Measurement of Antineutrophil Antibodies by Flow Cytometry: Simultaneous Detection of Antibodies Against Monocytes and Lymphocytes

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We describe a flow cytometric technique which detects the presence of antineutrophil antibodies (NABs) in human serum. The technique provides a qualitative as well as a semiquantitative screen and provides an excellent method for monitoring the presence of antineutrophil antibodies in patients with suspected autoimmune neutropenia. Using light-scatter gating, individual populations consisting of neutrophils, monocytes, and lymphocytes can be examined simultaneously for the presence of antibody. The methodology utilizes an indirect immunofluorescence technique with FITC-labeled goat antihuman F(ab')$_2$ antibody and fixed leukocyte suspensions. Furthermore, by utilizing class-specific FITC-labeled second antibody, significant information can be ascertained regarding the class, cell specificity, and quantity of detected antibody. Formalin fixation of neutrophils prevented pinocytosis of the fluorochrome, significantly reducing background fluorescence. Twenty-five normal subjects provided baseline antibody levels for each class. Of 92 patients with suspected autoimmune neutropenia, 27 had class IgG alone and seven were positive for both IgG and IgM class NABs. During treatment, IgG levels varied. IgM NABs alone were detected in four patients. Fifty-four patients had undetectable antibody. Antibodies were detected against monocytes in several of the IgG-positive patients. Two sera contained both IgA and IgG NABs. One serum contained IgA and IgG antibodies against monocytes. No IgD antibodies were detected in any sera tested. Some sera tested contained antibodies against lymphocytes—however, only in those sera which also contained antibodies to other cell types.

INTRODUCTION

Antineutrophil antibodies (NABs) are important in the pathogenesis of immune neutropenia [1, 2]. Several techniques have been developed for the detection of NABs. Complement fixation [3], agglutination [4], antiglobulin consumption [5], cytotoxicity [6], and leukocyte opsonization [7] have been used. More recent techniques include radiolabeled staphylococcal protein A [8] and cell elastimetry [9]. With the development of an immunofluorescence method [10] flow cytometric evaluation of NABs was also demonstrated. Most methods currently available for the detection of circulating NABs are time consuming and often require large volumes of blood. An advantage of flow cytometry is the ability to rapidly determine the presence, specificity, and quantity of NABs by using very small quantities of blood or serum. Previous measurements have been made by flow cytometry

[10,11], but few patients were studied and significant improvements in flow technology have occurred since that time. This report describes several new approaches to the measurement of antineutrophil antibodies, including the ability to simultaneously assess multiple populations of cells for the presence of those antibodies.

MATERIALS AND METHODS
Buffers and Lysing Solution

Phosphate-buffered saline (PBS) contained 0.147 M NaCl, 4.1 mM Na$_2$HPO$_4$·7H$_2$O, 2.3 mM NaH$_2$PO$_4$, 15 mM NaN$_3$, pH 7.4. Erythrocyte lysing solution consisted

of 0.15 M NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA, pH 7.4.

Preparation of Cells

Human blood was drawn into either heparinized (1 U/ml) or EDTA-containing Venoject tubes (Becton Dickinson, Sunnyvale, CA). Erythrocytes were lysed by 30-fold dilution of whole blood with lysing solution in a 50-ml sterile polypropylene tube (Corning Glass Works, Corning, NY). The tubes were gently mixed on a bench rotor at room temperature for 10 min. After centrifuging at 250g for 10 min, the supernatant was decanted and tubes were inverted to drain. Cell pellets were washed twice by using PBS and suspended in 5 ml PBS. Leukocytes were fixed for 5 min at room temperature in a final concentration of 1% w/v buffered formalin solution. Following fixation, leukocytes were washed twice in PBS and resuspended at a concentration of 2×10^6 leukocytes/ml.

Antisera

Fluorescein isothiocyanate (FITC)-labeled goat anti-human serum [F(ab')$_2$] class specific for IgG, IgM, IgA, IgD, and goat antiserum against human total immunoglobulins were obtained from Kallestad (Austin, TX). Antisera were centrifuged at 30,000g for 15 min before being diluted 1:50 for use in the assay. This dilution was determined to be the optimal dilution by titration.

