A Human Granulocyte-Specific Antigen Characterized by Use of Monoclonal Antibodies

By Keith M. Skubitz, Yong-su Zhen, and J. Thomas August

Hybridoma cell lines secreting monoclonal antibodies that bind to a surface antigen of human neutrophils have been prepared by fusion of mouse myeloma cells with spleen cells from mice immunized with human neutrophils. Several of the monoclonal antibodies (AHN 1-6) were specific for a neutrophil surface antigen and did not bind lymphocytes, monocytes, red blood cells, platelets, or basophils. All of the granulocyte-specific antibodies immunoprecipitated a polypeptide of 145,000 daltons and an isoelectric point of about 4.5 and other heterogeneous polypeptides of 105,000 daltons. These same components were the major lactoperoxidase-labeled proteins precipitated by hyperimmune mouse serum. The antibodies were further characterized for binding to several human myeloid leukemia cell lines and cells from patients with myeloid or lymphoid leukemia. All antibodies bound the HL-60, ML1, ML2, ML3, K562, and U937 myeloid leukemia cell lines. None of the antibodies bound the RPMI 6410, Raji, RPMI 8226, MOLT 4, or Daudi lymphoid cell lines. All of the hybridoma cell lines (AHN 1-6) produced IgM antibodies that were cytotoxic.

MATERIALS AND METHODS

Preparation of Peripheral Blood Cells

Neutrophils were prepared from heparinized (2 U/ml) human venous blood by the previously described modification of the method of Böyum12-13 and were suspended at appropriate concentrations in phosphate-buffered saline, pH 7.4, (PBS) or Hank’s balanced salt solution (HBSS) (M.: A. Bioproducts, Bethesda, Md.) containing 1 mg/ml bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, Mo.) (HBSS-BSA). Differential cell counts performed on Wright’s stained cells revealed greater than 95% neutrophils.

Red blood cells were obtained by centrifuging heparinized venous blood at 400 g for 5 min, removing the upper one-half of the red cell pellet, suspending the remaining pellet with PBS, and centrifuging at 100 g for 5 min.

Lymphocytes and monocytes were prepared by a modification of the method of Böyum.14 Two milliliters of heparinized venous blood was mixed with 2 ml of PBS, layered on 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.), and centrifuged at 400 g for 35 min at 20°C. Cells in the interface were removed and washed 3 times by suspending in HBSS-BSA and centrifuging at 400 g for 5 min at 4°C. Leukemic blast cells were prepared by the same method.

Platelets were prepared by centrifuging venous blood anticoagulated with 1/10 volume of 3.8% sodium citrate at 400 g for 10 min. The supernatant was removed and centrifuged at 2400 g for 20 min, and the pellet washed twice in PBS.

Normal human basophils, prepared as described,15 were a generous gift of Dr. Donald McGlashan (Jons Hopkins Medical School). This preparation contained 70% basophils, 4% polymorphonuclear cells (PMNs), 14% lymphocytes, and 12% monocytes.

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19
Immunization and Production of Hybridoma Cell Lines

Eight-week-old female C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me.) were immunized by intraperitoneal injection of 10⁶ neutrophils in Freund's complete adjuvant (GIBCO Laboratories, Grand Island, N.Y.), and subsequently, at weekly intervals, intraperitoneally with 10⁶ neutrophils in PBS and subcutaneously with 10⁶ neutrophils in incomplete Freund's adjuvant (GIBCO). One week following the third immunization, mice received 10⁵ neutrophils in PBS intraperitoneally. Three days later, their spleen cells were fused with murine myeloma P3-X63-Ag8.653 cells at a ratio of 5 spleen cells per myeloma cell, by a modification of the method described by Koprowski et al.²³ Hybridoma cell cultures secreting antibodies that bound to neutrophils were twice cloned in soft agar as described.²³ Only one final clone was selected from each initial well seeded with the fusion cell mixture. The antibodies described here were produced from three separate fusions, each using the spleens of two mice.

