

Characterization of Monoclonal Antibodies Directed Against the α -Subunit of the Human IgE High-Affinity Receptor

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ABSTRACT

A panel of monoclonal antibodies (8H10/D11, 6F9/H8, 6F9/G9, 5F2/F8/H11, 5F2/F8/G10, 8A4/G12/F9, and 8H10/F12) was raised in mice against the recombinant 20-kDa extracellular part of the α -chain of the human IgE high affinity receptors (ecFc ϵ RI α) produced in insect cells. The antibodies secreted by hybridomas were selected for specific binding to ecFc ϵ RI α , by enzyme-linked immunosorbent assay (ELISA). The selected clones were further characterized in surface plasmon resonance (SPR) experiments with ecFc ϵ RI α covalently immobilized on the surface of a sensor chip. The generated hybridomas can be divided into three groups. Hybridoma supernatants 8A4/G12/F9 and 8H10/F12 inhibited binding of human IgE to immobilized ecFc ϵ RI α in SPR (Group 1). Isotyping revealed that 8A4/G12/F9 and 8H10/F12 were of the IgE/ κ type. Antibodies present in the remaining supernatants were noninhibitory and bound to ecFc ϵ RI α in ELISA with intensities comparable to each other. Isotype analysis of antibodies secreted by these hybridomas showed that the antibodies 6F9/H8, 6F9/G9, 5F2/F8/H11, 5F2/F8/G10, and 8H10/D11 were IgG1/ κ . The hybridoma supernatants were purified via protein A chromatography. In a SPR experiment, ecFc ϵ RI α , displayed by immobilized human IgE, was still recognized by 6F9/H8 and 6F9/G9 (Group 2) as expected for noninhibitory antibodies. Surprisingly, 8H10/D11, 5F2/F8/H11, and 5F2/F8/G10 (Group 3) did not bind to this complex although they do not inhibit the binding of human IgE to ecFc ϵ RI α . All purified monoclonal antibodies gave positive signals in Western blotting.

INTRODUCTION

THE HUMAN RECEPTOR WITH HIGH AFFINITY for IgE (Fc ϵ RI) plays a pivotal role in mediating immediate hypersensitivity reactions. Association of a multivalent antigen with receptor-bound IgE leads to crosslinking of receptor molecules on the cell membrane. This triggers degranulation of basophils and mast cells resulting in rapid release of preformed mediators like histamine and the synthesis and secretion of biomolecules that activate inflammatory reactions.⁽¹⁾ Fc ϵ RI consists of a tetrameric complex of noncovalently linked polypeptides with the stoichiometry $\alpha\beta\gamma_2$. The β -chain and the disulfide-linked γ -chains appear to function primarily in signal transduction.⁽²⁾ For binding of human IgE to its high-affinity receptor the extracellular part of the Fc ϵ RI α -chain (ecFc ϵ RI α , Val¹-Leu¹⁷⁹, numbering according to Shimizu *et al.*, 1988, is sufficient.⁽⁴⁾ Although ecFc ϵ RI α is heavily glycosylated the carbohydrate component is not required for IgE binding.⁽⁵⁾ The ecFc ϵ RI α contains two immunoglobulin-like domains⁽⁶⁾ each containing one disulfide bridge: domain $\alpha(1)$ containing (Cys²⁶-Cys⁶⁸), which is distal, and domain $\alpha(2)$ containing (Cys¹⁰⁷-Cys¹⁵¹), which is proximal to the cell membrane. Domain $\alpha(2)$ was

shown to be sufficient for binding of IgE although indications exist that high-affinity binding may require at least part of the $\alpha(1)$ domain.⁽⁷⁾ Monoclonal antibodies (MAb) are appropriate tools for studying the functional relationship between hIgE and ecFc ϵ RI α . MAbs that inhibit the binding of hIgE to the receptor have been generated and used by several research groups^(8,9) to identify receptor regions involved in IgE binding. To provide additional means for further studying the functional properties of ecFc ϵ RI α , we have generated a panel of MAbs whose properties are described here.

