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Dynamics of the Interaction of Human IgG Subtype Immune Complexes with Cells Expressing R and H Allelic Forms of a Low-Affinity Fcγ Receptor CD32A

Rangaiah Shashidharamurthy,* Fang Zhang,† Aaron Amano,* Aparna Kamat,* Ravichandran Panchanathan,* Daniel Ezekwudo,* Cheng Zhu,† and Periasamy Selvaraj2*

CD32A, the major phagocytic FcγR in humans, exhibits a polymorphism in the ligand binding domain. Individuals homozygous for the R allelic form of CD32A (CD32AR allele) are more susceptible to bacterial infections and autoimmune diseases as compared with H allelic CD32A (CD32AH) homozygous and CD32AR/H heterozygous individuals. To understand the mechanisms behind this differential susceptibility, we have investigated the dynamics of the interaction of these allelic forms of CD32A when they are simultaneously exposed to immune complexes (IC). Binding studies using Ig fusion proteins of CD32A alleles showed that the R allele has significantly lower binding not only to human IgG2, but also to IgG1 and IgG3 subtypes. Competition assays using purified molecules demonstrated that CD32AH-Ig outcompetes CD32AR-Ig for IC binding when both alleles simultaneously compete for the same ligand. CD32AH-Ig blocked the IC binding mediated by both the allelic forms of cell surface CD32A, whereas CD32AR-Ig blocked only CD32AR and was unable to cross-block IC binding mediated by CD32AH. Two-dimensional affinity measurements also demonstrated that CD32AR has significantly lower affinity toward all three subtypes as compared with CD32AH. Our data suggest that the lower binding of CD32AR not only to IgG2 but also to IgG1 and IgG3 might be responsible for the lack of clearance of IC leading to increased susceptibility to bacterial infections and autoimmune diseases. Our further data suggests that in humans, inflammatory cells from CD32AR/H heterozygous individuals may predominantly use the H allele to mediate Ab-coated target cell binding during phagocytosis and Ab-dependent cellular cytotoxicity, resulting in a phenotype similar to CD32AH homozygous individuals. The Journal of Immunology, 2009, 183: 8216–8224.

Among the receptors for the Fc domain of immune complexes (IC),3 the low-affinity FcγR play a central role in many types of Ab-dependent cellular cytotoxicity and immunophagocytosis (1–5). In humans, CD32A, which is a FcγRII, is the major phagocytic Fc receptor (6). Human CD32A has low affinity for monomeric IgG, but it binds stably to IC. CD32A has been shown to exhibit a polymorphism in the ligand binding domain. This single nucleotide polymorphism in the ligand binding domain causes a substitution of amino acid arginine (CD32AR) to histidine (CD32AH) at position 131. Both CD32A alleles bind to human IgG1 and IgG3, but the CD32AH allotype shows a lower binding for human IgG2 when compared with CD32AR (7–9). Evidence suggests that CD32AR allele is associated with increased susceptibility to bacterial infections (10–15). Human IgG2 is the major subclass of Ab elicited by encapsulated bacteria in humans including human pathogens such as Neisseria meningitidis, Haemophilus influenzae, and Streptococcus pneumoniae (16–18). Yee et al. (19) showed that 90% of CD32AR homozygous individuals are more susceptible to pneumococcal infection.

Apart from bacterial infections, CD32AR allele is also associated with susceptibility to the development of certain autoimmune disease such as systemic lupus erythematosus (SLE) (16, 20–24). Various clinical studies have shown that patients with SLE who are CD32AR have a higher likelihood of developing proteinemia, hemolytic anemia, antinuclear RNAp Abs, glomerulonephritis and hypocomplementemia (25). Development of SLE at a younger age was reported in patients with the CD32AR genotype, with an earlier incidence of arthritis, sicca syndrome, nephritis, lymphadenitis, hematologic abnormalities, lupus anticoagulant, cryoglobulinemia, and hypocomplementemia (25).

Taken together, these studies suggest that the CD32A polymorphism plays a pivotal role in certain infectious and autoimmune diseases. The increased susceptibility of CD32AR homozygous individuals for the observed diseases may be due to the poor clearance of IC. Homozygous CD32AH individuals, in contrast, are not susceptible to certain bacterial infections and autoimmune diseases because IC is cleared efficiently (20, 21, 26, 27). Interestingly, CD32AR/H heterozygous individuals are also resistant to certain bacterial infections even though they express the CD32AR allele. It is not clear why the coexpression of CD32AR in heterozygous individuals is not reducing the efficiency of CD32AH allele. We hypothesize that in heterozygous individuals, CD32AR outcompetes CD32AR for ligand binding when both alleles are expressed on the same cell. To test our hypothesis, we have analyzed the interaction of IC with cells expressing R and H allelic forms of...
CD32A and their competition for ligand binding using recombinant dimeric forms of soluble R and H forms of CD32A alleles. The results presented in this study demonstrate that CD32A R outcompetes CD32A H when they simultaneously compete for the same ligand. Such a dominance of CD32A R allele in heterozygous individuals may be due to the higher affinity of CD32A R for all human IgG isotypes as compared with CD32A H, which is demonstrated in this study by cell binding assays and two-dimensional affinity measurement studies.

