Fcγ Receptor III on Human Neutrophils

Allelic Variants Have Functionally Distinct Capacities

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Abstract

As a model system to explore the functional consequences of structural variants of human Fcγ receptors (FcγR), we have investigated FcγR-mediated phagocytosis in relation to the NA1-NA2 polymorphism of FcγRIII (CD16) on neutrophils ($Fc\gamma RIII_{PMN}$). The neutrophil-specific NA antigen system is a biallelic polymorphism with codominant expression demonstrating a gene dose effect with the anti–NA1 MAb CLB-gran 11 in a large donor population. To explore the impact of this allelic variation of $Fc\gamma RIII_{PMN}$ on phagocytosis, we used two FcγRIII-dependent probes, IgG-sensitized erythrocytes (EA) and concanavalin A-treated erythrocytes (E-ConA). Comparison of FcγR-mediated phagocytosis by PMN from NA1 subjects and from NA2 subjects showed lower levels of phagocytosis of both probes by the NA2 individuals. The difference was most pronounced with lightly opsonized EA: at the lowest level of sensitization the phagocytic index was 72% lower for NA2 donors, whereas at the highest level of sensitization it was 21% lower ($P < 0.003$). Blockade of FcγRII with MAb IV.3 Fab amplified by threefold the difference between NA1 and NA2 donors.

NA1 and NA2 individuals had identical phagocytic capacities for the non-FcγRIII probes, serum-treated and heat-treated zymosan. These individuals did not show differential quantitative cell surface expression of $Fc\gamma RIII_{PMN}$ measured by a panel of anti-CD16 MAb (3G8, CLB FcR-gran 1, VEP13, BW209/2) and by Scatchard analysis of ^{125}I-IgG dimer binding. The difference in FcγR-mediated phagocytosis was not explicable on the basis of differential collaboration of $Fc\gamma RIII_{PMN}$ alleles with FcγRII, since (*a*) the difference in phagocytic capacity between NA1 and NA2 individuals was readily apparent with the E-ConA probe (which is independent of FcγRII) and (*b*) the difference in phagocytosis of EA was magnified by FcγRII blockade. The demonstration that allelic polymorphisms in FcγR can have significant consequences for physiological functions implies that within the structural complexity of human FcγRs, including both allelic forms and cell type-specific isoforms, there will be differences in quantitative, and perhaps qualitative, function with potential importance for disease processes. (*J. Clin. Invest.* 1990. 85:1287–1295.) Fcγ receptor • neutrophil • phagocytosis

Introduction

Receptors for the Fc portion of IgG are essential participants in many immune system effector functions including phagocytosis of opsonized and certain nonclassically opsonized particles, release of inflammatory mediators, and antibody-dependent cellular cytotoxicity (1, 2). In humans three structurally distinct families of Fcγ receptors (FcγR),[1] defined by molecular weights, ligand binding properties, and certain monoclonal antibodies (MAbs), represent distinct gene products and are present on characteristic populations of leukocytes (3, 4). Within these families of FcγR, several structural polymorphisms have been recognized. For example, two different forms of FcγRII, which influence the affinity of binding of various murine immunoglobulin isotypes to the human receptor, have been demonstrated by isoelectric focusing of isolated receptor from normals (5, 6). This polymorphism of FcγRII impairs the ability of the FcγRII with the "nonresponding" phenotype to provide sufficient cross-linking of murine anti-CD3 IgG1 MAb for in vitro T-cell mitogenesis (7–9). Another FcγR polymorphism, identified on FcγRIII as the neutrophil-specific NA antigen system by serological typing with alloantisera and by certain MAbs (10–12), reflects differences in protein sequences and number of glycosylation sites (13, 14) and has no known functional significance.

Systematic differences in FcγR-specific phagocytosis in certain immunogenetically defined disease-free individuals with normal numbers of surface FcγR (15–17) have raised the possibility that structural variations in FcγR may lead to functional differences. At present some differences in FcγR function between cell types have been defined (18–22), but few data about structure–function relationships for polymorphic FcγR within a single cell type are available (5). As a model system to explore the relationships between structural polymorphisms and functional properties of FcγR, we have investigated the NA polymorphism of FcγRIII on neutrophils ($Fc\gamma RIII_{PMN}$).

Using quantitative flow cytometry in a large donor population, we have demonstrated that the NA polymorphism of $Fc\gamma RIII_{PMN}$ shows a clear allelic gene dose effect and is a suitable model for study of the functional implications of allelic structural forms. We have examined the functional conse-

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1. *Abbreviations used in this paper:* ANOVA, analysis of variance; B_{max}, maximum binding; E, erythrocytes; EA, IgG-sensitized bovine erythrocytes; E-ConA, concanavalin A-treated rabbit erythrocytes; FcγR, receptors for Fc portion of IgG; $Fc\gamma RIII_{PMN}$, the 50–78-kD receptor on human neutrophils for the Fc portion of IgG; HTZ, heat-treated zymosan; K_a, association constant; mIgG, murine IgG; %P, percentage of neutrophils with internalized particles; PE, phycoerythrin; PI, phagocytic index; STZ, serum-treated zymosan.

quences of this NA1-NA2 polymorphism for quantitative phagocytosis using IgG-sensitized erythrocytes (EA) and concanavalin A-treated erythrocytes (E-ConA). These $Fc\gamma RIII_{PMN}$-dependent probes are internalized by classical ligand-mediated and nonclassical lectin-carbohydrate-mediated mechanisms, respectively (2). Our results show that neutrophils from NA2 donors have a lower capacity to mediate phagocytosis compared with that of NA1 donors, whether the FcγR is ligated by its classical IgG ligand-binding site or by lectin–carbohydrate interactions. Since these different functional capacities cannot be explained on the basis of altered numerical expression of $Fc\gamma RIII_{PMN}$, the allelic polymorphism per se appears to have direct impact on quantitative receptor function.

Methods

Subjects. 49 disease-free adults participated in the study. They ranged in age from 20 to 56 yr (34±9 [mean±SD]). Protocols for all studies were approved by the Institutional Committee on Human Rights in Research.

