AUTOIMMUNE MICE MAKE ANTI-Fc_γ RECEPTOR ANTIBODIES

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Hallmarks of autoimmune diseases such as SLE are hypergammaglobulinemia, the production of autoantibodies, and elevated levels of circulating immune complexes. Several mouse strains are used as models of SLE (see reference 1 for review), including NZB, NZB/NZW F_1, MRL/lpr, and BXSB. In human and murine SLE, there is frequently a pronounced inhibition of macrophage binding and phagocytosis of IgG-sensitized erythrocytes and immune complexes (2–4). One possible reason for this inhibition is the downmodulation of the macrophage $Fc_\gamma R$ after binding of immune complexes. An alternate explanation for the inhibition of $Fc_\gamma R$ function might be the binding of anti-$Fc_\gamma R$ autoantibody to the macrophage $Fc\gamma R$.

In this study, we have used a recombinant truncated $Fc_\gamma R$ ($tFc_\gamma R$)[1] to screen murine sera and mAbs for naturally occurring anti-$Fc_\gamma R$ antibody. The $tFc_\gamma R$ is derived from murine $Fc_\gamma RII\beta$, and consists of the two external Ig-like domains of mu$Fc_\gamma RII\beta$, but lacks the transmembrane and cytoplasmic domains of the receptor (5). We report here the presence and characterization of anti-$Fc_\gamma R$ antibodies in sera of some mice genetically prone to autoimmune diseases and anti-$Fc_\gamma R$ hybridomas derived from NZB and motheaten (me^v) mice.

Materials and Methods

mAbs and Sera. The mAbs that we screened for anti-$Fc_\gamma R$ activity were derived from fusions with spleen cells from old NZB mice or young NZB mice after stimulation with LPS (6). Other hybridomas screened were from unstimulated viable me^v mice (7). The IgM mAbs were all purified by affinity chromatography on an anti-κ IgG-Sepharose column and were eluted from the column with 0.1 M glycine HCl, pH 2.3. Samples of sera from NZB, NZB/NZW F_1, MRL/lpr, and BXSB mice were kindly given to us by Dr. Argyris Theofilopoulos (Scripps Clinic, La Jolla, CA). Sera from me^v, tightskin mouse (TSK), C57B16pa/pa, and C58/J were obtained from animals purchased from The Jackson Laboratory (Bar Harbor, ME). Anti-DNP hybridomas DHK109.3 (IgG1) and DHK10.12 (IgG2b) were a gift from Dr. Milton Schlessinger (Washington University Medical School, St. Louis, MO); anti-DNP hybridomas U7.6 (IgG1) and U7-27 (IgG2a) were a gift from Dr. Zelig Eschar (Weitzmann Institute, Rehovot, Israel).

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[1] *Abbreviations used in this paper:* AchR, acetyl choline receptor; EigG, IgG-sensitized erythrocytes; me^v, motheaten; $tFc_\gamma R$, truncated $Fc\gamma R$; TSK, tightskin mouse.

$tFc_\gamma R$. $tFc_\gamma R$ was purified by affinity chromatography on an IgG-Sepharose column from conditioned medium of the D1959 CHO cell line as previously described (5). The purified protein in PBS was denatured, for some experiments, by reduction with 10 mM dithiothreitol at 56°C followed by alkylation with 25 mM iodoacetamide. The alkylated receptor was then dialyzed against PBS.

ELISA. Anti-$Fc_\gamma R$ Ig was assayed by ELISA. Flat-bottomed microtiter plates (Immulon-2; Dynatech Laboratories Inc., Alexandria, VA) were coated overnight with either native or denatured $Fc_\gamma R$ (2.5 μg/ml in 0.1 M $NaCO_3$ buffer, pH 9.5) and then blocked with 1.0% NP-40 in PBS. $Fc_\gamma R$-coated plates were then incubated with serum samples diluted in 1% NP-40/PBS. Anti-$Fc_\gamma R$ antibodies were detected by sequential addition of biotinylated goat $F(ab')_2$ anti-mouse IgM or biotinylated goat anti-mouse IgG (0.5 μg/ml) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and streptavidin-horseradish peroxidase (0.5 μg/ml) (Kirkegaard & Perry Laboratories, Inc.), and developed as described previously (5). The rat mAb 2.4G2 was detected using a horseradish peroxidase goat anti-rat IgG reagent (Kirkegaard & Perry Laboratories, Inc.). For competition assays, protocols were similar except that the mAbs were incubated (1 h, room temperature) with serial dilutions of either native $tFc_\gamma R$ or mAb 2.4G2 before they were adsorbed onto the $tFc_\gamma R$-coated plates. All experiments were done in triplicate and the variation between the triplicates was <10%.

Binding Assays. Binding of mAbs to cells was determined by FACS analysis. J774 cells (a macrophage-like cell line) were incubated in suspension ($0.5–1.0 \times 10^6$ in 100 μl) with mAbs directly conjugated to FITC, sera, or affinity-purified Ig diluted in PBS containing 1% BSA (1 h, 4°C), and washed with PBS-1% BSA. For experiments with mouse sera or affinity-purified mouse Ig, bound Ig was visualized after incubation (1 h, 4°C) with either biotinylated goat $F(ab')_2$ anti-mouse IgM or biotinylated goat anti-mouse IgG followed by streptavidin-FITC (Kirkegaard and Perry Laboratories, Inc.) used at manufacturer's recommended dilution. After fixation in 1% neutral buffered formalin, the cells were analyzed by flow cytometry on a Epics cytofluorograph (Coulter Electronics Inc., Hialeah, FL) with three decades of amplification. Mean fluorescence peak channels were converted to relative fluorescence to compare the samples.

