). For competition assays, antibody samples were preincubated (1 h) with the cell suspensions prior to the addition of ¹²⁵I-labeled human IgE (19 ng) or rat IgE (250 ng).

Soluble complexes of ¹²⁵I-IgE cross-linked to ch-α-protein (¹²⁵I-IgE-ch-α) were prepared as described previously (25) except that the cells were solubilized in ice-cold 10 mM CHAPS/PBS buffer (10⁷ cells/ml). ¹²⁵I-IgE-ch-α complex was confirmed by SDS-PAGE (26) and autoradiography or by immunoprecipitation with antibodies before analysis by SDS-PAGE and autoradiography.

Preparation, Characterization, and Purification of Hybridoma Antibodies—Balb/c mice (Charles River Laboratories) were immunized by the intraperitoneal route with partially purified ch-α-protein (from 7 × 10⁸ cell-equivalents) mixed with an equal volume of Freund's complete adjuvant. 2 weeks later, the mice were injected intraperitoneally with a similar amount of purified ch-α-protein mixed with Freund's incomplete adjuvant. 7 weeks later, one mouse was injected intraperitoneally and intravenously with ch-α-protein (from 5.5 × 10⁹ cell-equivalents) on 2 successive days starting 4 days prior to the cell fusion. Spleen cells were isolated from this mouse and fused with NSO cells (27) at a ratio of 1:1 (spleen cells:NSO cells) with 35% (v/v) polyethylene glycol 4000 (E. Merck) (28). Hybrid supernatants were grown in the presence of 15% P388D1 supernatant (29) and screened for specific α-subunit antibodies by: 1) immunoprecipitation of ¹²⁵I-labeled ch-α-protein, 2) immunoprecipitation of ¹²⁵I-IgE-ch-α

complex, and 3) inhibition of ¹²⁵I-IgE binding to CHO-3D10 cells. Hybridoma cell lines were cloned by limiting dilution.

For the immunoprecipitation assays, hybridoma supernatants (0.1–0.5 ml) or purified antibody samples (5–20 μg), IP buffer (4 mM CHAPS/PBS, pH 7.4, 1 mg/ml bovine serum albumin), and either ¹²⁵I-labeled ch-α-protein or ¹²⁵I-IgE-ch-α complex were mixed together and allowed to stand for 30 min. The immune complexes were harvested by addition of goat anti-mouse antibodies coupled to agarose beads (Sigma) or protein G coupled to agarose (GammaBind G, Genex). Bound proteins were released by addition of SDS-PAGE sample buffer (26) and were analyzed by SDS-PAGE and autoradiography. Antibodies were also tested by Western blot analysis for binding to reduced and denatured ch-α-protein (30).

Antibodies were purified from large-scale hybridoma cultures by affinity chromatography on G protein bound to cross-linked agarose according to the manufacturer's protocol (GammaBind G, Genex).

Binding of Antibodies to Synthetic Peptides—16 overlapping peptides spanning the entire extracellular sequence of the α-subunit (amino acids 26–204) were prepared by standard solid-phase synthesis (31). The purified peptides were dissolved (1 mg/ml) in distilled water or dimethyl sulfoxide. Wells of enzyme immunoassay (EIA) plates were coated with 200 ng of each peptide, blocked with 1% bovine serum albumin, PBS, pH 7.4, and incubated with antibody for 2 h at room temperature. The antibody-peptide complexes were detected with goat anti-mouse IgG coupled to peroxidase (Boehringer Mannheim) and visualized with o-phenylenediamine (0.4 mg/ml) dissolved in 0.1 M citrate buffer, pH 4.5, containing 0.012% H₂O₂ and read at 492 nm. The binding of ¹²⁵I-labeled antibody to synthetic peptide coated to microtiter plates was performed as above except the bound antibody was released with 0.5 M acetic acid and counted in a γ counter. Nonspecific binding of ¹²⁵I-labeled antibody was determined in the presence of a 50-fold molar excess of unlabeled antibody.

Binding Assays with ¹²⁵I-Labeled Antibodies—Antibodies were radiolabeled with ¹²⁵I by a modification of the IODO-GEN method (Pierce Chemical Co.) as described (32). The binding of labeled antibodies to CHO-3D10 cells was determined as described for ¹²⁵I-IgE. Nonspecific binding was determined in the presence of 100-fold molar excess of unlabeled antibody or IgE. Competition assays with unlabeled purified antibodies and hybridoma supernatants were performed as described for ¹²⁵I-IgE, except that ¹²⁵I-labeled antibody is added at the end of the 1-h preincubation step. The concentration of antibody which inhibited 50% of the binding of labeled ligand was designated as the IC₅₀.

Binding of Antibodies to Native FcεRI on Human Basophils—Human basophils were enriched from heparinized peripheral blood by sedimentation in Ficoll-Paque (Pharmacia LKB Biotechnology Inc.) as previously described (33). Washed basophils (1.5 × 10⁶ cells) were incubated with 2.5–40 μg/ml purified antibodies in PBS containing 1% fetal bovine serum and 0.02% sodium azide for 20 min at room temperature. The cells were washed and then incubated with goat anti-human IgE labeled with fluorescein and (Fab)₂-goat anti-mouse IgG labeled with rhodamine (Tago Labs, Burlingame, CA). Cells were washed and dispersed on a slide using a Cytospin apparatus (400 rpm for 5 min). The slides were overlaid with 90% glycerol containing 0.22 M 1,4-diazabicyclo[2.2.2]octane (Sigma) as described previously (34). The cells were observed on a Leitz Diaplan fluorescent microscope and photographed with Kodak Ektachrome 400 film.

