Extracellular Mutations of Non-obese Diabetic Mouse FcγRI Modify Surface Expression and Ligand Binding*

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The non-obese diabetic mouse (NOD) expresses a unique form of the high affinity receptor for IgG (FcγRI), containing multiple mutations that result in substitutions and insertions of amino acids and a truncated cytoplasmic tail. As a result of these major changes, receptor affinity for IgG increases 10-fold over that of wild-type FcγRI from BALB/c mice, while the specificity for ligand is retained. Kinetic analysis revealed that the association rate of IgG with FcγRI from NOD mice (FcγRI-NOD) and FcγRI from BALB/c mice (FcγRI-BALB) is similar, IgG bound much more tightly to FcγRI-NOD as revealed by a profoundly diminished dissociation rate.

Transfection studies demonstrated that FcγRI-NOD was expressed at one-tenth of the level of FcγRI-BALB. Although mouse FcγRI was previously not known to associate with the FcγRI γ-subunit, transfection of C57Bl/10SnJ cells demonstrates that like human FcγRI, mouse FcγRI is also able to associate with this signaling subunit. Furthermore, expression levels of FcγRI-NOD were not restored by the presence of the FcγRI γ-subunit. The difference in the levels of expression was mapped to mutations in the extracellular region of FcγRI-NOD as replacement of the extracellular domains with those of human FcγRI or FcγRI-BALB restored expression to that of human FcγRI or FcγRI-BALB.

The mouse high affinity receptor for IgG, FcγRI (CD64), consists of three Ig-like extracellular domains and is the only Fcγ receptor that binds monomeric IgG (1, 2). Expressed on monocytes and macrophages and induced by interferon-γ (IFN-γ) on neutrophils (3–5), FcγRI functions by linking the humoral and cellular responses. Although functions mediated by mouse FcγRI are less well characterized, cross-linking of human FcγRI on myeloid cells leads to events such as tyrosine phosphorylation (6), Ca²⁺ flux (7), superoxide generation (8), inflammatory mediator release (9), antibody-dependent cellular cytotoxicity (10), and internalization of small immune complexes (11). Many of these sequelae may also be true in the mouse. While human FcγRI is associated with a homodimer of the γ-subunit from FcγRI (12–14), receptor co-association has not been shown in the mouse, and indeed, there are some differences between the function of human and mouse FcγRI (e.g. mouse FcγRI is constitutively phosphorylated, while human FcγRI is not) (15, 16).

Mouse FcγRI is structurally homologous to human FcγRI and exhibits high affinity binding of monomeric IgG (2). Genetic mapping studies revealed that the single gene encoding mouse FcγRI (Fcg1) is situated on chromosome 3, and studies in the non-obese diabetic mouse (NOD) revealed a linkage with the Idl-3 diabetes susceptibility genetic marker on chromosome 3 (17–19). The Fcg1 allele found in NOD mice has 24 single base differences (compared with the BALB/c allele), 17 of which encode changes in the predicted amino acid sequence, including a four-amino acid insertion between domains 2 and 3 and a four-nucleotide deletion leading to a frameshift and premature translation termination codon that essentially eliminates the cytoplasmic domain (truncated by 73 amino acids) (19). Studies on NOD peripheral blood MAC-1 cells demonstrated a lower surface expression of FcγRI compared with cells from C57BL/10SnJ mice. Binding and turnover studies revealed that FcγRI from NOD cells demonstrated a 73% reduction in the turnover of bound mIgG2a compared with C57BL/10SnJ mice (19).

This study characterizes FcγRI from NOD mice (FcγRI-NOD) and investigates the influence the mutations have on the cell-surface expression of FcγRI-NOD, association with the FcγRI γ-subunit, and interaction with ligand.

EXPERIMENTAL PROCEDURES

FcγRI cDNA Constructs and Generation of Chimeric Receptors—The FcγRI-NOD cDNA was generated by reverse transcription-PCR from RNA isolated from NOD/Lt spleen cells. Briefly, total RNA was isolated from 10⁷ spleen cells using guanidinium thiocyanate (20), and first-strand cDNA was synthesized using reverse transcription (Pharmacia Biotech Inc.). PCR was used to generate FcγRI-NOD and FcγRI-BALB cDNA clones as described (21) using 500 ng of oligonucleotide primers 1 and 9, and interaction with ligand. The cDNA sequences were checked by PCR was performed to splice the two fragments together and to amplify the spliced product. The cDNA sequences were checked by dyeexonucleotide sequencing (25). The receptor construct containing FcγRI-BALB extracellular domains and transmembrane and cytoplasmic...
mic domains of FcγRI-NOD origin was designated BALB-NOD. The chimeric receptors containing human FcγRI extracellular domains and transmembrane and cytoplasmic domains of either FcγRI-NOD or FcγRI-BALB origin were designated Hu-NOD and Hu-BALB, respectively.

