

Upregulation of FcεRI on human basophils by IgE antibody is mediated by interaction of IgE with FcεRI

Donald MacGlashan, Jr, MD, PhD,^a Lawrence M. Lichtenstein, MD, PhD,^a Jane McKenzie-White, MS,^a Kristin Chichester, BS,^a Alistair J. Henry, PhD,^b Brian J. Sutton, PhD,^b and Hannah J. Gould, PhD^b *Baltimore, Md, and London, United Kingdom*

Background: IgE is now known to upregulate the expression of FcεRI on human basophils. It is not known which receptor on basophils mediates this process of upregulation.

Objective: We sought to determine whether galectin-3, FcεRII (CD23), or FcεRI were involved in the upregulation of FcεRI by IgE.

Methods: The role of galectin-3 was examined by measuring the influence of α-lactose on upregulation. Basophils were examined for expression of FcεRII (CD23) by flow cytometry and messenger (m)RNA expression. Functional discrimination between binding to FcεRII or FcεRI was examined through the use of mutant IgE-Fc fragments or anti-FcεRII antibody. **Results:** Upregulation of FcεRI on basophils in the presence of IgE was not altered by coinubation with α-lactose, eliminating a role for galectin-3. Basophils were not found to express FcεRII, as determined by flow cytometry with enriched basophil preparations or RT-PCR with highly purified basophil preparations. A mutant of the Fc fragment of IgE (IgE-Fc), which binds to FcεRI with a greater than 10-fold lower affinity than IgE or wild-type IgE-Fc but exhibits no change in affinity for FcεRII, allowed us to distinguish between the functions of the two Fc receptors. The mutant (R334S; Henry et al 1997) was required at about 30-fold higher concentration than the wild-type IgE-Fc for the same stimulation of FcεRI expression on basophils, thus excluding a role for FcεRII in the response. In addition, treatment of basophils with anti-FcεRII antibody (MHM6), which is known to be competitive with IgE, had no effect on the expression of FcεRI or the ability of IgE to upregulate expression of FcεRI. **Conclusion:** Collectively, these data indicate that IgE interacts with FcεRI to upregulate its expression on human basophils. (*J Allergy Clin Immunol* 1999;104:492-8.)

Key words: IgE receptors, basophils, IgE antibody

Recent studies have demonstrated the regulation of cell surface FcεRI expression on basophils and mast cells obtained from either mice or humans by IgE antibody.¹⁻⁵ In the absence of IgE, cell surface FcεRIα decreases, and in its presence cell surface FcεRI accumulates. In either

Abbreviation used

EC50:	Effective concentration for 50% of the maximum effect
PIPES:	Piperazine-N,N-bis-2-ethanesulfonic acid
SPR:	Surface plasmon resonance

instance the total cellular mass of FcεRIα also decreases and increases.³ The increase in mass during incubation with IgE indicates the synthesis of FcεRIα. Inhibition of the increase in FcεRIα by cycloheximide in studies with mouse mast cells supports this conclusion.⁴ It is not yet clear whether synthesis is induced or constitutive. Upregulation occurs in purified cell preparations, and therefore it is unlikely that IgE induces upregulation indirectly through another cell type.³ Denaturing IgE with heat (56°C for 90 minutes) or blocking the Cε2/Cε3 region with specific mAbs blocks its ability to induce upregulation.³ However, the upregulation induced by IgE is slow, and the concentration dependence on IgE does not directly support the conclusion that upregulation occurs through binding of IgE to FcεRIα. On the basis of the estimates of the equilibrium constant for human IgE interacting through FcεRIα, one would expect that if upregulation depended on the equilibrium conditions of binding, then the effective concentration for 50% of the maximum effect (EC50) would be between 10 and 50 ng/mL of IgE antibody. In human basophils the EC50 for upregulation occurs at approximately 250 ng/mL, and the concentration dependence is quite broad.³ This result raised some concerns about which IgE-binding receptor might be mediating the effects of IgE. The current studies were performed to test the conclusion that upregulation results from a direct interaction between IgE and FcεRIα. Three possibilities were explored, namely that IgE interaction occurs through FcεRI, FcεRII, or εBP (galectin-3). In a previous report we eliminated a fourth possibility for interaction of IgE through CD32 on human basophils by noting that high concentrations of IgG (1 mg/mL) neither induced upregulation of FcεRI nor altered the upregulation induced by IgE.³

