INFECTION AND IMMUNITY, Jan. 1995, p. 73–81
0019-9567/95/$04.00+0
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Vol. 63, No. 1

Human Immunoglobulin G (IgG) Fc Receptor IIA (CD32) Polymorphism and IgG2-Mediated Bacterial Phagocytosis by Neutrophils

LIEKE A. M. SANDERS,[1]* ROBERT G. FELDMAN,[2]† MARLEEN M. VOORHORST-OGINK,[1] MASJA DE HAAS,[3] GER T. RIJKERS,[1] PETER J. A. CAPEL,[4] BEN J. M. ZEGERS,[1] AND JAN G. J. VAN DE WINKEL[4]

Department of Immunology, University Hospital for Children, "Het Wilhelmina Kinderziekenhuis," [1] and Departments of Microbiology[2] and Immunology,[4] University Hospital, Utrecht, and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam,[3] The Netherlands

Received 16 August 1994/Accepted 11 October 1994

Human immunoglobulin G (IgG) Fc receptor IIa (FcγRIIa; CD32) is expressed on phagocytes, triggers phagocytosis, and represents the sole Fc receptor for IgG (FcγR) capable of interaction with IgG2, the main IgG subclass induced in response to bacterial capsular polysaccharides. The two genetically determined structurally different allotypes of human FcγRIIa, the products of the FcγRIIa-R131 and IIa-H131 alleles, have functionally different reactivities with human IgG2. In humans, the FcγRIIa-H131 allotype is known to interact efficiently with complexed human IgG2, whereas the IIa-H131 allotype does so only poorly. This polymorphism may therefore have implications for IgG2-mediated phagocytosis of encapsulated bacteria and susceptibility to bacterial infections. Phagocytosis of IgG2-opsonized bacteria by homozygous FcγRIIa-R/R131, heterozygous IIa-H/R131, and homozygous IIa-H/H131 polymorphonuclear cells (PMN) was compared. A higher phagocytic capacity of IgG2-opsonized group B type III streptococci by PMN of homozygous H/H131 individuals compared with PMN from homozygous R/R131 individuals was observed ($P = 0.001$), while heterozygous IIa-H/R131 PMN showed intermediate phagocytosis. In this model system, IgG2-mediated phagocytosis was independent of the FcγRIIIb-NA1/NA2 allelic polymorphism.

Fc receptors for immunoglobulin G (IgG) (FcγR) form an essential bridge between the humoral branch and the effector cells of the immune system. They are involved in multiple biological processes, including phagocytosis, antibody-dependent cellular cytotoxicity, and release of inflammatory mediators (36, 48). In humans, three major classes of FcγR are recognized: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). These can be distinguished from one another on the basis of size, primary structure, monoclonal antibody (MAb) reactivity, affinity and specificity for ligands, and cellular distribution pattern. FcγRI represents a high-affinity receptor capable of binding monomeric human IgG1, IgG3, and IgG4. FcγRII and -III are low-affinity receptors, interacting only with IgG in complexed or aggregated form. Both FcγRII and -III interact with human IgG1 and IgG3, whereas FcγRII is the sole FcγR class capable of binding human IgG2 complexes (32, 50).

Human neutrophils (PMN) constitutively express two FcγR: FcγRIIa, with a classical membrane-spanning domain and cytoplasmic tail, and FcγRIIIb, which is linked to the cell membrane via a glycosyl-phosphatidylinositol anchor. Both these receptors exhibit genetically determined structural and functional polymorphisms, the biallelic FcγRIIa-R131 and -H131 polymorphism (previously known as the high-responder [HR]/low-responder [LR] FcγRIIa polymorphism), and the neutrophil antigen 1 (NA1)/NA2 FcγRIIIb polymorphism (5, 30, 31, 49). The two allelic forms of the PMN-specific FcγRIIIb differ by four amino acids and two potential glycosylation sites (30, 31, 48). No differences in binding of human IgG1 or IgG3 have been observed, but individuals homozygous for NA1 or NA2 exhibit distinct phagocytic capacities which are independent of the FcγRIIa allotypes (39, 40, 48). The FcγRIIa-H131 and -R131 allelic forms differ by two amino acids in their extracellular regions, only one of which, at amino acid position 131, was found to be critical for the binding of human IgG2 complexes: histidine at position 131 results in effective interaction with IgG2, whereas arginine results in poor binding of IgG2 (5, 6, 50). Moreover, PMN and monocytes of homozygous H/H131 individuals were found to internalize human IgG2-opsonized erythrocytes more efficiently than cells from R/R131 individuals (40).

Since human IgG2 is the predominant IgG subclass containing antibodies to bacterial capsular polysaccharides (11), we investigated IgG2-mediated phagocytosis of encapsulated bacteria by granulocytes from donors with either the homozygous FcγRIIa H/H131, the heterozygous H/R131, or the homozygous R/R131 phenotype. From previous experience, we used group B streptococcus type III (GBSIII), opsonized with affinity-purified IgG2 antibodies to the capsule of GBSIII, as a model with which to study the specific interaction between IgG2 and FcγRIIa.

MATERIALS AND METHODS

Subjects. Serum and heparin-anticoagulated blood samples were collected from randomly chosen healthy volunteers of Caucasian origin. In control exper-

* Corresponding author. Mailing address: Department of Immunology, University Hospital for Children, "Het Wilhelmina Kinderziekenhuis," P.O. Box 18009, 3501 CA Utrecht, The Netherlands. Phone: (31) 30 320911. Fax: (31) 30 320712.

† Present address: Department of Infectious Diseases and Bacteriology, The Royal Postgraduate Medical School, London, United Kingdom.

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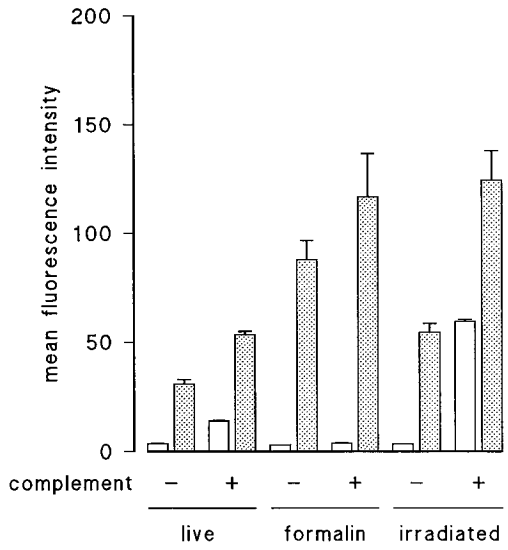

FIG. 1. Phagocytosis of GBSIII: effect of IgG2 anti-GBSIII carbohydrate antibodies and of complement. FITC-labeled live, Formalin-killed, or radiation-killed GBSIII were opsonized with IgG2 anti-GBSIII carbohydrate antibodies (shaded bars) or not opsonized (open bars) in the presence or absence of exogenous added complement. Uptake of bacteria by H/H131 PMN was measured by flow cytometry. Data shown are mean FITC fluorescence intensity values (triplicates, ± 1 standard deviation) for one of three independent experiments.

iments, PMN were derived from two FcγRIIIb-deficient donors known to lack both FcγRIIIb alleles (22).

