Characterization of Polymorphic Forms of Fc Receptor III on Human Neutrophils

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Abstract

We characterized Fc receptor III (FcR III) on human neutrophils and found it to be heavily glycosylated and polymorphic. In some individuals, FcR III that had been digested with N-glycanase appeared after SDS-PAGE under reducing conditions as two bands with apparent molecular masses of 33 and 29 kD. In other individuals, N-glycanase-treated FcR III appeared as a single band with an M of either 33 or 29 kD. After SDS-PAGE of N-glycanase-treated FcR III under nonreducing conditions, the apparent M of each structural type was decreased, suggesting the presence of intramolecular disulfide bonds. Digestion of the 33-kD band and the 29-kD band with *Staphylococcus aureus* V8 protease yielded similar, but not identical, peptide maps. Thus, at least two polymorphic forms of FcR III are expressed on human neutrophils. The structural polymorphism of neutrophil FcR III correlated with previously described antigenic polymorphisms detected by monoclonal antibody Gran 11 and by alloantisera which recognize epitopes of the biallelic, neutrophil antigen (NA) system. Individuals whose neutrophils expressed the two-band structural type of FcR III were NA1NA2 heterozygotes. Individuals whose neutrophils expressed the single 33-kD band structural type were NA2NA2 homozygotes, and individuals whose neutrophils expressed the single 29-kD band structural type were NA1NA1 homozygotes. These findings indicate that antigenic and structural polymorphisms of human neutrophil FcR III are related and can be accounted for by differences at the level of primary protein structure.

Introduction

Receptors for the Fc region of IgG (FcRs) play a key role in removing antigen-antibody complexes from the circulation. There are at least three FcRs for IgG on human cells. One of these, FcR III (also termed CD16 by the International Workshop on Human Leukocyte Differentiation Antigens), is present on neutrophils, tissue macrophages, natural killer cells, and eosinophils (1). FcR III binds immune complexes but not monomeric IgG, and appears to be an important mediator of immunophasagocytosis both in vitro (2) and in vivo (3, 4). FcR III from human neutrophils appears as a broad band from 50 to 73 kD after SDS-PAGE, and has been characterized as a complex sialoglycoprotein (1, 2, 5). In previous studies, we determined that the electrophoretic heterogeneity of neutrophil FcR III is due, in part, to abundant N-linked carbohydrate (2). We also noted a previously undescribed structural polymorphism of neutrophil FcR III. After digestion with N-glycanase, FcR III from neutrophils of some individuals appeared after SDS-PAGE under reducing conditions as two bands with apparent molecular masses of 33 and 29 kD. FcR III from neutrophils of other individuals appeared as a single band at 33 kD.

Antigenic polymorphisms of FcR III have been reported previously. Lalezari (6–8) described a neutrophil antigen (NA) system with three allotypes defined by alloantisera. Werner et al. (9), more recently demonstrated by indirect immunofluorescence and sequential immunoprecipitation using monoclonal antibodies (MAbs) 3G8, Leu 11, and Gran 11 that NA epitopes were allotypic forms of FcR III. Whereas MAbs 3G8 and Leu 11 recognized FcR III on neutrophils from all individuals, MAb Gran 11 (anti-NA1) recognized FcR III on neutrophils from only 46% of individuals (6, 7). Neutrophil surface components bearing NA epitopes are target antigens in autoimmune neutropenia (10–13).

In this report, we describe the relationship between the previously reported structural and antigenic polymorphisms of FcR III and demonstrate that differences between the various structural types of neutrophil FcR III reflect differences at the level of primary protein structure.

Methods

Preparation of peripheral blood neutrophils. Blood was obtained from normal human volunteers, anticoagulated with acid-citrate-dextrose, and then subjected to centrifugation on Hypaque-Ficoll (density 1.077) to separate neutrophils and erythrocytes from mononuclear cells (14). Neutrophils were then separated from erythrocytes by sedimentation with 6% dextran (14), and washed three times with phosphate (10 mM)-buffered saline, pH 7.4. Preparations usually contained > 95% neutrophils as determined by Wright staining.