Histamine release was performed as described (35).

RESULTS

Characterization of Monoclonal Anti-α-protein Antibodies—

Mice injected with purified, active soluble ch-α-protein produced serum antibodies that effectively blocked ¹²⁵I-IgE binding to CHO-3D10 cells and immunoprecipitated both the ¹²⁵I-labeled ch-α-protein and ¹²⁵I-IgE-ch-α complex (data not shown). A fusion with spleen cells isolated from one mouse yielded 46 hybridomas secreting anti-α-protein antibodies (Table I). All the antibodies immunoprecipitated a radiolabeled ~47-kDa protein from the ¹²⁵I-labeled ch-α-protein preparation (Fig. 1). The two major radiolabeled proteins of ~47 kDa and ~65 kDa present in the ¹²⁵I-ch-α-protein preparation represent labeled ch-α-protein and bovine serum albumin, respectively (Fig. 1). All of the prototype antibodies listed in Table I reacted with denatured and reduced ch-α-protein on Western blots (data not shown).

² N. Topkes, J. Hakim, and J. Kochan, manuscript in preparation.

TABLE I
Characterization of monoclonal FcεRI(α-subunit) antibodies

mAb ^a class (with prototype mAbs)	IP assay ^b			Receptor binding assay ^c				Epitope ^d
	¹²⁵ I-α	¹²⁵ I-de-α	¹²⁵ I-IgE/α	CHO-3D10 cells			RBL, ¹²⁵ I-IgE	
				¹²⁵ I-IgE	¹²⁵ I-15A5	¹²⁵ I-22E7		
Inhibitory (18) ^e								
15A5, 12E7, 6F7	+	+	—	+	+	—	—	Domain 2 (possibly amino acid 125–140)
4B4	+	+	—	+	+	—	+	Domain 2 (overlaps 15A5 epitope)
Noninhibitory (28)								
22E7, 11B4, 5D5, 8C8	+	+	+	—	—	+	—	Domain 1 (possibly amino acid 43–58)
29C6	+	+	+	—	+	+	—	Overlaps 22E7 and 15A5 epitopes
39D5	+	+	+	—	—	—	—	Unknown

^a mAb, monoclonal antibody.

^b IP assay, immunoprecipitation assay performed as described under "Materials and Methods" with ¹²⁵I-labeled purified ch-α-protein (¹²⁵I-α), ¹²⁵I-labeled deglycosylated purified ch-α-protein (¹²⁵I-de-α), and the soluble complex of ¹²⁵I-IgE cross-linked to ch-α-protein (¹²⁵I-IgE/α). Results were scored positive (+) if an antibody bound 5 × the radioactivity of the control antibody or negative (—) if an antibody bound the same radioactivity as the control antibody.

^c Receptor binding assay performed as described under "Materials and Methods" with CHO cells expressing chimeric α-protein (CHO-3D10) and RBL cells. The radiolabeled ligands included in the assays were ¹²⁵I-IgE, ¹²⁵I-15A5, and ¹²⁵I-22E7. Results were scored positive (+) if an antibody could inhibit binding of the respective ligand by 100% or negative (—) if an antibody could not inhibit binding of the respective ligand in a dose-dependent manner.

^d The domain of the α-subunit listed as the location of the epitope recognized by each prototype antibody was estimated from peptide EIA, double antibody competition assays, and binding to mutant chimeric α-protein. Domains 1 and 2 comprise sequences 26–109 and 110–205, respectively. "Unknown" means that the epitope of the prototype antibody could not be localized by the synthetic peptide EIA or double antibody competition assay.

^e The numbers in parentheses represent the number of antibodies in each class of antibody. Prototype antibodies are listed for each epitope in the inhibitory and noninhibitory classes.

^f The IC₅₀ is ~160 μg/ml.

^g The IC₅₀ is 0.18 μg/ml.

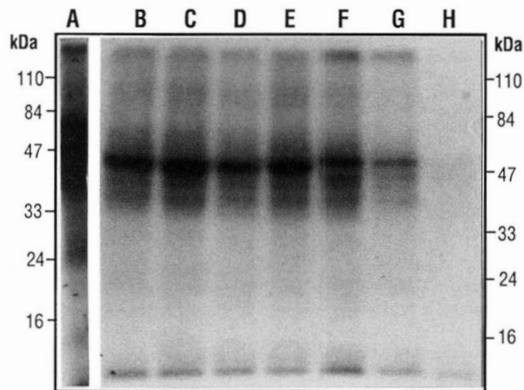


FIG. 1. Immunoprecipitations of ¹²⁵I-ch-α-protein (A) by purified monoclonal antibodies 5D5 (B), 22E7 (C), 8C8 (D), 29C6 (E), 12E7 (F), 15A5 (G), and control antibody (H). All immunoprecipitations were performed as described under "Materials and Methods." Bound proteins were separated by reducing SDS-PAGE on 12% gels and visualized by autoradiography as described under "Materials and Methods." The molecular masses indicated in the margins were estimated from standards (low molecular weight standards, Bio-Rad) electrophoresed in parallel tracks.