The oligonucleotide sequences (5'-3') used as primers in PCR are listed below, and nonhomologous sequences are underlined: MDH3, TTTTGTCGACATGATTCTTACCAGCTT; TISM6, TTTGAATTCCAGTCTGTATATTTGC; T-9, TTTGGATCCATGTGGTTCTTGACAACTCT; T-10, GAGCTCCAACTCAGGGCT; T-11, AGCCCTGAGTTGGAGCTC; T-12, Pharmacia). Polyclonal rabbit anti-sheep erythrocyte serum was used to sensitize sheep erythrocytes for EA rosetting studies of complexes as monomericaftersizefractionationchromatography (Superose 12, Pharmacia). Assays were performed on the complete cDNA of FcγRI-BALB (2) or γ-actin cDNA (26) and autodiographed.

Total DNA was harvested 48 h post-transfection from COS-7 cells by incubating cells in lysis buffer (50 mM Tris-Cl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 500 μM protease K) for 4 h at 55 °C prior to phenol and chloroform extractions and precipitation of DNA with an equal volume of isopropanol. The DNA was recovered by centrifugation and overnight digestion with EcoRI. 10 μg of DNA was electrophoresed and transferred to a nylon membrane as described (32). Northern blots were hybridized with the complete cDNA of FcγRI-BALB (2) and autoradiographed.

Radiodination of Cells and Immunoprecipitation—COS-7 cells transfected with various cDNA constructs were surface-radioiodinated using Na125I and lactoperoxidase (Sigma) as described (32). After washing with phosphate-buffered saline, 5 x 10⁶ cells were lysed on ice with 1 ml of Brij 96 lysis buffer containing 0.5% Brij 96 (Sigma), 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Sigma), and nuclear and cellular debris were removed by centrifugation. Cell lysates were incubated with 30 μl of packed Sepharose beads coupled with either whole mouse IgG2a or Fab' fragments of mouse IgG2a for 1 h at 4 °C with rotation before seven washes with lysis buffer. Samples were boiled in the presence of β-mercaptoethanol and analyzed by SDS-PAGE.

Western Blotting and FcγRI γ-Subunit Association—Transfected COS-7 cells lysates were incubated with whole mouse IgG2a-Sepharose and mouse IgG2a Fab' fragment-Sepharose as described above, washed, and denatured under nonreducing conditions prior to SDS-PAGE and Western blotting onto an Immobilon-P membrane (Millipore Corp., Bedford, MA). After transfer, the membrane was blocked with 2% casein in Tris-buffered saline (10 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 2 h before overnight incubation with polyclonal rabbit anti-FcγRI γ-subunit antisemur in Tris-buffered saline containing 1% casein. After washing with Tris-buffered saline containing 0.1% Tween 20, the blocks were autoradiographed.
were incubated with peroxidase-conjugated swine anti-rabbit IgG (DAKO-PATTS) and then washed again prior to chemiluminescent detection (ECL, Amersham International).

Association and dissociation kinetics of IgG binding were performed at 4°C with monomeric 125I-hIgG1 on COS-7 cells transfected with the FcRI constructs. The association of IgG was determined using transfected COS-7 cells (1 x 10^5 cells/ml) and incubating with 125I-hIgG1 (5 μg/ml) for the indicated times before assaying cell-bound 125I-hIgG1 by pelleting cells through phthalate oils. To compensate for the various levels of surface expression of the two receptors, the data are presented as percent maximum bound IgG, where 100% is the level of binding demonstrated after 3 h of incubation on ice.

The dissociation of bound human 125I-hIgG1 was measured by incubating either transfected COS-7 cells or mouse bone marrow-derived macrophages (±IFN-γ) with 125I-hIgG1 (5 μg/ml) for 2 h before adding 200-fold excess unlabeled hIgG (1 mg/ml) to the cells at 0°C. Cell-bound 125I-hIgG1 was assayed at the indicated times by pelleting cells through phthalate oils. To compensate for the various levels of surface expression, the data are presented as percent maximum bound IgG, where 100% is the level of binding demonstrated at time 0. The dissociation of bound 125I-hIgG1 from FcRI-transfected COS-7 cells was performed at both 4 and 22°C, while the dissociation of 125I-hIgG1 from FcRI on bone marrow-derived macrophages was measured at 4°C.

