Heterogeneity of Neutrophil Antibodies in Patients With Primary Sjögren’s Syndrome

By Armelle Lamour, Rozenn Le Corre, Yvon-Louis Pennc, Jeanine Carton, and Pierre Youinou

Our aims were to determine the prevalence of neutrophil antibodies in patients with primary Sjögren’s syndrome (pSS), identify their target antigen(s), and evaluate their functional significance. Neutrophil antibodies were detected using an indirect immunofluorescence (IIF) test and an enzyme-linked immunosorbent assay (ELISA), using recombinant human Fc-gamma receptor (FcyRIIIB) as a capture agent. Luminol-dependent chemiluminescence was then measured by an established technique. Antibodies to neutrophils were detected in 30 of 66 patients (45%) and categorized on the basis of positivity for the two assays: IIF+/ELISA+ (group A: five patients), IIF+/ELISA− (group B: five patients), and IIF−/ELISA+ (group C: 20 patients). All positive sera contained antibodies directed to the neutrophil-specific FcyRIIib, and none of them bound to NAnt neutrophils. The titer of neutrophil-reactive antibodies (groups A and B) showed no correlation with the neutrophil count, but these autoantibodies did reduce the cell ability to generate a respiratory burst. Thus, neutrophil antibodies are common in patients with pSS. Their main target appears to be FcyRIIIB, and this may partly account for the dysfunction in FcγR-mediated clearance by the reticuloendothelial system reported in these patients.

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Materials and Methods

Patients and controls. Sera were collected from 66 nontransfused patients with pSS, all fulfilling the European preliminary criteria for the classification of SS. Twenty males and 58 females ranging in age from 31 to 73 years (mean, 49 years) were enrolled in the study. Neutropenia was defined by a neutrophil count below 2 × 10^9/L for longer than 4 weeks. Normal controls (NC) consisted of 20 medical students and 14 members of the staff. None of these subjects was taking neutropenia-inducing drugs. The serum of a patient with idiopathic neutropenia served as a positive control in the IIF assay.

Cell preparation. Neutrophils from 15 volunteers were phenotyped for NA1/NA2 and NB1 antigens using alloantisera. Neutrophils from NA1′NA2′NB1′ individuals were taken as the substrate in the IIF test. Cell suspensions containing over 90% neutrophils were obtained by Dextran TS00 (Pharmacia, Uppsala, Sweden) sedimentation, followed by Ficoll-Hypaque density gradient centrifugation (Eurobio, Paris, France) and hypotonic lysis of residual erythrocytes. To reduce nonspecific staining, neutrophils were prefixed with paraformaldehyde (PFA). The fixation was performed by a 5-minute incubation at room temperature (RT) with 1% PFA in phosphate-buffered saline (PBS). To further prevent the binding of immune complexes (IC) to the cells, circulating IC were first precipitated with 2% polyethylene glycol (PEG), as described previously. Suspensions of NA1′NA2′NB1′, NA1′NA2′NB1′, NA1′NA2′NB1′, or NA1′NA2′NB1′ neutrophils were adjusted to 5 × 10^6/mL and incubated with serum, diluted 1/10 in PBS, for 30 minutes at 37°C. The cells were washed three times and incubated with decreased neutrophil count, but the autoantibodies were shown to depress the ability of these cells to generate a respiratory burst.

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Table 1. Characteristics of Neutrophil-Reactive Sera in Patients With pSS

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<th>Serum Donor</th>
<th>Antineutrophil Antibody Isotype (reciprocal titer)</th>
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<td>IgG</td>
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<tr>
<td>10</td>
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Antibodies were detected by IIF using control heterozygous NA1"NA2"NB1" neutrophils as a substrate.

