Identification of Neutrophil Subpopulations
With Monoclonal Antibodies

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Two monoclonal antibodies have been produced by the hybridoma technique that recognize subpopulations of human neutrophils. The antibodies, termed 1B5 and 4D1, react with a mean percentage of 57% and 51% of peripheral blood granulocytes, respectively. The antigens recognized appear to be neutrophil specific in that these antibodies do not react with eosinophils, platelets, erythrocytes, monocytes, or nonadherent peripheral blood mononuclear cells. Although the neutrophil subpopulations recognized by these antibodies are nearly identical (coinclusive), the antigenic determinants recognized appear to be different. These monoclonal antibodies to neutrophil subpopulations may prove useful to studying functional heterogeneity among neutrophils as well as for investigations of normal and abnormal myeloid differentiation.

PERIPHERAL BLOOD NEUTROPHILS perform a number of diverse functions that are important in maintaining the integrity of host defenses against microbial infections and in developing an inflammatory response. In many instances, the functional response of neutrophils to activating agents has been linked to the binding of a particular ligand to a specific receptor on the neutrophil membrane, thereby inducing an activating signal that can result in chemotactic mobility, adherence to surfaces, phagocytosis, degranulation, and the generation of superoxide anion and peroxide. Hence, the characterization of neutrophil membrane components can provide important insight into the mechanisms by which neutrophils are functionally activated.

Although their functional capabilities are diverse, neutrophils have generally been considered to be a relatively homogeneous cell population. However, studies from several laboratories have recently indicated that functional heterogeneity can be demonstrated among neutrophils. Furthermore, the observed functional heterogeneity was in some instances linked to differences in membrane receptor expression, indicating that membrane markers might be useful in defining neutrophil subpopulations.

The recent development of monoclonal antibody production by the hybridoma method has proven to be a powerful tool for obtaining monospecific antibodies to cell surface antigens that identify cell subpopulations. We have used this approach for the study of neutrophil heterogeneity and report the production of two monoclonal antibodies that react with subpopulations of neutrophils. These antibodies thus provide direct evidence that there is phenotypic heterogeneity of membrane composition among human neutrophils. This heterogeneity provides a basis for the identification of membrane markers for human neutrophil subpopulations.

MATERIALS AND METHODS

Isolation of Peripheral Blood Cells

Heparinized peripheral venous blood specimens were obtained from normal volunteers with informed consent in accordance with an institutionally approved protocol. Peripheral blood mononuclear cells (PBMC) containing the lymphocytes and monocytes were isolated by density gradient centrifugation over Ficoll-Hypaque. Monocytes were isolated from the PBMC by adherence to plastic tissue culture plates at 37°C for 60–90 min in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, N.Y.). After vigorous washing with cold medium to remove nonadherent cells, monocytes were harvested with a rubber policeman.

Granulocytes were isolated as previously described. Briefly, following Ficoll-Hypaque separation and removal of mononuclear cells as described above, the erythrocyte-granulocyte pellet was resuspended in phosphate-buffered saline (PBS) and mixed with an equal volume of 3% dextran in saline. Following sedimentation of the bulk of the erythrocytes, the remaining erythrocytes were lysed by osmotic shock and the nucleated cells were washed twice with PBS. The purified granulocyte fraction contained 91%–94% neutrophils, 5%–6% eosinophils, and 1%–3% mononuclear cells.

For preparation of purified eosinophils, the blood of a patient with neutropenia was processed as described above, but the purified granulocyte population contained 89% eosinophils and 11% neutrophils.

Platelets and erythrocytes were obtained from heparinized peripheral blood. Following centrifugation at 900 rpm, platelet-rich plasma was removed, diluted 1:50 with RPMI 1640, and cytocentrifuge slides of the platelets were prepared. In some experiments, platelets were briefly fixed in cold ethanol-acetic acid before immunofluorescent staining. Erythrocytes were isolated by repeated washing of the cell fraction with removal of the buffy coat following each centrifugation.