Bone marrow-derived macrophages—NOD/Lt and BALB/c bone marrow-derived macrophages were generated as adherent cells from their nonadherent progenitors essentially as described before (33), but with minor modifications so that they could be removed from the culture surface. The macrophages were eventually grown on Petri dishes for 4 days in RPMI 1640 medium supplemented with 50 μg/ml 2-mercaptoethanol, 20 μg/ml HEPES, 15% fetal calf serum, and 20% L-cell conditioned medium (as a crude source of colony-stimulating factor-1 or macrophage colony-stimulating factor). The bone marrow-derived macrophages were a relatively pure and homogenous population, with >95% of adherent cells binding colony-stimulating factor-1. Cells were washed twice with phosphate-buffered saline and where stated were treated with 1000 units/ml recombinant murine IFN-γ (Nippon-Roche, Tokyo, Japan) 18 h before harvesting by vigorous washing.

The bone marrow-derived macrophages—Bone marrow-derived macrophages that had been stimulated with IFN-γ were incubated with mAb 2.4G2 (anti-FcRI/IIA/IIIB) and F4/80 (anti-macrophage marker), and/or human IgG1 for 30 min on ice prior to washing and incubation with specific FITC-conjugated secondary antibodies. Since there are no mAbs that detect mouse FcγRI, the "specific" staining of mouse FcγRI antibodies was performed by blocking peripheral binding with mAb 2.4G2 (100 μg/ml) prior to the addition of monomeric human IgG (20 μg/ml). Human IgG bound by FcγRI was detected using FITC-conjugated sheep anti-human IgG, which does not cross-react with rat IgG. Binding of antibodies was measured as surface fluorescence using the FACSscan (Becton Dickinson).

RESULTS

Biochemical Characterization of FcγRI-NOD—To establish the molecular mass of FcγRI-NOD, COS-7 cells were transfected with either FcγRI-NOD or FcγRI-BALB cDNAs, and cells were radiolabeled and lysed 48 h post-transfection (Fig. 2). Immunoprecipitation experiments were performed on cell lysates from COS-7 cells transfected with FcγRI-NOD (Fig. 2, lanes a and b) and FcγRI-BALB (lanes c and d) using whole mouse IgG2a-Sepharose (lanes a and c) and mouse IgG2a Fab fragment-Sepharose (lanes b and d). The 70-kDa moiety precipitated by whole mouse IgG2a-Sepharose (Fig. 2, lane c) was the expected size for FcγRI-BALB (16). The molecular mass of FcγRI-NOD, however, was expected to be smaller than 70 kDa due to the truncation of the cytoplasmic domain and was predicted to be ~45 kDa. No such moiety was observed from immunoprecipitations with whole mouse IgG2a-Sepharose (Fig. 2, lane a), even after prolonged exposure of the autoradiograph. Fab fragments derived from mouse IgG2a (lanes b and d). Samples were then electrophoresed on SDS-PAGE under reducing conditions and autoradiographed.

Expression of FcγRI and Binding of IgG—To assess if ligand binding differences were the reason for the observed immunoprecipitation difference between FcγRI-NOD and FcγRI-BALB, binding studies using monomeric 125I-mIgG2a and 125I-hIgG1 were performed (Fig. 3). COS-7 cells transfected with FcγRI-NOD (Fig. 3A) or FcγRI-BALB (Fig. 3B) were incubated with various concentrations of 125I-hIgG1 or 125I-mIgG2a. It is clear that both FcγRI-NOD and FcγRI-BALB are able to bind IgG; however, the total saturable binding of IgG2a to FcγRI-NOD was one-tenth that of IgG2a binding to FcγRI-BALB. This difference was apparent in the binding of both hIgG1 and mIgG2a by FcγRI-NOD and was ~0.5 ng bound per 5 x 10^4 cells, 10-fold less than cells transfected with FcγRI-BALB (5–6 ng bound per 5 x 10^4 cells). This 10-fold difference in expression levels was reproducible in all 10 experiments performed.

Transcription and Translation Efficiency—To establish that the difference in surface expression was due to FcγRI-NOD receptor difference and not variability in the assay systems, the efficiency of transcription and mRNA levels were investigated. Total RNA (treated with DNase I) was harvested from COS-7 cells transfected with FcγRI-NOD (Fig. 4A, lane a), FcγRI-BALB (lane b), or the FcγRI γ-subunit (negative control) (lane c) and hybridized with FcγRI-BALB cDNA (upper panel) and γ-actin cDNA (lower panel). Hybridization of the mouse FcγRI cDNA revealed equivalent amounts of RNA in cells transfected with either FcγRI-NOD (Fig. 4A, lane a) or FcγRI-BALB (lane b). Moreover, hybridization was specific as no FcγRI mRNA was detected in cells transfected with FcγRI γ-subunit cDNA only (Fig. 4A, lane c). RNA probed with γ-actin cDNA revealed that similar amounts of total RNA were present in each sample.