METHODS

Buffers

Piperazine-N,N-bis-2-ethanesulfonic acid (PIPES; Sigma Chemical Co, St Louis, Mo) stock buffer (25 mmol/L PIPES con-

From ^athe Johns Hopkins Asthma and Allergy Center, Baltimore; and ^bThe Randall Institute, King's College London, London.

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Reprint requests: Donald MacGlashan, Jr, MD, PhD, Johns Hopkins Asthma and Allergy Center, 5501 Hopkins Bayview Circle, Baltimore, MD 21224.

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taining 110 mmol/L NaCl, 5 mmol/L KCl, and 40 mmol/L NaOH adjusted to pH 7.3) was stored at 10 times the above concentration. We also used PAG (PIPES [1×] containing 0.003% human serum albumin [Miles Laboratories Inc, Elkhart, Ind] and 0.1% glucose), PAGCM (PAG with 1.0 mmol/L CaCl₂ and 1.0 mmol/L MgCl₂), and PAG-EDTA (PAG with 1.0 mmol/L EDTA).

Reagents

Purified IgE-PS myeloma was a gift from Dr T. Ishizaka.⁶ Recombinant human IL-3 was obtained from Biosource (Camarillo, Calif). IgE-Fc(WT) and IgE-Fc(R334S) were obtained by procedures previously described and represent materials shown to be free of aggregates by HPLC.^{7,8} The so-called IgE-Fc(WT) is only considered a wild type with respect to R334S. Both IgE-Fcs bear 2 mutations, Asn265Gln and Asn371Gln, which were made to make the IgE more homogeneous by eliminating the exposed carbohydrates. The conserved (buried) glycosylation site N394 was retained because it was thought that it may be important in the structure. MHM6 antibody for flow cytometry of FcεRII was obtained from Dako (Carpinteria, Calif). The messenger (m)RNA primer pair for analysis of FcεRII mRNA by RT-PCR was obtained from Operon (Alameda, Calif) with the following sequences: 5'-CGTGATGATGCGGGGCTC and 5'-AGAGGAGCGGGAGATGTG. Conditions for using these primers were worked out on Ramos cells (kindly provided by Dr Farhad Imani, Johns Hopkins University Asthma and Allergy Center), which express high levels of this receptor. The PCR procedure used standard reagents (PCR kits; Perkin-Elmer, Foster City, Calif) with thermal cycling set for denaturing (94°C for 45 seconds), annealing (66°C for 45 seconds), and extension (72°C for 45 seconds) for 32 cycles.

Cell preparation

Two types of basophil preparations were used. Most of the studies used cells obtained from leukapheresis and were prepared as previously described.⁹ Basophil purity in these preparations ranged from 15% to 95% with a median of 33%. In some experiments cells were isolated from peripheral blood by using the double Percoll method.¹⁰ The blood was diluted with EDTA-saline and centrifuged at 500*g* for 15 minutes to obtain a buffy coat. The buffy coat cells were diluted in saline and layered onto a 2-step Percoll gradient (upper layer, 1.065 g/mL; lower layer, 1.079 g/mL), as described previously.¹¹ After centrifugation at 450*g* for 15 minutes, the interface between the 1.065 Percoll/plasma upper layer and the 1.079 lower Percoll layer was harvested and washed as above (basophil purity of 8% to 45%). The specific cell preparations used will be noted in the text. One of the preparations used to examine expression of CD23 mRNA in human basophils was performed on basophils further purified by positive selection. The procedure has been described elsewhere.⁹ Briefly, cells were incubated for 10 minutes with 1 μg/mL mouse anti-human IgE (TES-19; kindly provided by Dr Frances Davis at Tanox Corp) in the presence of 4 mg/mL normal human IgG to block FcγR; the entire procedure was carried at a temperature below 4°C in PAG with 50 μmol/L EDTA. After a subsequent 20-minute incubation with rat anti-mouse IgG2a+b paramagnetic beads (8 μL/10⁷ cells), the cells were passed through a MACS mini-column (MACS system; Miltenyi Biotec Inc, Sunnyvale, Calif). Both flow-through cells (contaminating mononuclear cells) and column-bound cells (basophils) were collected.