Isolation of Thymocytes

Thymocytes were isolated from thymus tissue removed from patients undergoing cardiac surgery. The tissue was minced with...
scissors and forceps in RPMI 1640, and a single cell suspension was obtained by passage of this medium through a fine-mesh nylon screen.

**Cell Lines**

All cell lines used in these studies, kindly provided by Dr. Charles Balch, were maintained by serial passage in RPMI 1640 medium supplemented with glutamine, penicillin, streptomycin, and 10% FCS. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

**Monoclonal Antibody Production**

Monoclonal antibodies were produced by a modification of previously published methods. Female BALB/c mice were immunized with 20 × 10⁶ granulocytes in normal saline by the intraperitoneal route. Mice were given three booster immunizations in similar fashion at 2-wk intervals. Two days after the final injection, the spleen was removed and a single cell suspension was prepared. Somatic cell hybridization of the spleen cells with nonsecretor P3/NS1/1-Ag4 myeloma cells was accomplished with 37% polyethylene glycol (PEG) 4000. Cells were then cultured at 2 × 10⁶/ml in 24-well cluster plates (Costar, Cambridge, Mass.) in hypoxanthine-aminopterin-thymidine (HAT) selection medium at 37°C in a humidified atmosphere containing 7% CO₂. All cultures contained mouse peritoneal washout feeder cells at a concentration of 1–2 × 10⁶/ml. Cultures were fed fresh aminopterin-free medium at 7-day intervals.

Once hybridoma selection and growth was accomplished, culture supernatants were harvested and screened by indirect immunofluorescence using a fluorescent-activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, Calif.) equipped with a logarithmic amplifier. Peripheral blood granulocytes and PBMC were isolated, and 5 × 10⁶ cells of each population were treated with 100 μl of each culture supernatant. Following a 20-min incubation on ice, cells were washed twice, then incubated with 10 μl of a 1:40 dilution of F(ab')₂ fragments of fluorescein-conjugated goat anti-mouse immunoglobulin (Cappel, Cochranville, Pa.). Cells were again washed, then analyzed for fluorescent staining with the FACS. Those wells having supernatants with antibodies reacting with granulocytes but not PBMC were selected, and the hybridoma cells were cloned by limiting dilution in 96-well microtiter plates using RPMI 1640 medium containing penicillin, streptomycin, glutamine, 15% FCS, and peritoneal feeder cells as before. Wells with monoclonal cell growth were again analyzed by indirect immunofluorescent staining of granulocytes and FACS analysis. The monoclonal hybrids were then expanded in vitro for the production of antibody-containing supernatants. Hybridoma cells were also injected into pristane-primed BALB/c mice for the production of antibody in ascitic form.

**Antibody Characterization**

The heavy chain isotype and light chain type of the monoclonal antibodies from culture supernatants were determined by a solid-phase ELISA as previously described using alkaline phosphatase-conjugated, affinity-purified goat antibodies specific for murine μ, γ₁, γ₂a, γ₂b, or γ₃ heavy chain isotypes and κ or λ light chains.

**Immunofluorescent Analyses and Cell Sorting**

Analyses of the reactivity of monoclonal antibodies with various cell lines and other cell types were performed by FACS analysis. Fluorescence was excited using the 488 nm line from an argon ion laser operated at 400 mW. The photomultiplier tube (operating voltage 550 V) signals were amplified logarithmically (T. Nozaki, Stanford, Calif.) and displayed on the 256-channel analyzer. Optical filters consisted of a 520-nm long-pass interference filter, a 535-nm long-pass yellow glass, and a 560-nm short-pass filter. Reactivity was also confirmed by standard fluorescent microscopy. For morphological assessment of cells comprising the subpopulations, granulocytes were stained by indirect immunofluorescence, then sorted into positive and negative fractions with the FACS. Cytocentrifuge slide preparations of cells from each fraction were prepared and stained with Wright's stain or Giemsa and May-Grünwald stains.

