Allelic Polymorphisms of Human Fcγ Receptor IIA and Fcγ Receptor IIIB

Independent Mechanisms for Differences in Human Phagocyte Function

Jane E. Salmon, Jeffrey C. Edberg, Nina L. Brogle, and Robert P. Kimberly
Department of Medicine, The Hospital for Special Surgery and The New York Hospital,
The Cornell University Medical College, New York 10021

Abstract

Two different allelic polymorphisms among the isoforms of human Fcγ receptors have been defined: the low-responder (LR)–high-responder (HR) polymorphism of huFcγRIIA expressed on both PMN and monocytes and the NA1–NA2 polymorphism of the neutrophil FcγRIII (huFcγRIIIB). To address the issues of whether the LR–HR polymorphism has a significant impact on FcγR-mediated functions in human blood cells and whether any differences in LR–HR might be related to higher FcγR-mediated phagocytosis in NA1 donors, we examined FcγR-specific binding and internalization by donors homozygous for the two huFcγRIIA alleles. PMN from LR homozygotes showed consistently higher levels of internalization of erythrocytes opsonized with pooled human IgG (E-hIgG). The absence of an LR–HR phagocytic difference with erythrocytes opsonized with either anti-FcγRIIA MAb IV.3 or rabbit IgG, as opposed to E-hIgG, suggested that the Fc piece of the opsonin might be important for this LR–HR difference. Accordingly, we studied HR and LR homozygotes with human IgG subclass-specific probes. Both PMN (independent of huFcγRIIIB phenotype) and monocytes from LR donors bound and internalized erythrocytes coated with human IgG2 (E-hIgG2) efficiently, whereas phagocytes from HR donors did so poorly. E-hIgG2 internalization was completely abrogated by blockade of the ligand binding site of huFcγRIIA with IV.3 Fab, indicating that huFcγRIIA is essential for the handling of hIgG2 and that the mechanism of the LR–HR phagocytic difference is at the level of ligand binding to huFcγRIIA. In contrast, the difference in internalization of E-hIgG between NA1 and NA2 homozygous donors was independent of the huFcγRIIA phenotype and did not manifest differences in ligand binding. Thus, the two known allelic polymorphisms of human FcγR have distinct and independent mechanisms for altering receptor function, which may influence host defense and immune complex handling. (*J. Clin. Invest.* 1992. 89:1274–1281.) Key words: IgG subclasses • phagocytosis • monocyte • neutrophil

Introduction

Two different allelic polymorphisms among the isoforms of human Fcγ receptors have been defined. The NA1–NA2 poly-

morphism of the neutrophil FcγRIII (huFcγRIIIB)[1] reflects several differences in amino acid sequence and two potential glycosylation sites (1–5). The low-responder (LR)–high-responder (HR) polymorphism of huFcγRIIA reflects two amino acid substitutions at positions 27 and 131, with an arginine residue at amino acid position 131 being critical for efficient binding of mIgG1 (6–9). For each of these allelic systems, there has been substantial interest in identifying their functional consequences and the molecular mechanisms underlying any differences. We have shown that individuals homozygous for the NA1 and NA2 alleles of huFcγRIIIB have distinct phagocytic capacities (10).

The recent observation by Warmerdam et al. (8) that position 131 of FcγRIIA expressed in transfected fibroblasts influences the capacity for binding human IgG2 (hIgG2) suggests that this polymorphism may also be important in human systems. Using a large donor population with defined huFcγRIIA alleles, we examined the effect of this polymorphism on quantitative phagocytosis by human phagocytes. Our results revealed that homozygous LR donors have a higher capacity to internalize erythrocytes (E) opsonized with human IgG (E-hIgG) than homozygous HR donors. Further analysis of these homozygous donors indicated that, when studied with human IgG (hIgG) subclass-specific probes, PMN and monocytes from both LR and HR subjects are able to bind and internalize E-IgG1 comparably. With E-IgG2, both cell types from homozygous LR subjects bind and internalize efficiently whereas cells from HR donors do so poorly. Thus, with intact human phagocytes, the LR–HR polymorphism influences both ligand binding to huFcγRIIA and subsequent FcγR-mediated phagocytosis.

This difference in FcγR-mediated phagocytosis, coupled with recent data localizing the genes for both huFcγRII and huFcγRIII to human chromosome 1 (11–13), raised the possibility that an association between the NA alleles of huFcγRIIIB and the LR–HR alleles of huFcγRIIA might explain the difference in FcγR-mediated phagocytosis between NA1 homozygous and NA2 homozygous donors. Thus, we also examined

the differences in phagocytosis for NA1 and NA2 homozygotes matched for expression of the LR–HR polymorphism. Phagocytosis of E coated with hIgG was higher for NA1 donors independent of the LR–HR phenotype. Furthermore, there were no differences in E-hIgG binding by cells expressing either NA1 or NA2 alleles. The mechanism of the phagocytic difference, therefore, appears to be distal to ligand binding. These data demonstrate that the two recognized allelic polymorphisms of human Fcγ receptors have independent mechanisms for mediating functionally apparent differences in FcγR-mediated phagocytosis. These differences may have important implications for human disease.

Methods

Subjects. Peripheral blood was collected from 32 disease-free volunteers who ranged in age from 20 to 56 yr (34±8 yr [mean±SD]). Protocols for these studies were approved by the Institutional Committee on Human Rights in Research.

Determination of NA antigens by leukoagglutination. Typing for neutrophil-specific antigens, NA1 and NA2, was performed by leukoagglutination as described by Lalezari (2, 14), with a panel of anti–NA1 and anti–NA2 allosera kindly provided by Dr. David Stroncek of the American Red Cross Neutrophil Serology Reference Laboratory, St. Paul, MN. The assignment of NA type was confirmed by immunoprecipitation and flow cytometry with MAbs CLB-FcR gran 1, CLB-gran 11, and GRM1 (15, 16).

Determination of LR–HR alleles by flow cytometry. Phenotyping of donors for the LR–HR alleles of huFcγRIIA was performed by quantitative flow cytometry using MAbs 41.H16 and IV.3 as described by Gosselin et al. (17). Phenotypic assignment was corroborated by anti-CD3 mitogenesis assays as described below.

Reagents. HBSS, RPMI 1640, and FCS were from Gibco Laboratories, Grand Island, NY. FCS was heat inactivated at 56°C for 60 min before use. FITC-conjugated rabbit anti–human IgG F(ab')₂, FITC-conjugated goat anti–rabbit IgG F(ab')₂, and phycoerythrin (PE)-conjugated goat anti–mouse IgG F(ab')₂ was purchased from Tago, Inc., Burlingame, CA. All conjugated reagents were absorbed against human mononuclear cells before use. Sulfosuccinimidobiotin (Sulfo-NHS-biotin), sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin), and streptavidin were obtained from Pierce Chemical Co., Rockford, IL. FMLP (Sigma Chemical Co., St. Louis, MO) was dissolved in American Chemical Society grade DMSO for a stock concentration of 10^{-3} M and stored at −20°C.

