HLA-DR−, CD33+, CD56+, CD16− Myeloid/Natural Killer Cell Acute Leukemia: A Previously Unrecognized Form of Acute Leukemia Potentially Misdiagnosed as French-American-British Acute Myeloid Leukemia-M3


We have identified and characterized a previously unrecognized form of acute leukemia that shares features of both myeloid and natural killer (NK) cells. From a consecutive series of 350 cases of adult de novo acute myeloid leukemia (AML), we identified 20 cases (6%) with a unique immunophenotype: CD33+, CD56+, CD11a+, CD13+, CD15−, CD34+, HLA-DR−, CD16−. Multicolor flow cytometric assays confirmed the coexpression of myeloid (CD33, CD13, CD15) and NK cell-associated (CD56) antigens in each case, whereas reverse transcription polymerase chain reaction (RT-PCR) assays confirmed the identity of CD56 (neural cell adhesion molecule) in leukemic blasts. Although two cases expressed CD4, no case expressed CD2, CD3, or CD8 and no case showed clonal rearrangement of genes encoding the T-cell receptor (TCR β, γ, δ). Leukemic blasts in the majority of cases shared unique morphologic features (deeply invaginated nuclear membranes, scant cytoplasm with fine azurophilic granularity, and finely granular Sudan black B and myeloperoxidase cytochemical reactivity) that were remarkably similar to those of acute promyelocytic leukemia (APL); particularly the microgranular variant (FAB AML-M3v). However, all 20 cases lacked the t(15;17) and 17 cases tested lacked the promyelocytic/retinoic acid receptor (RARα) fusion transcript in RT-PCR assays; 12 cases had 46,XX or 46,XY karyotypes, whereas 2 cases had abnormalities of chromosome 17q: 1 with del(17)(q25) and the other with t(11;17)(q23;q21) and the promyelocytic leukemia zinc finger/RARα fusion transcript. All cases tested (6/20), including the case with t(11;17), failed to differentiate in vitro in response to all-trans retinoic acid (ATRA), suggesting that these cases may account for some APLs that have not shown a clinical response to ATRA. Four of 6 cases tested showed functional NK cell-mediated cytotoxicity, suggesting a relationship between these unique CD33+, CD56+, CD16− acute leukemias and normal CD56−, CD16− NK precursor cells. Using a combination of panning and multiparameter flow cytometric sorting, we identified a normal CD56−, CD33+, CD16− counterpart cell at a frequency of 1% to 2% in the peripheral blood of healthy individuals. Our studies suggest that this form of acute leukemia may arise from transformation of a precursor cell common to both the myeloid and NK cell lineages; thus we propose the designation myeloid/NK acute leukemia. Recognition of this new leukemic entity will be important in distinguishing these ATRA-nonresponsive cases from ATRA-responsive true APL.

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MYELOID/NATURAL KILLER CELL ACUTE LEUKEMIA

MATERIALS AND METHODS

Sample acquisition and morphologic review. This study was based on specimens of bone marrow (BM) or peripheral blood (PB) submitted to the SWOG Reference Laboratory and Myeloid Repository at the UNM Cancer Center according to standard procedures of SWOG. Briefly, pretreatment specimens of BM and/or PB are submitted to the Repository for patients who are candidates for SWOG AML treatment studies. Upon initial receipt, leukemic blasts are enriched on Ficoll-Hypaque gradients (Pharmacia, Piscataway, NJ) and the leukemic blast percentage is determined by analysis of Wright-stained cytospins. All cases are immediately analyzed for the expression of a panel of hematopoietic cell surface antigens using multicolor flow cytometry. Residual leukemic cells are cryopreserved as sterile, viable cell suspensions at -135°C; these cryopreserved specimens were used for the additional biologic assays undertaken in this study.

Pretreatment Wright-stained BM aspirates and PB specimens as well as a panel of submitted or centrally performed cytochemical stains (Sudan black B [SBB], alpha naphth butyrate esterase, myeloperoxidase [MPO], and chloracetate esterase) were reviewed by members of the SWOG Leukemia Review Panel. Additional morphologic and cytochemical studies were also performed on air-dried aspirates and on thawed, previously cryopreserved cell suspensions and were reviewed by three of the authors (A.S., D.H., C.W.) and Dr Kathy Foucar (University of New Mexico, Albuquerque, NM). All cases were classified using standard FAB criteria and were alternatively classified as myeloid/NK acute leukemia if they had a distinct set of reproducible morphologic and cytochemical features different from those detailed in the current FAB classification system (see Results).

Immunophenotypic/flow cytometric analysis. The cell surface antigen profile of leukemic blasts in each case initially were determined on fresh cells at the time of sample submission using standard two-color flow cytometric analysis with a panel of monoclonal antibodies (MoAbs) as previously described. The monoclonal cell component after Ficoll-Hypaque gradient separation was then incubated in staining media with directly-conjugated [either fluorescein isothiocyanate (FITC) or phycoerythrin (PE)] MoAbs for 30 minutes at 4°C (Leu19 [CD56], MY10 [CD34], LeuM1 [CD15], LeuM2 [CD12]) [CD19], Leu20 [CD7], Leu5b [CD10], CR1 [CD35], CD11a, LeuM5 (CD11c), GPllbIIIa (CD41), Leu 5b (CD2), Leu4 (CD3), and HLA-DR (all supplied by Becton Dickinson, San Jose, CA); and MY9 (CD33), MY4 (CDw4), and MY7 (CD13) (all supplied by Coulter, Inc, Hialeah, FL). Background staining was determined using isotype controls (FITC- or PE-conjugated IgGl and PE-conjugated IgG2). After washing, samples were analyzed immediately with a Becton Dickinson FACScan flow cytometer. Although the percent viability of both fresh and thawed, previously cryopreserved leukemic cell suspensions usually exceeded 90%, dead cells were excluded from flow cytometric analysis with propidium iodide gating. Flow cytometric data were analyzed with FACScan software (Becton Dickinson) and results expressed as units of mean channel fluorescence relative to background control levels; composite two-color dot plots and contour plots were generated using the Lyse II analysis system (Becton Dickinson).