Affinity Chromatography. A $tFc_\gamma R$ affinity column was made by coupling 4 mg of $tFc_\gamma R$ to 1 ml of Reactigel-HW65 (Pierce Chemical Co., Rockford, IL) according to manufacturer's instructions. The efficiency of coupling was >90%. Serum (0.6 ml) was passed over a 3 × 100-mm column, which was then washed with 0.1 M $NaPO_4$, pH 7.4. Bound Ig was eluted with 0.1 M glycine HCl, pH 2.3, neutralized with .1 vol of 1 M Tris HCl, pH 8.5, and the amount of IgM and IgG was analyzed by ELISA with IgM and IgG standards.

Inhibition of Binding Immune Complexes by Anti-$Fc_\gamma R$ mAbs and Mouse Sera. 10 mg BSA in 1 ml of PBS was incubated with 1 mg trinitrobenzene sulfonic acid to introduce the TNP moiety. To remove the uncoupled hapten, the mixture was passed over a small Sephadex G25 column. The molar ratio of BSA/TNP was 1:20 (8). Anti-DNP antibody U7.6 (IgG1) was conjugated with FITC, and an optimum ratio of FITC-U7.6/DNP_{20}-BSA for binding of immune complexes to J774 cells was determined by FACS. In experiments here, we used a 4:1 ratio of FITC-U7.6/DNP_{20}-BSA. Inhibition of the binding of FITC-immune complexes to J774 cells by anti-$Fc_\gamma R$ antibodies and sera from different mouse strains was examined by FACS. J774 cells were preincubated (1 h, room temperature) with dilutions of test sera or mAbs, washed, and incubated with the FITC-immune complexes (1 h, 4°C).

Results

Detection of Anti-$Fc_\gamma R$ Antibodies in Mouse Serum. To detect the presence of anti-$Fc_\gamma R$ antibodies in sera, we developed an ELISA using microtiter plates coated with a recombinant truncated murine $Fc_\gamma RII\beta$ secreted by CHO cells (5). The protein consists solely of the two Ig-like extracellular domains. Since the native $tFc_\gamma R$ binds IgG, $tFc_\gamma R$ used to coat the ELISA plates was reduced and alkylated, a procedure that destroys the epitope recognized by mAb 2.4G2 and also ablates the binding of immune complexes.

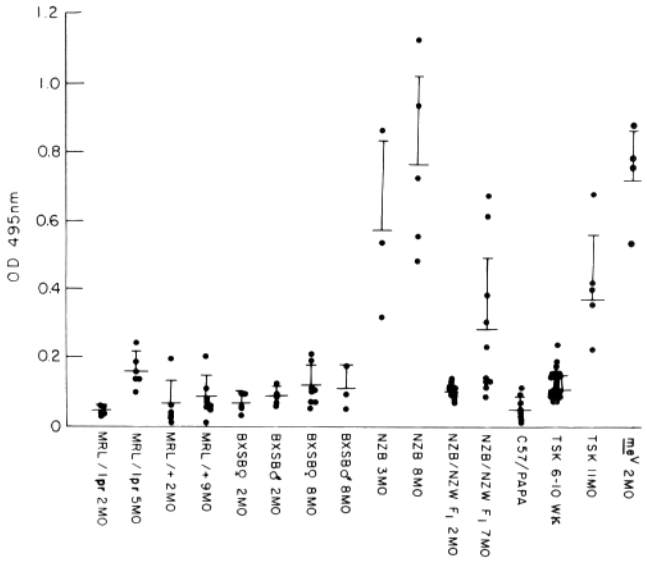

FIGURE 1. Anti-FcγR IgM in sera of autoimmune mice. Individual serum samples diluted 1:100 in 1% NP-40/PBS were assayed on microtiter plates coated with reduced and alkylated tFcγR, and developed with biotinylated anti-IgM antibody as described in Materials and Methods.

We found that several strains of mice, most notably NZB, NZB/NZW F_1, me^v, and TSK, have readily detectable anti-FcγR IgM in their serum (Fig. 1). The titer of anti-FcγR antibody for TSK, NZB/NZW F_1, and, to a lesser degree, MRL/lpr mice, increased with the age of the mice. Although in this set of sera the titers of anti-FcγR IgM in 3-mo-old vs. 8-mo-old NZB mice were not significantly different (Fig. 1), a second set of sera from NZB mice showed a clear correlation between anti-FcγR IgM titer and age (Fig. 2). Surprisingly, the older BXSB males, which develop a severe form of SLE, did not have a significant level of anti-FcγR IgM.

Titration of two serum samples from autoimmune mice and a normal C58/J serum for anti-FcγR IgM on tFcγR-coated and control microtiter plates is shown in Fig. 3. The C58/J serum gives no signal either on tFcγR or control wells at any dilution. Although the autoimmune sera show a slightly higher background than the C58/J serum, there is a >10-fold difference between the tFcγR-coated and the control wells. We chose as controls C58/J mice since old animals produce small amounts of anti-

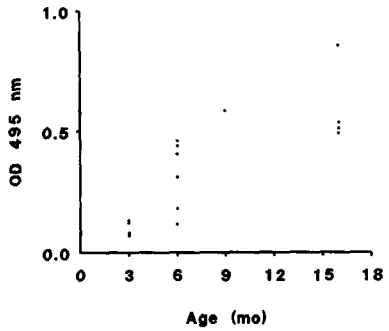

FIGURE 2. A set of sera from NZB mice of different ages was assayed for anti-FcγR IgM titer as described in Fig. 1.

DNA antibodies but do not develop a lupus-like disease. The IgM levels of the autoimmune mice are at most threefold higher than levels of normal mice (1). Therefore, results in Fig. 3 could not be due to elevated levels of IgM in sera of autoimmune mice compared with normal controls. To exclude the possibility that the binding of immune complexes in the serum of mice with autoimmune disease might explain these results, we centrifuged normal and autoimmune sera in an airfuge (Beckman Instruments) for 20 min at 100,000 *g*. The resultant decrease of 30–50% in the titer of anti-$Fc_\gamma R$ IgM is probably due to sedimentation of IgM in the short path length of the airfuge rotor (data not shown).