MAbs. Anti-FcγRI (CD64) MAb 197 (IgG2a [18]), anti-FcγRII (CD32) MAb IV.3 (IgG2b [24]) and F(ab′) fragments, and anti-FcγRIII (CD16) MAb 3G8 (IgG1 [13]) and $F(ab')_2$ fragments were provided by Medarex (Annandale, N.J.). $F(ab')_2$ fragments of anti-FcγRII MAb AT10 (IgG1 [17]) were provided by M. Glennie (Tenovus Research Laboratory, Southampton, United Kingdom). Anti-FcγRII MAb 41H16 (IgG2a [16]) was generously provided by B. M. Longenecker (University of Alberta, Edmonton, Canada). Anti-FcγRIII MAb CLB GRAN 11 (IgG2a [21, 52]), CLB GRAN 1 (IgG2a [21, 52]), and anti-complement receptor 3 MAb B2.12 (IgM [47]) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Anti-FcγRIII MAb BW209/2 (21) was a generous gift of R. Kurrle (Behringwerke AG, Marburg, Germany). Anti-CD16 MAb GRM1 (IgG2a [20, 21]) was provided by F. Garrido (Hospital Las Nievas, Granada, Spain). Anti-CD16 MAb 1D3 (IgG1 [34]) was provided by J. Griffin (Dana-Farber Cancer Institute, Boston, Mass.). $F(ab')_2$ fragments of anti-CD11b MAb MO1 (IgG2a [46]) were provided by R. Todd III (University of Michigan Medical Center, Ann Arbor).

Preparation of cells. PMN were isolated from whole blood by sedimentation with 5% dextran (Serva, Heidelberg, Germany) solution (4 volumes of blood, 1 volume of dextran) for 20 min at 37°C. Prior to the phagocytic assays, PMN were washed twice in ice-cold minimal essential medium and resuspended in Hanks' balanced salt solution with 0.1% gelatin (GHBSS) at 5×10^6 PMN/ml and used immediately. Mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation for use in anti-CD3-induced T-cell mitogenesis assays, as described previously (32).

Determination of H131 and R131 FcγRIIa phenotypes. Phenotyping of PMN and monocytes for H131 and R131 alleles of FcγRIIa was performed by quantitative flow cytometry with CD32 MAb IV.3, reacting with both FcγRIIa-H131 and -R131 (24), and MAb 41H16, which selectively reacts with FcγRIIa-R131 (16). To avoid cytophilic binding, all incubations were performed in the presence of 25% heat-inactivated human serum. Phenotypic assignments were confirmed by studying anti-CD3-induced T-cell mitogenesis using human IgG2 and mouse IgG1 anti-CD3 MAb (32).

Determination of the FcγRIIIb NA1/NA2 allotypes. Neutrophil-specific antigens NA1 and NA2 were determined serologically by radioimmunoassay (20). Briefly, MAb CLB GRAN 1 (anti-pan-FcγRIII) was covalently coupled to CNBr-activated Sepharose 4B beads (Pharmacia) and diluted to 0.1% (wt/vol) in phosphate-buffered saline (PBS) containing EDTA (10 mM) and 0.025% (vol/vol) Tween 20 (PET buffer). Then 500 μl of the suspension was incubated with plasma or serum for 18 h at room temperature by head-over-head rotation. A standard curve was constructed from pooled plasma from 80 volunteers. Two dilutions of donor plasma or serum in PET buffer were tested in triplicate. Following incubation with plasma, the Sepharose beads were washed and incubated with saturating amounts of ^{125}I-labeled MAb BW209/2 (anti-pan-FcγRIII) or CLB GRAN 11 (anti-FcγRIIIb-NA1) for 5 h at room temperature. The beads were washed again, and radioactivity was measured. Using serum from five donors known to be FcγRIIIb-NA1/NA1, the binding of CLB GRAN 11 relative to BW209/2 was determined and set as 100% relative binding. The NA phenotype of soluble FcγRIIIb was assigned as FcγRIIIb-NA1/NA1 (>80% relative binding of CLB GRAN 11), FcγRIIIb-NA1/NA2 (10 to 80%), or FcγRIIIb-NA2/NA2 (<10%).

Phenotyping was also performed by flow cytometry with CD16 MAb 1D3, selectively reactive with FcγRIIIb; MAb CLB GRAN 11, which recognizes FcγRIIIb-NA1; and MAb GRM1, which detects FcγRIIIb-NA2. PMN were incubated with MAb CD16 for 30 min at 4°C, washed, and incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig antiserum (Becton Dickinson). PMN were either positive only for CLB GRAN 11 (FcγRIIIb-NA1/NA1), only for GRM1 (FcγRIIIb-NA2/NA2), or for both of these MAbs (FcγRIIIb-NA1/NA2).

Bacteria. The well-encapsulated strain M732 GBSIII was kindly provided by Dennis Kaspar (Channing Laboratory, Boston, Mass.). It was first grown on blood agar plates and then cultured overnight in Todd-Hewitt broth (Difco) supplemented with 2.4 g of Na_2HPO_4 per liter. The pH was checked before harvesting and remained >6.5. The bacteria were resuspended in PBS (4×10^7 CFU/ml). Formaldehyde (1%, vol/vol) was added, and the bacteria were incubated for 48 h at 4°C, washed extensively, and labeled with FITC I (Sigma) (53). After being washed, the bacteria were resuspended in GHBSS and stored in aliquots at −20°C until use. There was no significant difference in the FITC labeling efficiency between live and formaldehyde-killed bacteria (data not shown). Furthermore, the binding of IgG2 anti-GBSIII carbohydrate antibodies (as assessed in a whole-cell enzyme-linked immunosorbent assay [ELISA]) to either live, formaldehyde-killed, or FITC-labeled bacteria did not differ significantly (0.46 ± 0.06, 0.46 ± 0.07, and 0.43 ± 0.04 arbitrary ELISA units, respectively).

Anti-GBSIII IgG2 antibodies. IgG antibodies were isolated from 200 ml of blood drawn from a donor known to have high levels of IgG2 against GBSIII, trace amounts of specific IgG1, and no detectable specific IgG3 or -4, as determined by GBSIII subclass ELISA (8). Antibodies directed against GBSIII were subsequently affinity purified as described previously (10). Any remaining specific IgG1 was removed by passing the preparation over an anti-human IgG1 column (murine anti-human IgG1 MAb, clone HP-6001; Sigma) and Immobilization Kit 1 (Pierce). The IgG2 was dialyzed against dilute PBS (1:10) and concentrated threefold by partial lyophilization. The final concentration of IgG2 in the preparation was 21 μg/ml, measured by a GBSIII ELISA. The final preparation contained IgG1 at the limit of detection in a GBSIII subclass ELISA, but no IgG3 or IgG4 was detectable. The IgG2 preparation was stored in small aliquots at −80°C until use.