Materials and Methods

Cell lines and reagents
PKH-26 labeling kit, rabbit anti-DNP IgG, HRP-conjugated anti-human Fc Ab, HRP- and FITC-conjugated goat anti-human IgG (F(ab')2), specific for L chain of human IgG, and cyanogen bromide-activated Sepharose were from Sigma-Aldrich. Human IgG subtypes were from Sigma-Aldrich. As per the manufacturer’s data sheet, the purity of the IgG molecules is >95%. To avoid the problems associated with the storage of IgG molecules in solution, the molecules were immediately aliquoted and stored at –20°C. The ICs were made fresh and used for the experiments. We have also used IgG molecules from two different lots that were found to have similar binding to human FcyR. The following IgG molecules (with cat-

ological number) were purified from human plasma by combination of chromatographic techniques as per Sigma-Aldrich: IgG1 (I5154), IgG2 (I5404), IgG3 (I5654), and IgG4 (4639). FITC- and Cy5-conjugated goat anti-human Fc-specific IgG F(ab')2, from Jackson ImmunoResearch Laboratories. Lipofectamine 2000 was from Invitrogen. The Mero BCA-protein assay kit was purchased from Pierce and the HRP substrate from Bio-Rad.

QuikChange II Site-Directed Mutagenesis kit was from Stratagene. Chi-

nese hamster ovary (CHO) cells (clone K1) were from American Type Culture Collection. Human RBC were isolated from healthy volunteers. SRBC were from Colorado Serum Company. Cell culture reagents were from Life Technologies.

Construction, expression, and purification of recombinant soluble CD32A-Ig molecules
The construction and expression of the dimeric form of CD32A R-Ig was conducted by ligating the extracellular domain of human CD32A R to the Fc domain of the human IgG1 H chain as described earlier (28). The mutated IgG1 Fc CH2-CH3 domains were obtained from Dr. P. Linsley (Bristol-Meyers Squibb, Seattle, WA). Mutations in the Fc domain are L267F, L268E, G270A, and A363T (numbered as in accession number AA960920.1). These mutations have been shown to abolish the binding of FcyR (29–31). The CD32A R-Ig was constructed by QuikChange II Site-directed Mutagenesis kit (Stratagene) using CD32A R-Ig DNA as a template. The recombinant molecules were purified from CHO cell transfectants using a protein G-Sepharose column (Pharmacia Biotech) (32). The immunofluorescence purified CD32A R-Ig molecules were analyzed using 10% SDS-PAGE under reducing and nonreducing conditions and the protein bands were visualized by silver staining and Western blot analysis. Protein concentration was measured by Micro BCA protein assay kit (Pierce) using BSA as a standard.

Establishment of CHO stable transfectants expressing cell surface CD32A alleles
CHO cells were transfected with pUB6A plasmid vectors containing CD32A R and CD32A H gene using Lipofectamine. Forty-eight hours after transfection, cell culture medium containing 20 µg/ml blasticidin (Life Technologies) was used as the selection medium to establish the stable transfectants of CHO-CD32A. Cells expressing high levels of transfected molecules were selected by a panning procedure (33).

Soluble IC binding assay
Soluble IC was prepared by mixing with either HRP- or FITC-conjugated F(ab')2, goat anti-human IgG specific for L chain (κ) with human IgG subtypes (1:1 molar ratio) for 4 h at 4°C. HRP- and FITC-conjugated F(ab')2, goat anti-human IgG were used for ELISA and cell binding assays, respectively. The complex was centrifuged at 15,000 rpm for 30 min at 4°C and the supernatant was used for the soluble IC binding assay. For ELISA, CD32A-Ig molecules (10 µg/ml) were coated on plates overnight and wells were blocked for 1 h with binding buffer (PBS/0.1% BSA, pH 7.4). The HRP-IC of human IgG subtypes (50 µl of 10 µg/ml) was then added, and incubation continued for 1 h at 4°C. The wells were washed and HRP substrate was added. The reaction was stopped by adding 1 N H2SO4 and read at 450 nm. The HRP-IC of human IgG sub-

types bound to IV.3, a blocking mAb for human CD32A molecule, treated CD32A-Ig coated wells were taken as nonspecific binding.