Southern blot analysis of total DNA was performed to determine the levels of plasmid DNA in the transfected cells (Fig. 4B). Total DNA, digested with EcoRI (which linearizes transfected plasmid to 4.76 kilobases) and hybridized with FcγRI-BALB cDNA, indicated that approximately equivalent amounts of FcγRI plasmid DNA were present in samples from cells transfected with FcγRI-NOD (Fig. 4B, lane a) and FcγRI-
BALB (lane b). Specificity was demonstrated by the failure to detect any hybridizing material in cells transfected with the FcγRI-g-subunit (Fig. 4, lane c). No difference in levels of mRNA or transfected DNA was observed.

Localization of Region Involved in Diminished Surface Expression—Chimeric Fcγ receptors were generated to localize the region of FcγRI-NOD affecting the level of cell-surface expression. These receptors were generated using splice overlap extension-PCR, wherein the extracellular domains of FcγRI-NOD were replaced with either FcγRI-BALB sequence, to generate chimeric BALB-NOD, or human FcγRI sequence, to generate Hu-NOD (Fig. 5A) (see also “Experimental Procedures”). These sequence exchanges were made at the conserved leucine (positions 268, 263, and 263 of NOD/Lt, BALB/c, and human FcγRI, respectively) within the conserved sequence LELQVLG on the N-terminal side of the predicted transmembrane region of each receptor (Figs. 1 and 5A).

Surface expression of chimeric receptors was tested by assessing the binding of monomeric human 125I-IgG1 (Fig. 5B).

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Fig. 3. Specific binding of radiolabeled human IgG1 (●) or mouse IgG2a (■) to FcγRI-NOD (A) or FcγRI-BALB (B) on transfected COS-7 cells. Background binding to untransfected cells has been subtracted. Note the different scales in the data shown for binding IgG by FcγRI-NOD (A) compared with FcγRI-BALB (B).

Fig. 4. Northern analysis of total RNA (A) and Southern analysis of DNA (B) from COS cells transfected with FcγRI-NOD (lane a), FcγRI-BALB (lane b), or the FcγRI-g-subunit (lane c). Total RNA was harvested 48 h post-transfection (A) and was probed with FcγRI cDNA (upper panel) or γ-actin cDNA (lower panel). DNA was harvested 48 h post-transfection (B) and was digested with EcoRI before Southern transfer and hybridization with mouse FcγRI cDNA. kb, kilobases.

Fig. 5. Construction and IgG binding properties of chimeric FcγRI. A, schematic representation of cDNAs used. The regions of FcγRI are indicated as follows: L, leader; D1, D2, and D3, domains 1, 2, and 3, respectively; TM, transmembrane region; CYT, cytoplasmic tail. Translated regions are boxed, and the origin of the appropriate template sequence is indicated by various shading (white boxes, NOD; hatched boxes, BALB/c gray boxes, human). Oligonucleotides used in the construction of the chimeras are labeled, and nonhomologous restriction enzyme sites used in the cloning are indicated (see “Experimental Procedures” for details). B, titration of radiolabeled human IgG1 binding to chimeric receptors. Shown is the binding of 125I-IgG1 by FcγRI-BALB (●), FcγRI-NOD (●), BALB-NOD (■), and Hu-NOD (■) expressed on transfected COS-7 cells. Values are expressed as amount bound (nanograms), and background binding has been subtracted.
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Fig. 6. Immunochemical characterization of FcγRI and FcγRI γ-chain association in transfected cells. A, COS-7 cells transfected with FcγRI-NOD and FcγRI γ-chain cDNA (lanes a and b), with the FcγRI γ-chain only (lanes c and d), or with FcγRI-BALB and the FcγRI γ-chain (lanes e and f) were radiolabeled, lysed, and incubated with mIgG2a-Sepharose (lanes a, c, and e) or Fab' fragment-Sepharose (lanes b, d, and f) prior to SDS-PAGE under reducing conditions. B, the FcγRI γ-chain in whole cell lysates was detected by Western blotting. Whole cell lysates of COS-7 cells transfected with FcγRI-BALB and the FcγRI γ-chain (lane a), with the FcγRI γ-chain alone (lane b), or with FcγRI-NOD and the FcγRI γ-chain (lane c) were subjected to SDS-PAGE under nonreducing conditions, blotted onto polyvinylidene difluoride membranes, and probed with rabbit anti-FcγRI γ-chain antibody. C, shown are the results of the Western blot analysis of FcγRI γ-chain association with mouse FcγRI. Lysates of COS-7 cells transfected with FcγRI-BALB and the FcγRI γ-chain (lanes a and b), with FcγRI-NOD and the FcγRI γ-chain (lanes c and d), or with the FcγRI γ-chain only (lanes e and f) were incubated with whole mouse IgG2a-Sepharose (lanes a, c, and e) or Fab' fragment-Sepharose (lanes b, d, and f) and subjected to SDS-PAGE under nonreducing conditions before the Western blotting. D, shown are the results of the Western blot analysis of the association of the FcγRI γ-chain with chimeric FcγRI proteins. COS-7 cells were transfected with the FcγRI γ-chain and chimeric receptors containing the human FcγRI extracellular domains fused to the membrane-spanning region and cytoplasmic tails of FcγRI from either BALB/c (lanes a and b) or NOD/Lt (lanes c and d) mice or transfected with the FcγRI γ-chain only (lanes e and f). Lysates of these cells were incubated with whole mouse IgG2a-Sepharose (lanes a, c, and e) or Fab' fragment-Sepharose (lanes b, d, and f), and Western blots were probed with rabbit anti-FcγRI γ-chain antibody.

