

CHARACTERIZATION OF A MONOCLONAL ANTIBODY DIRECTED AGAINST THE MURINE B LYMPHOCYTE RECEPTOR FOR IgE¹

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A rat hybridoma producing a high-affinity IgG2a monoclonal antibody (B3B4) directed against the murine lymphocyte IgE receptor (FcεR) was established by using purified FcεR from FcεR⁺ murine hybridoma B cells as immunogen. The monoclonal and polyclonal anti-FcεR inhibited the binding of IgE to the murine lymphocyte FcεR and were also used to isolate the FcεR. B3B4 specifically recognized only the 49-Kd FcεR on murine B lymphocyte as determined by immunoprecipitation and SDS-PAGE analysis. In addition to its reaction with intact FcεR, B3B4 also recognized FcεR fragments that were present in the culture media of FcεR⁺ hybridoma cells. The predominant fragments isolated were 38 Kd and 28 Kd by SDS-PAGE analysis. When tested for reactivity with other cell types, B3B4 was highly specific for murine B lineage cells in that it did not significantly react with FcεR on macrophages and T cells and, in addition, did not react with the high affinity mast cell FcεR. B3B4 completely blocked IgE rosetting, and a reciprocal inhibition of binding was seen in a dose-dependent fashion between IgE and B3B4, indicating a close proximity of the IgE and B3B4 binding sites. Saturation binding analysis indicated that the Fab' fragment of B3B4 bound to twice as many sites/cell as IgE, suggesting that there are two identical B3B4 determinants per 49-Kd FcεR or that the IgE binding site is formed by the association of at least two 49-Kd FcεR. However, unlike IgE, neither B3B4 nor F(ab')₂-B3B4 nor Fab'-B3B4 were very effective in causing FcεR upregulation on murine hybridoma B cells; in fact, B3B4 prevented this upregulation when added in combination with IgE. These results suggest that a site-specific interaction provided only by IgE may be essential for ligand-specific upregulation. Both polyclonal and monoclonal antibodies will be useful in further studies concerning the functional relationship between the membrane FcεR and the soluble FcεR fragments.

have been identified on the surface of a variety of cells, including T and B lymphocytes, macrophages, and platelets (1). In contrast to the high affinity IgE receptor on mast cells and basophils that plays a role in allergic degranulation, the functional role of the low affinity lymphocyte FcεR is less well understood; one possibility for its function is involvement in isotype-specific immunoregulation (2). Although the low affinity FcεR was first described in the human system (1), the rodent model is a more attractive system to study. The lymphocyte FcεR in mice (3) and rats (4) has a relatively high affinity for IgE ($K_a = 10^8 \text{ M}^{-1}$). The FcεR has been characterized on murine B lymphocytes (5-7), and recent studies in this laboratory (8) have led to the development of several hybridoma B cell lines that bear FcεR in high numbers and exhibit identical properties to those previously described for normal B cells. In addition, these hybridoma cells possess the ability to upregulate their FcεR numbers in response to culture with IgE. In continuation of these studies on the structure and biological function of lymphocyte FcεR, polyclonal goat and rat antisera to the murine B lymphocyte FcεR have been developed, and one high affinity monoclonal (mab) anti-FcεR was also developed. This is the first report of a monoclonal antibody specific for the murine B lymphocyte FcεR. This study describes the characterization of these antibodies with regard to their specificity and binding characteristics.

MATERIALS AND METHODS

Cells. The murine B hybridoma line (O1.2B2), possessing a relatively high FcεR level, produced in this laboratory (8) by the fusion of nematode infected BALB/c splenocytes with a pre-B lymphoma, M12.4.5 (9), was used in these studies. The following other cell lines were used in some experiments: the mouse macrophage cell line P388D1, the mouse mast cell line PT-18 (10), the human B lymphoblastoid cells RPMI-8866, and the murine T hybridoma cell lines 229D3 and 230D3 (11) (generously provided by Dr. K. Ishizaka); IR983F rat myeloma (12) and mouse hybridoma cells producing anti-rat-κ (Mark-1) (13) were gifts from Dr. H. Bazin. All cell lines were grown in enriched RPMI1640 (RPMI/c) medium (8). Purified splenic B cells were prepared from BALB/c mice infected with *Nippostrongylus brasiliensis* larvae (5), and resident peritoneal macrophages were purified by adherence to plastic (14). A breeding nucleus of Lou/m rats was obtained from NIH, and the animals were maintained in our facilities.

Immunoglobulins. Rat IgE was purified from IR162 ascites (15), and purified mouse IgE from hybridoma H-1-DNP-26 (16) was a gift from Dr. K. Ishizaka. The hybridoma 2.4G2, producing a monoclonal antibody against the murine receptor for the IgG2b subclass (Fcγ_{2b}R) (17), was a gift from Dr. J. Unkeless. The anti-Fcγ_{2b}R was purified from ascites as described (17). Where indicated, protein preparations were coupled to Affi-gel 10 (Affi) by procedures described by the manufacturer (Bio-Rad Laboratories, Richmond, CA), and purified proteins were radiolabeled by the chloramine-T procedure (18).

