Identification of the Low Affinity Receptor for Immunoglobulin E on Mouse Mast Cells and Macrophages as FcγRII and FcγRIII

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Summary

In addition to their well characterized high affinity immunoglobulin E (IgE) receptors (FcεRI) mast cells have long been suspected to express undefined Fc receptors capable of binding IgE with low affinity. In this paper, we show that FcγRII and FcγRIII, but not Mac-2, on mouse mast cells and macrophages bind IgE-immune complexes. This binding is efficiently competed by 2.4G2, a monoclonal antibody against the extracellular homologous region of both FcγRII and FcγRIII. Furthermore, IgE-immune complexes bind specifically to FcγRII or FcγRIII transfected into COS-7 cells. The association constants of IgE binding estimated from competition experiments are about $3.1 \times 10^{7} \text{M}^{-1}$ for FcγRII, and $4.8 \times 10^{8} \text{M}^{-1}$ for FcγRIII. Engagement of FcγRII and FcγRIII with IgE-immune complexes (after blocking access to FcεRI) or with IgG-immune complexes triggers C57.1 mouse mast cells to release serotonin. This release is inhibited by 2.4G2, and at maximum, reaches 30–40% of the intracellular content, about half of the maximal release (60–80%) obtained after FcεRI engagement. These data demonstrate that mouse FcγRII and FcγRIII are not isotype specific, and that the binding of IgE-immune complexes to these receptors induces cell activation.

The molecular cloning of IgE-receptors has helped to classify these receptors into two very different families of proteins. The high affinity IgE receptor (FcεRI) is a non-covalent tetrameric complex of an α chain, a β chain, and two disulfide-linked γ chains (1, 2). The high affinity binding site of FcεRI is on the α chain, a structure homologous to other Fc receptors and a member of the Ig superfamily. By contrast, low affinity IgE-binding structures are part of the lectin-like receptor family. One of these, FcγRII or CD23, belongs to the calcium-dependent class of animal lectins (3, 4) and the other, Mac-2, also called CBP35 and cBP (5, 6), is of the thiol-dependent class of animal lectins.

The tissue distribution and function of these receptors are also very different. The high affinity IgE receptor is found on the surface of mast cells, basophils (1), and Langerhans cells (7). Crosslinking of this receptor via IgE and antigen results in release of the mediators that cause the symptoms of allergic diseases. CD23 in humans is expressed on a wide variety of hemopoietic cells, among them B and T cells, but in mice, the expression of CD23 appears to be restricted to B cells (3). The role of CD23 is not yet completely understood, but it has been proposed to regulate IgE synthesis, to endocytose IgE-immune complexes in B cells, and to serve as an adhesion molecule. Mac-2 was originally described as a macrophage cell surface marker (8), but it is also expressed in mast cells and fibroblasts (5, 6). In spite of the lack of a typical transmembrane domain, a proportion of Mac-2 molecules in these cells are attached to the cell surface. The mechanism of membrane anchoring may involve binding to cell surface glycoconjugates, because incubation of cells with lactose disrupts the attachment (9). In solution, Mac-2 binds IgE, but this binding is carbohydrate dependent and has not been demonstrated for surface-expressed molecules, so its physiological relevance is at best speculative.

More than a decade ago, two molecules with different molecular weight were isolated by affinity chromatography on IgE-Sepharose from extracts of rat basophilic leukemia cells and rat mast cells (10, 11). Then rat basophilic leukemia cells were shown to express IgG receptors also capable of binding IgE (12). In fact, these receptors seemed to have a greater affinity for rat IgE than for rat IgG (12, 13). Other reports
indicated that rat IgE could inhibit the binding of IgG to rat lymphocytes (14) or to rat macrophages (15). However, this inhibition was characterized as unidirectional and was thought to be unique for rat cells (16).

Our original goal was to characterize the putative low affinity IgE/IgG receptor on rodent mast cells by molecular methods. Despite the restriction of earlier studies to the rat system, we decided to use mouse mast cells because antibodies are available against the various structures known to bind IgE or IgG. We found that the two low affinity receptors for IgG (FcγRII/III) known to be expressed on the surface of mouse mast cells (17, 18) and macrophages (19), are also low affinity receptors for IgE.