For FITC-labeled IC binding assay, the FcγR-positive cells (50 µl of 10 5/ml) were incubated with FITC-labeled IC of human IgG subtypes (10 µg/ml) in binding buffer for 1 h at 4°C in the absence or presence of purified CD32A-Ig molecules or blocking mAbs. Cells treated with IV.3 (40 µg/ml) for 30 min at 4°C served as a specificity control. Cells were then washed and resuspended in 150 µl of binding buffer and fixed by adding 150 µl of 2% formalin in PBS. Binding of FITC-labeled IC to FcγR-positive cells was analyzed by flow cytometry.

Particular IC binding assay
SRBC were coupled with human IgG subtypes (referred to in the work as EA) by the chromium chloride method (34). PKH labeling of EA was performed using a PKH labeling kit. Binding of FcγR-positive cells to PKH-labeled EA was analyzed by flow cytometry (28, 32). Briefly, PKH-

labeled EA were washed and resuspended in binding buffer. If aggregates of EA were noticed, they were removed using 75-µm nylon filters before the assay. FcγR-positive cells (50 µl of 10 5/ml) in binding buffer were incubated with PKH-labeled EA (50 µl of 1.5 × 10 5/ml) for 2 h at 4°C. Binding assays were performed in the absence or continuous presence of CD32A-Ig molecules or blocking mAbs for CD32A-Ig (IV.3) during the incubation with EA. EA bound to cells were analyzed by flow cytometry. PKH-labeled unopsonized erythrocytes were used as a control in all the experiments. Mean fluorescent intensity at FL2 channel and the percentage of FcγR-positive cells bound to EA were determined using FACScan flow cytometer (BD Biosciences). The binding index was calculated using the formula for the percentage of cells bound to EA × mean fluorescence divided by 100.

Competition assay
Saturating concentration of CD32A-Ig molecules for rabbit anti-DNP IgG opossum sheep erythrocytes (EA) binding was determined using flow cytometry. Briefly, sheep erythrocytes were coated with DNP as described (35) and opsonized with rabbit anti-DNP IgG (EA). EA (50 µl of 1 × 10 5 cells/ml) were incubated with various concentrations of CD32A-Ig mole-

ules for 1 h at 4°C. After washing twice with binding buffer the FITC-conju-
gated F(ab')2, goat anti-human Fc-specific IgG (portion of CD32A-Ig molecules is a human IgG1 Fc domain) was added and incubated for another 1 h at 4°C. The cells were washed twice and analyzed for CD32A-Ig molecules binding. For the competition assay, EA (50 µl of 1 × 10 5 cells/ml) were incubated with 50 µl of CD32A-Ig molecules either alone or in combination (equimolar or saturating concentration) for 1 h at 4°C. The CD32A-Ig molecules were precipitated with Protein G-Sepharose (100 µl) and washed ten times with binding buffer. For the FITC-conjugated F(ab')2, goat anti-human Fc-specific IgG (Ig portion of CD32A-Ig molecules is a human IgG1 Fc domain) was added and incubated for another 1 h at 4°C. The cells were washed twice and analyzed for CD32A-Ig molecules binding. For the competition assay, EA (50 µl of 1 × 10 5 cells/ml) were incubated with 50 µl of CD32A-Ig molecules either alone or in combination (equimolar or saturating concentration) for 1 h at 4°C. The CD32A-Ig molecules were precipitated with Protein G-Sepharose (100 µl) and washed ten times with binding buffer.

Micropipette adhesion frequency assay
To determine two-dimensional affinity (the affinity of a receptor-ligand pair expressed on opposing cell surface) of CD32A alleles for the human IgG subtypes, the micropipette adhesion frequency assay was conducted as described (34). Briefly, human RBC were coated with human IgG subtypes using CrCl3 coupling method as described. The site density of human IgG subtypes on human RBCs and Fc receptors on CHO cells was determined by flow cytometry using fluorescently coupled standard beads (Quantum 25 FITC High Level; Bangs Laboratory).

The micropipette apparatus used in this experiment has been previously described (34, 36, 37). Briefly, a micropipette-aspirated RBC coated with human IgG subtypes was driven by a piezoelectric trans-

lator to make a 10 s contact with a CHO cell expressing CD32A alleles held stationary by another pipette. At the end of the contact duration, the two cells were separated by retracting the RBC. Upon retraction, an adhesion event between the two cells was indicated by stretched RBC membrane (see Fig. 6A). This contact test cycle was repeated 100 times using the same pair of cells, keeping the contact duration (t) and the apparent area (A, A + 3 µm 2) constant, and the number of adhesion event was counted to obtain an adhesion frequency (P). Nonspecific binding is determined using RBC coated with BSA. The effective binding affinity (κ, kr) and off-rate (k2) were extracted by fitting the equation, P = 1 − exp(−mrkknRk1 − exp(−k2)), in which mr and m2 are receptor and ligand site density, respectively. For experiments in this study, the adhesion
frequency was determined at a sufficiently long contact duration ($t = 10 \times e^{\ln(1/Kd)}$) without measuring a full binding curve to simply estimate the effective binding affinity ($A/K_d$) from the equation, $A/K_d = 1/m, n_t (\ln(1 - P_b))^{-1}$, which is derived from the steady state version (i.e., $t \rightarrow \infty$) of the adhesion frequency ($P_b$) equation.