membrane region and residual cytoplasmic domain of FcγRI-NOD did not appear to influence surface expression of FcγRI.

Association of FcγRI γ-Subunit with Mouse FcγRI-Mouse FcγRI is known to associate with protein subunits. However, human FcγRII, FcγRIII, and FcγRI all share a common subunit (FcγRI γ-subunit) (12–14, 34–37). The association with the FcγRI γ-subunit has been found to be dependent upon membrane-spanning regions of FcγRI and FcγRIII (34–37). While expression of human FcγRI is not dependent on the association with the FcγRI γ-subunit, FcγRII and FcγRI both require the FcγRI γ-subunit for expression. Thus, the FcγRI γ-subunit may be required to "optimize" mouse FcγRI expression, especially that of FcγRI-NOD.

Cotransfection and immunoprecipitation experiments were performed with FcγRI-BALB or FcγRI-NOD and the FcγRI γ-subunit to demonstrate any association of the FcγRI γ-subunit with mouse FcγRI. The cotransfection of the FcγRI γ-subunit and FcγRI-NOD did not increase cell-surface expression of FcγRI-NOD. Mouse IgG2a failed to immunoprecipitate any labeled material from the transfected COS-7 cells (Fig. 6A, lane a). However, FcγRI-BALB was clearly precipitated by IgG2a from cells cotransfected with the FcγRI γ-subunit (Fig. 6A, lane e). No material was precipitated by IgG2a from cells transfected with the FcγRI γ-subunit only (Fig. 6A, lanes c and d) or by Fab' fragment-conjugated beads (lanes b, d, and f). It was clear, however, that the transfected cells were expressing the FcγRI γ-subunit as Western blots of whole cell lysates indicated the presence of the 22-kDa dimer protein (Fig. 6B) in cells cotransfected with FcγRI-BALB and the FcγRI γ-subunit (lane a) and with FcγRI-NOD and the FcγRI γ-subunit (lane c) and in cells transfected with the FcγRI γ-subunit cDNA only (lane b).

To establish if the FcγRI γ-subunit was capable of association
with mouse FcRI, COS-7 cells were transfected with FcRI-BALB and FcRI γ-subunit cDNA (Fig. 6C, lanes a and b), with FcRI-NOD and the FcRI γ-subunit (lanes c and d), or with the FcRI γ-subunit alone (lanes e and f). Samples were incubated with either intact mouse IgG2a-Sepharose (Fig. 6C, lanes a, c, and e) or Fab’-fragment-Sepharose (lanes b, d, and f). Following SDS-PAGE under nonreducing conditions, Western blotting revealed that the FcRI γ-subunit was specific for FcRI-BALB (Fig. 6C, lane a); however, no FcRI γ-subunit was detected in precipitates from cells transfected with FcRI-NOD and the FcRI γ-subunit (lane c) or with the FcRI γ-subunit alone (lane e). As expected, precipitation was specific for FcRI as no material was precipitated from these cells after incubation with Fab’-fragments of mouse IgG2a (Fig. 6C, lanes b, d, and f). Thus, the observation that FcRI-NOD did not appear to associate with the FcRI γ-subunit was presumably due to the low surface expression of this receptor (Fig. 6A) and, consequently, the low level of communoprecipitation of the FcRI γ-subunit.