Materials and Methods

Immunglobulins. Igs were purchased from the following sources: rabbit IgG and goat anti-rabbit IgG F(ab')2-FITC from Organon Teknika (West Chester, PA); mouse monoclonal anti-Dansyl IgE from Pharmingen (San Diego, CA); mouse anti-rat IgG (Fc-specific) from Jackson Immunoresearch Laboratories (West Grove, PA); and mouse monoclonal IgG2b from Southern Biotechnology Associates (Birmingham, AL). The FITC-labeled anti-mouse FcεRII antibody B3B4 (20) was a gift of Dr. D. H. Conrad (Medical College of Virginia, Richmond, VA). The anti-rat Mac-2 antiserum, which crossreacts with the mouse protein, was a gift of Dr. F. T. Liu (Scripps Clinic, La Jolla, CA). Mouse monoclonal anti-DNP IgE (H1 DNP-e.26.82) (21) was purified from ascites as previously described (22) or it was purified by ion exchange chromatography on DEAE-Trisacryl (IBF Biotechnics, Columbia, MD) from culture supernatant of the hybridoma grown in Protein-Free Hybridoma Medium (Gibco BRL, Grand Island, NY) in a Mini Flo-Path bioreactor (Amicon, Beverly, MA). Mouse anti-DNP IgE was labeled with FITC by standard procedures (23). Rabbit anti-mouse IgE (22) and mouse anti-DNP IgG (24) were prepared as described. The rat mAb 2.4G2 (anti-mouse FcγRII and -III) (25) was purified from tissue culture supernatant by Protein-G affinity chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ). To make oligomers, 100 mg/ml rabbit IgG or mouse monoclonal IgE were chemically crosslinked using dimethyl suberimidate (Pierce Chemical Co., Rockford, IL) at a crosslinker/protein molar ratio of 10:1 at 30°C for 3 h. Rabbit IgG dimers were obtained as a consequence of inhibition. Saturation binding assays with increasing amounts of radiolabeled IgG dimers were performed by using 10 μg/ml radiolabeled rabbit IgG dimer (corresponding to 6.7 x 10^-8 M IgG molecules) and varying concentrations of unlabeled monomeric mouse monoclonal IgG2b, rabbit IgG, or mouse monoclonal anti-DNP IgE (molar ratios of monomer/dimer 0.6:1–150:1, based on IgG molecules). The cell density was 2 x 10^5/ml, the buffer was PBS containing 0.2% BSA, and the incubation was at 4°C for 2 h. Cells were centrifuged through phthalate oil (6 volume parts dibutyl phthalate, 4 volume parts bis(ethylhexyl)phthalate) to separate bound from unbound ligand. Percent cell-bound radioactivity was calculated by dividing cpm in the cell pellet by total cpm. Nonspecific binding was determined with transfectants expressing γ alone and subtracted from the percent binding value to give percent specific binding. The degree of inhibition by various monoclonic Iggs was calculated by setting the specific binding in the absence of inhibitors equal to 0% inhibition. 100% inhibition was defined as absence of specific binding as a consequence of inhibition. Saturation binding assays with increasing amounts of radiolabeled IgG dimers were performed before the competition binding experiments to verify expression of the receptors and to determine optimal conditions.

Serotonin Release. COS-7 cells were cultured with 1 μCi/ml of 5-[1,2-3H(N)]-hydroxytryptamine binoxalate ([3H]-serotonin) (DuPont Co., Wilmington, DE) for 16 h. All procedures after labeling were performed in buffer containing 25 mM Na2Pipes (pH 7.1), 100 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 1 mM CaCl2, 5.6 mM d-glucose, and 0.1% BSA (30). Cells were incubated with monoclonic IgG, 2.4G2, or both at 37°C for 1 h and washed. The cells were challenged by rabbit anti-mouse IgE or multivalent antigen (DNP-albumin) for triggering through FcεRI.
For triggering through FcγR4, receptor-bound 2.4G2 was cross-linked with anti-rat IgG (Fc-specific). When IgE and/or 2.4G2 were used as inhibitors, these inhibitors were resupplemented after saturation and washing, and the cells were challenged by IgG- or IgE-immune complexes. The reaction was stopped after 30 min by placing the tubes on ice and sedimenting the cells for 3 min at 4,000 rpm in a microfuge. Radioactivity was determined separately in the pellet and the supernatant. Percent release was calculated by dividing counts per minute in the supernatant by total counts/min.