Monoclonal antibodies. A panel of MAbs that recognize epitopes on FcγRIII (CD16) was used (10, 23): MAb 3G8 (mIgG1) was kindly provided by Dr. Jay Unkeless, Mt. Sinai Medical Center, New York; CLB-FcR gran 1 and CLB-gran 11 (both mIgG2a) by Dr. Pedro Tetteroo and GRM1 (mIgG2a) by Dr. Tom Huizinga, both investigators from the Central Laboratory of the Netherlands Red Cross, University of Amsterdam, Amsterdam, The Netherlands; BW209/2 (mIgG2a) by Dr. R. Kurrle (Behringwerke, Hanover, Federal Republic of Germany); VEP 13 (mIgM) and B73.1-PE (mIgG1) were obtained commercially from Calbiochem-Behring Corp. (San Diego, CA) and Becton, Dickinson & Co. (Mountain View, CA), respectively. The MAb IV.3 (mIgG2b) and IV.3 Fab fragments recognizing FcγRII (CD32) were kindly provided by Drs. Clark Anderson (Ohio State University, Columbus, OH) and Paul Guyre (Dartmouth Medical Center, Hanover, NH) (24). Silver stain analysis of the IV.3 Fab fragment preparation indicated that there was no intact IgG. The MAb 41H.16 (mIgG2a) recognizing FcγRII was obtained from Dr. Thomas Zipf, University of Texas Cancer Center, Houston, TX (25). Murine IgG1 and IgG2a myeloma proteins (Sigma Chemical Co., St. Louis, MO), and mIgG1-PE (Becton, Dickinson & Co.) were used as IgG subclass controls for the anti-FcγR MAbs.

Preparation of neutrophils. Peripheral blood, obtained by venipuncture from healthy volunteers, was drawn into heparinized syringes. PMN were separated by discontinuous two-step centrifugation on Ficoll-Hypaque density gradients (d = 1.077 and d = 1.119) followed by hypotonic lysis of contaminating erythrocytes. Microscopic examination of the cells demonstrated > 98% PMN.

Human neutrophil antigen typing. Serologic typing for the neutrophil-specific antigens, NA1 and NA2, was performed using the granulocyte agglutination assay described by Lalezari (26) and a panel of anti-NA1 and anti-NA2 allosera generously provided by Dr. David Stroncek of the American Red Cross Neutrophil Serology Reference Laboratory, St. Paul, MN. Among the 49 unrelated healthy volunteers, 25 individuals were heterozygotes positive for both NA1 and NA2, 7 individuals were positive only for NA1, and 17 individuals were positive only for NA2. The phenotype frequency of this population (NA1, 65%; NA2, 86%) is similar to that reported by others (10–12). The demographic characteristics of each group were comparable.

Preparation of particles. Antibody-coated E (EA) were prepared by incubating bovine E with rabbit IgG anti-bovine E antibody (Cappel Laboratories, Cochranville, PA) for 1 h at 37°C. The cells were washed and resuspended at 10^8 cells/ml in RPMI and 20% FCS (2). The standard amount of antibody used was a fourfold dilution of the minimum agglutinating titer. Serial dilutions of the standard antibody concentration were used to vary the degree of opsonization of the E for dose-response experiments. The phagocytic capacity of PMN for EA varied inversely with the degree of opsonization of the erythrocytes with the rabbit anti-E antibody.

E-ConA were prepared by a modification of the method of Goldman and Cooper as previously described (2, 27, 28). Rabbit E at 10^8 cells/ml in PBS were incubated with ConA (Sigma Chemical Co.), 0.1 μg/ml, for 30 min at 37°C. The cells were then washed and resuspended at 10^8 cells/ml in RPMI and 20% heat-inactivated FCS.

Serum-treated zymosan (STZ) was prepared by incubating zymosan A (Sigma Chemical Co.) at 1 mg/ml in serum from normal volunteers for 30 min at 37°C with vortexing at 5 min intervals (2). Heat-treated zymosan (HTZ) was prepared by boiling zymosan at 1 mg/ml for 10 min with constant stirring. The STZ or HTZ was washed in PBS and resuspended at 2.5×10^8 particles/ml in RPMI with 20% FCS.

Assay of phagocytosis. Quantitation of PMN phagocytosis was performed as previously described (2). Briefly, PMN were resuspended in RPMI with 20% heat-inactivated IgG-free FCS (Gibco Laboratories, Grand Island, NY) at 5×10^6 cells/ml. In certain experiments, the PMN were preincubated with either 3G8 or IV.3 MAb (10 μg/ml) or their Fab fragments (250 μg/ml) for 10 min; the MAbs were present throughout the assay of phagocytosis.

To assess internalization of E target particles, PMN (100 μl) were combined with EA or E-ConA (250 μl). The PMN-erythrocyte mixtures were centrifuged at 44 g for 3 min and then incubated at 37°C for 30 min to allow for maximum internalization. After lysis of noninternalized E with ammonium chloride, phagocytosis was quantitated by light microscopy. At least 400 cells per slide were counted in duplicate without knowledge of the donor NA type. The data are expressed as percent phagocytosis (%P, percentage of PMN with one or more internalized E) and phagocytic index (PI, number of ingested E per 100 PMN).

To assess internalization of the zymosan, PMN (200 μl) were combined with STZ or HTZ (20 μl), centrifuged at 44 g for 3 min, and then incubated for 10 min at 37°C. Phagocytosis was quantitated by light microscopy as described above.

Quantitation of FcγR number and affinity by IgG oligmer binding. Saturable binding of IgG oligomers to PMN was assessed by the method of Kurlander and Batker (29) as described previously (16). A single batch of human IgG oligomers, cross-linked with bis-diazotized benzidine, was sized by gel filtration with AcA22 (Pharmacia-LKB, Piscataway, NJ) and stored at −70°C, and an aliquot was iodinated by the lactoperoxidase method every 2 wk. Sucrose-density gradient ultracentrifugation demonstrated predominantly dimeric complexes with 10% larger aggregates. Specific activity of the oligomers ranged from 6 to 12×10^7 cpm/nmol IgG. IgG molarities were calculated by assuming a mol wt of 160,000 according to Segal and Hurwitz (30), regardless of the degree of polymerization.