Statistical analysis

A statistical comparison of the CD32A$^R$ and CD32A$^H$ alleles was performed using the Student $t$ test. Values for $p < 0.01$ were considered as significant and $p < 0.001$ considered highly significant.

Results

Recombinant Ig fusion proteins of CD32A alleles are secreted as a homodimers

The extracellular domain of CD32AR was ligated to the mutated CH2 and CH3 regions of the Fc domain of human IgG1 molecule using a strategy similar to the construction of CD16A-Ig (28). The CD32AR-Ig was created by site-directed mutagenesis using CD32AR-Ig DNA as a template. The resultant chimeric cDNA was transfected in CHO cells. The culture supernatant obtained from CHO transfectants was analyzed by immunoaffinity chromatography using protein G-Sepharose. The yield was nearly 1.5 mg for CD32AR-Ig and 2.0 mg for CD32AH-Ig per liter of culture supernatant. SDS-PAGE analysis of immunoaffinity chromatography purification showed a major band of 141 kDa and a minor band of ~120 kDa under nonreducing conditions and 68 kDa under reducing conditions, suggesting that the recombinant CD32AR-Ig molecules are secreted as a disulfide-linked homodimer. The minor protein band of 120 kDa (under nonreducing conditions) may be the precursor or degradation product of CD32A-Ig because it reacted with anti-human Fc-specific IgG in Western blot analysis (Fig. 1B).

The IgG binding properties of CD32A-Ig molecules are similar to cell surface expressed CD32A alleles

The purified dimeric CD32A-Ig molecules were assayed for their functional ability to bind soluble IC of human IgG subtypes. Soluble IC of human IgG subtypes conjugated with HRP were prepared as described in Materials and Methods and assayed for binding to FcγR-Ig coated on the plates. As expected, CD32A$^H$-Ig bound to human IgG1, IgG2 and IgG3, whereas CD32A$^R$-Ig bound to IgG1 and IgG3 but not to IgG2 (Fig. 2). Both alleles were unable to bind to IgG4. The wells with CD32A-Ig molecules and treated with IV.3 mAb (a mAb that specifically blocks binding of IC to CD32A) before the addition of HRP-F(ab')$_2$-labeled IC were used as a specificity control. The results show that HRP-F(ab')$_2$-labeled IC binding to CD32A is specific and can be completely blocked by IV.3 mAb. This also rules out the possibility of unconjugated HRP-F(ab')$_2$ binding to CD32A-Ig molecules.

Next, we determined whether the dimerized CD32A alleles had a ligand binding pattern similar to cell surface CD32A alleles. To test this possibility, we assayed the CHO transfectants expressing the CD32A$^R$ and CD32A$^H$ alleles separately. Flow cytometry analysis using IV.3 mAb confirmed that both alleles of CD32A were expressed to almost similar levels on CHO cell surface (Fig. 3A). Soluble IC of human IgG subtypes conjugated with FITC was prepared as described in Materials and Methods. Flow cytometry
analysis of FITC-conjugated IC showed that CHO cells expressing CD32AH allele (CHO-CD32AH) bound to human IgG1, IgG2, and IgG3, whereas CHO-CD32AR bound to IgG1 and IgG3 but not to IgG2 (Fig. 3B, filled histogram). As a specificity control for FITC-conjugated F(ab')2 binding, the CHO-CD32A cells were treated with IV.3 Ab before the addition of FITC-labeled IC. Results show that the FITC-labeled IC was completely blocked by IV.3, suggesting that the uncomplexed FITC-conjugated F(ab')2 does not bind nonspecifically to CD32A expressed on CHO cells (Fig. 3B, open histogram). Fig. 3C represents the level of human IgG subtypes IC bound to CHO-CD32A alleles. Thus, as we observed in the previous experiment with dimerized CD32A-Ig (Fig. 2), both of the cell surface CD32A alleles bind to IgG1 and IgG3, but CD32AH binds better than CD32AR (Fig. 3C).