Cell culture

Enriched basophil preparations were cultured in Iscove's modified Dulbecco's medium media (Life Technologies) containing 2% FCS, 40 μg/mL gentamycin, and 10 ng/mL IL-3. The total cell density was 2 × 10⁶/mL, and culturing was done in 96-, 24-, or 6-

well tissue culture-treated plates (Costar, Cornell, NY) as appropriate.

Flow cytometry

A flow cytometric technique incorporating light scatter characteristics was used to quantify FcεRIα chain expression on basophils as described.¹² Cell surface expression of FcεRIα chain was detected by using a mouse IgG1 anti-human FcεRIα chain mAb (22E7; generously provided by J Kochan, Roche Pharmaceuticals¹³) and was compared with labeling with an identical concentration of irrelevant mouse IgG1 (Coulter, Hialeah, Fla). The 22E7 antibody has been shown to recognize an epitope that is unaffected by FcεRIα occupancy.¹³ Aliquots of cells were labeled in PAG containing 0.2% human serum albumin with 1 mg/mL human IgG to minimize non-specific binding to FcγR.¹² Each of the mAbs were used at concentrations predetermined to be optimal for labeling. Titration of the MHM6 antibody was performed on preparations of lymphocytes or Ramos cells (kindly provided by Dr Farhad Imani, JHU-AAC). Dilutions of 1:100 were used for these experiments. Binding of mAbs was detected by using saturating concentrations of R-phycoerythrin-conjugated polyclonal goat anti-mouse IgG (Tago, Burlingame, Calif). An EPICS Profile flow cytometer (Coulter, Inc) was used to analyze fluorescent signals after excitation at 488 nm. "Bitmap" gates, which are intermediate between the forward and side scatter characteristics of lymphocytes and monocytes, were used to select for a population of cells that were predominantly basophils. Because the cells were already enriched in basophils, these bitmaps can select a population of cells that is generally greater than 80% basophils, with the primary contaminants being lymphocytes. Data are expressed as the mean fluorescence in labeled cells minus the mean fluorescence of IgG1 controls. Day-to-day variability in the sensitivity of the flow cytometer was corrected by noting or adjusting the photomultiplier tube voltage to generate the same signal for a set of standard calibration beads (Immunochek, Coulter). For the analysis of MHM6 binding, the basophil preparations were also incubated with rabbit FITC-labeled anti-IgE antibody, and the bitmap selected cells also gated on green fluorescence to focus on cells that were exclusively basophils. The photomultiplier tube voltages for red fluorescence (phycoerythrin staining) were set very high so that a full distribution of IgG1 control antibody staining could be appreciated. This high setting should also enhance the likelihood of detecting low levels of binding (eg, with the settings used for basophils, Ramos cells were found to give off-scale distributions), although these settings also introduced considerable noise.

In previously published studies the flow cytometric measurements were calibrated, examining the fluorescence staining of 6 donors' basophils that spanned a moderate range of staining intensities (7 to 120 fluorescent units or 8000 to 140,000 FcεRI per basophil) and simultaneously assessing receptor or IgE density by the acetate elution method. 22E7 staining (ordinate) compared with total FcεRI density by acetate elution (after sensitizing with PS myeloma IgE as described above) was linear, with a slope of 0.00084 (ie, a fluorescence measurement of 100 represents approximately 120,000 receptors) and an *r* value of 0.963.