All binding assays were performed in triplicate at 4°C for 2 h, in PBS containing 0.2% BSA and 0.1% sodium azide. 5×10^5 PMN per sample were incubated with varying concentrations of iodinated oligomers ($5–150 \times 10^{-8}$ M), both with and without a large excess of unlabeled heat-aggregated human IgG (100-fold excess). Specific binding, in molecules of IgG per PMN, was calculated after subtraction of nonspecific binding (30) and the data were used for Scatchard analysis (16, 29). Specific binding of IgG oligomers reached a plateau within 2 h at 4°C. After incubation with a large excess of unlabeled IgG oligomer, 50% of the saturably bound radiolabeled IgG oligomers was displaced at 5 min and at least 70% was displaced by 120 min.

Quantitation of FcγR expression by flow cytometry. Fresh PMN (5×10^5 cells in PBS with 0.1% FCS) were incubated with saturating doses of MAb at 4°C for 30 min and, after washing twice with PBS-FCS at 4°C, were further incubated with saturating concentrations of FITC-conjugated rabbit anti-mouse IgG $F(ab')_2$ fragments (Cappel Laboratories) or phycoerythrin (PE)-conjugated goat anti-mouse IgG $F(ab')_2$ fragments (Tago, Inc., Burlingame, CA). For MAb B73.1, which was directly conjugated to PE, the second step was omitted, and PE-conjugated mIgG1 was used as a control. Cell-associated immunofluores-

cence was quantitated in a Cytofluorograf IIs and 2151 computer system using a three and one half decade logarithmic amplifier (Becton, Dickinson & Co.). For each experiment, the instrument was calibrated with FITC-conjugated calf thymus nuclei (Fluorotrol-GF, Becton, Dickinson & Co.) and quantitative FITC and PE microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC). Data were recorded in log fluorescence units and, for selected experiments, in linear fluorescence units (see Fig. 1).

Data analysis. For the assessment of phagocytic capacity and oligomer binding, all experiments were performed in a matched pairs experimental design with an NA1 and NA2 subject studied simultaneously. The %P, PI (studied under the "standard" opsonization conditions), maximum binding (B_{max}), and association constant (K_a) were compared using a paired t test (two-tailed). Differences between the two groups studied under varying opsonization conditions were compared using a one-way analysis of variance (ANOVA). The mean fluorescence channel data, obtained with each MAb for each subject, were averaged for each allotypic grouping (NA1 only, NA1/NA2, NA2 only) and compared by Student's t test. A probability of 0.05 was used to reject the null hypothesis that there is no difference between the groups.

The percent inhibition of phagocytosis in experiments using MAb or Fab fragments of 3G8 and IV.3 to block FcγRIII or FcγRII, respectively, was calculated by the following formula: (phagocytosis$_{control}$ − phagocytosis$_{MAb}$)/phagocytosis$_{control}$.

Results

Allotypic characterization for NA1 and NA2. We analyzed the allotypic characteristics of our serologically typed population by quantitative flow cytometry using a panel of anti-FcγRIII MAb including MAbs B73.1 and GRM1 and the neutrophil-specific MAb CLB-gran 11 which recognizes only the NA1 allele. Neutrophil preparations from all donors were > 98% positive with the multicell-reactive anti-FcγRIII MAb, such as CLB-FcR gran 1, which recognizes both NA alleles. Those