The 46 antibodies were divided into inhibitory and noninhibitory classes depending on their ability to inhibit ¹²⁵I-IgE binding to CHO-3D10 cells or to immunoprecipitate ¹²⁵I-IgE·ch-α complex, respectively (Table I). Inhibitory antibodies, such as 15A5 and 12E7 (Fig. 2), and 4B4 and 6F7 (Table II), blocked ¹²⁵I-IgE binding to CHO-3D10 cells at concentrations comparable to unlabeled IgE. Five noninhibitory antibodies did not block radiolabeled IgE binding to ch-α-protein-bearing cells (Fig. 2 and Table II) at concentrations of 100 μg/ml. The ¹²⁵I-IgE·ch-α-protein complex was immunoprecipitated by all

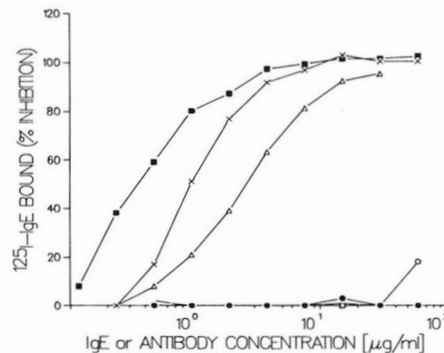


FIG. 2. Inhibition of ¹²⁵I-IgE binding to CHO-3D10 cells by monoclonal antibodies. The data are expressed as % inhibition of ¹²⁵I-IgE binding in the presence of the indicated concentrations of antibody when compared to the specific binding in the absence of antibody. Symbols: unlabeled IgE (×) and antibodies 15A5 (■), 12E7 (●), 5D5 (○), 29C6 (□), and 8C8 (▲).

the noninhibitory antibodies, while the inhibitory antibodies did not immunoprecipitate this radiolabeled complex (Table I).

The binding of radiolabeled IgE and inhibitory and noninhibitory antibodies to CHO-3D10 cells was saturable and specific (data not shown). Scatchard plot analysis (36) of the equilibrium binding data indicated that the apparent dissociation constants (K_D s) for each ligand were approximately equal (Table II).

The apparent molecular mass of the CHO cell-expressed ch-α-protein was approximately 47 kDa as determined by SDS-PAGE and autoradiography of the ¹²⁵I-labeled purified ch-α-protein preparation (Fig. 1) and Western blot analysis (data not shown). This apparent molecular mass indicated

TABLE II
Antibody affinities and competition studies

Antibody	IC ₅₀ ^a				K _D ^b CHO-3D10
	CHO-3D10 cells			RBL	
	IgE ^c	15A5 ^c	22E7 ^c	IgE ^c	
	μg/ml				nM
Inhibitory					
IgE	0.89 ^d	2.41 ^d	>50 ^d	0.29 ^d	3.4
15A5	0.33	1.09	>50	>1200	1.6
12E7	2.43	13.7	>50	ND	ND
6F7	0.1	ND ^e	ND	>1200	3.2
4B4	0.6	1.49	>50	330	3.8
Noninhibitory					
22E7	>100	>120	0.26	ND	1.7
5D5	>100	>120	0.79	ND	ND
8C8	>100	>120	0.29	>1200	ND
29C6	>100	160 ^f	0.18	ND	3.2
11B4	>50	>100	5.9	ND	10.4

^a IC₅₀ determined as described under "Materials and Methods" from the type of data illustrated in Fig. 2.

^b Apparent dissociation constant (K_D) of IgE and each antibody for binding to CHO-3D10 cells was determined by transformation of equilibrium binding data by the method of Scatchard (32).

^c Ligands for the competition assays were radiolabeled human IgE, 15A5, and 22E7 with CHO-3D10 cells and radiolabeled rat IgE with RBL cells.

^d Unlabeled human IgE and rat IgE were used for the competition assays with CHO-3D10 and RBL cells, respectively.

^e Although antibody 6F7 blocked 15A5 binding to CHO-3D10 cells, an IC₅₀ value has not been determined for this antibody. ND, not determined.

^f An IC₅₀ value was estimated from the slope of the inhibition curve. At the highest concentration tested (120 μg/ml), the inhibition was 38%.

that the ch-α-protein is highly posttranslationally modified, since the coding sequence predicts a size of approximately 24 kDa (17). To determine if the antibodies recognize carbohydrate residues or peptide sequence, each antibody was tested in an immunoprecipitation assay with *N*-glycanase-digested ¹²⁵I-labeled ch-α-protein (Fig. 3). The *N*-glycanase-digested receptor migrated with an apparent molecular mass of ~25 kDa (Fig. 3, lane 6, b). The mock-digested ¹²⁵I-ch-α-protein sample did not contain the ~25-kDa protein (Fig. 3, lane 6, a). Both noninhibitory (11B4 and 39D5; Fig. 3, lanes 1 and 2) and inhibitory (15A5, lane 3) antibodies immunoprecipitated the ~25-kDa deglycosylated ¹²⁵I-labeled ch-α-protein. Antibodies present in the serum from the mouse immunized with purified ch-α-protein also immunoprecipitated the glycosylated and deglycosylated forms of ¹²⁵I-labeled ch-α-protein (Fig. 3, lane 4). Most (43 of 46) of the monoclonal antibodies immunoprecipitated deglycosylated ¹²⁵I-labeled ch-α-protein (data not shown).