Determination of Antibody Class

Immunoglobulin isotype was determined using radiolabeled class-specific rabbit anti-mouse immunoglobulins in the live cell binding assay as described below.

Immunofluorescent Antibody Binding Assay

Fifty microliters of a suspension of 5 × 10⁶ target cells/ml in HBSS were placed on microscope slides, dried at room temperature, and then fixed in the vapor above 0.5% formaldehyde for 20 min at room temperature. Antibodies were assayed for cell-binding activity by indirect immunofluorescence using fluorescein-labeled goat anti-mouse immunoglobulin (obtained from the Division of Cancer Cause and Prevention, NCI) diluted 1:80 in PBS containing 50 mg/ml BSA.

Solid-Phase Cell Extract Binding Assay

Cell extracts were solubilized by suspending target cells in a buffer solution containing 5 mM Tris-HCl (pH 9.2), 1 mM EDTA, 400 mM KCl, 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride as described.²² The extract was diluted in PBS containing 0.02% NaN₃ to various concentrations, and 50 μl aliquots were applied to wells of Linbro 96-well microtiter plates (Linbro, Flow Laboratories, Inc., Hamden, Conn.) and dried overnight at 37°C. Two-hundred microliters of PBS containing 50 mg/ml BSA and 0.02% NaN₃, was then added to each well and incubated 4 hr at 37°C. This solution was then removed, and 50 μl of supernatant to be tested was added and incubated 1 hr at 4°C. Excess antibody was removed by washing 4 times with 200 μl of ice-cold PBS containing 1 mg/ml BSA and 0.1% Triton X-100. ²¹ I-labeled goat anti-mouse immunoglobulin (10 ng, 15–25 μCi/μg) was then added in 50 μl of buffer containing 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl, and 20 mg/ml BSA and incubated for 1 hr at 4°C. After washing as above, 100 μl of 2 M NaOH was added and after 15 min at 65°C, the solution was transferred to glass tubes and the radioactivity counted.

Solid-Phase Whole Cell Binding Assay

Target cells were suspended at various concentrations in PBS and 0.02% NaN₃, and 50 μl aliquots were applied to wells of a Linbro 96-well microtiter plate and dried overnight at 37°C. Two-hundred microliters of PBS containing 50 mg/ml BSA and 0.02% NaN₃ was then added to each well and incubated for 4 hr at 37°C. Assay of antibodies for cell-binding activity was then performed exactly as described for the solid-phase cell extract binding assay.

Live Cell Binding Assay

A quantity of 5 × 10⁶ target cells in 50 μl HBSS-BSA was incubated with 50 μl of hybridoma culture supernatant for 1 hr at 4°C in a siliconized glass test tube. Two milliliters of ice-cold HBSS-BSA was then added and the cells centrifuged at 400 g for 10 min. This washing procedure was repeated, 50 μl of ¹²⁵I-labeled rabbit or goat anti-mouse immunoglobulin (2.5–5 μCi/μg) was added, and the cells were incubated at 4°C for 1 hr. The cells were washed twice with ice-cold HBSS-BSA as above and the radioactivity of the cell pellet counted.

Cell Culture

HL-60 cells²² were kindly provided by Dr. R. C. Gallo (National Institutes of Health, Bethesda, Md.); the ML1, ML2, ML3,²⁶ and the MOLT 4, Daudi, RPMI 6410, RPMI 8226, and Raji²⁷ cell lines by Dr. C. Civei (Johns Hopkins Medical School); the K562²⁵ cell line by Dr. J. Spivak (Johns Hopkins Medical School); and the U937²⁷ cell line by Dr. Giovanni Rovera (Wistar Institute, Philadelphia, Pa.). The cells were grown in 25 cm² T flasks (Corning, Corning, N.Y.) in Dulbecco's modified Eagle medium (GIBCO, Grand Island, N.Y.) with 10% heat inactivated fetal calf serum (HY-Clone, Sterile Systems, Inc., Logan, Utah), 10 mM N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (Sigma), 0.05 mg/ml sodium pyruvate (GIBCO), 0.150 mg/ml oxalacetic acid (Aldrich), and 0.2 U/ml bovine insulin (Sigma).³⁶ The cells were seeded at 2.5–5 × 10⁶/ml and split approximately once a week, before the cell concentration reached 2 × 10⁶/ml. Cell viability was determined by trypan blue dye exclusion.