Monoclonal antibodies. The cell line producing MAb 3G8 (murine IgG2a) (1) was a generous gift of Dr. David Segal (NIH, Bethesda, MD). Ascites containing MAb Gran 11 (murine IgG1) (9) was a generous gift of Dr. Pedro A. T. Tetteroo (Central Laboratory of the Netherlands Red Cross, Amsterdam). A cell line producing MOPC 21 (murine IgG, myeloma) was obtained from American Type Culture Collection, Bethesda, MD. RPC 5 (murine IgG2a, myeloma) was obtained from Bionetics, Charleston, SC. MAbs were purified from acetics by precipitation with 45% saturated ammonium sulfate, followed by precipitation with caprylic acid (1.5%, vol/vol) (Sigma Chemical Co., St. Louis, MO) (15). Fab fragments were prepared by digestion with immobilized pepsin (Sigma Chemical Co.), and purified by passage over protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at pH 8.3. No contamination with immunoglobulin heavy chains was detected by SDS-PAGE.
Alloantisera. Polyclonal anti-NA1 and anti-NA2 alloantisera, obtained from pooled blood of transfused patients and pregnant women, were generously provided by Dr. Parviz Lalezari (Albert Einstein Medical Center, Bronx, NY). Alloantisera and normal human serum were subjected to centrifugation at 160,000 g for 30 min to remove aggregates.

**Immunoprecipitation.** Purified human neutrophils (3.0–5.0 x 10^7 cells) were surface-labeled with 125I using Iodogen (100 µg/tube) (Pierce Chemical Co., Rockford, IL), and lysed in PBS containing 10 mM octyl-2-d-thioglucose, 1.0 mM diisopropylfluorophosphate, 1.0 mM PMSF, and 0.02% (wt/vol) sodium azide (Sigma Chemical Co.). After centrifugation at 10,000 g for 10 min, lysates were incubated at 4°C for 2 h with BSA coupled to Sepharose 4B and then for 2 h with MAB 3G8 coupled to Sepharose 4B. Sepharose beads were washed extensively and resuspended in PBS containing 0.5% (wt/vol) SDS. After boiling for 3 min, eluted FcR III was treated with either N-glycanase (see below) or PBS, and then subjected to SDS-PAGE and autoradiography.

**Sequenial immunoprecipitation.** Lysates of radiolabeled neutrophils were prepared as described above. Following incubation of lysates with 3G8 coupled to Sepharose 4B, FcR III was eluted with 0.1 M glycine, pH 2.5, and neutralized with 1.0 M Tris, pH 8.5. FcR III was then treated with N-glycanase (20 µM/µl) as described below to remove all N-linked carbohydrate. FcR III digests were incubated with goat anti-mouse Ig (G) (Cappel Laboratories, Malvern, PA) that had been coupled to Sepharose 4B and previously incubated for 2 h at 4°C with either MAB 3G8, MAB Gran 11, or the irrelevant murine myeloma proteins MOPC 21 and RPC 5. The Sepharose beads were washed extensively and resuspended in PBS containing 0.5% (wt/vol) SDS. FcR III was eluted by boiling for 3 min, and then subjected to SDS-PAGE and autoradiography.

**Treatment with glycosidases.** After immunoprecipitation with MAB 3G8 coupled to Sepharose, FcR III was solubilized by boiling for 3 min in PBS containing 0.5% (wt/vol) SDS. Samples were then treated with either: N-glycanase (Genzyme, Boston, MA) in PBS, pH 7.4, containing 10 mM 1,10 phenanthroline (Sigma Chemical Co.) and 1.0% (vol/vol) NP-40; endoglycosidase H (Endo H; Boehringer-Mannheim, Indianapolis, IN) in 0.1 M sodium citrate, pH 5.5, containing 0.1% (wt/vol) SDS; or endoglycosidase D (Endo D) in 0.1 M sodium phosphate, pH 6.5, containing 0.1% SDS (wt/vol) (16, 17). Reaction mixtures were incubated at 37°C overnight. After boiling for 3 min to inactivate enzymes, samples were subjected to SDS-PAGE and autoradiography. One unit of Endo D and Endo H is defined as the amount of enzyme required to release 1.0 µmol of carbohydrate from appropriate substrates. One unit of N-glycanase is defined as the amount of enzyme required to release 1.0 nmol of carbohydrate from appropriate substrates.

