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INTRODUCTION

Antineutrophil antibodies have been detected in the sera of patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), idiopathic thrombocytopenic purpura (ITP), human immunodeficiency virus disease (HIV), Coombs' positive hemolytic anemia, chronic hepatitis, and lymphoproliferative disorders (1). In addition, antineutrophil antibodies may be associated with chronic neutropenias in infants and children (2). Reliable methods of detecting antineutrophil antibodies are important for discriminating among the potential causes of neutropenia and in planning management.

Numerous assays have been developed to detect antineutrophil antibodies, including the leukocyte agglutination technique (3,4), neutrophil opsonization (5,6), indirect granulocyte immunofluorescence using fluorescence microscopy (7), quantitative antiglobulin consumption (8), radiolabeled antiglobulin binding (9), and staphylococcal protein A binding (10). These methods, however, can be time-consuming, tedious, difficult to quantitate, and of limited reproducibility. Flow cytometry offers the potential for rapid evaluation of large numbers of cells with small volumes of serum in a more quantitative and objective manner. Flow cytometric assays have been reported to be useful for detecting antineutrophil antibodies, but earlier studies have not defined standards for positivity (7). In contrast to previous reports (11), we found that the use of paraformaldehyde-fixed neutrophils as antigenic targets resulted in increased background fluorescence, so we have used unfixed cells as antigenic targets for these studies. Sera from neutropenic patients with a variety of associated clinical conditions were studied for evidence of enhanced binding of IgG to normal target neutrophils using a modified flow cytometry assay. Results are discussed and compared with studies of sera from normal individuals and from nonneutropenic patients with RA and SLE. In addition, we studied the binding of patients' IgG to stimulated target neutrophils and observed changes in antibody binding, compared to unstimulated neutrophils.

MATERIALS AND METHODS

Serum Preparation

Blood samples were obtained from patients with suspected autoimmune neutropenia and from normal volunteers by ven-

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epuncture into sterile glass tubes, allowed to clot, and centrifuged for 10 minutes at 400g. Serum was heat-inactivated at 56°C for 30 minutes and the serum specimens were frozen at −70°C prior to use. Blood samples were obtained according to a protocol approved by the Walter Reed Army Medical Center Human Use Committee.

Neutrophil Preparation

Neutrophils were purified from heparinized venous blood of normal volunteers by a density gradient/dextran sedimentation technique (12). Blood (20 ml) was collected by venepuncture into 50 ml heparinized polypropylene tubes (10 units heparin/ml whole blood) and diluted with 20 ml of Hank's Balanced Salt Solution, without calcium or magnesium (HBSS-, Gibco Laboratories, Grand Island, NY); 12 ml of Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) were underlayered; and the tubes were centrifuged for 30 minutes at 400g at room temperature. From each tube, 10 ml of plasma were collected and saved, and the remainder of the supernatant including the mononuclear layer was aspirated to the red cell pellet and discarded. The 10 ml of plasma were returned and 3% dextran was added to a total volume of 40 ml. The tubes were mixed and the red cells allowed to sediment for 25 minutes at room temperature; then the supernatant was transferred to a clean tube and the RBC pellet was discarded. Tubes were centrifuged at 400 g at 4°C for 10 minutes and the supernatant was aspirated and discarded. Residual erythrocytes were removed by hypotonic lysis as follows. The pellet containing neutrophils and residual erythrocytes was resuspended in 10 ml of cold 0.2% saline with gentle pipetting for 20 sec; then 10 ml of 1.6% saline were added to restore isotonicity. HBSS- was added to a total volume of 40 ml, and the cells were centrifuged again. The hypotonic lysis was repeated once and the cells resuspended in 10 ml HBSS-. Neutrophils were counted using a Baker 9000 cytometer (Baker Instruments, Bethlehem, PA), or manual white blood cell counts were done using a hemocytometer. The cells were then centrifuged at 400g for 10 minutes and resuspended in PBS with 2% fetal calf serum and 0.1% sodium azide (PBS/FCS buffer) to a final cell concentration of 10×10^6 cells per ml. Cell viability was evaluated using trypan blue exclusion, and neutrophils were found to be consistently > 95% viable following separation. The average yield from this procedure varies depending on the donor's white blood cell count and percent neutrophils, but it is usually 2×10^6 cells per ml of donor blood. For stimulation studies, neutrophils were suspended in HBSS with calcium and magnesium (Gibco Laboratories, Grand Island, NY), stimulated with 10^{-7} mol/L fMet-Leu-Phe (fMLF) for 15 minutes at 37°C, then cooled to 4°C, and resuspended in PBS/FCS buffer prior to incubation with sera.