Opsonic Assay for Antineutrophil Antibodies

The opsonic assay of Boxer and Stossel [7] has been used as a laboratory standard for the measurement of NABs. Test serum was used to opsonize random donor neutrophils. Neutrophils will attempt to ingest other neutrophils sensitized with antibody. The attempt at ingestion activates the oxidative metabolism of the ingesting (indicator) leukocytes which can be monitored by measurement of glucose oxidation [7]. The test was considered positive if the glucose oxidation rates exceeded 2 standard deviations above the control mean. This test was used as a standard for establishing the positive and negative NAB sera for standardization of the flow-based assay.

Flow Cytometry

All fluorescence measurements were made on Coulter EPICS C or EPICS V flow cytometers (EPICS Division, Coulter Corp., Hialeah, FL) utilizing an argon ion laser emitting 600 mW at 488 nm. Green fluorescence was measured by using a 525-nm band-pass filter. Leukocytes were gated electronically after simultaneous measurement of forward and right-angle light scatter and the green fluorescence of each population was collected. The signal collected was the light-scatter gated integral log (scale) green fluorescence (ILGF). For calibration of the flow cytometer, Coulter 12.5% bright calibration beads were used. High voltage to the photomultiplier tube (PMT) was

adjusted so that the beads produced a mean fluorescent peak at channel number 177 on a logarithmic green fluorescence scale of 256 channels.

Fluorescence Assay Technique

Patient or control sera (50 μl) were added to 100 μl formalin-fixed leukocyte suspension (2 $\times$ 10^6/ml) in 10 $\times$ 75 mm polyethylene tubes (Sarstedt, W. Germany) and incubated for 30 min at 4°C. Thereafter, 2 ml PBS (4°C) was added and the cells were washed three times by centrifugation at 200g for 5 min. After the final wash, PBS was decanted and the cells were resuspended by gentle vortexing; 50 μl of the appropriate FITC-labeled goat antihuman antiserum was added and the tubes were incubated for 15 min at 4°C. The cells were washed once in 3 ml ice-cold PBS (200g for 5 min) and resuspended in 100 μl PBS. If cell preparations could not be run on the flow cytometer immediately, 100 μl of 2% w/v paraformaldehyde was added to each tube while gently vortexing and cells were stored at 4°C until analysis.

Assay Rationale

Figure 1 provides an overview of the assay system. A significant feature of a flow-cytometric-based assay is the ability to measure circulating NABs (indirect) and also antibodies bound to patient neutrophils (direct). The indirect procedure, described exclusively in the present communication, is a measure of the free-circulating antineutrophil immunoglobulins which bind to control leukocytes. The direct assay allows the determination of leukocyte-bound immunoglobulins on the patient's leukocytes. The direct path in Figure 1 begins by binding of the fluorescent label to the patient leukocytes followed by cytometric procedures. Because of the unique ability of flow cytometry to measure individual cell characteristics, sufficient leukocytes can be obtained from very small quantities of blood from neutropenic patients.

Calibration and Data Reduction

Figure 2 describes the pathway from calibration of the flow cytometer to the analysis and interpretation of data by three alternate methods. To ensure identical cytometer operating conditions, the mean fluorescence histrogram of a calibration bead was placed at a preset value. Controls (known negative and positive NAB sera) were run to validate the assay setup procedure. Patient samples were then run, followed by data analysis. Figure 2 indicates there are at least three options (A, B, or C) for data analysis of fluorescence histograms where quantitative assessment is desired.