Cytogenetic analysis. Pretreatment BM and/or PB samples were prepared for cytogenetic analysis by direct and/or short term (24 to 72 hour) unstimulated cultures at approved SWOG cyogenetics laboratories. A minimum of 20 G-banded metaphases were analyzed in each case, and chromosomes were identified and clonal abnormalities classified according to the International System for Human Cyogenetic Nomenclature (ISCN) (1985) and ISCN (1991). Representative original karyotypes were submitted for central review to the SWOG Cyogenetics Working Group (R. Ellen Magenlis, Chair) and were reviewed by one of us (K.T.).

Reverse-transcriptase polymerase chain reaction (RT-PCR) and oligonucleotide probe hybridization assays for CD56 neural cell adhesion molecule (NCAM). To detect CD56 NCAM transcripts in leukemic blasts, RT-PCR assays were performed on total RNA isolated from cases 8 and 15 (Table 1), as previously described. The CD56+ myeloma cell line 8226Dox40 was used as a high-level positive control. RNA from a case of AML that was not a myeloid/NK leukemia case and that exhibited 1% expression of CD56 by flow cytometric analysis was used as a negative control. Briefly, cDNA was synthesized from 1 μg of total cellular RNA in 20 μL of a solution containing 5 mmol/L MgCl2, 50 mmol/L KCL, 10 mmol/L TRIS-HCL (pH 8.3), 20 U RNase inhibitor, 50 U reverse

Table 1. Morphologic, Immunophenotypic, and Cytogenetic Characteristics of Myeloid/NK Precursor Cell Leukemia Cases

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<th>FAB*</th>
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<th>Karyotype</th>
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<td>46,XY</td>
</tr>
</tbody>
</table>

*positivity is defined as greater than 20% of fluorescence intensity compared with the background isotope control fluorescence intensity.

Abbreviations: My/NK, myeloid/natural killer cell morphologic features; idem, same as previous clone; ND, not determined or unsuccessful.

If morphologic features were consistent with a particular FAB subtype, then that subtype is provided. Alternatively, if features were most consistent with the myeloid/NK My/NK subtype, then that designation is provided.
transcriptase, 0.75 \mu M of downstream primer, and 1 \mu M of each deoxynucleotide triphosphate (dNTP). The samples were incubated in a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) at 42°C for 30 minutes and 99°C for 5 minutes. To amplify the cDNA, 55 \mu L of a PCR master mix was added to each tube to yield a total concentration of 2 mmol/L MgCl\(_2\), 50 mmol/L KCl, and 10 mmol/L TRIS-HCl (pH 8.3), along with one AmpliWax PCR Gem (Perkin Elmer-Cetus) to enable hot-start PCR. Tubes were heated to 70°C for 3 minutes and cooled to 15°C for 3 minutes. A final 25 \mu L of PCR mix that now included AmpliTaq DNA polymerase (2.5 U/100 \mu L) and the upstream primer (final concentration = 0.15 \mu M/\mu L). PCR was performed for 45 cycles as follows: 1 cycle at 95°C for 3 minutes, 60°C for 3 minutes, and 72°C for 3 minutes; 43 cycles at 45 seconds at 95°C, 1 minute at 60°C, and 1 minute at 72°C; and finally, 1 cycle of 95°C for 45 seconds, 60°C for 1 minute and 72°C for 5 minutes.

The NCAM primers yielding a 493-base product were as follows: upstream primer, 5'-GGCCATCTCAAATACAAAGC-3' (NCAM residues 2,156 through 2,176). Ten microliters of the RT-PCR product was electrophoresed in agarose gels and standard Southern blot transfer was performed.\(^{18}\) Nitrocellulose membranes were prehybridized for 1 hour at 65°C in 5 \mu L of Rapid-Hyb buffer (Amersham, Arlington Heights, IL). Final hybridization was performed with a 525-bp PCR-synthesized NCAM DNA probe spanning the region initially amplified in the patient samples [5'- primer: NCAM residues 1,667 through 1,686 (5'-GAGCCACAGCAGGGTGCC-3')]; 3' primer: NCAM residues 2,175 through 2,194 (5'-GGCTGGAGC-CTGGGCGAGG-3'). The probe was random primed (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and the membrane was hybridized for 1 hour at 65°C. Stringency washes included one wash of 15 minutes at room temperature in 0.1 \times SSC, 0.1% sodium deoxycholate (SDS), followed by two 15-minute washes in 0.1 \times SSC, 0.1% SDS at 65°C followed by autoradiography.