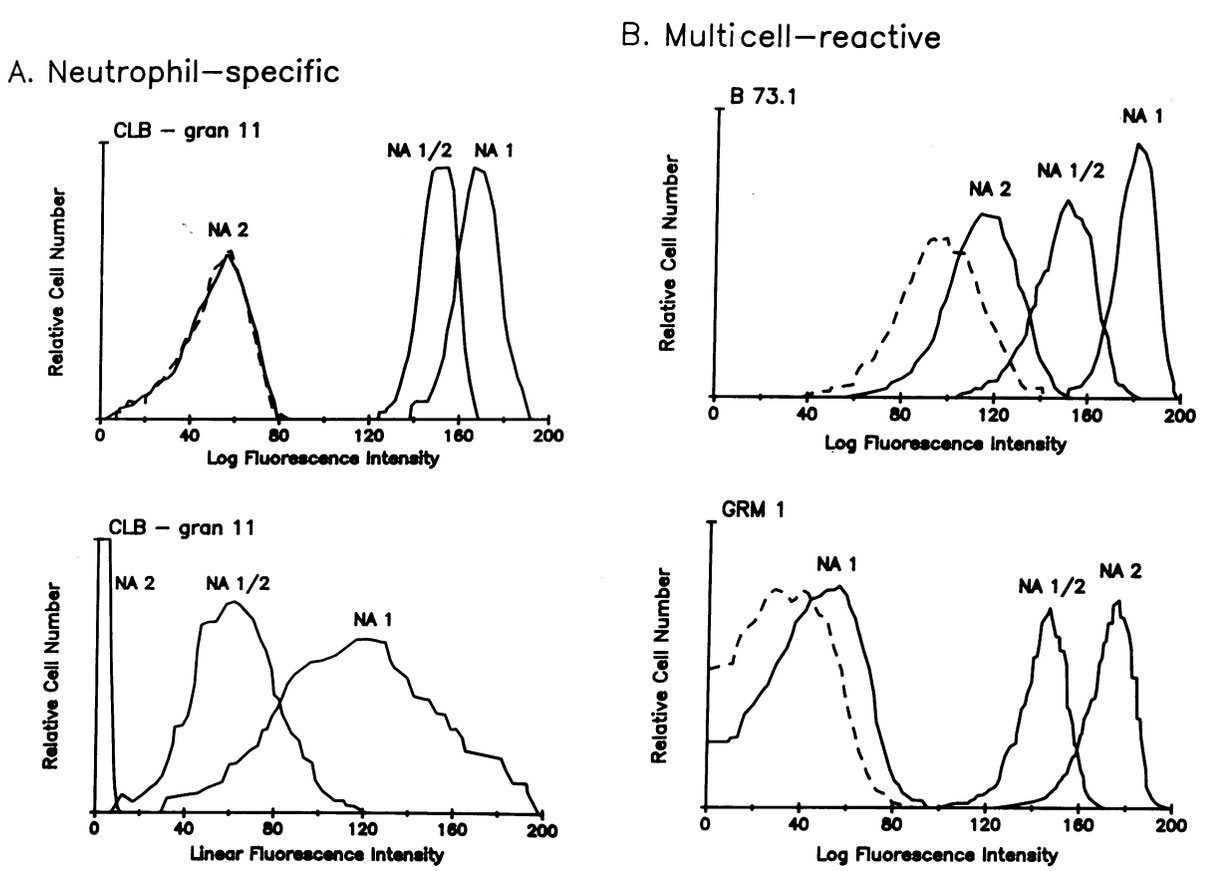

Figure 1. Quantitative expression of FcγRIII$_{PMN}$. (*A*) Neutrophils from representative NA1-, NA1/2-, and NA2-positive individuals were incubated with the NA1-specific anti-CD16 MAb CLB-gran 11 (*solid line*) or mIgG2a (*dashed line*), followed by FITC-conjugated rabbit anti-mouse IgG F(ab')$_2$ fragments. Cell-associated immunofluorescence was assayed by flow cytometry and expressed as log fluorescence intensity (*above*) and linear fluorescence intensity (*below*) in arbitrary units. For NA2 donors, fluorescence intensity with CLB-gran 11 was identical to the mIgG2a control. There was twice as much fluorescence with CLB-gran 11 on PMN from the NA1 donor compared with PMN from the NA1/NA2 donor (mean channel linear fluorescence 120.3 vs. 60.5 units). PMN from individuals in all three allotypes had equal fluorescence intensity with the other multicell-reactive anti-CD16 MAbs 3G8, CLB-FcR gran 1, BW209/2, and VEP13 (cf. Table II). (*B*) Neutrophils were incubated with multicell-reactive anti-CD16 MAbs B73.1-PE (*solid line, above*) and control mIgG1-PE (*dashed line, above*) or with GRM1 (*solid line, below*) and control mIgG2a (*dashed line, below*) followed by PE-conjugated goat anti-mouse IgG F(ab')$_2$. B73.1-PE preferentially reacted with PMN from NA1 donors, whereas GRM1 preferentially reacted with PMN from NA2 donors. For both B73.1-PE and GRM1, there were on average nearly twofold differences in staining between heterozygotes and homozygotes (cf. Table I). However, heterozygotes showed more variability with each of the MAbs than with CLB-gran 11. It should be noted that B73.1-PE reacts weakly with NA2 donors and GRM1 reacts weakly with NA1 donors. Neither of these MAbs is neutrophil-specific or absolutely NA allotype-specific.

individuals typed by leukoagglutination as only NA2-positive did not stain with CLB-gran 11 (< 2% positive cells), stained only dimly with B73.1, but were brightly stained with GRM1. Subjects typed as only NA1-positive stained with CLB-gran 11 (98±2.4% positive [mean±SD]) and with B73.1 but were only minimally reactive with GRM1 (Table I, Fig. 1).

Although differences in the quantitative epitope expression (defined by some MAbs with preferential allelic staining) have been noted between individuals seropositive for either one or both NA alleles (10, 12, 31), the lack of a demonstrated gene dose effect has raised some question about the correspondence among genotype, expressed phenotype, and MAb epitopes. Analysis of $Fc\gamma RIII_{PMN}$ with MAbs B73.1 and GRM1, which react with PMN and mononuclear cells (12, 23, 32), demonstrated allelic preferences in binding. However, reactivity was not restricted to specific NA epitopes, and although some study pairs showed near twofold differences between NA1/2 individuals compared with individuals positive for NA1 or NA2 (Fig. 1 *B*), not all study pairs showed such a relationship (Table I). In contrast, analysis with MAb CLB-gran 11 showed that individuals serotyped as NA1/2 had half as many cell surface FcγRIII bearing the NA1 epitope than the individuals typing positive only for NA1, despite equivalent numbers of FcγRIII defined by several MAbs (Table II). Whether analyzed in log fluorescence units (19-channel log fluorescence difference = twofold difference in epitope expression—NA1 vs. NA1/NA2, 163.8±7.8 mean channel log fluorescence units vs. 145.8±5.9, $P < 0.00001$) or linear fluorescence units (120.3 vs. 60.5 mean channel linear fluorescence units; Fig. 1 *A*) a twofold difference in density of the NA1 epitope was evident between the NA1-positive and NA1/NA2-positive donors. These observations of a gene dose effect, coupled with correspondence to immunoprecipitation data (31, 32), indicate that $Fc\gamma RIII_{PMN}$ alleles are codominantly expressed.

FcγR-mediated phagocytosis is lower in NA2 subjects compared with that in NA1 subjects. To examine the functional consequences of this NA1-NA2 allelic polymorphism of $Fc\gamma RIII_{PMN}$, we compared the quantitative phagocytic capacity of NA1 homozygous and NA2 homozygous individuals for EA prepared at four different levels of opsonization. For the assay of phagocytosis, PMN from an NA1 and from an NA2 donor were studied simultaneously with the four different EA particles to maximize the ability to detect subtle differences between the two groups. In each of the four pairs studied, the NA2 donor had a lower phagocytic capacity. When analyzed by group, the NA2 donors had a significantly lower %P and PI for EA (%P, $P < 0.025$; PI, $P < 0.05$, ANOVA; Fig. 2). This difference in phagocytic capacity became more pronounced as the degree of opsonization of EA decreased. With the most lightly opsonized EA, the PI was 72±16% lower for NA2 donors compared with that for the NA1 donors.

To confirm the difference in FcγR phagocytosis between individuals from the two NA allotypes, we studied an expanded population. For these experiments, we used maximally opsonized EA ("standard" rabbit antibody concentration) which gave the highest absolute PI but also showed the smallest difference between the groups (Fig. 2). In 12 of 14 study pairs the NA2-positive subject had a lower phagocytic capacity for EA than the corresponding NA1-positive subject. For the entire study group the %P of EA by PMN from NA2 subjects was 15% lower than in NA1 subjects ($P < 0.04$) and the PI was 21% lower ($P < 0.003$; Table III). Thus, under stringent conditions allowing for maximum phagocytosis, a consistent and reproducible difference in phagocytic capacity between NA1 and NA2 donors was apparent.

Lower phagocytic capacity in NA2 subjects is FcγR-specific. To evaluate the possibility that the difference in phagocytosis between NA2 and NA1 individuals might reflect differences in general phagocytic ability, we compared internalization of FcγR-independent ligands by PMN from both groups. Previous work has shown that neutrophil phagocytosis of STZ and HTZ is dependent upon complement receptor type 3 (CR3) and independent of FcγR (2, 33, 34). STZ binds to the site on the α-chain of CR3 for fixed iC3b, whereas HTZ binds to a different site on α-chain of CR3 by way of lectin–carbohydrate interactions (35). Accordingly, we examined the capacity of NA2 and NA1 individuals to phagocytize STZ and HTZ.