Measurements were recorded as the mean fluorescence channel number (CH#) between CH#1 and 255 of the ILGF histogram. Sera lacking NAB resulted in very low mean channel numbers, usually between CH#1 and 10. It was usually necessary to collect log, rather than lin-
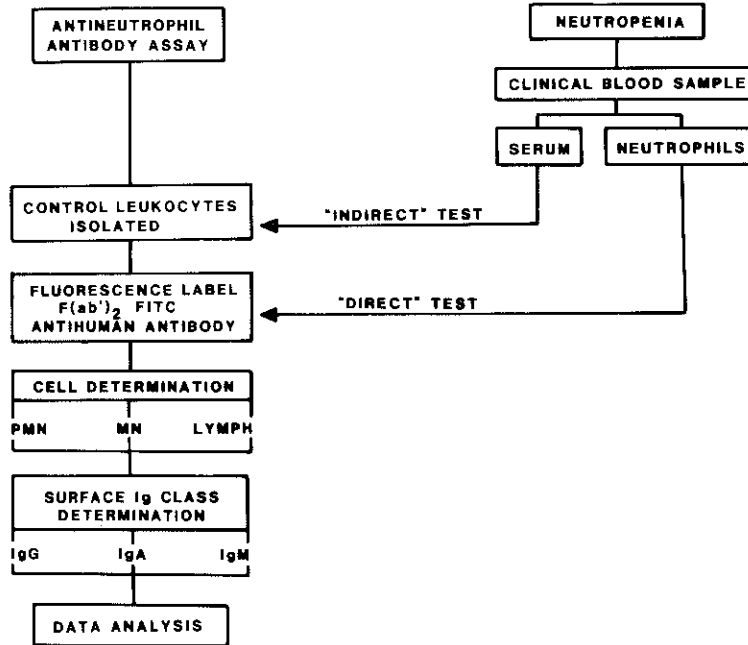
Fig. 1. An overview of the antineutrophil antibody (NAB) assay showing the measurement of serum NABs by using the direct test described in this report. Flow cytometry also provides the ability to utilize minute quantities of neutropenic blood to measure membrane-bound NAB on patient leukocytes using a direct form of the assay.

ear signals because of the large range of fluorescence distributions for these cell populations. Everything below CH#10 was defined as negative; thus a mean fluorescence histogram generated between CH#10 and 255 gave the relative brightness of positive cells. Multiplication of the percent positive times the mean channel number gave a number which was an effective estimate of positivity [12] which we have called a relative fluorescence value (RFV). Due to occasional variations in cell source, technician performance or cytometer alignment, this did not always occur. When negative control mean CH# was > 10, a normalization procedure for control fluorescence histograms was performed. A transformation algorithm was used to transform all histograms of a particular assay relative to the negative control. By translocating the fluorescence histogram distributions so that 90% of the control cells were below CH#10, and then translocating all other histograms relative to that transformation, data from day to day were directly comparable. Alternatively, the RFV was determined by simply excluding 90% of control cells and using that channel number as the cutoff for negative/positive fluorescence, a common method presently employed with flow cytometric immunofluorescence assays.

The third alternative for analysis of fluorescence histograms (Fig. 2C) was a linear transformation method which utilized the difference between negative controls and patient samples to provide an index which was a measure of the increase in fluorescence brightness of un-

known samples over controls [13]. This was determined by measuring, on a linear scale, the mean channel number of a standard bead and then increasing the PMT high voltage until the channel number doubled, representing a doubling in fluorescence. By measuring the fluorescence on a logarithmic scale simultaneously, we calculated that a doubling in fluorescence on the EPIC V flow cytometer was represented by a change of 25.2 logarithmic channels. The relative fluorescence index (RFI) was then used to convert the log green fluorescence signal into a linear scale by the equation $RFI = 2 \exp ((p-c)/25.2)$ where p is the patient sample mean log fluorescence CH# and c is the negative control mean log fluorescence CH#.