MATERIALS AND METHODS

Receptor

The recombinant extracellular part (Val¹-Leu¹⁷⁹, numbering according to Shimizu *et al.*, 1988)³ of the human Fc ϵ RI α was produced in Sf9 insect cells (Invitrogen, San Diego, CA) using the baculovirus expression system. After purification at Sandoz Basle, Batch EN 24/460 was used for immunization of mice.

Control antibodies

Human IgE, named sus-11, was obtained from B. Stadler, University Bern. sus 11 was detected with a goat anti-human IgE horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO). MAb 5H5F8, a mouse MAb, raised against a fusion protein consisting of a κ -light chain fused to the amino terminus of ecFc ϵ RI α , has been produced at our institute and was used as positive control MAb for binding to ecFc ϵ RI α . The MAb 15/1, raised against the Fc ϵ RI α γ complex,⁽⁹⁾ prevents IgE binding to the receptor and was used as positive control in inhibition experiments. As negative control the mouse-anti-human IgE antibody BSW17 (B. Stadler, University Bern) was used. IgG antibodies were detected with a goat anti-mouse IgG HRP conjugate (BioRad, Hercules, CA) and developed as described next.

Production of hybridomas

At Day 0, 100 μ g ecFc ϵ RI α in 300 μ l PBS (150 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM K₂PO₄, pH 7.5) was emulsified 1:1 with complete Freund's adjuvant and injected IP into female Balb/c mice. At Day 7 and 28 after the first injection, the mice were injected once more with 100 μ g ecFc ϵ RI α in 300 μ l PBS emulsified 1:1 with incomplete Freund's adjuvant. At Days 33, 34, 35, and 36, the mice were boosted by injecting IV 50 μ g antigen in 150 μ l PBS. At day 37, spleens were removed and the splenocytes were fused with the mouse myeloma cell line SP2 using polyethylene glycol (PEG) as fusogen.⁽¹¹⁾

ELISA experiments

100 ng ecFc ϵ RI α were immobilized in Costar Strip Plate-8 wells (Cambridge, MA) in 100 μ l coating buffer (0.1 M NaHCO₃, 0.01% NaN₃, pH 9.6) in a humidified chamber at 4°C over night. Wells were washed twice with PBS and unspecific binding sites were saturated for 1 h at room temperature with 300 μ l PBS containing 5% BSA (Sigma). Wells were washed twice with PBS containing 0.05% Tween 20 (Serva, Heidelberg) and incubated with 100 μ l of hybridoma culture fluids or purified MAbs for 2 h at room temperature. After washing the wells twice with PBS containing 0.05% Tween 20, bound antibodies were incubated with a goat anti-mouse IgG HRP conjugate (Biorad, Hercules, CA) for 1 h at room temperature. The wells were washed three times with PBS containing 0.05% Tween 20 and further incubated with 100 μ l of the mixed components (according to the manufacturer) of the ABTS substrate (BioRad). The reaction was stopped with 100 μ l of 2% oxalic acid and color intensities were read at 405 nm in a spectrophotometer. Hybridoma culture fluids which gave strong signals were diluted 1:2, 1:4, and 1:8 with PBS and the specific ELISA was repeated with these dilutions. Hybridomas which gave strong signals even at the 1:8 dilution were selected and further characterized by SPR experiments.

The ELISA procedure for screening for MAbs that inhibit IgE/Fc ϵ RI binding was the same except the following changes: after coating the wells with ecFc ϵ RI α and washing, 100 μ l of each hybridoma supernatant was incubated under the same conditions, as just described. Subsequently, the plates were washed and further incubated with 900 ng sus-11 IgE per well. After

washing, bound IgE was quantitated by incubation with goat-anti-human IgE HRP and chromogenic substrate as described. Supernatants that inhibited IgE binding at least as strongly as the control antibody 15/1 were selected and further characterized.