<table>
<thead>
<tr>
<th>IgM</th>
<th>p&lt;0.001</th>
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<tr>
<td>IgG</td>
<td>p&lt;0.001</td>
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Fluorescein isothiocyanate (FITC)-conjugated F(ab')2 anti-human immunoglobulins (Ig) for 30 minutes at RT. The cells were washed another three times and analyzed by flow cytometry. Before use, all reagents were absorbed with a pellet of normal neutrophils and tested by direct immunofluorescence to ensure that they did not bind to the cells. NA"neutrophils were also collected from a blood donor, reagents were absorbed with a pellet of normal neutrophils and tested cells (prepared as described elsewhere) for 15 minutes to remove homozygous NA1 or NA2 cells before testing. This was obtained using FITC-conjugated F(ab')2 anti-human IgG, IgM, or IgA (Stago, Asnières, France). The positive sera, referred to as IIF-positive, were absorbed with pooled human umbilical vein endothelial cells (prepared as described elsewhere for 15 minutes to remove any irrelevant autoantibody and re-tested in the IIF assay. To further characterize their antigenic targets, antibodies were absorbed with homozygous NA1 or NA2 cells before testing. This was obtained by incubating 500 μL of serum, diluted 1/10 in PBS, with 10^5 neutrophils for 18 hours at 4°C. IgG were purified from one of these IIF-positive serum using protein G chromatography and were pepsin-digested. The digest was dialyzed against PBS and F(ab')2 fragments purified on diethyl aminoethyl (DEAE)-cellulose, analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and retested in the IIF assay.

Inhibition with monoclonal antibodies (MoAbs). NA1"NA2"NB1" neutrophils from NC were used to test the remaining sera. Sera from 34 NC were also examined, and the mean percentage of positive neutrophils was 5.5%. This allowed us to define a normal threshold as 20% of stained cells. To determine the optimal dilution of MoAb for 30 minutes at 37°C, the plates were washed four times with PBS containing 0.05% Tween (PBS-T) and blocked with PBS containing 5% BSA for 1 hour at 37°C. After another four washes, serum samples, diluted 1/100 in PBS-T, were dispensed into the wells and left for 90 minutes at RT. Bound antibodies were detected by 8-fluorocitrate (8FC), the plates were washed four times with PBS containing 0.05% Tween (PBS-T) and blocked with PBS containing 5% BSA for 1 hour at 37°C. After another four washes, serum samples, diluted 1/100 in PBS-T, were dispensed into the wells and left for 90 minutes at RT. Bound antibodies were detected by flocculating the wells with horseradish-peroxidase (HRP)-conjugated F(ab')2 anti-human IgG or IgM (Dako, Dako, Copenhagen, Denmark). IgM MoAb (Becton Dickinson) directed toward Fcy-Rllb was used as a positive control and revealed with HRP-conjugated goat F(ab')2 anti-mouse IgM antibody.

DEGCGOXYLATION OF FcγRllb. Neutrophils obtained from a homozygous NA1 donor were suspended at 5 x 10^6/mL in RPMI 1640 containing 0.25% bovine serum albumin (BSA) and incubated for 3 hours at 37°C with PI-specific phospholipase (Sigma, Chemical Co, St Louis, MO) at a final concentration of 0.1 U/mL. Neutrophils were pelleted, and the cell-free FcγRllb-containing supernatant was poured into PBS supplemented with protease inhibitors. This preparation was treated with 20 U/mL N-glycanase (Genzyme, Cambridge, UK) for 36 hours at 37°C. Deglycosylation was confirmed by comparing the digested molecule with a control aliquot and a nonglycosylated recombinant human (rhu) FcγRllb by SDS-PAGE.