Clinical Study

For data presented hereafter, sera taken from 25 healthy adult males were used as the negative controls. Undiluted sera were used repeatedly to establish the standardization procedures for the flow cytometer. A known positive serum (determined by opsonic assay procedure) was initially used to discriminate between negative and positive fluorescence. Heat inactivation of sera did not alter results of a range of negative and positive sera which were compared. Subsequently, sera were not heat inactivated prior to assays. Ninety-two sera were available from patients who were neutropenic and whose clinical picture was suggestive of possible immune neutropenia.
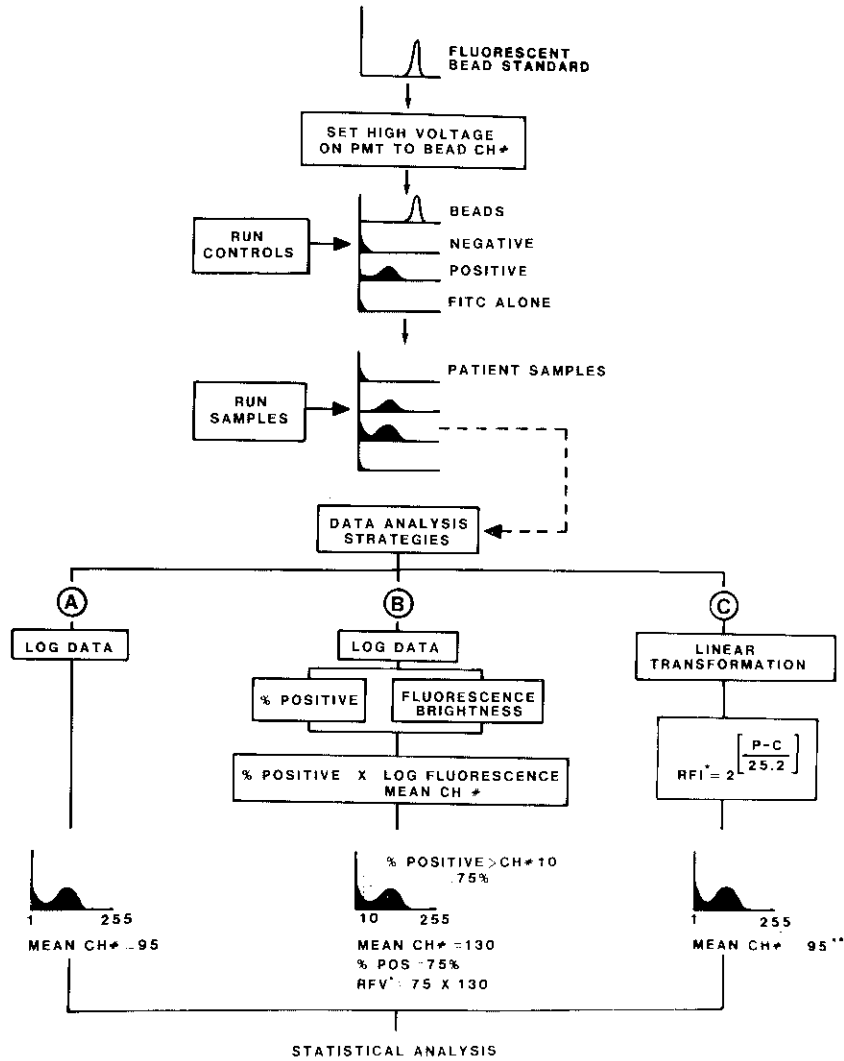
Fig. 2.   RFI = relative fluorescence index, p = positive sera, c = negative control sera (ILGF values). RFV = relative fluorescence value. ILGF = integral log (scale) green fluorescence (the rationale behind the quantitative assessment of NAB using flow cytometry). Method B was used for quantitation of data; however, two alternate methods, A and C, are shown.

RESULTS

Flow Cytometric Determination of NAB

Figure 3A shows the fluorescence histograms of neutrophils incubated with several of the negative control sera. Greater than 90% of neutrophils, monocytes, and lymphocytes demonstrated no measurable fluorescence. Sera from 25 normal controls were used to determine normal ranges for each antibody class for three cell types: neutrophils, monocytes, and lymphocytes. Any samples with an RFV greater than two SDs from the mean were considered positive.

Several sera were borderline positive (Fig. 3B) due to a low percent positive fluorescence in the neutrophil gate and low mean fluorescence intensity. These would be considered as sera from patients with possible immune neutropenia. Usually, less than 50% of the target neutro-

phils were positive in borderline sera. The flow cytometric data from a number of IgG NAB-positive sera are shown in Figure 3C. There was significant variation in the fluorescence histograms (log green fluorescence), although all sera described in this figure stained greater than 90% of the neutrophils.