Preparation of anti-FcεR polyclonal antisera. FcεR was isolated from O1.2B2 cells that had been incubated overnight with 10 μg/ml rat IgE (induced cells). The isolation procedure, performed with

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² Abbreviations used in this paper: FcεR, receptor for the Fc portion of IgE; Fcγ_{2b}R, receptor for the Fc portion of IgG2b; Affi, Affi-gel-10; HAT, hypoxanthine, aminopterin, thymidine; mab, monoclonal antibody; Nb, *Nippostrongylus brasiliensis*.

affinity chromatography on IgE-Affi, was as described (8) except that larger amounts of cells were used. The IgE-Affi containing bound Fc ϵ R was then suspended in a total volume of 1 ml with the use of PBS, and a 1:1 emulsion was prepared with complete Freund's adjuvant. For subsequent injections, the emulsion was made with incomplete Freund's adjuvant. One goat and four Lou/m rats were immunized with this antigen preparation. The goat received Fc ϵ R from 1 to 2×10^9 cells per injection, and each rat received about one-fourth of this amount. Individual serum samples were tested for antibodies directed against the cell surface by a radioimmunoassay (RIA) and for anti-Fc ϵ R activity by using the inhibition of IgE binding assay as described below. The IgG from the goat antisera was purified by precipitation with 40% ammonium sulfate and ion-exchange chromatography on DE-52 performed with 0.01 M phosphate, 0.05 M NaCl, pH 7.35; the IgG does not adhere in this buffer.

Cell surface RIA. Polyclonal antisera and hybridoma supernatants were initially screened for the presence of antibodies directed against the cell surface. Induced O1.2B2 cells were acid stripped and suspended in modified HBSS (Hanks' balanced salt solution containing 20 mM HEPES and 2% FCS). One hundred microliters of the cell suspension (1×10^6 cells) were added to dilutions of antisera or hybridoma culture supernatant or medium alone (500 μ l total reaction volume), and the mixtures were incubated on ice for 1 hr. The cells were washed twice in modified HBSS and resuspended in 300 μ l of the same buffer. Two hundred microliters (2 μ g) of ^{125} I-F(ab')₂ mouse anti-rat IgG (Fc) (Pelfreeze Laboratories, Rogers, AR) were added, and incubation was carried out for another 45 min. The amount of labeled antibody bound to the cell pellet was determined by centrifuging duplicate samples containing 3×10^5 cells through a phthalate oil mixture (19) and counting the cell pellet (phthalate oil procedure). Hybridoma supernatants that contained antibodies directed against the cell surface were then screened for their specificity to Fc ϵ R by using the inhibition assay described below.

Inhibition assay. Antibodies specific to Fc ϵ R were detected by their ability to inhibit binding of rat IgE to O1.2B2 cells. Acid-stripped O1.2B2 cells (1×10^6) were incubated for 30 min on ice with 100 μ l of appropriate dilutions of test preparation. ^{125} I-rat IgE (2 μ g) was added to each of the tubes (500 μ l final reaction volume), and the preparations were incubated for an additional 60 min on ice. The amount of ^{125} I-rat IgE bound to the cell pellet was determined by the phthalate oil procedure. Where applicable, nonspecific binding was determined by the addition of a 100-fold excess of the unlabeled ligand before the addition of the specific labeled antibody, and zero inhibition was defined by preincubation with buffer alone. In later experiments with mab, the converse inhibition, that being IgE-mediated inhibition of ^{125} I-mab binding, was also studied. The protocol was the same except that the cells were then preincubated with increasing concentrations of IgE before subsequent addition of 2 μ g/ml of ^{125} I-mab and continuation as above. The percentage of inhibition of binding was calculated as follows: % inhibition = $100 \times [1 - (\text{cpm bound in the presence of the inhibitor} / \text{cpm bound in the absence of the inhibitor})]$.

Fusion protocol. Spleen cells from one of the animals displaying the highest inhibition titer was used for fusion. The HAT- (hypoxanthine, aminopterin, and thymidine) sensitive Lou rat myeloma cell line IR938F (12) was used as the neoplastic parent. For 10 days before fusion, IR938F cells were grown in RPMI/c medium containing 50 μ g/ml 8-azaguanine to control for revertants. Equal numbers of splenocytes (1×10^6) and IR938F cells were subjected to fusion by using the protocol described by Lane et al. (20). After fusion, the cells were plated in HAT medium in 96-well plates at 3×10^5 , 1×10^5 , and 0.33×10^5 tumor cells/well. The plates contained 2×10^3 peritoneal macrophages from normal Lou/m rats as a feeder layer. The first clones were evident by day 14. As hybrids became confluent, they were tested for binding to O1.2B2 cells by RIA, and potential positives were maintained for an additional 14 days in HT medium (HAT medium minus aminopterin) and after 28 days in RPMI/c medium. The line that remained positive and produced anti-Fc ϵ R antibody (B3B4) as detected by the inhibition assay was subcloned by limiting dilution (21), again with macrophages used as the feeder layer. A subclone of B3B4 that was positive was selected and identified as rat IgG2a by using an enzyme-linked immunosorbent assay (ELISA) kit from Zymed Laboratories. B3B4 was purified from Lou/m rat ascites by ammonium sulfate precipitation and DE-52 column chromatography as described by Rousseaux and Bazin (22). The purified fractions were pooled, concentrated, and stored at -70°C until further use. F(ab')₂ fragments of B3B4 were obtained by pepsin digestion (23) and gel filtration on an ACA-34 column. Fab' fragments from this were obtained by mild reduction and alkylation (24). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) analysis (data not shown) indicated that 99% of the initial IgG was converted to F(ab')₂ fragments and that the Fab' preparations were free of F(ab')₂. The

activity of all the preparations was tested by determining the ability of the radiolabeled antibody to bind to O1.2B2 cells. Seventy percent, 40%, and 30% of the total purified radiolabeled B3B4, F(ab')₂, and Fab' bound to O1.2B2 cells, respectively.