**RT-PCR assays for promyelocytic (PML)/retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) mRNA transcripts.** RNA was isolated from 17-20 myeloid/NK leukemia cases with residual cryopreserved cells (all cases except cases 6, 9, and 13; Table 1) and from 3 AML-M3 cases with a confirmed t(15;17).\(^{19}\) The RT-PCR amplification method used was similar to that of Biondi et al.\(^{20}\) One microgram of total cellular RNA was incubated for 10 minutes at 23°C, 15 minutes at 42°C, and 5 minutes at 99°C in a total volume of 20 \mu L containing 2 \mu L of 10 \times buffer (100 mmol/L TRIS pH 8.3, 0.5 mol/L KCl, and 50 mmol/L MgCl\(_2\)), 2 \mu L of 10 mmol/L dNTPs, 0.5 \mu L of 100 pmol/\mu L random hexamers (Boehringer-Mannheim), 0.5 \mu L of RNAse (20 U/0.5 \mu L) (Promega, Madison, WI), 2 \mu L of 50 mmol/L dithiothreitol, 0.25 \mu L of Moloney murine leukemia virus (MMLV) reverse transcriptase (50 U/0.25 \mu L) (GIBCO-BRL, Gaithersburg, MD), and 7.75 \mu L of diethylpyrocarbonate-treated H\(_2\)O. Five microliters of this reaction was then used for nested RT-PCR assays using primer sequences to detect PML/RAR\(\alpha\) fusion mRNAs as previously described: M28R (M2 derived from PML exon 6 and R8 from RAR\(\alpha\) exon 3) to analyze breakpoint cluster regions (bcr) 1 and 2 and M48R (M4 derived from PML exon 3) to analyze bcr region 3.\(^{21}\) After amplification, 15 \mu L of the PCR reaction was fractionated and visualized in ethidium bromide-stained agarose gels. Amplification of 32-microglobulin mRNA, using 5 \mu L of the cDNA was performed as an internal control for RNA integrity and PCR reaction fidelity in each sample, using an annealing temperature of 55°C.\(^{22}\)

**RT-PCR assays for the promyelocytic leukaemia zinc finger (PLZF)/RAR\(\alpha\) mRNA transcripts.** RT-PCR was performed to detect the PLZF/RAR\(\alpha\)-fusion transcript arising from the t(11;17), as previously described.\(^{23}\) Total RNA (4 \mu g) from each leukemia sample was hybridized to a primer (5'-TGGGATTTCTGCCTCAGGCTAAGCCCTTGTCCAG-3') complementary to the B region of the RAR\(\alpha\) gene and reverse transcribed by incubation with 200-U MMLV reverse transcriptase (GIBCO-BRL) at 37°C for 45 minutes. One tenth of the cDNA product was then amplified by PCR using Taq polymerase (Promega) in 100-\mu L volume with a primer complementary to the B region of the RAR\(\alpha\) [5'-GGGCACTATCTC-TTCAG-3'] and a primer complementary to the B region of PLZF (5'-CTGTTCTCATGGACTTT-3'), with two cycles at 98°C for 50 seconds, 47°C for 1.5 minutes, and 72°C for 2 minutes, 32 cycles at 98°C for 25 seconds, 47°C for 1.5 minutes, and 72°C for 2 minutes, and a final extension for 12 minutes at 72°C. Ten microliters of the PCR products were electrophoretically separated through a 1.4% agarose gel, transferred to nitrocellulose, prehybridized, and hybridized overnight in 5 \times salinity phosphate and ethylenediamine tetra-acetate (ETTE, 5 \times Denhardt's, 1 mg/mL salmon sperm DNA at 63°C with a radiolabeled oligonucleotide complementary to PLZF (5'-TGAGACAGCAAACTGACGTCG-3'), located 3' to the PLZF amplification primer. After washing, the filters were autoradiographed. A previously identified AML patient with t(11;17) and a PLZF/RAR\(\alpha\)-fusion product was used as a positive control.\(^{21,22}\)

**In vitro differentiation studies with ATRA.** Previously cryopreserved leukemia blasts from four patients (cases 11, 14, 15, and 16; Table 1) and from two AML-M3 patients with a confirmed t(15;17) were thawed in 37°C defrosting medium [RPMI 1640; 20% fetal calf serum (FCS)], centrifuged at 1,100 rpm for 5 minutes and then resuspended in complete culture medium (RPMI 1640, 20% FCS, antibiotics). Cells were cultured with or without 1 \mu M/LATRA (Sigma Chemical Co, St Louis, MO) for 7 days as previously described.\(^{23,24}\) Aliquots of cells removed at time 0 and days 2, 5, and 7 were analyzed by cytoligic evaluation and differential count of Wright and SBB-stained cytopsins. Maturation was defined by a gain in cytoplasmic volume, loss of cytoplasmic granularity after the promyelocytic stage, and nuclear segmentation.

**In vitro cytotoxicity assays.** The cytotoxicity of previously cryopreserved leukemia cell suspensions from six myeloid/NK leukemia cases with residual cells (cases 3, 10 [a posttreatment relapse specimen was analyzed as pretreatment cells were not available], 12, 14, 15, and 19, Table 1) was tested in a \(^{51}\)C Chromium (Cr) release functional assay.\(^{25}\) An NK leukemia cell line (IMC-1; recently developed in our laboratory) and an AML-M3 sample with a confirmed t(15;17) were used as controls.\(^{26}\) Target cells included the NK cell-susceptible K562 (human erythroleukemia) line. Target cells were incubated with \(^{51}\)Cr (New England Biolabs, Boston, MA) in 1 mL of bovine calf serum (BCS) (50 \mu Ci/1 \times 10\(^6\) cells) for 2 hours at 37°C in air/\(\text{CO}_2\), 37°C. After incubation and removal of the IL-2, the supernatant was collected using a harvesting press (Skatron, Sterling, VA) and counted for radioactivity for 20 minutes at 37°C in a gamma counter. Specific lysis was determined as follows: (Spontaneous cpm)/(maximum cpm)/(Spontaneous cpm) = (maximum cpm)/(Spontaneous cpm) 