Immunofluorescence Flow Cytometry

Serum (100 μl) diluted 1:5 with PBS was added to 100 μl cell suspension containing 10^6 neutrophils (final serum dilution 1:10) in polypropylene tubes and incubated at 4°C unless otherwise specified. The incubation was stopped after 30 minutes by the addition of 1 ml of ice-cold PBS/FCS. The cell suspensions were then centrifuged at 400g for 10 minutes. Tubes were manually decanted, then washed a second time. Following the second centrifugation step, the supernatant was aspirated using a pasteur pipette without disrupting the cell pellet. Samples were aspirated rather than decanted at this step in order to more completely dry the cell pellet, thereby reducing variability between replicate samples during the next incubation. Cells were then incubated with 100 μl of fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgG, $F(ab')_2$ fragment, gamma chain specific, (anti-IgG-FITC) (Sigma Chemical CO., St. Louis, MO) diluted 1:100 with PBS/FCS for 30 minutes at 4°C. Cells were washed twice, then resuspended in 1 ml cold PBS containing 1% paraformaldehyde prior to analysis. Cells were analyzed on a Becton Dickinson FACSCAN flow cytometer integrated with a Hewlett-Packard computer using FACSCAN or LYSYS software (Becton Dickinson, Mountain View, CA). Results were expressed in terms of computer-generated histograms of cell number vs. fluorescence, at a wavelength of 488 nm (FL1) for FITC labeled cells. Cells were analyzed with the forward angle light scatter and side scatter gated for the neutrophil population. For each sample, 10^4 cells were counted and all samples were run in quadruplicate. Day-to-day variability was minimized by recalibrating the cytometer daily with standardized, fluorescent microspheres (Becton Dickinson, Mountain View, CA.) and by further calibrating with a sample of unstained neutrophils as recommended by the manufacturer. For consistency between experimental days, the FL1 detector was calibrated such that the mean fluorescence channel for serum-free control neutrophils was 300 ± 10. The FACSCAN system has a logarithmic scale of 1,024 channels, representing 4 log decades of fluorescence intensity, on the x-axis. The mean channel number, calculated for each fluorescence histogram, was used for numerical comparisons of study samples.

RESULTS

Range of Normal and Reproducibility

Serum specimens (144) from healthy, nonneutropenic adult volunteers were evaluated for IgG binding to normal neutrophils. Since the distribution of mean fluorescence values appeared to be gaussian on the logarithmic fluorescence scale, we applied statistical analyses on that scale. When the flow cytometer was calibrated as described in Methods, the average of the mean fluorescence measurements for neutrophils incubated with these normal sera was 409, with 95% confidence limits of 345–473. Positive antineutrophil antibody activity was defined as mean fluorescence >2SD (on the logarithmic scale) above the mean for normal controls. Applying these criteria, we identified sera with enhanced IgG binding to normal target neutrophils and observed <3% false

positives with normal sera. To evaluate reproducibility, 20 replicate specimens of serum from three healthy, nonneutropenic adults were studied on separate occasions. The mean fluorescence measurements were serum A: 388 ± 1 (SEM) and 393 ± 3; serum B: 409 ± 5 and 418 ± 2; serum C: 370 ± 4 and 381 ± 3, reflecting slight day-to-day and test-to-test variability for identical normal samples. Patient samples with enhanced IgG binding had 2–10-fold more fluorescence on the linear scale than an average control sample, and >80% of fluorescent events were brighter than average control specimens. Examples of histograms generated with neutrophils unexposed to serum, incubated with normal serum, and incubated with antineutrophil antibody positive serum are shown in Figure 1. Histograms of neutrophils incubated with antineutrophil antibody positive sera were usually symmetrical, without evidence for neutrophil subpopulations.

