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THE BINDING OF IgE TO MURINE FcεRII IS CALCIUM-DEPENDENT BUT NOT INHIBITED BY CARBOHYDRATE[1]

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Despite extensive study, little is known about the functions of the moderate affinity IgE receptors (FcεRII) on B cells. Recent cDNA and genomic cloning studies have demonstrated that, in contrast to other FcR, FcεRII is not a member of the Ig gene superfamily. Moreover, it uniquely expresses a region that is highly homologous with a membrane-associated, calcium-dependent binding lectin, the asialoglycoprotein receptor. We now report that the interaction between IgE and the FcεRII of murine B cells and macrophages requires calcium. Furthermore, as might be expected of asialoglycoprotein lectins, this binding was pH-dependent and resulted in ligand internalization. However, although ^{125}I-FcεRII bound in a calcium-dependent manner to monosaccharide-agarose beads, high concentrations of mono- and disaccharides did not inhibit the interaction between either ^{125}I-IgE and intact B cells or ^{125}I-FcεRII (from surface-labeled B cells) and IgE-Sepharose. These results suggest that although murine FcεRII is a lectin, it is not strictly dependent upon IgE oligosaccharides for its binding to IgE.

IgE receptors have been distinguished functionally, structurally, and by their affinity for IgE. The high affinity FcεRI[3] is located on mast cells (1) and basophils (2) and plays a key role in the degranulation of these cells during allergic reactions. The low-to-moderate affinity FcεRII is found on monocytes (3), eosinophils (4), B lymphocytes (5), and T lymphocytes (6). FcεRII on monocytes and eosinophils functions in combating parasitic infections (7, 8) but its role on B lymphocytes remains somewhat enigmatic. Much interest has focused on IgE self-regulation via FcεRII (9–17) and, more recently, FcεRII has been associated with the differentiation of human B cells (18) and the regulation of Ia Ag on mouse B lymphocytes (19).

The recent cloning of human B cell FcεRII revealed some unusual characteristics. Previously reported FcR nucleotide sequences, including Fcγ2b/γ1R (20, 21), poly IgR (22), and FcεRIα (23), show that they have common Ig domains and belong to the Ig gene superfamily. In contrast, FcεRII is not a member of the Ig gene superfamily and, in fact, its predicted membrane orientation places the amino terminus in the cytoplasm (24). Additionally, a segment of the putative extracellular (carboxy-terminal) portion of the receptor is highly homologous with several animal lectins; i.e., chicken, human, rat hepatic lectins (or asialoglycoprotein receptors (25–27)), and rat mannose binding protein (28). This suggests the possibility that FcεRII might be binding to IgE via a carbohydrate determinant. Indeed, IgE is the most heavily glycosylated of the Ig, having a carbohydrate content of 13% vs 3% for IgG. Moreover, the homologs of another, but distinct, IgE-binding protein appear to bind IgE in a galactose-specific manner (29). Nonetheless, previous attempts to characterize the carbohydrate dependence of the IgE-FcεRII binding event by using anti-IgE mAb (30) or synthetic IgE peptides (31) have yielded conflicting conclusions.

We now report that the binding of IgE to FcεRII of murine B cells and macrophages is entirely dependent upon the presence of calcium. Moreover, as with C-type (calcium-dependent binding) lectins (32), FcεRII-IgE binding is pH-dependent and associated with internalization of the bound protein. Additionally, FcεRII binds immobilized carbohydrate in a calcium-dependent manner. However, the inability to block the interaction between FcεRII and IgE with high concentrations of monosaccharides suggests that the binding of IgE to FcεRII is either independent of or only partially dependent on IgE oligosaccharides. Alternatively, the lectin domain of FcεRII might be responsible for interacting with other membrane-bound or extracellular glycolipids or glycoproteins.

MATERIALS AND METHODS

Reagents. BSA, EGTA, EDTA, L-fucose-agarose, L-isoleucine-agarose, D-glucose-agarose, and all mono- and disaccharides except D-glucosamine were obtained from Sigma Chemical Co. (St. Louis, MO). Agarose conjugates of D-galactose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and D-mannose were purchased from Pierce Chemical Co. (Rockford, IL). Other reagents include: D-glucosamine (Calbiochem, La Jolla, CA), Nonidet P-40 (Bethesda Research Laboratories, Rockville, MD), and CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). DNP_{15}-OVA conjugates were prepared as described (33). IgE-Sepharose 4B was prepared by using DNP-specific IgE mAb (33). Guinea pig complement (Pel-Freeze Biologicals, Rogers, AR) was adsorbed at 4°C with mouse spleen cells before use.

Antibodies. DNP-specific IgE, IgM, and IgG1 mAb were affinity-purified from mouse ascites (33). B3B4 (anti-mouse FcεRII mAb (34)) was generously provided by Daniel Conrad (Medical College of Virginia, Richmond, VA). Goat anti-mouse IgM was a gift of Donald Mosier (Medical Biology Institute). Monoclonal anti-Thy-1.2 was de-

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[3] Abbreviations used in this paper: NCS, newborn calf serum; DME, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; εBP, epsilon binding protein; ASF, asialofetuin; RBL, rat basophilic leukemia; FcR, Ig Fc receptor; FcεRII, moderate affinity FcR for IgE; FcεRI, high affinity FcR for IgE.

rived from F7D5e7 hybridoma clone (35), obtained as ascites and centrifuged. Ig were radiolabeled with $Na^{125}I$ (New England Nuclear, Boston, MA) by using the chloramine-T method.

Cells. Hybridoma ascites were produced in 12- to 16-wk-old male (BALB/c × A/J) F1 (CAF_1) mice. Spleen cells were obtained from male BALB/c mice, aged 8 to 12 wk. Both mouse strains were obtained from the Medical Biology Institute breeding colonies. M12.4.5 mouse B lymphoma cells ($Fc\epsilon RII^+$) were obtained from Richard Asofsky (National Institutes of Health, Bethesda, MD) and maintained in DME (Whittaker MA Bioproducts, Walkerville, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (HyClone Laboratories, Logan, UT). RBL cells were generously provided by Keith Albrandt (Medical Biology Institute).