The ICs formed between autoantibodies and target cells are particulate in nature. Therefore, we determined the binding pattern of CD32A alleles to particulate IC of human IgG subtypes using CHO cells expressing CD32AR and CD32AH forms. As shown in Fig. 3D, CHO cells expressing CD32AH alleles bound to human IgG1, IgG2, and IgG3, whereas CHO-CD32AR cells bound to IgG1 and IgG3 but not to IgG2. Neither of the alleles bound to IgG4. These results show that CD32AR and CD32AH alleles have a similar binding pattern for both soluble and particulate IC of human IgG subtypes. Soluble and particulate IC binding to CD32A alleles was completely blocked by IV.3 mAb (a mAb to CD32A) and served as a specificity control. These results suggest that dimeric recombinant CD32A-Ig molecules and cell surface CD32A alleles interact similarly with human IgG subtypes. Furthermore, dimerization of the recombinant CD32A-Ig molecules did not change its binding specificity to human IgG subtypes. Interestingly though, both CD32A alleles bound to IgG1 and IgG3, CD32AH allele binding was better.

FIGURE 3. Flow cytometry analysis of expression of CD32A alleles in CHO cells. Stable transfectants of CHO cells expressing CD32A alleles were stained with anti-hCD32A mAb (IV.3). A, Isotype control (open histogram) and the binding of IV.3 Ab to CD32A alleles (filled histogram) are indicated. Dotted line indicates the expression level of CD32A alleles. B, Binding of cell surface CD32A alleles to soluble IC (sIC) of human IgG subtypes. CHO cells expressing CD32A alleles were incubated with FITC-conjugated soluble IC of human IgG subtypes (10 μg/ml) in the presence and absence of IV.3 (10 μg/ml) and analyzed for FITC-labeled IC binding to CHO-CD32A alleles using flow cytometry (filled histogram). IV.3-treated cells served as a negative control (open histogram). Data are representative of three individual experiments. C, Representation of human IgG subtype soluble IC bound to CHO-CD32A alleles is shown. Results are mean ± SD from three experiments. *, p < 0.01, **, p < 0.001. D, Binding of cell surface CD32A alleles to human IgG subtypes coated with sheep erythrocytes (EA). CHO cells expressing CD32A alleles were incubated with human IgG subtype coated, PKH-labeled EA (50 μl of 1.5 x 10^7 cells/ml) for 2 h at 4°C. Results are mean ± SD from three experiments. EA bound to the cells was analyzed by flow cytometry. Cells incubated with PKH-labeled unopsonized-E and IV.3-treated cells served as specificity control. The binding index was calculated using the formula for the percentage of cells bound to EA x mean fluorescence divided by 100. Results are mean ± SD of data from three experiments. *, p < 0.01 and **, p < 0.001.
A simple in vitro competition assay was performed to directly test the allelic form for ligand binding using purified CD32A-Ig molecules. We studied the competition between these alleles for ligand binding when both the alleles are exposed simultaneously. We determined the competition of both the alleles to EA to study the competition of these molecules for binding to EA. The cells treated with secondary Ab alone served as a specificity control. EA treated with secondary Ab alone (panel I), EA incubated with 50 μg/ml FITC-CD32AH-Ig (panel II), EA incubated with 50 μg/ml Cy5-conjugated CD32AH-Ig (panel III), EA incubated with CD32AH-Ig (50 μg/ml) and CD32AR-Ig (25 μg/ml) (panel IV), EA incubated with CD32AH-Ig (25 μg/ml) and CD32AR-Ig (25 μg/ml) (panel V), and EA incubated with CD32AH-Ig (50 μg/ml) and CD32AR-Ig (50 μg/ml) (panel VI). Unstained cells (lower left quadrant), CD32AR-Ig bound EA (lower right quadrant), CD32AH-Ig bound EA (upper left quadrant), and EA bound to both the CD32AH-Ig molecules (upper right quadrant) are shown. Data are representative of three individual experiments.

FIGURE 4. Competition of CD32AH-Ig and CD32AR-Ig for binding to rabbit IgG opsonized SRBCs. A, To determine the saturating concentration of CD32AH-Ig molecules binding to rabbit IgG-coated erythrocytes (EA), varying concentrations of CD32A-Ig molecules were incubated separately with 50 μl of EA (2 × 10⁶ cells/ml) for 1 h at 4°C. After washing three times with binding buffer, the incubation was continued with F(ab')₂, goat anti-human Fc-specific Ab conjugated with FITC for 1 h at 4°C. Cells were analyzed for CD32A-Ig molecules binding by flow cytometry. B, For competition assay, an equal amount or saturating concentration of both the molecules was mixed and incubated with EA. The CD32AH-Ig molecules were preincubated with F(ab')₂, goat anti-human Fc-specific Ab conjugated with Cy5 (for CD32AH-Ig) or FITC (for CD32AR-Ig) for 1 h at 4°C and then added to EA to determine the competitive binding of both the alleles to EA. Cells were washed and analyzed for CD32A-Ig binding by flow cytometry. The cells treated with secondary Ab alone served as a specificity control. EA treated with secondary Ab alone (panel I), EA incubated with 50 μg/ml FITC-CD32AH-Ig (panel II), EA incubated with 50 μg/ml Cy5-conjugated CD32AH-Ig (panel III), EA incubated with CD32AH-Ig (50 μg/ml) and CD32AR-Ig (25 μg/ml) (panel IV), EA incubated with CD32AH-Ig (25 μg/ml) and CD32AR-Ig (25 μg/ml) (panel V), and EA incubated with CD32AH-Ig (50 μg/ml) and CD32AR-Ig (50 μg/ml) (panel VI). Unstained cells (lower left quadrant), CD32AR-Ig bound EA (lower right quadrant), CD32AH-Ig bound EA (upper left quadrant), and EA bound to both the CD32AH-Ig molecules (upper right quadrant) are shown. Data are representative of three individual experiments.