MAb 3G8 (mIgG1) recognizing huFcγRIII (CD16) was kindly provided by Dr. J. Unkeless, Mt. Sinai Medical Center, New York (18). The MAb IV.3 (mIgG2b) and IV.3 Fab fragments recognizing huFcγRII (CD32) and 3G8 F(ab')₂ were purchased from Medarex, Inc., (West Lebanon, NH) (19). Silver stain analysis of the IV.3 Fab fragment preparation and the 3G8 F(ab')₂ preparation indicated that there was no intact IgG. The MAb 41H.16 (mIgG2a) recognizing huFcγRII was generously provided Dr. Thomas Zipf, University of Texas Cancer Center, Houston, TX (20). Leu4 and OKT3 (IgG1 and IgG2a anti-CD3, respectively) were provided by Dr. Robert Evans, Memorial Sloan-Kettering Cancer Center, New York. Isotype controls (mIgG1 [MOPC21] and mIgG2a [UPC10]) were purchased from Sigma Chemical Co.

Purified hIgG1 and hIgG2 myeloma proteins were obtained from Dr. Elliot Osserman of Columbia University, New York. hIgG3 and hIgG4 myeloma proteins were obtained from The Binding Site, Inc. (San Diego, CA). A second set of preparations of hIgG1, hIgG2, hIgG3, and hIgG4 were obtained from Calbiochemic-Behring Corp., San Diego, CA. Normal pooled hIgG was obtained from Sigma Chemical Co.

Preparation of cells. Fresh anticoagulated human peripheral blood was separated by centrifugation through a discontinuous two-step Ficoll-Hypaque gradient (10). PMNs were isolated from the lower interface and washed with HBSS. Contaminating E were lysed with hypotonic saline (0.02% NaCl) for 20 s followed by 0.16% NaCl and a final wash with HBSS. Mononuclear cells were isolated from the upper Ficoll-Hypaque interface and washed with HBSS. The percent monocytes within the mononuclear cell layer was determined by peroxidase staining. After final washes, all cells were resuspended to 5×10^6 PMNs or monocytes/ml.

Preparation of E. Antibody-coated erythrocytes (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 (21). The standard amount of antibody used was a fourfold dilution of the minimum agglutinating titer and resulted in maximum phagocytosis of EA by monocytes.

E were coupled to pooled or subclass-specific hIgG by a biotin–avidin technique (22). To prepare biotinylated E (E_B), 0.5 ml of E (1×10^9 cells/ml) in 0.1 M carbonate buffer (pH 8.6) was incubated with sulfo-NHS-biotin (500 μg/ml) for 20 min at 4°C with mixing, followed by three washes with buffer containing divalent cations as follows (mM): (2.5 veronal buffer, pH 7.4; 146 NaCl, 0.05% gelatin, 0.15 CaCl₂, and 0.05 MgCl₂). E_B at 1×10^9/ml were incubated with an equal volume of streptavidin (250 μg/ml) for 30 min at 4°C with mixing. The streptavidin-coated E_B (E_BA) were then washed three times with the buffer and resuspended to 1×10^9 E/ml for immediate use. Pooled hIgG or purified human myeloma proteins of specific IgG subclasses were biotinylated with NHS-LC-biotin (0.01 mg biotin/mg protein) for 60 min at room temperature with occasional mixing. To bind the biotinylated hIgG to the E_BA, E_BA (12.5 μl at 1×10^9/ml) were combined with varying amounts of biotinylated protein (200 μg/ml to 1 mg/ml) for 30 min at 4°C with mixing. After three washes, the hIgG-coated E_BA (E-hIgG) were then resuspended in 125 μl (1×10^8 E/ml) and used immediately. For certain experiments, the E_BA were coupled to biotinylated IV.3 Fab. The results of experiments using E-IV.3 prepared by the biotin–avidin sandwich technique were comparable to those using E-IV.3 Fab heteroantibodies (23–25).

Assay of phagocytosis. Quantitation of phagocytosis by PMNs and monocytes was performed as previously described (10, 21, 26). Briefly, cells were resuspended in RPMI with 20% heat-inactivated IgG-free FCS (Gibco Laboratories), at 5×10^6 cells/ml. In certain experiments, the PMNs were preincubated with either IV.3 Fab or 3G8 F(ab')₂ (30 μg/ml) for 5 min; the MAbs were present throughout the assay of phagocytosis. For experiments with FMLP-treated PMN (10^{-7} M), FMLP was added simultaneously with the phagocytic particle.

To assess internalization of E target particles, cells (100 μl) were combined with EA (250 μl), E-IV.3 (250 μl), or E-hIgG (125 μl). The leukocyte–erythrocyte mixtures were centrifuged at 44 g for 3 min and then incubated at 37°C for 15 min to allow for maximum internalization. After hypotonic lysis of noninternalized E, phagocytosis was quantitated by light microscopy. At least 400 cells/slide were counted in duplicate without knowledge of the donor huFcγRIIA or huFcγRIIIB allotype. The data are expressed as phagocytic index (PI, number of ingested E/100 phagocytes). For assays with monocytes, PI was corrected for the percent peroxidase positivity of mononuclear cells.

Assay of adherence. To quantitate adherence, PMN and E target particles were prepared and combined as described in the assay of phagocytosis above. After centrifugation at 44 g for 3 min, the PMN–E mixtures were maintained at room temperature for 10 min and then gently resuspended. Adherence of E to PMN was quantitated by light microscopy. Data are expressed as adherence index (AI, number of adherent or internalized erythrocytes/100 PMN). Under these conditions, < 1% of phagocytes had internalized E.

Immunofluorescent flow cytometry. Fresh leukocytes (5×10^5 in PBS with 0.1% FCS) were incubated with saturating amounts of specific MAb or isotype controls for 30 min at 4°C. After two washes with cold PBS containing 1% FCS, cells were incubated with saturating concentrations of phycoerythrin (PE)-conjugated goat anti–mouse IgG F(ab')₂ for 30 min at 4°C followed by washing twice with cold PBS/1%

FCS. E-hIgG were stained with FITC-conjugated rabbit anti–human IgG F(ab')$_2$, E coated with IV.3 Fab were stained with PE-conjugated goat anti–mouse IgG F(ab')$_2$, and E opsonized with rabbit anti–ox E antibodies were stained with FITC-conjugated goat anti–rabbit IgG F(ab')$_2$, followed by washes with cold PBS/1% FCS.

After staining, cell-associated immunofluorescence was quantitated on a Cytofluorograf IIS (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with a 2151 computer as previously described (10, 15, 16). For each experiment, the instrument was calibrated with FITC-conjugated calf thymus nuclei (Fluorotrol-GF, Becton, Dickinson & Co., Mountain View, CA) and quantitative PE microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC) to allow for assessment of both absolute and relative levels of immunofluorescence.

Anti-CD3 proliferation assay. Peripheral blood mononuclear cells, separated by Ficoll-Hypaque as described above, were resuspended at 1 × 10^6 cells/ml in RPMI 1640 with 10% FCS (supplemented with glutamine, penicillin, and streptomycin) and aliquoted into 96-well plates at 1 × 10^5 cells/well. OKT3 (IgG2a anti-CD3, 5 μg/ml final concentration), Leu4 (IgG1 anti-CD3, 5 μg/ml final concentration), nonspecific control isotype antibodies, or media alone were added to triplicate wells and the cells were incubated for 4 d at 37°C. For the final 8 hr, [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL), 2 μCi/well was added, after which the cells were harvested, washed, and [^{3}H]-thymidine incorporation measured in a beta counter (27–29).