Preliminary studies of the internalization of HTZ and STZ showed that phagocytosis increased as the ratio of target parti-

Table I. Preferential Binding to NA Alleles by Anti-FcγRIII MAbs

NA phenotype	MAbs					
	Control		Multicell-reactive			
	mIgG2a	CLB-gran 11*	mIgG1-PE	B73.1-PE‡	mIgG2a	GRM1§
NA1	53.2±3.3	163.8±7.8	73.3±23.9	167.2±11.9	38.6±2.2	43.2±4.9
NA2	52.9±5.1	52.7±5.5	74.9±17.7	93.7±26.2	35.3±5.3	168.1±3.4
NA1/2	53.9±5.6	145.8±5.9	73.1±21.7	149.0±10.4	34.6±4.2	154.5±10.4

Expression of FcγRIII on PMN was analyzed with anti-CD16 MAbs showing preferential binding to NA alleles. Neutrophils were incubated with saturating doses of CLB-gran 11 or GRM1 followed by incubation with FITC-conjugated rabbit anti-mouse IgG $(Fab')_2$ or PE-conjugated goat anti-mouse IgG $(Fab')_2$, respectively. For B73.1-PE the second step was omitted. Murine myeloma proteins were IgG subclass-specific controls. Values represent the mean±SD of 6–12 donors of each phenotype. In addition, individuals studied at several different points in time showed stable values. Subsets of these data are presented in Edberg et al. (32). * NA1 donors had significantly brighter staining than both NA2 donors and NA1/2 donors with CLB-gran 11 ($P < .00001$; $P < .0001$, respectively, Student's *t* test, two-tailed). For NA2 donors, fluorescence with CLB-gran 11 was the same as mIgG2a control. ‡ NA1 donors had significantly brighter fluorescence with B73.1-PE than either NA2 or NA1/2 donors ($P < 0.001$ and $P < 0.02$, respectively). For NA2 donors, fluorescence with B73.1-PE was greater than mIgG1-PE control levels ($P < 0.02$). § NA2 donors had significantly brighter fluorescence intensity with GRM1 than either NA1 or NA1/2 donors ($P < 0.0001$ and $P < 0.003$, respectively). For NA1 donors, fluorescence with GRM1 mAb was greater than mIgG2a control ($P < 0.05$).

Table II. Quantitative FcγRIII Expression on PMN

NA phenotype	MAbs					
	Control		Multicell-reactive			
	mIgG1	mIgG2a	3G8	CLB-FcR gran 1	Vep13	Bw209/2
NA1	49.9±5.8	53.2±3.3	153.1±10.0	167.9±9.8	107.6±11.3	153.7±17.0
NA2	53.6±6.5	52.9±5.1	155.1±9.0	169.8±8.4	107.1±8.0	156.3±7.9
NA1/2	54.8±4.8	53.9±5.6	155.8±8.1	170.6±8.9	104.5±10.8	158.8±10.0

Expression of FcγRIII on PMN was analyzed with anti-CD16 MAb. Neutrophils (5×10^5) were incubated with saturating doses of anti-FcγRIII MAb or murine myeloma proteins (mIgG) as IgG subclass-specific controls for 30 min at 4°C. After washing, cells were stained with saturating amount of FITC-conjugated rabbit anti-mouse IgG $(Fab')_2$ fragments. Cell-associated immunofluorescence was assayed by flow cytometry and expressed as mean channel log fluorescence. Values represent mean±SD of 7 NA1 donors, 11 NA2 donors, and 10 NA1/2 donors. Individuals studied at several different points in time showed stable values. No differences in cell associated immunofluorescence among the donor groups was evident for MAbs 3G8, CLB-FcR gran 1, VEP13, and BW209/2.

cles to PMN increased. At ratios of HTZ or STZ to PMN of 10:1 or greater, there was nearly 100% phagocytosis by all donors examined. Therefore, to maximize the likelihood of detecting subtle differences among individuals all assays were performed at a zymosan to PMN ratio of 5:1. Experiments examining the kinetics of internalization of HTZ and STZ demonstrated that the percentage of PMN ingesting the particles reached a peak after 10 min. To maximize the potential for identifying differences in phagocytic capacity, internalization of HTZ and STZ quantitated after a 10-min incubation period. All experiments were performed with paired NA1 and NA2 donors using HTZ, STZ, and EA.

As shown in Fig. 3, neither the %P nor the PI for HTZ and STZ was different for NA1 compared with NA2 donors. Simultaneous assessment of EA phagocytosis with the same pairs of subjects showed the NA2 donor with lower phagocytic capacity than the NA1 donor in each experiment (Fig. 2). Thus, the difference in phagocytosis between NA1 and NA2 individuals is not due to a generalized difference in phagocytic capacity.

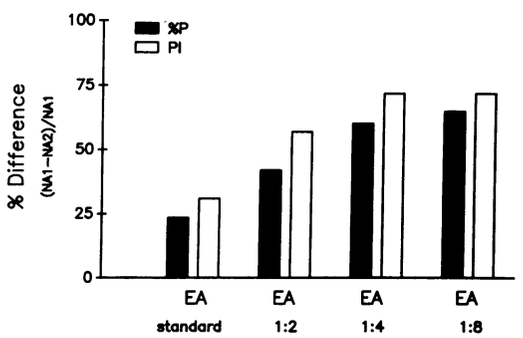

Figure 2. FcγR-mediated phagocytosis is lower in NA2 subjects compared with that in NA1 subjects. PMN from NA1 and NA2 donors were incubated with EA sensitized with serial dilutions of rabbit anti-ox E antibody. Phagocytosis was quantitated by light microscopy and the percent difference between the donors calculated. For each assay, an NA1 and NA2 donor was studied simultaneously with the four different EA particles. In each of the four paired studies, the NA2 donor had lower phagocytic capacity. The NA2 donors as a group had significantly lower %P and PI ($P < 0.025$ and $P < 0.05$, ANOVA).