Isolation of Fc ϵ R and electrophoretic analysis. Induced O1.2B2 cells or purified BALB/c splenic B lymphocytes (1 to 3×10^6) from *Nippostrongylus brasiliensis*- (Nb) infected mice were acid stripped to remove cytophilic immunoglobulin (8) and were labeled with ^{125} I by using the lactoperoxidase procedure (25). The labeled cells were disrupted in lysis buffer (PBS containing 0.5% Nonidet P-40 [PBS/NP-40] and protease inhibitors) (8); the lysate was applied to IgE-Affi or to mab-Affi (5 and 1 mg protein/ml Affi, respectively). After mixing (3 hr to overnight), the Affi was washed as described (5) and was eluted with SDS-PAGE sample buffer. Biosynthetically labeled Fc ϵ R was prepared by culturing O1.2B2 cells (1×10^7 /ml) in methionine-deficient RPMI/c medium containing 200 μ Cl/ml [^{35} S]methionine for 4 hr. The cells were washed, then were disrupted with lysis buffer, and the lysate was applied to IgE-Affi and mixed overnight. The IgE-Affi was then washed and eluted with 0.5 N acetic acid with immediate neutralization (5). The eluate was then applied to the goat anti-Fc ϵ R-Affi, and after mixing for 4 hr (4°C), the Affi was washed and eluted with SDS-PAGE sample buffer. SDS-PAGE was performed on 1.5-mm 8 to 18% gradient slab gels (5) by using the Laemmli system (26). Molecular weights were determined on Coomassie Blue-stained gels by using Bio-Rad m.w. standards. Gels containing ^{35} S-labeled proteins were fluorographed, and radioautography was performed as described (5).

Rosette assay. This assay is described in detail elsewhere (3). Briefly, for IgE rosettes, trinitrophenol- (TNP) coated ox erythrocytes (ORBC) were sensitized with a subagglutinating amount of mouse IgE anti-DNP antibody, and IgG rosettes utilized ORBC coated with rabbit IgG anti-ORBC antibody (3). For rosette inhibition, O1.2B2 cells or nonadherent splenic lymphocytes (1×10^6) were first incubated with 10 μ g/ml of the potential inhibitor or with buffer alone for 60 min at 4°C , followed by the respective indicator cells. The cells were counted as described (3) the next day.

B3B4 binding analysis. For determination of B3B4 reactivity with Fc ϵ R on other cell types, three procedures were used. i) acid-stripped cells (1×10^6) were washed and incubated with 10 μ g/ml of biotinylated B3B4-F(ab')₂ for 60 min at 0°C in modified HBSS containing 0.1% sodium azide. The cells were then washed three times in the above buffer, resuspended to 1×10^6 cells/ml, and incubated with either 10 μ g/ml fluorescein isothiocyanate (FITC) conjugated avidin (Vector Laboratories, Burlingame, CA) or 2 μ g of phycoerythrin-avidin (Biomed Corp., Foster City, CA) or with buffer alone for 30 min at 0°C . The cells were then washed once in buffer, were resuspended to 1×10^6 cells/ml, and were analyzed with a FACS II system (Becton Dickinson, Mountain View, CA). ii) Cells (1×10^6) were incubated with 5 μ g/ml of ^{125} I-B3B4 for 60 min at 0°C , and the bound label was determined by the phthalate oil procedure. Positive binding was indicated by comparison to a control containing a 100-fold excess of unlabeled B3B4. iii) Inhibition of IgE rosette formation was as described above.

Stoichiometry of B3B4 binding was determined by incubating a constant number of acid-stripped O1.2B2 cells (2×10^6 /ml) with increasing concentrations of ^{125} I-rat IgE, ^{125} I-B3B4, ^{125} I-B3B4-F(ab')₂, or ^{125} I-B3B4-Fab' in modified HBSS for 90 min at 0°C . The radioactivity associated with the cell pellet was assessed by using the phthalate oil procedure and was corrected for nonspecific binding. The number of ligand molecules bound was determined by using 180,000, 150,000, 100,000, and 50,000 as the m.w. for rat IgE, B3B4, B3B4 F(ab')₂, and B3B4 Fab', respectively.

Fc ϵ R upregulation experiments. O1.2B2 cells were cultured for 12 hr in the presence of medium alone or in the presence of the various ligands described in Results. Subsequently, the cells were collected, and bound IgE or bound mab was removed by acid stripping (acetate buffer, pH 4.0 (5) for IgE removal and 0.05 M glycine, 0.1 M NaCl, 1% FCS, pH 2.5, for B3B4 removal, with cell viability remaining $>90\%$). Cells (1×10^6) were then incubated with 5 μ g of ^{125} I-rat IgE for 90 min in a total reaction volume of 500 μ l. In some experiments the low pH step was omitted, and the Fc ϵ R number was determined by adding 5 μ g of monoclonal ^{125} I-anti-rat- κ for 60 min at 0°C . The cell bound cpm were determined by the phthalate oil procedure, and after correction for nonspecific binding, the data were expressed as the fold increase in receptor number compared with that of noninduced cells [cells cultured in media alone]. The two procedures gave similar Fc ϵ R values, indicating that only one anti- κ can bind per IgE or B3B4.

RESULTS

Analysis of polyclonal antisera. Sera from rats and purified IgG from the goat injected with IgE-Affi/Fc ϵ R

preparations exhibited an excellent capacity to inhibit binding of IgE to O1.2B2 cells (Fig. 1). In both situations the sera or the IgG fraction was first absorbed with IgE-Affi to remove any anti-IgE activity before testing in the binding assay. The polyclonal antisera was also effective with respect to isolation of the Fc ϵ R. Figure 1 (inset) demonstrates that the antisera can be used to isolate highly purified Fc ϵ R. Biosynthetically labeled Fc ϵ R was partially purified on an IgE-Affi column and was subsequently isolated to apparent homogeneity on a goat anti-Fc ϵ R column (lane a). The rat antiserum was also capable of isolating the Fc ϵ R (lane b). The Fc γ receptor at 58 kilodaltons (Kd) (lanes b and c) is isolated due to Fc interaction. Separate experiments (data not shown) demonstrated that both the goat and the rat polyclonal antiserum were reactive with several other cellular constituents, although in two-stage purification procedures, as indicated in Figure 1, the polyclonal antisera is very effective in isolating highly purified Fc ϵ R.