**Biochemical Characterization of the Membrane Antigens**

Granulocytes (7 × 10⁶) were surface labeled with ¹²⁵I by the lactoperoxidase-catalyzed method of Goding. The radiolabeled cells were lysed by addition of 1 ml of 1% Nonidet P-40 (NP-40; BDH Chemicals Ltd., Poole, England) in 0.15 M NaCl, 0.02% NaN₃, 0.05 M Tris-HCl, pH 7.5, for 30 min on ice, and the nuclei and debris were removed by 15-min centrifugation at maximum speed in an Eppendorf microcentrifuge. A preliminary clearing precipitation was performed by adding to the lystate 25 μl of goat anti-mouse immunoglobulin, followed by 250 μl of a suspension of heat-killed, Protein-A-bearing S. aureus (Pansorbin, Calbiochem-Behring Corp., La Jolla, Calif.). After incubating 30 min on ice, the lystate was centrifuged to remove the S. aureus. The supernatant was then divided into 3 aliquots, which were treated with 50 μl of hybridoma culture supernatants containing either IB5, 4D1, or negative control antibody. After 30 min on ice, 100 μl of the Pansorbin suspension was added and incubated an additional 30 min. The suspension was then centrifuged, and the pellets were washed twice with 0.05 M Tris-HCl, pH 8.0, containing 0.4 M NaCl, 1% deoxycholate, 1% NP-40, and 0.1% SDS (Bio-Rad Laboratories, Richmond, Calif.), and then once with 0.02 M Tris-HCl, pH 8.0. The bound antigen-antibody complexes were removed by boiling the pellets for 5 min in 2% SDS with or without 0.05% 2-mercaptoethanol, and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5%–12% acrylamide gradient gel according to the method of Laemmli. Following electrophoresis, gels were analyzed by autoradiography. Bovine serum albumin, ovalbumin and chymotrypsinogen were used as molecular weight markers.

**RESULTS**

**Monoclonal Antibodies Recognize Neutrophil Subpopulations**

Although most of the monoclonal antibodies recognizing antigenic determinants on granulocytes reacted with virtually all of the cells in this population, two monoclonal antibodies, hereafter termed IB5 and 4D1, were consistently noted to react with distinct subpopulations of granulocytic cells. These results are depicted in Fig. 1. IB5 reacted with 55% of the peripheral blood granulocytes, while 4D1 reacted with 49% of these cells. These findings were confirmed in comparable analyses of granulocytes isolated from the peripheral blood of 14 additional people, as IB5 reacted with 49%–61% of the neutrophils from these donors, while 4D1 recognized 44%–55%. No differences were noted between results obtained using conventional immunofluorescent microscopy and those obtained using the
FLUORESCENCE INTENSITY

1B5 4D1

Fig. 3. 1B5 and 4D1 do not react with eosinophils. A granulocyte preparation containing 89% eosinophils and 11% neutrophils was stained with 1B5 or 4D1, then analyzed by FACS. Both antibodies react with 6% of the cells in this preparation, all of which were neutrophils. The unstained cells contain the remaining neutrophils (5% of the total granulocyte population) and all eosinophils.

FACS. Furthermore, the consistent demonstration of neutrophil subpopulations with these antibodies could not be attributed to any reduction of reactivity imposed by limiting antibody concentrations, since identical results were obtained using a tenfold excess of antibody (in ascitic form) over that shown to be saturating (data not shown). Hence, the monoclonal antibodies 1B5 and 4D1 recognize antigenic determinants present on distinct subpopulations of granulocytes that were present in all persons tested.

The Antigens Recognized by 1B5 and 4D1 are Neutrophil Specific

Because many antigens on myeloid cells have also been found on monocytes or macrophages, culture supernatants from the monoclonal hybridomas were reanalyzed for reactivity with either nonadherent PBMC or with monocytes isolated by adherence, and these results are shown in Fig. 2. Both 1B5 and 4D1 failed to stain the nonadherent PBMC, which are predominately lymphocytes, or the adherent monocyte population. Similarly, both antibodies did not react with human thymocytes (data not shown).