Complement source. Human serum from a donor with undetectable anti-GBSIII anticapsular antibody (determined by ELISA) was obtained. Serum was absorbed twice with freshly harvested GBSIII, and no detectable levels of either IgG subclass were observed in a whole-cell ELISA for anti-GBSIII antibodies. The serum exhibited 63% functional hemolytic complement activity compared with a pooled internal standard and was stored in aliquots at −80°C until use. Hemolytic complement activity in the stored serum was well preserved (about 60%).

Phagocytic assays. The phagocytic assay was based on a previously described method (3). A total of 25 μl of bacterial GBSIII suspension (equivalent to 4×10^6 bacterial particles) was opsonized by incubation with 100 μl of anti-GBSIII IgG2 (final concentration, 2.1 μg/ml). In some experiments, 100 μl of complement source was added to a final concentration of 6% (vol/vol). Assay mixtures were incubated at 37°C in a shaking water bath for 30 min, and reactions were stopped by addition of 3 ml of ice-cold PBS. After washing and resuspension in 100 μl of GHBSS, 100 μl of freshly isolated PMN (5×10^6 cells per ml) in GHBSS was added. In some experiments, PMN were preincubated for 20 min on ice with anti-FcγRI MAb 197 (IgG, 20 μg/ml), anti-FcγRII MAb IV.3 (IgG, 5 μg/ml) or F(ab′) fragments (5 μg/ml), MAb AT10 $F(ab')_2$ fragments (1 μg/ml), or anti-FcγRIII MAb 3G8 (IgG, 20 μg/ml) or $F(ab')_2$ fragments (5 μg/ml). Alternatively, PMN were preincubated with MAb B2.12 (IgM, 10 μg/ml), which interferes with CR3-C3bi binding (47), or $F(ab')_2$ fragments of anti-CD11b MAb MO1 (0.5 μg/ml) (46). Bacteria were then added, and the suspension was further incubated for 12 min at 37°C, after which the reaction was stopped by addition of 3 ml of ice-cold PBS. PMN were washed twice in PBS and resuspended in 400 μl of GHBSS–1% paraformaldehyde (pH 7.35). The fluorescence of extracellular bacteria was quenched prior to flow cytometry with trypan blue (37).

PMN were analyzed on a flow cytometer (FACStar Plus; Becton Dickinson), and in each sample, data from 10,000 cells were acquired. PMN were gated according to their characteristic light scatter pattern, and logarithmic FITC intensity was plotted versus relative cell number. Because the fluorescence intensity of a given cell was proportional to the number of ingested bacteria, the mean fluorescence of a given sample reflects the overall uptake of bacteria. Logarithmic FITC intensity values were transformed to linear units by using the phagocytic index (PI); $PI = 10(\frac{\text{mean FL1}}{64})$. The IgG2-stimulated phagocytic activity (stimulation index [SI]) was calculated by dividing the mean PI of IgG2-opsonized bacteria ($PI_{\gamma 2}$) by the mean PI of nonopsonized (PI_0) bacteria.

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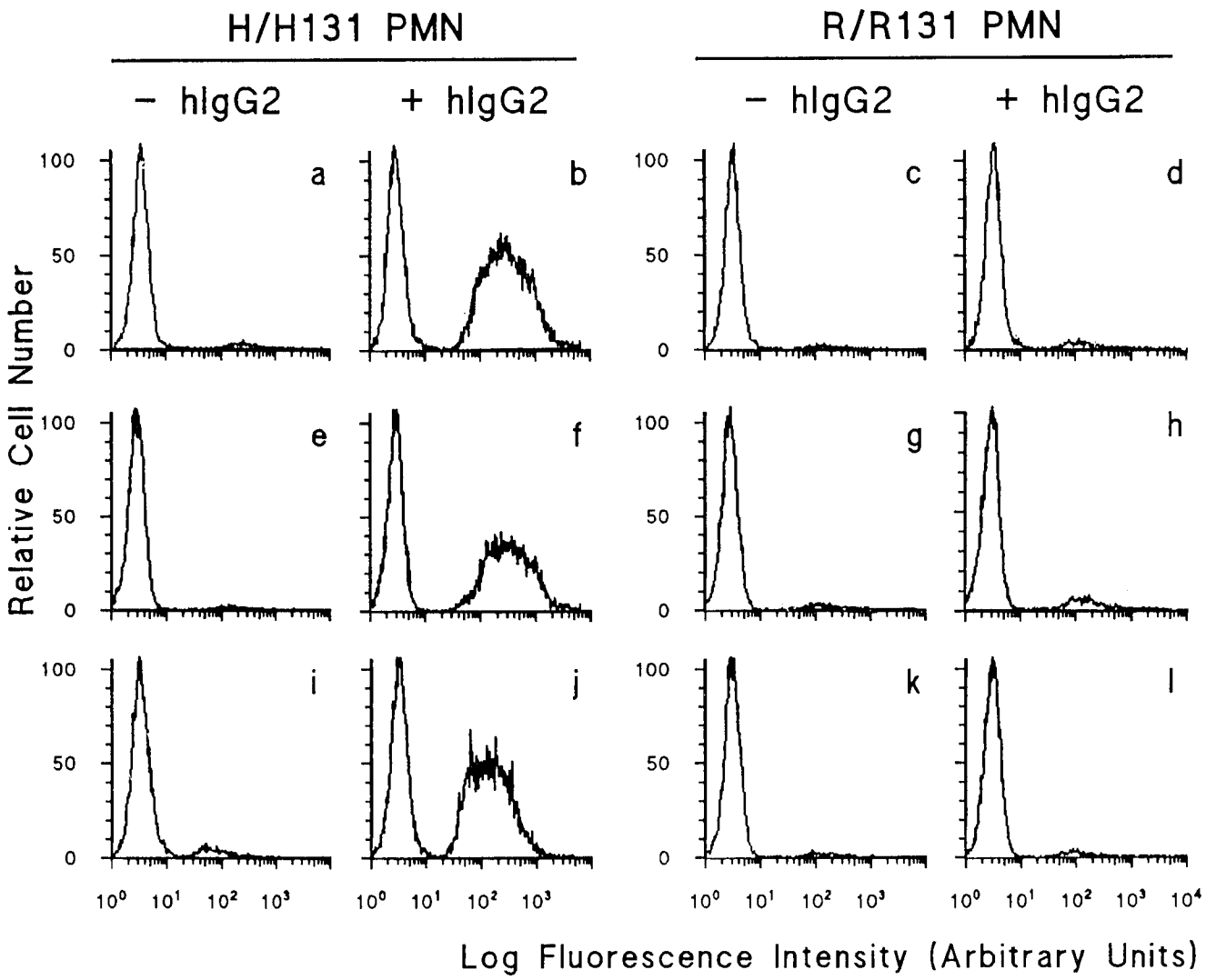

FIG. 2. Phagocytosis of unopsonized and IgG2-opsonized GBSIII by FcγRIIa-H/H131 and -R/R131 PMN. PMN from three H/H131 (a, b, e, f, i, and j) and R/R131 (c, d, g, h, k, and l) donors were incubated with GBSIII alone (a, e, i, c, g, and k) or with IgG2-opsonized GBSIII (b, f, j, d, h, and l) for 12 min at 37°C. No exogenous complement was added. Cells were washed, and the FITC fluorescence intensity of PMN was measured by flow cytometry. H/H131 and R/R131 PMN were assayed in parallel and were matched for NA1/NA2 FcγRIIIb allotypes. hIgG2, human IgG2.