Antibody Binding to α Subunit Peptides—The binding of each antibody to peptides corresponding to amino acid sequences 26–51, 43–58, 51–65, 59–74, 73–88, 66–80, 81–103, 93–110, 104–117, 111–124, 118–132, 125–140, 134–158, 151–167, 160–197, and 185–204 of the α-subunit (10, 14) was determined by EIA. Of the 43 antibodies tested, two reacted with specific peptides. 15A5 specifically bound the 125–140 peptide, but did not react with the other 15 peptides, as for example the 83–101 peptide (Fig. 4A). The binding of 15A5 to the 125–140 peptide was inhibited in a dose-dependent manner by purified extracellular domain of the α-subunit (Fig. 4B). Scatchard plot analysis of the equilibrium binding of ¹²⁵I-15A5 to the 125–140 peptide immobilized on the microtiter plates indicated an apparent dissociation constant of 16.9 nM (data not shown).

The binding of ¹²⁵I-15A5 and ¹²⁵I-human IgE to CHO-3D10

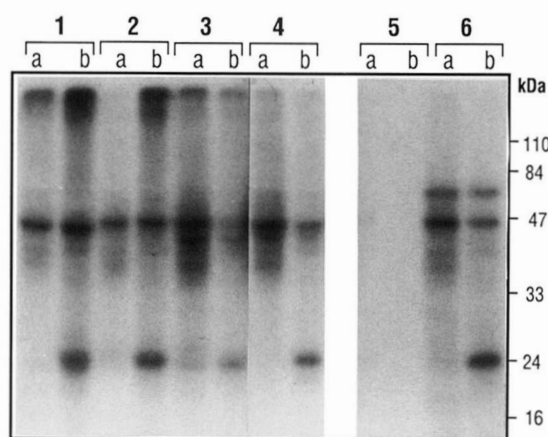


FIG. 3. Immunoprecipitation of deglycosylated ¹²⁵I-ch-α-protein. Affinity-purified ¹²⁵I-ch-α-protein was either digested with *N*-glycanase (b) or mock-digested (a) as described under "Materials and Methods." Mock-digested α-protein was treated identically to the *N*-glycanase-digested protein, except that enzyme was not added to the mock digest. Both *N*-glycanase-digested and mock-digested samples were immunoprecipitated with antibodies 11B4 (lane 1), 39D5 (lane 2), 15A5 (lane 3), mouse polyclonal anti-α-protein antiserum (lane 4), and control antibody (lane 5). The deglycosylated ch-α-protein appears as a single band at approximately 25 kDa in the *N*-glycanase-digested sample (lane 6, b) and is not present in the mock-digested sample (lane 6, a). The molecular sizes indicated in the margins were estimated from standards run in parallel lanes.

cells or to the extracellular domain of the α-protein was not inhibited by the 125–140 peptide or any of the other synthetic peptides (data not shown). Peptide 125–140 is visibly insoluble at high concentrations in aqueous buffers (>10 μg/ml) and sufficiently high concentrations of peptide could not be tested for inhibitory activity in the ¹²⁵I-15A5 and ¹²⁵I-IgE binding assays. However, 15A5 inhibited the binding of a rabbit anti-125–140 peptide antiserum to CHO-3D10 cells (data not shown).

Noninhibitory antibody 11B4 that was present in the parental hybridoma supernatant bound the 43–58 peptide in a dose-dependent manner, but did not bind to the other 15 peptides (data not shown). However, upon cloning the 11B4 hybridoma line, the reactivity to the 43–58 peptide could not be recovered in any of the cloned 11B4 sublines. The parental 11B4 and each of the cloned 11B4 hybridoma lines maintained reactivity with the ch-α-protein.

Competition between Pairs of Antibodies for Binding to CHO-3D10 Cells—To identify the epitopes of the antibodies which did not bind to the synthetic peptides, competition studies were performed between pairs of antibodies for binding to the CHO-3D10 cells. All inhibitory antibodies and unlabeled human IgE blocked ¹²⁵I-15A5 binding to CHO-3D10 cells (Tables I and II). Only one noninhibitory antibody, 29C6, could inhibit ¹²⁵I-15A5 binding at concentrations that were 10–160-fold higher than for inhibitory antibodies (Table II). The other noninhibitory antibodies did not block ¹²⁵I-15A5 binding at these concentrations (Table II).