**Results and Discussion**

*Cell Surface Receptors on C57.1.* Our first goal was to establish unequivocally whether IgE binds to a second site on mast cells other than FcεRI. This, and the identification of the binding site, requires antibodies against cell surface proteins known to bind IgG or IgE. For murine cells, several useful antibodies are available, such as 2.4G2, which binds to the extracellular domains of the highly homologous FcγRII and -III receptors and blocks the binding of IgG (25), or B3B4, which recognizes mouse FcεRII (20). Comparable anti-rat reagents are not known. Therefore, we analyzed the mouse mast cell line C57.1 rather than the rat cell line RBL, which had been studied previously. We first characterized relevant cell surface proteins on this cell line (Table 1). C57.1 is FcεRI⁺, FcεRII⁺, FcγRII/III⁺, and Mac-2⁺. Mac-2 can be removed from the cell surface by lactose, as has been described for RBL (9). The cell line responds to engagement of FcεRI in the same way as RBL (30a).

**2.4G2-inhibitable Binding of IgE-immune Complexes to Mast Cells.** To investigate binding of IgGs to their surface, C57.1 cells were incubated with monomeric antibody or with immune complexes and analyzed by flow cytometry. Monomeric IgE strongly binds to C57.1 (Fig. 1 A). This binding is completely inhibited by excess unlabeled monomeric IgE (50 μg/ml) (Fig. 1 B). There is no further inhibition when both excess IgE and 2.4G2 are employed (Fig. 1 C). This is consistent with the presence of a single class of high affinity binding sites for IgE on these cells, i.e., FcεRI receptors. IgE-immune complexes also strongly bind to C57.1 (Fig. 1 D). In contrast to the binding of monomeric IgE, the binding of immune complexes is not completely inhibited by excess monomeric IgE (Fig. 1 E), indicating that IgE-immune complexes do indeed bind to a second site in addition to FcεRI. However, the binding of immune complexes is completely blocked by the combination of excess monomeric IgE and FcγRII/III-specific 2.4G2 (Fig. 1 F). This demonstrates that the staining not inhibited by monomeric IgE is due to a cell surface structure recognized by 2.4G2. Further binding, which could be attributed to other structures such as Mac-2, is not detected. These experiments were performed with monoclonal anti-DNP IgE purified from serum-free bioreactor supernatant to ensure there is no contamination with other Ig classes and repeated with a preparation of the same antibody from ascites. No difference between the two preparations was detected. Oligomerized IgG binds to C57.1 (Fig. 1 G). It is not surprising that its binding is inhibited by 2.4G2 (Fig. 1 H), and also by IgE-immune complexes (Fig. 1 I). Thus, on C57.1, IgE and IgG bind to the same low affinity binding site, which reacts with 2.4G2 and is therefore likely an FcγRII/III receptor. These findings confirm those of Segal et al. (12), who described mutually inhibitable low affinity binding of IgE and IgG to the RBL mast cell line, and extend them by pointing towards FcγRII/III as the responsible entity.

**Binding of IgE-immune Complexes to Macrophage Cell Lines and to a FcεRI-deficient Mast Cell Line.** We investigated whether this low affinity, 2.4G2-reactive binding site was also present on cell types that express FcγRII/III receptors but not FcεRI. J774, a mouse monocyte-macrophage cell line (31), does not bind monomeric IgE (Fig. 2 A), but does bind IgE-immune complexes to a site that is blocked by 2.4G2 (Fig. 2 B). In the same way, 2.4G2 blocks binding of oligomeric IgG (Fig. 2 C). J774 stains with an anti-rat Mac-2 antiserum, which crossreacts with the mouse protein. The staining is abolished by prior incubation of the cells with 25 mM lactose, indicating that Mac-2 binds to cell surface conjugates on J774. However, the binding of IgE-immune complexes to J774 is not affected by this treatment (data not shown). Together with the lack of a third binding site for IgE on C57.1, this observation excludes Mac-2 from the ranks of functional cell surface IgE receptors. The ability of cell surface Mac-2 to bind IgE has also been questioned on theoretical grounds (9), because the protein, which does not have a transmembrane region, apparently attaches to the cell surface by binding a membrane glycoconjugate and cannot bind IgE simultaneously. Essentially the same results were obtained with P388D1, (macrophage-like) (32), and the mastocytoma-like P815 (33) (data not shown), which express FcγRII and -III but not FcεRI (18, 34). This is further evidence that the low affinity binding site for IgE on mast cells and macrophages is an FcγRII/III receptor.