Variation of Donor Neutrophils

For individual sera, there appeared to be only minor differences in binding to neutrophils from different donors. To evaluate variability of a positive sample, aliquots of a single serum sample from a patient with suspected autoimmune neutropenia were studied in quadruplicate using target neutrophils from 4 different donors. The average mean fluorescence measurements for these 4 assays were 524 ± 5 (SEM), 492 ± 2, 556 ± 5, and 504 ± 19, reflecting consistently positive results, with slight variation between donor target neutrophils in degree of positivity. Since neutrophil phenotypes have been defined using sera from mothers of infants with alloimmune neonatal neutropenia, we obtained antisera of known specificities from the American Red Cross (see Acknowledgments).

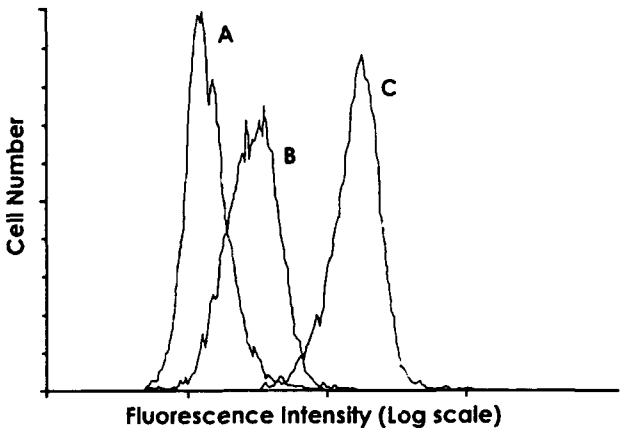

Fig. 1. Flow cytometric histograms of neutrophils after incubation with anti-IgG-FITC, as described in Methods. **A.** "Serum-free" neutrophils exhibit moderate background fluorescence. **B.** Neutrophils incubated with normal serum show an increase in this background fluorescence. **C.** Neutrophils incubated with serum from a neutropenic patient illustrate enhanced IgG binding, interpreted as "positive" for antineutrophil antibodies. For each histogram, 10^4 cells were counted, with forward and side scatter gates set for the neutrophil population.

Using antisera to the neutrophil antigens NA1, NA2, NB1, and Mart, the flow cytometric assay was able to clearly distinguish positive and negative donor neutrophil phenotypes. In contrast, we did not identify any sample from our studies of adults with autoimmune neutropenia that appeared to distinguish between these neutrophil phenotypes.

Effect of Concentration of Serum and Anti-IgG-FITC

The conditions of the assay were evaluated by varying the concentrations of serum and anti-human IgG as shown in Table 1. Results are expressed as the difference in mean fluorescence channel between a serum specimen positive for antineutrophil IgG antibodies and serum from a normal control donor. The reagent dilutions that gave the greatest separation between the positive and control measurements were 1:10 for serum and 1:100 for anti-IgG-FITC, and these dilutions were used for the remainder of the study.