Since the membrane-spanning region of the FcRI α-subunit and FcRIIA have been shown to be crucial for the association with the FcRI γ-subunit (34–36), the question of whether the membrane-spanning region of FcRI-NOD and amino acid changes found therein would influence association with the FcRI γ-subunit was assessed. Chimeric receptors (see Fig. 5A) with extracellular domains of human FcRII origin and transmembrane and cytoplasmic domains from either mouse FcRI-BALB (Hu-BALB) (Fig. 6D, lanes a and b) or FcRI-NOD (Hu-NOD) (lanes c and d) were cotransfected into COS-7 cells with the FcRI γ-subunit (lanes e and f, FcRI γ-subunit alone). Radiolabeled cell lysates were incubated with whole mIgG2a-Sepharose (Fig. 6D, lanes a, c, and e) or mIgG2a Fab’-fragment-Sepharose (lanes b, d, and f) prior to SDS-PAGE under nonreducing conditions. Western blotting revealed that the 22-kDa homodimer of the FcRI γ-subunit was coprecipitated with Hu-BALB chimeric FcRI (Fig. 6D, lane a) and also with Hu-NOD FcRI (lane c), which contains only the membrane-spanning sequence and residual cytoplasmic tail of FcRI-NOD. No material was precipitated from lysates from cells transfected with the FcRI γ-subunit alone (Fig. 6D, lane e) or from samples incubated with Fab’-fragment-Sepharose (Fig. 6D, lanes b, d, and f). It is clear from these results that the FcRI γ-subunit is able to associate with mouse FcRI and that this association can take place in the presence of BALB/c- or NOD-derived membrane-spanning and cytoplasmic tail sequences. Thus, it is likely that the reduced expression of FcRI-NOD is due to the mutations in the extracellular domains.

Specificity and Affinity of IgG Interactions with FcRI Forms—Since mutations in the extracellular domains affected cell-surface expression but not FcRI γ-subunit association, possible additional effects on ligand interactions were tested using both complexed and monomeric IgG (Table I). First, the binding of immune complexes was tested on FcRI-NOD- and FcRI-BALB-transfected COS-7 cells. Both FcRI-NOD and FcRI-BALB exhibited binding of complexed rabbit IgG-sensitized sheep erythrocytes assessed by rosetting, but could not bind complexed mouse IgG1-sensitized sheep erythrocytes thereby confirming that the extracellular changes did not affect the specificity of mouse FcRI. While the specificity of FcRI-BALB and FcRI-NOD was identical, repeated Scatchard analysis demonstrated that although both receptors bound monomeric IgG, FcRI-NOD bound 125I-mlG2a or 125I-hlG1 with a 10-fold increase in affinity compared with FcRI-BALB (Table I).

The reasons for the increased affinity were addressed by kinetic analysis of IgG binding (Fig. 7). The association of monomeric 125I-hlG1 with FcRI-NOD or FcRI-BALB showed identical kinetics, with 95% of maximum ligand bound by 30 min (4°C) (Fig. 7A). By contrast, the dissociation of monomeric 125I-hlG1 from FcRI-NOD and FcRI-BALB was remarkably different (Fig. 7B). Whereas IgG showed a biphasic dissociation from FcRI-BALB with a rapid initial phase and a slower second phase, the dissociation from FcRI-NOD was extremely slow. Only a single phase was apparent, with <10% of bound IgG dissociated from FcRI-NOD after 45 min compared with ~75% from FcRI-BALB. The differences in disso-
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FIG. 8. FACScan analysis of FITC fluorescence from bone marrow-derived macrophages from BALB/c mice (gray and stippled) and NOD/Lt mice (white). IFN-γ-stimulated macrophages were incubated with a mAb that binds FcγRI/FcγRII (2.4G2) (A) or with a mAb that binds a macrophage-specific marker (F4/80) (B), and the binding of these antibodies was detected with FITC-conjugated sheep (Fab) anti-mouse Ig. The binding of monomeric human IgG (C), with prior blocking of FcγRII/FcγRII sites with mAb 2.4G2, was detected using FITC-conjugated sheep (Fab) anti-human Ig. Background fluorescence (stippled histograms) was measured on BALB/c-derived macrophages by incubation with FITC-conjugated antibody alone (sheep anti-mouse Ig (A and B) and sheep anti-human Ig (C)).

