Neutrophil Antigens (NAs) are involved in several clinical conditions, such as blood transfusion reactions and immune-mediated neutropenia. The NA system has been extensively investigated and is located on IgG Fc receptor type IIIb (FcγRIIIB; CD16). NA1- and NA2-FcγRIIIB differ by four amino acids in the membrane-distal Ig-like domain. These differences lead to distinct glycosylation patterns of NA1- and NA2-FcγRIIIB. Although the amino acid differences are not located in the membrane-proximal, ligand-binding domain of the receptor, the FcγRIIIB gene encoding NA2-FcγRIIIB and, to a lesser extent, NA2-FcγRIIIB can be detected in a large proportion of patients suffering from neonatal immune-mediated neutropenia.

Recently, Bux et al described four cases of alloimmune neutropenia in which alloantibodies recognizing a thus far unknown antigen on FcyRIIB were identified. The newly identified alloantigen was termed SH and has a gene frequency of 4% in the German population. Nucleotide sequence analysis showed that the SH-FcγRIIIB gene differed from NA2-FcγRIIIB by a single base pair (266C→A), encoding an Ala→Asp substitution at amino acid position 60. The authors concluded that SH-FcγRIIIB is the product of an NA2-FcγRIIIB polymorphism.

In this report, we analyzed individuals whose neutrophils were phenotyped as NA(1+,2+)SH(+). Genomic analysis showed that in these individuals, an FcγRIIIB gene encoding SH-FcγRIIIB exists alongside an NA1- and an NA2-FcγRIIIB gene. All three FcγRIIIB genes were transcribed into mRNA. Furthermore, in all three donors we observed a clear gene-dosage effect on the level of expression of neutrophil FcγRIIIB.

Materials and Methods

Antibodies and antisera. Anti-pan-FcγRII (CD16) monoclonal antibodies (MoAbs) used were CLBFcRgran1 (mIgG2a), 3G8 (mIgG1), BW209/2 (mIgG2a), and MEM154 (mIgG1). CLBgran11 (mIgG2a) and MG38 (mIgG1) recognize NA1-FcγRIIIB, whereas GRM1 (mIgG2a) recognizes NA2-FcγRIIIB and FcγRIIIA. PEN1 (mIgG2a) reacts with a small oligosaccharide moiety of NA2-FcγRIIIB and with FcγRIIIA. BW209/2 was a generous gift from Dr Kurrle (Behring Werke, Marburg, Germany) and GRM1 was provided by Dr Garrido (Hospital des Nieves, Granada, Spain). CLBFcRgran1, CLBgran11, and irrelevant control MoAbs were from our own institute. The other CD16 MoAbs were obtained via the Fifth International Workshop on Leukocyte Antigens (Boston, MA; November 1993). A human antiserum recognizing SH-FcγRIIIB was obtained via the Second International Granulocyte Serology Workshop (Helsinki, Finland; May 1996). Sera from healthy AB-positive individuals were used as controls. Fluorescein isothiocyanate (FITC)-labeled goat-antimouse–Ig and FITC–goat-anti-human–Ig from our institute were used to detect MoAb and human antibody binding, respectively.

Isolation of cells. EDTA-anticoagulated blood was centrifuged over a Ficoll-Hypaque gradient with a specific gravity of 1.076 g/mL (Pharmacia Fine Chemicals, Uppsala, Sweden). Mononuclear cells were harvested from the interphase for DNA isolation and the pellet was treated with ice-cold NH4Cl solution (155 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA, pH 7.4) to lyse erythrocytes. The remaining cells were more than 95% neutrophils.