BALB/c mouse spleen cells were prepared by dispersing in MEM (Whittaker) by using a tissue grinder. After centrifuging, RBC were lysed by treating with ammonium chloride solution (8.3 g/L NH_4Cl, 1 g/L $KHCO_3$ and 0.37 g/L EDTA, pH 7.4) for 5 min followed by washing twice in MEM. Depletion of T cells was accomplished by incubating cells with anti-Thy-1.2 mAb followed by guinea pig complement (36).

Peritoneal macrophages were harvested immediately after killing of BALB/c mice. Five milliliters of MEM/5% NCS were injected i.p. and, after massaging the abdomen, removed with a sterile syringe. After washing the peritoneal cells twice in MEM, macrophages were isolated by incubating in 24-well sterile plastic dishes (Costar, Cambridge, MA) in DME/10% FCS for 90 min at 37°C, 10% CO_2. Each culture well was then washed several times with PBS/0.1% azide/10% NCS at 4°C until only adherent cells remained. The washings were saved and the nonadherent cells were recovered by centrifugation. IgE-binding studies were performed with the adherent cells in the culture wells after the washing procedure. After the binding incubation (2 h, 4°C), macrophages were removed from the culture wells by scraping and then centrifuged through oil (19).

Labeling cell surface molecules with radioiodinated antibody. With some modification, these procedures have been described previously (19). Briefly, the radiolabeled antibody (1 μg/ml) was incubated with 2×10^6 cells in PBS containing 0.1% sodium azide and either 10% NCS or 0.1% BSA (for calcium-free incubations) in a final vol of 200 μl for 2 h at 4°C. Cell-associated radioactivity was determined by centrifuging through oil. Nonspecific binding was determined in the presence of 200-fold excess unlabeled antibody. All binding experiments were performed at least twice, with similar results. Sampling was routinely performed in triplicate.

Cell surface radioiodination. After isolation of the appropriate population, the cells were washed twice and resuspended in PBS/10 μM potassium iodide (250 μl per 2×10^8 lymphocytes or 5×10^7 RBL cells). To each 250 μl of cell suspension, 6 μg lactoperoxidase (Worthington Biochemical Corp., Freehold, NJ) and 1 mCi $Na^{125}I$ (New England Nuclear) were added. H_2O_2 (10 μl of a 0.03% solution) was added three times, each followed by 1-min incubations at room temperature with mild shaking. After addition of 100 μl of 10% NCS in PBS, cells were washed twice in PBS (4°C).

SDS-PAGE analysis of surface proteins. Except where noted otherwise, cells were lysed by adding 0.5 ml of PBS/0.5% NP-40 containing 1% aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.2 μM phenylmethylsulfonyl fluoride (all from Sigma) and 1 mM $CaCl_2$. Samples were incubated on ice for 20 min with occasional vortexing, and centrifuged in an Eppendorf microcentrifuge for 15 min at 4°C. Lysate supernatants were incubated with Sepharose beads overnight at 4°C, washed four to six times with 1 ml PBS/0.5% NP-40/10% NCS followed by four to six washes in PBS/0.5% NP-40/1 mM $CaCl_2$ at 4°C. After elution from the Sepharose beads, the eluates were either incubated with a second solid support or dialyzed for 8 h in water (4°C), lyophilized overnight, and reconstituted in SDS sample buffer (2% SDS, 10% glycerol, 0.01% bromphenol blue, and 61.25 mM Tris, pH 8). Proteins remaining on the beads after the initial elution procedure were removed by boiling the Sepharose beads for 5 min in SDS sample buffer. Immediately before loading on the gel, 2-ME (5%) was added to all samples followed by placement in a boiling water bath for 3 min. Electrophoretic separation was performed in 10 or 12.5% polyacrylamide slab gels (PAGE) (37) in vertical Bio-Rad (Richmond, CA) gel boxes for approximately 5 h. Gels were dried at 80°C for 2 h and exposed to Kodak OMAT XAR-5 film with intensifying screen at −70°C.

RESULTS

EGTA inhibits IgE binding to murine B cells. The binding of several radiolabeled Ig to murine B cells was tested in the presence of 2 mM EGTA (pH 7). As shown in Figure 1, the binding of IgM to its FcR was unaffected by EGTA. Conversely, IgE binding to murine B cells was completely inhibited in the presence of EGTA, usually to a level slightly lower than that permitted by a 200-fold excess of unlabeled IgE. This observation was extended to ^{125}I-IgE binding in the presence of an equal quantity of specific Ag (1 μg/ml DNP-OVA). The binding of Ag-complexed IgG1 mAb to the low affinity FcγR was not inhibited but rather increased by EGTA (data not shown). Displacement of ^{125}I-IgG1 binding with excess mouse IgG indicated that the interaction of γ1 Ig and their FcR was not calcium-dependent. The binding of polyclonal goat antibodies to surface IgM was not affected by EGTA (Fig. 1). Additionally, EGTA did not affect the binding of anti-FcεRII mAb (B3B4) despite the fact that B3B4 and IgE compete for the same binding sites on murine B cells (34). Hence, at least during the 2-h, 4°C binding assay, EGTA selectively affected the interaction of IgE with B cells.

EGTA inhibition of IgE binding to B cells is reversed by calcium. To establish that the observed effects of EGTA were related to depletion of calcium, cell-associated radioactivity was quantitated after a 2-h incubation at 4°C with and without calcium and EGTA. As shown in Figure 2, the presence of calcium increased IgE binding to B cells in cultures devoid of EGTA and reversed the inhibiting effects of low-to-moderate concentrations of EGTA. However, when the concentration of EGTA (2 mM) exceeded that of calcium, specific IgE binding to B cells was completely eliminated. Similar results were obtained by using EDTA (data not shown). These results support the likelihood that the inhibitory effect of EGTA is related to its effective chelation of calcium, and thus establish that the binding of IgE to murine B cells is calcium-dependent.