A number of cell lines were also tested with both monoclonal antibodies. Neither 1B5 nor 4D1 showed any reactivity with T-cell lines (MOLT-3, MOLT-4, and HSB), B-cell lines (Raji, Daudi, SB, and WIL2), a histiocytic cell line (U937), a promyelocytic line (HL-60), or an erythroleukemia-derived line (K562).

Initially, attempts were made to assess the reactivity of 1B5 and 4D1 with eosinophils by sorting immuno-fluorescent-stained granulocyte samples into positive and negative cell fractions with the FACS and analyzing Wright’s stained preparations of each for the presence of eosinophils. Although neither of these antibodies appeared to react with eosinophils, the small number of these cells (<6%) in the starting granulocyte samples made it difficult to obtain conclusive data regarding this question. The opportunity to study eosinophils in a more definitive fashion was provided by a granulocyte preparation from a patient with unexplained neutropenia, which contained 89% eosinophils and 11% neutrophils. Immunofluorescent analysis of these cells, shown in Fig. 3, revealed that both 1B5 and 4D1 failed to react with 94% of the cells. In both instances, the 6% of the granulocytic cells that were recognized by the monoclonals were shown to be neutrophils by Wright’s staining of the positive cells. The remaining neutrophils (representing 5% of the total granulocyte population) and all eosinophils were present in the unstained cell fraction. Consequently, these monoclonals again recognized a subpopulation of neutrophils but did not react with the eosinophils of this patient. Although we cannot be certain that the eosinophils from this patient were normal in their membrane composition, the apparent nonreactivity of these antibodies with eosinophils from several normal donors previously cited suggests that 1B5 and 4D1 do not react with peripheral blood eosinophils.

These monoclonal antibodies were also tested on
platelets by immunofluorescent staining and did not show detectable reactivity. Similarly, both antibodies were tested against erythrocytes by both indirect immunofluorescent staining with FACS analysis, as well as by hemagglutination (with or without the addition of rabbit anti-mouse immunoglobulin antiserum as an enhancing reagent). In both assays, neither antibody could be shown to react with erythrocytes. Consequently, in the case of peripheral blood cells, IB5 and 4D1 appear to be neutrophil specific.

The characterization of antibodies IB5 and 4D1 and their reactivity with peripheral blood cells and cell lines is summarized in Table 1. IB5 is a γ_{2a}, κ antibody that reacts with 49%–61% of peripheral blood granulocytes (mean 57%). It does not react with eosinophils, platelets, erythrocytes, thymocytes, monocytes, or other PBMC, nor with any of the cell lines tested. 4D1 is a γ_{1}, κ antibody that recognizes 44%–55% of peripheral blood granulocytes (mean 51%) but is not reactive with eosinophils, platelets, erythrocytes, thymocytes, PBMC, or the cell lines.

**1B5 and 4D1 React With Nonidentical Antigenic Determinants Present on Similar Neutrophil Subpopulations**

Because neutrophils have generally been considered to comprise a relatively homogeneous cell population, the production of two hybridoma clones secreting antibodies to neutrophil subpopulations raised several questions. First, since both monoclonal antibodies reacted with approximately 45%–60% of peripheral blood neutrophils, it was possible that these antibodies both reacted with a single membrane antigen on one neutrophil subpopulation, i.e., that both IB5 and 4D1 recognized the same cell surface component. Alternatively, it was also possible that IB5 and 4D1 recognized different membrane antigens serving as markers for separate neutrophil subsets. Furthermore, these subsets could be mutually exclusive, could overlap to a variable extent, or could be virtually identical, i.e., both markers could be present on the same cells.