Flow cytometry. Neutrophils were tested for reactivity with a panel of CD16 MoAbs and with the SH antiserum by indirect immunofluorescence. Briefly, neutrophils were fixed with 1% paraformaldehyde in 0.1% sodium azide and permeabilized with ice-cold NH4Cl solution (155 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA, pH 7.4) to lyse erythrocytes. The remaining cells were more than 95% neutrophils.
30 minutes at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (w/v) vol the cells were stained with FITC-labeled F(ab')2 fragments of goat-antimouse-Ig or FITC–goat-antimouse-Ig. Binding of the conjugate was assessed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Soluble FcγRIII enzyme-linked immunosorbot assay (ELISA). The plasma level of sFcγRIII was measured by a sandwich ELISA essentially as previously described. Briefly, ELISA plates were incubated with CD16 MoAb CLBFcRgran1 and blocked with PBS containing 2% (v/v) milk. The plates were incubated with plasma that was diluted in High Performance ELISA (HPE) buffer (CLB, Amsterdam, The Netherlands). Subsequently, a biotinylated polyclonal rabbit-antihuman–FcγRIII antibody diluted in HPE buffer was added. After incubation with horseradish peroxidase-labeled streptavidin, a substrate was added to measure the amount of bound antibody. Plasma from 90 healthy individuals was pooled and used to construct a calibration curve. The level of sFcγRIII in this pool was set at 100 arbitrary units (AU).

FcγRIIB-NA(1,2) genotyping assays. Genotyping for the FcγRIIb-NA(1,2) polymorphism was performed as described before. In brief, two sets of primers specifically annealing to either an NA1-FcγRIIB or an NA2-FcγRIIB fragment were used. NA1-FcγRIIB- and NA2-FcγRIIB-specific fragments were separately amplified from gDNA in a Perkin Elmer Cetus Cyclor (Norwalk, CT) in a total volume of 50 µL. Amplification of a fragment of the p22-phox (CYBA) gene served as an internal control in each polymerase chain reaction (PCR). The nucleotide sequence of FcγRIIB-NA2-specific fragments was determined by cycle sequencing of purified PCR products with 32P-labeled terminators with the Thermo Sequenase kit, according to the manufacturer’s instructions (Amersham Life Sciences, Cleveland, OH).

Southern blot-based restriction fragment length polymorphism (RFLP) assay. Southern blot-based restriction fragment analysis was performed as previously described. Briefly, 10 µg of genomic DNA was digested overnight with BamHI and EcoR1 (Promega, Madison, WI). After gel electrophoresis and transfer to nylon sheets, the blot was hybridized with 32P-labeled pGP5, a probe that contains the entire coding region of NA1-FcγRIIB (Dr. G. Peltz, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). This probe hybridizes to both isoforms of FcγRIIB, as well as to FcγRIIA. The double digestion resulted in an FcγRIIB- and an FcγRIIA-specific fragment of 2.0 and 5.5 kb, respectively. The labeling intensities of the two fragments were measured and compared by phospho-imaging (Fuji, London, UK). NA(1,2) genotyped donors and known hemizygous FcγRIIB gene-deficient donors served as controls.

Sequence analysis of FcγRIIB-encoding cDNA. Messenger RNA was isolated from purified neutrophils with a CsCl gradient and was reverse transcribed into cDNA. The entire coding region of FcγRIIB was amplified by PCR, after which the products were cloned into a pGEM-T vector according to the manufacturer’s instructions (Promega). After transformation of Escherichia coli, inserts were amplified by PCR and sequenced with 32P-end-labeled primers (Amersham) with the BRL cycle sequencing kit (BRL, Gaithersburg, MD).

RESULTS

Genomic analysis. In a population of 55 healthy laboratory workers, we identified three healthy donors whose neutrophils reacted positively with the SH antiserum giving a phenotype frequency of approximately 5%, in concordance with previous findings. Neutrophils from all three donors were phenotyped as NA1(1,2)+SH(+) and NA2-FcγRIIB insert, 21 carried the NA2-FcγRIIB insert, and 3 carried the SH-FcγRIIB insert.

Neutrophil FcγRIIB expression. We determined the reactivity of NA1(1,2)+SH(+) neutrophils with a panel of CD16

fragment of the NA2-FcγRIIB gene from gDNA by an allele-specific primer-annealing (ASPA) PCR. As shown in Fig 2A to C, direct sequencing of these products showed that the individuals were heterozygous at nucleotide position 266. At this position, a C as well as an A were detected, suggesting that two different fragments were amplified with the NA2-FcγRIIB–specific primer set. Sequence analysis of NA2-FcγRIIB-specific fragments amplified from genomic DNA from two NA(1,2)+SH(+) controls showed a single band at position 266 (Fig 2D to E).