Statistical analysis

The Student *t* test was used for most statistical comparisons, whereas other comparisons were done with a nonparametric Wilcoxon signed-rank statistic or ANOVA. If errors or error bars are shown, they represent SEM unless otherwise indicated.

RESULTS

A role for galectin-3 (εBP) was excluded by performing upregulation experiments in the presence or absence

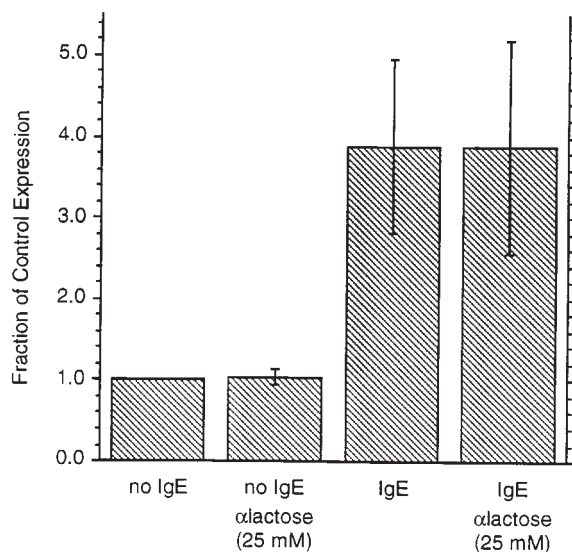


FIG 1. Effect of α -lactose on the IgE-mediated upregulation of Fc ϵ RI on human basophils. Enriched basophils (average of 46% purity) were cultured for 7 days in the presence or absence of IgE antibody at 1 μ g/mL and in the presence or absence of α -lactose at 25 mmol/L ($n = 3$). Cell surface Fc ϵ RI was detected by flow cytometry by using mAb 22E7. The control mean fluorescent intensity was 28 ± 5 units.

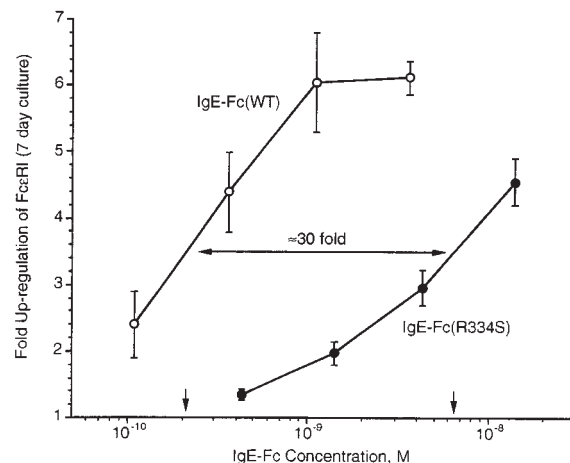


FIG 2. Upregulation of Fc ϵ RI on basophils cultured in the presence or absence of IgE-Fc(WT) or IgE-Fc(R334S) ($n = 4$). Basophils were cultured with several concentrations of either fragment, noted on the abscissa, for 7 days. Upregulation is expressed as the ratio of Fc ϵ RI expression on day 7 to its expression on day 1 as detected by flow cytometry with 22E7. The starting average mean fluorescent intensity for 22E7 staining was 25 ± 3 units, which is similar to the starting Fc ϵ RI densities for the previously published experiments with PS myeloma IgE (see "Methods" section).³

TABLE I. Previously published binding constants for recombinant IgE-Fc binding to Fc ϵ RI or Fc ϵ RII