Hybridoma supernatants containing 22 of the 25 noninhibitory antibodies blocked ¹²⁵I-22E7 binding to CHO-3D10 cells (data not shown). These antibodies blocked ¹²⁵I-22E7 binding in a dose-dependent manner similar to unlabeled 22E7 (Table II). The epitopes recognized by the remaining three noninhibitory antibodies (i.e. 39D5 class; Table I) did not block radiolabeled 22E7 binding to these cells. Unlabeled IgE and inhibitory antibodies (i.e. 15A5, 12E7, and 4B4) could not inhibit radiolabeled 22E7 binding to these cells at concentrations of 50 μg/ml (Table II). Noninhibitory antibodies

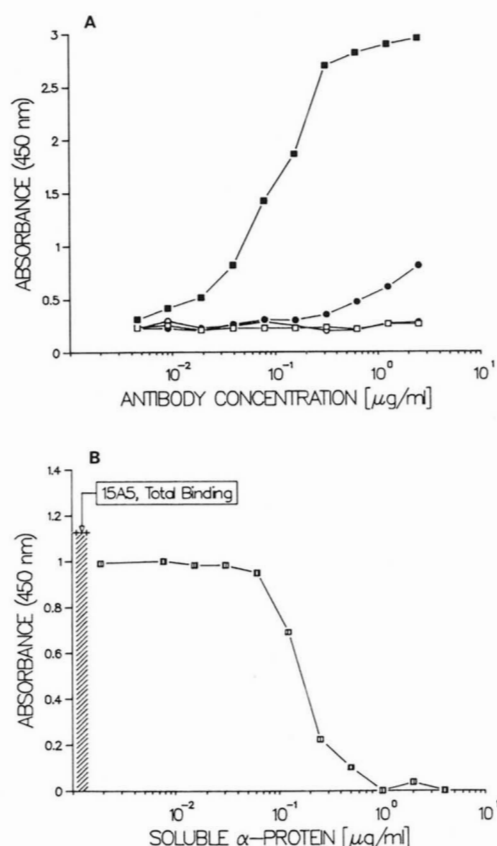


FIG. 4. A, binding of purified antibody 15A5 and control antibody to peptides corresponding to α -subunit sequences 81–103 and 125–140 were determined in an EIA as described under "Materials and Methods." 15A5 binding to peptides 125–140 (■) and 81–103 (□). Control antibody binding to peptides 125–140 (●) and 81–103 (○). B, inhibition of antibody 15A5 binding to the 125–140 peptide by purified extracellular domain of α -subunit. The indicated concentrations of extracellular domain of the α -subunit were preincubated with 50 ng/ml of 15A5 for 30 min at room temperature before addition to the EIA plate coated with peptide 125–140. The binding of 15A5 to peptide 125–140 in the absence (15A5 Total Binding) and the presence of extracellular domain of α -subunit was determined as described under "Materials and Methods."

(22E7 and 11B4) also did not inhibit binding of a rabbit anti-125–140 peptide antiserum to CHO-3D10 cells (data not shown).

Antibody Binding to the Native Receptor (FcεRI) on Human Basophils—Two-color immunofluorescence assays were performed to evaluate the ability of the antibodies to recognize the native α -subunit in the FcεRI complex on human basophils. Human basophils isolated from normal donors usually have IgE bound to the majority of their FcεRI receptors. Therefore, fluorescein isothiocyanate-labeled anti-IgE will identify the basophils within the population of cells on the slide (Fig. 5, C and D). Inhibitory antibody 15A5 very faintly stains the basophils (Fig. 5E). This antibody also shows only faint staining to CHO-3D10 cells that were pretreated with IgE (data not shown). Noninhibitory antibody 5D5 brightly stained the same cells to which IgE is bound (Fig. 5F), indicating that IgE and 5D5 can simultaneously bind to the native FcεRI receptor. Since 15A5 brightly stains CHO-3D10 cells with unoccupied receptors (data not shown), the data also indicate that the 15A5 epitope is blocked by IgE on the majority of FcεRI receptors on the basophils.

Bivalent antibody molecules can promote degranulation of basophils by cross-linking the native FcεRI receptor. The ability of antibodies to induce degranulation can be measured

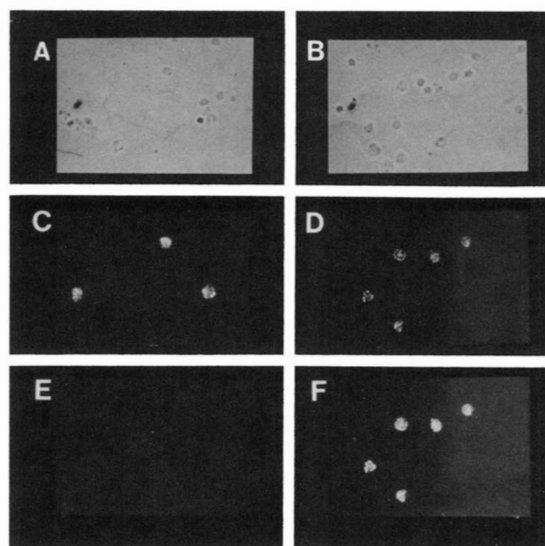


FIG. 5. Two-color immunofluorescence to detect the binding of IgE, 15A5, and 5D5 to an enriched population of human basophils. Enriched peripheral blood basophils were prepared (phase-contrast micrographs; A and B) and incubated with either inhibitory antibody 15A5 (C and E) or noninhibitory antibody 5D5 (D and F) as described under "Materials and Methods." After washing the cells, IgE bound to basophils was visualized with goat anti-IgE labeled with fluorescein (C and D). The binding of antibodies 15A5 (E) or 5D5 (F) to the same basophils depicted in C and D, respectively, were visualized with goat F(ab)₂-anti-mouse IgG labeled with rhodamine as described under "Materials and Methods."