Additionally, NA2-FcγRIIB–specific PCR products were digested with Smal, which recognizes a 5'-GATGC-3' sequence present only in the SH-FcγRIIB-derived fragment. Figure 3 shows the results of this digestion. Lane 1 shows Smal treatment of a fragment amplified from a plasmid containing SH-FcγRIIB-encoding cDNA, resulting in a single band of 137 bp, confirming that complete digestion is obtained by Smal analysis. Lanes 2 to 4 contain Smal-treated DNA fragments from NA1(1,2)+SH(+) controls. Only the undigested fragment of 168 bp is present. In lanes 5 to 7 DNA fragments from three NA(1,2)+SH(+) individuals are shown. Digested (137 bp) and undigested (168 bp) fragments are visible, indicating the presence of an SH-FcγRIIB and an NA2-FcγRIIB sequence.

To confirm the hypothesis that more than two FcγRIIB genes are present in the genome of NA(1,2)+SH(+) individuals, a Southern blot-based RFLP assay was performed. The number of FcγRIIB genes was determined by comparing the labeling intensities of an FcγRIIB-specific and an FcγRIIA-specific fragment. Genomic DNA from two of the three individuals was available for testing. Table 1 shows the quantitative results of the Southern blot, obtained with a phospho-imager. For these two NA(1,2)+SH(+) individuals, the ratio between the FcγRIIB- and FcγRIIA-specific band is 1.16 and 1.14, respectively. This is approximately three times higher than the ratio obtained for three individuals with only one FcγRIIB gene (0.37 ± 0.17), and 1.5-fold the ratio found for six NA(1,2)+SH(+) controls (0.69 ± 0.12).

cDNA analysis. Messenger RNA was isolated from purified neutrophils from one NA1(1,2)+SH(+) individual and reversely transcribed into cDNA to investigate whether all three FcγRIIB genes were transcribed. The entire coding region of FcγRIIB was amplified by PCR and cloned into E coli. Figure 4, panel 1, shows part of the sequence of an NA1 transcript, whereas the nucleotide sequence shown in panel 2 is derived from an NA2 transcript. The NA1-NA2 difference at nucleotide position 227 is depicted (A→G), and all other described nucleotide differences were normally present (not shown). The sequence shown in panel 3 only differed from a normal NA2 sequence at nucleotide position 266 (C→A). This substitution predicts the Ala→Asp substitution in SH-FcγRIIB, described by Bux et al. Sequence analysis of the complete coding region showed no other nucleotide substitutions. In a total of 30 sequenced clones with an FcγRIIB-encoding insert from this NA(1,2)+SH(+) individual, 6 were found to carry the NA1-FcγRIIB insert, 21 carried the NA2-FcγRIIB insert, and 3 carried the SH-FcγRIIB insert.
MoAbs in comparison with neutrophils from donors who were NA(1+,2+)SH(−) in three separate experiments (Table 2). Neutrophils from SH(−) and either homozygous NA1- or NA2-positive individuals were simultaneously analyzed in two of these experiments. Compared with neutrophils from NA(1+,2+)SH(−) individuals, SH(+) neutrophils showed a higher reactivity with anti–pan-FcγRIII MoAbs CLBFcRgran1, 3G8, MEM154, and BW209/2 (170%, 119%, 140%, and 163%, respectively). The NA(1+,2+)SH(−) neutrophils reacted approximately twice as strong with the NA2-specific MoAb GRM1 as did control neutrophils (Table 2). However, the reactivity of the NA(1+,2+)SH(+) neutrophils was comparable to that of NA(1−,2+) genotyped donors, who carried two NA2 genes, as determined by Southern blot analysis,13 with mean fluorescent intensities (MFIs) of 1,480 and 1,585 ± 568, respectively. However, reactivity of the NA2-specific MoAb PEN1 was about sixfold higher for NA(1+,2+)SH(+) neutrophils compared with NA(1+,2+)SH(−) neutrophils and threefold higher compared with NA(1−,2+)SH(−) neutrophils. Reactivity with NA1-specific MoAbs CLBFcRgran11 and MG38 was similar between SH(−) and SH(+) NA(1+,2+) neutrophils, suggesting.