than the CD32AH allele. Taken together, these results suggest that CD32A alleles not only differ in binding to IgG2 but also to IgG1 and IgG3.

**CD32AH allele outcompetes CD32AH allele for ligand binding when both alleles are exposed to IC simultaneously**

Because previous ligand binding experiments suggest that CD32AH binds to IC more efficiently than CD32AR allele, next we studied whether CD32AH allele competes with CD32AR allele for ligand binding under situations in which both the alleles are exposed to the same ligand simultaneously. We studied the competition between these alleles for ligand binding using purified CD32A-Ig molecules. A simple in vitro competition assay was performed to directly determine whether CD32AH competes with CD32AR for ligand binding. In this experiment, we used rabbit IgG coated SRBC (EA) as a model IC and anti-human Fc specific Ab for detection. First, we determined the saturating concentration of the dimers binding to rabbit IgG coated SRBC by incubating rabbit IgG coated SRBC with various concentration of CD32AH-Ig and CD32AH-Ig separately. FITC-conjugated F(ab')₂, goat anti-human Fc-specific Ab was used to detect binding via flow cytometry. As shown in Fig. 4A, CD32AH-Ig reaches saturation around 25 μg/ml, whereas CD32AH-Ig requires 50 μg/ml. These results suggest that these molecules differ significantly ( p < 0.001) in their affinity for rabbit IgG binding, which is consistent with our previous report (38). Then we determined the competition between these molecules for binding to rabbit IgG when both the alleles are exposed simultaneously to IC. In this experiment, we incubated the EA with either equimolar (25 μg/ml of each CD32A-Ig molecules) or saturating concentrations (CD32AH-Ig: 50 μg/ml, CD32AR-Ig: 25 μg/ml) of CD32A-Ig molecules. CD32AH-Ig was pre-complexed with Cy5-conjugated goat anti-human Fc-specific F(ab')₂, Ab, whereas CD32AR-Ig was pre-complexed with FITC-conjugated goat anti-human Fc-specific F(ab')₂, Ab. Both molecules (either equimolar or saturating concentration) were then added to the EA to study the competition of these molecules for ligand binding. The secondary Ab was titrated, and excess dimers were added to avoid cross-binding of the secondary Ab to the dimers during the incubation of the dimers with EA. Both CD32AH-Ig molecules (Fig. 4B, panel II) and CD32AH-Ig molecules (Fig. 4B, panel III) bind to rabbit IgG-coated SRBC when incubated separately. When both molecules were incubated together at their saturation concentrations (CD32AH-Ig: 50 μg/ml, CD32AH-Ig: 25 μg/ml) (Fig. 4B, panel IV), 60% of the EA were Cy5-positive, indicating that 60% of EA bound only to CD32AH-Ig. Only 40% of the EA were both Cy5- and FITC-positive, suggesting that 40% of EA bound to both the dimers. At equimolar concentrations (25 μg/ml of both molecules) (Fig. 4B, panel V) (50 μg/ml of both molecules) (Fig. 4B, panel VI) of the dimers, more than 80% of EA were Cy5-positive, indicating that more than 80% of EA was bound only to CD32AH-Ig. Only less than 20% of the EA were both Cy5- and FITC-positive, suggesting that less than 20% of EA bound to both the dimers. The EA treated with secondary Ab alone served as a specificity control (Fig. 4B, panel I). These results suggest that CD32AH allele has a higher binding efficiency for IC and thus out-competes the R allelic form for ligand binding under situations in which both alleles are exposed to the same ligand simultaneously, as may happen in R/H heterozygous individuals.
CD32AH-Ig is able to cross-block IC binding mediated by cell surface CD32AR

Next we determined whether the CD32AH-Ig molecules can cross-block the ligand binding mediated by cell surface CD32AR alleles. The cross-blocking studies were conducted using CHO transfectants expressing CD32AR and CD32AH alleles separately. In this experiment, we have used human IgG1 and IgG3 for CHO-CD32AR, whereas IgG1, IgG2, and IgG3 were used for CHO-CD32AH for both soluble and particulate IC. As shown in Fig. 5A, at 25 μg/ml, CD32AH-Ig was able to block more than 90% of human IgG subtype binding to both the cell surface CD32A alleles. At the same concentration, CD32AR-Ig was able to block up to 90% of human IgG binding to CHO-CD32AR, but only cross-blocked 10% of human IgG binding to CHO-CD32AH (Fig. 5A, compare top and bottom panel). Similar results were observed when EA was used as an IC (Fig. 5B). These data suggest that the CD32AH-Ig cross-blocks the ligand binding mediated by cell surface CD32AR alleles, whereas CD32AR-Ig is unable to cross-block the ligand binding mediated by cell surface CD32AH allele.