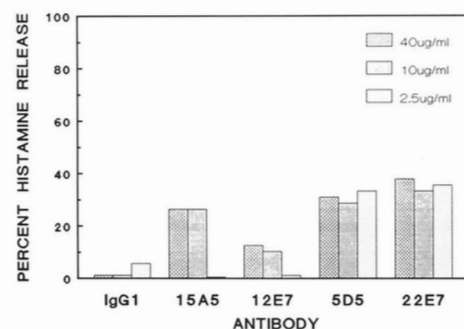


FIG. 6. Stimulation of histamine release from human basophils by treatment with anti-FcεRI(α) antibodies. Human peripheral blood basophils were prepared and treated with the indicated concentrations of anti- α -subunit antibodies as described under "Materials and Methods." Histamine release was measured as described (35).

by the amount of histamine released from the basophils (Fig. 6). Anti-IgE antibodies induced histamine release from the basophils in a dose-dependent manner (data not shown). The noninhibitory antibodies 5D5 and 22E7 induced maximal histamine release at the lowest antibody concentration (2.5 μg/ml; Fig. 6). However, inhibitory antibodies 15A5 and 12E7 did not induce histamine release at 2.5 μg/ml, but did induce histamine release at higher concentrations (10 and 40 μg/ml). Most likely, the high concentrations of inhibitory antibodies are necessary to cross-link the few receptors not occupied by IgE.

Antibody Binding to Native FcεRI Receptor on RBL Cells—Inhibitory and noninhibitory antibodies were tested for their ability to inhibit ¹²⁵I-rat IgE binding to the FcεRI receptor on RBL cells (Table II) or to immunoprecipitate the soluble complex of ¹²⁵I-rat IgE cross-linked to the rat α -subunit (data not shown). Only one inhibitory antibody, 4B4, blocked ¹²⁵I-rat IgE binding to RBL cells, although it was approximately

1100-fold less potent than unlabeled rat IgE. No other antibody could block ^{125} I-rat IgE binding to the RBL cells, although these same antibodies were very potent at blocking ^{125} I-human IgE binding to CHO-3D10 cells (Table II). None of the noninhibitory antibodies could immunoprecipitate the soluble complex of ^{125} I-rat IgE cross-linked to rat α -subunit (data not shown).

DISCUSSION

The high affinity Fc receptor for IgE, Fc ϵ RI, is present on mast cells and basophils. The ~50-kDa α -subunit of the tetrameric receptor complex contains the binding site for IgE. In this study, we report on a series of monoclonal antibodies specific for the human α -subunit. These antibodies fall into two main categories: 1) antibodies which block radiolabeled IgE binding to Fc ϵ RI bearing cells (inhibitory class) and 2) those which do not block this ligand-receptor interaction (noninhibitory class). Both classes of antibodies bind to the α -subunit expressed as a recombinant chimeric protein on CHO cells and as part of the Fc ϵ RI on human basophils.

The extracellular domain of the human α -subunit contains two potential disulfide bonded loops and seven potential N-linked glycosylation sites (10, 14). The recombinant chimeric α -protein expressed in CHO cells has an apparent molecular mass of ~45 kDa (this study), while the predicted mass from the cDNA is 24 kDa (17). Removal of N-linked carbohydrates from the chimeric recombinant α -protein by N-glycanase digestion resulted in a shift in size from approximately 47 kDa to 25 kDa. The 25-kDa protein was recognized by the monoclonal antibodies. These data indicated that most, if not all, of the glycosylation on the chimeric α -protein is N-linked and that the antibodies described in this report can recognize either linear or conformational determinants present on the polypeptide core of the α -protein.

Both inhibitory and noninhibitory antibodies bound to the ch- α -protein with affinities which were nearly equal to or greater than the affinity of IgE for the α -subunit. The affinities were estimated by Scatchard analysis of equilibrium binding data and by comparison of IC_{50} values for competition with radiolabeled IgE, 15A5, and 22E7. These data indicate that the difference between the two classes of antibodies was not based on affinity but on the epitope bound by each antibody. Five epitopes on the extracellular region of the α -subunit of the human Fc ϵ RI receptor have been identified with our panel of monoclonal antibodies; two inhibitory and three noninhibitory epitopes (Table I). Previous reports have described the development and characterization of monoclonal antibodies specific for high affinity immunoglobulin receptors, for example the rat Fc ϵ RI receptor (37–39), the human Fc ϵ RI receptor (40), and the human Fc γ RI receptor (41). Cross-competition studies with pairs of antibodies identified two inhibitory epitopes on the rat Fc ϵ RI- α -subunit (37) and two noninhibitory epitopes and one inhibitory epitope on the human Fc γ RI- α -subunit (40). The locations of inhibitory and noninhibitory epitopes on the α -subunits of the high affinity immunoglobulin receptors were not identified.