Of the 92 patient samples tested in the present study, 38 or 41% demonstrated some NAB positivity for any immunoglobulin type against neutrophils. Figure 4 demonstrates the typical ranges of fluorescent histograms seen for neutropenic patient sera for class IgG, IgA, and IgM antibodies.

Table I shows the normal ranges calculated for use in determining positive antibody response to neutrophils. Table I also provides an analysis of four sera from neutropenic patients. Three sera (patients 1, 2, and 4) were positive for IgG against neutrophils. All four demon-
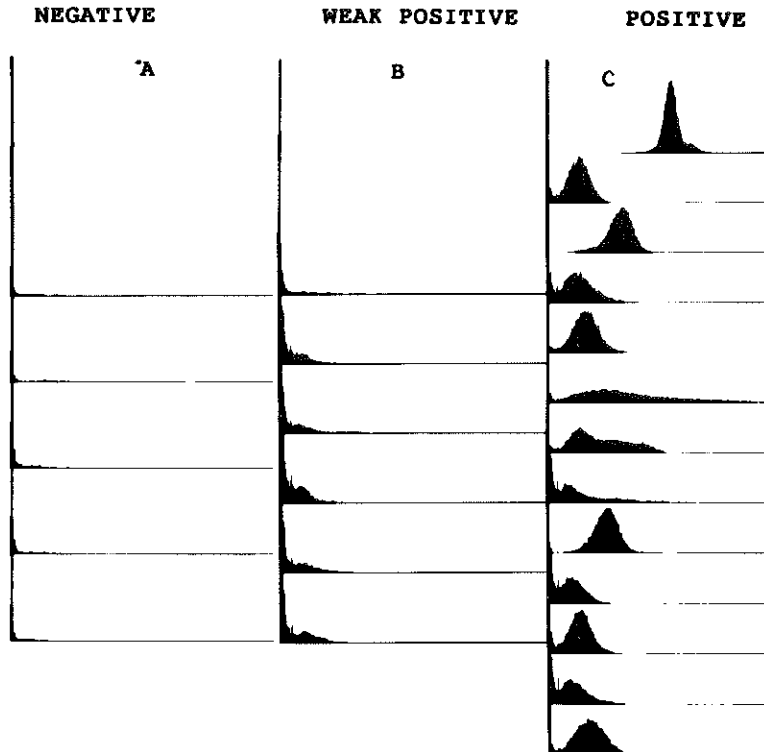
NEGATIVE   **WEAK POSITIVE**   **POSITIVE**

Fig. 3.   A: Fluorescence histograms showing five representative neg-
ative control sera. B: Six sera which exhibited borderline positivity.
C: Thirteen positive NABs containing sera. Measurements are all
ILGF of gated neutrophils with cell number on the ordinate.

strated neutrophil positivity when stained with an anti-
serum against total immunoglobulins. High background
values made use of this reagent of little value and no fur-
ther data are presented using this reagent. Lymphocyte
populations were IgM positive in three cases (patients 1,
3, and 4) and in one case for IgG (patient serum 4). One
serum (patient 3) was negative for IgG but positive for
IgM. This patient (SP) was tested several times over a 5-
month treatment period during which the patient received
IV gammaglobulin and a 10-day course of antithymocyte
globulin. At times, neutrophils were positive for both IgM
and IgG NAB.

Comparison With the Opsonic Assay

Test sera from 34 patients were evaluated for circulat-
ing NABs by using both the opsonic assay and the flow-
cytometry-based fluorescence assay. There was agree-
ment between 16 samples. Thirteen samples were found
to be positive and three samples were negative in both as-
say systems (Table II). Nine samples were positive with
the fluorescence assay but negative with the opsonic as-
say. Similarly, nine other sera were positive by the op-
sonic assay while negative by fluorescence.