Preparation of monoclonal anti-Fc ϵ R. To circumvent the polyspecificity exhibited by the antisera, a mab was prepared. Spleen cells from a rat exhibiting anti-Fc ϵ R activity in the serum were fused to the IR983F myeloma cells, and the hybridoma supernatants were screened by the RIA for their ability to bind to hybridoma O1.2B2 cells. Of the 300 growth-positive hybrids, only two were consistently cell surface positive by RIA. These two hybridomas were further screened for their capacity to inhibit binding of ^{125} I-rat IgE to O1.2B2 cells. Only one of the hybridomas (B3B4) inhibited rat IgE binding to O1.2B2 cells. B3B4 was cloned by limiting dilution and

was injected into rats. The mab was purified from the ascites, and F(ab') $_2$ and Fab' fragments were prepared.

Immunochemical isolation of Fc ϵ R and Fc ϵ R fragments by using B3B4. O1.2B2 cells were surface labeled, and the cell extract was applied to a B3B4-Affi column or to a F(ab') $_2$ -B3B4-Affi column. SDS-PAGE analysis of the bound proteins is shown in Figure 2. It is evident that the 49-Kd Fc ϵ R is bound by the mab columns. Fc γ_{2b} R at 58 Kd (Fig. 2, lane a) is also isolated unless the F(ab') $_2$ fragment is used (lane b). In addition, the same 49-Kd component can be isolated by using extracts from surface labeled splenic B lymphocytes (lane e); an IgE-Affi eluate from surface labeled B cells is shown for comparison (lane d). Frequently, acid stripping does not completely remove all the cytophilic IgE present on O1.2B2 cells; thus, some labeled IgE is isolated (band at $\approx$ 70 Kd) because of the multivalency of the Fc ϵ R (6).

To determine if Fc ϵ R-like fragments are released from the cell, ^{125} I-surface labeled O1.2B2 cells were cultured in RPMI/c medium for 18 hr, and the supernatant was collected and subjected to F(ab') $_2$ -B3B4 affinity chromatography. The bound material was eluted and the SDS-PAGE analysis is shown (Fig. 2, lane c). Two major Fc ϵ R fragments, 38 Kd and 29 Kd, which still retained B3B4 binding activity, were recovered from the media after the 18-hr culture. The degradation appears to follow a sequential pattern, since at 4 hr, only the 38 Kd fragment is seen. No Fc ϵ R fragments were seen if the culture medium was applied to an irrelevant immunoglobulin column (data not shown).

Reactivity of B3B4 with various cell types. To determine the cell specificity of B3B4, various cell lines or cell preparations were analyzed for B3B4 binding. The results are summarized in Table I. From this data it is evident that B3B4 has a high specificity for the Fc ϵ R on murine B lymphocytes. It is species specific in that it does not react with the B cell Fc ϵ R from rats or humans. A very weak and variable reaction was obtained with 10% of the peritoneal macrophages from Nb-infected mice; however,

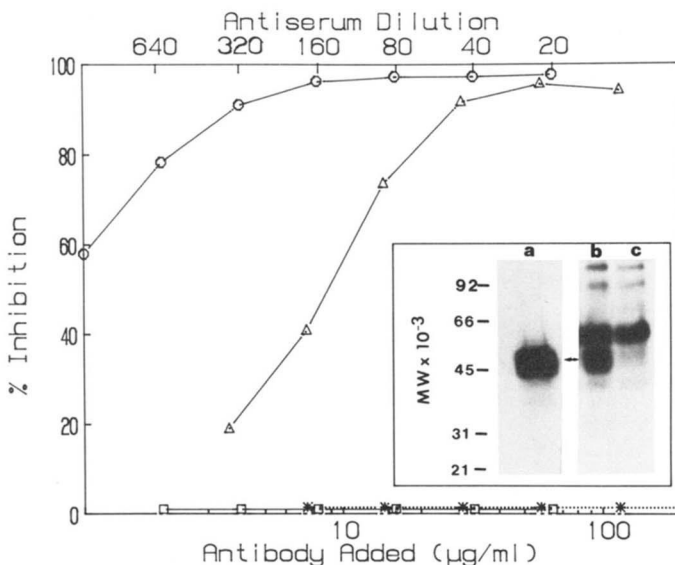


Figure 1. Inhibition of IgE binding by polyclonal goat and rat anti-Fc ϵ R antibodies. O1.2B2 cells (1×10^6 /sample) were incubated with serial dilutions of a rat antiserum (top legend) or with different amounts of purified goat anti-Fc ϵ R IgG (bottom legend). After a 30-min incubation, ^{125} I-IgE (2 μ g/sample) was added, and the cells were incubated for an additional 60 min. Cell bound cpm was determined, and percent inhibition of binding was calculated as described in *Materials and Methods*. (Δ - Δ) goat and ($\circ$ - $\circ$) rat anti-Fc ϵ R, ($\square$ - $\square$) preimmune goat IgG fraction, and ($\ast$ - $\ast$) preimmune rat antiserum, respectively. *Inset.* Immunochemical characterization of polyclonal antisera. (a) [^{35} S]Methionine-labeled lysate (see *Materials and Methods*) was first applied to an IgE-Affi column, and the acid eluate was applied to the goat anti-Fc ϵ R column. In lane (b) 5 μ l of rat antiserum or in (c) 5 μ l of normal rat serum were added to ^{125} I-lysate, and subsequently the immune complexes were isolated on a monoclonal anti-rat- κ column. All isolations used lysates from 2 to 5×10^7 cell equivalents. The arrow indicates the Fc ϵ R. The migration position of m.w. standards is shown on the left.