Fixation of Target Neutrophils

In order to assess the effect of prior paraformaldehyde fixation of target neutrophils on non-specific background fluorescence, positive sera and normal control sera were tested with fixed and nonfixed targets. Neutrophils were fixed by incubation in PBS containing 1% paraformaldehyde for 30 minutes at room temperature. Neutrophils fixed with paraformaldehyde, without exposure to serum, exhibited increased background fluorescence compared to unfixed neutrophils, as shown in Table 2. After incubation with serum from healthy nonneutropenic donors, fixed neutrophils were again found to have increased fluorescence compared to fresh, unfixed cells. In contrast, paraformaldehyde-fixed neutrophils incubated with serum from a patient known to have antineutrophil antibodies, were found to have less fluorescence than living cells incubated with the same patient's serum. Paraformaldehyde fixation of FITC-labeled neutrophils performed after both in-

TABLE 1. Effect of Dilutions of Patient's Serum and Antihuman IgG-FITC Second Label on Fluorescence of Target Neutrophils

Antihuman IgG-FITC dilution	Serum dilution			
	1:1	1:10	1:20	1:50
1:50	72[a] (1.9)[b]	67 (1.8)	61 (1.7)	55 (1.6)
1:100	191 (5.6)	245 (9.1)	228 (7.8)	105 (2.6)
1:200	157 (4.1)	128 (3.2)	198 (5.9)	61 (1.7)
1:400	NP	81 (2.1)	116 (2.8)	50 (1.6)

[a]Results are expressed as the difference between mean fluorescence channel (log scale) for a known antineutrophil antibody positive serum and a previously defined normal serum of average background intensity, and represent the average of 4 replicate measurements.
[b]Numbers in parentheses represent the difference in fluorescence intensity on a linear scale as (patient ÷ control) (26).
NP = not performed.

TABLE 2. Effect of Paraformaldehyde Fixation of Target Neutrophils on Antineutrophil Antibody Binding

Sample	Condition: With paraformaldehyde		Condition: Without paraformaldehyde	
Serum-free	345 ± 4[a]	[22.2][b]	310 ± 10	[16.3]
Normal serum	404 ± 10	[37.9]	349 ± 7	[23.0]
Antineutrophil antibody positive patient serum	498 ± 20	[88.2]	525 ± 26	[112.4]
Difference between normal and positive sera	94	(2.3)[c]	176	(4.9)

[a]Results represent the mean fluorescence channel ± SEM for quadruplicate measurements.
[b]Numbers in brackets represent absolute fluorescence values on a linear scale (26).
[c]Number in parentheses represent the difference in fluorescence intensity on a linear scale as (patient ÷ control).

cubation steps had been completed had no effect on the mean channel or on the shape of the fluorescence histograms.

Studies of Patients

Sera from 159 patients with neutropenia of suspected immune etiology were studied using immunofluorescence flow cytometry, for evidence of enhanced binding of patients' IgG to normal neutrophils. All patients had absolute neutrophil counts of < 1,500, and no other clinical reason for neutropenia, such as cytotoxic chemotherapy or malignancy involving the bone marrow. Clinical data for patients with positive assay results are presented in Table 3. Patients with collagen vascular disorders (SLE, RA, Felty's syndrome, or other disorders) and suspected immune neutropenia had a higher percentage of positive assay values than patients with other diagnoses. For patients with positive assay results, there were more females with collagen vascular disease or adult chronic neutropenia, whereas there were more males with "other" associated diagnoses. The absolute neutrophil counts for patients with positive antineutrophil antibody assays were generally lower for children than for adults. Overall, 37% of sera submitted by clinicians for evaluation of suspected autoimmune neutropenia were found to have antineutrophil antibodies by flow cytometry.

In order to evaluate the role of a clinical diagnosis of collagen vascular disease, we studied sera from 37 patients with typical seropositive RA and from 251 other patients seen in a rheumatology clinic for a variety of collagen vascular disorders, primarily SLE, but also including Sjögren's syndrome, mixed connective tissue disease, and other conditions. Whereas 0/37 nonneutropenic patients with typical RA had enhanced IgG binding to neutrophils, 51/244 (21%) of sera from nonneutropenic patients with SLE or other collagen vascular disorders also showed enhanced IgG binding to neutrophils. This enhanced binding of IgG to target neutrophils was not correlated with the presence of rheumatoid factor, nor with the presence of immune complexes, in these sera.