NA1 and NA2 individuals do not differ in numerical expression of FcγRIII on PMN. Among the potential mechanisms to explain the FcγR-specific differences in phagocytic capacity between NA1 and NA2 individuals, we considered a systematic variation in numerical expression of $Fc\gamma RIII_{PMN}$ linked to the structural polymorphism. However, examination of this possibility by quantitative flow cytometry demonstrated that NA1 and NA2 subjects had the same number of surface $Fc\gamma RIII_{PMN}$ as measured by a panel of anti-CD16 MAb (MAbs 3G8, CLB-FcR gran 1, VEP 13, and BW 209/2; Table II).

Since we could find no evidence for quantitative differences in $Fc\gamma RIII_{PMN}$ expression between NA1 and NA2 individuals, we considered the possibility that qualitative differences might exist. For example, structurally determined heterogeneity in IgG ligand-binding site corresponding to the different allelic forms of $Fc\gamma RIII_{PMN}$ might result in variations in phagocytic capacity for EA. To quantitate the capacity of PMN FcγR from NA allotype-defined donors to bind ligand, we examined the specific binding of ^{125}I-labeled IgG oligomers at multiple ligand concentrations and estimated both the B_{max} and K_a by Scatchard analysis. Five pairs of NA1 and NA2 donors were examined, each at two different points in time.

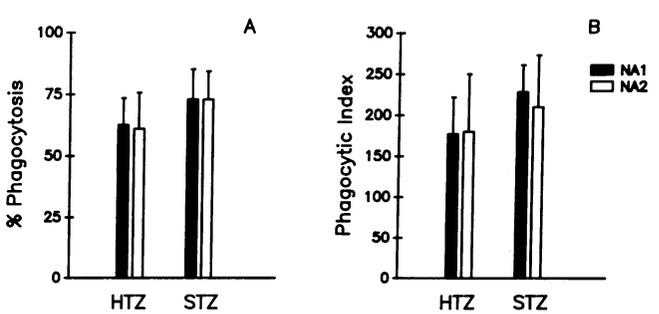

Figure 3. Phagocytosis of FcγR-independent particles is similar for NA1 and NA2 individuals. PMN from NA1 and NA2 donors were incubated with HTZ or STZ at a ratio of target particle to PMN of 5:1 for 10 min at 37°C. Thereafter, %P and PI were quantitated by microscopy. There were nine paired studies performed with HTZ and five paired studies performed with STZ. No significant difference was found in phagocytosis of HTZ or STZ between the NA allotype-defined groups.

The estimated B_{max} for the NA1 donors was similar to that of the NA2 donors (5.5±1.6 vs. 4.9±1.7 × 10^5 molecules IgG/PMN) (Fig. 4). These estimates of B_{max} were not significantly different whether analyzed as two populations ($P > 0.63$) or as matched pairs (mean difference±SEM: 0.5±0.8; $P > 0.52$). Although we cannot categorically exclude a contribution of FcγRII to the binding of the IgG oligomers, these data are in general agreement with other estimates of the number of FcγRIII sites/cell (29, 36–38) and confirm the results of comparable receptor epitope expression on PMN in different NA allotypic groups measured by flow cytometry (Table II).

Estimates of the association constant of FcγRs for oligomeric IgG, derived from the Scatchard transformation, showed some variability among the donors. The mean value for K_a (2.0±0.7 × 10^6 M^{-1}) was in agreement with previous reports (29). Although there was some suggestion of a lower K_a for the NA2 group (Fig. 4), the mean K_a was not significantly different for the NA1 donors compared to the NA2 donors (2.4±0.6 × 10^6 M^{-1} vs. 1.6±0.7 × 10^6 M^{-1}, respectively, $P > 0.10$).

The difference in phagocytic capacity between NA1 and NA2 subjects is independent of FcγRII. Since PMN also express FcγRII which may participate in FcγR-mediated phagocytosis (24), we considered the possibility that FcγRII might influence the data on $FcγRIII_{PMN}$ polymorphisms and phagocytosis. To address this possibility, we took three approaches: we compared (*a*) quantitative FcγRII expression in our population of NA allotype-defined subjects, and both (*b*) phagocytosis of EA in the presence of FcγRII blockade and (*c*) phagocytosis of E-ConA (an $FcγRIII_{PMN}$-dependent but FcγRII-independent probe) in the same groups.

With the anti-CD32 MAbs IV.3 and 41H.16, we examined quantitative expression of FcγRII in the two homozygous study groups. NA1 individuals showed the same quantitative staining with IV.3 and the same distribution of staining with 41H.16 as the NA2 individuals (MAb IV.3, 113.8±4.6 vs. 109.4±5.0; MAb 41H.16, 89.4±28.2 vs. 104.1±24.8 mean channel log fluorescence units; $P > 0.1$ and $P > 0.3$, respectively).

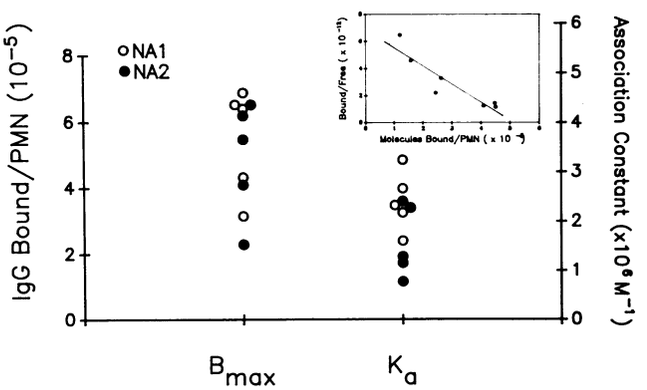

Figure 4. Scatchard analysis of binding of ^{125}I-labeled IgG oligomers to PMN. PMN were allowed to equilibrate with ^{125}I-labeled IgG oligomers at varying concentrations in the presence or absence of a large excess of unlabeled aggregated IgG. Cell-bound and-free IgG oligomers were separated by centrifugation through oil. Specific binding was calculated after subtraction of nonspecific binding at each ligand concentration (30). Best fit of experimental points was determined by least squares linear regression. The x intercept is the estimate of B_{max} and the slope is the estimate of association constant (*inset*). Five pairs of NA1 and NA2 donors were each studied twice. Scatter plots of PMN binding parameters are shown. The estimated B_{max} and association constants were similar for the NA1 and NA2 groups ($P > 0.05$).