Data analysis. For assessment of phagocytic capacity, all experiments were performed in a matched-pairs experimental design. Accordingly, each subject, homozygous for a given FcγR isoform (huFcγRIIA or huFcγRIIIB), was studied in comparison to a second subject, homozygous for the other allele of the same isoform and matched for the same phenotype of the other FcγR (e.g., HR–NA1 vs. LR–NA1).

Data are displayed as mean±SEM. PI for specific probes were compared using a paired t test (two tailed). Differences in phagocytic capacity between the groups for E opsonized over a range of densities were compared by repeated measures analysis of variance (ANOVA). A probability of 0.05 was used to reject the null hypothesis that there is no difference between the groups.

Results

Characterization of huFcγRIIA and huFcγRIIIB alleles. The allotypic characteristics of our normal population for huFcγRIIIB were determined by serological typing and quantitative flow cytometry of PMN using NA1- and NA2-specific MAbs (10, 15, 16). The LR–HR alleles of huFcγRIIA in our population were identified by quantitative flow cytometry with MAb 41H.16, which recognizes the HR allele of huFcγRIIA, and MAb IV.3, which recognizes both HR and LR alleles (17). Using the ratio of fluorescence intensity of 41H.16/IV.3 in both monocytes and PMN, we assigned the homozygous HR phenotype to donors having a ratio of 0.88–1.1 ($n = 8$), the heterozygous HR/LR phenotype to donors having a ratio of 0.42–0.59 ($n = 11$), and the homozygous LR phenotype to donors having a ratio of < 0.13 ($n = 13$). The phenotypic assignment of homozygous individuals was corroborated by proliferation assays with anti-CD3 MAbs of both mIgG1 and mIgG2a isotypes. In all cases, the results of the mitogenesis assays were in agreement with the flow cytometry assignment for huFcγRIIA.

Quantitative expression of huFcγRII as determined by flow cytometry using MAb IV.3 showed small differences between the phenotypes (PMN: HR 129±2, HR/LR 128±3, LR 121±2 mean channel log fluorescence units [MCF]; monocytes: HR 130±4, HR/LR 122±3, LR 118±4 MCF; for PMN, HR vs. LR $P < 0.02$, Mann-Whitney U test; all other comparisons, $P > 0.1$). The expression of huFcγRIII as determined by flow

cytometry using MAb 3G8, which recognizes the NA1 and NA2 alleles, was the same for all phenotypes (NA1 154±4; NA1/NA2 150±2; NA2 152±6; $P > 0.1$ for all comparisons).

Phagocytosis by PMN from LR homozygotes is higher than HR homozygotes. Given the difference in ligand binding by FcγRIIA alleles transfected into fibroblasts or COS cells (7–9), we considered the possibility that the FcγR-mediated phagocytic capacity of blood cells expressing these alleles might differ. Accordingly, we examined quantitative phagocytosis by PMN from normal donors who are homozygous for the LR or HR alleles. To control for differences due to huFcγRIIIB, each pair of homozygous donors was matched for NA alleles. Although not different when probed with anti-FcγRII Fab-coated E (E-IV.3, a ligand-independent huFcγRII-specific probe), LR homozygotes showed significantly higher phagocytosis than HR homozygotes when probed with E-hIgG (PI [E-hIgG internalized/100 PMN]: LR vs. HR, 50±7 vs. 27±10, $P < 0.003$; Fig. 1).

Over a broad range of opsonization densities, LR subjects had a greater level of phagocytosis of E-hIgG ($P < 0.02$, repeated measures ANOVA). In contrast, phagocytosis EA and E-IV.3 prepared at a broad range of opsonization densities was indistinguishable between the two groups ($P > 0.1$, repeated measures ANOVA). The absence of the difference in phagocytosis between LR and HR donors with EA and E-IV.3 suggested that the nature of the ligand might be important in defining the LR–HR functional difference.

Higher phagocytosis by FcγRIIA LR homozygotes: relationship to hIgG subclasses. Since the alleles of huFcγRIIA differentially bind murine IgG1 (9, 27–32) and hIgG2 in a transfected fibroblast system (8), we examined the capacity of the LR–HR alleles expressed on blood cells to recognize and bind subclasses of hIgG. In each of seven pairs of homozygous LR and HR subjects matched for huFcγRIIIB alleles, there was a dramatic difference between the HR and LR individuals in
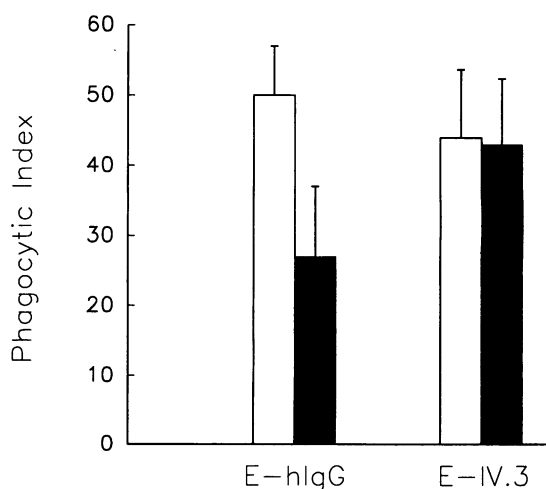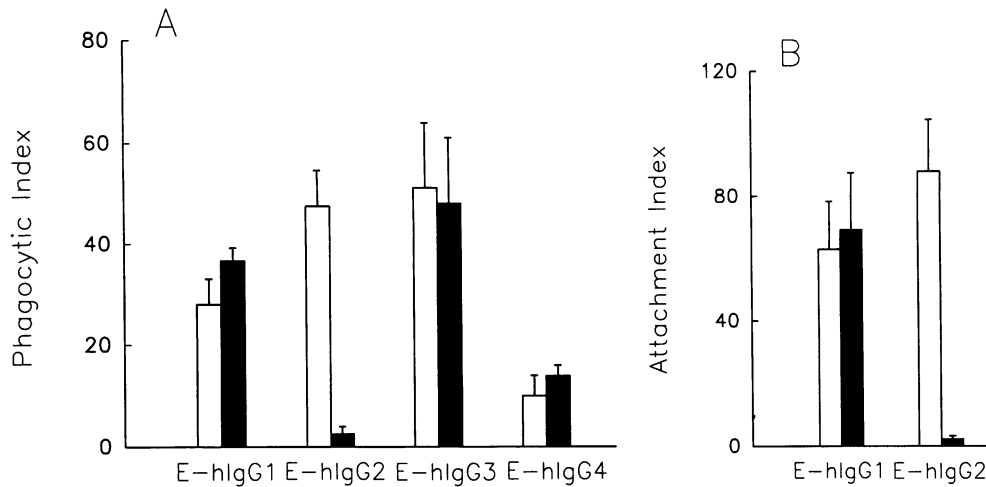

Figure 1. Phagocytosis of E-hIgG by PMNs from LR homozygotes is higher that by HR homozygotes. PMNs from normal donors, homozygous for the LR or HR alleles and matched for identical NA alleles of huFcγRIIIB, were studied simultaneously for internalization of E-IV.3 and E-hIgG in a matched-pairs design. PI was quantitated by light microscopy. In each of six pairs, internalization of E-IgG was greater for the homozygous LR subjects ($P < 0.003$, paired t test). In contrast, phagocytosis of E-IV.3 was indistinguishable between the two groups (P = NS). (□) LR; (■) HR.