**Peptide mapping by limited proteolysis.** Human neutrophil FcR III was immunoprecipitated with MAB 3G8, treated with N-glycanase, and subjected to SDS-PAGE as described above. Peptide mapping was then carried out as described by Cleveland et al. (18). Samples were digested with Staphylococcus aureus V8 protease (Sigma Chemical Co.) and then analyzed by SDS-PAGE.

**Immunonanaysis.** Purified human neutrophils were fixed with 1.0% (vol/vol) paraformaldehyde, washed with PBS, and then incubated for 30 min at 4°C with either MAB Gran 11 antibodies (1:10,000 dilution), MAB 3G8 (10 µg/ml), anti-NA1 or anti-NA2 alloantisera (undiluted), normal human serum (undiluted), or the irrelevant murine myeloma protein MOPC-21 (10 µg/ml). Cells were washed and then incubated with either fluorescein-conjugated goat anti-human IgG, IgM, and IgA, or goat anti-mouse IgG and IgM (Jackson Immunoresearch, Westwood, PA). Neutrophils were examined by fluorescence microscopy and/or flow cytometry using either an Ortho Cytofluorograph II (Ortho, Westwood, MA) or a FACS IV (Becton Dickinson, Palo Alto, CA).

To measure binding of radiolabeled MAB 3G8, neutrophils (0.5–1.0 x 10^7 cells/ml) were incubated at 4°C with equal volumes of 125I-labeled Fab fragments of MAB 3G8 (2.5 µg/ml) and either PBS containing 1.0 mg/ml BSA (PBS/BSA) or unlabeled MAB (1.0 mg/ml). After 30 min, cells were suspended in 0.5 ml cold PBS/BSA, layered over 0.5 ml Versilube FS 15 silicone oil (General Electric, Waterford, NY) in 1.5 ml polypropylene microtubes, and centrifuged for 5 min at 10,000 g. After aspiration of the aqueous and oil phases, the bottoms of tubes containing cell pellets were excised and subjected to gamma counting. Specific binding was determined by subtracting radioactivity that became cell-associated in the presence of excess unlabeled antibody from total radioactivity.

**Results**

**FcR III on human neutrophils appears to be polymorphic.** Treatment of immunoprecipitated FcR III with N-glycanase, which cleaves N-linked oligosaccharides at sugar-amino acid linkages, changed its electrophoretic mobility under reducing conditions from a broad band extending from 50 to 73 kD to either two major bands with apparent molecular masses of 33 and 29 kDa (Fig. 1) or a single major band at either 33 or 29 kDa (see below). In contrast, neither Endo H, which removes high mannose oligosaccharide residues (16), nor Endo D, which removes complex glycans (17), affected the electrophoretic mobility of human neutrophil FcR III after SDS-PAGE.

In a survey of 22 normal individuals, digestion of neutrophil FcR III with N-glycanase resulted in the appearance of a major band with an M_f of 33 kDa in 10 individuals, the appearance of a major band with an M_f of 29 kDa in two individuals, and the appearance of two major bands with approximate molecular masses of 29 and 33 kDa in 10 individuals. The results of treating neutrophil FcR III from three representative individuals with N-glycanase are shown in Fig. 2. Digestion of FcR III from neutrophils of one individual consistently yielded two bands of M_f 35 and 29 kDa (data not shown).

Small differences also were noted in the electrophoretic mobility of native (undigested) FcR III (Fig. 2). Native FcR III from all individuals exhibited broad electrophoretic mobility after SDS-PAGE. However, native FcR III that yielded the single 33-kD band after digestion with N-glycanase migrated to a position corresponding to a greater M_f than did native FcR III that yielded the single 29-kD band after digestion with N-glycanase. Native FcR III that appeared as two bands after digestion with N-glycanase migrated to a position that was intermediate as compared to the two single-band structural types.