IgE requires calcium for binding to B cells and macrophages, but not to RBL cells. The calcium-dependence of IgE binding to other FcεR-bearing cells was tested (Fig. 3). The equivalent findings in T cell-depleted and unfractionated splenic lymphocytes were expected inasmuch as unstimulated T cells express few FcεRII (11, 38). Similar results also were obtained with peritoneal macrophages. Despite the high background, EGTA blocked ^{125}I-IgE binding to adherent peritoneal cells at least as well as that permitted by 200 μg/ml unlabeled IgE. Nonadherent peritoneal cells, comprised primarily of B cells (J. Riggs, personal communication), and $Fc\epsilon RII^+$ M12.4.5 B lymphoma cells also did not bind IgE in the presence of EGTA. These findings are consistent with human FcεRII sequence analyses that predict that the extracellular domains of FcεRII on B cells and macrophages are identical. On the other hand, RBL cells bound IgE at least as well in the presence of EGTA than in its absence. The latter result documents previous findings with basophilic FcεRI (39) and further distinguishes the interaction of IgE with FcεRI and FcεRII.

FcεRII isolated from B cells binds to IgE-Sepharose in a calcium-dependent manner. To test whether the calcium-dependent interaction of IgE with B cells reflected a simple ligand-receptor interaction, B cells were surface radiolabeled, lysed, and the lysate incubated with IgE-Sepharose. Elution of the bound material was carried out with acid or EGTA-containing buffer (Fig. 4). Acidic solutions of pH 4 or lower have been reported to strip cytophilic IgE from B cells (40) as well as remove FcεRII

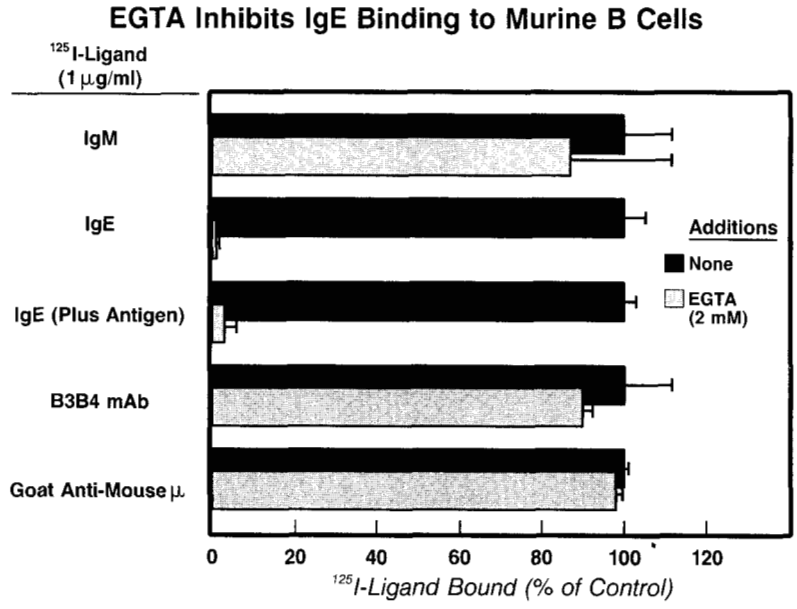

Figure 1. EGTA inhibits IgE binding to murine B cells. Binding was carried out with BALB/c mouse splenic B cells (10^7/ml). Radioligand incubations were performed in the presence or absence of 2 mM EGTA for 2 h at 4°C in PBS with 0.1% azide and 10% NCS. The DNP-specific murine mAb, IgM and IgE, were used to label their respective FcR. Anti-DNP IgE was incubated in the presence and absence of its specific Ag, DNP-OVA (1 μg/ml). B3B4 is a rat IgG mAb specific for murine FcεRII. Goat anti-mouse IgM are polyclonal, affinity-purified antibodies. Nonspecific binding was determined by co-incubation of the radioligand (1 μg/ml) with a 200-fold excess of unlabeled antibody (200 μg/ml) and subtracted from all values. Nonspecific binding comprised approximately 50% (IgM) or less than 10% (IgE ± Ag, B3B4 mAb, goat anti-mouse IgM Ab) of total binding.

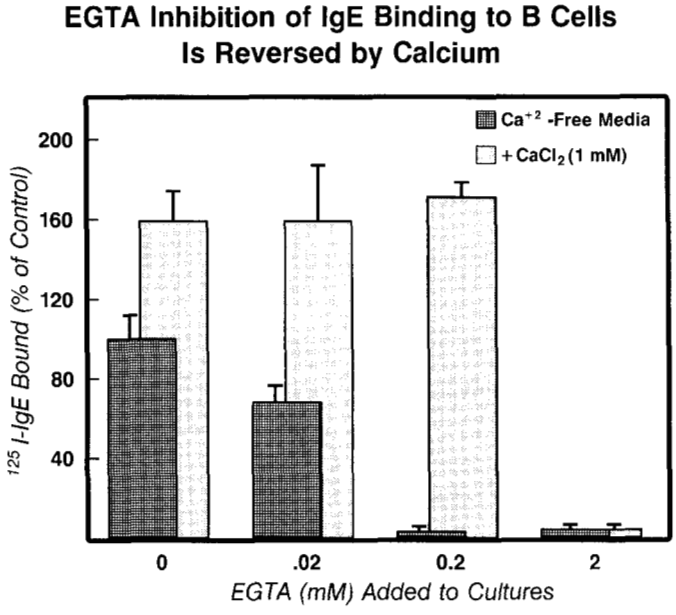

Figure 2. EGTA inhibition of IgE binding to B cells is reversed by calcium. Equilibrium binding data was generated by using BALB/c B cells in calcium-deficient media (McCoy's media, Sigma) containing 0.1% azide and 0.1% BSA with or without 1 mM $CaCl_2$. Cell samples were prewashed in their respective binding buffer and co-incubated with ^{125}I-IgE (1 μg/ml) and varying concentrations of EGTA. Nonspecific binding (less than 10% of total) was determined in the presence of 200 μg/ml IgE and subtracted from all values.

from IgE beads (41). The 68-kDa fragment isolated on IgE-Sepharose probably represents nonspecifically bound BSA as it was used in excess to halt cell surface radiolabeling. More extensive washing removed the 68-kDa protein while leaving FcεRII unaffected (e.g., Fig. 8). A protein of expected size (49 kDa) for murine FcεRII (41) was observed in both the acid (Fig. 4, *lane 1*) and EGTA (Fig. 4, *lane 3*) elution buffers. Moreover, as with intact B cells, EGTA did not cause dissociation of IgE and FcεRII in the presence of excess calcium (Fig. 4, *lane 4*).