The phagocytic capacities of PMN from homozygous FcγRIIa-R/R131 (n = 14), homozygous FcγRIIa-H/H131 (n = 14), and heterozygous FcγRIIa-H/R131 (n = 7) individuals were compared. All experiments were performed in duplicate and in a matched experimental design, which means that each FcγRIIa-H/H131 donor was studied in parallel with an FcγRIIa-R/R131 donor and an FcγRIIa-H/R131 donor and the donors were matched for their FcγRIIIb-NA1/NA2 phenotype. Results are expressed as means ± standard errors of the means.

Statistics. The significance of observed differences was determined by using a paired Student's t test.

RESULTS

Validation of the flow cytometric GBSIII phagocytosis assay. In order to study the effect of FcγR heterogeneity on phagocytosis of encapsulated bacteria, we have set up a model system consisting of GBSIII bacteria opsonized with affinity-purified IgG2 anti-capsular polysaccharide antibodies. PMN phagocytosis of live GBSIII bacteria that were opsonized with IgG2 antibodies and complement was significantly better ($P <$ 0.01) than phagocytosis of GBSIII opsonized with IgG2 only (Fig. 1). The increased phagocytosis, however, was largely due to an antibody-independent effect of complement. A similar phenomenon was observed with GBSIII bacteria that were killed by gamma irradiation; in this case, opsonization with complement alone was also sufficient to cause significant phagocytosis of GBSIII by PMN. Phagocytosis of Formalin-killed GBSIII could not be induced by opsonization with complement only. Moreover, complement did not significantly enhance phagocytosis of IgG2-opsonized, Formalin-killed GBSIII (Fig. 1).

Phagocytic capacities of PMN from FcγRIIa-H/H131, -H/R131, and -R/R131 individuals using IgG2-opsonized GBSIII. To examine the functional consequences of the FcγRIIa-H/R131 polymorphism, we compared the phagocytic capacities of PMN from homozygous H/H131, homozygous R/R131, and heterozygous donors. In a first series of experiments, the phagocytic capacities of homozygous H/H131 and R/R131 PMN were compared. Incubation of PMN with nonopsonized,

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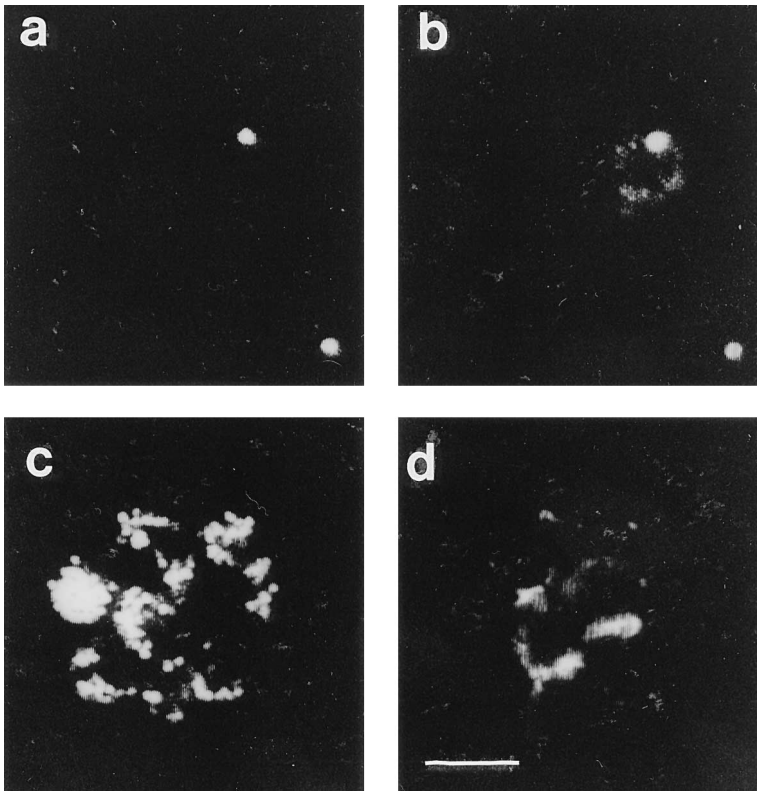

FIG. 3. Confocal laser fluorescence microscopy of GBSIII phagocytosis by H/H131 PMN and R/R131 PMN. R/R131 PMN (a and b) and H/H131 PMN (c and d) were incubated with IgG2-opsonized FITC-labeled GBSIII for 12 min at 37°C. Cells were then washed and stained for 30 min at 4°C with RITC-conjugated goat anti-human IgG. After being washed, cells were fixed in 1% paraformaldehyde and spun onto microscope slides. Slides were examined with a Bio-Rad System 1000 confocal laser fluorescence microscope. (a and c) FITC-fluorescent GBSIII; (b and d) RITC fluorescence of the anti-human IgG antiserum. Bar = 12.5 μm.

Formalin-killed GBSIII resulted in only limited phagocytosis, irrespective of the FcγIIa allotype (Fig. 2). When GBSIII opsonized with specific IgG2 were used, this situation changed dramatically: IgG2-opsonized GBSIII were readily phagocytosed by FcγRIIa-H/H131 PMN, but FcγRIIa-R/R131 PMN were hardly active in this respect (Fig. 2). No complement was added in this series of experiments.

Phagocytosis of GBSIII was also assessed microscopically (Fig. 3). To that end, H/H131 PMN and R/R131 PMN were incubated with IgG2-opsonized GBSIII as described above, washed, and incubated with RITC-labeled goat anti-human IgG antiserum. Cytospin preparations were analyzed by confocal laser microscopy. In preparations of R/R131 PMN, only limited numbers of GBSIII were found, which, because they displayed both FITC and RITC fluorescence, were cell associated but not ingested (Fig. 3a and b). H/H131 PMN, on the other hand, contained FITC-labeled GBSIII; the absence of RITC fluorescence indicated that the bacteria actually were phagocytosed (Fig. 3c and d).