Type of assay	Receptor	Constant	IgE-Fc(WT)	IgE-Fc(R334S)
Cell binding	Fc ϵ RI	k_f	$9.9 \pm 1.1 \times 10^5$	$7.5 \pm 2.6 \times 10^5$
		k_r	$1.7 \pm 0.2 \times 10^{-5}$	$2.0 \pm 0.1 \times 10^{-4}$
		K_a	5.8×10^{10}	3.7×10^9
SPR	Fc ϵ RI	k_{f1}	$1.2 \pm 0.8 \times 10^6$	$3.9 \pm 3.2 \times 10^5$
		k_{f2}	$2.5 \pm 1.8 \times 10^5$	$9.3 \pm 3.8 \times 10^4$
		k_{r1}	$1.0 \pm 0.1 \times 10^{-2}$	$4.6 \pm 0.4 \times 10^{-3}$
		k_{r2}	$2.0 \pm 0.1 \times 10^{-4}$	$9.9 \pm 0.4 \times 10^{-3}$
		K_{a1}	1.1×10^8	8.6×10^6
		K_{a2}	1.2×10^9	1.0×10^7
		K_a	$3.2 \pm 2.4 \times 10^7$	$2.4 \pm 3.9 \times 10^7$
Cell binding	Fc ϵ RII	K_a		

of α -lactose. Fig 1 demonstrates the absence of an effect of 25 mmol/L lactose on upregulation, a concentration which should completely inhibit the interaction of IgE oligosaccharide chains with galectin-3.^{14,15}

Interaction with Fc ϵ RII was examined by 4 different approaches. First, basophils were cultured with 2 fragments of the IgE molecule generated by recombinant techniques. Both fragments contain the epsilon second through fourth constant regions (C ϵ 2 to C ϵ 4) as a disulphide-linked dimer, which is referred to as the IgE-Fc, but they differ in that the IgE-Fc(R334S) has the arginine at residue 334 in the wild-type sequence replaced by serine.⁸ The Fc ϵ RI binding characteristics, as determined in cell-binding and surface plasmon resonance (SPR) assays, have been previously published,⁸ and the results are summarized in Table I. The important distinguishing characteristic is that IgE-Fc(R334S) has a significantly lower affinity for Fc ϵ RI than IgE-Fc(WT) while retaining its

native affinity for Fc ϵ RII. The relative decrease in binding affinity of IgE-Fc(R334S) compared with IgE-Fc(WT) differed between the cell-binding assay (a factor of 15) and the SPR assay, which displayed biphasic binding kinetics (a factor of 12 for K_{a1} and a factor of 120 for K_{a2}) but can be regarded as a difference greater than 10-fold in all assays. The previously published measurements of K_a for Fc ϵ RII in a cell binding assay (Table I) have been confirmed by SPR and show that the mutation had no effect on binding to this receptor. Fig 2 shows the concentration dependence of Fc ϵ RI upregulation on basophils cultured with either IgE-Fc(WT) or IgE-Fc(R334S). These data show that there is an approximately 30-fold difference in EC50 for upregulation of Fc ϵ RI, as would be expected if IgE were interacting with Fc ϵ RI α .

Whereas the R334S mutation in IgE-Fc(R334S) might not have altered the binding affinity for Fc ϵ RII, this mutation might alter any functional consequence of this bind-