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ous cpm), with maximum release determined by adding 100 µL of target cells to wells containing 100 µL of 10% Triton X-100.\textsuperscript{27} Isolation of a nonleukemic CD33\textsuperscript{+}, CD56\textsuperscript{+}, CD16\textsuperscript{+} normal counterpart cell. NK cells were isolated from PB buffy coats of four healthy blood donors, with a pooled average cell count of 4.7 × 10\textsuperscript{6}/L, as previously described.\textsuperscript{24-26} Mononuclear cells were collected by Ficoll-Hypaque centrifugation at 1,360 rpm (400g) for 25 to 30 minutes resuspended in 2 mL of RPMI, and layered on top of an equal amount of FCS and centrifuged again at 800 rpm for 5 minutes to remove platelets. The cell pellet was resuspended in complete medium (RPMI 1640, 20% FCS), plated on plastic petri dishes and incubated overnight at 37°C in a CO\textsubscript{2} incubator to remove adherent monocytes. The nonadherent cell fraction was washed and T lymphocytes were removed by incubation with mouse anti-CD3 antibody, followed by rosetting with sheep red blood cells coated with rabbit-antimouse IgG. A second Ficoll-Hypaque centrifugation was performed to remove rosetted T cells and the nonrosetted cell layer was harvested. This NK cell-enriched population was then directly labeled for three-color flow cytometric analysis; cells were stained with purified CD56 antibody (Becton Dickinson), washed, and then labeled with biotinylated goat-antimouse IgG (Fab\textsubscript{b}). After a blocking step with goat serum, the cells were labeled with FITC-conjugated CD3 (Coulter) and PE-conjugated CD16 (Becton Dickinson). After washing with Hank's Balanced Salt Solution (HBSS), the cells were labeled with streptavidin PE-conjugated Texas Red (RED613) (Life Technologies, Becton Dickinson, San Jose, CA) and then washed and resuspended in HBSS. Cells were initially sorted using forward-angle light-scatter and side-scatter parameters on a Coulter 753 Cell Sorter to select for a small to medium-sized, nongranular population; cells stained with mouse isotype control antibodies were used as a negative control to establish gating parameters. Additional gating was performed on CD56\textsuperscript{+} and CD33\textsuperscript{+} cells to obtain CD16\textsuperscript{+} and CD16\textsuperscript{+} populations for cytospin morphologic and cytochemical evaluation.

Clinical and outcome data. Clinical data for patients registered on treatment studies SWOG 8600 or 9031 were obtained from SWOG central data base and were subjected to standard SWOG procedures for reporting and quality control. Seventeen of these patients were registered on SWOG AML frontline studies (11 on SWOG 8600, 3 on SWOG 9031, and 2 on SWOG 9034); three patients were not ultimately registered on any SWOG treatment study (cases 12, 14, and 15, Table 1). Data for patients registered on study SWOG 9034, an intergroup study coordinated by Eastern Cooperative Oncology Group (ECOG), were obtained from the ECOG central data base. Data for patients who were not registered on SWOG treatment studies (cases 12, 14, and 15, Table 1) were obtained from the hematologist-oncologist responsible for the patient's care. Overall survival was measured from the day of registration on SWOG treatment study (or the start date of induction therapy for non-SWOG patients) until death from any cause. Relapse-free survival (RFS) was measured from the onset of complete response until relapse or death from any cause, with observation censored at the date of last contact for living patients with no report of relapse. Median durations of survival and RFS were obtained from distributions estimated by the method of Kaplan and Meier.\textsuperscript{31} The BM differential data listed in Table 2 is based on the local institutional pathology.

RESULTS

Flow cytometric immunophenotypic studies. Pretreatment BM and PB specimens from patients who were candidates for frontline AML treatment studies SWOG 8600, 9031, and 9034 were reviewed for possible inclusion in this study. Specimens from 350 consecutive patients were examined and flow cytometric analysis of hematopoietic cell surface antigen expression showed 20 cases (6%) with a unique immunophenotypic profile. These 20 cases were characterized by a lack of expression of HLA-DR and by the coexpression of various myeloid (CD33, CD13, CD15) and NK cell-associated (CD56) surface antigens; the detailed immunophenotypic profile in each case is provided in Table 1. Like AML cases of the FAB M3 subtype, all cases consis-

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<th>Case No.</th>
<th>Age/Sex</th>
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<th>BM promyelocytes (%)</th>
<th>Extramedullary Disease</th>
<th>Bleeding Diathesis</th>
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Abbreviations: Extramedullary disease: Ax, axillary lymphadenopathy; Cx, cervical lymphadenopathy; ing, inguinal lymphadenopathy; ANOS, adenopathy site unspecified; S, splenomegaly; H, hepatomegaly; CNS, central nervous system. Response to induction therapy (TX): CR, complete remission; ED, early death; NASS, not adequately assessed; NR, no response; ND, not done; Unk, unknown.
tentatively lacked expression of HLA-DR, an antigen that is preferentially expressed by myeloblasts and lost during maturation to the promyelocytic stage.5,22 Although two cases were positive for CD4, this antigen is not T-cell lineage specific and may frequently be expressed on AML blasts and on a subset of PB monocytes.5,8 The absence of T-cell lineage commitment was further confirmed by the lack of expression of the T-cell lineage-associated antigens CD2, CD3, and CD8 and by a lack of clonal rearrangement of genes encoding the T-cell receptor (TCRβ, γ, δ1, and δ2; data not shown). All cases were characterized by the expression of the myeloid-associated antigens CD33, CD13, and/or CD15; the hematopoietic stem and progenitor cell antigen CD34 was expressed at low antigen density in 6/20 cases (Table 1). All cases expressed CD56 (a variably spliced form of the NCAM)33,34 at high antigen density and did not express CD16 (the immunoglobulin Fcy receptor). The CD11a adhesion molecule required for NK cell-mediated cytotoxicity,35,36 was also expressed at high antigen density in all cases in which it was assessed (Table 1, Cases 1 through 10). Coexpression of CD56 and myeloid-associated antigens (CD33, CD13, and CD15) was confirmed in each case by two- and three-color flow cytometric studies (Fig 1).