The quantitative differences in phagocytosis of IgG2-opsonized GBSIII were determined by parallel analysis of FcγRIIa-H/H131 and FcγRIIa-R/R131 PMN that were matched for FcγRIIIb-NA1/NA2 alleles (Table 1). In addition, PMN from seven heterozygous H/R131 individuals were studied (Table 2). In all cases, R/R131 PMN showed a low or negative phagocytic capacity (PI_0 = 4.1 ± 0.2; $PI_{\gamma 2}$ = 6.3 ± 0.9; mean SI = 1.6 ± 0.3). Phagocytosis was highest in homozygous H/H131 PMN (PI_0 = 4.6 ± 0.4; $PI_{\gamma 2}$ = 48.0 ± 9.1; mean SI = 10.4 ± 2.0; P = 0.001), while heterozygous PMN displayed intermediate activity (PI_0 = 5.3 ± 0.6; $PI_{\gamma 2}$ = 30.0 ± 7.9; mean SI = 6.0 ± 1.9). These data therefore indicate that the FcγRIIa allotype quantitatively determines the phagocytosis of IgG2-opsonized GBSIII.

Role of complement. Phagocytosis of encapsulated bacteria has been previously observed to involve complement activation and complement receptor type 3 (CD11b/CD18). We analyzed phagocytosis of nonopsonized (PI_0) and IgG2-opsonized ($PI_{\gamma 2}$) Formalin-killed GBSIII with and without addition of exogenous complement. In the presence of complement, the difference in phagocytic capacities between FcγRIIa-H/H131 and -R/R131 PMN became less pronounced because of enhanced uptake of non-IgG2-opsonized GBSIII (Fig. 4): H/H131 PMN (n = 3) without complement, PI_0 = 3.4 ± 0.29, versus 8.4 ± 1.7 in the presence of complement; R/R131 PMN (n = 3) without complement, PI_0 = 3.0 ± 0.29, versus 7.7 ± 0.7 in the presence of complement. When IgG2-opsonized bacteria were used, no difference in uptake in either the presence or absence of complement was observed: H/H131 PMN, $PI_{\gamma 2}$ = 41.6 ± 7.1 without complement versus 42.3 ± 2.5 in the presence of complement; R/R131 PMN, $PI_{\gamma 2}$ = 7.8 ± 3.6 without complement versus 10.7 ± 2.6 with complement. The addition of complement thus increased the uptake of unopsonized GBSIII at least twofold. The phagocytosis of IgG2-opsonized GBSIII was also independent of complement when limiting amounts of IgG2 anti-GBSIII carbohydrate were used (data not shown). Phagocytosis in this model system with Formalin-killed GBSIII therefore proved to be primarily dependent on IgG2 anticapsular antibodies, which was further confirmed in blocking studies (see below, Fig. 5). Moreover, the difference in phagocytosis between FcγRIIa-H/H131 and -R/R131 PMN

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TABLE 1. Phagocytosis of IgG2-opsonized GBSIII by FcγRIIa-H/H131 and -R/R131 PMN[a]

Expt no.	PMN type		Mean ± SD		
	FcγRIIa	FcγRIIIb	PI_0	$PI_{\gamma 2}$	SI
1.1	H/H131	NA1/NA2	4.0	55.4	11.9
1.2	R/R131	NA1/NA1	2.4	3.7	1.5
2.1	H/H131	NA1/NA2	3.0 ± 0.1	36.5 ± 3.2	11.2 ± 1.2
2.2	R/R131	NA1/NA2	3.4 ± 0.0	4.9 ± 0.3	1.40 ± 0.1
2.3	H/H131	NA1/NA1	3.0 ± 0.0	32.9 ± 1.0	11.0 ± 0.3
2.4	R/R131	NA1/NA1	3.2 ± 0.1	14.9 ± 2.3	4.70 ± 0.9
3.1	H/H131	NA2/NA2	4.1 ± 0.0	47.0 ± 11.4	11.5 ± 2.8
3.2	R/R131	NA2/NA2	4.2 ± 0.3	4.20 ± 0.2	1.10 ± 0.1
3.3	H/H131	NA2/NA2	3.5 ± 0.1	13.4 ± 3.6	3.90 ± 1.1
3.4	R/R131	NA2/NA2	4.3 ± 0.1	4.90 ± 0.2	1.10 ± 0.1
3.5	H/H131	NA2/NA2	3.6 ± 0.1	12.3 ± 3.7	3.40 ± 1.1
3.6	R/R131	NA2/NA2	3.9 ± 0.2	5.30 ± 0.8	1.40 ± 0.3
3.7	H/H131	NA1/NA2	4.6 ± 0.0	30.7 ± 3.2	6.70 ± 0.7
3.8	R/R131	NA1/NA2	3.8 ± 0.1	4.10 ± 0.1	1.10 ± 0.0

[a] The results of phagocytosis of nonopsonized GBSIII (PI_0) and IgG2-opsonized GBSIII ($PI_{\gamma 2}$) are shown. The SI of opsonization with IgG2 anti-GBSIII capsular antibodies was calculated by dividing $PI_{\gamma 2}$ by PI_0. Each H/H131 donor was studied in comparison with an R/R131 donor, and H/H131 and R/R131 PMN were matched for FcγRIIIb-NA1/NA2 expression except in experiments 1.1 and 1.2. Experiments 1.1 and 1.2, 2.1 to 2.4, and 3.1 to 3.8 were performed on the same day.

remained significant in the presence of complement ($P < 0.01$).

Role of FcγRIIa in phagocytosis of IgG2-opsonized GBSIII by PMN. Previous studies pointed to a role for FcγRIIa in binding of complexed human IgG2. The role of FcγRIIa in phagocytosis of IgG2-opsonized GBSIII was next assessed by using blocking antireceptor MAb. H/H131 PMN from two individuals were assayed for uptake of IgG2-opsonized GBSIII in the presence of different blocking MAbs. In the presence of CD32-specific (anti-FcγRII) MAb IV.3 [IgG or purified F(ab′) fragments] or AT10 [$F(ab')_2$ fragments], a nearly complete inhibition of IgG2-mediated phagocytosis was observed (Fig. 5). Incubation with CD16 (anti-FcγRIII) MAb 3G8 [IgG or $F(ab')_2$ fragments] also led to diminished phagocytosis of IgG2-opsonized GBSIII. However, $F(ab')_2$ fragments of MAb 3G8 always showed a significantly lower inhibition of phagocytosis than did 3G8 IgG. CD64 (anti-FcγRI) MAb 197 did not bind to PMN (data not shown) and did not affect phagocytosis (Fig. 5). Control experiments using monocytes and equivalent amounts of MAb 197 showed that this treatment completely prevented binding of monomeric and complexed IgG to FcγRI (32).