**Fig 1.** Reactivity of neutrophils from an NA(1+,2+)SH(+) donor and an NA(1+,2+)SH(−) control with human anti–NA1- and anti–NA2-FcγRIIIb antisera and the SH antiserum. AB serum was used as control.

**Fig 2.** Nucleotide sequence (GATC) of FcγRIIB-NA2-specific genomic DNA fragments. (A to C) Fragments from three NA(1+,2+)SH(+) individuals. Two bands (A and C) are visible at nucleotide position 266, indicating the presence of a normal (266C) as well as a mutated (266A) FcγRIIB-NA2 gene. (D and E) Sequences of NA(1+,2+)SH(−) controls.
an equal membrane expression of NA1-FcγRIIib (Table 2). Figure 5 shows the results of a representative experiment.

Because the level of soluble (s)FcγRII in plasma correlates with the number of FcγRIIIB genes, we determined the amount of sFcγRII in plasma from the three SH(+) individuals. In concordance with the high membrane expression levels, we found plasma sFcγRII levels of 197, 279, and 137 AU (mean, 204 ± 71 AU). However, this value was not significantly different from the mean sFcγRII level in 24 NA(1+) donors phenotyped as NA(1+)SH(+). 10 Moreover, three NA(1+,2+)SH(+) donors were reanalyzed and were found to carry three FcγRIIIB genes as well (J. Bux, personal communication). Only confirmation by PCR and/or Southern blotting can settle the question as to whether any SH positivity without gene duplication exists. However, with these methods the possibility of one FcγRIIIB gene-deficient chromosome and two FcγRIIIB genes on the other one cannot be ruled out. 14 If we assume that the SH-FcγRIIIB gene is only present in association with a NA2-FcγRIIIB–specific PCR. Digestion of these NA2-FcγRIIIB–specific fragments with SfaNI confirmed the presence of two NA2 genes. Furthermore, a Southern blot-based RFLP assay showed that three FcγRIIIB genes are present in the genome of these NA(1+,2+)SH(+) individuals. All three FcγRIIIB genes were transcribed, because three distinct transcripts encoding either NA1-, NA2-, or SH-FcγRIIib were isolated from neutrophil mRNA. These data indicate that these three donors each carry three FcγRIIIB genes, namely, NA1-FcγRIIIB, NA2-FcγRIIIB, and SH-FcγRIIIB. Six of 14 SH(+) individuals described by Bux et al were phenotyped as NA(1+,2+), and the remaining eight were NA(1+,2+). 10 Moreover, three NA(1+,2+)SH(+) donors were reanalyzed and were found to carry three FcγRIIIB genes as well (J. Bux, personal communication). Only confirmation by PCR and/or Southern blotting can settle the question as to whether any SH positivity without gene duplication exists. However, with these methods the possibility of one FcγRIIIB gene-deficient chromosome and two FcγRIIIB genes on the other one cannot be ruled out. 14 If we assume that the SH-FcγRIIIB gene is only present in association with a second FcγRIIIB gene on the same chromosome, then the presence of NA(1+,2+)SH(+) phenotyped donors in the study of Bux et al might indicate that NA2-FcγRIIIB is located on the same chromosome as SH-FcγRIIIB. It is conceivable that