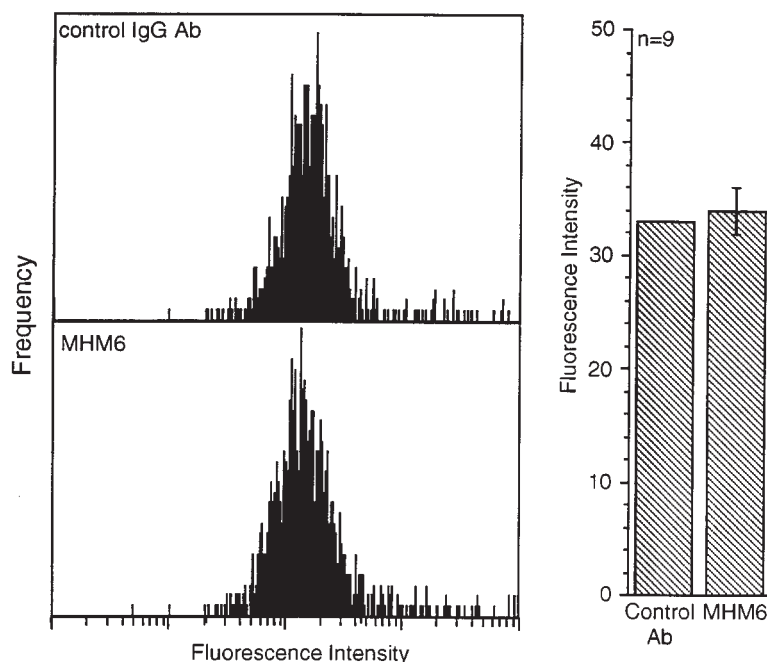


FIG 3. Lack of FcεRII expression on the basophil cell surface as detected by flow cytometry with MHM6. Enriched basophil preparations (average purity of 35% ± 10%) were examined by flow cytometry by using a 1:100 dilution of MHM6. The *left panel* shows the results for one experiment. The *right panel* shows the average of results from 9 different preparations of basophils, with the data expressed as the ratio of mean fluorescence for cells incubated with MHM6 versus control antibody (irrelevant mouse IgG at 50 µg/mL).

ing. Therefore additional studies were carried out to test for FcεRII expression on human basophils. Basophils are not known to express CD23, as tested by flow cytometry. However, flow cytometry is not a sensitive technique when used on basophils obtained by dextran sedimentation or single step density gradients (purities <2%); expression levels less than 5000 molecules per cell would not be detected.¹² However, by using more enriched basophil preparations (>20%), flow cytometry is somewhat more sensitive and able to detect IgE densities at around 1000 per cell. Therefore partially enriched basophil preparations were tested for FcεRII expression by using MHM6 antibody. As demonstrated in Fig 3, there was no difference in the flow cytometric distributions when control mouse IgG or MHM6 were used as the primary antibodies.

Fig 4 demonstrates that FcεRII could not be detected in basophil mRNA preparations examined by RT-PCR. Basophils were purified to near homogeneity by positive selection, and their mRNA was extracted. The mRNA from contaminating cells remaining after positive selection was also examined after extraction. Fig 4 shows 3 preparations of purified basophils compared with the contaminating cells. The right halves of the gels show the results for serially diluted samples of the contaminating cell mRNA. The left halves of the gels show results for serially diluted samples from basophils. In 2 of the lower purity basophil preparations (95% and 94%), a close inspection reveals a faint band in the lane representing undiluted samples, the density of which is consistent

with the expected density of a band derived from the contaminating cells diluted to this extent. In data not shown, the house-keeping mRNA for hypoxanthine phosphoribosyl transferase was readily detected in both samples obtained from basophils and contaminating cells by using the appropriate primers.

MHM6 antibody has been found to induce biologic responses in systems in which FcεRII is known to play a role,¹⁶⁻²⁰ and it is known to be competitive with IgE.²¹ Therefore we examined whether this antibody could influence the upregulation of FcεRI on basophils in a 7-day culture with or without IgE antibody. As shown in Fig 5, MHM6 caused no significant changes in the expression of FcεRIα at any of the concentrations tested; no effect on FcεRIα expression in the absence or presence of IgE was observed. Parenthetically, although the absolute concentration of the commercial MHM6 antibody is not known, as noted in the flow cytometric studies (see "Methods" section), titration of the antibody showed that a 1:100 to 1:300 concentration provided optimal labeling of Ramos cells.