Assessment of Expression and Dissociation Rate of FcγRI on NOD Macrophages—It was of some concern that the differences between FcγRI-BALB and FcγRI-NOD had been defined using a somewhat artificial transfection system. Thus, a series of experiments were performed to analyze FcγRI from macrophages derived from mice, and indeed, the results of the transfections were confirmed using macrophages. FACScan analysis of expression of cell-surface markers and FcγRI was performed on BALB/c or NOD/Lt bone marrow-derived macrophages treated with IFN-γ (to up-regulate mouse FcγRI). Using the 2.4G2 antibody, it was found that the expression of the low affinity IgG receptors (FcγRI/FcγRII) was approximately the same on both BALB/c- and NOD/Lt-derived macrophages (Fig. 8A). Similarly, both BALB/c- and NOD/Lt-derived macrophages expressed nearly identical levels of a macrophage marker detected by antibody F4/80 (Fig. 8B). In contrast, NOD/Lt-derived macrophages bound less monomeric human IgG via FcγRI than did BALB/c-derived macrophages (Fig. 8C). Since no monoclonal anti-mouse FcγRI is available, FcγRI was detected by using monomeric human IgG in the presence of excess mAb 2.4G2 (which blocks any binding to FcγRII/FcγRII) (Fig. 8C). The expression of FcγRI on NOD/Lt-derived macrophages was ~5-fold lower than that of BALB/c-derived macrophages with (see above) or without IFN-γ (data not shown), indicating that

the expression defect observed in the COS-7 transfection system is also observed in vivo on macrophages, although not to quite the same extent (10-fold versus 5-fold expression difference).

The slower dissociation of ligand from FcγRI-NOD expressed on COS-7 cells was also observed using NOD/Lt-derived macrophages (Fig. 9). As previously observed, monomeric human IgG dissociates quickly from BALB/c FcγRI and less quickly from NOD/Lt FcγRI. Treatment of the macrophages with or without IFN-γ did not appear to influence the dissociation rate of monomeric IgG from macrophages from either strain of mouse (although FcγRI expression levels were altered (data not shown)). As noted previously with the expression phenotype of FcγRI from NOD/Lt-derived macrophages, the difference in the dissociation rate of IgG from FcγRI on NOD/Lt- and BALB/c-derived macrophages was also not as dramatic as that seen in the COS-7 transfection system; however, the interaction of FcγRI on NOD/Lt-derived macrophages was still dramatically different than that of FcγRI on BALB/c-derived macrophages.

FIG. 9. Kinetics of dissociation of radiiodinated human IgG1 from FcγRI on bone marrow-derived macrophages incubated with or without IFN-γ and derived from either BALB/c or NOD/Lt mice. Data represent the average of duplicate samples, and error bars are 1 S.D. from the mean.

Discussion

The mutations of FcγRI from the diabetes-prone NOD mouse have profound effects on receptor function. Whereas many protein mutations may result in loss of function, the FcγRI-NOD mutations result in a 10-fold increase in receptor affinity. Since artificially introduced mutations in mouse FcγRI are known to alter affinity and specificity (21), the binding of IgG to FcγRI-NOD and FcγRI-BALB was tested. The specificity of FcγRI-NOD and FcγRI-BALB was identical, with both receptors binding human IgG1, mouse IgG2a, and rabbit IgG, but not mouse IgG1 (Table I). The 10-fold increase in affinity was observed for both human IgG1 and mouse IgG2a. Through the use of chimeric receptors, the increased affinity of FcγRI-NOD for ligand could be attributed to mutations in the extracellular (ligand-binding) domains rather than to a secondary effect of the transmembrane region and residual cytoplasmic tail.

Previous studies of mouse FcγRI indicate that extracellular domain 3 is important in modulating the affinity and specificity of the receptor and that the first two domains interact with ligand with low affinity (21). It is interesting to note that there is a four-amino acid insertion between domains 2 and 3 of
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FcγRI-NOD. In addition, the FcγRI-BALB His231 to FcγRI-NOD Tyr236 mutation occurs in the body of domain 3, but is unlikely to affect binding as human FcγRI contains tyrosine in this position (see Fig. 1). Many of the amino acid changes found in FcγRI-NOD either are identical to those in human FcγRI or are conservative changes. However, the insertion of Glu350 between domains 1 and 2 and the substitution of Asp335 (FcγRI-BALB) for Gly336 in FcγRI-NOD are potentially more disruptive. Indeed, the Asp335→Gly336 mutation falls within a region homologous to Ig-interactive regions of FcγRI (38-40), FcγRII (41), and FcγRII (27). It is also possible, however, that the change in binding characteristics is due to a combination of the mutations.