Mice that make IgM anti-$Fc_\gamma R$ antibody also make IgG anti-$Fc_\gamma R$ antibody, a fact that indicates that the response is not limited to IgM. There is a positive correlation between titers of IgM anti-$Fc_\gamma R$ antibody and IgG anti-$Fc_\gamma R$ antibody in individual sera (Fig. 4). Internal IgM and IgG controls confirm the IgG and IgM specificity of the reagents used in these assays.

Affinity Purification of anti-$Fc_\gamma R$ Antibody. To determine with more precision the levels of anti-$Fc_\gamma R$ Ig and to confirm the ELISA data indicating that there is little anti-$Fc_\gamma R$ Ig in normal mouse serum, we subjected serum pooled from 8-mo-old NZB female and 12-mo-old C58/J female mice to affinity chromatography on a $tFc_\gamma R$-Reactigel-HW65 column. After elution with 0.1 M glycine HCl, pH 2.3, the eluate was analyzed for IgM and IgG by ELISA with appropriate standards. We found 16 μg/ml of anti-$Fc_\gamma R$ IgM in pooled serum from aged NZB female mice, but no IgM was eluted after affinity chromatography pool of serum from C58/J female mice. The eluted material from the $tFc_\gamma R$ column was also assayed for IgG. Eluates from both the C58/J and the NZB serum samples contained 90 μg of IgG/ml of serum. However, since the $tFc_\gamma R$ coupled to the column was not denatured, we interpret this as reflecting primarily binding of IgG to the $tFc_\gamma R$, and not antigenic specificity of the IgG.

To examine the specificity of the IgG eluted from the $tFc_\gamma R$ column, we assayed fractions on microtiter plates coated with reduced and alkylated $tFc_\gamma R$. IgG affinity purified from the NZB serum bound to the denatured $tFc_\gamma R$, and showed a decrease in OD 495 nm with increasing dilution. In contrast, the IgG eluted from the C58/J serum barely exceeded the background of the IgG2a myeloma control, and the OD

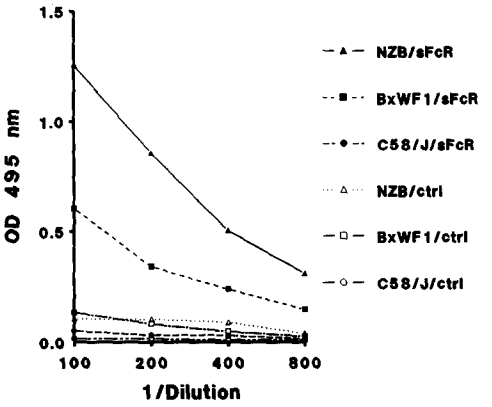

FIGURE 3. Titration of anti-$Fc_\gamma R$ IgM. Sera from an 8-mo-old NZB mouse (△, ▲); a 7-mo-old NZB/NZW F_1 mouse (□, ■), and a 12-mo-old C58/J mouse (○, ●) were diluted in 1% NP-40/PBS and assayed on control microtiter plates (△, □, ○) or plates coated with reduced and alkylated $tFc_\gamma R$ (▲, ■, ●).

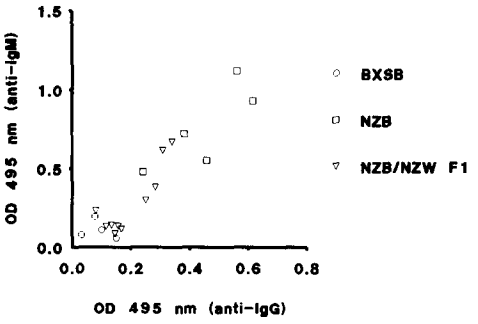

FIGURE 4. Correlation of anti-$Fc_\gamma R$ IgM and IgG. Individual sera (diluted 1:100 in NP-40/PBS) from BXSB (○), NZB (□), or NZB/NZW F_1 (△) mice were assayed on plates coated with reduced and alkylated $tFc_\gamma R$ and developed with specific IgG or IgM reagents as described.

495 nm did not vary with dilution (Fig. 5). Since the same amount of IgG was isolated from the C58/J serum as from the NZB serum, we assume most of the IgG is nonspecific. Therefore, the binding we detect in this experiment reflects high affinity anti-$Fc_\gamma R$ IgG present at concentrations <2.5 μg/ml.

In addition to binding to $tFc_\gamma R$-coated plates, the affinity-purified Ig from the NZB serum bound to J774 macrophages to a much greater extent than either affinity-purified Ig from C58/J serum or isotype controls (Table I, Fig. 6). We also examined the staining of macrophages by serum from C58/J, NZB and me^v mice. The autoimmune sera, but not the C58/J serum, contained IgM that bound to J774 cells. Surprisingly, we did not find any staining of J774 cells by NZB or me^v sera when the cells were stained with an anti-IgG reagent.