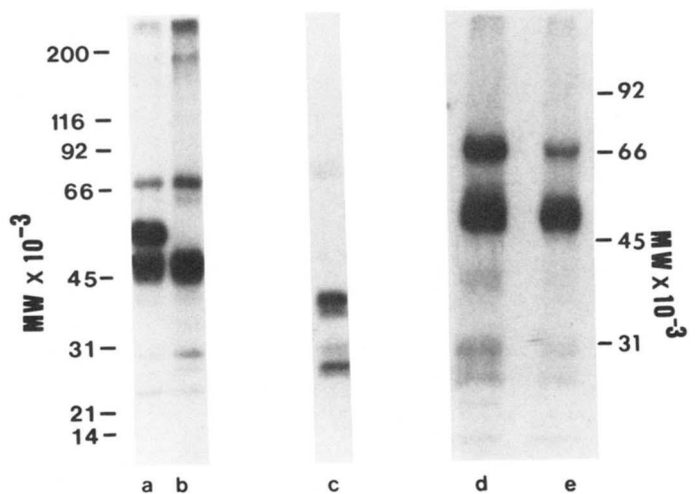


Figure 2. SDS-PAGE analysis of Fc ϵ R and Fc ϵ R fragments by using B3B4. Surface labeled O1.2B2 cells (5 to 10×10^7) were disrupted in lysis buffer, and the lysate was applied to either B3B4-Affi (lane a) or B3B4-F(ab') $_2$ -Affi (lane b). In lane c, surface labeled O1.2B2 cells were cultured overnight, and the cellfree supernatant was applied to B3B4-F(ab') $_2$ -Affi. Lysate from surface labeled splenic B lymphocytes was applied to IgE-Affi (lane d) or B3B4-F(ab') $_2$ -Affi (lane e). The washed Affi columns were eluted with sample buffer, and the eluates were examined by SDS-PAGE. Molecular weight standards for lanes a through c are at left and for lanes d and e are at right.

TABLE I
Reactivity of B3B4 with FcεR from other cell types

Cell Type	Assay ^a	Reactivity of mab with FcεR ^b
Hybridoma O1.2B2	F, R, RIA	+
Murine B lymphocyte	F, R	+
SP2/0	RIA	-
RPMI-8866	RIA	-
Rat spleen cells	F	-
Murine macrophages	F	±
P388D1	RIA	-
Murine T lymphocytes	F, RIA	-
229D3, 220D3 ^c	RIA	-
PT-18	F, RIA	-

^a The reactivity of B3B4 with other cell types was assayed by fluorescence (F), by inhibition of IgE rosette formation (R), or by a direct binding assay (RIA) as described in *Materials and Methods*.

^b +, Indicates a strong reactivity with B3B4; -, indicates no reactivity with B3B4, and ±, indicates a weakly positive reaction.

^c T hybridoma cells (11).

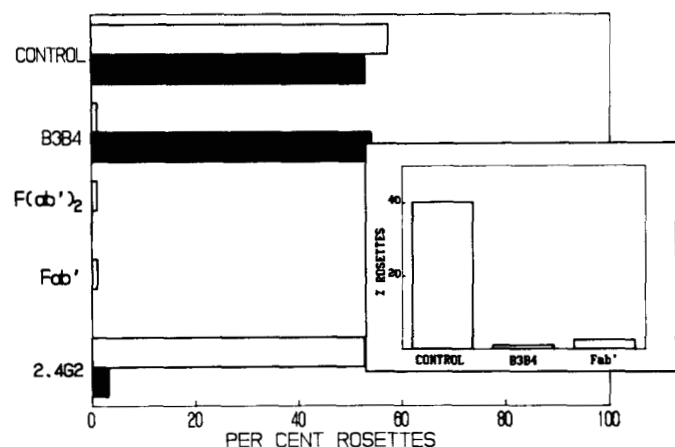


Figure 3. Inhibition of IgE-specific rosettes by monoclonal anti-FcεR. O1.2B2 cells were incubated with 10 μg/ml of B3B4, B3B4-F(ab')₂, B3B4-Fab', or 2.4G2 for 60 min at 4°C. IgE-ORBC (open bars) or IgG-ORBC (solid bars) were then added, and the corresponding IgE-specific or IgG-specific rosettes were determined. Inset, Nonadherent splenic B lymphocytes from BALB/c mice were acid stripped, and the IgE-specific rosettes were determined after preincubation with 10 μg/ml of the B3B4 or B3B4-Fab'.

no reaction was seen with the macrophage line P388D1, which is reportedly FcεR⁺ (27). It is also completely unreactive with the high affinity IgE receptor, as exemplified by its lack of binding to the murine mast cell line PT-18, which has the classical four-subunit IgE receptor (28, 29). We have also been unable to demonstrate any reactivity with either murine T cells from Nb-infected or normal mice or with murine T-T hybridoma cells; the latter are weakly FcεR⁺ (5 to 10%) by rosetting procedures (11). Finally, sorting of BALB/c spleen cells for B3B4-positive cells by the fluorescence-activated cell sorter (FACS) results in the isolation of cells that are 100% surface immunoglobulin positive and 0% Thy-1.2 positive, again supporting the concept that B3B4 is specific for B cells.