Use of Frozen Neutrophil Targets

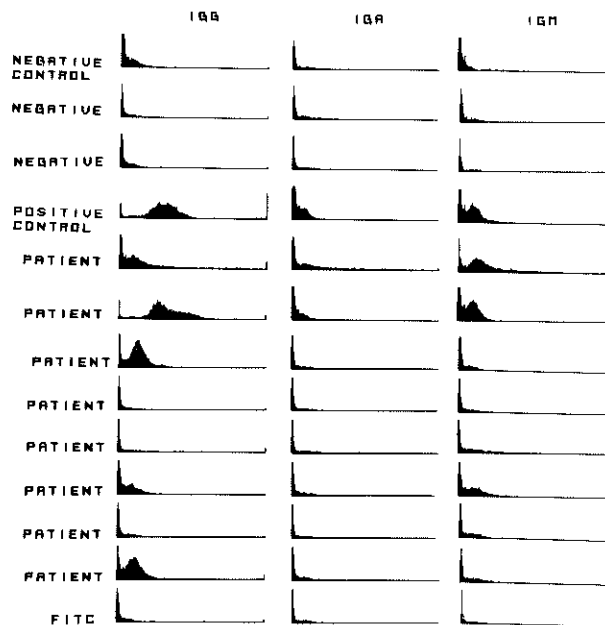It would be advantageous to utilize a constant source

Fig. 4.   A typical panel of control and patient sera measured for three
classes of NAB—IgG, IgA, and IgM. All histograms are of the
neutrophil population and are ILGF. Four of the eight patient sera
tested positive for IgG NABs, two for IgA NABs, and three for IgM
NABs. Ordinate values are cell numbers with ILGF on the abscissa.

TABLE I. Values From a Series of Normal Sera From Controls (12 samples) and From 4 Neutropenic Patients*

	IgG			IgA			IgM		
	N	M	L	N	M	L	N	M	L
Control values									
	676	778	357	890	659	233	1,094	4,844	663
SD	468	589	243	304	422	54	711	3,634	325
Patient sera									
1.	13,300	3,168	160	4,960	920	150	944	363	1,450
2.	6,960	3,168	147	900	175	299	1,705	7,335	912
3.	966	4,710	386	1,220	2,190	371	2,830	7,163	1,448
4.	7,140	11,528	908	804	580	191	11,160	10,368	2,988

*Shown are the values obtained from a series of normal sera from controls (12 samples). Each value is the mean for an individual cell type when tested against each fluorescent antibody. Included are the values for the standard deviation from the mean for each control. The second group of data represent four patients' sera. Data from four neutropenic patients (1–4) were derived by using the calculation method (B) from Figure 2. Each datum is the product of the mean channel number of the fluorescence histogram and the percent positive cells greater than CH#10 (RFV). Values underlined were positive based upon being more than 2 standard deviations from the appropriate control values shown at the top of Table I. N = neutrophils; M = monocytes; L = lymphocytes.

TABLE II. Comparison Between the Opsonic Assay [7] and the Fluorescence Assay*

	Positive opsonic	Negative opsonic
Positive fluorescence	13	9
Negative fluorescence	9	3

Sixteen of 34 samples produced similar results. Eighteen samples (53%) produced different results. Of 22 samples positive with the opsonic assay, nine were negative with the fluorescence assay. Similarly, nine samples were determined positive by fluorescence but negative by the opsonic assay.
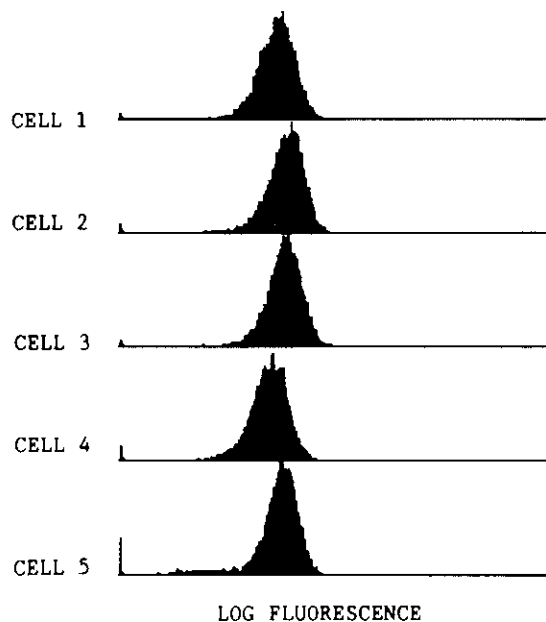