ELISA detection of autoantibodies. The rhuFcy-Rllb was produced in Escherichia coli, and its purity was demonstrated by high power liquid chromatography and DNA sequence analysis. It was encoded by an NA1-FcγRllb gene supplied by M. Colonna (La Pitie-Salpetriere Hospital, Paris, France). To obviate nonspecific binding of IgG via its Fc domain in the autoantibody test, rhuFcy-Rllb was heated for 5 minutes at 95°C and treated with 15% β-mercaptoethanol. Microtiter plates (Dynatech, Oslo, Norway) were coated either with 100 μL of this preparation (1 μg/mL in citrate buffer, pH 3.5) or with 100 μL of NA1-FcγRllb (1 μg/mL in carbonate bicarbonate buffer, pH 9.6) extracted from neutrophils and treated or not with N-glycanase. After an overnight incubation at 4°C, the plates were washed four times with PBS supplemented with 0.05% Tween (PBS-T) and blocked with PBS containing 5% BSA for 1 hour at 37°C. After another four washes, serum samples, diluted 1/100 in PBS-T, were dispensed into the wells and left for 90 minutes at RT. Bound antibodies were detected by flocculating the wells with horseradish-peroxidase (HRP)-conjugated F(ab')2 anti-human IgG or IgM (Dako, Dako, Copenhagen, Denmark). IgM MoAb (Becton Dickinson) directed toward Fcy-Rllb was used as a positive control and revealed with HRP-conjugated goat F(ab')2 anti-mouse IgM antibody.

Table 2 for the definition of the patient groups) and 34 NC (open circles) with rhuFcy-Rllb used as a capture agent in the ELISA. The cut-off values are indicated.
Measurement of chemiluminescence. Blood was taken from five NA1"NA2" normal volunteers, and neutrophils were prepared as above. After three washes in Hanks' balanced salt solution (HBSS), each cell suspension was divided into aliquots that were incubated for 1 hour at 37°C with HBSS, control sera (n = 4), IIF-positive/ELISA-positive sera (n = 3), or IIF-negative/ELISA-positive sera (n = 3). The cells were pelleted, washed twice with HBSS, and suspended at 10^6 neutrophils per milliliter in the same medium. Chemiluminescence (CL) was then measured. As previously described, the reaction mixture consisted of 0.5 mL neutrophil suspension and 0.9 mL HBSS containing 2 x 10^-4 mol/L luminol in a polystyrene vial. Complement-opsonized zymosan particles (100 µL; Sigma Chemicals) were added, and the vial was placed in the chamber of the luminometer (LKB, Bromma, Sweden). The resulting light output in millivolts was continuously recorded until the CL peaked and demonstrated a definite decline. A background subtraction control zeroed the instrument before addition of the opsonized particles. Assays were performed in triplicate, with greater than 95% reproducibility between the results, which were averaged.

Various serologic tests. The ELISA to measure IC has been described in detail previously. Briefly, IC were allowed to bind to Clq-coated plates, and IgG- and IgM-containing IC were detected by flooding the wells with HRP-conjugated F(ab'), antihuman IgG or IgM (Dakopatts), respectively. To evaluate the content of IgA in sera, one isotype of anti-FcyRIIIb autoantibody. None of these sera contained neutrophil agglutinins by standard methods (Laboratory of Immuno-Hematology, Blood Bank, Kremlin-Bicêtre Hospital, Paris, France). Overall, three antibody profiles could be defined (Table 2): five sera were positive in both assays (group A), five were positive in the IIF test and negative in the ELISA (group B), and 20 were positive in the ELISA and negative in the IIF test (group C). IgG IIF titers correlated strongly with OD for IgG (r = 0.80, P < .02) in the group A sera. The proportions of stained neutrophils from two NC were similar to those of autologous neutrophils when incubated with two group A, two group B, or two group C sera, diluted 1/80 and revealed with FITC-conjugated F(ab')2 antihuman IgG (Table 3). These results demonstrate a definite decline. A background subtraction control zeroed the instrument before addition of the opsonized particles. Assays were performed in triplicate, with greater than 95% reproducibility between the results, which were averaged.