Several experiments were performed to investigate these possibilities. First, to assess the degree of overlap of the neutrophils recognized by IB5 and 4D1, double fluorescent staining experiments were performed. Neutrophils were sequentially treated with monoclonal IB5 culture supernatant, fluorescein-conjugated affinity-purified goat anti-mouse γ_{2a} antibodies, 4D1 culture supernatant, then affinity-purified rhodamine-conjugated goat antibodies specific for mouse γ_{1} antibodies. The neutrophils were then examined for double staining by immunofluorescent microscopy, which revealed that >90% of the neutrophils stained with 4D1 were also recognized by IB5. Conversely, >85% of the neutrophils stained with IB5 were also recognized by 4D1 (data not shown). Similar results were obtained when neutrophils stained with IB5 and fluorescein-conjugated antibodies to mouse immunoglobulin were separated into positive and negative fractions with the FACS, then treated with 4D1 and the rhodamine-conjugated antibodies against the γ_{1} subclass. Hence, the neutrophil subpopulations recognized by IB5 and 4D1 show a very high degree of overlap.

Nonetheless, several lines of evidence suggested that IB5 and 4D1 recognized different antigenic determinants present on neutrophils rather than sharing reactivity against a common antigenic epitope. First, in all granulocyte samples tested, IB5 consistently reacted with a higher percentage of the cells than did 4D1 (mean difference 6%, with a range of 4%–10%). Secondly, clear differences were noted in the reactivity of IB5 and 4D1 against neutrophils that were fixed in 95% ethanol–5% acetic acid prior to immunofluorescent staining, as 4D1 reacted brightly with fixed neutrophils, whereas IB5 reacted significantly less well. More direct evidence was provided by experiments designed to assess the inhibition of binding of one antibody by pretreatment with the other mono-

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**Table 1. Reactivity of IB5 and 4D1 With Peripheral Blood Cells and Cell Lines**

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*Heavy chain isotype and light chain type determined by solid-phase ELISA.
†Values shown are the mean percent from 15 individuals with the range in parentheses.
clonal reagent. In one representative experiment, shown in Fig. 4, neutrophils were pretreated with a substantial excess of 1B5 in ascitic form (Fig. 4A) or with a comparable, nonimmune control ascites (Fig. 4B). After washing, each sample was stained with 4D1 and fluorescein-conjugated goat anti-mouse γ1 antibodies, then analyzed with the FACS. It is evident that pretreatment of the cells with 1B5 does not reduce the subsequent staining intensity of 4D1 with the neutrophils. Similar results were obtained with neutrophils pretreated with 4D1 and subsequently analyzed with 1B5 and fluorescein-conjugated goat antibodies to murine γ2a antibodies (data not shown). While these data do not exclude the possibility that these two antibodies recognize separate antigenic epitopes present on the same membrane molecule, they do strongly suggest that 1B5 and 4D1 recognize different antigenic determinants that are expressed to a large extent on the same neutrophils.

**Biochemical Characterization of the Membrane Antigens**

To analyze the membrane components recognized by these monoclonal antibodies, granulocyte membrane components were surface radioiodinated by the lactoperoxidase method. Following detergent solubilization, aliquots of the lysate were treated with 1B5 or 4D1 and *S. aureus*, Cowan I strain, to precipitate immune complexes. The isolated antigens were then analyzed by SDS-PAGE (under reducing and nonreducing conditions) and autoradiography. The results under reducing conditions are depicted in Fig. 5. 4D1 precipitates a molecule with a molecular weight of approximately 59,000 daltons (under both reducing and nonreducing conditions). In contrast, no radioiodinated antigen could be demonstrated from 1B5-treated samples.

There are several possible explanations for our inability to demonstrate radioiodinated antigens recognized by 1B5. First, it is known that lactoperoxidase-catalyzed surface iodination does not label all membrane components present on the cell exterior. It is possible that the 1B5 antigen is either not a protein or does not have tyrosine residues accessible for surface radioiodination. Consequently, the antigen recognized by 1B5 may not be radioiodinated by this method, and biosynthetic internal labeling techniques may be required to characterize this antigen biochemically. Alternatively, the 1B5 antigen may escape solubilization with the nonionic detergents employed or may be altered in some fashion during solubilization such that it loses its antigenicity. Finally, the monoclonal 1B5 antibodies may have a relatively low affinity for the antigen (before or after solubilization) such that antigen is not retained by the antibody during immunoprecipitation. At present, we cannot distinguish which of these (or other) possibilities might account for this
finding. In conjunction with the previous data, however, the SDS-PAGE differences noted for 1B5 and 4D1 lend support to the conclusion that the membrane antigens recognized by these antibodies are dissimilar.