Figure 2. PMNs from LR homozygotes internalize E-hIgG2. PMNs from pairs of homozygous HR and LR donors with identical NA alleles were studied simultaneously with all probes. (A) For the assay of phagocytosis, PMNs were incubated with E-hIgG1, E-hIgG2, E-hIgG3, or E-hIgG4 opsonized with similar intermediate densities of IgG (MCF 150–160). In each of seven pairs, there was minimal internalization of E-hIgG2 by HR donors (LR vs. HR, P < 0.0004). No significant difference in phagocytosis of the other probes was noted. (B) For the assay of attachment, PMNs were incubated with E-hIgG1 or E-hIgG2. In each of six pairs studied, there was minimal binding of E-hIgG2 to HR PMNs (LR vs. HR, P < 0.003), whereas binding of E-hIgG1 was similar for LR and HR. (□) LR; (■) HR.

phagocytosis of E-hIgG2 (PI [E internalized/100 PMN]: LR vs. HR, 47±7 vs. 3±1, P < 0.0004), whereas internalization of E-hIgG1, E-hIgG3, and E-hIgG4 was similar for both donor types (Fig. 2 A). The lack of internalization of E-hIgG2 reflected an inability of the E-hIgG2 to bind to PMNs from HR subjects (AI for E with intermediate density of opsonization: 0–3 E attached/100 PMN; Fig. 2 B). Identical results were obtained with a second and different myeloma preparation of each hIgG subclass. A series of blocking experiments confirmed that huFcγRII was responsible for the phagocytosis of E-hIgG2. Pretreatment of PMN with MAb IV.3 Fab (30 μg/ml) to block huFcγRII completely abrogated internalization of E-hIgG2 (Fig. 3). Indeed, pretreatment with 3G8 F(ab')₂ to block huFcγRIIIB did not block, but rather enhanced, phagocytosis of E-hIgG2 consistent with our previous findings that cross-linking huFcγRIIIB primes phagocytosis mediated by huFcγRII in PMN (PI: control vs. 3G8 F(ab')₂, 47±16 E internalized/100 PMN vs. 72±16, P > 0.05) (25).

The capacity of the LR allele of huFcγRIIA to bind mIgG1 and form subclass-specific rosettes or induce T cell proliferation (32, 33) is dependent in part on the concentration of MAb presented to receptor-bearing cells. A similar relationship is apparent for the capacity of the HR allele of huFcγRIIA on PMN to bind hIgG2. As shown in Figure 4 A, higher densities of hIgG2 on E resulted in a higher PI in both LR and HR donors. At all levels of hIgG2, however, a marked difference between the LR and HR groups was evident (P < 0.001, repeated measures ANOVA). In contrast, the phagocytosis of E-hIgG1 showed no difference between the two groups at any level of E surface hIgG1 (Fig. 4 B). Similarly, there was no difference in internalization of E-hIgG4 opsonized over a range of densities between both groups. For example, the PI for E-hIgG4 with MCF 170–179 was 29±10 E internalized/100 PMN for LR donors and 27±6 for HR donors.

Interestingly, internalization of E-hIgG and E-hIgG1 by HR PMN was identical over most of the range of opsonization, whereas internalization of E-hIgG by LR PMN was higher than that of E-hIgG1 with similar densities of opsonization. This difference is presumably due to recognition of hIgG2 on the E-hIgG by the LR allele, and this observation suggests that functional differences between the huFcγRIIA alleles may be apparent even with polyclonal antibody responses.

Detection of the differential capacity of huFcγRIIA alleles to bind hIgG2 is not restricted to PMNs. Human monocytes, which express both FcγRIIA and FcγRI, show efficient internalization of E-hIgG2 when derived from LR subjects but little internalization when derived from HR subjects (PI [E internalized/100 monocytes]: LR vs. HR, 145±14 vs. 21±3, P < 0.0005). In contrast, phagocytosis of both E-hIgG and E-hIgG1 was comparable for both groups (PI [E internalized/100 monocytes]: LR vs. HR, 152±10 vs. 146±25 and 149±13 vs. 153±37, respectively, P > 0.1). Blockade of huFcγRII with IV.3 Fab blocked internalization of E-hIgG2 by monocytes in LR subjects (PI [E internalized/100 monocytes]: 169±43 vs. 10±6, P < 0.03), similar to the findings in PMN (Fig. 3).
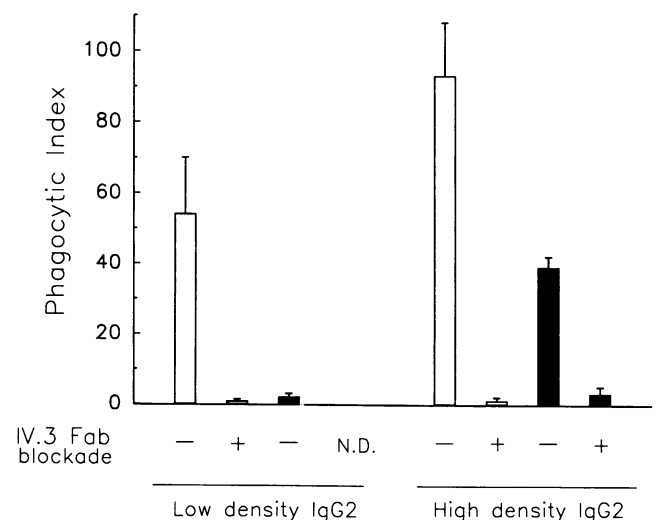

Figure 3. Blockade of huFcγRIIA with IV.3 Fab completely inhibits internalization of E-hIgG2 in both LR and HR PMNs. PMNs from HR and LR donors were pretreated with IV.3 Fab (30 μg/ml) or control medium for 10 min and then combined with E-hIgG2 prepared at lower (intermediate density; MCF 150–160) or higher (MCF 170–180) density opsonization. In each experiment both an HR and an LR donor were studied simultaneously (n = 3–4 pairs). Pretreatment of PMN with IV.3 Fab blocked phagocytosis of both E-hIgG2 probes in LR donors (control vs. IV.3 Fab, P < 0.03). In HR donors, IV.3 Fab blocked phagocytosis of the high density E-hIgG2 (control vs. IV.3 Fab, P < 0.006). (□) LR; (■) HR.