Our study indicates a location for the epitopes of inhibitory and noninhibitory antibodies. Results from a direct peptide EIA indicates that the epitope recognized by inhibitory antibody 15A5 may be localized to the sequence 125–140 within the predicted second domain (amino acids 110–205) of the α -subunit. Preliminary data also suggest that noninhibitory antibody 11B4 bound to a peptide (43–58) located within the predicted first domain (amino acids 26–109) of the α -protein. The specificity of the interaction between 15A5 and the 125–140 peptide bound to a solid phase could be demonstrated by

the dose-dependent inhibition produced by the soluble extracellular domain of the α -subunit. However, the 125–140 peptide did not inhibit the binding of 15A5 or IgE to the ch- α -protein expressed on CHO cells or to the extracellular α -subunit coated to EIA plates. Most likely, this was a consequence of the poor solubility of the 125–140 peptide and the 10-fold lower affinity of 15A5 for the peptide. It is unlikely that the binding of 15A5 to the 125–140 peptide is nonspecific, since this peptide was not recognized by 11 other purified anti- α -subunit antibodies or three unrelated anti-interleukin 2 and anti-human c-myc protein antibodies. In addition, 15A5 did not bind to any other peptide from the α -subunit sequence or to peptides from the human c-myc and IL-2 sequences. The ability of 15A5 to block the rabbit anti-125–140 peptide antiserum from binding to CHO-3D10 cells indicates that these two antibodies bind to overlapping epitopes. However, our data cannot show whether the overlap is caused by the two antibodies binding to identical epitopes or to sterically adjacent epitopes. The simultaneous binding of 22E7 and rabbit anti-125–140 peptide antiserum to CHO-3D10 cells indicates that the epitopes recognized by these antibodies do not overlap.

In preliminary studies, we have measured the binding of radiolabeled inhibitory (15A5 and 4B4) and noninhibitory (22E7) antibodies to ch- α -protein with mutated first (amino acids 26–109) or second (amino acids 110–201) domains.³ The results demonstrate that 15A5 and 4B4 bind to mutant proteins that contain the native sequence in the second domain, while 22E7 only binds to mutant proteins that have an intact first domain. These observations support the results presented in this study that indicate 15A5 binds to an epitope located within the second domain of the α -protein, and 22E7 most probably binds to an epitope within the first domain of the α -protein.

Paired antibody competition studies demonstrate that all the inhibitory antibodies bound to sites that either directly or sterically overlapped the 15A5 epitope. IgE also competed with 15A5 for binding to the ch- α -protein, confirming the IgE and 15A5 bind to overlapping but probably not identical domains in the receptor (see below). Only one inhibitory antibody, 4B4, blocked IgE binding to the rat α -protein, indicating that the 4B4 epitope overlaps but is not identical to the 15A5 epitope.

Noninhibitory antibodies of the 22E7 class do not block IgE or 15A5 binding, indicating that the 22E7 epitope is sufficiently separated from the 15A5 epitope to allow both antibodies to simultaneously bind the α -protein. One noninhibitory antibody, 29C6, blocked both 22E7 and 15A5 binding to the ch- α -protein (IC_{50} s of 0.18 μ g/ml and 160 μ g/ml, respectively) and identified a third non-inhibitory epitope that may be located between the 22E7 and 15A5 epitopes. The data also suggest that the 29C6 epitope is closer to the 22E7 epitope than the 15A5 epitope since approximately 900-fold more 29C6 was needed for the same degree of inhibition.

Our results suggest that the epitopes recognized by the noninhibitory 22E7 and inhibitory 15A5 classes of antibodies are located within the first and second domains, respectively, of the α -protein. However, the separation of noninhibitory and inhibitory epitopes to the first and second domains does not localize the binding site for IgE to a single domain. An antibody bound to an inhibitory epitope on the α -protein could block IgE binding by three different mechanisms. The antibody could: 1) directly overlap the contact points between the α -protein and IgE; 2) sterically interfere with IgE binding

³ M. Mallamaci, R. Chizzonite, and J. Kochan, unpublished observations.

by binding to an epitope distant from the IgE contact points; and 3) indirectly inhibit receptor binding by inducing a conformational change within the IgE binding site of the α-protein. Our data do not indicate which possibility is the most probable. However, our data combined with the mutagenesis studies suggest that a portion of the first domain is not necessary for IgE binding since noninhibitory antibodies (22E7) and IgE can simultaneously bind the α-protein. Currently, additional mutagenesis studies are underway to delete and replace very small segments of each domain. These studies may help pinpoint the epitopes of both classes of antibodies and elucidate the contributions of each domain to the binding of IgE.

Acknowledgments—We are grateful to J. Kondas and T. Truitt for excellent technical assistance and J. Farruggia for preparation of the manuscript.