Inhibition of IgE binding. A common method for visualizing lymphocyte FcεR activity is by rosette analysis. B3B4, whether intact or in fragment form, completely blocked IgE-specific rosettes both to O1.2B2 cells (Fig. 3) and to splenic lymphocyte preparations (Fig. 3, inset). The data were obtained by using splenic lymphocytes from BALB/c mice, but similar results were obtained with BDF1 and B6 mice. Specificity is seen in that B3B4 had no effect on IgG rosetting, whereas the control anti-

Fcγ_{2b}R mab (2.4G2) blocked the lymphocyte IgG rosettes. The dose dependence of this inhibition of binding was examined in a reciprocal fashion by using ¹²⁵I-rat IgE, ¹²⁵I-B3B4, or ¹²⁵I-Fab'-B3B4 (Fig. 4). It is evident that reciprocal inhibition is seen in that B3B4, whether intact or as a Fab' fragment, inhibited IgE binding in a dose-dependent manner, and in addition, IgE inhibited B3B4 binding. The intact antibody was impressively effective in inhibiting IgE binding; ~50% inhibition of 2 μg/ml ¹²⁵I-IgE binding was achieved with only 0.1 μg/ml of B3B4.

Analysis of the stoichiometry of B3B4 binding. The inhibition results suggested that B3B4 has a higher affinity for the FcεR than does IgE, and this was tested by direct binding analysis. Increasing amounts of ¹²⁵I-B3B4, ¹²⁵I-Fab'-B3B4, or ¹²⁵I-IgE were added to O1.2B2 cells, and after equilibrium was achieved, the resulting specifically bound label was determined. The binding results were linearized by using the Scatchard method (Fig. 5), and the affinity (K_a) for the respective interaction was calculated from the regression lines. The affinity for B3B4 binding is about 10-fold higher than the affinity for the binding of IgE with the Fab'-B3B4, having an intermediate affinity. F(ab')₂-B3B4 behaved essentially identically to the intact mab (data not shown). The maximum number of binding sites for B3B4, Fab'-B3B4, and IgE was determined by extrapolation to the x axis, and as shown, IgE and divalent B3B4 bound to virtually the same number of sites. In contrast, Fab'-B3B4 bound to twice as many sites per cell (2 × 10⁵ vs 1 × 10⁵ for intact B3B4 and IgE). Possible reasons for this interesting finding will be discussed.

Effects of B3B4 on FcεR upregulation. Previous results from our laboratory demonstrated that murine FcεR⁺ hybridoma cell lines, such as the O1.2B2 used in this study, increase their FcεR level when cultured with rat or mouse IgE (8). In view of the close association of IgE and B3B4 binding determinants, as visualized by inhibition studies (Fig. 4), it was of interest to see what effect B3B4 would have on FcεR upregulation. Thus, O1.2B2 cells were cultured overnight with B3B4, F(ab')₂-B3B4,

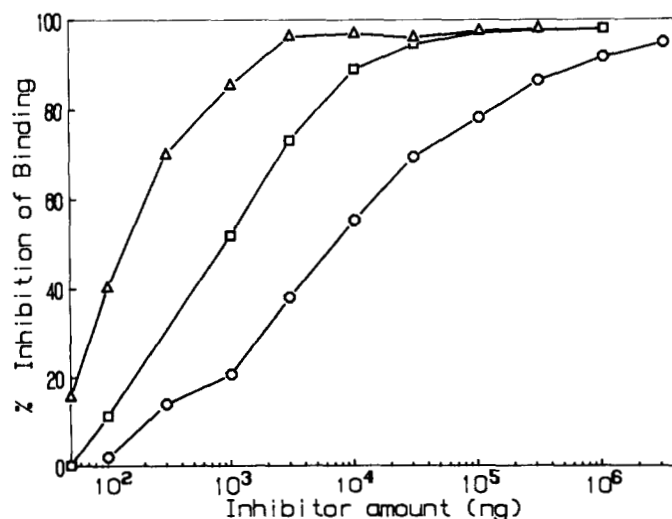


Figure 4. Reciprocal inhibition of binding of IgE and B3B4. O1.2B2 cells (2 × 10⁶ cells/ml) were incubated for 30 min with the indicated amount of inhibitor (0 to 2 mg/ml). Subsequently, ¹²⁵I-mab or ¹²⁵I-rat IgE (2 μg/ml) was added, and after 60 min at 4°C, the cell bound cpm was determined. Inhibition of: (Δ—Δ) IgE binding by B3B4; (□—□) IgE binding by B3B4-Fab'; (○—○) B3B4 binding by IgE.

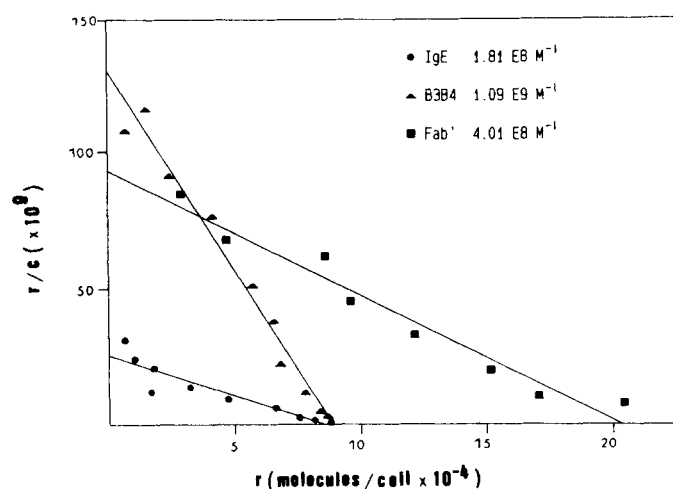


Figure 5. Scatchard analysis of B3B4 and IgE binding. O1.2B2 cells (2×10^6 cells/ml) were incubated with increasing concentrations of ^{125}I -rat IgE (○—○), ^{125}I -B3B4 (△—△), or ^{125}I -B3B4-Fab' (□—□) for 90 min at 0°C . Cell bound cpm was then determined on duplicate aliquots of cells, and specific bound cpm was determined by appropriate correction by using control samples containing a 100-fold excess of unlabeled ligand. The lines shown are derived by linear regression analysis.