RESULTS

Detection of autoantibodies. Ten of the 66 sera from patients with pSS (15%) and none from the NC were positive (P < .001) in the IIF test. The IgG titers ranged from 1/40 to 1/1,280 (Table 1); three patients had anti-neutrophil IgG and IgM (1/160 to 1/320), while one had IgG and IgA (1/80). Prior incubation with endothelial cells did not modify the titers. To address the question as to whether PFA may destroy epitopes, three sera from controls and three sera from patients (patients 2, 6, and 9 in Table 1) were tested in parallel with PFA-fixed and unfixed neutrophils from the same donor. The cut-off values were set at 20% and 26% of stained fixed and unfixed cells, respectively. The patients' IgG autoantibodies titers were 1/1,280 using fixed cells compared with 1/640 using unfixed cells, 1/640 compared with 1/640, and 1/320 compared with 1/160, respectively.

IgM and IgG autoantibodies were detected in 24 and 17 pSS sera, and one and two NC sera, respectively (Fig 1) by ELISA. Thirty sera from patients (IgM only in 14, IgG only in 6, and both in 10) and two from NC contained at least one isotype of anti-FcyRIIIb autoantibody. None of these sera contained neutrophil agglutinins by standard methods (Laboratory of Immuno-Hematology, Blood Bank, Kremlin-Bicêtre Hospital, Paris, France). Overall, three antibody profiles could be defined (Table 2): five sera were positive in both assays (group A), five were positive in the IIF test and negative in the ELISA (group B), and 20 were positive in the ELISA and negative in the IIF test (group C). IgG IIF titers correlated strongly with OD for IgG (r = 0.80, P < .02) in the group A sera. The proportions of stained neutrophils from two NC were similar to those of autologous neutrophils when incubated with two group A, two group B, or two group C sera, diluted 1/80 and revealed with FITC-conjugated F(ab')2 antihuman IgG (Table 3). These results demonstrate a definite decline. A background subtraction control zeroed the instrument before addition of the opsonized particles. Assays were performed in triplicate, with greater than 95% reproducibility between the results, which were averaged.

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cannot be explained by binding of IC to FcγR, given that their removal by PEG precipitation was shown to be effective (Table 4). Furthermore, F(ab')₂ fragments obtained from one group A serum recognized 30% of autologous neutrophils, while its native IgG bound to 35%.

Identification of the autoantibody target. Sera from groups A and B were tested by IIF after incubation of the cells with anti-CR MoAbs. The antibody titers remained virtually the same. In contrast, the binding of five sera to neutrophils was significantly reduced (P < 0.05) after incubation of the cells with anti-FcγRIII IgG or IgM MoAbs (Fig 2). Selected group A sera (serum of donors 1 through 3 in Table 1; squares at the top of Fig 1) were absorbed onto a pellet of NAI+NA2+ neutrophils and then re-tested in the IIF assay. OD decreased from 0.681 to 0.258, from 0.654 to 0.310, and from 0.333 to 0.187, respectively, suggesting that the two types of tests are directed to the same antigen.

To determine whether the IIF antineutrophil antibodies react with alloantigens, sera 1 through 5 from group A and sera 6 through 10 from group B were evaluated on NAI+NA2+NB1+, NAI+NA2+NB1−, NAI+NA2+NB1+, NAI+NA2+NB1−, and NAI−NA2+NB1− cells. All the sera reactive with NAI+NA2+ cells recognized NB1+ and NB1− cells (Table 5). Group A sera recognized also NA2+ homozygous cells, while group B did not. There seems to be sera directed specifically toward the NA1 isoform (group B). To substantiate this interpretation, sera were absorbed with either NA1 or NA2 homozygous cells before being tested. Group B sera showed a lowered binding to NA1 cells after absorption with NA1 neutrophils, whereas the reaction was not influenced by incubation with NA2 (Table 6). In contrast, group A sera showed a reduced reactivity with NA1, as well as NA2 homozygous neutrophils, after incubation with either type of neutrophils. To further confirm that the NA allotype-specific group B sera do not bind to FcγRIIIb in the ELISA, neutrophil-extracted receptor was deglycosylated because of the absence of sugar residue on rheumatoid FcγRIIIb. Five group B sera bound to native NA1+ receptor but did not bind to this deglycosylated NA1+ receptor in the ELISA (Fig 3), while results remained positive with three group A sera, two group C sera, and the positive control serum. Patients' neutrophils were then phenotyped. Interestingly, there were four NA1+NA2+ and one NA1+NA2− in group A and five NA1+NA2− in group B.