Surface plasmon resonance (SPR)

SPR experiments were performed in a BIAcore instrument (Pharmacia). A sensor chip CM5 (research grade) was prepared using the amine-coupling procedure as described by the manufacturer. Briefly, the glass support was washed with flow buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.05% BIAcore surfactant P₂₀ from Pharmacia) at a flowrate of 5 μ l/min. The sensor chip was then activated by injection of 35 μ l of a v/v mixture of 200 mM N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC) in H₂O and 50 mM N-hydroxysuccinimide (NHS) in H₂O. The reactive surface was coated with ecFc ϵ RI α at 50 μ g/ml and free reactive groups were saturated with ethanolamine. The coated surface was used to select hybridoma culture fluids that bind to ecFc ϵ RI α and to determine the binding characteristics of the purified MAbs. After each run, the sensor chip was regenerated with a pulse of 10 mM hydrochloric acid.

Isotyping of hybridoma culture fluids

Isotypes of MAbs in hybridoma culture fluids were determined with a mouse isotype kit (BioRad) according to the manufacturers' protocol.

Protein A purification of hybridoma culture fluids

Two hundred ml of each hybridoma culture fluid was clarified by centrifugation and then purified using the AvidChrom Protein Antibody Purification Kit (Sigma) according to the manufacturer's protocol. Briefly, culture supernatant was first

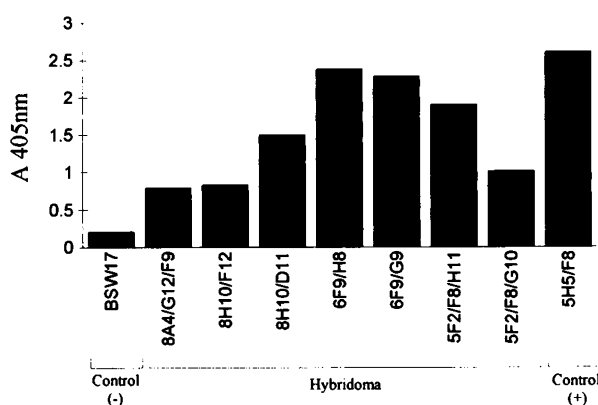


FIG. 1. Binding of MAbs to ecFc ϵ RI α (ELISA). One hundred ng ecFc ϵ RI α was coated to each well in a humidified chamber at 4°C over night. After blocking for 1 h at room temperature (RT), 100 μ l of each hybridoma supernatant were added and incubated for 2 h at RT. Bound MAbs were detected with HRP-conjugated goat-anti-mouse IgG by incubation for 1 h at RT and color reaction using chromogenic ABTS substrate. The absorbance values shown represent the mean values of duplicates and were corrected for unspecific binding.

TABLE 1. ELISA EXPERIMENTS COMPARING BINDING TO ecFceRI α OF HYBRIDOMA SUPERNATANTS VERSUS PURIFIED SAMPLES

MAbs	Binding to ecFceRI α of hybridoma supernatant	Binding to ecFceRI α of purified sample	Inhibition of hIgE/RI α binding by hybridoma supernatant	Inhibition of hIgE binding by purified sample
8A4/G12/F9	+	—	yes	no
8H10/F12	+	—	yes	no
8H10/D11	++	+++	no	no
6F9/H8	+++	+++	no	no
6F9/G9	+++	+++	no	no
5F2/F8/H11	++	+++	no	no
5F2/F8/G10	++	+++	no	no
5H5/F8	n.d	+++	no	no
15/1	n.d	+++	yes	yes

Purified MAbs 15/1 and 5H5/F8 were taken as references for inhibition of the IgE/ecFceRI α interaction and binding, respectively. ELISA: +++: binding comparable to 5H5/F8 ($A_{405} = 3.0-2.0$), ++: $A_{405} = 2.0-1.0$, +: $A_{405} = 1.0-0.5$ and —: $A_{405} \leq 0.25$ (background absorbance). The degree of IgE/ecFceRI α binding inhibition was compared with them of 15/1 and only scored yes or no. Abbreviation—hIgE-human IgE.