In addition to the observed altered affinity, FcγRI-NOD was expressed at approximately one-tenth of the level of FcγRI-BALB in the COS-7 cell system. The mechanism of this difference remains unclear and could not be explained by the level of transient transfection efficiency or steady-state RNA amounts. Mouse FcγRI is able to associate with the FcγRI γ-subunit (Fig. 6); however, cotransfection of FcγRI-NOD and FcγRI γ-subunit cDNA does not rescue the expression of FcγRI-NOD. The region in the transmembrane domain thought to be involved in the FcγRI γ-subunit association is conserved between human FcγRI and FcγRI-NOD and was not expected to be disrupted by the truncation of the cytoplasmic tail of FcγRI-NOD. This was formally demonstrated when chimeric FcγRI mutants containing this sequence were shown to be able to associate with the FcγRI γ-subunit.

The mutations within the extracellular domains of FcγRI-NOD were shown to be involved in altering cell-surface expression as constructs containing FcγRI-NOD transmembrane and residual cytoplasmic tail domains were expressed at levels comparable to those of human FcγRI or FcγRI-BALB. However, it is not clear how the extracellular domain mutations can affect the surface expression and whether the mutations affect processes such as translation, the assembly of the polypeptide, transport to the cell surface, or even stability of the protein.

By measuring the level of surface expression using total saturable ligand binding, it is possible that the reduced expression of functional FcγRI-NOD is due to alterations in the receptor’s configuration that allow only a small fraction (10%) of the total receptor pool to bind ligand rather than to a decrease in total receptor protein. Currently, this possibility cannot be directly tested as there are no mouse FcγRI-specific antibodies (monoclonal or polyclonal) to evaluate total cell-surface receptor levels. Nonetheless, it is clear that the mutations in the extracellular domains of FcγRI-NOD affect the levels of functional receptor on the cell surface.

Transfection of the FcγRI cDNAs into COS-7 cells was chosen primarily as a way to study mouse FcγRI in the absence of other Fc receptors with which it is normally coexpressed (necessary because of the lack of monoclonal antibody reagents); however, the disadvantage of the system is that COS-7 cells may lack other proteins necessary for mouse FcγRI function. Indeed, although bone marrow-derived macrophages from NOD/Lt mice expressed less functional FcγRI on their surface (Fig. 8), the expression difference varied between 3- and 5-fold less than that of FcγRI from BALB/c-derived macrophages, not the reproducible 10-fold difference observed in the COS-7 system. This implies that other molecules, possibly even other Fc receptors, may play a role in the surface expression of functional FcγRI. Indeed, the dissociation of IgG from FcγRI on NOD/Lt-derived macrophages was again slower than that of FcγRI on BALB/c-derived macrophages; however, the effect was less dramatic than that seen in the COS-7 system, again pointing to other molecules being involved with FcγRI in macrophages. Despite this, however, the trend of higher affinity, slower dissociation, and lower expression was still observed in the NOD/Lt-derived macrophages.

The initial study of mutated FcγRI-NOD also demonstrated a lower surface expression of functional FcγRI on peripheral blood MAC-1+ cells from NOD mice (19). This study reported that immune complexes bound to this receptor were still present on the surface of NOD-derived cells and not on C57BL/10SnJ-derived cells after incubation of the cells at 37 °C for a few minutes. This observation indicates either that FcγRI from NOD mice was unable to internalize the complexes or that the immune complexes bound well and had not dissociated from the receptor on NOD cells. The fact that FcγRI-NOD demonstrated markedly reduced dissociation of ligand in the COS-7 system, demonstrated herein, directly supports the latter hypothesis.

The functional capacity of mouse FcγRI on cells from NOD mice must be questioned as it appears that the mutations in the extracellular domains lead to reduced expression of functional receptor, and receptors that are expressed exhibit higher affinity for monomeric ligand. Not only is ligand bound, but it does not appear to dissociate normally, implying that mouse FcγRI on NOD cells would be permanently saturated or perhaps continually signaling. The fact that FcγRI-NOD can associate with the FcγRI γ-subunit implies that signaling through this pathway may be intact. However, there are many other defects, including aberrant protein kinase C function (42), and this, in conjunction with the FcγRI-NOD phenotype, may affect immune complex and antigen-immune complex handling. As signaling via human FcγRI in macrophages can lead to inflammatory mediator release such as interleukin-8 (43), interleukin-6 (44), and tumor necrosis factor (9), the role of dysfunctional mouse FcγRI in the exacerbation of the NOD mouse pathology requires further investigation.

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