Cytotoxicity Assay

Hybridoma culture supernatants were tested for cytotoxicity against various target cells as previously described³⁹ with minor modifications. Two microliters of culture supernatant and 2 μl of HBSS-BSA containing 6 × 10⁵ cells were mixed under a drop of mineral oil in a 96-well microtiter plate, and the samples were incubated for 15 min at room temperature. Five microliters of rabbit complement (Pel Freeze Biologicals, Rogers, Ark.), appropriately diluted in PBS containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), was added and incubated at room temperature for 90 min. Ten microliters of 0.1% Trypan blue in PBS was then added and the samples were incubated for 10 min at room temperature. The cells were examined for dye exclusion following the addition of 25 μl of HBSS-BSA.

Antibodies

Mouse anti-human neutrophil antiserum was prepared from C57BL/6J mice immunized weekly for 4 mo with 10⁵ neutrophils in Freund's incomplete adjuvant given intraperitoneally. Affinity purified goat IgG against mouse immunoglobulin, antibody 128C3/3, an IgM monoclonal antibody that binds Schistosoma mansoni, and antibody 114C6/1, an IgM monoclonal antibody that binds human ferritin, were gifts of Dr. M. Strand (Johns Hopkins Medical School). Monoclonal antibody P3, an IgG1 with no known specificity, was obtained from the supernatant of the P3 × 63Ag8 cell line. Radiolabeled class-specific rabbit anti-mouse immunoglobulins were a gift of Dr. R. J. Johnson (Johns Hopkins Medical School). The monoclonal antibodies used were either the hybrid cell culture supernatants or culture supernatants concentrated ten-fold by ammonium sulfate precipitation. All sera were inactivated at 56°C for 30 min and clarified by centrifugation at 100,000 g for 1 hr.


Radiolabeling

Goat anti-mouse immunoglobulin was iodinated using chloramine-T as described.14 Neutrophils were surface labeled with 125I using lactoperoxidase25 following treatment with diisopropylfluorophosphate (DFP) (Sigma) as follows: 4 x 10^7 cells were suspended in 3 ml of PBS on ice and DFP to 5 mM was added.25 After incubation for 10 min, the cells were washed twice with 50 ml of ice-cold PBS. All reactions involving DFP were performed in a fume hood and all articles contacting DFP were washed in 5 M NaOH before removing from the hood. The cells were suspended in 2 ml of cold PBS at 2 x 10^7 cells/ml and 10 u of lactoperoxidase (Sigma) in PBS, 2 mCi of Na125I (carrier-free sodium salt, Amersham), and 20 uL of 0.06% H2O2 were added. After 5 min, an additional 20 uL of 0.06% H2O2 was added and the reaction was terminated 5 min later by adding 500 uL of 0.4 mg/ml tyrosine in PBS. The cells were then washed with ice-cold PBS. All steps were performed at 0-4°C.