Anti-$Fc_\gamma R$ mAbs. Since the sera of NZB mice had a high titer of anti-$Fc_\gamma R$ antibody, we decided to screen by ELISA a panel of mAbs from autoimmune mice (6). Out of 20 IgM mAbs from fusions with LPS-stimulated spleen cells from 3-mo-old NZB mice, one anti-$Fc_\gamma R$ IgM, ZK2H5-3, was found; out of 20 mAbs isolated from fusions with spleen cells from unstimulated 16-mo-old NZB mice, two more IgM mAbs, ZL37-9 and ZL173-7, were identified (Fig. 7). We found, in addition, an anti-$Fc_\gamma R$ IgM mAb, UN40-6, from a fusion with me^v mouse spleen cells (7). Fig. 7 shows the binding of these mAbs to native and reduced and alkylated $tFc_\gamma R$, as well as the binding of monomeric IgG1 and mAb 2.4G2 under the same conditions. Because IgM does not bind to $Fc_\gamma R$, we could use native $Fc_\gamma R$ to coat ELISA plates without fear of artifactual binding of IgM. We found that ZL37-9 and ZL173-7 bound

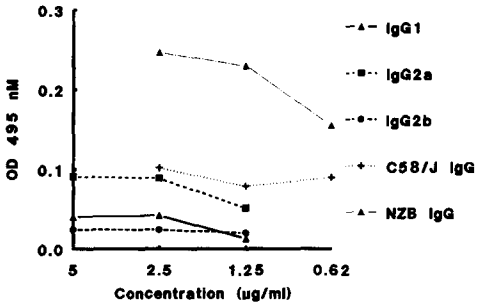

FIGURE 5. Titration of affinity-purified IgG. Isotype controls and affinity-purified Ig were adsorbed onto microtiter plates coated with reduced and alkylated $tFc_\gamma R$, and assayed with biotinylated anti-mouse IgG. The amount of IgG in the protein eluted from the $tFc_\gamma R$ column from the NZB and C58/J serum pools was assayed by ELISA.

TABLE I

Binding of Affinity-purified IgG and Sera to J774 Cells

Sample	Percent positive cells	
	Anti-IgG	Anti-IgM
IgG1 (DHK109.3)	2.6	
IgG2a (U7-27)	2.9	
IgG2b (DHK10.12)	2.5	
C58/J Ig (affinity purified)	8.4	
NZB Ig (affinity purified)	32.2	
C58/J serum (12 mo)	2.6	1.8
NZB serum (3 mo)	1.4	10.8
NZB serum (8 mo)	3.3	16.6
me^v serum (2 mo)	1.3	19.4

Cells were incubated with isotype controls (5 μg/ml), with affinity-purified IgG (2.5 μg of IgG/ml), or with serum (1:100 dilution) diluted in PBS containing 1% BSA. The cells were stained with biotinylated reagents as described in Materials and Methods. The percentage of cells that were positive was estimated by setting the cursor so that ~1% of the control without mAb were positive.

better to native tFc$_\gamma$R than to reduced and alkylated tFc$_\gamma$R, whereas ZK2H5-3 bound equally to both forms. The 2.4G2 epitope is completely inactivated by reduction and alkylation, and the binding of IgG1 is also strongly inhibited.

We examined the avidity of binding of the IgM anti-Fc$_\gamma$R mAbs to native tFc$_\gamma$R-coated microtiter plates (Fig. 8). In several experiments the maximal binding of mAb ZK2H5-3 was less than ZL37-9 and ZL173-7. ZL37-14 is an IgM mAb control. ZL37-9 had the poorest avidity, ~10^8 M^{-1}, while the other two have an apparent K_a of 10^9

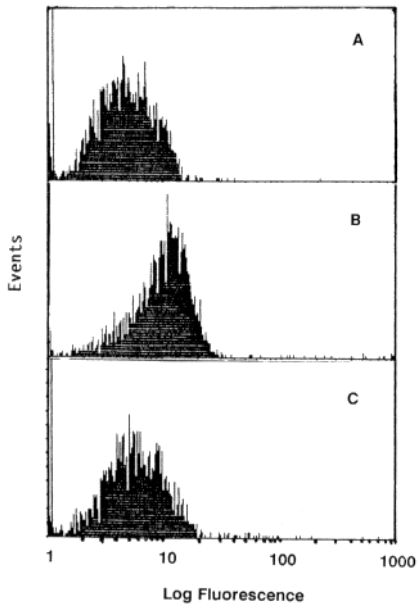

FIGURE 6. FACS analysis of J774 cells incubated with Ig purified by affinity chromatography. Cells were stained with 5 μg/ml of IgG2a (*A*), or 2.5 μg/ml of total IgG isolated by affinity chromatography on a tFc$_\gamma$R column from either old NZB mice (*B*) or C58/J mice (*C*), and bound IgG was visualized with biotinylated goat anti-mouse IgG and streptavidin-HRP as described. The mean peak channel numbers were: (*A*) IgG2a, 56; (*B*) NZB, 88; (*C*) C58/J, 61.

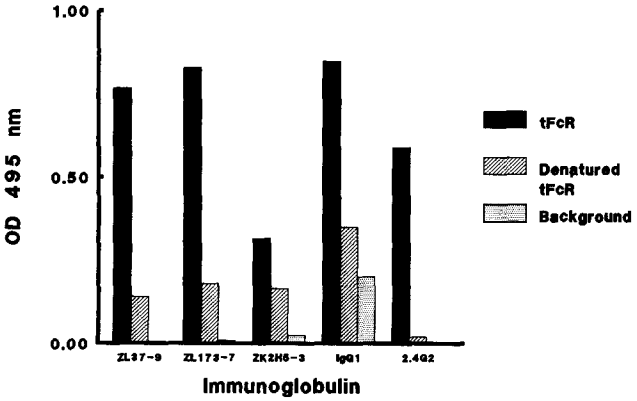

FIGURE 7. Binding of mAbs to tFcγR-coated microtiter plates. The concentrations of mAbs used were: ZL37-9, 2.5 μg/ml; ZL173-7, 0.63 μg/ml; ZK2H5-3, 2.5 μg/ml; DHK-109.3 IgG1, 10 μg/ml; 2.4G2, 10 μg/ml. Secondary antibodies were specific for either mouse IgM, mouse IgG, or rat IgG as appropriate.

M^{-1}. This high avidity probably reflects the dense coating of the ELISA plates with FcγR, which results in multivalent cooperative binding of IgM.