Stimulation of Target Neutrophils

The use of living neutrophils as targets allows the study of IgG binding to membrane antigens that may be upregulated or downregulated by cellular activation. We studied the binding of patients' IgG to unstimulated neutrophils and to neutrophils that had been stimulated with the chemotactic peptide

TABLE 3. Clinical Characteristics of Patients With Suspected Immune Neutropenia Evaluated by Flow Cytometric Antineutrophil Antibody Assay

Diagnostic category	IgG antineutrophil antibody assay: number of specimens positive/number studied (%positive)	Patients with positive assay result: Sex (M:F)	Age in years, mean (range)	Absolute neutrophil count:[a] mean (range)
Collagen vascular disorders; includes rheumatoid arthritis (18), systemic lupus erythematosus (4), and other collagen vascular diseases (4)	20/26 (77%)	7:13	56 (30–72)	845 (0–1,500)
Adult chronic neutropenia	12/37 (32%)	4:8	52 (26–86)	830 (40–1,500)
Childhood chronic neutropenia	8/29 (28%)	4:4	1 (0–2)	330 (50–800)
Immune thrombocytopenic purpura	6/12 (50%)	5:1	10 (1–17)	270 (0–880)
Other diagnosis; includes HIV (5), drug administration (8), lymphoproliferative disorders (8), febrile transfusion reactions (6), others (28)	13/55 (24%)	10:3	46 (20–64)	480 (0–1,500)
All Patients	59/159 (37%)	30:29	41 (0–86)	574 (0–1,500)

[a]All patients had absolute neutrophil counts < 1,500 and no other clinical reason for neutropenia, such as cytotoxic chemotherapy or malignancy involving the bone marrow.

fMLF (10^{-7} mol/L) for 15 minutes at 37°C. This amount of chemotactic peptide stimulus has been shown to upregulate membrane molecules such as integrins that are found in neutrophil secondary granules. IgG from normal individuals consistently exhibited less binding to stimulated neutrophils than to unstimulated neutrophils (illustrated in Fig. 2A). Sera from 80% of patients with neutropenia and antineutrophil antibodies in our study population, also demonstrated this same pattern. In contrast, sera from 12/59 (20%) of neutropenic patients with antineutrophil antibodies showed an increase in IgG binding to stimulated neutrophils (illustrated in Fig. 2B): 4 patients had an associated clinical diagnosis of rheumatoid arthritis, one had SLE, one had Sjögren's syndrome, one had ITP, and five had chronic neutropenia. Four of these patients have been previously described (13) as having other evidence for antibody specificity for the neutrophil adhesion glycoprotein complex CD11b/CD18, a neutrophil membrane receptor that is known to be upregulated after fMLF stimulation. We also studied 50 sera from neutropenic patients that were initially classified as normal for antineutrophil antibodies. In 2 cases, sera could be reclassified as positive for antineutrophil antibodies when studied using activated neutrophils. Both of these cases had high-normal amounts of IgG binding when studied using unstimulated target neutrophils (e.g., between 1 and 2 standard deviations above the mean for normal controls).