Immunoprecipitation of Cellular Proteins

A quantity of 4 x 10^7 cells treated with DFP and labeled with 125I were suspended in 1.0 ml of cell lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP-40, 0.02% NaN3, and 2 mM phenylmethylsulfonylfluoride) and incubated on ice for 45 min.14 The cell extract was dialyzed overnight at 4°C against a buffer containing 20 mM Tris-HCl, pH 8.2, 100 mM NaCl, 1 mM EDTA, and 0.2% Triton X-100, and then centrifuged at 100,000 g for 1 hr at 4°C. Prior to immunoprecipitation, 1 mL of cell extract was incubated on ice for 15 min with a Staph A (Cowan Strain I) pellet derived from 200 uL of 10% Staph A;19 the supernatant obtained after centrifuging in an Eppendorf centrifuge was used for immunoprecipitation. Cell proteins radiolabeled with 125I were immunoprecipitated in reaction mixtures containing cell extract (approximately 10^6 cpm of acid-insoluble protein), antibody, and lysis buffer with 0.125 mg/ml gelatin (final concentration); the total volume was 0.25 ml in 10 x 75 mm siliconized glass tubes. After incubating the suspension overnight at 0°C, 5 ml of goat anti-mouse IgM antiserum (Litton Bionetics, Kensington, Md.) was added and the mixture incubated at 0°C for 4 hr. Fifty microliters of 10% Staph A was then added. After 15 min at 0°C, the mixture was washed twice by adding 2 mL of buffer containing 20 mM Tris, pH 8.2, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 2.5 M KCl, and 0.25 mg/ml gelatin,18 and centrifuging at 2000 g for 20 min. The pellet was suspended in 2 mL of 20 mM Tris, pH 8.2, transferred to Eppendorf tubes and pelleted for 5 min in an Eppendorf centrifuge. The precipitate was suspended in sample buffer and analyzed by one- or two-dimensional polyacrylamide gel electrophoresis.27,18 Molecular weight standards were a gift of Dr. D. Kiehart (Johns Hopkins Medical School). Gel slabs were stained, dried, and examined by autoradiography using Kodak X-Omat XR film.

RESULTS

Production of Monoclonal Antibodies Reacting With Human Neutrophils

Six independent hybridoma cell lines (AHN-1 to AHN-6) secreting IgM antibodies reactive with neutrophils were cloned in soft agar. All six monoclonal antibodies bound to live cells, implying that the antigens detected by these antibodies were expressed on the surface of the cells. These antibodies were all of the IgM class as determined by Ouchterlony immunodiffusion and by the use of class-specific antisera in the live cell binding assay (data not shown). All of the antibodies were strongly reactive with neutrophils when assayed by the immunofluorescent assay, while none was reactive with normal human lymphocytes, monocytes, red blood cells, or basophils (Table 1). P3, a control supernatant, or two monoclonal IgM antibodies with reactivity to irrelevant antigens (128C3/3 and 114C6/1) did not bind any of the cells. The specificity of binding was examined by using the whole cell plate assay as described (data not shown). This assay confirmed the results of the immunofluorescent assay except that basophils could not be assayed. Binding of monoclonal antibody AHN-1 was dependent on target cell concentration and was proportional to target cell concentration under conditions of primary antibody excess. Fluorescent activated cell sorter analysis of peripheral blood cells (Rovera et al., unpublished data) also documented granulocyte-specific binding.

Immunoprecipitation of a Neutrophil-Specific Antigen

The specificity of the monoclonal antibodies was analyzed by immunoprecipitation and NaDodSO4 polyacrylamide gel electrophoresis of proteins in detergent extracts of human neutrophils labeled at the cell surface with 125I by the lactoperoxidase method (Fig. 1).

The predominant reactivity of the polyclonal serum from mice hyperimmunized with intact neutrophils was against components of 145,000 and 105,000 daltons (Fig. 1, lane B). These immunoprecipitated polypeptides were highly enriched from the other components of the neutrophil cell surface labeled with 125I (lane A). Two additional hyperimmune mouse sera precipitated bands of the same apparent molecular weights plus additional proteins of 62,000 and 94,000 daltons (not shown).