A   E-hIgG2

A

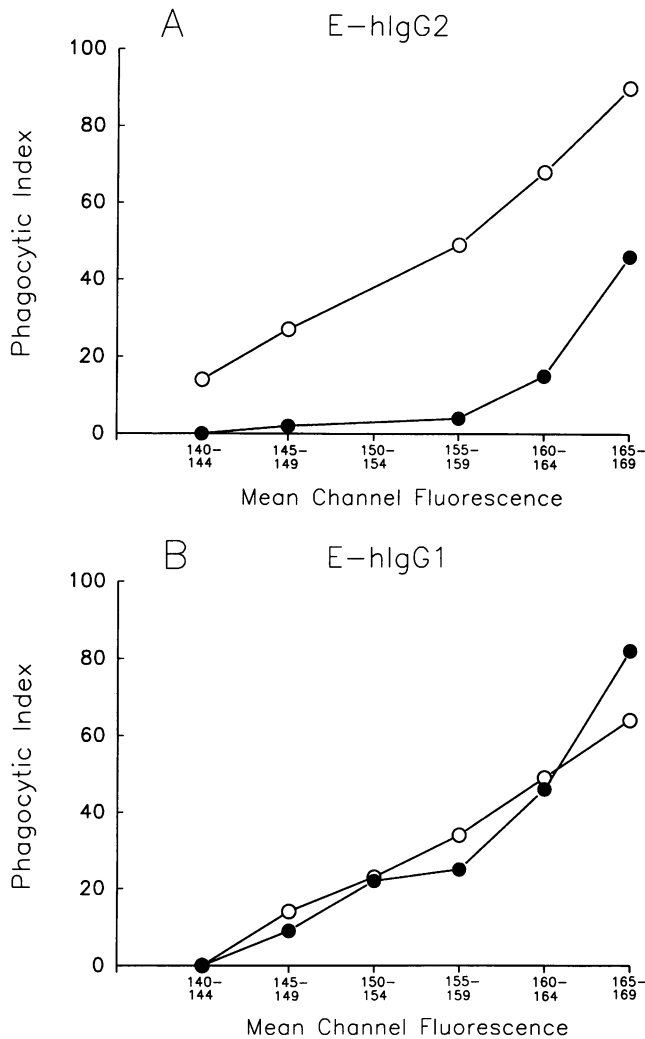Low density G2

B   E-hIgG1

B

High density G2

Figure 4. The lack of internalization of E-hIgG2 by PMNs from homozygous HR subjects is relative, not absolute. PMNs from HR and LR were combined with E opsonized with varying amounts of hIgG2 (*A*) or hIgG1 (*B*), as determined by flow cytometry (*n* = 3–5 pairs/level). Phagocytosis of all probes was directly related to degree of opsonization. (*A*) Internalization of E-hIgG2 was higher for LR donors at all levels of opsonization (*P* < 0.001, repeated measures ANOVA), (*B*) whereas that for E-hIgG1 remained similar for both groups. (o) LR; (•) HR.

Figure 5. The difference in E-hIgG2 internalization between LR and HR donors persists in primed PMN. PMN from HR and LR donors were treated with FMLP (10^{-7} M), 3G8 F(ab')$_2$ (30 μg/ml), or control medium before quantitation of phagocytosis of E-hIgG2 prepared at lower (intermediate density; MCF 150–160) and higher (MCF 170–180) density opsonization. Phagocytosis of the (*A*) lower density E-hIgG2 (*A*) and the (*B*) higher density E-hIgG2 was greater for LR donors (*n* = 3–9). Low density E-hIgG2, LR vs. HR: control *P* < 0.01, FMLP *P* < 0.03, 3G8 F(ab')$_2$ *P* < 0.01; High density E-hIgG2, LR vs. HR: control *P* < 0.001, FMLP *P* < 0.03, 3G8 F(ab')$_2$ *P* < 0.03. (□) LR; (■) HR.

The difference in E-hIgG2 internalization between LR and HR donors persists in primed PMNs. Simultaneous engagement of several receptors may be the most relevant physiological stimulus for phagocytes. For example, it is unlikely that microbes opsonized in vivo or endogenously formed immune complexes would be composed exclusively of hIgG2 and thus ligate only huFcγRII. The observation that treatment of PMNs with 3G8 F(ab')$_2$ to crosslink huFcγRIIIB augmented internalization of E-hIgG2 raised the possibility that primed or activated PMNs found in sites of infection or inflammation might not show a difference between LR and HR donors. To examine this possibility, PMNs from LR and HR donors were pretreated with 3G8 F(ab')$_2$, FMLP, or control medium. In spite of an increase in E-hIgG2 internalization, phagocytosis of E-hIgG2 by LR PMNs remained significantly higher than that by HR PMNs (Fig. 5). The increase in E-hIgG2 phagocytosis was associated with an increase in E-hIgG2 binding in both LR and
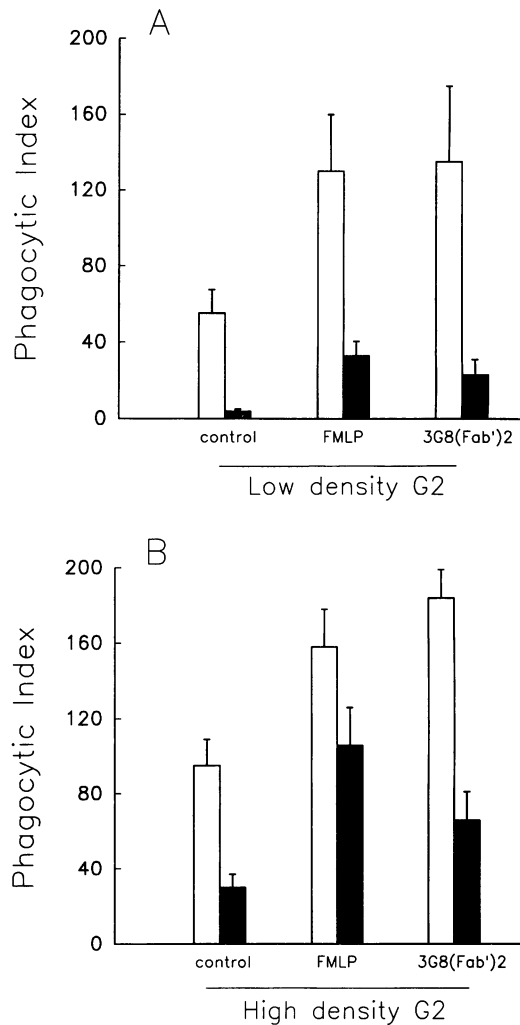
HR donors, although this may not be only mechanism for the increase in internalization (AI for LR [E-hIgG2 adherent/100 PMN]: control 134±34, FMLP 219±36, 3G8 F(ab')$_2$ 150±42, *n* = 5–6, FMLP vs. control *P* < 0.02; AI for HR: control 8±3, FMLP 72±15, 3G8 F(ab')$_2$ 40±10 *n* = 3–4, FMLP vs. control *P* < 0.04). Phagocytosis of E-hIgG2 by FMLP-stimulated PMNs from homozygous HR donors was also completely inhibited by blockade of huFcγRII with IV.3 Fab (PI [E internalized/100 PMN]: FMLP-treated PMNs = 37±9, FMLP-treated PMNs in the presence of IV.3 Fab = 5±2; *n* = 4; *P* < 0.05). Thus, in primed PMNs, hIgG2 binding and internalization is predominantly mediated by huFcγRII.