loaded onto the protein A cartridge. The Protein A cartridge was then washed to remove excess, unbound protein and connected to a desalting cartridge equilibrated with HEPES buffer. After addition of the elution buffer, the purified IgG was obtained as 5 ml eluate at physiological pH. Purified samples were concentrated using a Microsep 10 K Spin Column device (Filtron, Northborough, MA) and concentration of the MAbs was measured in a spectrophotometer by absorbance at 280 nm. 1 μ g of concentrated, purified IgG was applied to reducing SDS-PAGE to control purity and molecular weight of heavy and light chains.

Western blotting

Two hundred ng ecFceRI α in 1.25% SDS and 5% β -mercaptoethanol was applied in each well of a 1 mm 14% Tris-glycine minigel (Novex, San Diego, CA) and electrophoresed for 90 min at constant voltage of 125 volts. The separated proteins were transferred to a 0.2 μ m polyvinylidene difluoride (PVDF) membrane (Novex). The membrane was blocked with 5% nonfat dry milk (BioRad) in TBS (20 mM Tris, pH 7.5, 500 mM NaCl), for 1 h and then washed twice for 10 min with TBS_T (0.05% Tween 20-TBS, pH 7.5). The membrane was sliced into strips, placed into trays, and incubated with 1% nonfat dry milk in 20 ml TBS containing 3 μ g/ml purified MAbs for 1 h at room temperature on an orbital shaker. Strips were washed twice for 10 min with TBS_T and incubated with HRP-conjugated goat anti-mouse IgG for 1 h at room temperature on an orbital shaker. After washing four times for 10 min with TBS_T, blots were developed using the ECL substrate (Amersham, instruction protocol) and were exposed to ECL hyperfilm (Amersham) for 1 min.

RESULTS

After cell fusion, hybridomas were selected for binding to immobilized ecFceRI α by ELISA. Strongly positive clones

were further subcloned by limiting dilution. After repeated testing in ELISA and SPR experiments the following 7 stable MAb-secreting clones were obtained that specifically recognized ecFceRI α : 8H10/D11, 6F9/H8, 6F9/G9, 5F2/H11, 5F2/G10, 8A4/G12/F9, and 8H10/F12 (Fig. 1). The highest readout in ELISA was obtained with hybridomas secreting MAbs 6F9/G9 and 6F9/H8, whereas the MAbs 8A4/G12/F9 and 8H10/F12 gave only weak signals. All other MAbs bound moderately to FceRI α in this test. The binding intensities can be compared qualitatively under the assumption that the amount of antibody secreted by a hybridoma is about 20–50 μ g/ml.⁽¹²⁾

When hybridoma supernatants were assayed for antibodies that inhibit the IgE/FceRI α interaction, two of the selected clones, 8A4/G12/F9 and 8H10/F12, were found to inhibit binding of hIgE to ecFceRI α (Table 1, Column 3). The selected hybridoma supernatants were purified by protein A chromatography and binding properties of the purified antibodies were compared with those of crude cell supernatants (Table 1). Binding of all purified MAbs to ecFceRI α were similar to each other except for the samples from hybridoma supernatants previously found to inhibit IgE/RI α binding: the protein A eluates from 8A4/G12/F9 and 8H10/F12 were no longer able to give positive signals in ELISA. This can be explained by the subtyping experiment shown in Table 2. All selected hybridomas produced antibodies of IgG1/ κ isotype except 8A4/G12/F9 and

TABLE 2. ISOTYPE DETERMINATION OF ANTIBODIES SECRETED BY HYBRIDOMA SUPERNATANTS

MAbs	Isotype
8A4/G12/F9	IgE/ κ
8H10/F12	IgE/ κ
8H10/D11	IgG1/ κ
6F9/H8	IgG1/ κ
6F9/G9	IgG1/ κ
5F2/F8/H11	IgG1/ κ
5F2/F8/G10	IgG1/ κ