Table 1. Monoclonal Antibody Binding to Normal Peripheral Blood Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antibody (% Positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>2 ± 0.5†</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0</td>
</tr>
<tr>
<td>RBC</td>
<td>0</td>
</tr>
<tr>
<td>Platelets</td>
<td>0</td>
</tr>
<tr>
<td>Basophils</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0</td>
</tr>
</tbody>
</table>

*Antibody binding was determined by the immunofluorescent assay using an optical microscope with undiluted cell culture supernatant for the primary incubation.
†Antibodies produced by clones AHN-2 to AHN-6 gave results similar to those seen with antibody AHN-1.
‡Mean ± SD.

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The polypeptides of 145,000 and 105,000 daltons were also precipitated by the monoclonal antibodies AHN-1 and AHN-2 (lanes D and E) and by antibodies AHN-3 to AHN-6 (not shown). This immunoprecipitation was specific in that normal mouse serum (lane C) or two control IgM monoclonal antibodies with no reactivity against neutrophils (lanes F and G) did not precipitate these polypeptides. NaDodSO₄ polyacrylamide gel electrophoresis of the immunoprecipitated proteins under nonreducing conditions also revealed two polypeptides with apparent molecular weights similar to those seen under reducing conditions (not shown).

The observation that the molecules migrate as separate bands in the nonreduced state during polyacrylamide gel electrophoresis suggests that they are not disulfide linked. Whether these two molecules contain an identical antigenic determinant or are unique but associated with a common antigenic determinant was analyzed by treating the ¹²⁵I-labeled cell extract with 1% NaDodSO₄ and 5% 2-mercaptoethanol at 100°C for 2 min, followed by dialysis before immunoprecipitation. Both the 145 and 105 kd proteins were still immunoprecipitated by the monoclonal antibodies following this treatment (not shown), suggesting the presence of the antigen on both molecules.

Further definition of the polypeptides reacting with AHN-1 and AHN-2 was obtained by two-dimensional gel electrophoresis. Proteins labeled at the cell surface by ¹²⁵I were resolved into several components. Most of the ¹²⁵I was present in a single major component composed of several isomorphic subunits of about 68,000 daltons, corresponding to the major band seen on the one-dimensional gel (Fig. 2A). A single polypeptide of 145,000 daltons and pI 4.5 was enriched by immunoprecipitation by AHN-1 (Fig. 2B). Upon longer exposure of the autoradiogram, a heterogeneous series of other proteins of 105,000 daltons and pI 3.5–6.3 were also observed, corresponding to the

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**Fig. 1.** Immunoprecipitation and polyacrylamide gel electrophoresis of ¹²⁵I-labeled neutrophil surface proteins. 10⁶ cpm of ¹²⁵I-labeled cell extract was used for each immunoprecipitation reaction. (Lane A) Cell extract before immunoprecipitation. (Lanes B–G) Immunoprecipitates: (B) hyperimmune mouse serum; (C) normal mouse serum; (D) monoclonal antibody AHN-1; (E) monoclonal antibody AHN-2; (F) antibody 128C3/3; and (G) antibody 114C6/1. Proteins used as molecular weight standards were: myosin heavy chain, 200,000; β-galactosidase, 116,000; phosphorylase-a, 95,000; bovine serum albumin, 68,000; catalase, 60,000; and actin, 43,000.

**Fig. 2.** Immunoprecipitation and two-dimensional gel electrophoresis of ¹²⁵I-labeled neutrophil surface proteins. 10⁶ cpm of ¹²⁵I-labeled cell extract was used for each immunoprecipitation reaction. (A) Cell extract before immunoprecipitation. (B) Immunoprecipitate with monoclonal antibody AHN-1. Molecular weight standards were the same as Fig. 1 with the addition of carbonic anhydrase, 29,000.
105,000 dalton band seen on the one-dimensional gel. The same results were obtained with AHN-2 (not shown).