Next, we examined the contribution of FcγRII to the phagocytosis of EA. Blockade of FcγRII by IV.3 IgG at saturating concentrations (10 μg/ml) inhibited the phagocytosis of EA by an average of 64% (range 49–80%) (Fig. 5). Similarly, IV.3 Fab fragments at supersaturating concentration (250 μg/ml) inhibited ingestion of EA by an average of 68% (range 44–88%) (Fig. 5). IV.3 Fab at lower concentrations was significantly less effective in inducing inhibition of EA ingestion. These data, taken together with our previous evidence that MAb 3G8 IgG and 3G8 Fab inhibit EA phagocytosis by 94% and 97% (2), respectively, demonstrate that both FcγRs on PMN participate in EA phagocytosis in a synergistic manner.

To study the relative phagocytic capacity of the two alleles of FcγRIII in the absence of FcγRII, we quantitated EA phagocytosis in the presence of IV.3 Fab. In all three paired studies of NA1 and NA2 donors, the difference in internalization between the two NA alleles was amplified in the presence of FcγRII blockade with MAb IV.3 Fab (percent difference in %P: control vs. IV.3, 26±12% vs. 172±90%) (Fig. 6). Thus, the differential ability of $FcγRIII_{PMN}$ alleles to mediate internalization is independent of IgG-mediated engagement of FcγRII.

Finally, we examined the phagocytosis of E-ConA, a non-IgG ligand binding site probe. E-ConA engages $FcγRIII_{PMN}$ by a ConA–carbohydrate interaction, and we have previously shown that its internalization is inhibited by both MAb 3G8 IgG and 3G8 Fab (percent inhibition, 80±7% and 73±6%, respectively, $P < 0.001$) (2, 39). In contrast, ConA does not ligate affinity-isolated FcγRII (39). In a series of experiments using different donors, MAb IV.3 IgG blockade of FcγRII had no effect on the internalization of E-ConA (control vs. IV.3, 46±6% vs. 48±6% phagocytosis). Furthermore, the addition of MAb IV.3 IgG to MAb 3G8 did not result in any further decrement of E-ConA internalization relative to that induced by 3G8 alone ($n = 3$). Thus, E-ConA is $FcγRIII_{PMN}$-dependent but independent of ligand-mediated engagement of FcγRII.

Using this FcγRIII-specific probe, we compared phagocy-

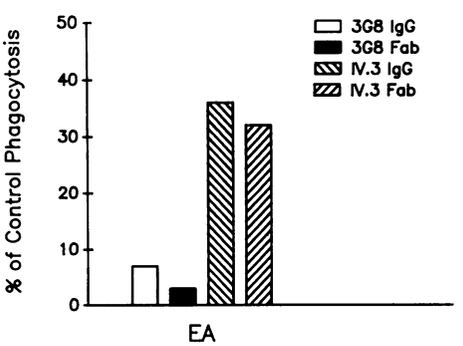

Figure 5. FcγRII and FcγRIII participate in the internalization of EA. PMN were pretreated with MAb 3G8 or IV.3 IgG (10 μg/ml) or their Fab fragments (250 μg/ml), and then incubated with EA for 30 min. Noninternalized erythrocytes were lysed and phagocytosis by PMN was quantitated by light microscopy. Results are expressed as percentage of control phagocytosis and represent the means of four to five experiments with different donors. Internalization of EA was inhibited by blockade of FcγRIII (3G8 IgG and 3G8 Fab; $P < 0.001$) and by blockade of FcγRII (IV.3 IgG and IV.3 Fab; $P < 0.005$).

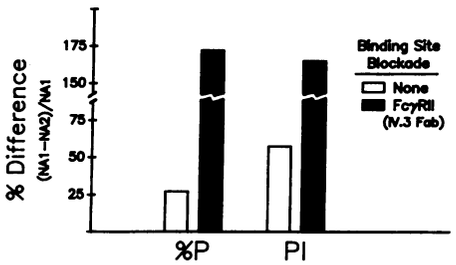

Figure 6. FcγRII blockade magnifies the difference in phagocytosis of EA between NA1 and NA2 subjects. PMN from NA1 and NA2 donors were pretreated with MAb IV.3 Fab (250 μg/ml) or control medium and then incubated with EA (1:2 sensitization, see Fig. 2). Phagocytosis was quantitated by light microscopy and percent difference between donors was calculated. For each assay, NA1 and NA2 donors were studied simultaneously. In each of three paired studies, the difference in phagocytic capacity between the NA1 and NA2 donors was greatly enhanced in the presence of FcγRII blockade by MAb IV.3 Fab.

tosis of E-ConA by our NA1 and NA2 subjects. As with EA, the kinetics of E-ConA ingestion approach a plateau after 10 min of incubation. Therefore, PMN were incubated with E-ConA for 30 min to allow time for maximum internalization. In 13 of the 14 study pairs, the NA2 donor had a lower phagocytic capacity than the NA1 donor. When analyzed as a group (Table III), the NA2 subjects had significantly decreased E-ConA internalization compared to the NA1 individuals. The percent phagocytosis in NA2 subjects was 19% lower ($P < 0.007$) and the phagocytic index was 21% lower ($P < 0.002$) than in NA1 subjects. Interestingly, this difference in phagocytosis defined by E-ConA indicates that the functional distinction is not simply a reflection of differing affinities of the allelic forms for IgG ligand but rather a reflection of some other intrinsic property of $Fc\gamma RIII_{PMN}$.

Discussion

Using the neutrophil-specific NA polymorphism of $Fc\gamma RIII_{PMN}$, we have demonstrated a consistent quantitative difference in FcγR-mediated phagocytosis between NA1 and NA2 individuals. This difference indicates that allelic polymorphisms in FcγR can have significant consequences for physiologic functions. Structural variations of FcγRs, whether inherited allelic polymorphisms (10), cytokine-regulated receptor isoforms (40), or cell type-specific receptor isoforms (32), may provide an important mechanism in the determination of FcγR function.

Table III. Phagocytosis of EA and E-ConA by PMN

	Target particles			
	EA (standard Ab concn.)		E-ConA	
NA phenotype	%P	PI	%P	PI
NA1 (n = 7)	71±5	132±16	50±7	76±10
NA2 (n = 14)	62±9	109±15	42±4	63±5
P (paired t)*	0.04	0.003	0.002	0.007

PMN from NA1 donors and NA2 donors were incubated with EA which were maximally opsonized (see Fig. 2) or E-ConA for 30 min. Noninternalized erythrocytes were lysed. Phagocytosis by 400 PMN was quantitated by microscopy. Results are expressed as %P phagocytosis and PI and represent the mean±SD of 14 different experiments. In each experiment, one NA1 and one NA2 donor together were studied simultaneously.