TABLE 3. SURFACE PLASMON RESONANCE EXPERIMENTS

	Binding of hybridoma supernatants to <i>ecFceRIα</i>	Binding of purified MABs to <i>ecFceRIα</i>	Binding to <i>ecFceRIα</i> displayed by immobilized IgE
BSW17	—	—	—
8A4/G12/F9	+	$\pm$	—
8H10/F12	+	$\pm$	—
8H10/D11	++	+	—
6F9/H8	+++	++	+
6F9/G9	+++	++	+
5F2/F8/H11	+++	++	—
5F2/F8/G10	++	++	—
5H5/F8	n.d.	+++	++
15/1	n.d.	+	—

Column 1: 30 μ l of each hybridoma supernatant was tested. SPR readings: +++: 500 RU/30 μ l supernatant (6F9/H8, 6F9/G9, and 5F2/F8/H11); ++: 300 RU/30 μ l supernatant (8H10/D11, 5F2/F8/G10); +: 100 RU/30 μ l supernatant (8A4/G12/F9, 8H10/F12). Column 2: Binding of 30 μ l purified MABs to immobilized *ecFceRI α* was measured after 90 s of dissociation in flow buffer and data were normalized for the different concentrations of the various antibodies to yield RU/ μ g antibody: +++: >150, ++: >50, +: >15; $\pm$: 5–3. Column 3: hIgE was immobilized on the sensor chip and *ecFceRI α* was bound in flow buffer. Then 30 μ l of each purified MAB (50 μ g/ml) was injected and monitored for binding to the IgE/receptor complex. ++: binding as good as 5H5/F8; +: significant binding.