REFERENCES

- Metzger, H., Alcaraz, G., Hahman, R., Kinet, J. P., Publuda, V., and Quarto, R. (1986) *Annu. Rev. Immunol.* **4**, 419–470
- Conrad, D. H., Berczi, I., and Froese, A. (1976) *Immunochemistry* **13**, 329–332
- Kulczycki, A., Jr., McNearney, T. A., and Parker, C. W. (1976) *J. Immunol.* **117**, 661–665
- Holowka, D., Hartmann, H., Kanellopoulos, J., and Metzger, H. (1980) *J. Recept. Res.* **1**, 41–68
- Holowka, D., and Metzger, H. (1982) *Mol. Immunol.* **19**, 219–227
- Perez-Montfort, R., Kinet, J.-P., and Metzger, H. (1983) *Biochemistry* **22**, 5722–5728
- Perez-Montfort, R., Fewtrell, C., and Metzger, H. (1983) *Biochemistry* **22**, 5733–5737
- Alcaraz, G., Kinet, J.-P., Kumar, N., Wank, S. A., and Metzger, H. (1984) *J. Biol. Chem.* **259**, 14922–14927
- Kinet, J.-P., Metzger, H., Hakimi, J., and Kochan, J. (1987) *Biochemistry* **26**, 4605–4610
- Shimizu, A., Tepler, I., Benfey, P. N., Berenstein, E., Siraganian, R. P., and Leder, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1907–1911
- Kinet, J.-P., Blank, U., Ra, C., White, K., Metzger, H., and Kochan, J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 6483–6487
- Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J.-P. (1989) *Nature* **337**, 187–189
- Ra, C., Jouvin, M.-H. E., and Kinet, J.-P. (1989) *J. Biol. Chem.* **264**, 15323–15327
- Kochan, J., Pettine, L. F., Hakimi, J., Kishi, K., and Kinet, J.-P. (1988) *Nucleic Acids Res.* **16**, 3584
- Kuster, H., Thompson, H., and Kinet, J.-P. (1990) *J. Biol. Chem.* **265**, 6448–6452
- Miller, L., Blank, U., Metzger, H., and Kinet, J.-P. (1989) *Science* **244**, 334–337
- Hakimi, J., Seals, C., Kondas, J. A., Pettine, L., Danho, W., and Kochan, J. (1990) *J. Biol. Chem.* **265**, 22079–22081
- Ishizaka, K. (1985) *Methods Enzymol.* **116**, 76–94
- Bazin, H., Querinjean, P., Beckers, A., Heremans, J. F., and Dessy, F. (1974) *Immunology* **26**, 713–723
- Bayer, E. A., Skutelsky, E., and Wilchek, M. (1979) *Methods Enzymol.* **62**, 308–315
- McConahey, P., and Dixon, F. J. (1986) *Int. Arch. Allergy Appl. Immunol.* **29**, 185–189
- Urdal, D. L., Call, S. M., Jackson, J. L., and Dower, S. K. (1988) *J. Biol. Chem.* **263**, 2870–2877
- Kulczycki, A., Jr., Isersky, C., and Metzger, H. (1974) *J. Exp. Med.* **139**, 600–616
- Kilian, P. L., Kaffka, K. L., Stern, A. S., Woehle, D., Benjamin, W. R., Dechiara, T. M., Gubler, U., Farrar, J. J., Mizel, S. B., and Lomedico, P. T. (1986) *J. Immunol.* **136**, 4509–4514
- Chizzonite, R., Truitt, T., Kilian, P. L., Stern, A. S., Nunes, P., Parker, K. P., Kaffka, K. L., Chua, A. O., Lugg, D. K., and Gubler, U. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8029–8033
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Galfre, G., and Milstein, C. (1981) *Methods Enzymol.* **73**, 3–46
- Fazekas de St. Groth, S., and Scheidegger, D. (1980) *J. Immunol. Methods* **35**, 1–21
- Nordan, R. P., Pumphrey, J. G., and Rudikoff, S. (1987) *J. Immunol.* **139**, 813–817
- Burnette, W. H. (1981) *Anal. Biochem.* **112**, 195–200
- Barany, G., and Merrifield, R. B. (1980) in *The Peptides: Analysis Synthesis, Biology* (Gross, E., and Meienhofer, J., eds) Vol. 2, pp. 1–255, Academic Press, New York
- Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 332–333, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Leonard, E. J., Roberts, R. L., and Skeel, A. (1984) *J. Leukocyte Biol.* **35**, 169–174
- Krenik, K. D., Kephart, G. M., Offord, K. P., Dunnette, S. L., and Gleich, G. J. (1989) *J. Immunol. Methods* **117**, 91–97
- Helm, B., Kebo, D., Verchelli, D., Glorsky, M., Gould, H., Ishizaka, K., Gehe, R., and Ishizaka, T. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 9465–9469
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- Basciano, L. K., Berenstein, E. H., Kmak, L., and Siraganian, R. P. (1986) *J. Biol. Chem.* **261**, 11823–11831
- Baniyash, M., Alkalay, I., and Eshhar, Z. (1987) *J. Immunol.* **138**, 2999–3004
- Stracke, M. L., Basciano, L. K., Fischler, C., Berenstein, E. H., and Siraganian, R. P. (1987) *Mol. Immunol.* **24**, 347–356
- Miroli, A. A., and Spitz, M. (1987) *J. Immunol. Methods* **97**, 185–190
- Guyre, P. M., Graziano, R. R., Vance, B. A., Morganelli, P. M., and Fanger, M. W. (1989) *J. Immunol.* **143**, 1650–1655