Binding of FcεRII to IgE-Sepharose is blocked by B3B4 mAb. To establish that the 49-kDa protein eluted from IgE-Sepharose by EGTA was the same protein described by Rao et al. (34) as FcεRII, IgE-Sepharose was then incubated with lysed, radiolabeled B cells in the presence of varying concentrations of B3B4 (anti-FcεRII) mAb and eluted with EGTA-containing buffer (Fig. 5). The EGTA-elutable protein did not bind to IgE-Sepharose in the presence of excess B3B4 mAb. These results establish that calcium is directly involved in IgE recognition of FcεRII and that this interaction seems to be independent of other membrane components.

The α-subunit of FcεRI, isolated from RBL cells, binds to IgE in a calcium-independent manner. Similar procedures for isolating B cell FcεRII were applied to isolate FcεRI from RBL cells. The α-subunit of FcεRI typically appears as a broad band on SDS-PAGE from approximately 50 to 60 kDa (42). As with FcεRII proteins, FcεRI-α dissociates from IgE beads in the presence of acid (Fig. 6). However, EGTA does not elute FcεRIα from IgE-Sepharose, thus, corroborating the specificity of the calcium dependence of ^{125}I-IgE binding to FcεRII.

IgE binding to B cells results in IgE internalization. Recent cloning of the human FcεRII has revealed a segment of sequence homology with several hepatic (or calcium-dependent binding) lectins (24). Among the hallmark characteristics of these membrane lectins are that they do not bind carbohydrate in buffers below a pH of 6.5 and they are efficiently internalized after binding of a specific carbohydrate or glycoprotein ligand (32). Similar to a previous report (43) and as shown in Figure 7, a significant fraction of IgE is internalized (not susceptible to elution with pH 4 buffer) after incubation in DME/10% FCS at 37°C for at least 2 h. When complexed with specific Ag (DNP-OVA), slightly more IgE was maintained intracellularly. Four- and six-hour incubations yielded similar results (data not shown). As expected, substantially less ^{125}I-IgE was internalized by B cells incubated at 4°C or at 37°C in the presence of EGTA, indicating the importance of surface receptor and cytoskeletal mobility in these processes.

Both the calcium-dependent binding and internalization of IgE are consistent with a lectin (FcεRII)-glycoprotein (IgE) interaction and could play an important role in IgE-mediated Ag focusing as proposed by Kehry and Yamashita (44). However, whereas ligand binding to previ-

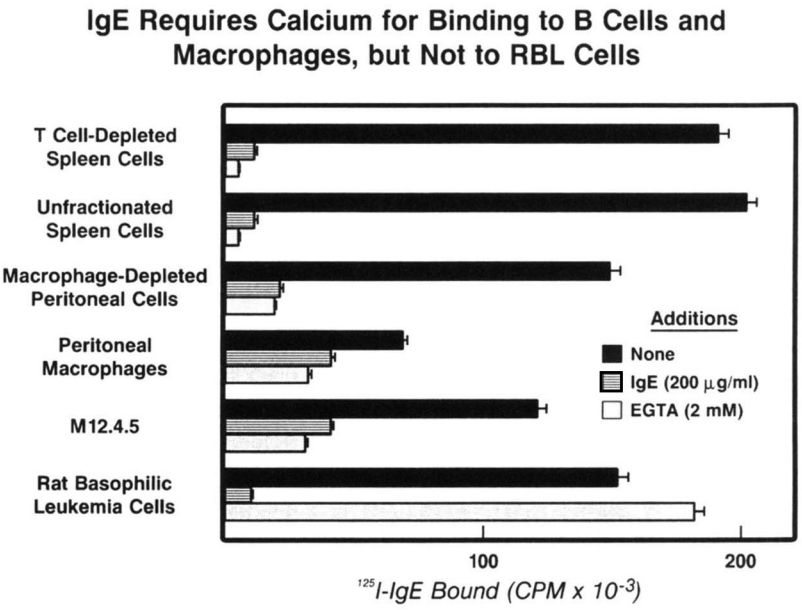

Figure 3. IgE requires calcium for binding to B cells and macrophages, but not to RBL cells. EGTA susceptibility of IgE binding was tested in the following cell types: RBC- and T lymphocyte-depleted BALB/c spleen cells (10^6 cells/sample), RBC-depleted spleen cells (2×10^6 cells/sample), adherent cell-depleted BALB/c peritoneal cells (2×10^6 cells/sample), macrophage-enriched BALB/c peritoneal cells (2×10^6 cells/sample), M12.4.5 mouse B lymphoma cells (2×10^6 cells/sample) and RBL cells (10^5 cells/sample). Incubations with ^{125}I-IgE (1 μg/ml) were carried out in PBS/0.1% azide/10% NCS for 2 h at 4°C in the presence or absence of EGTA (2 mM) or 200-fold excess unlabeled IgE.