**RT-PCR and oligonucleotide probe hybridization assays for CD56 (NCAM).** To confirm that CD56, and not a cross-reactive epitope, was expressed in the myeloid/NK acute leukemia cases, RT-PCR assays were performed to detect CD56 (NCAM) mRNA transcripts in leukemic blasts. A major 493-bp CD56 (NCAM) PCR product was identified in each of two cases tested (Cases 8 and 15; Table 1) (Fig 2A). The analysis also included the CD56+ myeloma cell line 8226/Dox40 used as a high-level positive control and an AML case with very low CD56 expression (1% by flow cytometry) as a negative control. The identity of this 493-bp product has been previously confirmed by DNA sequencing.18 Hybridization of the RT-PCR products with an NCAM DNA probe confirmed the specificity of the RT-PCR products (Fig 2B).

**Review of morphologic and cytochemical features.** The referring institutional FAB diagnoses were as follows for the 20 cases: 1 M0/L2, 10 M1, 5 M2, 2 M3, and 2 M4. Upon rereview of BM aspirates and PB smears in each case by the SWOG Leukemia Review Panel, the majority of the cases (13/20) were noted to have distinct, yet similar morphologic and cytochemical features (which we have chosen to designate myeloid/NK acute leukemia; Table 1). These features included strikingly invaginated nuclear membranes, scant to moderate cytoplasm with fine to moderately coarse azurophilic granules, and weak to moderately finely granular SBB and/or MPO cytochemical reactivity (Fig 3, A and C). Although these features were highly reminiscent of AML-M3, particularly the microgranular variant (AML-M3v) (Fig 3B), distinct differences were apparent. Whereas occasional myeloid/NK blasts had conspicuous cytoplasmic granules, they were not as prominent as typical AML-M3 blasts; moreover, the pattern of weak or moderate finely granular SBB or MPO positivity was distinct from the intense and dense SBB and MPO staining of AML-M3 and M3v (Fig 3D). Rare myeloid/NK blasts contained Auer rods; bundles of Auer rods (faggot cells) characteristic of FAB M3 were not present. All cases were characterized by a high marrow-blast percentage (median blast cell percentage, 92%; range of 47% to 99%); however, in contrast to cases of AML-M3, increased numbers of marrow promyelocytes were not seen (Table 2). Increased numbers of cells with large granular

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**Fig 1.** Two-color contour plots derived from three-color flow cytometric analysis of gated blasts from a representative case of myeloid/NK acute leukemia with PE, FITC, and Texas Red (TR)-conjugated antibodies. (A) Coexpression of CD33-PE (Y-axis) and CD56-TR (X-axis). (B) Expression of CD33-PE (Y-axis) and lack of expression of HLA-DR-TR (X-axis). (C) Expression of CD33-PE (Y-axis) and lack of expression of CD16-FITC (X-axis). (D) Expression of CD56-TR (X-axis) and lack of expression of CD16-FITC (Y-axis).

**Fig 2.** (A) Ethidium-stained gel of NCAM RT-PCR products. DNA size markers (in bp) are shown in lane S. A major 493-bp NCAM RT-PCR product is detected in the CD56+ 8226/Dox40 cell line used as a positive control (lane 1) while an AML sample lacking CD56 (<1% by flow cytometry) essentially lacks this product (lane 4). Two CD56+ myeloid/NK cell acute leukemia cases in lane 2 (Case 8; Table 1) and lane 3 (Case 15; Table 1) show the major 493-bp NCAM RT-PCR product. (B) Autoradiograph of the gel in A hybridized with an NCAM-specific DNA probe. Hybridization confirms the specificity of the NCAM RT-PCR products; lanes as identified in Fig 2A.
Fig 3. Morphologic and cytochemical features of myeloid/NK acute leukemias in comparison to AML-M3v blasts. (A) Wright-stained myeloid/NK leukemic blasts; note deeply invaginated nuclear membranes and fine azurophilic granularity of cytoplasm. Original magnification × 1,000. (B) Wright-stained AML-M3v; note similarities to A. Original magnification × 1,000. (C) SBB-stained myeloid/NK leukemic blasts; note finely granular staining pattern. (D) SBB-stained AML-M3v blasts; note intense and dense staining pattern.

Fig 6. Isolation of a CD33+, CD56+, CD16- nonleukemic PB counterpart cell. (A) After a three-step isolation process to enrich for NK cells fromuffy coats, a population of small to intermediate-sized cells with low side scatter was selected for flow cytometric sorting (the population selected for sorting is indicated by the electronic gates). (B) From the population of cells obtained in (A), a subpopulation of cells with strong coexpression of CD33 and CD56 was obtained. (C) Next, the CD33+, CD56+ copositive cells shown in (B) were analyzed for expression of CD16. A population of CD33+, CD56+, CD16- cells were then selected in the final sort. (D) Morphologic features of the CD33+, CD56+, CD16- sorted cells; these cells display large granular lymphocyte morphology with irregular or indented nuclei with coarsely clumped chromatin and a moderate amount of cytoplasm with small azurophilic granules. Original magnification × 1,000.
lymphocyte (LGL) morphology associated with the NK cell lineage were not seen in the BM aspirates and PB smears of some cases (data not shown); whether these cells were derived from the leukemic clone or represented residual normal NK cells is not known. Whereas seven of the remaining cases (cases 1, 3, 8, 10, 12, 16, and 19; Table 1) had morphologic and cytochemical similarities to cases designated as myeloid/NK acute leukemia, the overall features were equally consistent with an M1, M2, or M7 designation in the FAB classification system and these cases are designated in this fashion (Table 1).