<table>
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<th>Table 1. Quantitative Results of Southern Blot-Based RFLP Assay as Measured by Phospho-Imaging</th>
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<tbody>
<tr>
<td><strong>No.</strong></td>
</tr>
<tr>
<td>One FcγRIIIB gene</td>
</tr>
<tr>
<td>Two FcγRIIIB genes</td>
</tr>
<tr>
<td>Donor 1†</td>
</tr>
<tr>
<td>Donor 2</td>
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*Mean value ± standard deviation.
†Neutrophils of donor 1 and donor 2 were phenotyped as NA(1+,2+)SH(+).
before or after the SH mutation occurred, an unequal crossing-over event between two chromosomes carrying FcγRIIIB genes has led to the supposed NA2-SH allele. The counterpart of this unequal crossing-over is an FcγRIIIB gene deletion, which has been described. The genotype frequencies of SH- and FcγRIIIB gene deletion, being 4% and 3% to 9%, respectively, are not contradicting this theory. Theoretically, SH(−) donors with three FcγRIIIB genes (gene duplication without mutation) should exist. Southern blot analysis of gDNA from a large group of donors could settle this question.

Neutrophils from healthy individuals carry 100,000 to 300,000 copies of FcγRIIIb per cell. Previously, we described that the amount of FcγRIIIb on the neutrophil membrane correlates with the number of FcγRIIIB genes. Individuals who are hemizygous FcγRIIIB-gene–deficient have approximately half the neutrophil FcγRIIIb expression and half the plasma soluble

### Table 2. Reactivity of SH-Positive and SH-Negative Neutrophils With a Panel of CD16 MoAbs.

<table>
<thead>
<tr>
<th>MoAb</th>
<th>NA1NA1 (n = 2)</th>
<th>NA1NA2 (n = 3)</th>
<th>NA2NA2 (n = 2)</th>
<th>Relative to NA1+2+SH−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>15 ± 5</td>
<td>12</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>Anti-pan FcγRIIIb</td>
<td>1,764</td>
<td>1,970 ± 609</td>
<td>2,409</td>
<td>3,345 ± 324</td>
</tr>
<tr>
<td>CLBFcRgran1</td>
<td>1,427</td>
<td>1,309 ± 820</td>
<td>1,426</td>
<td>1,555 ± 535</td>
</tr>
<tr>
<td>3G8</td>
<td>767</td>
<td>989 ± 298</td>
<td>956</td>
<td>1,611 ± 563</td>
</tr>
<tr>
<td>BW209/2</td>
<td>1,075</td>
<td>1,117 ± 568</td>
<td>1,198</td>
<td>1,636 ± 445</td>
</tr>
<tr>
<td>MEM154</td>
<td>415</td>
<td>311 ± 130</td>
<td>16</td>
<td>248 ± 95</td>
</tr>
<tr>
<td>Anti-NA1 FcγRIIIb</td>
<td>1,934</td>
<td>1,141 ± 317</td>
<td>17</td>
<td>994 ± 149</td>
</tr>
<tr>
<td>MG38</td>
<td>30</td>
<td>914 ± 439</td>
<td>1,480</td>
<td>1,585 ± 568</td>
</tr>
<tr>
<td>CLBgran11</td>
<td>24</td>
<td>149 ± 153</td>
<td>270</td>
<td>834 ± 153</td>
</tr>
</tbody>
</table>

Results of three separate experiments with neutrophils of different donors are shown.