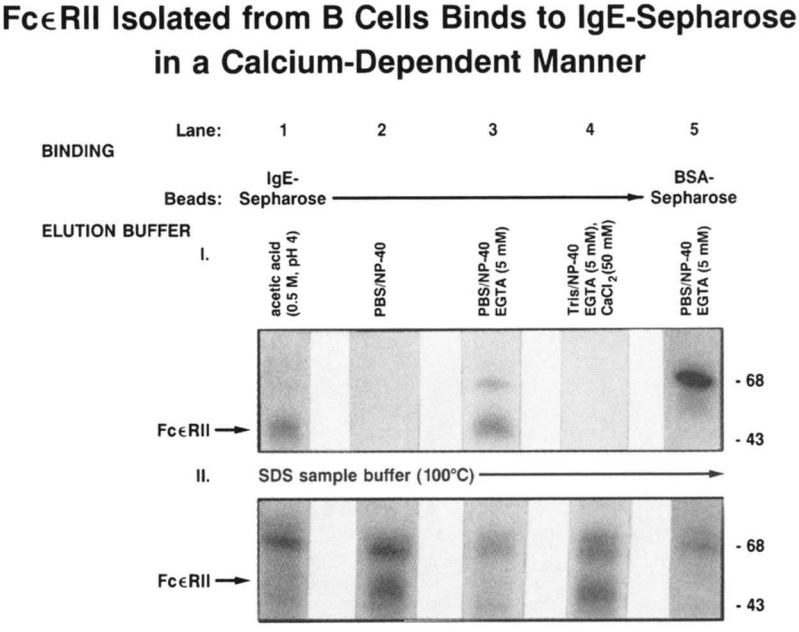

Figure 4. FcεRII isolated from B cells binds to IgE-Sepharose in a calcium-dependent manner. Fresh B cells from BALB/c mice were surface-labeled, lysed, and incubated with either IgE-Sepharose or BSA-Sepharose (100 μl). After washing, the beads were eluted with four washes of 200 μl each of 0.5 M acetic acid (pH 4) (*lane 1*) or binding buffer (PBS/0.5% NP-40) at pH 7 with (*lanes 3* and *5*) and without EGTA (5 mM) (*lane 2*). The elution buffer containing 50 mM $CaCl_2$ used 75 mM Tris rather than PBS (*lane 4*). The *upper frame* shows the radiolabeled proteins after elution from Sepharose beads, dialysis, lyophilization, and reconstitution in SDS sample buffer. The *lower frame* (elution buffer II) shows PAGE analysis of the proteins remaining on the Sepharose beads after elution with buffer I that were subsequently removed by boiling the beads in SDS sample buffer. All samples were boiled 3 min in sample buffer with 5% 2-ME before loading on the gel. Molecular weight markers are shown to the right.

ously characterized C-type lectins is completely inhibited at pH 6.5 (32), only 40% of IgE binding to B cells is inhibited at pH 6.5 (data not shown). This suggests that either FcεRII does not act as a lectin with regard to its interaction with IgE or its pH-dependent binding profile differs from C-type lectins.

Carbohydrates are less important for binding of IgE by FcεRII than by the 35-kDa IgE-binding protein (εBP). To ascertain whether IgE-FcεRII binding is oligosaccharide-dependent, FcεRII proteins were isolated from lysates of surface radiolabeled B cells on IgE-Sepharose beads and eluted with either EGTA or one of several sugars (Fig. 8, *upper frame*). After elution with EGTA or carbohydrate, beads were boiled in SDS sample buffer to elute the remaining proteins (Fig. 8, *lower frame*). Only EGTA dissociated FcεRII completely from IgE-Sepharose (Fig. 8, *lane 1*) whereas FcεRII proteins were incompletely removed by elution with 200 mM D-glucose (Fig. 8, *lane 2*), sucrose (Fig. 8, *lane 3*), D-fructose (Fig. 8, *lane 4*), and D-galactose (Fig. 8, *lane 6*). Substantially less FcεRII was recovered when eluting IgE beads with 20 mM of each sugar (data not shown). Attempts to elute FcεRII with other sugars or organic compounds (10 to 200 mM), i.e., D-mannose, D-glucosamine, *N*-acetyl-D-glucosamine, D-fucose, L-fucose, L-isoleucine, and uridine, yielded similar results (data not shown). Conversely, 200 mM α-lactose, which was the least effective sugar for eluting FcεRII from IgE-Sepharose, efficiently removed the 35-kDa εBP (Fig. 8, *lane 5*). D-galactose (200 mM) also was able to elute εBP from IgE-Sepharose (Fig. 8, *lane 6*), but less efficiently than lactose. This corroborates findings described in a recent report where εBP was proposed to interact with IgE via galactose residues and could be eluted from glycoprotein-Sepharose (including IgE-Sepharose) columns by lactose (29). Moreover, these results suggest that if FcεRII associates with IgE oligosaccharides, it does so with lesser dependence on carbohydrate than demonstrated for εBP.

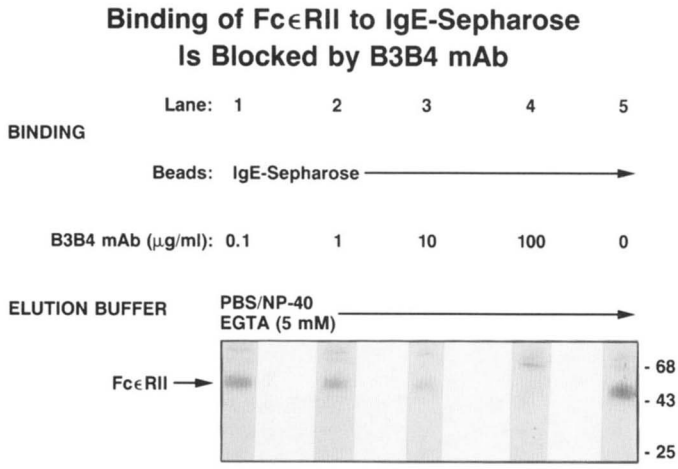

Figure 5. Binding of FcεRII to IgE-Sepharose is blocked by B3B4 mAb. B cell lysates were incubated with IgE-Sepharose in the absence or presence of varying concentrations of B3B4 antibody. Elution of each sample was performed with binding buffer containing EGTA (5 mM). Eluants were dialyzed, lyophilized, and reconstituted in SDS sample buffer. All samples were boiled 3 min in sample buffer with 5% 2-ME before loading on the gel.

The α Subunit of FcεRI, Isolated from RBL Cells, Binds to IgE in a Calcium-Independent Manner

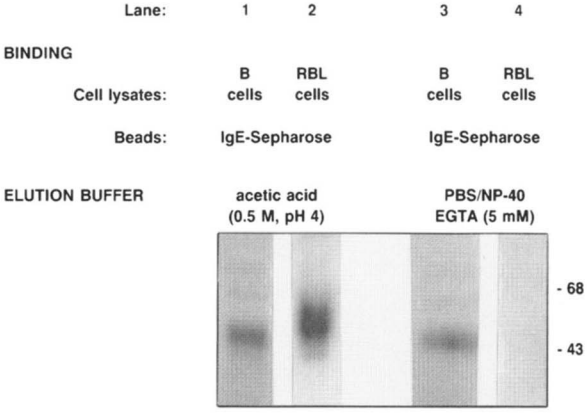

Figure 6. The α-subunit of FcεRI, isolated from RBL cells, binds to IgE in a calcium-independent manner. RBL cells (5×10^7) were surface-radiolabeled and lysed by using a similar procedure as outlined for B cells. IgE-Sepharose was incubated with the lysate, washed, and eluted by using an identical procedure as outlined for isolating FcεRII proteins from B cells. B cell and RBL lysates were split into two fractions and then eluted with acid or EGTA buffers.