**Antibody Binding to Human Leukemia Cells**

The expression in hematologic malignancies of the myeloid-specific antigen recognized by the monoclonal antibodies was examined with a variety of human leukemia cells, using the immunofluorescent assay. Each of the AHN-1–6 antibodies reacted with the human promyelocytic leukemia cell line, HL-60, and the recently described human myeloid leukemia cell lines, ML1, ML2, and ML3 (Table 2). These antibodies exhibited only slight binding to leukemic blast cells (designated AML-EB) isolated from a patient with acute nonlymphocytic leukemia (ANLL). None of the antibodies exhibited binding to cells from a patient with chronic lymphocytic leukemia (CLL), to the human lymphoid leukemia cell lines MOLT 4, Raji, or Daudi, the normal human B-cell line RPMI 6410, or the human myeloma cell line RPMI 8226. Neither control monoclonal antibody P3 nor 128C3/3 exhibited significant binding to any of the cells tested. No antibody binding to K562 or U937 cells could be detected with this assay. However, using a cytotoxic assay and a fluorescent second antibody directed against both immunoglobulin heavy and light chains, we could detect low intensity binding to both K562 and U937 cells (Rovera et al., unpublished observation).

**Cytotoxicity of Monoclonal Antibodies and Complement to Normal and Leukemic Cells**

The potential complement-dependent cytotoxicity of the antibodies was analyzed by assays for their cytotoxic effects on a variety of normal and malignant cells. Antibodies AHN-1 to AHN-6 were cytotoxic to normal human neutrophils under conditions were lymphocytes were not killed (Table 3). A control IgM hybridoma supernatant (128C3/3) and the P3X63Ag8 myeloma supernatant were not cytotoxic in this system. All six IgM antibodies were also cytotoxic to the HL-60, ML1, ML2, ML3, K562, and U937 cell lines. Significant cytotoxicity against leukemic blasts from a patient with ANLL was also observed. None of the antibodies was cytotoxic to leukemic cells from a patient with CLL, nor the RPMI 6410, MOLT 4, Raji, RPMI 8226, and Daudi cell lines.

**DISCUSSION**

This article describes the production and characterization of a defined set of monoclonal antibodies that

### Table 2. Monoclonal Antibody Binding to Human Leukemia Cells*

<table>
<thead>
<tr>
<th>Cells†</th>
<th>Antibody (%) Positive</th>
<th>P3</th>
<th>AHN-1</th>
<th>128C3/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>0.5 ± 0.5§ 92 ± 2 2 ± 2</td>
<td>92 ± 2</td>
<td>2 ± 2</td>
<td></td>
</tr>
<tr>
<td>ML1</td>
<td>2 ± 2             99 ± 1 2 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML2</td>
<td>2 ± 2             100 2 ± 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML3</td>
<td>2 ± 2             99 ± 1 2 ± 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML-EB</td>
<td>0 ± 2             0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>0 ± 0             0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI 6410</td>
<td>0 ± 0    0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raji</td>
<td>0 ± 0             0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI 8226</td>
<td>0 ± 0   0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOLT 4</td>
<td>0 ± 0             0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAUDI</td>
<td>0 ± 0             0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>0 ± 1             1 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U937</td>
<td>0 ± 1             1 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Antibody binding was determined by the immunofluorescent assay using an optical microscope with undiluted cell culture supernatant.
†HL-60, ML1, ML2, and ML3 are human myeloid cell lines; AML-EB, leukemic blast cells obtained from peripheral blood of a patient with acute myeloid leukemia; CLL, chronic lymphocytic leukemia cell; RPMI 6410, normal human B-cell line; Raji, human Burkitt’s lymphoma cell line; RPMI 8226, human IgGλ chain secreting myeloma cell line; MOLT 4, human T-cell lymphoid leukemia cell line; Daudi, human B-cell lymphoid leukemia cell line; K562, human erythroleukemia cell line; and U937, human histiocytic lymphoma cell line.
§Monoclonal antibodies AHN-2 to AHN-6 gave results similar to antibody AHN-1.