Higher phagocytic capacity of PMNs from NA1 subjects is not related to allelic polymorphisms of huFcγRIIA. Homozygous NA1 donors have higher phagocytosis of EA than homo-

zygous NA2 donors, which is FcγR specific rather than due to differences in generalized phagocytic capacity (10). Given the differences in FcγRIIA-mediated phagocytosis and the recent data localizing the genes for both huFcγRII and huFcγRIII to human chromosome 1 (11–13), we determined whether an association between the alleles of huFcγRIIA and the NA1–NA2 alleles of huFcγRIIIB might explain the higher phagocytic capacity of NA1 homozygotes. In each of six pairs of NA1 and NA2 homozygous individuals, each with identical huFcγRIIA alleles, internalization of EA and of E-hIgG was higher in the NA1 donor, independent of the huFcγRIIA phenotype of the donor pair (Fig. 6). The difference in phagocytic capacity was not related to alterations in binding of E-hIgG (AI for standard level of opsonization [E-hIgG adherent/100 PMN]: NA1 vs. NA2 = 265±69 vs. 280±66, n = 5). Simultaneous analysis of huFcγRII-specific phagocytosis in the NA1 and NA2 homozygous donors with E-IV.3 showed no significant differences (PI [E internalized/100 PMN]: NA1 vs. NA2 = 32±12 vs. 29±10, n = 5; Fig. 6). Thus, an huFcγRIIIB-specific mechanism, unrelated to the quantitative binding of ligand and unrelated to huFcγRIIA alleles, appears to be responsible for the difference in phagocytosis between NA1 and NA2 homozygous donors.

Discussion

Allelic polymorphisms of human FcγR have functionally distinct capacities that are important in mediating interactions between human phagocytes and IgG ligands. Using E-hIgG, we show consistently higher PMN phagocytosis by individuals homozygous for the LR allele of huFcγRIIA and by individuals homozygous for the NA1 allele of huFcγRIIIB. This difference
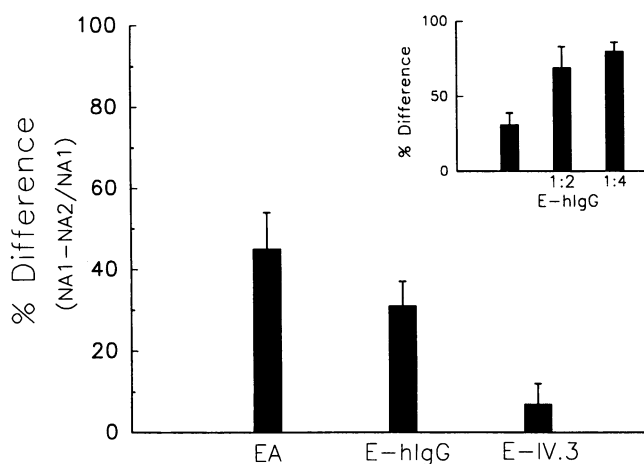

Figure 6. FcγR-mediated phagocytosis of EA and E-hIgG, but not E-IV.3, is higher in homozygous NA1 subjects compared with that of NA2 subjects. PMN from NA1 and NA2 homozygous donors with identical HR–LR alleles were assayed simultaneously with EA, E-hIgG, and E-IV.3, and the percent difference between the donors was calculated. In each of six pairs studied, the NA2 donor had a lower phagocytic capacity for EA and E-hIgG (PI for EA: 45±9% lower, P < 0.03; PI for E-hIgG: 33±8% lower, P < 0.003). Phagocytosis of E-IV.3 was similar for both groups (P = NS). As with EA (9), the difference in phagocytosis of E-hIgG became more pronounced with less opsonization of the E, such that a fourfold dilution of the standard concentration of hIgG showed an 80±10% difference in PI (insert). The dilutions of biotinylated hIgG used to opsonize E_{BA} corresponded to MCFs of 164 (std), 158 (1:2), and 144 (1:4).

is even more pronounced in LR homozygous subjects for hIgG2 subclass-specific phagocytosis, since, in contrast to HR, the LR allele of huFcγRIIA binds hIgG2 efficiently.

The differences in functional capacity for the two alleles of huFcγRIIA appear to be mediated by a mechanism distinct from that involved in the differences between the two alleles of huFcγRIIIB. The biallelic HR–LR polymorphism of huFcγRIIA alters the affinity of the ligand binding site for some subclasses of IgG ligand. Originally distinguished by differences in binding of mIgG1 to human monocytes as assessed by T cell proliferation induced by anti-CD3 MAb (27–32), the functional HR–LR phenotype is determined by a single amino acid present at position 131, which plays a critical role in the binding capacity for mIgG1 (6, 7, 9). Warmerdam and colleagues (8) also showed that fibroblast transfectants expressing the LR allele (histidine residue at position 131) lack the capacity to bind mIgG1 but can bind hIgG2, whereas transfectants expressing the HR allele (arginine residue at position 131) bind mIgG1 but do not recognize hIgG2. Importantly, the functional impact of this difference is not restricted to transfected fibroblasts expressing a single receptor family but is evident in resting and activated human phagocytes. Our data indicate that for human PMN, which express both huFcγRII and huFcγRIIIB, submaximally opsonized E-hIgG are internalized more efficiently by LR donors. This difference reflects the ability of LR donors to bind hIgG2, which comprises ~ 20% of pooled hIgG (34, 35). E-hIgG2 are internalized efficiently by individuals homozygous for LR allele of huFcγRIIA but are internalized poorly by individuals homozygous for the HR allele (Fig. 2). Even in "primed" PMNs, which demonstrate enhanced binding and internalization of E-hIgG2, the difference between HR and LR donors persists (Fig. 5). This difference in E-hIgG2 phagocytosis is also evident in human monocytes that express both huFcγRI and huFcγRII. The inability of HR PMN to bind hIgG2 is relative rather than absolute, and at high levels of opsonization there is a modest degree of internalization of E-hIgG2 (Fig. 4). A similar discriminative preference for the other human subclasses of IgG was not identifiable. Although absolute comparisons of efficiency of binding and internalization for each of the hIgG subclasses are not possible, in part because determinations of "comparable densities" of opsonization may be influenced by unique properties of individual myeloma proteins and by properties of the second antibody, our results suggest that in LR PMN the relative efficiency is hIgG1 = hIgG3 = hIgG2 > hIgG4 and, in HR PMN, hIgG1 = hIgG3 > hIgG4 > hIgG2.

The biallelic NA1–NA2 polymorphism of huFcγRIIIB, expressed only on human PMNs, is related to several amino acid differences, two of which alter potential N-linked glycosylation sites (1–5). Since huFcγRIIIB may function in part as a binding molecule to present ligand to huFcγRIIA (24), we considered the contribution of the LR–HR alleles of huFcγRIIA to the functional consequences of the huFcγRIIIB polymorphism. The current results demonstrate higher phagocytosis of E-hIgG by NA1 donors compared with NA2 donors, despite matching of donors for huFcγRIIA alleles (Fig. 6) and no difference in phagocytosis of E-IV.3 (an huFcγRII-specific probe) by NA1 and NA2 donors (Fig. 6). These data, along with our previous observation that blockade of huFcγRII with IV.3 Fab amplifies the difference in phagocytosis between NA1 and NA2 (10), support the conclusion that the difference in EA and E-hIgG phagocytosis between NA1 and NA2 homozygous donors is

independent of huFcγRIIA phenotype. The mechanism underlying the difference between NA1 and NA2 homozygotes remains undefined, although IgG-independent, allele-specific interactions with other surface membrane molecules provide provocative possibilities.