Pretreatment clinical characteristics and therapeutic response. Detailed pretreatment clinical characteristics and therapeutic responses are provided in Table 2. Median age at time of treatment was 48 years (range, 18 to 72 years) with an equal sex distribution (10 women, 10 men). The majority of patients presented with high white blood cell counts at presentation with a median of 75.0 \times 10^9/L (range of 1.0 \times 10^9/L to 328.0 \times 10^9/L; Table 2). The median PB lymphocyte count was 3,800/µL (range, 400 to 15,300/µL); 9/18 cases had counts greater than 4,000/µL (data not shown). Extramedullary disease, including hepatosplenomegaly and lymphadenopathy, was noted in only 5 patients; among 18 patients in whom central nervous system (CNS) involvement was assessed, none displayed evidence of CNS involvement at presentation or relapse. In contrast to AML-M3 patients, less than half of the myeloid/NK leukemia patients had a bleeding diathesis at presentation. Eight cases of patients remain alive without report of relapse at 28 days, 9/18 cases had counts greater than 4,000/µL (data not shown), confirming that myeloid/NK acute leukemia cases are genetically and molecularly distinct from APL/AML-M3.

RT-PCR screen for the PLZF/RARα fusion transcript. The AML-associated t(11;17)(q23;q21)1 has recently been cloned and characterized. Like cases of true APL with a t(15;17), the t(11;17) involves the RARα gene in the same intron on chromosome 17q21; but in contrast to APL, the t(11;17) results in a fusion of RARα with a novel gene designated PLZF on chromosome 11q23. As one of our cases had a cytogenetically detectable t(11;17)(q21;q23) (case 16; Table 1), we wanted to test for the presence of the PLZF/RARα fusion transcript in this case, and to determine whether any of the other cases (particularly those with 46,XY or 46,XX karyotypes) contained a PLZF/RARα fusion mRNA. RT-PCR for the PLZF/RARα fusion mRNA, as shown in Fig 4D, detected a 319-bp PLZF/RARα product in our case with a t(11;17) (Fig 4D, lane 12) as well as in a positive control sample. However, none of the eleven other myeloid/NK acute leukemia cases tested expressed this fusion product (Fig 4D). Thus, although the t(11;17)(q23;q21) appears to be associated with the myeloid/NK leukemia cell phenotype, other genetic abnormalities, yet to be defined, must be present in the majority of the myeloid/NK acute leukemia cases.
observed in none of the six myeloid/NK acute leukemia cases, including case 16 (Table 1) with a t(11;17) (data not shown). In contrast, both control APL/AML-M3 cases showed myelocytic differentiation with decreased blast percentages at days 5 and 7 (data not shown). These results further support the distinct biologic nature of myeloid/NK acute leukemia and confirm the nonresponsiveness of these cases to ATRA in vitro.

Assessment of NK cell-mediated functional cytotoxicity. Because the myeloid/NK acute leukemia cases coexpressed the CD56 and CD11a antigens essential for functional NK cell-mediated cytotoxicity in addition to sharing the CD56+, CD16+ phenotype of NK precursor cells, we sought to determine whether these leukemic cases displayed functional NK cell-mediated cytotoxicity. Cytotoxicity was assessed in a standard 51Cr release functional assay using the NK cell-susceptible line K562 as a target cell (Fig 5). Leukemic blasts from six cases [cases 3, 10 (results obtained with relapse material), 12, 14, 15, and 19; (Table 1)] were added as effector cells; in each case selected for analysis, the leukemic blast cell percentage in the enriched cryopreserved cell suspensions exceeded 90% and no cells with LGL morphology were evident in the preassay or postassay samples. The levels of cytotoxicity in these leukemic samples were compared with those of a positive control cell line, the IL-2–dependent IMC-1 NK leukemia cell line that displays equal or increased cytotoxicity compared with freshly isolated PB NK cells28 (Fig 5). NK cell-mediated cytotoxicity was also analyzed in two cases of APL with a confirmed t(15;17) (Fig 5). Four cases (cases 3, 10, 14, and 19; Table 1) displayed significant cytotoxic activity with IL-2, as compared with the positive NK leukemia control cell line (Fig 5; showing the data from case 14). In contrast, both cases of APL/FAB M3 lacked cytotoxic activity (Fig 5). This demonstration of functional cell-mediated cytotoxicity in the leukemic cells further supports a relationship between myeloid/NK acute leukemias and cells derived from the NK lineage.

Isolation of a CD33+, CD56−, CD16− nonleukemic counterpart cell. Although our myeloid/NK acute leukemia cases expressed the CD56+, CD16− phenotype of NK precursor cells, they also coexpressed the myeloid-associated antigen CD33. As previous studies characterizing CD56+, CD16− NK precursor cells have not included an analysis of myeloid antigens such as CD33, we sought to determine whether the coexpression of CD33 in our cases reflected an aberrant leukemic phenotype or whether a CD33+, CD56−, CD16− myeloid/NK cell was present in normal BM and PB. Using PB buffy coats from four healthy donors, a three-step isolation process was performed to enrich for NK cells. Cells obtained by these methods were labeled with CD33, CD56, and CD16 antibodies and sorted using multiparameter flow cytometry. From plots of forward-angle light scatter-
ter versus side scatter, a population of intermediate-sized cells with low side scatter (Fig 6A, see page 249) was selected for flow cytometric sorting using a Coulter 753 flow cytometer. From these sorting experiments, a population of CD33+, moderately bright CD56+, CD16- cells was obtained in each sample (Fig 6, B and C). Previous studies that used FACscan flow cytometry with log scale of 4 have shown that CD16+ NK cells are predominantly CD56 brightly positive. The use of log 3 data in our study, as compared with log 4 data, tends to compress the data toward the X-axis and therefore the fluorescence intensity shift does not appear to be as great. Based on the sensitivity of the Coulter 753 flow cytometer and the log 3 scale setting, although our data cannot be directly compared with Lainer’s study, we believe that our data is consistent with the findings of that study. We calculate these cells to be present at a frequency of approximately 1% to 2% of PB mononuclear cells in healthy adults. The morphologic and cytochemical features of the sorted CD33+, CD56+, CD16- population showed features of NK or LGLs with irregular or invaginated nuclear membranes with coarsely clumped chromatin and abundant slightly basophilic cytoplasm containing scattered small azurophilic granules. These cells did not stain with nonspecific esterase stains (data not shown), distinguishing them from CD56+ monocytic cells. Comparison of the morphologic features of these normal sorted CD33+, CD56+, CD16- PB cells (Fig 6D) to those of the CD33+, CD56+, CD16- leukemic cells (Fig 3A) show striking similarities: moderately high nuclear to cytoplasmic ratios, irregular nuclear membrane contours, and scant azurophilic cytoplasmic granularity. These data confirm the presence of a normal CD33+, CD56+, CD16- myeloid/NK cell population in the PB of normal individuals.