To address the role of FcγRIIa in IgG2-mediated phagocytosis of GBSIII in more detail, we next studied PMN from two individuals who do not express FcγRIIIb because of absence of both FcγRIIIB alleles (22). Both $FcγRIIIb^{neg}$ PMN and control PMN matched for FcγRIIa phenotypes (H/R131) mediated phagocytosis of IgG2-opsonized GBSIII (Fig. 5). Incubation of the $FcγRIIIb^{neg}$ PMN with CD32 MAb IV.3 [IgG or F(ab′) fragments] abrogated IgG2-opsonized GBSIII phagocytosis. IgG or $F(ab')_2$ fragments of CD16 MAb 3G8, however, had no effect on these cells, in contrast to control PMN. These analyses supported a role for FcγRIIa in internalization of IgG2-opsonized GBSIII and furthermore pointed at the involvement of FcγRIIIb in this process. Little influence of anti-CR3 MAb B2.12 (IgM) and MAb MO1 $F(ab')_2$ fragments was observed (Fig. 5). Both MAbs were used at a concentration that prevents EA-C3bi rosetting completely (23).

DISCUSSION

Although the molecular structure and the genomic organization of FcγR genes have been resolved in considerable detail

TABLE 2. Phagocytosis of IgG2-opsonized GBSIII by FcγRIIa-H/H131, -H/R131, and -R/R131 PMN[a]

Expt no.	PMN type		PI_0	$PI_{\gamma 2}$	SI
	FcγRIIa	FcγRIIIb			
1.1	H/H131	NA1/NA1	7.6	72.2	9.5
1.2	H/R131	NA2/NA2	4.7	15.5	3.3
1.3	R/R131	NA1/NA1	4.7	5.3	1.1
2.1	H/H131	NA2/NA2	4.5	28.8	6.4
2.2	H/R131	NA2/NA2	4.6	14.3	3.1
2.3	R/R131	NA2/NA2	5.0	5.7	1.1
3.1	H/H131	NA1/NA2	4.3	140	132.6
3.2	H/R131	NA1/NA2	4.1	63.2	15.4
3.3	R/R131	NA1/NA2	4.5	9.2	2.0
4.1	H/H131	NA1/NA2	4.8	38.2	8.0
4.2	H/R131	NA1/NA2	5.4	22.1	4.1
4.3	R/R131	NA1/NA2	3.8	4.7	1.2
5.1	H/H131	NA1/NA1	5.0	39.5	7.9
5.2	H/R131	NA1/NA1	4.8	21.9	4.6
5.3	R/R131	NA1/NA1	4.4	4.4	1.0
6.1	H/H131	NA1/NA2	7.1	77.6	10.9
6.2	H/R131	NA1/NA2	7.9	42.8	5.4
6.3	R/R131	NA1/NA2	6.1	10.5	1.7

[a] The results of phagocytosis of unopsonized GBSIII (PI_0) and IgG2-opsonized GBSIII ($PI_{\gamma 2}$) are shown. The SI of opsonization with IgG2 anti-GBSIII capsular antibodies was calculated by dividing $PI_{\gamma 2}$ by PI_0. Each H/H131 donor was studied in comparison with an H/R131 and an R/R131 donor, and each set of donors was matched for FcγRIIIb-NA1/NA2 expression except in experiments 1.1 to 1.3. Experiments 1.1 to 2.3, 3.1 to 3.3, 4.1 to 5.3, and 6.1 to 6.3 were performed on the same day.

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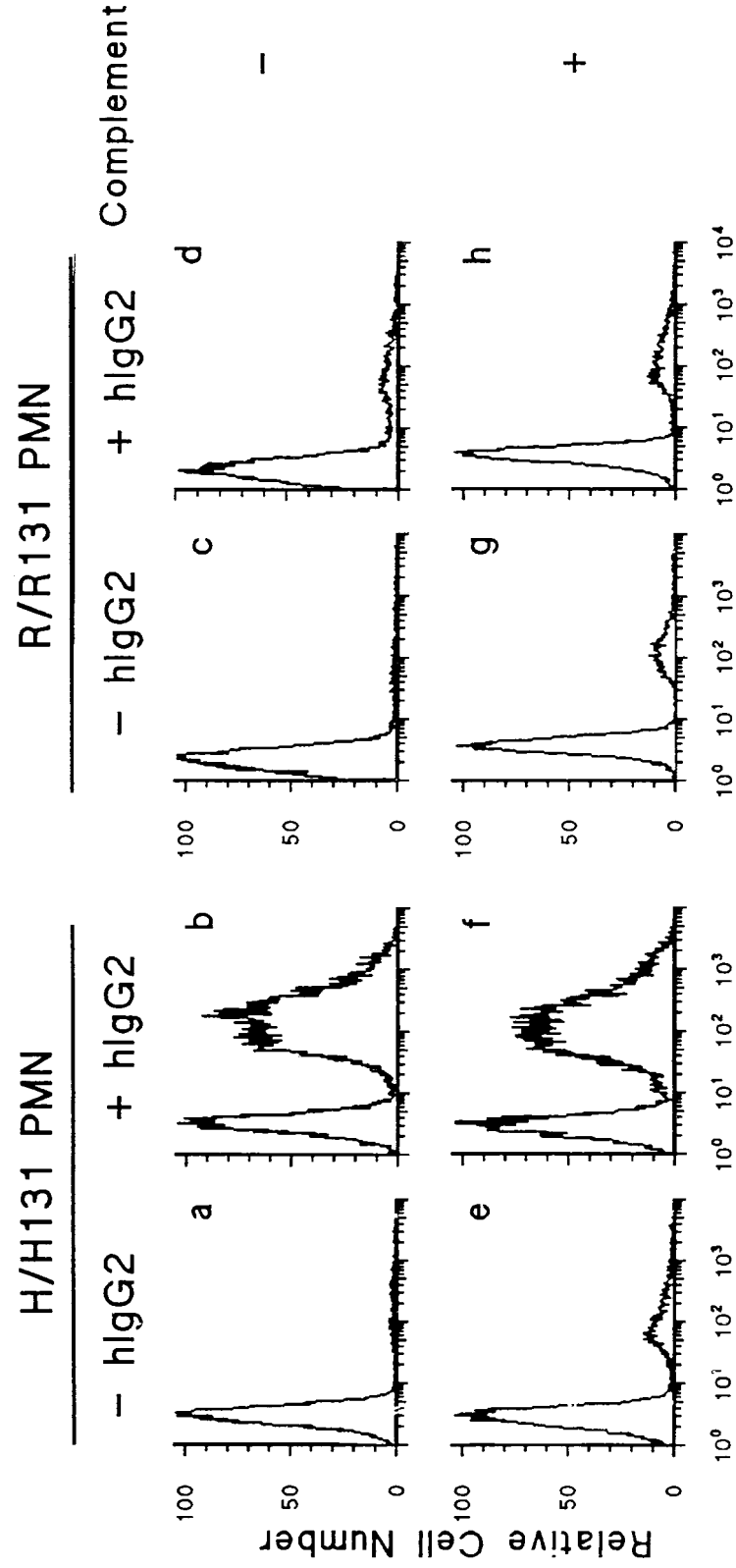

FIG. 4. Effect of exogenous complement on phagocytosis of GBSIII and IgG2-opsonized GBSIII by FcγRIIa-H/H131 and -R/R131 PMN. PMN from two homozygous H/H131 and R/R131 donors were incubated with GBSIII alone (e and g), GBSIII with exogenous complement (a and c), or IgG2-opsonized GBSIII in medium alone (f and h) or with exogenous complement (b and d). Uptake of bacteria was quantitated by flow cytometry. The results for H/H131 (a, b, e, and f) and R/R131 (c, d, g, and h) PMN, assayed simultaneously, are shown.