The implications of these two allotypic systems are intriguing. The NA1–NA2 functional polymorphism appears to be IgG-ligand independent. No differences in opsonized target binding have been demonstrated, and no subclass preference has been identified since, among HR homozygous donors, the inability to bind hIgG2 is equally apparent for both the NA1 homozygous and NA2 homozygous phenotypes. The difference in internalization is evident with concanavalin-treated E, which engage huFcγRIIIB through nonclassical (IgG-ligand independent) carbohydrate-mediated interactions (10). The fact that the NA1–NA2 alleles are functionally distinct indicates, however, that huFcγRIIIB serves as an integral participant in phagocytosis by PMNs. In contrast, the HR–LR functional polymorphism is directly ligand dependent, and the ability of LR PMNs and monocytes to bind hIgG2 efficiently suggests that a reexamination of the role of hIgG2 humoral response may be appropriate. For example, since the antibody response to bacterial polysaccharide antigens is predominantly hIgG2 isotype (36), the relationship between hIgG2 levels and susceptibility to infection with encapsulated bacterial pathogens (34, 35, 37) may be influenced by the HR–LR phenotype of the host. Thus, the increased risk of invasive *Hemophilus influenzae* infection in certain populations that have impaired antibody responses to *H. influenzae* and low hIgG2 levels (38, 39) may be greater for HR homozygotes than for LR homozygotes. Similarly, in cystic fibrosis, where bacterial colonization of the lungs by *Pseudomonas aeruginosa* is associated with high serum levels of hIgG2 anti-*Ps. aeruginosa* antibodies and immune complexes containing hIgG2 anti-*Ps. aeruginosa* antibodies, the potential for the hIgG2 antibodies to act as poor opsonins and inhibit clearance of the organisms (40–42) may vary in accord with the HR–LR phenotype of the host. Importantly, activation of PMNs does not abrogate this allelic difference in function. Indeed, one might speculate that the evolutionary pressure for the presence of the LR allele of huFcγRIIA (29) is related to more effective defense against microbial agents eliciting an hIgG2 humoral response.

The precedent for allelic differences in receptor structure having a significant impact on phagocyte function is of potential importance given the structural diversity of huFcγR. Definition of further such structure–function relationships among huFcγR may provide insights into both disease susceptibility and pathogenesis.

Acknowledgments

References

1. Werner, G., A. E. G. Kr. von dem Borne, M. J. E. Bos, J. F. Tromp, C. M. van der Plas-van Dalen, F. J. Visser, C. P. Engelfriet, and P. A. Tetteroo. 1985. Localization of the human NA1 alloantigen on neutrophils to Fcγ receptors. *In* Leucocyte Typing II. Vol. 3. E. L. Reinherz, B. F. Haynes, L. M. Nadler, and I. D. Bernstein, editors. Springer-Verlag, New York, 109–121.

2. Tetteroo, P. A. T., C. E. Van der Schoot, F. J. Visser, M. J. E. Bos, and A. G. E. Kr. von dem Borne. 1987. Three different types of Fcγ receptors on human leukocytes defined by workshop antibodies: FcγR$_{low}$ of neutrophils, FcγR$_{low}$ of NK/K lymphocytes and FcγRII. *In* Leucocyte Typing III. A. J. McMichael, editor. Oxford University Press, Oxford, UK. 702–706.

3. Ory, P., I. M. Goldstein, E. E. Kwoh, and S. Clarkson. 1989. Characterization of polymorphic forms of FcγRIII on human neutrophils. *J. Clin. Invest.* 83:1676–1681.

4. Ory, P., M. R. Clark, E. E. Kwoh, S. B. Clarkson, and I. M. Goldstein. 1989. Sequences of complementary DNAs that encode the NA1 and NA2 forms of Fcγ receptor III on neutrophils. *J. Clin. Invest.* 84:1688–1691.

5. Ravetch, J. V., and B. Perussia. 1989. Alternative membrane forms of FcγRIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J. Exp. Med.* 170:481–497.

6. Clark, M. R., S. B. Clarkson, P. A. Ory, N. Stollman, and I. M. Goldstein. 1989. Molecular basis for a polymorphism involving Fc receptor II on human monocytes. *J. Immunol.* 143:1731–1734.

7. Warmerdam, P. A. M., J. G. J. van de Winkel, E. J. Gosselin, and P. J. A. Capel. 1990. Molecular basis for a polymorphism of human Fcγ receptor II (CD32). *J. Exp. Med.* 172:19–25.

8. Warmerdam, P. A. M., J. G. J. van de Winkel, A. Vlug, N. A. C. Westerdal, and P. J. A. Capel. 1991. A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. *J. Immunol.* 147:1338–1343.

9. Clark, M. R., S. G. Stuart, R. P. Kimberly, P. A. Ory, and I. M. Goldstein. 1991. A single amino acid distinguishes the high-responder from low-responder form of Fc receptor II on human monocytes. *Eur. J. Immunol.* 21:1911–1916.

10. Salmon, J. E., J. C. Edberg, and R. P. Kimberly. 1990. Fcγ receptor III on human neutrophils. Allelic variants have functionally distinct capacities. *J. Clin. Invest.* 85:1287–1295.

11. Peltz, G. A., H. O. Grundy, R. V. Lebo, H. Yssel, G. S. Barsh, and K. W. Moore. 1989. Human FcγRIII: cloning, expression, and identification of the chromosomal locus of two Fc receptors for IgG. *Proc. Natl. Acad. Sci. USA.* 86:1013–1017.

12. Grundy, H. O., G. Peltz, K. W. Moore, M. S. Golbus, M. L. G. Jackson, and R. V. Lebo. 1989. The polymorphic Fcγ receptor II gene maps to the human chromosome 1q. *Immunogenetics.* 29:331–339.

13. Brooks, D. G., W. Q. Qiu, A. D. Luster, and J. V. Ravetch. 1989. Structure and expression of human IgG FcRII (CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J. Exp. Med.* 170:1369–1385.

14. Lalezari, P. 1977. Neutrophil antigens: immunology and clinical implications. *In* The Granulocyte: Function and Clinical Utilization. T. Greenwalt and G. S. Jamieson, editors. Alan R. Liss, New York, 209–225.

15. Edberg, J. C., P. B. Redecha, J. E. Salmon, and R. P. Kimberly. 1989. Human FcγRIII (CD16). Isoforms with distinct allelic expression, epitopes displays and membrane anchors on PMN and NK cells. *J. Immunol.* 143:1642–1649.