Fig. 5. Five different donor populations of neutrophils were tested with the same positive NAB serum. Very little variation was observed with target cells from different normal donors, when tested with the same serum samples on the same day.

of neutrophils, thus reducing the number of variables. An attempt was made to freeze leukocyte suspensions at a controlled rate by using standard cryopreservation methods and store them in liquid nitrogen. Leukocyte suspensions were resuspended in RPMI 1640 medium containing 10% DMSO and 10% FCS. Thawing was rapid at 37°C; leukocytes were washed and resuspended in PBS. The results indicated that neutrophils often stained positive when incubated with negative control sera. In addition, a significant reduction in neutrophil number and 90° light scatter was observed compared with freshly prepared cells normally indicating structural damage of the neutrophil (data not shown).

Presence of NABs in Sera From Transfused Patients

Four sera from patients who had received multiple blood transfusions demonstrated some variation in NABs. Two of the four showed positivity with IgG class NABs and two were negative. No data were collected for monocytes or lymphocytes.

Titration of Positive Sera

We titrated some positive sera to determine the optimum dilution for use in the fluorescence assay. Two

highly positive sera were titered with twofold serial dilutions to 1:32 dilution. With increasing dilution to 1:8, the mean channel of the fluorescence increased without any alteration in the percentage of positive cells. After 1:8 dilution the mean channel of fluorescence of positive cells was reduced. For positive NAB sera which also stained positive for monocytes, a similar result was observed. Mean fluorescence increased with dilutions down to 1:8 and fluorescence reduced thereafter. No positive histograms were observed with lymphocytes at any dilution.

Variability in Random Donor Target Neutrophils

In order to determine the effect of different donor control neutrophils, five negative and five positive sera were

run simultaneously on five different donor cells. Mean fluorescence histograms were almost identical despite the different donor cells. Similarly, negative control sera fluorescence histograms demonstrated a high degree of reproducibility with different donor cells when run at the same time. Negative fluorescence histograms were observed for all monocyte and lymphocyte populations with these sera (Fig. 5).

DISCUSSION

This is the first systematic study of neutrophil antibodies measured on neutrophils while simultaneously monitoring the presence of antibodies on monocytes and lymphocytes using flow cytometry. We have found that we can rapidly determine the presence of neutrophil antibodies (NABs) in sera by using the flow-cytometric immunofluorescence method. In addition, we have observed positive fluorescence on monocytes but infrequently on lymphocytes.

The basis for our selection of positive sera controls for this study was determined by previous measurement with the opsonic assay [7]. The results of our comparison between the opsonic assay and the fluorescence assay poses a number of possibilities. There was failure in agreement in 53% of the sera tested in both flow cytometry and functional opsonic assays. In part, an explanation for the failure of the opsonic assay to detect some NABs may relate to insufficient amounts of opsonins on the surface of the neutrophils or an activation of complement during the transportation of serum samples. On the other hand, the opsonic assay may have detected either antibodies or immune complexes that could have fixed complement to the neutrophil surface which would promote phagocytosis but not be detected by immunofluorescence. Both the antibodies and immune complexes have a low affinity for the neutrophils and dissociated rapidly from the surface of the neutrophil, but still retain the ability to fix complement. Under these conditions, the flow-cytometric assay would not detect antibody on the surface of neutrophils unless it were developed to detect C3 fixation. Therefore it may be important to monitor this important phagocytic opsonin in the flow cytometric assay. Others have shown that under some circumstances NABs are indeed capable of fixing C3 onto the surface of neutrophils [14]. Clearly, the implementation of C3 into the flow cytometric assay may have significant advantages, particularly since a number of false negatives may be determined without the measurement of C3.