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**Figure 1. Neutrophil FcR III is susceptible to digestion with N-glycanase.** Lysates of surface-labeled neutrophils were immunoprecipitated with MAB 3G8 coupled to Sepharose 4B. FcR III was treated with sample buffer (lane 1); N-glycanase, 10 U/ml (lane 2); Endo H, 100 U/ml (lane 3); and Endo D, 150 U/ml (lane 4). Reaction mixtures were then subjected to SDS-PAGE on a 10% gel under reducing conditions, followed by autoradiography.

**Polymorphic Forms of Fc Receptor III on Neutrophils**

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We next attempted to determine the basis for the apparent structural heterogeneity of FcR III. We considered four possibilities. First, the two-band structural type may represent degradation products of the one-band structural types. According to the manufacturer, N-glycanase copurifies with a zinc-dependent protease. Accordingly, removal of N-linked sugars from FcR III with N-glycanase was always carried out in the presence of 1,10 phenanthroline, an inhibitor of zinc-dependent proteases. Patterns observed after N-glycanase treatment of FcR III from each individual tested were consistent from experiment to experiment. Each individual was studied at least three times. Patterns were not affected by prolonged incubation nor by an excess of N-glycanase. Furthermore, N-glycanase did not cleave BSA (data not shown). Therefore, it is unlikely that the difference between structural types is due to proteolysis.

Another possible explanation for differences between structural types of FcR III is the presence of an interchain disulfide bond in the two-band structural type that is absent in the one-band structural types. SDS-PAGE under nonreducing conditions of FcR III treated with N-glycanase revealed decreased apparent molecular masses of each structural type (Fig. 3). An increase in apparent Mr following reduction suggests the presence of intramolecular disulfide bonds (19), but does not explain the observed differences between the structural types. Differences between structural types were observed after SDS-PAGE under both reducing and nonreducing conditions. It should be noted that a minor band was observed on nearly all gels. Under reducing conditions, it migrated slightly faster, while under nonreducing conditions, it migrated slightly slower than did the major bands (Figs. 2 and 3).

A third possibility is that the structural types differ in degree of O-linked glycosylation. Although we did not exclude this possibility, the differences in patterns seen after SDS-PAGE are not typical of those seen with differences in glycosylation.

Finally, differences between structural types may reflect differences in primary protein structure. Using peptide mapping, we examined relationships between different structural types and relationships of bands within each structural type. Shown in Fig. 4 are peptide maps from two representative individuals. The 33-kD band in the two-band structural type and the 33-kD band in the single 33-kD band structural type yielded similar peptide maps. Peptide mapping of the 29-kD band of the two-band structural type exhibited a pattern that was similar to that of the 33-kD band. However, a peptide with an approximate Mr of 23 kD appeared to be missing in all three individuals examined. These results suggest that differences between FcR III structural types reflect differences at the level of primary protein structure.

Structural differences among FcR III core protein(s) are related to differences in reactivity with MAb Gran 11 and anti-Na-1 and anti-Na-2 alloantisera. Previous work by Werner et al. (9) and by Lalezari (6–8) demonstrated that FcR III on human neutrophils exhibits antigenic heterogeneity. Both MAb 3G8 and Gran 11 precipitated a 50–73-kD protein (1, 9). However, MAb 3G8 recognized FcR III on neutrophils from all individuals, whereas Gran 11 recognized FcR III on neutrophils from ~50% of individuals. Monoclonal antibody Gran 11 recognizes NA1 of the neutrophil-specific, biallelic NA antigen system (9). In a normal caucasian population, NA1 and NA2 were found on neutrophils from 46 and 88% of individuals, respectively (6, 7).

Analyses by flow cytometry and/or fluorescence microscopy were carried out on cells from 22 individuals. Neutrophils from all individuals reacted with MAb 3G8 (Fig. 5 and Table I). Reactivity of neutrophils with MAB Gran 11 corre-
Mab Gran 11 correlates with the appearance of the 29-kD band. All cells reacted with MAb 3G8, but no cells reacted with the control myeloma proteins, MOPC 21 or RPC 5.