Three sera from group A, three from group B, and two from group C were then tested, diluted 1/80, against NA(Ig) neutrophils. The proportions of stained cells ranged from 0.4% to 3.9%. Five normal sera were also evaluated, and the mean percentage of positive neutrophils was 2.6% ± 2.0%.

Effects on neutrophil count and CL. There was no correlation between the presence of autoantibody and the number of circulating neutrophils. Fourteen patients were neutropenic; of these, only three (patients 4, 5, and 6 in Table 1) had neutrophil-reactive antibodies. However, neutrophil CL, ie, the ability to generate a respiratory burst, was depressed (Fig 4) after incubation of NA1+NA2+ neutrophils from five normal donors with IIF-positive sera (group A), while control sera and IIF-negative sera (group C) did not influence this reaction. It is of note that two of the four normal sera were the two that were weakly positive in the anti-FcγR ELISA (Fig 1 and Table 2). There were no associations between the levels of IgG-, IgM-, or IgA-containing IC, IgG, IgM, and IgA RF, anti-Ro/SSA, and anti-La/SSB antibodies on the one hand, and the presence of anti-FcγRIIIb autoantibodies on the other.

**DISCUSSION**

Autoantibodies to neutrophils were detected in the sera of 45% of the patients with pSS. To determine this, two assays were developed: IIF of neutrophils and ELISA using rheumatoid FcγR.

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**Table 5. Allospecificities Recognized by Neutrophil-Reactive Antibodies, as Determined by an IIF Assay in 10 Patients With pSS**

<table>
<thead>
<tr>
<th>Phenotype of the Target Neutrophils</th>
<th>Serum Sample</th>
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<tr>
<td></td>
<td>NA1+NA2+</td>
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γRIIib. In the former test, it is essential to distinguish neutrophil-reactive autoantibodies from nonspecific IgG bound to the FcγR through their Fc portion. The cells were, therefore, fixed with PFA. This treatment does not destroy epitopes, as experiments were repeated with nonfixed neutrophils and gave the same results. To exclude false-positives caused by the interaction of cells with IC, circulating IC were PEG-precipitated before testing, and their absence was ascertained using in-house tests. In contrast to that used in previous studies, our recombinant molecule was of human origin and complete, rather than of murine origin and truncated. Hence, this capture agent had to be denatured to prevent nonspecific capture of free or IC-included IgG.

Our central finding is that autoantibodies may be categorized on the basis of results obtained in the IIF test and the ELISA: group A antibodies were IIF-positive/ELISA-positive (n = 5); group B, IIF-positive/ELISA-negative (n = 5); and group C, IIF-negative/ELISA-positive (n = 20). Absorption experiments were accomplished. While not an absolute proof, the reduction of the OD established that IIF and ELISA were directed to the same antigen. Alternatively, steric hindrance may be incriminated. Another potential explanation for the results given in Table 2 relates to the general sensitivity of the two assays. This is, however, unlikely to be correct, insomuch as the titers of antineutrophil antibodies of group A patients were similar to those of group B in the IIF assay (Table 1), although the latter antibodies were undetectable in the ELISA. Furthermore, none of three sera from group A, three sera from group B, and two sera from group C bound to NAEx null neutrophils in the flow cytometry test. In support of our hypothesis that the antibodies are indeed autoantibodies, one may also argue that neutrophils

![Fig 3. Reactivity of eight sera with neutrophil-extracted FcγRIIib before and after treatment of this receptor with N-glycanase (squares, group A sera; triangles, group B sera; and circles, group C sera). Note that group B sera bound to native receptor (left) but did not recognize its nonglycosylated form (right).](image-url)
of all patients with anti-NA1 antibodies were positive for NA1 antigen.