Carbohydrates do not block IgE-FcεRII Binding. Lysates of surface radiolabeled B cells were incubated with IgE-Sepharose in the presence of various sugars (100 mM), including the principal carbohydrates found on mammalian membranes (45). All bead samples were then eluted with EGTA to remove IgE-bound FcεRII. The eluants were dialyzed in water, lyophilized, and separated by PAGE (Fig. 9, *upper frame*). Subsequent to EGTA elution of the beads, the 35-kDa εBP was eluted from IgE-Sepharose by boiling the beads in SDS sample buffer (Fig. 9, *lower frame*). Whereas lactose and, to a lesser extent, galactose blocked the binding of εBP to IgE-Sepharose, none of the carbohydrates substantially inhibited the binding of FcεRII. Alternatively, the binding of ^{125}I-IgE to intact B cells was tested in the presence of both 10 and 100 mM: D-glucosamine, α-lactose, D-mannose, D-fucose, D-galactose, *N*-acetyl-D-glucosamine, D-glucose, L-fucose, sucrose, D-mannitol, and L-isoleucine (data not shown). None of the substances significantly reduced the binding of ^{125}I-IgE to B cells. These results further suggest that FcεRII does not interact with IgE exclusively via its

IgE Binding to B Cells Results in IgE Internalization

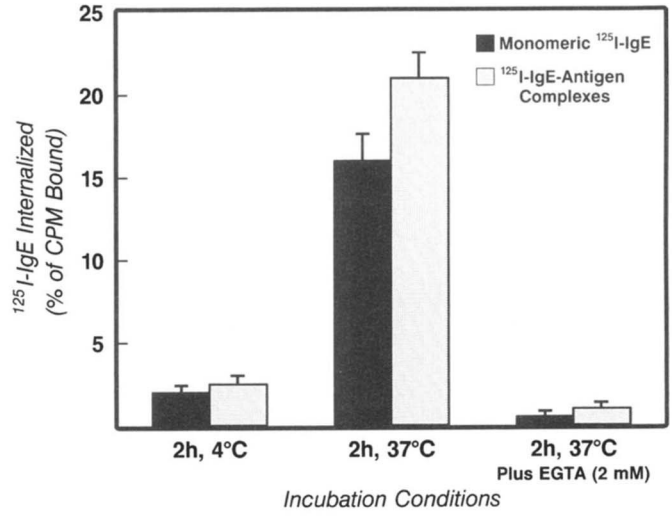

Figure 7. IgE binding to B cells results in IgE internalization. B cells were isolated from BALB/c mouse spleen and incubated with radiolabeled IgE (1 μg/ml) ± DNP-OVA (1 μg/ml) in DME/10% FCS for 2 h at 4°C. EGTA (2 mM) was included in duplicate samples. After the initial 2 h, 4°C incubation, samples were either transferred to 37°C water bath or kept at 4°C for an additional 2-h incubation. Half of each treatment group were either directly centrifuged through oil (total cell-associated cpm) or the cell surface IgE was first removed by centrifuging the cells and exposing to DNP-saturated MEM (pH 4) (19) before centrifuging through oil (internalized cpm). The difference between the cpm obtained in the two groups represents membrane-bound IgE.

Carbohydrates Are Less Important for Binding of IgE by FcεRII Than by the 35 kD IgE Binding Protein (εBP)

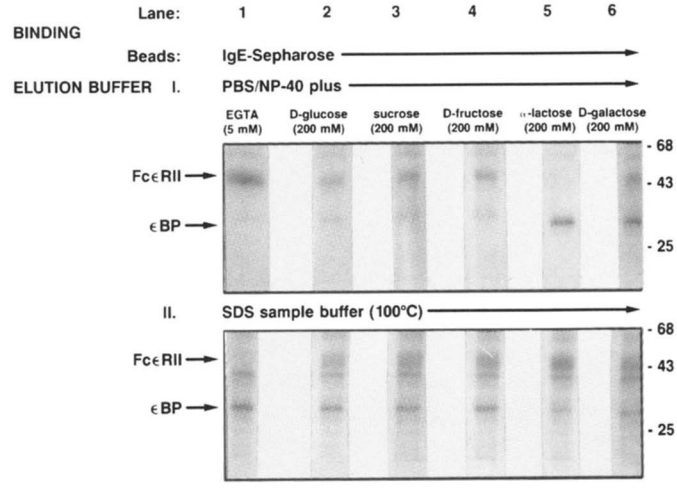

Figure 8. Carbohydrates are less important for binding of IgE by FcεRII than by the 35-kDa IgE-binding protein (εBP). Murine B cells were surface radioiodinated, and incubated with IgE-Sepharose (600 μl) overnight. The beads were then washed as described in *Materials and Methods*, aliquoted to separate tubes, and eluted with various carbohydrates (200 mM) in PBS/0.5% NP-40 (4×200 μl). The *upper frame* shows the proteins eluted by EGTA or carbohydrate and the *lower frame* shows the proteins subsequently eluted from the corresponding beads by boiling in SDS sample buffer.