Reactivity of neutrophils with anti-NA alloantisera also correlated with differences in structural types (Table I). FcR III on neutrophils that reacted with both anti-NA1 and anti-NA2 alloantisera yielded the two-band structural type after digestion with N-glycanase. FcR III on neutrophils that reacted only with anti-NA-2 alloantisera yielded the 33-kD band and FcR III on neutrophils that reacted only with anti-NA1 alloantisera yielded the single 29-kD band. Therefore, the heterogeneity in reactivity of neutrophils with anti-NA alloantisera correlates with the structural heterogeneity of neutrophil FcR III. Individuals with the two-band structural type of FcR III are NA1NA2 heterozygotes, individuals with the single 33-kD band are NA2NA2 homozygotes, and individuals with the single 29-kD band are NA1NA1 homozygotes. The one individual whose neutrophil FcR III was of the two-band structural type but with slightly increased molecular masses of the two bands was a heterozygote.

Deglycosylated FcR III exhibits antigenic heterogeneity. We examined further whether differences in reactivity of the structural types of FcR III with MAB 3G8 and MAB Gran 11 were due to differences in glycosylation. Sequential immunoprecipitation (Fig. 6) revealed that although both MABs 3G8 and Gran 11 recognized undigested FcR III of the two-band structural type (lanes 1 and 3, respectively), only MAB 3G8 recognized undigested FcR III of the single 33-kD-band structural type (lane 9). After digestion with N-glycanase, MABs 3G8 and Gran 11 also recognized the 29-kD band in the two-band structural type (lanes 5 and 7, respectively). Although MAB 3G8 appeared to recognize the 33-kD band noted after N-glycanase digestion of the single 33-kD band structural type (lane 13), faint 33-kD bands also appeared after incubating cell

**Table I. Reactivity of Neutrophils with Monoclonal Antibodies and Alloantisera**

<table>
<thead>
<tr>
<th>MABs and alloantisera*</th>
<th>33-kD band type</th>
<th>Two-band type</th>
<th>29-kD band type</th>
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<tr>
<td></td>
<td><em>n = 10</em></td>
<td><em>n = 10</em></td>
<td><em>n = 2</em></td>
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<tr>
<td>3G8</td>
<td>+*</td>
<td>+</td>
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<tr>
<td>MOPC 21</td>
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<td>GRAN 11</td>
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<td>RPC 5</td>
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<tr>
<td>Anti-NA1</td>
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<tr>
<td>Anti-NA2</td>
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*Freshly isolated neutrophils were either fixed with 1.0% paraformaldehyde and suspended in PBS or plated at a density of 1 x 10^6 cells/well on Terasaki plates and allowed to adhere for 30 min at 37°C. Nonspecific binding was blocked by incubating the cells with PBS containing 1% (vol/vol) fetal calf serum for 30 min at 4°C. Cells were washed and then incubated with either a MAB or alloantisera followed by incubation with either FITC-labeled goat anti-mouse or FITC-labeled goat anti-human immunoglobulin for 30 min at 4°C. Neutrophils were then examined using fluorescence microscopy and/or flow cytometry.

* (+) indicates that > 20% of the neutrophils reacted with the antibody and (-) indicates that < 3% of neutrophils reacted with the antibody.
lysatcs with myeloma proteins MOPC 21 and RPC 5. The latter probably represent nonspecific binding. MAB Gran 11 did not appear to recognize either undigested FcR III (lane 11) or deglycosylated FcR III (lane 15) of the single 33-kD band structural type. This further supports our conclusion that antigenic and structural differences are probably due to differences at the level of primary protein structure.

Different structural types of FcR III are expressed with similar densities on human neutrophils. We next investigated whether structural differences in FcR III correlate with differences in surface expression of FcR III. Neutrophils from six individuals that expressed the single 33-kD band and neutrophils from four individuals that expressed the two-band structural type were investigated with respect to binding of radiiodinated Fab fragments of MAB 3G8. There was no significant difference between the two structural types with respect to binding of MAB 3G8. Mean specific binding to the single 33-kD band structural type and two-band structural type was 10,769 cpm (range 6,260–14,365 cpm) and 11,133 cpm (range 9,546–12,935 cpm), respectively. Although we have not yet investigated the surface expression of neutrophil FcR III of the single 29-kD band structural type, these data suggest that the different structural types of neutrophil FcR III are expressed similarly.