CD32AH allele has higher affinity for all human IgG subtypes than CD32AR allele

The above ligand binding studies and cross-blocking studies suggest that the CD32A alleles might differ in their affinity not only for human IgG2 but also for IgG1 and IgG3. Using a micropipette adhesion frequency assay, we measured the two-dimensional binding affinity of the two CD32A alleles (CHO-CD32AR and CHO-CD32AH) for four human IgG subtypes (IgG1, IgG2, IgG3, and IgG4). The adhesion frequency of CHO cells expressing either CD32A allele to RBC coated with one of the four subtypes of human IgG were measured and the effective binding affinity was calculated as described in Materials and Methods. The typical binding is represented in Fig. 6A. The results showed that CD32AH has higher binding affinity than CD32AR for IgG1, IgG2, and IgG3 (Fig. 6B). Both alleles have a similarly low-binding affinity for IgG4. We observed that CD32AH has 1.8-, 5.8-, and 2.22-fold higher affinity for IgG1, IgG2, and IgG3 when compared with CD32AR (Table I). For both CD32A alleles, the binding affinity for human IgG subtypes ranked as IgG3>IgG1>IgG2>IgG4. These results indicate that the
CD32A alleles not only differ in human IgG2 binding, but also differ substantially in their affinity for IgG1 and IgG3.

**Discussion**

The importance of low-affinity FcγR in the development of autoimmune diseases and defense against bacterial infections has recently been well documented (16, 22, 23, 25, 27). The consequences of polymorphisms in CD32A, particularly their different ligand binding characteristics, are being intensely investigated due to their role in certain infectious and autoimmune diseases. CD32A<sup>H</sup> has been shown to bind to IgG2 with high affinity, whereas CD32A<sup>R</sup> shows little or no binding to IgG2 (16–18, 39–41). Several studies have documented that CD32A<sup>R</sup> homozygous individuals are more susceptible to bacterial infections and have a phenotype similar to CD32A<sup>H</sup> individuals. Our observation with IgG1 binding contrasts but agrees on IgG3 binding with a previous report by Bredius et al. (44) that shows that CD32A alleles differ in binding to IgG2 and IgG3 but not IgG1. A recent report by Bruhns et al. (9) has shown that CD32A alleles do not differ in binding to IgG1 and IgG3. At present, the reason for these discrepancies is not clear but it may be due to the use of different cell types and molecules. The report by Bredius et al. (44) used neutrophils treated with anti-CD16 mAb, whereas Bruhns et al. (9) used CD32A fused with FLAG peptide either as expressed on CHO cells or purified molecules. In this report, we have used CHO cells expressing the unmodified CD32A alleles and purified dimeric CD32A-Igs. Furthermore, we have used a two-dimensional affinity measurement technique (33), which directly measures the affinity of a receptor-ligand pair expressed on cell membranes when they are mediating cell-cell adhesion as happens during FcγR-expressing cells binding to Ab-coated target cells.

Interestingly, CD32A<sup>R/H</sup> heterozygous individuals are not susceptible to bacterial infections and have a phenotype similar to CD32A<sup>H</sup> individuals. To determine whether this effect is due to the dominant effect of CD32A<sup>R</sup> binding to IC, we studied the competition between these alleles for ligand binding using purified molecules. The results of the competition assay using rabbit IgG-coated SRBC suggest that when both alleles are exposed to the same ligand simultaneously, CD32A<sup>H</sup> outcompetes CD32A<sup>R</sup> for

![FIGURE 6. Representation of two-dimensional affinity measurement by micropipette method. The typical experimental setup is represented. A. Briefly, two glass micropipettes and the chamber were filled with binding buffer. A micropipette-aspirated RBC coated with human IgG subtypes was driven by a piezoelectric translator to make a 10-s contact with a CHO cell expressing CD32A alleles, held stationary by another pipette (cell contact). At the end of the contact duration, the two cells were separated by retracting the RBC. Upon retraction, an adhesion event between the two cells was indicated by stretched RBC membrane (cell binding). Nonspecific binding is determined by using RBC coated with BSA (no binding). B. CD32A<sup>R</sup> allele has a higher affinity for human IgG subtypes than the CD32A<sup>H</sup> allele as measured by micropipette method. The micropipette adhesion frequency assay was conducted as described in Materials and Methods. The contact test cycle as described in A was repeated 100 times using the same pair of cells, and the number of adhesion events was counted to obtain an adhesion frequency (P<sub>a</sub>). The effective binding affinity (A<sub>eff</sub>) was calculated by fitting the data into the equation described in Materials and Methods. Data are representative of two individual experiments. *, p < 0.01 and **, p < 0.001.](http://www.jimmunol.org/)