We examined the binding of the mAbs to J774 cells. To eliminate uncertainties inherent in the use of second antibody reagents, we derivatized the mAbs directly with FITC. The mAbs ZL37-9 and ZL173-7 stain murine macrophage cell lines poorly. This probably reflects a low intrinsic affinity of the antibodies. ZK2H5-3 and UN40-6 stain J774 cells intensely, but since these mAbs have so many specificities, we cannot unequivocally state that the mAbs are binding to the FcγR. The positively stained population examined by fluorescence microscopy showed a surface membrane fluorescence pattern typical of membrane antigens.

The specificity of three anti-FcγR mAbs was examined further by competition assays with both tFcγR (Fig. 9 *A*) and mAb 2.4G2 (Fig. 9 *B*) (9), which is directed against muFcγRII. All mAbs were inhibited by tFcγR. The inhibition by mAb 2.4G2 varies for the different mAbs. ZK2H5-3 is inhibited by 50–100-fold less 2.4G2 IgG than are ZL37-9 and ZL173-7. However, from these results, one cannot conclude that the antibodies ZL37-9 and ZL173-7 recognize similar epitopes since steric hindrance could account for the results. The data, however, confirm the anti-FcγR specificity of the IgM anti-FcγR mAbs.

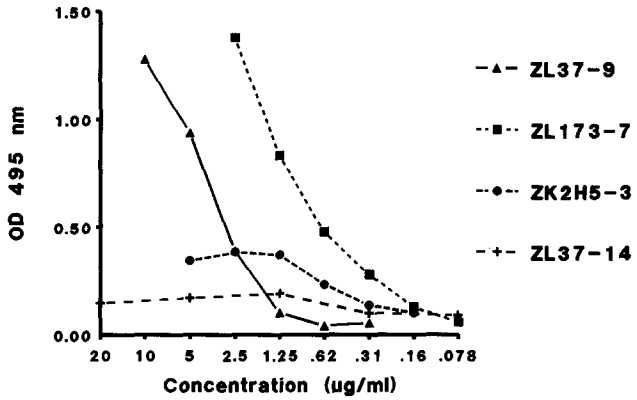

FIGURE 8. Titration of anti-FcγR IgM antibodies. Assays were performed on tFcγR-coated microtiter plates. ZL37-14 is an IgM mAb with specificity for DNA and histone.

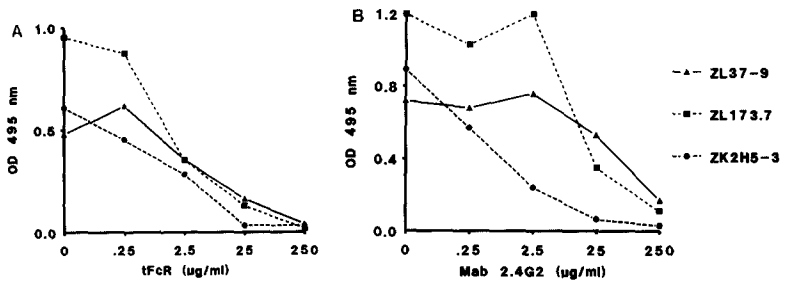

FIGURE 9. Competition by $tFc_\gamma R$ or mAb 2.4G2 of the binding of anti-$Fc_\gamma R$ mAbs to $tFc_\gamma R$-coated microtiter plates. (*A*) Competition by $tFc_\gamma R$. Samples were preincubated for 1 h at room temperature before assay. (*B*) Competition by anti-$Fc_\gamma R$ mAb 2.4G2. mAb 2.4G2 was added to the wells at the start of the assay.

The 40 mAbs that we screened had been examined previously for reactivity with a variety of autoantigens, including DNA, histones, type I-IV collagens, an IgG2a myeloma protein, myelin basic protein, intrinsic factor, thyroglobulin, thyroid-stimulating hormone receptor, acetyl choline receptor (AchR), transferrin, murine bromelain-treated RBC membranes, and topoisomerase I (6, 7). ZL173-7, in addition to binding $Fc_\gamma R$, also binds to procaryotic and eukaryotic dsDNA. ZL37-9 binds to dsDNA and to fish AchR. ZK2H5-3 is a multispecific antibody, binding to histones 1, 2a, 2b, and 4, AchR, and thyroglobulin. We found that the binding of the DNA-specific mAbs ZL173-7 and ZL37-9 to DNA-coated plates is inhibited by $tFc_\gamma R$ and, reciprocally, the binding to $tFc_\gamma R$-coated plates is inhibited by preincubation with DNA (data not shown). These observations imply that complexes of IgM mAbs ZL173-7 and ZL37-9 with DNA could also bind to $Fc_\gamma R$ molecules on the plasma membranes of phagocytes, mesangial cells, and $Fc_\gamma R$-bearing lymphocytes. Such multivalent complexes might have a higher avidity for $Fc_\gamma Rs$ on the cells than the IgM alone. Similarly, if mAb ZK2H5-3 formed an immune complex with nucleoprotein (since it binds to histones), the immune complex could also bind to $Fc_\gamma R$ molecules present on cells.

Inhibition of Immune Complex Binding to J774 Cells. We have shown that antibodies in sera from autoimmune mice and some mAbs bind to $tFc_\gamma R$ and also to macrophages. To demonstrate that the anti-$Fc_\gamma R$ antibodies could alter function, we next looked for inhibition of immune complex binding to J774 cells (Table II, Fig. 10). ZL37-9 and ZL173-7, both of which bind to dsDNA, significantly inhibited immune complex binding to macrophages. ZL37-9 inhibits immune complex binding better than does ZL173-7, although the former binds with lower avidity to $tFc_\gamma R$-coated microtiter wells. ZK2H5-3 and UN40-6, although they bind far better to macrophages (Table III), are poorer inhibitors than either of the two anti-$Fc_\gamma R$ mAbs with DNA specificity.