Discussion

In this report, we describe a structural polymorphism of FcR III on human neutrophils. At least two forms of FcR III are expressed on the surface of human neutrophils (Figs. 2 and 3). Deglycosylated FcR III on neutrophils from 10 individuals appeared as a single major band with an apparent Mr of 33 kD after SDS-PAGE under reducing conditions. Deglycosylated FcR III on neutrophils from another 10 individuals appeared as two major bands with molecular masses of 33 and 29 kD. Deglycosylated FcR III on neutrophils from two individuals appeared as a single major band with an Mr of 29 kD. A minor band was observed after electrophoresis of each structural type under both reducing and nonreducing conditions (Figs. 2 and 3). This additional band may represent a polypeptide that is noncovalently associated with FcR III on the surface of human neutrophils. Alternatively, the minor band may reflect incomplete digestion of FcR III by N-glycanase, the presence of variable amounts of O-linked oligosaccharides, or an artifact.

Peptide mapping by limited proteolysis with S. aureus V8 protease revealed differences between the structural types of neutrophil FcR III (Fig. 4). Peptide maps of the 33-kD band in the single 33-kD band and two-band structural types of FcR III were similar. Peptide mapping of the 29-kD band yielded a pattern that was similar to that of the 33-kD band, except that a 23-kD peptide was missing. Although it is possible that the “missing” 23-kD peptide simply was not radioiodinated because a tyrosine residue was rendered inaccessible by some posttranslational effect, these results are consistent with the conclusion that the 33-kD polypeptide differs from the 29-kD polypeptide with respect to its amino acid sequence.

Sequential immunoprecipitation of FcR III as well as fluorescence microscopy and flow cytometry showed that there were differences in reactivity with MAB Gran 11 among the structural types (Figs. 5 and 6). Both MABs 3G8 and Gran 11 recognized undigested FcR III of the two-band structural type as well as the 29 kD band noted after digestion with N-glycanase. Only MAB 3G8 recognized undigested FcR III of the single 33 kD-band structural type. Differences in reactivity with MABs also were probably due to differences in primary protein structure.

Neutrophil FcR III of the different structural types also exhibited different reactivities with anti-NA alloantisera (Table I). Individuals whose neutrophils expressed the two-band structural type appeared to be heterozygous for both NA1 and NA2, whereas individuals whose neutrophils expressed the single 33-kD band structural type appeared to be homozygous for NA2. The two individuals whose neutrophils expressed the single 29-kD band structural type appeared to be homozygous for NA1. As would be expected, cells from NA1-NA1 homozygotes bound more MAB Gran 11 than did cells from NA1-NA2 heterozygotes (Fig. 6). Thus, the structural polymorphism of FcR III correlates with the neutrophil-specific NA antigen system.

FcR III has been cloned recently from human placenta, sequenced, and found to be the product of a single gene (20). Unlike other FcRs, FcR III is anchored to the membrane via a glycosyl-phosphatidylinositol linkage (20–22). In light of these recently reported observations, and the results of our own experiments, it is possible that there are two alleles of the FcR III gene, one which encodes a 29-kD polypeptide and another which encodes a 33-kD polypeptide. It also is possible that the 29- and 33-kD polypeptides are products of alternatively spliced transcripts from a single gene.

Polymorphisms have been reported for other Fc receptors. Human FcR II, a 40-kD protein on neutrophils, monocytes, and platelets, as well as FcR I, a 72-kD protein on monocytes and macrophages have been shown to be polymorphic (23–27). Although the functional significance of structural polymorphisms of FcR III is unknown, a wide range in rates of Fc-mediated clearance of antigen-antibody complexes has been demonstrated. Prolonged Fc-mediated clearance of antigen-antibody complexes has been demonstrated in systemic lupus erythematosus (28, 29), SJogren’s syndrome (30), dermatitis herpetiformis (31), and normal individuals with the HLA-B8/DRw3 haplotype (31). Because of the role played by FcR III in mediating immunophagocytosis in vivo (3, 4), it is possible that differences in rates of clearance of circulating immune complexes are related to structural polymorphisms of FcR III.

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References


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