**Binding of IgE-immune Complexes to Transfected Low Affinity Fcy Receptors.** So far, the identification of the low affinity binding site for IgE as FcγRII/III rests entirely on its reactivity with 2.4G2. However, if there exists an IgE/IgG receptor unrelated to FcγRII/III which either crossreacts with 2.4G2 or binds this antibody via its Fc part, it would simulate the results we have observed. The problem of Fc-binding can be

| Surface antigen | Antibody       | Staining |
|-----------------|----------------|----------|
| FcεRI           | IgE (Monomeric)| ++ +     |
| FcεRII          | B3B4           | -        |
| FcγRII/III      | 2.4G2          | + +      |
| Mac-2           | Antiserum      | +        |

Cells were stained with the antibodies shown above, and analyzed by flow cytometry. Anti-Mac-2 staining could be abolished by incubation of C57.1 with 25 mM lactose in flow cytometry buffer.
circumvented with F(ab')2 fragments of 2.4G2, but even low levels of contaminating whole antibody molecules can interfere (35). Furthermore, the question of crossreactivity is not addressed by this approach. Therefore, we decided to positively identify the receptors by transiently transfecting the genes for the single chain of mouse FcγRII and the two chains (α and γ) required for surface expression of mouse FcγRIII (34) into COS-7 cells. Control cells which were transfected with the γ subunit alone bound neither oligomeric IgG nor IgE-immune complexes (Fig. 3 E and F). Cells which transiently expressed FcγRII bound IgE-immune complexes (Fig. 3 B), as well as oligomeric IgG (Fig. 3 A). In both cases the binding was completely inhibited by 2.4G2. A similar staining was observed with cells expressing FcγRIII (Fig. 3, C and D). This result shows that the so-called IgG receptors FcγRII and -III are not isotype specific; they bind IgE and are thus functional Fce receptors.

Affinity of FcγRII and FcγRIII for IgE. Because the binding of monomeric antibody to FcγRII/III is too weak to measure, our experiments have relied on the increased avidity of oligomers and complexes of antibodies to visualize binding to the Fcγ receptors. The avidity of such a complex depends on its size, therefore it cannot be used in measurements of affinity constants. In this situation, the relative affinities of different antibodies can be determined by allowing the monomeric antibody to compete with rabbit IgG dimers, whose binding is sufficiently strong to be measurable.

COS-7 cells, which transiently expressed either FcγRII or FcγRIII, were incubated with radiolabeled IgG dimers and either monomeric mouse IgG2b, rabbit IgG, or mouse IgE.
Table 2. Serotonin Release from C57.1 Mast Cells through FcγRII/III or FcεRI

| Preincubation          | Challenge          | Percent serotonin release |
|------------------------|--------------------|---------------------------|
| (-)                    | (-)                | 0.9 ± 0.2                 |
| 2.4G2                  | (-)                | 1.0 ± 0.1                 |
| (-)                    | Anti-rat IgG       | 1.2 ± 0.1                 |
| 2.4G2                  | Anti-rat IgG       | 33.2 ± 0.2                |
| (-) Mouse IgG immune   | Mouse IgG immune   | 25.2 ± 1.0                |
| complex                | complex            | 25.2 ± 1.0                |
| Anti-Dansyl IgE (-)    | Anti-Dansyl IgE (+) | 62.6 ± 1.3               |
| Anti-mouse IgE         | Anti-mouse IgE     | 1.1 ± 0.2                 |

Cells were preincubated with buffer alone, or with 2.4G2 (25 μg/ml), or with monomeric mouse anti-Dansyl IgE (25 μg/ml) and washed, and then challenged by either mouse anti-DNP IgG immune complexes (5 μg/ml IgG), or anti-rat IgG (5 μg/ml), or rabbit anti-mouse IgE (5 μg/ml). Values are means ± SD of triplicate determinations.