DISCUSSION

We have characterized a previously unrecognized form of acute leukemia with unique morphologic, immunophenotypic, cytogenetic, and functional biologic features. These 20 adult de novo acute leukemia cases were characterized by the coexpression of myeloid-associated (CD33, CD13, CD15) and NK cell-associated (CD56) antigens and the lack of expression of HLA-DR and the T-cell–associated antigens CD3 and CD8. Despite their morphologic and immunophenotypic similarity to cases of APL/FAB M3y, these cases lacked the t(15;17) and the resultant PML/RARα fusion transcript. Furthermore, no case showed a differentiation-induction response to ATRA in vitro, in contrast to cases of true APL with the PML/RARα fusion transcript. We suggest that these acute leukemia cases may account for some cases of APL that have not responded to ATRA. Because these CD33+, CD56+, CD16- acute leukemias showed functional NK cell-mediated cytotoxicity and because we were able to identify a normal CD33+, CD56+, CD16- counterpart cell in the PB of healthy individuals, we further suggest these acute leukemia cases may arise from transformation of a precursor cell common to the myeloid and NK cell lineages. Therefore, we have chosen to designate this type of leukemia as myeloid/NK acute leukemia.

Historically, NK cell leukemia has been considered a subset of LGL lymphoproliferative disorders, characterized by an indolent or chronic clinical course. It seems inappropriate to include myeloid/NK cell acute leukemia, a more aggressive disease with a distinct non-T-, non-B-cell immunophenotype, as a subtype of this disease. True human NK cell-lineage acute leukemias are rare; to date, only a few well-characterized cases have been reported in the literature. Until recently, the literature regarding LGL disorders has been confusing, partly because of numerous proposed classification schemes and to a paucity of detailed molecular, genetic, and immunophenotypic data. In a recent review, Loughran provides a basis for a uniform classification of LGL disorders that is based on reproducible clinical and clonal features: LGL disorders are divided into three distinct subgroups designated T-LGL leukemia (clonal CD3+), NK-LGL leukemia (clonal CD3+), and LGL lymphocytosis (nonclonal CD3- or CD3+). The myeloid/NK acute leukemias that we describe in this report are most similar to the NK-LGL subgroup. However, whereas the acute clinical presentation, equal sex distribution, and CD3- clonality of NK-LGL leukemia may resemble myeloid/NK acute leukemia, comparison of other features shows that these two diseases are distinct. Although 9/18 of the myeloid/NK leukemia cases had a mild to moderate absolute increase in PB lymphocytes, only rare cases showed an absolute increase in PB and BM cells with LGL morphology. In contrast, NK-LGL leukemia consistently presents with an absolute lymphocytosis (greater than 4,000/μL) and displays frequent and rapid increases in LGL counts (greater than 50,000/μL). Coagulopathy is also more common and severe in NK-LGL leukemia. Other features not shared with myeloid/NK acute leukemia include massive hepatosplenomegaly and involvement of the gastrointestinal tract with jaundice and ascites. The characteristic morphologic feature

![Graph](https://example.com/graph.png)
of NK-LGL leukemia is the presence of increased PB and BM cells with LGL morphology with or without coarse cytoplasmic granules, rather than the unique blast cell features of myeloid/NK acute leukemia. The immunophenotype of NK-LGL leukemia (CD3-, CD56+, CD25-, CD16+, and plasmic granules, rather than the unique blast cell features) is also distinctly different from myeloid/NK acute leukemia. Although expression of stem cell, myeloid-associated, and adhesion molecules have not yet been thoroughly studied in NK-LGL leukemia, the HLA-DR+/CD56+ NK-LGL leukemia cases reported by Imamura were CD13- and CD33-negative, supporting our theory that NK-LGL leukemia and myeloid/NK acute leukemia are separate and distinct diseases. In contrast to myeloid/NK acute leukemia, many clonal NK-LGL cases have been reported to have simple or complex abnormalities of chromosome 5. Finally, in contrast to the majority of our myeloid/NK acute leukemia cases, most NK-LGL leukemia patients have had rapidly progressive clinical courses and have died within several months of presentation despite aggressive treatment. Thus, although the myeloid/NK acute leukemias that we present in this report and cases of NK-LGL leukemia have some shared features and both may be related to the NK cell lineage, distinct biologic and clinical differences are apparent.

All myeloid/NK acute leukemia cases expressed the CD56 antigen at high density. CD56 is an isoform of the well-characterized NCAM that mediates cell/cell adhesive interactions. CD56, CD16+ NK cells express a variably spliced form of CD56 that mediates NK cell-target binding. Expression of CD56, in addition to the CD11a adhesion molecule also expressed on myeloid/NK acute leukemia cases, is required for NK cell-mediated cytotoxic effector function. In addition to the characteristic expression of CD56 on normal NK cells, CD56 is also expressed on a subset of CD3+ cytotoxic T cells, and a subset of PB monocytes. In hematopoietic neoplasms, CD56 has also been detected on clinically aggressive T-cell lymphomas, plasma cell myelomas, and de novo AMLs with t(8;21), t(8;16), and other monocytic leukemia-associated cytogenetic abnormalities. The forms of CD56 expressed by various hematopoietic neoplasms are highly variable as a result of both alternative splicing and differential glycosylation; these alternative isoforms may account in part for the different biologic behaviors of CD56+ neoplasms.