Sera from old female NZB mice inhibit binding of immune complexes. The inhibition is apparent even at a dilution of 1:400 (Fig. 10, *B*, *C*, and *D*). The me^v sera inhibited even more strongly than the NZB serum (Fig. 10, *F*, *G*, and *H*). In contrast, a 1:100 dilution of C58/J serum had no effect on the binding of immune complexes to macrophages (Fig. 10 *E*).

TABLE II
Inhibition of Immune Complex Binding to J774 Cells

Completing mAbs	Concentration	Mean fluorescence peak channel	Inhibition
	μg/ml		%
Control J774 cells with complexes		158	-
ZK2H6-2	5	155	6.1
	1	156	4.4
	0.2	160	-6.8
ZL37-9	5	106	75.3
	1	116	67.5
	0.2	133	48.2
ZL173-7	5	109	73.2
	1	135	55.7
	0.2	160	-6.7
ZK2H5-3	5	99	79.5
	1	151	16.4
	0.2	163	-15.4
UN40-6*	5	110	54.8
	1	133	15.8
	0.2	130	22.5

J774 cells were preincubated for 1 h at room temperature with mAbs diluted in PBS-1% BSA, washed, and incubated with FITC-U7.6/DNP_{20}/H/BSA complexes (20 μg/ml U7.6, 5 μg/ml DNP_{20}/H/BSA) at 4°C for 1 h, washed, and fixed.

* UN40-6 data comes from a different experiment in which the mean fluorescence peak channel for J774 cells incubated with immune complexes was 140.

Discussion

Mice that are used as models for SLE make autoantibodies against many different antigens (see reference 1 for review). Autoantibodies directed against specific proteins have been identified as pathologic agents in several diseases, including cyclic amegakaryocytic thrombocytopenia (blockade of granulocyte/macrophage-CSF action) (10), vasculitis (antibody to vascular endothelial antigens) (11), autoimmune hemolytic anemia in NZB mice (12), acquired angioedema (anti-C1 esterase inhibitor) (13), myasthenia gravis (14), and Graves' disease (15). This report identifies the presence, in the sera of several inbred strains prone to autoimmune diseases, of both IgG and IgM antibody directed against the external Ig-like domains of $Fc\gamma RII$. These antibodies were identified by an ELISA in which the plates were coated with either a recombinant $tFc\gamma RII$, consisting of the external Ig-like domains, or reduced and alkylated $tFc\gamma RII$. We believe that the recombinant $tFc\gamma R$ is a useful probe to screen for anti-$Fc\gamma R$ autoantibody since the recombinant protein is glycosylated, binds IgG, and bears epitopes recognized by a variety of monoclonal murine anti-$Fc\gamma R$ reagents (5, and unpublished results). Rabbit antisera elicited by the $tFc\gamma R$ immunoblot and immunoprecipitate murine $Fc\gamma R$ and will stain murine $Fc\gamma R$-bearing cells brightly (data not shown).

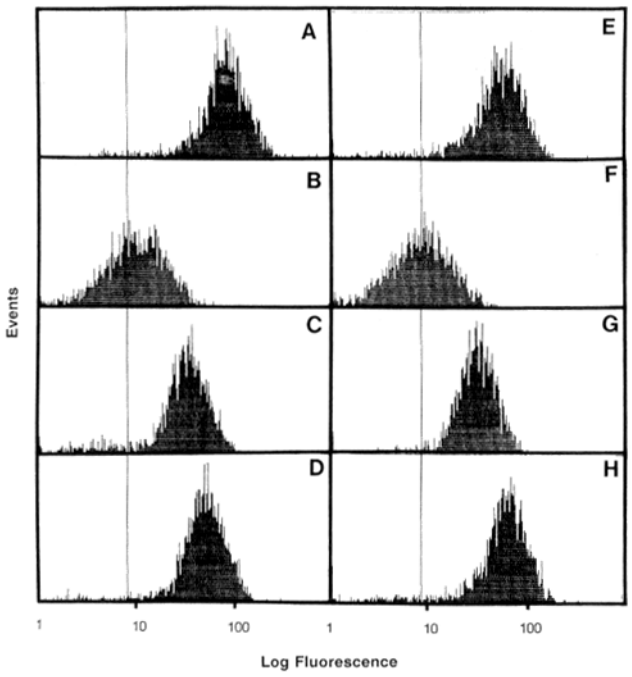

FIGURE 10. Inhibition of immune complex binding to J774 cells by mouse sera. (*A*) The binding of immune complexes to cells incubated in PBS/1% BSA. Cells were preincubated with 1:100, 1:200, and 1:400 dilutions of 6-mo-old female NZB mice sera (*B*, *C*, and *D*); 1:100, 1:400, and 1:800 dilutions of 2-mo-old me^v mice sera (*F*, *G*, and *H*); and a 1:100 dilution of C58/J serum (*E*). Mean peak channels were: *A*, 158; *B*, 80; *C*, 135; *D*, 149; *E*, 156; *F*, 81; *G*, 126; *H*, 155. Relative to the control in *A*, these correspond to the following inhibitions: *B*, 88%; *C*, 46%; *D*, 21%; *E*, 2.7%; *F*, 87%; *G*, 57%; *H*, 7.8%.