DISCUSSION

Collectively, these data indicate that there is no interaction of IgE with either galectin-3 or FcεRII. There are indications that galectin-3 is expressed in human basophils,²² although current thinking suggests that if some of this galectin-3 is found outside the cell, it is probably bound to cell surface carbohydrates by its

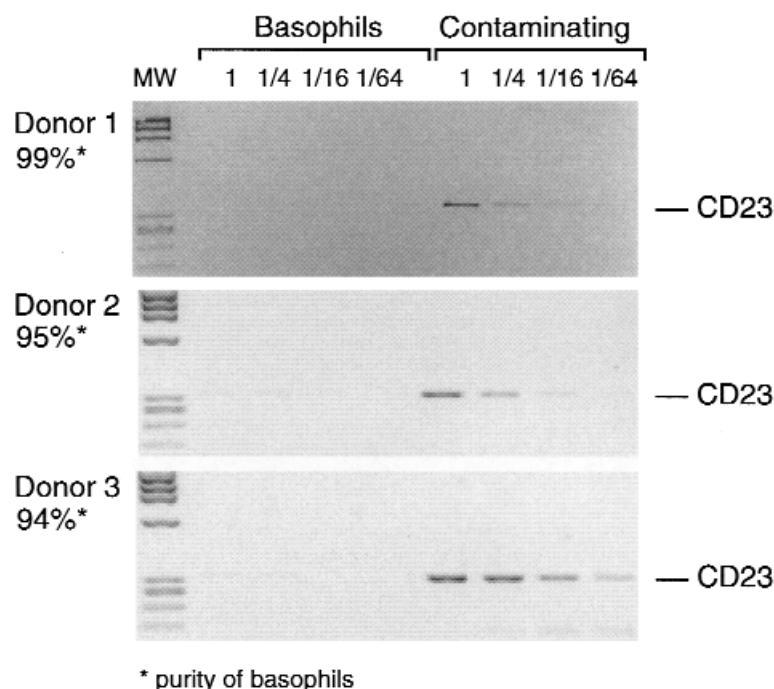


FIG 4. RT-PCR of mRNA derived from purified basophils or contaminating cells from the same preparations (see "Methods" section). The *left side* of the figure shows results for basophils, with 3 dilutions of the original sample, and the *right side* of the figure shows the results for the contaminating cells. The purity of the basophil preparations is noted on the left ($n = 3$).