Expression of CD16 is not uniform in cells of NK lineage and is not necessary for NK cell function. In contrast to initial reports of diminished cytotoxicity of CD56+, CD16+ NK cells, subsequent studies have shown that CD56+, CD16+ NK cells may have increased cytotoxicity. Thus, the lack of expression of CD16 in our cases does not preclude NK cell lineage and the cytotoxicity shown by our CD33+, CD56+, CD16+, CD11a− cases is not unexpected. Although the majority of PB NK cells coexpress CD56 and CD16, a CD56+, CD16− NK cell population has been identified at frequency of less than 2% in normal PB. It has been suggested that CD56− NK cells that are CD16+ or CD16− are NK cell precursors because these CD56−, CD16− cells comprise the majority of NK cells found in the BM and splenic pools. Natural killer cells with a similar phenotype have also been generated from primitive CD34+, HLA-DR− progenitors in long-term BM cultures supplemented with IL-2. Unfortunately, the coexpression of myeloid-associated antigens has not been examined in most NK cell studies; thus, we cannot determine whether the CD56+, CD16− NK precursor cells identified in those studies correspond to or are distinct from the CD33+, CD56+, CD16− leukemic cells that we have identified in this report. As NK cells are known to be BM-derived, we speculate that the normal CD33+, CD56+, CD16− cells that we have identified may be a precursor population common to the myeloid and NK cell lineage. It will be interesting to determine the growth factor dependence of these cells in future studies, and whether the CD33 antigen is lost upon culture of these cells with IL-2 that may promote further differentiation into the NK lineage. Finally, we speculate that the CD33+, CD56+, CD16− acute leukemias that we have identified may represent leukemic transformation of this normal CD33+, CD56+, CD16− normal counterpart cell.

Despite morphologic and immunophenotypic similarities, myeloid/NK acute leukemia cases lacked the t(15;17) and failed to show a differentiation-induction response to ATRA in vitro. Distinguishing these myeloid/NK acute leukemias from cases of true APL will be important for clinical studies of ATRA. De Rossi et al have proposed that immunophenotypic/flow cytometric analysis could be used to make a precise diagnosis of APL; the diagnostic profile was CD33+, CD13+, CD9+ (a B-cell and granulocytic marker), HLA-DR−, and CD7−. Unfortunately, the immunophenotypic studies presented in this report show that this profile would not distinguish cases of true APL from myeloid/NK cell acute leukemia cases. Our studies suggest that myeloid/NK cell acute leukemias could be distinguished from cases of true APL by inclusion of the CD56 and CD16 antigens in the proposed diagnostic panel. However, this immunophenotypic approach may still not allow precise diagnosis; in a review of the immunophenotypic data of 50 SWOG APL/AML-M3 cases [(with a confirmed t(15;17)], we identified two APL cases with expression of CD56 at high antigen density (data not shown). Thus, we propose that all acute leukemia cases with morphologic features similar to APL be initially analyzed by flow cytometric immunophenotyping using a panel that includes the HLA-DR, CD34, CD33, CD15, CD16, and CD11a antigens, and by careful morphologic examination with rigid adherence to criteria for AML-M3/M3v. Diagnostic confirmation of APL versus myeloid/NK acute leukemia should include RT-PCR studies to assess the presence or absence of the PML/RARα fusion transcript. With the morphologic and immunophenotypic similarity of myeloid/NK acute leukemia cases to APL, it is of interest that one of our myeloid/NK acute leukemia cases was associated with a t(11;17)(q23;q21) that results in fusion of the RARα and PLZF genes. PLZF has some homology with the zinc finger gene MZF1 and is a putative transcription factor preferentially expressed by myeloid cells. Although the t(11;17) was initially cloned from a case of APL, our myeloid/NK acute leukemia patient with t(11;17) did not have features of APL. Morphologically, this patient was classified as M2 by FAB criteria and had 10% promyelocytes in the BM. The patient did not respond to chemotherapy, nor did the patient’s cells respond to...
ATRA in vitro. Nevertheless, it is intriguing that the patient displayed a moderate bleeding diathesis. Why patients with the P14.2/RARα transcript arising from t(11;17) fail to respond to ATRA, in contrast to ATRA-responsive t(15;17) patients with PML/RARα fusion product, remains to be determined.

The myeloid/NK cell acute leukemias described in this report received various remission-induction therapies, primarily on SWOG treatment studies. For this reason and because of the limited number of cases, we cannot make definitive statements regarding prognosis. In general, the outcomes of our myeloid/NK acute leukemia cases were comparable with that expected for AML patients treated with daunorubicin and cytosine arabinoside, with a median survival of 30 months. However, several of the myeloid/NK acute leukemia cases had an aggressive clinical course (cases 2, 8, and 14; Table 1) without response to standard AML induction chemotherapy. The blasts in two of these three cases (cases 2 and 14, Table 1) displayed high levels of NK cell-mediated cytotoxicity. Although the therapeutic responsiveness of acute myeloid/NK cell acute leukemia must be determined in larger studies of uniformly treated patients, these initial data suggest that more effective treatment regimens may be required for this unique group of acute leukemia patients.

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HLA-DR-, CD33+, CD56+, CD16- myeloid/natural killer cell acute leukemia: a previously unrecognized form of acute leukemia potentially misdiagnosed as French-American-British acute myeloid leukemia-M3 [see comments]

AA Scott, DR Head, KJ Kopecky, FR Appelbaum, KS Theil, MR Grever, IM Chen, MH Whittaker, BB Griffith and JD Licht