To eliminate possible artifacts due to binding of IgG or immune complexes in the ELISA of murine sera, we coated the microtiter plates with $tFc_\gamma R$ that had been inactivated by reduction and alkylation. The presence of anti-$Fc_\gamma R$ IgM and IgG in sera from autoimmune mice was readily detectable at dilutions from 1:100 to 1:300 in the ELISA. There was a positive correlation between the levels of anti-$Fc_\gamma R$ IgM and IgG found in the sera. However, the titer of anti-$Fc_\gamma R$ antibodies varied considerably among the different autoimmune strains. NZB, NZB/NZW F_1, TSK, and me^v mice had high titers of anti-$Fc_\gamma R$ IgM that increased with age for the first three strains of mice. Young MRL/lpr had no anti-$Fc_\gamma R$ activity, but old MRL/lpr

TABLE III

Binding of IgM mAbs to J774 Cells

mAb	Specificity	Mean fluorescence peak channel
Control unstained cells		106
ZL37-9	$Fc_\gamma R$, DNA, AchR	108
ZL173-7	$Fc_\gamma R$, DNA	108
ZK2H5-3	$Fc_\gamma R$, histones, AchR, thyroglobulin	141
UN40-6	$Fc_\gamma R$, thyroglobulin, Sm, transferrin, myelin basic protein, IgG, RBC, thymocytes	199

Cell were stained with mAbs (5 μg/ml) (6, 7) as described and analyzed by FACS.

exhibited modest levels of anti-$Fc\gamma R$ antibodies. BXSB mice, including old males that have severe SLE, do not make anti-$Fc\gamma R$ Ig.

To quantify the amount of anti-$Fc\gamma R$ in autoimmune mice and to confirm its absence in sera of normal mice, we performed affinity chromatography of serum from both groups on a column coupled with $tFc\gamma R$. The amount of anti-$Fc\gamma R$ IgM in pooled sera from old NZB female mice was 16 μg/ml serum, ~2% of the total IgM. There was no IgM recovered after affinity chromatography of serum from old C58/J mice. The purified NZB anti-$Fc\gamma R$ IgM was of low avidity. We could not detect binding to $tFc\gamma R$-coated plates or to J774 cells at 0.2 μg/ml IgM (data not shown). However, the IgG fraction of the purified material bound well to $Fc\gamma R$ on macrophages and bound to $tFc\gamma R$-coated plates. Since we purified the Ig on a native $tFc\gamma R$ column, we do not know the absolute amount of high affinity anti-$Fc\gamma R$ IgG.

We also examined the binding of IgM and IgG in autoimmune and control sera to J774 cells. IgM, but not IgG, from serum of autoimmune mice with high titers of anti-$Fc\gamma R$ Ig bound to mouse macrophages. This paradoxical result may be due to occupation of the binding site of high affinity anti-$Fc\gamma R$ IgG with soluble $Fc\gamma R$, which is present in sera of mice and elevated in mice infected with schistosomes (16, 17). Results of Pure et al. (16) suggest that a major source of serum $Fc\gamma R$ may be released from activated B cells, and autoimmune mice might thus be expected to have high serum $Fc\gamma R$ levels. The soluble $Fc\gamma R$ level in normal mouse serum is $\sim 6 \times 10^{-8}$ M (15), which would be sufficient to neutralize ~1 μg/ml of high avidity anti-$Fc\gamma R$ IgG in serum.

Russell and Steinberg (3) have examined the binding and phagocytosis of IgG-sensitized erythrocytes (EIgG) by resident peritoneal macrophages isolated from SLE-prone mice. NZB, NZB/NZW F_1, and MRL/lpr mice all had profound defects in both binding and phagocytosis of EIgG relative to control inbred mice. The $Fc\gamma R$-defect was not reversible by culture in vitro nor by stripping with pronase (18). However, there is no intrinsic defect in the macrophages, since macrophages derived from bone marrow of autoimmune animals, or peritoneal macrophages elicited by inflammatory agents such as thioglycollate or *Corynebacterium parvum*, showed normal binding and phagocytosis of EIgG (18). There is clear evidence that in vitro culture of macrophages on immune complex–coated surfaces results in downmodulation of $Fc\gamma R$ function (19). Thus, the thesis that the $Fc\gamma R$ defect in autoimmune mice is due to immune complexes downmodulating the $Fc\gamma R$ is reasonable.

We would like to suggest, however, that anti-$Fc\gamma R$ autoantibodies rather than immune complexes, per se, are responsible for inhibition of $Fc\gamma R$ function in vivo. We find high levels of anti-$Fc\gamma R$ Ig in NZB, NZB/NZW F_1, TSK, and me^v mice, and somewhat elevated levels of anti-$Fc\gamma R$ in old MRL/lpr mice, all of which have impaired $Fc\gamma R$ function. Male BXSB mice, which have a severe form of SLE, show normal macrophage binding and phagocytosis of EIgG (3), and we find that these mice have no anti-$Fc\gamma R$ Ig. The compromised $Fc\gamma R$ function seen in other autoimmune strains may be related to the presence of anti-$Fc\gamma R$ antibody, or immune complexes containing anti-$Fc\gamma R$ IgM or IgG. In agreement with this thesis, we found that serum from old NZB and me^v mice dramatically inhibited binding of soluble immune complexes, whereas the C58/J serum had no effect. We show that IgM from NZB and me^v mice binds to J774 cells, but did not find binding of IgG from the same sera to the cells. We therefore believe that the inhibition of $Fc\gamma R$ function we

observed with these sera is not due to IgG immune complex binding but to the binding of IgM antibody with anti-$Fc_\gamma R$ specificity.

The anti-$Fc_\gamma R$ IgM mAbs that we identified by screening IgM NZB hybridomas (6, 7) were multispecific. Out of a total of 40 hybridomas, we found three anti-$Fc_\gamma R$ IgM mAbs. Two of these, ZL-37 and ZL-173, also had anti-DNA specificity, and a third, 2K2H5, was directed against a variety of antigens, including histones, acetylcholine receptor, and thyroglobulin. A fourth anti-$Fc_\gamma R$ IgM, UN40-6, from a hybridoma panel made from me^v, was directed against a variety of antigens, including thyroglobulin, Sm antigen, transferrin, myelin basic protein, and IgG2a (7). The isolation of IgM antibody with diverse specificities has been reported previously (see reference 20 for review), and is related either to similar conformation of the disparate antigens or to multiple binding sites of low affinity within the IgM molecule. Polyspecificity has also been reported in some IgG mAbs. An IgG anti-DNA mAb has been shown to bind to vimentin (21) and another IgG2b anti-DNA mAb was found to bind to renal mesangium and vascular endothelium (22), which resulted in an increase in albumin permeability in perfused rat kidneys.