IgE. The molar ratio of monomers/dimers (based on IgG molecules) varied between 0.6 and 150. IgE was less efficient than either rabbit IgG or mouse IgG2b at competing with dimers for binding to FcγRII (Fig. 4, left), and as efficient as rabbit IgG, but less so than mouse IgG2b, at competing for FcγRIII (Fig. 4, right). The approximate association constants for the different monomeric Ig classes are calculated from the reciprocals of the concentration required for half-maximal inhibition of dimer binding. From the inhibition values obtained, we estimate the association constant between FcγRII and mouse IgG2b, rabbit IgG, and mouse IgE as 7.7

Figure 3. Binding of IgE-immune complexes and IgG oligomers to COS-7 transfectants. COS-7 were transfected with the genes for FcγRII (A and B), the α and γ subunits of FcγRIII (C and D), or the γ subunit alone (E and F). Cells were incubated with rabbit IgG oligomers followed by FITC-conjugated goat anti-rabbit IgG F(ab')2 (A, C, and E) or with FITC-conjugated IgE-immune complexes (B, D, and F). The binding was either not inhibited (buffer control: solid lines) or inhibited with 2.4G2 (dashed lines). Background staining (dotted lines) was assessed using no antibody or only the secondary antibody.

Figure 4. Displacement of IgG dimers from FcγRII and FcγRIII by IgG and IgE. COS-7 were transfected either with FcγRII (left) or FcγRIII (α and γ subunits, right). They were incubated with 125I-labeled rabbit IgG dimers (10 μg/ml, corresponding to 6.7 × 10^-8 M IgG molecules) and increasing amounts of monomeric mouse IgG2b (●), monomeric rabbit IgG (○) or monomeric mouse IgE (▲). Inhibitors and labeled IgG dimers were premixed before the addition of cells. The degree of inhibition was calculated by setting percent specific binding of IgG dimers without inhibitors equal to 0% inhibition. 100% inhibition was defined as absence of specific binding as a result of competition. Values are means ± SEM. Error bars are omitted where they fall within the size of the data symbol.

Figure 5. Serotonin release from C57.1 mast cells triggered by IgE-immune complexes or chemically crosslinked IgE. Cells loaded with [3H]serotonin were triggered by IgE-immune complexes (left) or chemically crosslinked IgE (right). Triggering was either not inhibited (○), or inhibited by 2.4G2 alone (●), monomeric IgE alone (▲), or monomeric IgE and 2.4G2 (□). Values are means ± SD calculated from triplicate samples. Error bars are omitted where they fall within the size of the data symbol.
We have established that C57.1 can be activated through FcεRII, although in these earlier studies differentiation between the subclasses of Fcγ receptors was not possible.

We disagree with Segal et al. (12) on the relative affinities of IgG and IgE for the low affinity receptor. They estimated the affinity of IgE for the low affinity receptor on mast cells to be almost four times as high as that of rabbit IgG, whereas according to our experiments, IgG has the higher affinity. Here it is important to remember that Segal et al. (12) used mast cells for their experiments. Incomplete blocking of FceRI stimulation is much weaker than that to FcεRII/III. IgE can thus trigger mast cells through both FcεRI and FcεRII/III.

To summarize, we have unequivocally shown that: mouse IgE binds with low affinity to a site on mast cells other than FcεRI; that this site corresponds to the mouse IgG receptor FcεRII/III also found on cell types such as macrophages; that the affinity of IgE for the receptors is comparable with that of IgG; and that the binding is functional, because it contributes to mast cell activation. Our goal is now to investigate whether these findings are also relevant to the human system. In that case, our findings would have dramatic implications for explaining the role of IgE in parasitic diseases (39). It might be argued that in view of the serum levels of IgE, binding of IgE to FcεRII or FcεRIII will not occur under physiological conditions, and in any case will be competed by much higher levels of IgG. However, the affinity of these receptors is comparably low for IgG. In fact, normal macrophages are capable of binding IgG-immune complexes because most of the receptors are empty in physiological conditions. It is therefore probable that IgG- as well as IgE-immune complexes will bind to these receptors and activate relevant defence systems, particularly in situ, where the concentration of immune complexes relative to monomeric ligand might be much higher than in the serum.
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