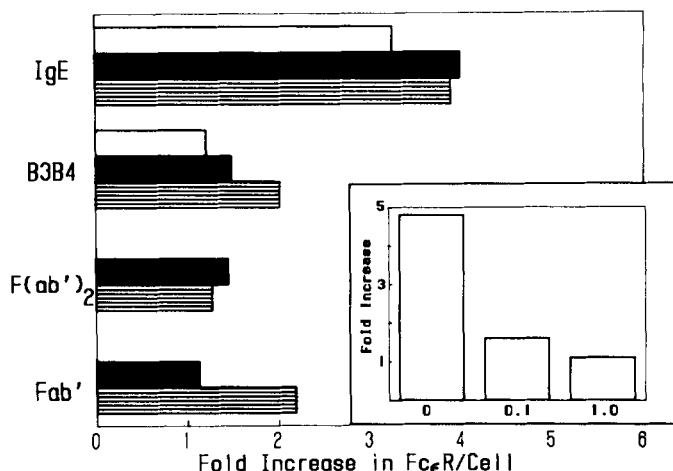


Figure 6. Ligand-specific FcεR upregulation. O1.2B2 cells were cultured overnight with the indicated agent; open bars, solid bars, and striped bars represent 1.0 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$ of this agent, respectively. The cells were then collected, the bound ligand was removed with pH 2.5 glycine buffer (see *Materials and Methods*), and the FcεR number was determined by incubation with ^{125}I -IgE. Alternatively, the low pH step was omitted and the bound ligand was determined by using monoclonal ^{125}I -anti-rat- κ . The results shown are composite averages from five experiments. *Inset*, Coculture experiment. O1.2B2 cells were cultured overnight with 10 $\mu\text{g/ml}$ rat IgE plus the indicated amount of B3B4. The FcεR number was determined by the ^{125}I -IgE method as above. All results are expressed as the relative receptor number; the FcεR level on control (media alone) cells is taken as one, and the fold increase is determined.

or Fab'-B3B4, and the resulting FcεR level was determined after the culture. B3B4 had a minimal effect on FcεR levels (Fig. 6). High doses of B3B4 (100 $\mu\text{g/ml}$) increased the FcεR level 1.5 to 2-fold, however, 1 $\mu\text{g/ml}$ of rat IgE increased the FcεR level more than 3-fold. Thus, the higher affinity of the mab does not translate into its being more effective in causing FcεR upregulation. This observation combined with the inhibition results (Fig. 4) suggested that B3B4 might actually prevent IgE-mediated upregulation. That this is true is shown in the inset of Figure 6; as little as 0.1 $\mu\text{g/ml}$ of B3B4, when added with 10 $\mu\text{g/ml}$ of IgE, almost totally blocked the IgE-mediated FcεR increase on O1.2B2 cells.

DISCUSSION

The initial observations of Vander-Mallie et al. (3) that the murine B lymphocyte had a relatively high affinity for IgE, and the fact that the amount of FcεR on B cells could be increased severalfold by parasite infection (3, 5), prompted us to use this as a model system to further study the low affinity IgE receptor (5-8). In the present study, polyclonal and monoclonal antibodies to the lymphocyte FcεR were characterized. After injection of IgE-Affi containing bound FcεR, the goat and the rats developed anti-FcεR activity. The polyclonal antibody was not monospecific for the FcεR. The polyclonal goat and rat antisera contained antibodies directed against a number of other components (data not shown). However, the major antibody activity was directed against the FcεR. Indeed, this antisera has proved useful in double-purification studies in which IgE Affi eluates from biosynthetically labeled O1.2B2 cells are further purified with goat anti-FcεR affinity chromatography (Fig. 1, *inset*, lane a); only the 49-Kd FcεR is isolated, suggesting that the antibodies against the contaminants resulted from immunization with very low levels of antigen. Single-stage affinity procedures, at least with biosynthetically labeled FcεR receptors, usually give many "bands" upon SDS-PAGE analysis (30), indicating that the preparations are still quite impure, and the lymphocyte FcεR is analogous to other FcR in this respect. This double-purification procedure is similar to the repetitive affinity chromatography procedure described by Kulczycki (30) and has been very useful in studies of posttranslational modification of the FcεR (A. Keegan and D. Conrad, manuscript in preparation).

The most obvious way to circumvent the polyspecificity of the antisera is via monoclonal anti-FcεR. In the two fusions performed with the Lou/m rats producing anti-FcεR antibody, one clone (B3B4) was obtained that reacted with the FcεR. The monoclonal anti-FcεR, when attached to Affi, facilitates the isolation of the 49-Kd FcεR from both O1.2B2 cells and splenic B lymphocytes (Fig. 2). Indeed, when F(ab')₂-B3B4 was employed, only the FcεR was isolated, proving that the mab B3B4 binds to the FcεR through its antigen binding site rather than due to any unusual Fc interaction.

FcεR receptors are antigenically related to immunoglobulin binding factors; this has been observed in both the IgE and the IgG systems (31, 32). Thus, it was of interest to see if FcεR shedding occurred, and B3B4-Affi was used to isolate any potential shed FcεR. As is evident (Fig. 2, lane c), FcεR fragments were released into the media. FcεR fragments were also observed with murine B lymphocytes.³ No intact FcεR was ever observed in the media; because detergents are necessary to solubilize the intact lymphocyte FcεR (5-8), the fragments observed had presumably lost the hydrophobic amino acids that anchored the FcεR in the membrane. Recent data suggest that the FcεR fragments do not bind to an IgE-Affi column.³ Current investigations are aimed at delineating the biological functions, if any, of the shed FcεR fragments.