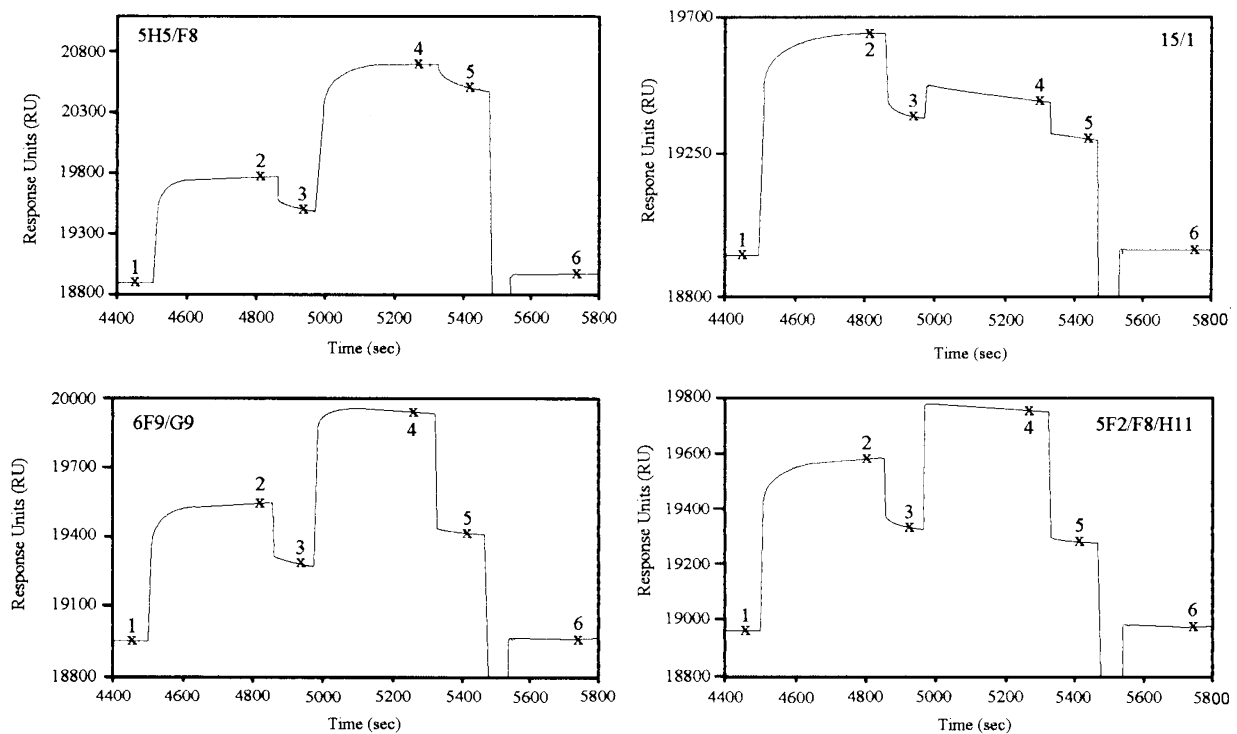


FIG. 2. Analysis of the interaction of MABs with the IgE/*ecFceRI α* complex with SPR. Each sensorgram are described by the following markers: Marker 1 indicates the baseline after immobilization of the ligand (hIgE). Marker 2 shows maximum association of injected *ecFceRI α* to hIgE. The steep rise of the baseline immediately after sample injection is due to the different refractive indexes of the dilution buffer and the flow buffer. Marker 3 shows bound material after 90 s of dissociation. Immediately after this point, the MAB to be tested is injected and association is monitored until Marker 4. Bound antibody is measured after 90 s of dissociation at Marker 5. MABs that were considered binders had to reach a significantly higher readout at marker 5 as compared to Marker 3. Marker 6 shows the hIgE surface after regeneration with a pulse of 10 mM hydrochloric acid. Ideally, Marker 6 has the same readout as Point 1. **Top left:** representative sensorgram which shows that the noninhibitory control MAB 5H5/F8 binds to the hIgE/*ecFceRI α* complex. The sensorgram at the **top right** shows that the inhibitory control MAB 15/1 does not bind to hIgE/*ecFceRI α* complex. **Bottom left:** sensorgram obtained with MAB 6F9/G9 indicating binding to the hIgE/*ecFceRI α* complex. An identical sensorgram was obtained with 6F9/H8 (data not shown). **Bottom right:** a representative sensorgram for a nonbinding MAB (5F2/F8/H11) is shown.

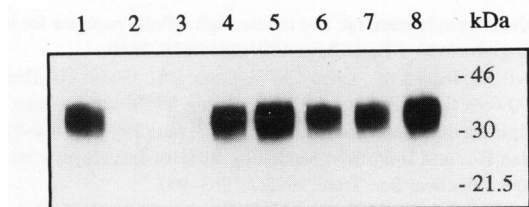


FIG. 3. Binding of purified MABs to ecFceRI α on Western blot. In each lane 200 ng of ecFceRI α was electrophoresed by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was sliced into strips and probed with the following MABs: 1) 5H5/F8, 2) BSW17, 3) 15/1, 4) 8H10/D11, 5) 5F2/F8/G10, 6) 6F9/G9, 7) 6F9/H8, and 8) 5F2/F8/H11. The relative molecular weight of approximately 35 kDa results from glycosylation of the insect cell-derived ecFceRI α .

8H10/F12 which were IgE/ κ . Therefore, purification by the IgG-specific protein A chromatography obviously resulted in an almost complete loss of the IgE/ κ antibodies from 8A4/G12/F9 and 8H10/F12 hybridoma. SPR experiments with hybridoma supernatants confirmed the ELISA data. As shown in Table 3, binding profiles obtained with the purified MABs to ecFceRI α covalently immobilized to the sensor chip were similar to data obtained with ELISA. The very weakly positive signals obtained with 8A4/G12/F9 and 8H10/F12 result from the small amount of the IgE antibodies obtained from Protein A chromatography that could be detected also by SDS-PAGE (data not shown).

When hIgE, immobilized on the sensorchip, was saturated with an excess of ecFceRI α only two antibodies (6F9/G9, 6F9/H8) were still able to recognize this complex (Table 3, Column 3).