* The absolute value of the difference in EA phagocytosis and EA PI between the NA1 and NA2 groups is 9±3 (mean±SEM) and 23±5, respectively. The absolute value of the difference in E-ConA phagocytosis and E-ConA PI is 9±2 and 12±3, respectively.

The structural complexity of human FcγR is becoming increasingly apparent with the identification of three families of receptors (FcγRI, FcγRII, and FcγRIII [4, 41]), structural variants or isoforms within these families (14, 31, 32, 42–44), and potential allelic polymorphisms (5–10). With both the allotypic NA polymorphism found on FcγRIII in neutrophils and the responder/nonresponder polymorphism on FcγRII in monocytes and platelets, individual subjects can be characterized either as one type alone, both types together, or the other type alone. These observations and family studies of the NA system have suggested a classical Mendelian inheritance pattern with codominant expression, but this interpretation has been confounded by the lack of an allelic gene dose effect for specific NA epitope expression (10). In our population, we have performed a series of carefully calibrated flow cytometry experiments which demonstrate a clear gene dose effect for the NA1 epitope recognized by the neutrophil-specific MAb CLB-gran 11 but not for the NA-related epitopes recognized by the multicell-reactive MAbs B73.1 and GRM1. The data that the density of NA1 epitope expression corresponds directly with both the serotyping and the allele(s) defined by immunoprecipitation in a population of donors (31, 32) indicates that the NA phenotypic polymorphism corresponds to codominantly expressed polymorphic proteins. Recent studies, demonstrating differences in the primary sequences of the genes encoding the NA1 and NA2 alleles in several donors (13, 14), also suggest a correspondence with genotype. Taken together with the absence of a third protein form on biochemical analysis in the immunoprecipitation studies (31, 32), these observations support the NA system as a truly biallelic system. Accordingly, we have referred to those individuals typing for NA1 alone as NA1 homozygotes and those typing for NA2 alone as NA2 homozygotes.

Using this biallelic polymorphism on $Fc\gamma RIII_{PMN}$, we have explored the functional consequences of these structural variations. Our data indicate that individuals defined as NA2 homozygotes have a lower level of quantitative phagocytosis of FcγR-specific probes than their NA1 homozygous counterparts (Fig. 2, Table III). This difference is not a generalized property of the PMN since it is not apparent using FcγR-independent probes (Fig. 3). It reflects an intrinsic property of $Fc\gamma RIII_{PMN}$ since the difference in phagocytic capacity for EA between NA1 and NA2 subjects is amplified in the presence of FcγRII blockade (Fig. 6). It is not a consequence of quantitatively different $Fc\gamma RIII_{PMN}$ expression on the cell surface (Table II), nor is it due to a difference in the association constant for IgG–ligand binding in the two different allotypically defined groups (Fig. 4). Not only are the association constants estimated from Scatchard analysis of ^{125}I-IgG oligomer binding similar in the two groups, but E-ConA, whose binding to $Fc\gamma RIII_{PMN}$ is mediated by carbohydrate interactions unlike the classical IgG ligand, is sensitive to the difference between the allotypes.

The exact mechanism of the quantitative difference in phagocytosis remains unclear. $Fc\gamma RIII_{PMN}$ has recently been

recognized as having a glycosyl-phosphatidyl inositol anchor (45–47). The role of such lipid-anchored molecules in signal transduction is not established, and a primary role for $Fc\gamma RIII_{PMN}$ as a focusing (or binding) molecule has been proposed (38, 45, 48). Indeed, $Fc\gamma RIII_{PMN}$ is the critical molecule for the binding of EA by neutrophils (2), and given the ability of MAb 3G8 Fab and MAb IV.3 Fab to partially inhibit phagocytosis, both $Fc\gamma RIII_{PMN}$ and $Fc\gamma RII$ appear to collaborate in the subsequent phagocytosis of IgG-opsonized particles. However, since E-ConA cannot be ligated by $Fc\gamma RII$ (39) and are fully ingested in the presence of $Fc\gamma RII$ blockade with MAb IV.3, $Fc\gamma RIII_{PMN}$ does not link in an obligatory ligand-dependent fashion with $Fc\gamma RII$ in order to mediate phagocytosis. This conclusion indicates that $Fc\gamma RIII_{PMN}$ is more than a simple focusing (or binding) molecule for $Fc\gamma RII$ (49). Either singly or through interactions with other surface molecules, $Fc\gamma RIII_{PMN}$ can mediate phagocytosis just as it can mediate cytotoxicity for some targets (19–22). Thus, rather than being restricted to differences in ligand binding (for which we could gather no supportive data), the NA polymorphism may influence receptor function distal to that initial binding event.

Among potential mechanisms underlying the influence of the NA polymorphism on receptor function, we must consider the participation of another, as yet unidentified, surface molecule which would collaborate with $Fc\gamma RIII_{PMN}$ in phagocytosis. The fact that MAb 3G8 completely blocks E-ConA phagocytosis without inhibiting binding of the probe to the cell surface (2) indicates that such a molecule, collaborating with $Fc\gamma RIII$, may facilitate phagocytosis without binding the phagocytic probe itself. Among potential candidates for interdomain interactions would be members of the integrin family which have been shown to facilitate internalization of targets recognized by multiple receptors (50–52).

Although the basis for the differences in phagocytic capacity of NA1 and NA2 homozygote individuals is unclear, the biological implications of this observation are manifest. Taken over time, the differences in phagocytic capacity could be compounded into a large and physiologically significant effect. This precedent for allelic differences in receptor structure having a significant impact on receptor function is of potential importance given the structural diversity of human $Fc\gamma R$. Similar structural polymorphisms might underlie the difference in quantitative phagocytosis seen in normal individuals with an HLA haplotype containing either DR2 or DR3 (15–17). Definition of these structure–function relationships may provide important insight into both inherited differences and regulatory strategies in $Fc\gamma R$ function both in health (15, 17) and human disease (16, 53–55).

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