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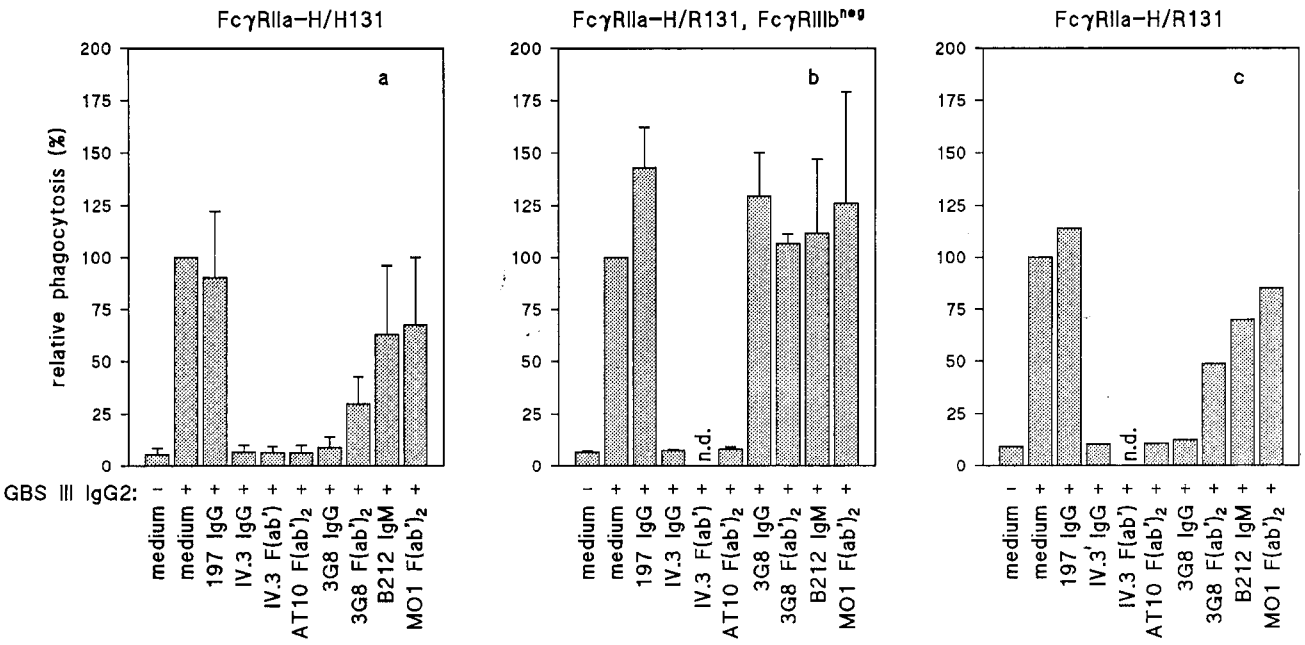

FIG. 5. Blocking of IgG2-opsonized GBSIII phagocytosis by MAbs. PMN were incubated with GBSIII in medium alone (control), with IgG2-opsonized GBSIII, or with IgG2-opsonized GBSIII in the presence of excess amounts of one of the following MAbs: CD64 MAb 197, CD32 MAb IV.3 [IgG and F(ab′)], MAb AT10 $F(ab')_2$, CD16 MAb 3G8 [IgG and $F(ab')_2$ fragments], anti-CR3 MAb B212 (IgM), or MAb MO1 [$F(ab')_2$ fragments]. Note that no exogenous complement was added. The phagocytosis of IgG2-opsonized GBSIII in medium alone was set at 100% for comparison. (a) Results for PMN from two homozygous FcγRIIa-H/H131 donors, assayed on the same day (PI_0 = 3.6; $PI_{\gamma 2}$ = 99.8; SI = 25.8). FcγRIIIb-deficient PMN (n = 2, panel b) and control PMN (FcγRIIa-H/R131; n = 1, panel c) were assayed in parallel. (FcγRIIIb^{neg} donors, PI_0 = 5.5, $PI_{\gamma 2}$ = 82.3, SI = 12.9; control PMN, PI_0 = 3.4, $PI_{\gamma 2}$ = 36.6, SI = 10.)

(48), many questions about the functioning of FcγR in vivo remain unanswered. A first observation that the FcγRIIa-H/R131 polymorphism may have in vivo relevance was the finding that kidney allograft recipients treated with mouse IgG1 anti-CD8 MAb showed differences in clearance of T cells between FcγRIIa-H/H131 and -R/R131 individuals (51). Recent studies revealed that the FcγRIIa polymorphism in humans also results in differences in binding of IgG2 immune complexes (32, 50) and different phagocytic capacities of IgG2-sensitized erythrocytes by PMN and monocytes (39). Bredius et al. showed that phagocytosis of *Haemophilus influenzae* type b and *Staphylococcus aureus* Wood 46 opsonized by natural antibodies that are present in purified IgG2 is higher in FcγRIIa-H/H131 PMN than in FcγRIIa-R/R131 PMN (4). The role of heterozygous FcγRIIa-H/R131 phagocytic cells in IgG2-mediated phagocytosis has not been addressed yet.

We studied IgG2-mediated phagocytosis by FcγRIIa-H/H131, -H/R131, and -R/R131 PMN in a model system using an encapsulated strain of GBSIII (M732). The Formalin-killed bacteria were opsonized with purified IgG2 anti-GBSIII capsular antibodies. In every experiment, homozygous H/H131 PMN exhibited higher phagocytic capacities for IgG2-opsonized GBSIII than R/R131 PMN (P = 0.001). Phagocytosis by heterozygous H/R131 PMN was intermediate between those by homozygous H/H131 and R/R131 PMN.