**DISCUSSION**

Although neutrophils perform a number of diverse functions in response to a wide variety of stimuli, these cells have generally been considered to comprise a relatively homogeneous cell population. In this article, the production of two monoclonal antibodies recognizing neutrophil subpopulations is described. These two antibodies, termed 1B5 and 4D1, react with a mean of 57% and 51% of peripheral blood granulocytes, respectively, but do not react with monocytes, nonadherent PBMC, eosinophils, platelets, erythrocytes, thymocytes, or a panel of cell lines. The antigenic determinants recognized by these two antibodies appear to be distinct from one another, yet are present on subpopulations of cells that show a high degree of overlap. These antibodies thus provide direct evidence of membrane antigen heterogeneity among neutrophils.

Additional evidence supporting the existence of neutrophil heterogeneity has been noted in studies of neutrophil function. For example, whereas all neutrophils from normal donors reduce the dye nitroblue tetrazolium (NBT) following stimulation with phorbol myristate acetate, a significant number of neutrophils fail to reduce NBT following activation with endotoxin or N-formyl-methionyl-leucyl-phenylalanine. Similarly, a sequence of membrane depolarization and subsequent partial repolarization in response to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine is observed in approximately 65% of neutrophils, while the remaining 35% show either hyperpolarization or no change in membrane potential.

Several laboratories have also reported heterogeneity in Fc receptor function or expression by neutrophils. Under appropriate conditions, only 60%-80% of human peripheral blood neutrophils form rosettes with 7S EA (IgG-coated) erythrocytes. These rosette-forming cells are functionally superior in bacterial phagocytosis, bactericidal activity, and chemotaxis in comparison with the nonrosetting neutrophils. Fc receptor expression has also been used to distinguish neutrophils that produce an active form of lactoferrin capable of suppressing the production of granulocyte-macrophage colony-stimulating activity by human monocytes. These reports of functional heterogeneity among neutrophils raise the intriguing possibility that monoclonal antibodies 1B5 and 4D1 may be useful in separating and identifying functionally distinct subpopulations of neutrophils, and studies of the functional capabilities of the subpopulations of neutrophils recognized (or not recognized) by the monoclonal antibodies described herein are in progress.

In view of the limited information available concerning phenotypic heterogeneity of human neutrophils, it is difficult to speculate on the nature or functional significance of the membrane components recognized by 1B5 or 4D1. As previously noted, heterogeneity of neutrophil Fc receptor expression has been reported. We cannot presently exclude the possibility that 1B5 or 4D1 recognizes the neutrophil Fc receptor, although neither antibody reacts with the Fc receptor found on monocytes or B cells. Although other neutrophil-specific antigens have been demonstrated using alloantisera or, more recently, with monoclonal antibodies, these antigens are expressed by virtually all neutrophils and hence are distinct from those recognized by 1B5 or 4D1.

Monoclonal antibodies have proven to be of significant value in studies of the differentiation and function of various mononuclear cell subpopulations. In view of recent reports demonstrating the usefulness of myeloid-specific monoclonal antibodies for effecting the inhibition of neutrophil chemotaxis and degranulation, the availability of reagents recognizing neutrophil subpopulations may prove to be of value in assessing functional heterogeneity among neutrophils. These antibodies may also be useful for studies of myeloid cell differentiation, and investigation of the ontogeny of the subpopulations recognized by 1B5 and 4D1 has begun. Finally, these antibodies may prove to be valuable in the characterization of human myelogenous leukemias, particularly if neutrophils derive from antigenically distinct precursors during myeloid differentiation.

**ACKNOWLEDGMENT**

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Identification of neutrophil subpopulations with monoclonal antibodies

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