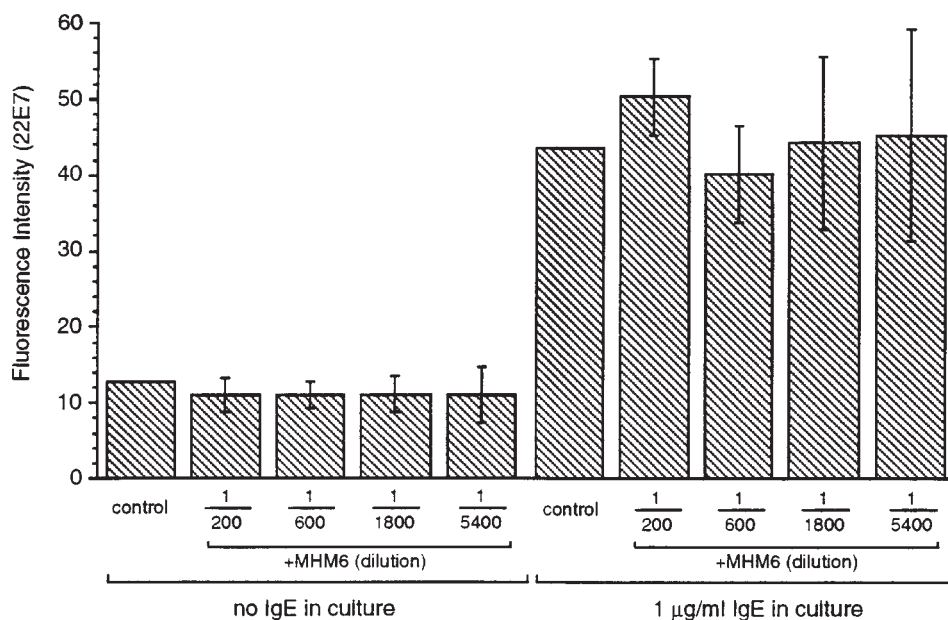


FIG 5. Effect of treating basophils with MHM6 antibody. Basophils were cultured with or without IgE antibody at 1 µg/mL in the presence or absence of several dilutions of MHM6 antibody. After 7 days of culture, the cells were harvested and analyzed by flow cytometry with 22E7. Data are expressed in arbitrary fluorescence units ($n = 3$).

lectin-binding domain. If so, it is more difficult to envision how it could also then interact with extracellular IgE. Nevertheless, we sought and found a more definitive test of galectin-3 involvement in the upregulation of

FcεRI. The lack of inhibition by α-lactose excludes any such involvement because α-lactose at the concentrations used prevents binding to IgE.^{14,15,23}

Four methods were used to test the likelihood that IgE

interacts with FcεRII on basophils. No evidence could be found for basophils expressing even low levels of FcεRII. Although the RT-PCR procedure detected a faint band of mRNA from purified basophil preparations, the intensity of the band from undiluted samples was consistent with it coming from the small number of contaminating cells in these basophil preparations. Indeed, the band was somewhat fainter than if 5% to 10% of contaminating cells were contributing to its presence, based on the dilutional RT-PCR data shown in the right half of Fig 4. Flow cytometric data and functional data obtained by using MHM6 supported this conclusion. Finally, the difference in the ability of IgE-Fc(WT) and IgE-Fc(R334S) to upregulate FcεRI expression was consistent with IgE interacting with FcεRIα because the 30-fold difference agrees with the observed difference in affinity of IgE-Fc R334S and wild-type for FcεRIα.

The conclusion that IgE antibody interacts with FcεRIα leads back to the original problem: the concentration-dependence for upregulation appears shifted to concentrations of IgE antibody that are higher than expected from a simple assumption of equilibrium binding between IgE and FcεRIα. Expectations for these results were based on binding constants for IgE to basophil FcεRI, which we have determined in recent *in vitro* studies.³ However, these values are not reflective of binding constant values found in the literature. The dissociation constant determined in our recent studies of IgE dissociation from basophils in culture³ was found to be low (of the order of 1×10^{-6} sec⁻¹) compared with other published studies.²⁴⁻²⁷ In addition, our calculations of the forward binding constants have been consistently lower than other published values.^{3,24-28} The source of the discrepancies is not currently known. Therefore a strong conclusion regarding an expected EC₅₀ for upregulation of FcεRI by IgE cannot be made; that is, the value of 250 ng/mL may very well be accounted for by a particular choice of published rate constants for human IgE antibody and human FcεRI. However, it is useful to note that we are currently exploring a model of this upregulation that predicts that the relatively slow binding of IgE to the receptor (regardless of which forward binding constants one accepts as correct) could cause an apparent requirement for higher concentrations of IgE to induce upregulation. The central tenets of the model are that the receptor is constitutively synthesized and that unoccupied receptor is susceptible to loss from the cell surface. This is akin to the currently accepted model of FcεRII expression in which a metalloprotease has been identified and shown to mediate the loss of unoccupied FcεRII from the cell surface.²⁹ Relatively specific inhibitors of this enzyme can be shown to upregulate expression of FcεRII. Future experiments will be needed to verify the basic tenets of a similar model for FcεRI regulation, namely that synthesis is constitutive rather than induced and that only unoccupied receptor is lost from the cell surface. The mechanism of this loss also needs exploration, and future experiments need to determine whether loss is a saturable process. In summary, our results indicate that IgE interacts with

FcεRI to upregulate its expression, but the precise explanation for the *in vitro* results concerning concentration dependence awaits further study.

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