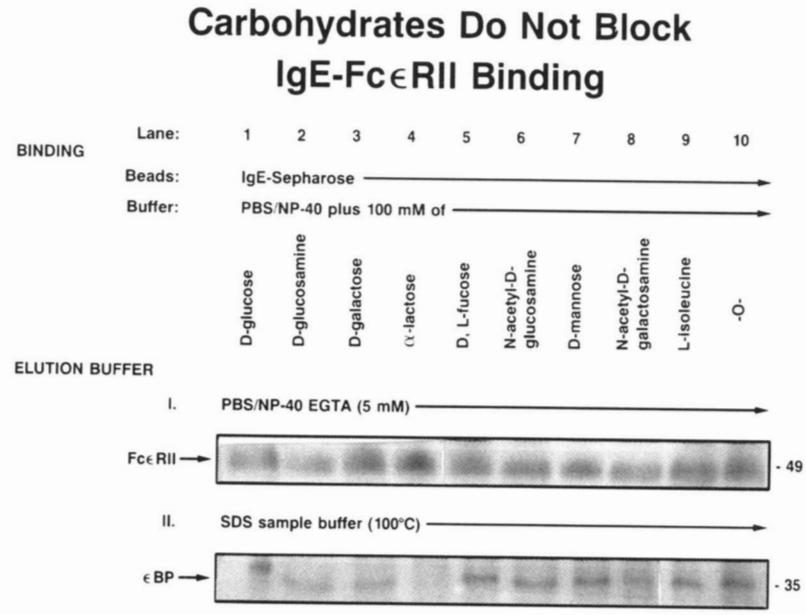

Figure 9. Carbohydrates do not block IgE-FcεRII binding. PAGE analysis was performed on radioiodinated B cell surface proteins that were incubated overnight with IgE-Sepharose in the presence of various carbohydrates/amino acids (100 mM). After incubation, IgE beads were eluted with PBS/0.5% NP-40/5 mM EGTA, dialyzed, lyophilized, and separated electrophoretically (*upper frame*). After elution of the beads with EGTA, εBP was eluted from the IgE-Sepharose samples by boiling in SDS sample buffer and separated by PAGE (*lower frame*).

oligosaccharides.

Murine B cell FcεRII binds differentially to immobilized carbohydrates. Murine B cell FcεRII was tested for its capacity to bind agarose-conjugated carbohydrates. Attempts to extract FcεRII from crude B cell lysates with monosaccharide-agarose beads were unsuccessful. Hence, FcεRII was first isolated by surface-radiolabeling B cells, incubating the lysate with IgE-Sepharose, and eluting FcεRII from IgE-Sepharose by washing the beads with 75 mM Tris/5 mM EGTA. The eluate was treated with $CaCl_2$ (20 mM), centrifuged to remove the EGTA, and then incubated with immobilized monosaccharides. After 8 h, agarose beads were isolated by centrifugation and the remaining unbound proteins were analyzed by PAGE. The monosaccharide-agarose beads completely removed the FcεRII as unbound FcεRII was found only in samples incubated with L-isoleucine-agarose (data not shown). The bead samples were washed with 75 mM Tris (pH 8) buffer containing either 2 mM $CaCl_2$ (Fig. 10, *odd-numbered lanes*) or 2 mM EGTA (Fig. 10, *even-numbered lanes*), eluted by boiling in SDS sample buffer and analyzed by PAGE. As illustrated in Figure 10, FcεRII bound most efficiently and in a calcium-dependent manner to IgE-Sepharose and to agarose beads conjugated with D-galactose, D-mannose, and *N*-acetyl-D-galactosamine. FcεRII also associated with D-glucose-, L-fucose-, *N*-acetyl-D-glucosamine- and L-isoleucine-agarose beads but with less efficiency and/or with less or no dependence on calcium. Interestingly, co-incubation of 200 mM mannose, galactose, or fucose with their agarose conjugates did not block ^{125}I-FcεRII binding to the sugar-coated beads (data not shown). Hence, although murine FcεRII binds to immobilized monosaccharides with an apparent preference for D-galactose and D-mannose residues, the lack of binding inhibition by soluble monosaccharides suggests either that the binding is nonspecific or that complexation of the carbohydrate as an oligosaccharide or glycoslated protein is required for its low affinity interaction with FcεRII.

DISCUSSION

We have described several features of the interaction between murine IgE and FcεRII that are consistent with the binding of oligosaccharides to certain lectins. Most notable of these is the absolute dependence upon calcium for the binding event to occur. The calcium-dependent

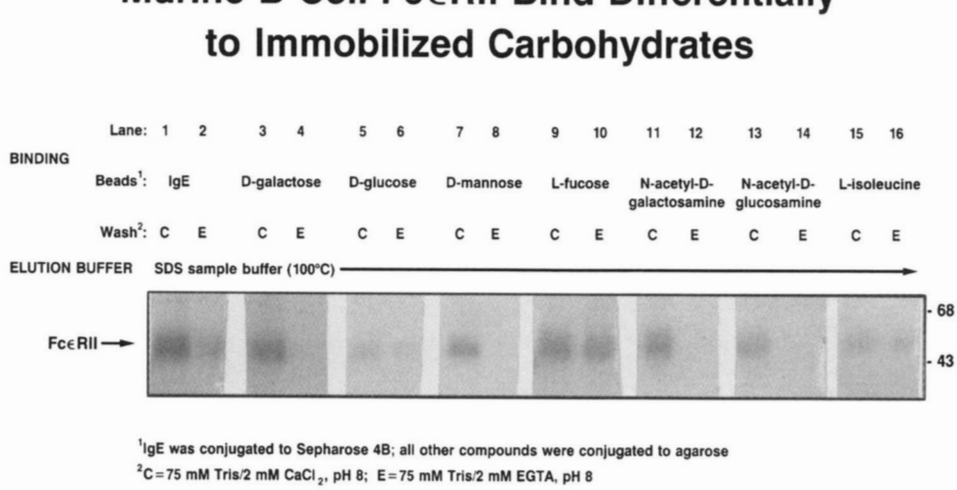

Figure 10. Murine B cell FcεRII binds differentially to immobilized carbohydrates. BALB/c mouse spleen cells were surface labeled, lysed, and incubated with IgE-Sepharose (2 ml) overnight at 4°C. The beads were washed 5 times with 8 ml PBS/0.5% NP-40/1 mM $CaCl_2$/0.5% BSA and 5 times with 8 ml PBS/0.5% NP-40/1 mM $CaCl_2$, followed by elution with 3 × 2 ml of 75 mM Tris (pH 8)/2 mM EGTA/0.5% BSA. Excess $CaCl_2$ (20 mM) was added to the eluant and the calcium/EGTA complexes were removed by centrifugation. The supernatant was aliquoted and incubated with 100 μl of the indicated beads overnight at 4°C. Beads were then washed 5 times with 1 ml of either 75 mM Tris/2 mM $CaCl_2$ (pH 8) (*odd-numbered lanes*) or 75 mM Tris/2 mM EGTA (pH 8) (*even-numbered lanes*). Samples were eluted by boiling in SDS sample buffer and analyzed by PAGE.

nature of the IgE-FcεRII interaction was conclusively demonstrated by measuring the calcium dependence of both IgE binding to intact cells as well as FcεRII binding to IgE beads. The effect of EGTA on IgE binding to FcεRII is corroborated functionally by EGTA inhibition of IgE-induced Ia and FcεRII expression on B cells (M.L. Richards and D.H. Katz, manuscript in preparation). These findings contrast to the negligible effects of EGTA on the binding of both IgG1 and IgM (Fig. 1) to B cells or on IgE-binding to FcεRI on RBL cells (Fig. 3). The FcR involved in the latter interactions are members of the Ig superfamily and are oriented, like most membrane proteins, with their carboxy terminus in the cytoplasm (20–23). However, nucleotide sequencing predicts that human FcεRII has an inverted membrane orientation. Murine B cell FcεRII recently was shown to share homology with the IgE receptor(s) found on human lymphocytes and monocytes (46). Thus, its sequence represents a departure from FcR of the Ig gene superfamily and might portend radical differences with regard to the interactions it has with its ligand.