In contrast, MABs 5F2/F8/H11, 5F2/F8/G10, and 8H10/D11 did not bind to the complex although they are noninhibitory. As expected, the control MAB 15/1, which is known to interfere with IgE/R1 α binding, also showed no binding to the complex whereas the noninhibitory MAB 5H5/F8 did still recognize its epitope on IgE-bound R1 α . A representative set of sensorgrams is shown in Fig. 2. In Western blot experiments, where ecFceRI α was separated by SDS-PAGE under reducing conditions, all MABs reacted as good as the control antibody

5H5/F8. MAB 15/1 and the control anti-human IgE MAB BSW17 gave no signals on Western blots (Fig. 3).

DISCUSSION

In this report, the production and characterization of a set of MABs raised in mice against the extracellular part of the human IgE high affinity receptor α -chain (ecFceRI α) is described. As a result of the presented experiments, the described MABs can be categorized into three groups (Table 4) according to their general binding characteristics.

The first group that can be clearly distinguished from the others are the MABs belonging to the IgE/ κ isotype that inhibited hIgE binding to the receptor. They could not be purified using Protein A chromatography because IgE does not bind to Protein A. Because mouse IgE binds to the human receptor, inhibitory character could either be a consequence of binding to the IgE antibodies to ecFceRI α via their complementary determining regions ("antibody-like") or via the Fc-parts ("IgE-receptor interaction"). A possibility to answer this question would be the generation of F(ab) fragments of these IgE antibodies and testing these fragments for receptor binding. Recognition of ecFceRI α by the F(ab) fragments would indicate the "antibody-like" interaction type whereas no binding would point toward the "IgE-receptor interaction." A prerequisite for such experiments would be the purification of the IgE/ κ MABs that could, for example, be achieved by affinity chromatography on immobilized FceRI α .

The second group consists of MABs 6F9/G9 and 6F9/H8. Both antibodies did bind to the hIgE/ecFceRI α complex in SPR experiments indicating that their epitopes are not involved in the hIgE/receptor interaction as expected from noninhibitory MABs. Furthermore, they can be distinguished from each other by their affinity for ecFceRI α as determined in ELISA experiments (data not shown).

Antibody Group 3 contains antibodies 5F2/F8/H11, 5F2/F8/G10, and 8H10/D11. They differ from the ones belonging to Group 2 by their inability to recognize receptor-bound IgE although they do not interfere with the Fce/FceRI α interaction. An explanation for this observation might be that the epitope(s) to which these antibodies bind in solution is (are)

TABLE 4. GROUPING OF ANTI-ecFceRI α MABs ACCORDING TO THEIR BINDING CHARACTERISTICS

Group (MAB)	(1) 8A4/G12/F9, 8H10/F12	(2) 6F9/H8 6F9/G9	(3) 5F2/F8/H11, 5F2/F8/G10, 8H10/D11
ELISA			
Binding of supernatants to ecFceRI α	+	+	+
Inhibition of ecFceRI α /hIgE-binding isotype	+	—	—
Surface plasmon resonance			
Binding of supernatants to ecFceRI α	+	+	+
Binding of purified MABs to ecFceRI α	—	+	+
Binding of purified MABs to ecFceRI α /hIgE complex	—	+	—
Western blot			
With purified MABs	n.d	+	+

distorted upon binding of IgE to the receptor. Evidence for conformational changes in both Fc ϵ and Fc ϵ RI α accompanying the binding process have been reported in literature.⁽¹³⁾ Such a structural rearrangement would most likely affect complex conformational epitopes rather than linear ones. However, the ability of the antibodies of Group 3 to give positive signals in Western blots argues against such complex epitopes.

Taken together, with respect to their properties summarized in Table 4, we think that the panel of MAb described in this report will serve as tools for future experiments aimed at the evaluation of the interaction of hIgE with its high-affinity receptor and to further characterize antigenic determinants on Fc ϵ RI α .

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