It is noteworthy that autoantibodies from all sera were directed to FcγRIIIb. It is interesting that antibodies to NA1 and NA2 are also important in other clinical conditions, including neutrophil-mediated transfusion reactions and alloimmune neonatal neutropenia. The presence of anti-FcγRIIIb activity in group A sera does not imply that all antineutrophil antibodies share this specificity. However, CR should not be targeted, as incubation of the cells with anti-CR MoAb did not reduce significantly the binding of autoantibodies, whereas the proportion of stained cells declined clearly after addition of anti-FcγRIII MoAb. This may reflect competition for similar epitopes or recognition of multiple epitopes by polyclonal autoantibodies. Unlike group A antibodies (which bind to NA1+ as well as NA2+ cells), group B antibodies are specific for the NA1 allotypic motif of FcγRIIIb. This epitope depends on the glycosylation status of the molecule and, consequently, is not expressed in the rhuFcyRIIIb. Group C antibodies are detectable in the ELISA but unreactive with cell-bound FcγRIIIb, irrespective of the NA and NB phenotype of the neutrophils. They also did not bind to FcγRIIla-carrying NK cells and various cell lines bearing FcγRI and/or FcγRII, such as U937 and K532 cells. This suggests that epitopes are either cryptic at the cell surface level or that membrane antigens are internalized on the cytoplasmic tail of FcγRIIIb. Mechanisms have been proposed that allow lymphocytes sensitized against cryptic peptides to initiate autoimmune processes. Alternatively, changes may occur to the receptor after shedding upon activation. This release is exaggerated in pSS, rheumatoid arthritis, and systemic lupus erythematosus, so that beyond a certain threshold, cell-free receptors may self-aggregate through lectin-sugar residue associations and trigger autoantibody production.

Intriguingly, the titer of neutrophil-reactive antibody in groups A and B did not correlate with the decreased neutrophil count. In fact, group studies of patients with systemic lupus erythematosus have also failed to show such a relationship between neutrophil count and the level of cell-binding IgG. This observation is difficult to reconcile with the role recently assigned to cell-bound FcγRIII in neutrophil apoptosis, which would be associated with the release of FcγRIIIb. Instead, autoantibodies may cause neutrophil dysfunction, which is consistent with their reduced efficacy in patients with pSS. In the present study, luminol-dependent CL appeared to be extremely reduced after incubation of the cells with anti-FcγR antibodies that bound to neutrophils, but not with those that did not bind to neutrophils. Notably, neutrophils did not aggregate after incubation with normal group A or group C sera, given that we were unable to detect neutrophil agglutinins. This effect has received little attention in pSS, whereas a statistically significant inverse correlation was demonstrated between superoxide radical generation and neutrophil-binding IgG in patients with rheumatoid arthritis. Nevertheless, CL tended to be higher in the patients with cell-free FcγRIIb and, therefore, related autoantibodies, but this difference did not reach significance. Finally, as demonstrated elsewhere, the possibility arises that the above autoantibodies crossreact with FcγRI and FcγRII and account for the defective FcγR-mediated clearance by the reticuloendothelial system described in patients with pSS. Neutrophils are generally not considered to be crucial for IC handling. However, additional data have been reported elsewhere. Not only were there anti-FcγRIIIb antibodies recognizing FcγRI and FcγRII, but some of them were also reacting with FcγRIIla eluted from large granular lymphocytes and expressed by tissue macrophages. The real pathogenic role of these autoantibodies, however, remains to be elucidated.

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