The high affinity of B3B4 makes FcεR detection quite straightforward. A panel of cell lines and cell preparations was screened to determine whether B3B4 recog-

³ W. T. Lee, M. Rao, and D. H. Conrad. 1986. The murine lymphocyte receptor for IgE. IV. The mechanism of ligand-specific receptor upregulation on B cells. *Submitted for publication*.

nized Fc ϵ R on these cells. With the methods employed in the present study, the mab did not react with any non-B cell phenotype. The weak reactivity found with peritoneal macrophages requires further study, but it could be due either to a low level of contaminating lymphocytes or to nonspecific trapping. The lack of reactivity with T lymphocytes is interesting. Various levels of Fc ϵ R⁺ T lymphocytes have been reported. Chen et al. (33) indicated that $\approx 50\%$ of Fc ϵ R⁺ lymphocytes were T cells, whereas Vander-Mallie et al. (3) found significant numbers of Fc ϵ R⁺ T lymphocytes only after parasite infection, and Katona et al. (34) were unable to observe any Fc ϵ R⁺ T cells. The negative results obtained herein suggest that the Fc ϵ R on T and B lymphocytes are different at least with respect to the epitope recognized by B3B4. In the human system, also, results are presently contradictory, with some groups of investigators reporting reactivity of monoclonal anti-Fc ϵ R with T cells (35, 36), whereas others have found, even with a panel of anti-receptor antibodies, no reactivity with T lymphocytes (37). Our data in the rodent system would conform to the lack of reactivity with T cells. In this regard, the T cells of IgE myeloma-bearing mice have a much higher Fc ϵ R level in that up to 30% of the T cells are Fc ϵ R⁺ (38). In future studies, we will test whether any of the T lymphocytes from such tumor-bearing mice will be both Fc ϵ R⁺ and B3B4⁺. Finally, note that the mab also did not react with the high affinity IgE receptors on murine mast cell lines; this is in line with the observation that the high affinity IgE receptors are structurally and functionally different from the low-affinity Fc ϵ R (7, 39).

B3B4 blocks IgE binding to murine B lymphocytes, whether the binding is measured by rosette formation (Fig. 3) or by direct inhibition of binding (Fig. 4). Indeed, the inhibition studies suggest that the antibody reacts relatively close to the IgE binding site in that B3B4 will effectively block IgE binding, and conversely IgE will block B3B4 binding. The low levels of B3B4 required to inhibit IgE binding indicated that it has a much higher affinity for the Fc ϵ R than IgE has, and this was borne out in the Scatchard analysis (Fig. 5). This analysis also demonstrated the interesting finding that the divalent antibody bound to essentially the same number of sites as IgE and that the Fab' fragment bound to twice as many sites per cell as IgE. A similar finding was seen with a murine mab against the high affinity IgE receptor found on rat basophilic leukemia cells (40), and this led to the suggestion that the receptor exhibited two symmetrical or identical sequences within the same polypeptide. A similar argument could be made here. However, an alternative explanation is that the IgE binding site requires two 49-Kd Fc ϵ R "units" to form one IgE binding site. Previous cross-linking studies have demonstrated that the murine lymphocyte 49-Kd Fc ϵ R associates with both itself and surface immunoglobulin (7). The occurrence of this association makes the latter hypothesis more likely as the correct explanation for the Scatchard results in the case of the lymphocyte receptor.

The close proximity of B3B4 and IgE binding sites indicated the interesting possibility that B3B4, like IgE, would cause Fc ϵ R upregulation. Figure 6 demonstrates that this was not the case. At high levels of F(ab')₂-B3B4 and Fab'-B3B4, a small and variable Fc ϵ R upregulation was seen; but a more consistent and considerably higher

upregulation was seen with 100-fold less IgE. Thus, the results indicate that upregulation is mediated by a site-specific interaction brought about only by rodent IgE. Whether this is due to IgE reacting with a slightly different site than B3B4 or whether the Fc-Fc ϵ R interaction is inherently different than an antigen-antibody interaction will require further study. The fact that B3B4 did not react with the rat B cell Fc ϵ R, whereas rodent IgE obviously does, argues in favor of the different-site explanation. In this context, polyclonal antibodies and mab reacting with IgE-binding factors did cause Fc ϵ R upregulation (31), indicating that antibody- (via antigen site interaction) mediated Fc ϵ R upregulation is possible. In any case, the inefficiency of B3B4 in causing Fc ϵ R upregulation, combined with the observation that it inhibited IgE binding (Fig. 4), suggested that the mab would actually prevent IgE-mediated Fc ϵ R upregulation. The inset of Figure 6 demonstrates this phenomenon in that B3B4 effectively prevented Fc ϵ R upregulation mediated by IgE. Surprisingly, overnight culture with divalent B3B4 did not cause any decrease in the Fc ϵ R numbers due to capping, endocytosis, etc. The mobility of the Fc ϵ R is a matter of current investigation, but these results suggest that B3B4 does not cause extensive Fc ϵ R cross-linking, giving some support to the symmetrical-determinant theory mentioned above. In this regard, preliminary results with the goat polyclonal antibody indicate that it also does not cause Fc ϵ R upregulation; however, the number of IgE-binding sites are decreased after overnight culture with the goat anti-Fc ϵ R (M. Rao, unpublished observations), indicating that extensive cross-linking does cause Fc ϵ R loss.

While this paper was under preparation, three groups (35, 37, 41) reported the production and characterization of mab to the human B lymphocyte Fc ϵ R. To our knowledge, this is the first report of a mab specific to the murine B lymphocyte Fc ϵ R. This mab will greatly aid in purifying large amounts of Fc ϵ R for further biochemical characterization. The function of the low affinity Fc ϵ R is currently the subject of investigation. To a large extent the function appears to depend on the type of cell on which it is expressed. On B lymphocytes, it may play a role in activation and differentiation. Hence, B3B4 will be extremely useful for studying the role of Fc ϵ R on B cells as well as its functional relationship with soluble Fc ϵ R molecules.

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