The present approach was to further develop an assay suitable for use in a laboratory with flow-cytometric facilities—thus the use of an indirect fluorescence (IF) assay. Previous attempts at using IF for detection of antineutrophil antibodies have identified the major problem as being unacceptably high backgrounds for negative control sera [10,15]. Fixed cells and F(ab')$_2$ FITC-

labeled second antibody were used to rectify these problems.

There are several advantages in the determination of antineutrophil antibodies by the present method over other methods. The use of lysed whole blood significantly reduces the time and effort required for the isolation of control cells. An added benefit is the ability to examine antibody levels on both monocytes and lymphocytes simultaneously with neutrophils. This is accomplished by electronic gating of the different cell populations based on light-scatter properties. Concomitant with this is the use of different class-specific FITC-labeled antisera, which allows the detection of all classes of antibodies. Although there appear to be few reports on IgM NABs, we have detected several. Previous reports have indicated a role for IgM antibodies in red blood cell cold agglutinating and lymphocytotoxic activities [16] and platelet antibodies [17].

Most techniques do not establish class specificity of NABs. In several cases in the present study, a high IgM antibody level was accompanied by positive IgG antibody. These sera were from a single patient and were collected over a prolonged period of time. We were able to observe variations in the levels of NABs over time. When IgG antibody level was very high, a low level of IgM NABs was often detected. The significance of this finding remains to be determined. However, it should be noted that IgM-related fluorescence could be due to IgM rheumatoid factor bound to IgG on the neutrophil surface. This possibility needs to be excluded in interpretation of sera which gives high IgM and IgG fluorescence. Similarly, the possibility that high levels of immune complexes may interfere with this assay has not been discounted.

The inclusion of IgD NABs may be a useful control for background since no fluorescence was ever associated with IgD. We dropped IgD from our routine protocol after these initial findings. There is no evidence for IgD NABs in the literature. Use of antisera specific for total immunoglobulins resulted in abnormally high background fluorescence levels which rendered it unsuitable as a screen indicator for the presence of NABs.

Antibodies against neutrophils are of primary importance when testing for the presence of NABs in sera from neutropenic patients. We have observed that in some instances, antibodies against mononuclear cells can also be detected. With some sera with high IgG serum antibody levels on neutrophils, antibodies of the same class can be detected on monocytes. Since the patients from which the sera were taken were neutropenic but not monocytopenic, it is not clear as to the meaning of this observation. A single case was observed where positive levels of IgG and IgA were detected on monocytes but not on neutrophils, although positive levels of IgM were detected on the same neutrophils. The presence of NABs on lympho-

cytes could have been due to some monocyte contamination into the lymphocyte light-scatter gate since it is difficult to totally eliminate all monocytes without a further cell purification step or the use of multicolor flow-cytometric gating techniques. On most occasions where antibody was detected on lymphocytes, there was a corresponding presence on monocytes. No previous studies have associated neutrophilic antibodies with monocytes or lymphocytes. Positive measurements with lymphocytes may also be a valuable control for the presence of HLA antibodies. In most of our studies lymphocytes consistently stained negative for NABs. Positive results may indicate HLA incompatibility between the random donor cells and the patient's serum. This requires further investigation.

A major difficulty with many NAB assays is quantitation. This problem is also common to flow-based IF assays. Recently, Weeks et al [13] presented an elegant solution which we have used as a basis for discrimination between positive and negative NABs. A significant advantage is that serum samples from a patient taken over a long time period can be accurately compared. This is particularly useful if monitoring a regimen for reversal of neutropenia [18]. Alternatively, by using a value such as the RFV described, both the density of fluorescence marker as well as the percentage of positive cells is taken into consideration.

The development of a pooled preserved target cell reagent might be of additional practical value in these assays, ensuring minimum day-to-day variation. It would also ensure that all possible epitopes involved in NAB production were on the targets. However, our data obtained by using the frozen cell preparation indicated that damage probably occurred to the neutrophil membrane resulting in false positives.

The combination of automated cytology, class-specific fluorescent antisera, and a quantitation algorithm make the present assay highly suitable for the routine detection of antineutrophil antibodies. It would be particularly useful if assessment were desired using autologous cells from neutropenic patients where few cells are available.

ACKNOWLEDGMENTS

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