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Fig 5. Staining pattern of NA(1-2+)SH(−) (dashed lines) and NA(1-2+)SH(+) neutrophils (continuous lines) with a panel of CD16 MoAbs. One representative experiment of three is shown. (A and B) The reactivity of SH(+) neutrophils with anti-pan-FcγRIIIb MoAbs (CLBFcRgran1 and 3G8) was higher than that of SH(−) neutrophils. (C and D) The reactivity of both types of neutrophils with NA1-FcγRIIIB–specific MoAbs CLBFcRgran11 and MG38 was comparable. (E) Reactivity of NA2-FcγRIIIB–specific MoAb GRM1 was approximately twice as high with SH(+) neutrophils compared with SH(−) neutrophils. (F) NA2-FcγRIIIB–specific MoAb PEN1 showed a reactivity with SH(+) neutrophils that was sixfold higher compared with the reactivity of SH(−) neutrophils.
(s)FcyRIII level of individuals carrying two FcγRIIIB genes. Moreover, reactivity of neutrophils from NA-homozygous individuals with NA-specific MoAbs is twice as high compared with NA(1+2+) neutrophils. The three NA(1+2+)SH(+) individuals that we tested had a higher neutrophil FcγRIIIb expression as measured with anti-pan-FcγRIII MoAbs, with the exception of 3G8. Furthermore, the reactivity with the NA2-specific MoAb GRM1 was approximately twice as high compared with SH(−) neutrophils, whereas the reactivity with NA1-specific MoAbs was similar. MoAb 3G8 recognizes an epitope in the membrane-proximal domain of FcγRIII, whereas binding of GRM1 is dependent on the presence of 47Ser. Whether the 60Ala→Asp substitution influences the affinity of 3G8 will have to be investigated in studies with SH-FcγRIIIb-transfected cells or with neutrophils from donors at the genomic level proven to carry only the SH-FcγRIIIB gene and no NA2-FcγRIIIB. Our data suggest that the substitution of the hydrophobic alanine to the negatively charged aspartic acid in SH-FcγRIIIb influences the epitope recognized by PEN1, which might be located in the membrane-distal Ig-like domain because of its NA2 specificity. Finally, SH(+) individuals seemed to have higher levels of plasma sFcγRIII compared with NA(1+2+)SH(−) control donors. This suggests that the gene-dosage effect observed for plasma sFcγRIII levels also holds for individuals with three FcγRIIIB genes. Therefore, our findings imply that the large interindividual variation in neutrophil FcγRIIIB expression and plasma levels of sFcγRIII might be partly caused by differences in the number of FcγRIIIB genes. One could hypothesize that the number of FcγRIIIB copies on the neutrophil membrane influences the effector functions of the cell. However, hemizygous and homozygous FcγRIIIB gene deficiency does not seem to be associated with an increased infection risk. Further experiments should elucidate whether the neutrophil response to opsonized particles correlates with the number of FcγRIIIB genes.

It should be further investigated whether the SH mutation influences the ligand-binding capacity of the receptor. The NA1-FcγRIIIB and NA2-FcγRIIIB isoforms have been shown to interact differently with IgG, although the amino acid differences are all in the membrane-distal domain. Moreover, mutations in the membrane-distal domain of FcγRIIa affected the ligand-binding capacity as well.

The Second International Granulocyte Serology Workshop agreed for the time being to term the new antigen SH. For the nomenclature of this gene/phenotype it is important to elucidate whether SH positivity will always be accompanied by NA2 positivity, either because of the close homology between the SH and NA2 isoforms, or because of genetic linkage between the two genes. Thus far, only the SH antisera, genomic analysis, and possibly CD16 MoAb PEN1 can distinguish between NA2- and NA2SH-FcγRIIIB. To underline the close connection of the SH antigen with the NA1(2) system and to emphasize the similarity to NA2-FcγRIIIB, we propose that the antigen be termed NA2SH-FcγRIIIB or NA3.

In conclusion, we detected the presence of three FcγRIIIB genes in three individuals whose neutrophils were phenotyped as NA(1+2+)SH(+) . These three genes, NA1-FcγRIIIB, NA2-FcγRIIIB, and SH-FcγRIIIB, were all transcribed and a clear gene-dosage effect regarding neutrophil FcγRIIIB expression was observed. Our data indicate that it may be possible that a chromosomal locus exists on which NA2-FcγRIIIB is located in tandem with SH-FcγRIIIB.

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REFERENCES

8. Bredius RGM, Fijen CAP, De Haas M, Kuijper EJ, Weening RS, Van de Winkel JGJ, Out TA: Role of neutrophil FcγRII (CD32) and FcγRIIIb (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. Immunology 83:624, 1994


**FKRRIIB Gene Duplication: Evidence for Presence and Expression of Three Distinct FKRRIIB Genes in NA(1+,2+)SH(+) Individuals**

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