The calcium dependence of IgE binding can be used to distinguish receptor types/subtypes. In rodents, calcium-dependent binding distinguishes IgE binding to the high affinity FcεRI of mast cells and basophils from the moderate affinity FcεRII of macrophages and B cells (Fig. 3). Moreover, it shows that FcεRII of macrophages and B cells interact with IgE in a similar manner. If murine FcεRII parallels that of human lymphoid cell lines, then these results would not be surprising. Sequence analysis predicts that the FcεRII of human macrophages, B cell, and T cell lines have virtually identical extracellular domains (47, 48). Although the putative cytoplasmic regions differ, it is unlikely that such differences would substantially change the interactions of membrane macromolecules with soluble proteins.

The finding that FcεRII cDNA have an extracellularly-located homologous sequence with several hepatic or asialoglycoprotein lectins (46, 49) provides an opportunity to study the potential interaction of this putative lectin with the oligosaccharides of IgE. Although the presence of carbohydrate on proteins has long been assumed to be important, delineation of their specific functions has been slow to develop. IgG oligosaccharides have been reported to be important for triggering complement-dependent lysis as well as binding to FcR on monocytes (50). However, no role has yet been ascribed to the oligosaccharides on the carbohydrate-rich IgE. Previous reports that have addressed the importance of IgE carbohydrates for binding to FcεRII have yielded seemingly conflicting results. mAb that bind to regions adjacent to glycosylated sequences of human IgE also blocked IgE binding to FcεRII (30). Conversely, binding of synthetic, oligosaccharide-free IgE peptides to FcεRII on human cells showed that carbohydrates are unnecessary for binding (31). However, interpretation of these results is difficult. Attachment of macromolecules to Ig Fc domains would be expected to have marked and unpredictable effects on the affinity of IgE for its receptor. Moreover, synthetic IgE peptide fragments likely would have differences in their tertiary structure compared with the complete native molecule. Hence, in an effort to simplify the system, carbohydrates were used in the present study to compete with IgE in its interaction with murine FcεRII.

Although murine FcεRII displays the hallmark characteristics of hepatic lectins, i.e., calcium- and pH-dependent (although partial) ligand (IgE) binding, location on the cell surface, cytoplasmic amino termini, and internalization after ligand association, carbohydrates did not significantly interfere with the binding of IgE to FcεRII. However, this does not definitively establish the absence of an interaction between FcεRII and the oligosaccharides of IgE. One might argue, for example, that the specific sugar with which FcεRII selectively associates was not used in the competitive binding assays. This is unlikely because of the over 100 monosaccharides found in nature, only a few are found on membrane glycoproteins; primarily galactose, mannose, fucose, galactosamine, glucosamine, and glucose (45). Characterization of human IgE oligosaccharides has demonstrated that it is comprised of mannose, galactose, *N*-acetyl-glucosamine, fucose, and sialic acid (51). Moreover, heretofore identified lectins generally associate with varying affinities to many carbohydrates. In spite of the ability of galactose-containing sugars to block εBP binding to IgE, none of the carbohydrates tested inhibited the interaction of IgE with FcεRII. Similar results were obtained whether binding radiolabeled IgE to intact B cells (data not shown) or radiolabeled FcεRII to IgE-Sepharose (Fig. 9). Furthermore, although a fraction of the IgE-Sepharose-bound FcεRII could be eluted by 200 mM of several sugars (Fig. 9), there was no carbohydrate selectivity, and a similar fraction of FcεRII protein was eluted with 200 mM of either isoleucine or uridine, suggesting a nonspecific effect of highly osmolar solutions.

Additionally, one must consider the possibility that mono- or disaccharides compete poorly with glycoproteins or complex carbohydrates for lectin binding. Consistent with this is the observation that FcεRII binds mannose- or galactose-agarose beads by a calcium-dependent mechanism that is not inhibited by soluble monosaccharides. However, FcεRII bound to carbohydrate-coated beads only after first isolating the protein from crude B cell lysates on IgE-Sepharose. This indicates that the interaction between FcεRII and monosaccharide-agarose occurs with much lower avidity than that observed between FcεRII and IgE-Sepharose. Furthermore, we (data not shown) and others (29), using similar procedures, have demonstrated that the IgE binding protein, εBP, can be eluted from IgE-Sepharose by its competing monosaccharide or disaccharide. Moreover, experiments performed in the laboratory of F.-T. Liu have shown that εBP in 150-fold excess molar concentration does not inhibit IgE binding to murine B cell FcεRII. Thus, in summary, although an interaction between FcεRII and carbohydrate cannot be definitively established by the results described herein, the likelihood of murine FcεRII binding IgE exclusively via a carbohydrate residue appears remote.

Alternatively, murine FcεRII probably has bifunctional binding properties. Lectins have been shown to have both protein- and carbohydrate-binding regions (52). Hence, it is quite possible that FcεRII binds IgE via both its protein- and carbohydrate-binding domains. This would explain the lack of binding inhibition by mono- and disaccharides and would obviate the need to propose that the calcium-dependent binding of FcεRII to both IgE and carbohydrate is fortuitous. The concept of FcεRII having both a carbo-

hydrate and protein binding region has intriguing implications for the functions the lectin activity might serve, possibly as a result of IgE binding. The role FcεRII plays in receptor internalization, receptor capping, transmembrane signaling, or B cell Ia modulation are possible candidates. Refocusing the studies of FcεRII functions on its putative lectin activity might aid in definitively establishing the function(s) of FcεRII on B cells.

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