### Table I. Affinity of cell surface CD32A alleles by two-dimensional affinity measurement

<table>
<thead>
<tr>
<th>Human IgG Subtypes</th>
<th>Human IgG1</th>
<th>Human IgG2</th>
<th>Human IgG3</th>
<th>Human IgG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-CD32A&lt;sup&gt;R&lt;/sup&gt;</td>
<td>7.98 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>2.34 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.45 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.31 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHO-CD32A&lt;sup&gt;H&lt;/sup&gt;</td>
<td>1.77 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.38 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>2.61 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1.39 x 10&lt;sup&gt;-9&lt;/sup&gt;</td>
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</tbody>
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ligand binding. Furthermore, our results show that purified CD32AH-Ig is capable of blocking both the cell surface CD32AR and H alleles from binding to human IgG subtypes, whereas purified CD32AH-Ig was unable to cross-block the binding mediated by cell surface CD32AH allele. These differences in the ability to compete for the ligand and cross-block each other may be due to differences in the strength of the cell-cell adhesion mediated by these molecules, as evidenced by differences in their two-dimensional affinity. For instance, CD32AH has a 2-fold higher affinity for IgG1 and IgG3 and a 5-fold higher affinity for IgG2 compared with the CD32AH allele. The results from binding and affinity studies suggest that individuals homozygous for the CD32AH allele have lower binding affinity for all human IgG subtypes and therefore may not be able to bind to Ab-coated bacteria effectively, have lower binding affinity for all human IgG subtypes and therefore compete for the ligand and cross-block each other may be due to differences in their two-dimensional affinity. For instance, CD32AH has a 2-fold higher affinity for human IgG1, IgG2, and IgG3 and a 5-fold higher affinity for IgG2 compared with the CD32AH allele. The results from binding and affinity studies suggest that individuals homozygous for the CD32AH allele have lower binding affinity for all human IgG subtypes and therefore may not be able to bind to Ab-coated bacteria effectively, becoming susceptible to infection as a result. In individuals homozygous for CD32AH allele, the H allele has a higher affinity for all human IgG subtypes and therefore may not be able to bind to Ab-coated bacteria leading to quicker clearance of infection. In heterozygous individuals, as the CD32AH allele has a higher affinity for all human IgG subtypes, it is able to outcompete the CD32AH allele for binding to Ab-coated bacteria. Consequently, the heterozygous individuals display a phenotype similar to the CD32AH homozygous individuals.

Apart from bacterial infections, studies have shown an association between CD32A and IC-mediated autoimmune diseases such as SLE (20, 21). In humans, individuals who express only both CD32AR and CD32AH are more susceptible to Ab-mediated autoimmune diseases than individuals expressing the CD32A allele, which binds to human IgG with higher affinity (16–18). This increased susceptibility in CD32AR individuals is suggested to be due to the inability of CD32AR to bind and clear IC from circulation leading to the accumulation and deposition of IC on tissues (26, 27, 45, 46). In humans, ICs formed are not only with IgG2 but also with other IgG subtypes and therefore the accumulation of IC in CD32AR individuals cannot be attributed to the inefficient binding of IgG2 alone. Our findings suggest that CD32AR individuals have a lower affinity not only for IgG2 but also for human IgG1 and IgG3 subtypes, and this might explain why IC from the circulation are not cleared efficiently. The IC deposited on tissues in CD32AH homozygous individuals may lead to the development or acceleration of the disease via complement activation and engagement of other activating FcRs such as CD16. Several studies suggest the involvement of complement proteins (47–52) and CD16 (16, 53, 54) in the development of autoimmune diseases.

In conclusion, our results demonstrate that the H allele of CD32A has a higher affinity for human IgG1, IgG2, and IgG3 subtypes and outcompetes the R alleleic form for binding to Ab-coated bacteria leading to quicker clearance of infection. In heterozygous individuals may predominantly use the H allele to mediate Ab-coated bacteria and become susceptible to infection as a result. In humans, ICs formed are not only with IgG2 but also with other IgG subtypes and outcompetes the R allelic form for ligand binding to human IgG subtypes, it is able to outcompete the CD32AR allele for binding to Ab-coated bacteria. Consequently, the heterozygous individuals display a phenotype similar to the CD32AH homozygous individuals.

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Disclosures

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References


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