### Table 3. Cytotoxicity of Monoclonal Antibodies and Complement Against Normal and Leukemic Human Cells*

<table>
<thead>
<tr>
<th>Cells†</th>
<th>Antibody (%) of Cells Killed§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P3</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HL-60</td>
<td>&lt;5</td>
</tr>
<tr>
<td>ML1</td>
<td>&lt;2</td>
</tr>
<tr>
<td>ML2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>ML3</td>
<td>&lt;2</td>
</tr>
<tr>
<td>AML-EB</td>
<td>5 ± 5 36 ± 5 63 ± 4</td>
</tr>
<tr>
<td>CLL</td>
<td>&lt;2</td>
</tr>
<tr>
<td>RPMI 6410</td>
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<td>Raji</td>
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<td>&lt;2</td>
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<tr>
<td>MOLT 4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>DAUDI</td>
<td>&lt;2</td>
</tr>
<tr>
<td>K562</td>
<td>&lt;2</td>
</tr>
<tr>
<td>U937</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

*The cytotoxic assay was performed using undiluted cell culture supernatants as the source of antibody. All experiments were performed at least twice.
†Cells are as described in Table 2. AML-EB cells are leukemic blasts isolated from a patient with ANLL.
§Monoclonal antibodies AHN-3 to AHN-6 gave results similar to those of antibody AHN-1.
§Determined by trypan blue exclusion.
Mean ± SD.
reacted specifically with a cell surface protein of normal human neutrophils. Mice were immunized with human venous blood neutrophils and the immune spleen cells were hybridized to the P3X63Ag8.653 myeloma cell line; these myeloma cells do not express immunoglobulin but form antibody-secreting hybrid cell lines. Appropriate hybridomas were selected on the basis of antibody binding to the surface of normal human neutrophils. One set of 6 independent hybridoma cell lines (AHN-1 to AHN-6) secreted antibodies that showed the same specificities of cell binding and protein immunoprecipitation. Characterization of the reaction, as measured by the whole cell and cell extract plate assays, showed that binding was dependent on both the antigen and antibody concentrations. These monoclonal antibodies were specific for peripheral blood neutrophils; no binding to normal human lymphocytes, monocytes, red blood cells, basophils, or platelets was detected. Each of these antibodies was IgM.

These monoclonal antibodies immunoprecipitated a major 125I-labeled neutrophil cell surface polypeptide of 145,000 daltons and an isoelectric point of 4.5. Several components of 105,000 daltons with heterogeneous isoelectric points between 3.5 and 6.3 were also present in the immunoprecipitate. These 145,000 and 105,000 dalton polypeptides were the major iodinated components immunoprecipitated by polyclonal sera from several different hyperimmune mice. They thus appeared to act as strong antigens in the immunized mouse. The relationship of the heterogeneous 105,000 dalton components to the major immunoprecipitate of 145,000 daltons is unknown; in general, the 105,000 dalton polypeptides could be components of a modified protein, coprecipitates, or cross-reactive proteins. The fact that both proteins are immunoprecipitated by these antibodies after the cell extract was reduced and denatured suggests the antigen may be present on both components. Bands of identical mobility were precipitated by each of the antibodies AHN-1 to AHN-6. Neutrophils are known to contain a large quantity of a variety of proteases, and others have emphasized the importance of adding diisopropylfluorophosphate (DFP) prior to solubilization of neutrophil in the study of neutrophil structural proteins. For this reason, experiments were conducted with neutrophils treated with DFP both before or after labeling with 125I by the lactoperoxidase method. A difference between the electrophoretic patterns of proteins precipitated from extracts of the labeled neutrophils was observed. The immunoprecipitates of antibody AHN-1 from neutrophils treated with DFP after iodination contained two lower molecular weight bands not seen in precipitates from neutrophils treated with DFP before iodination. More pronounced differences of other proteins precipitated with hyperimmune sera were observed. These results suggest that some degradation of neutrophil membrane proteins could occur during 125I labeling with lactoperoxidase. It is for this reason, in all results reported here, that the cells were treated with DFP prior to radiolabeling. It is still possible, however, that the heterogeneous 105,000 dalton components were derived from the 145,000 dalton polypeptide or some larger molecule.