16. Edberg, J. C., M. Barinsky, P. B. Redecha, J. E. Salmon, and R. P. Kimberly. 1990. FcγRIII expressed on cultured monocytes is an N-glycosylated transmembrane protein. Similarity to FcγRIII expressed on NK cells. *J. Immunol.* 144:4729–4734.

17. Gosselin, E. J., M. F. Brown, C. L. Anderson, T. F. Zipf, and P. M. Guyre. 1990. The monoclonal antibody 41H16 detects the Leu 4 responder form of human FcγRII. *J. Immunol.* 144:1817–1822.

18. Fleit, H. B., S. D. Wright, and J. C. Unkeless. 1982. Human neutrophil Fcγ receptor distribution and structure. *Proc. Natl. Acad. Sci. USA.* 79:3275–3279.

19. Looney, R. J., D. H. Ryan, K. Takahashi, H. B. Fleit, H. J. Cohen, G. N. Abraham, and C. L. Anderson. 1986. Identification of a second class of IgG Fc receptors on human neutrophils. A 40 kilodalton molecule also found on eosinophils. *J. Exp. Med.* 163:826–836.

20. Antoun, G. R., B. M. Longenecker, and T. F. Zipf. 1989. Comparison of the 40 kDa hematopoietic cell antigens bound by monoclonal antibodies IV.3, 41H.16 and KB61. *Mol. Immunol.* 26:333–338.

21. Salmon, J. E., S. Kapur, and R. P. Kimberly. 1987. Opsonin-independent ligation of Fcγ receptors: the 3G8-bearing receptors on neutrophils mediate the

phagocytosis of concanavalin A–treated erythrocytes and nonopsonized *E. coli*. *J. Exp. Med.* 166:1798–1813.

22. Edberg, J. C., and R. P. Kimberly. 1992. Receptor specific probes for the study of Fcγ receptor specific function. *J. Immunol. Methods.* In press.

23. Shen, L., P. M. Guyre, C. L. Anderson, and M. W. Fanger. 1986. Hetero-antibody-mediated cytotoxicity: antibody to the high affinity Fc receptor for IgG mediates cytotoxicity by human monocytes that is enhanced by interferon-gamma and is not blocked by human IgG. *J. Immunol.* 137:3378–3382.

24. Anderson, C. L., L. Shen, D. M. Eicher, M. D. Wewers, and J. K. Gill. 1990. Phagocytosis mediated by three distinct Fcγ receptor classes on human leukocytes. *J. Exp. Med.* 171:1333–1345.

25. Salmon, J. E., N. L. Brogle, J. C. Edberg, and R. P. Kimberly. 1991. Fcγ receptor III induces actin polymerization in human neutrophils and primes phagocytosis mediated by Fcγ receptor II. *J. Immunol.* 146:997–1004.

26. Salmon, J. E., R. P. Kimberly, A. Gibofsky, and M. Fotino. 1986. Altered phagocytosis by monocytes from HLA-DR2 and DR3-positive healthy adults is Fcγ receptor-specific. *J. Immunol.* 136:3625–3630.

27. Tax, W. J. M., H. W. Willems, P. P. M. Reekers, P. J. A. Capel, and R. A. P. Koene. 1983. Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. *Nature (Lond.).* 304:445–447.

28. Tax, W. J. M., F. F. M. Hermes, R. W. Willems, P. J. A. Capel, and R. A. P. Koene. 1984. Fc Receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody-induced T cell proliferation. *J. Immunol.* 133:1185–1189.

29. Abo, T., A. B. Tilden, C. M. Balch, K. Kumagai, G. M. Troup, and M. D. Cooper. 1984. Ethnic differences in the lymphocyte proliferative response induced by murine IgG1 antibody, Leu 4, to the T3 molecule. *J. Exp. Med.* 160:303–309.

30. Cueppens, J. L., and F. van Vaeck. 1987. Direct demonstration of binding of anti-Leu 4 antibody to the 40 kDa Fc receptor on monocytes as a prerequisite for anti-Leu 4 induced T cell mitogenesis. *J. Immunol.* 139:4067–4071.

31. van de Winkel, J. G. J., G. J. J. C. Boonen, P. L. W. Janssen, A. Vlug, N. Hoog, and W. J. M. Tax. 1989. Activity of two types of Fc receptors, FcγRI and FcγRII, in human monocyte cytotoxicity to sensitized erythrocytes. *Scand. J. Immunol.* 29:23–31.

32. Clement, L. T., A. B. Tilden, and N. E. Dunlap. 1985. Analysis of the monocyte Fc receptor and antibody-mediated cellular interactions required for the induction of T cell proliferation by anti-T3 antibodies. *J. Immunol.* 135:165–171.

33. Boot, J. H. A., M. E. J. Geerts, and L. A. Aarden. 1989. Functional polymorphisms of Fc receptors in human monocyte-mediated cytotoxicity towards erythrocytes induced by murine isotype switch variants. *J. Immunol.* 142:1217–1222.

34. Schur, P. H. 1987. IgG subclasses—a review. *Ann. Allergy.* 58:89–99.

35. Jefferis, R., and D. S. Kumararatne. 1990. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin. Exp. Immunol.* 81:357–367.

36. Yount, W. J., M. M. Corner, H. G. Kunkel, and E. A. Kabat. 1968. Studies on human antibodies. VI. Selective variations in subgroup composition and genetic marker. *J. Exp. Med.* 127:633–646.

37. Scott, M. G., D. E. Briles, and M. H. Nahm. 1990. Selective IgG subclass expression: biologic, clinical and functional aspects. *In* The Human IgG Subclasses: Molecular Analysis of Structure, Function and Regulation. F. Shakib, editor. Pergamon Press, Oxford, UK. 161–183.

38. Siber, G. R., M. Santosham, G. R. Reid, C. Thompson, J. Almeido-Hill, A. Morell, G. deLange, J. K. Ketchum, and E. H. Callahan. 1990. Impaired antibody response to *Haemphilus influenza* type b polysaccharide and low IgG2 and IgG4 concentrations in Apache children. *N. Engl. J. Med.* 323:1387–1392.

39. Ward, J. G. Brenneman, G. W. Letson, W. L. Heyward, and the Alaska *H. influenza* Vaccine Study Group. 1990. Limited efficacy of a *Haemophilus influenza* type b conjugate vaccine in Alaska infants. *N. Engl. J. Med.* 323:1393–1401.

40. Fick, R. B., Jr., J. Olchowski, S. U. Squier, W. W. Merrill, and H. Y. Reynolds. 1986. Immunoglobulin-G subclasses in cystic fibrosis. IgG2 response to *Pseudomonas aeruginosa* lipopolysaccharide. *Am. Rev. Respir. Dis.* 133:418–422.

41. Fick, R. B., Jr., G. P. Naegel, R. A. Matthay, and H. Y. Reynolds. 1981. Cystic fibrosis *Pseudomonas* opsonin. Inhibitory nature in an in vitro phagocytic assay. *J. Clin. Invest.* 68:899–914.

42. Hornick, D. B., and R. B. Fick, Jr. 1990. The immunoglobulin G subclass composition of immune complexes in cystic fibrosis. Implications for the pathogenesis of the *Pseudomonas* lung lesion. *J. Clin. Invest.* 86:1285–1292.