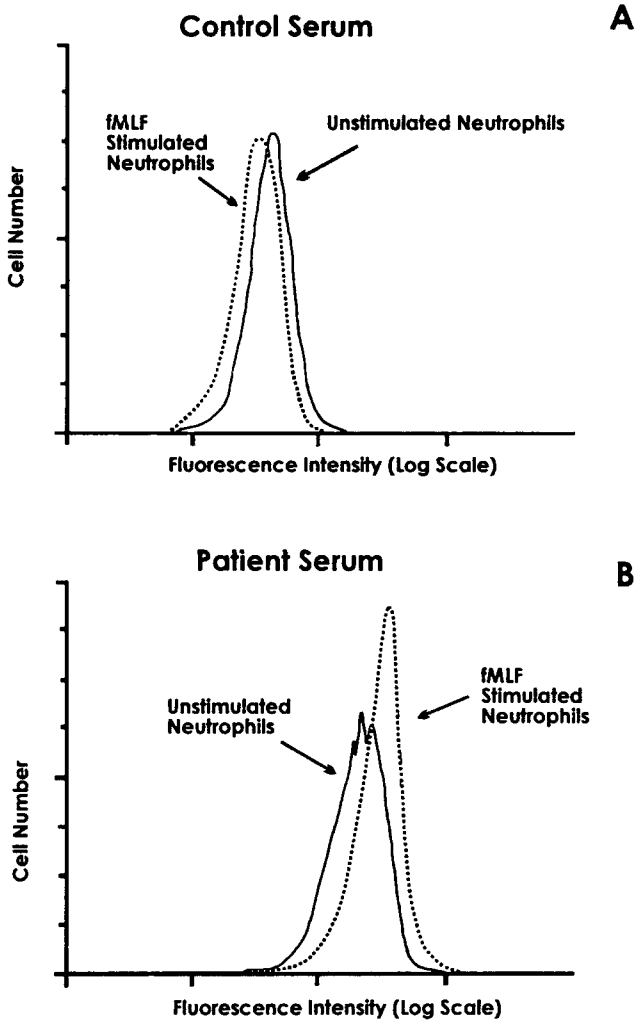

Fig. 2. Effect of fMLP-induced stimulation of target neutrophils on antibody binding. Unstimulated neutrophils were kept at 4°C, whereas stimulated neutrophils were incubated with the chemotactic peptide fMLF at 10^{-7} mol/L for 15 minutes at 37°C, then cooled to 4°C. Neutrophils were then incubated with sera from normal individuals or from patients with neutropenia and studied for binding of IgG using anti-IgG-FITC. When incubated with serum from a normal individual, stimulated neutrophils consistently demonstrated a loss of fluorescence compared to unstimulated neutrophils (**A**). When incubated with sera from a patient with neutropenia and antineutrophil antibodies, stimulated neutrophils demonstrated enhanced fluorescence compared to unstimulated neutrophils (**B**). An increase in IgG binding with stimulated neutrophils was observed in 12 of 59 sera that contained antineutrophil antibodies.

DISCUSSION

Neutropenia may occur as one manifestation of a systemic autoimmune disease, such as RA, SLE, Hashimoto's thyroiditis, Graves' disease, or Sjögren's syndrome. Neutropenia may also be associated with viral diseases such as Epstein-Barr virus and HIV, with lymphoproliferative disorders, and with the ingestion of drugs. In addition, chronic neutropenia may occur in adults and children as an isolated clinical and laboratory finding, in the absence of other disease manifestations. Many of the conditions associated with neutropenia involve disturbances of immunoregulation, and the immune basis for many cases of neutropenia has been clearly established. Humoral or cellular immune mechanisms may act alone or in concert to produce neutropenia, and other elements of the blood may likewise be affected. Moreover, therapies directed at modulating the immune response, in some cases, have been successful in moderating the neutropenia and in reducing associated morbidity.

Until recently, the autoimmune nature of certain cases of suspected immune neutropenia was not well established. However, multiple lines of evidence now support the involvement of an immunologic mechanism in the pathogenesis of many cases of otherwise unexplained neutropenia. Using a flow-cytometric assay, we were able to detect enhanced binding of patient's IgG to donor target neutrophils in 37% of sera submitted for evaluation of suspected immune neutropenia. These studies confirm previous reports that antineutrophil antibodies may be found in neutropenic patients respresenting a broad range of age groups and a variety of associated clinical diagnoses (1). In addition we found enhanced neutrophil-binding IgG in the sera of 21% of a group of nonneutropenic patients with SLE and other collagen vascular disorders. Certain patients with autoimmune disorders, particularly SLE, have been previously reported to have elevated neutrophil-binding IgG in the absence of neutropenia (9). This activity was not cor-

related with the presence of rheumatoid factor in our patients, nor with the presence of immune complexes, suggesting that specific antineutrophil autoantibodies may be present in some of these patients.