In spite of an avidity for binding of the anti-$Fc_\gamma R$ mAbs to tFc_γ-coated microtiter plates of 10^8–10^9 M^{-1}, we believe that the intrinsic affinity of the mAb binding site for $Fc_\gamma R$ of ZL37-9 and ZL173-7 is low because these mAbs do not stain $Fc_\gamma R$-bearing cells. Because the $Fc_\gamma R$ density on the cells is low compared with the $tFc_\gamma R$-coated ELISA plates, the mAbs do not bind with sufficient avidity to give good staining after washing. ZK25-3 and UN40-6 stain macrophages very well, but since these antibodies have so many specificities, we cannot be sure that the primary antigen recognized on the macrophage is $Fc_\gamma RII$. However, the apparent avidity of anti $Fc_\gamma R$ antibody could be greatly enhanced in vivo if the anti-$Fc_\gamma R$ IgM were to form a complex with other antigens, such as nucleoprotein coated with histones or DNA, both of which offer multiple epitopes to form large complexes. The high avidity anti-$Fc_\gamma R$ IgG we have isolated by affinity chromatography might by itself bind to and trigger $Fc_\gamma R$-bearing cells.

Although ZL37-9 and ZL173-7 do not stain cells effectively, either using directly fluoresceinated antibody (Table II) or anti-IgM reagents (data not shown), they do inhibit immune complex binding to J774 macrophages. ZL37-9 and ZL173-7 may selectively (since IgM is pentamer) bind to a small proportion of cell surface $Fc_\gamma Rs$ that are aggregated or patched, and to which immune complexes preferentially bind. Alternatively, since the J774 cells were preincubated for 1 h at room temperature with the anti-$Fc_\gamma R$ mAbs, the anti-$Fc_\gamma R$ IgM might trigger the internalization of the $Fc_\gamma R$.

The binding of immune complexes to $Fc_\gamma R$ on macrophages, neutrophils, and NK cells results in the activation of phagocytic mechanisms, elaboration of toxic metabolites of oxygen, release of prostaglandins and leukotrienes, and the synthesis of cytokines such as TNF, IFN-γ, and IL-2 by NK cells (23). The mesangial cell, which expresses $Fc_\gamma R$ (24, 25), might also be activated by immune complexes or anti-$Fc_\gamma R$ antibody. Stimulation of these receptors leads to release of platelet-activating factor, prostaglandin A2, H_2O_2, and enhanced secretion of IL-1 (26), all of which could contribute to kidney pathology seen in SLE. The anti-$Fc_\gamma R$ antibodies we describe here may play a role in triggering these responses. Other preliminary experiments show that 2K2H5 and UN40-6, when adsorbed to plastic, trigger

an oxidative burst from murine peritoneal macrophages elicited with thioglycollate medium (data not shown).

The impaired $Fc\gamma R$ function in the autoimmune mice has also been seen in patients with SLE, as well as other autoimmune diseases such as rheumatoid arthritis and Sjögren's syndrome (4, 27). It is possible that one component in the slow clearance of immune complexes seen in lupus patients and patients with other autoimmune diseases is the presence of anti-$Fc\gamma R$ antibody. The receptor that plays a predominant role in clearance of large immune complexes is $Fc\gamma RIII$ or CD16. Blockade of $Fc\gamma RIII$ in chimpanzees with the anti-$Fc\gamma RIII$ mAb 3G8 or its Fab fragment resulted in a 20-fold increase in clearance time for IgG-sensitized chimpanzee erythrocytes (28). Antibody against $Fc\gamma RIII$ allotypes NA1 and NA2 has been associated with juvenile neutropenia (29, 30), and anti-$Fc\gamma RIII$ antibody has also been identified in sera of patients with SLE (31). The role that the anti-$Fc\gamma R$ Ig we have detected in the autoimmune mice and that is present in sera of patients with SLE (31) plays in the pathology of the diseases remains to be established.

Summary

We demonstrate, using a recombinant truncated $Fc\gamma RII$ molecule as a probe, the presence of anti-$Fc\gamma R$ antibodies in several strains of autoimmune mice. Affinity chromatography on a truncated $Fc\gamma R$ column of pooled sera from aged NZB females resulted in isolation of 16 μg of IgM per ml of serum, ~2% of the total IgM; no anti-$Fc\gamma R$ IgM was found in sera from C58/J mice. Mice with high titers of anti-$Fc\gamma R$ IgM also had anti-$Fc\gamma R$ IgG. Affinity-purified anti-$Fc\gamma R$ IgG bound to $Fc\gamma R$-bearing cells. A good correlation was found between the presence of anti-$Fc\gamma R$ Ig and impaired phagocytosis of immune complexes in autoimmune strains such as NZB or NZB/NZW F_1. Sera with high titers of anti-$Fc\gamma R$ Ig from NZB and motheaten mice inhibited the binding of soluble immune complexes. Furthermore, BXSB, a lupus-prone mouse strain that does not produce anti-$Fc\gamma R$ Ig, shows normal macrophage binding and phagocytosis of immune complexes. A set of four IgM mAbs that bind to $Fc\gamma R$ was identified. These antibodies were polyspecific; some were directed against DNA, and others recognized a wide variety of antigens including histones, thyroglobulin, and transferrin, but all anti-$Fc\gamma R$ IgM antibodies effectively inhibited the binding of IgG1 anti-DNP/DNP_{20}BSA complexes to J774 macrophages. The role of anti-$Fc\gamma R$ Ig in autoimmunity remains to be established. It may act to crosslink and activate $Fc\gamma R$s on neutrophils, macrophages, NK, and mesangial cells, or it may desensitize $Fc\gamma R$ function of $Fc\gamma R$-bearing cells.

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