In our model system, the IgG2-mediated phagocytosis of Formalin-killed GBSIII was independent of complement, and antibodies to CR3 did not interfere with phagocytosis. Previously described GBSIII phagocytosis assays have found complement dependence (9, 29). Unlike our method, in all these assays, the ingestion and killing of live GBS were measured. When we used live GBSIII, ingestion of both unopsonized and IgG2-opsonized GBSIII was dependent on the addition of complement. At this moment, it is not clear why phagocytosis of Formalin-killed GBSIII is complement independent. Surface molecules will undoubtedly be rendered biologically inactive by formaldehyde treatment. However, with respect to the in vivo relevance of the FcγRIIa polymorphism in defense against encapsulated bacteria, it is important to note that when employing live GBSIII opsonized in the presence of complement, the difference in phagocytic capacity between H/H131 PMN (n = 11, PI_0 = 7.0 ± 0.5, $PI_{\gamma 2}$ = 21.0 ± 2.5, SI = 2.9 ± 0.24) and R/R131 PMN (n = 11, PI_0 = 6.3 ± 0.5, $PI_{\gamma 2}$ = 12.3 ± 1.4, SI = 1.9 ± 0.15) decreased but remained significant (P < 0.01).

The involvement of FcγRIIa in uptake of IgG2-opsonized GBSIII was supported by the finding that CD32 MAb IV.3 [IgG or F(ab′)] and MAb AT10 [$F(ab')_2$ fragments] block phagocytosis. Furthermore, the phagocytic capacity of PMN from two FcγRIIIb^{neg} donors was similar to that of control PMN and was fully inhibitable by the two CD32 MAbs. This confirmed that FcγRIIa is critical for phagocytosis of IgG2-opsonized GBSIII and is consistent with the observations of others (29, 39). An involvement of the neutrophil-specific FcγRIIIb was indicated by the reduced phagocytosis observed in the presence of CD16 MAb 3G8 [IgG and $F(ab')_2$ fragments]. This fits into the concept that the role of FcγRIII may be the attachment of IgG-coated particles to the PMN surface, while FcγRIIa mediates ingestion and triggers the respiratory burst (2). Furthermore, FcγRIIa has been found to affect triggering induced by FcγRIIIb, as observed in immune complex-induced actin assembly (38) and calcium flux (28). The latter study described inhibition of an aggregated IgG-induced calcium mobilization response by CD32 MAb KuFc79, postulating a role for FcγRIIa in cross-regulation between PMN FcγRIIa and FcγRIIIb signalling in PMN (28). However, the involvement observed for FcγRIIIb in phagocytosis of IgG2-opsonized GBSIII may also be attributable to a carbohydrate-

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lectin-like interaction between GBSIII and FcγRIIIb, such as is found for *Escherichia coli* (41). Alternatively, the residual amount of IgG1 in the antibody preparation may have accounted for this observation, but this seems unlikely, as 100% inhibition is seen with MAbs that bind only FcγRII (Fig. 5).

The FcγRIIIb-NA1/NA2 structural polymorphism has previously been shown to affect phagocytic capacities of PMN. FcγRIIIb-NA2/NA2 PMN exhibited a lower phagocytic capacity for human Ig-sensitized erythrocytes than NA1/NA1 PMN (39, 40). In addition, the functional H/R131 FcγRIIa polymorphism was found to be independent of NA1/NA2 FcγRIIIb expression (39). So far, no genetic linkage between the H/R131 FcγRIIa allotypes and NA1/NA2 FcγRIIIb alleles in different ethnic populations has been reported. In our study, PMN were matched for NA1/NA2 FcγRIIIb phenotypes to exclude any (uncontrolled) influence of the FcγRIIIb on FcγRIIa-mediated phagocytosis.

IgG2 is the main Ig isotype induced in response to streptococcal and pneumococcal bacterial capsular polysaccharide antigens in adults, while in children, IgG1 antibodies may be the predominant subclass (11, 35). The restriction to the IgG2 subclass of anticarbohydrate antibodies in adults does not seem to result from a simple downstream H-chain gene switching by a single B cell but rather from recruitment of new B-cell clones using different V regions and precommitted to a particular IgG subclass (7, 43–45). IgG1 and IgG2 subclass anticarbohydrate antibodies may have different affinities or avidities for polysaccharide antigens (33), whereas IgG2 may be superior to IgG1 in the case of polysaccharide antigens (33). Affinity and avidity rather than concentration seem to correlate with the protective capacities of IgG antibodies (19). Importantly, at high antigen concentrations, IgG2 antibodies seem to be more efficient than other IgG subclasses in complement-mediated cytolysis (27) and activation of the alternative complement route (26). Polysaccharide antigens typically are present in a repetitive form and usually in high concentrations on the surface of encapsulated bacteria. Furthermore, the opsonophagocytic capacity for GBSIII resides in the IgG2 rather than the IgG1 subclass (15). Interestingly, Lortan et al. found that both IgG1 and IgG2 antibodies were correlated with in vitro phagocytosis of serotype 14 *Streptococcus pneumoniae* by neutrophils in the presence of complement. However, after heat inactivation, the opsonic activity of the sera correlated only with specific IgG2 antibody (25). This indicates that IgG2-specific antibody is able to function by complement-independent interaction with FcγR on neutrophils (25). Of note is the fact that in older studies comparing opsonic activities of IgG1 and IgG2 anticapsular antibodies, the allotype of FcγRIIa was not taken into account, which is crucial in any conclusion (1). Unfortunately, we were not able to address the opsonophagocytic capacities of IgG1 versus IgG2 anti-GBSIII antibodies in our model system, because the antibodies from all 15 individuals with anti-GBSIII antibodies were primarily IgG2, with only minimal amounts of the IgG1 subclass. Clinical data, however, support the concept of the superiority of IgG2 antipolysaccharide antibodies over IgG1 antibodies. For example, otitis-prone children seem to have normal IgG1 but low IgG2 antibody levels for certain pneumococcal serotypes (14).

Because of the difference in phagocytosis of IgG2-opsonized encapsulated bacteria between homozygous FcγRIIa-H/H131 and -R/R131 PMN, we hypothesize that the FcγRIIa allotype on phagocytic cells may influence the susceptibility to infections with encapsulated bacteria. In support of this hypothesis is the recent observation by Fijen et al. (12). These authors analyzed 15 individuals with a complement component deficiency of the terminal pathway and observed that individuals with combined FcγRIIa-R/R131 and FcγRIIIb-NA2/NA2 allotypes experienced significantly more episodes of meningococcal disease. We recently evaluated 48 children with frequent bacterial infections of the respiratory tract and normal serum IgG2 levels. We found that in this group of patients, the frequency of individuals with the FcγRIIa-H/H131 phenotype was significantly lower than in a large healthy Caucasian control population. Both heterozygous H/R131 and homozygous R/R131 frequencies in the patient population were found to be increased (42).

In conclusion, this study supports the idea that the H/R131 FcγRIIa polymorphism has functional implications for IgG2-mediated phagocytosis of encapsulated bacteria. Moreover, the FcγRIIa polymorphism may have functional implications for susceptibility to diseases caused by encapsulated bacteria.

ACKNOWLEDGMENTS

We thank Marion Kleijer and Nomdo Westerdaal for excellent technical support. We also thank Dik van Wiegen and Roel Broekhuizen (Department of Pathology, Academic Hospital, Utrecht, The Netherlands) for assistance in confocal laser fluorescence microscopy.

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