In our evaluation of the immunofluorescent flow cytometric assay, we observed increased background fluorescence when normal control sera were incubated with neutrophils previously fixed with paraformaldehyde. In addition we found decreased specific fluorescence when positive control antisera were incubated with fixed neutrophils. Whereas previous investigators have used paraformaldehyde fixed neutrophils (6,7,11,14), others have found increased background fluorescence when target cells were fixed with aldehyde agents (15). A decreased signal-to-noise ratio with target cell fixation may result from denaturation of antineutrophil antibody target antigens and increased nonspecific antibody binding (16), therefore we used freshly prepared, unfixed neutrophils as target cells. Fixation of neutrophils after fluorescent staining but before flow cytometry had no effect upon results.

Neutrophil-specific allelic antigens have been defined using sera from mothers of infants with neonatal alloimmune neutropenia in agglutination assays (17). Thus the use of random donors as sources of target neutrophils introduces the potential for false negative results due to absence of specific allelic antigens on target neutrophils. Particularly in neonatal alloimmune neutropenia, phenotypic heterogeneity of target neutrophils may significantly influence the detection of antineutrophil antibodies (2,3,18–21). However, in our studies of adults with neutropenia, this has not been the case, as we and others (11) have demonstrated consistent results with multiple target cell donors. For the study of neonatal alloimmune sera, the use of target neutrophils that include most known phenotypes could eliminate this potential source of error.

ABO blood group antigens have been shown to be absent from neutrophils (22). In contrast, HLA antigens may be expressed on neutrophils, although the antigenic density has been reported to be variable and lower than that of lymphocytes (23). Pre-incubation of patients' sera with pooled platelets has been used by neutrophil serotyping laboratories as a method to eliminate this reactivity (17), although we did not use this procedure in our studies. Certain of the antineutrophil antibody positive results reported here may therefore be due to antibodies directed against HLA antigens.

The assay described here is specific for IgG antibodies; however, it is readily adaptable for the detection of IgM autoantibodies through the use of FITC-conjugated, class-specific antihuman IgM. The assay can also be used as a "direct" test for antineutrophil antibodies, by separating the patient's neutrophils using the method described, then incubating these neutrophils with antihuman IgG-FITC. This approach may be limited by the low number of neutrophils available for harvest from a neutropenic patient.

Stimulation of living neutrophils with agents such as chemotactic peptide receptor agonists results in dramatic changes in the composition of the surface membrane, including increased expression of β_2 integrins, shedding of L-selectin, and cleavage of terminal sialic acid residues (24,25). Antineutrophil antibodies that bound specifically to any of these molecular structures would be expected to have different binding to unstimulated versus stimulated neutrophils. When target neutrophils were stimulated with the chemotactic peptide fMLF, sera from normal control individuals had consistently less IgG binding than that observed with unstimulated target neutrophils. Most sera from neutropenic patients (that had previously been found to be positive for antineutrophil antibodies using unstimulated neutrophils) also had less IgG binding to stimulated neutrophils; however, serum IgG from 20% of these patients demonstrated increased binding to stimulated target neutrophils. These obsevations support the conclusion that these sera contain IgG specific for antigenic epitopes that are upregulated or modified as a result of cellular activation. Furthermore, 2 sera with high-normal amounts of IgG binding to unstimulated neutrophils were found to have enhanced IgG binding to stimulated neutrophils. This finding suggests that stimulated target neutrophils may assist in the identification of some patients with antineutrophil antibodies that are not detected using unstimulated targets.

The detection of autoantibodies to neutrophils has been difficult due to the high degree of nonspecific binding of immunoglobulins to the neutrophil surface and to the wide range of fluorescence found after incubation of neutrophils with normal sera. The standardization of cell preparation and incubation conditions, as well as of flow cytometer calibration described in this report allow for reproducible detection of specific antineutrophil binding IgG, in the evaluation of sera from patients with suspected autoimmune neutropenia. In addition, the use of stimulated target neutrophils may assist in the identification of antibodies directed against neutrophil antigens that are upregulated with activation.

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