Several recent reports have described granulocyte-specific monoclonal antibodies; however, in each case the antigens remain undefined and it is thus difficult to compare the antigenic specificity of these antibodies with AHN-1-6. It is noteworthy however that all of the 9 granulocyte-specific monoclonal antibodies recently reported by Perussia et al. were IgM and seemed to react with the same or closely associated antigenic determinants as determined by competition studies. Similarly, the first granulocyte-specific antibody described was also an IgM. Since the 145,000 dalton polypeptide is highly antigenic in mice, it is possible that some of these antibodies recognize the same antigen. There may also be a homology to the murine Mac 1 antigen, which was identified as two iodinated polypeptides of 190,000 and 105,000 immunoprecipitated from murine macrophages by the M1/70 monoclonal antibody.

All of these antibodies were reactive with the HL-60, ML1, ML2, and ML3 human myeloid leukemia cell lines, as determined by the immunofluorescence assay. These cell lines were derived from patients with acute nonlymphocytic leukemia. Antibody binding was not detected by the immunofluorescence assay when chronic lymphocytic leukemia cells or a variety of human lymphocytic leukemia cell lines were used as target cells. These included a normal B-cell, Burkitt's lymphoma, IgG-λ chain secreting myeloma, T-cell leukemia, and B-cell leukemia cell lines. The cell specificity of the antibodies was also tested by cytotoxicity, a more sensitive assay because binding of a single complement-fixing IgM per cell is sufficient to cause cell death by activation of the complement cascade. The results were the same as those of the immunofluorescence assay performed with an optical microscope except for the K562, U937, and AML-EB cells (leukemic blasts isolated from a patient with acute non-lymphocytic leukemia). Antibody binding to both K562 and U937 cells was detected by a more sensitive immunofluorescence assay using a cytofluorograph. AML-EB cells were no longer available for this analysis.

It is of interest that antibody binding to K562 cells was detected. K562 is a cell line derived from a patient
with chronic myelogenous leukemia in blast crisis and has some properties of early erythroid precursors. K562 cells grow as undifferentiated blast cells, do not stain with cytochemical reagents normally positive in granulocytes and monocytes, synthesize glycoporin A, and can be induced to synthesize hemoglobin. Recently, others have reported the presence of granulocyte antigens on K562 cells by using polyclonal antiserum with granulocyte specificity as well as a monoclonal antibody. The results suggest the potential use of these antibodies in the treatment of myelogenous leukemia. Others have reported cure of murine hematologic malignancy using in vitro purging of bone marrow with tumor-specific antibody followed by autologous bone marrow transplantation into a lethally irradiated host. Similar therapeutic trials of immunotherapy for lymphoblastic leukemia have recently been performed in man. Such therapy has not been reported for human myeloid leukemia, as specific antigens that are expressed in a large percentage of patients with ANLL have not been detected. Preliminary studies using an immunofluorescent second antibody assay indicate that these antibodies recognize antigens on leukemia cells from approximately 50% (11/20) of pediatric and 30% (3/10) of adult patients with ANLL (Civin et al., unpublished observations). It will be of interest to examine the expression of this antigen in a large number of cases of myeloid and nonmyeloid leukemias. Such studies are in progress. It is likely that the treatment of populations of leukemic cells will require a combination of antibodies recognizing different antigens. For the antibodies described here to be useful for such therapy, it is essential that they not exhibit cytotoxicity against human stem cells. Preliminary studies, in collaboration with Strauss and Civin, indicate that these antibodies do not kill bone marrow CFU-C. Myeloid leukemia may represent a system where differentiation-specific antigens such as described here could be utilized in place of tumor-specific antigens in therapeutic interventions.

REFERENCES

A human granulocyte-specific antigen characterized by use of monoclonal antibodies

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