An Immunofluorescent Assay for Acute Promyelocytic Leukemia Cells

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Sequential treatment with all-trans retinoic acid followed by chemotherapy significantly improves the long-term survival of patients who have acute promyelocytic leukemia (APL). Consequently, a simple and accurate test is needed to establish the diagnosis of APL and to identify those patients having a relapse of the disease. We describe an accurate, 2-hour indirect immunofluorescent assay for identifying APL cells in bone marrow specimens. The assay uses the PML (PG-M3) murine monoclonal antibody that is directed against the amino-terminal portion of the PML gene product. We observed a distinctive, finely speckled pattern of fluorescence in the NB4 cell line (a positive control), as well as in 15 clinical specimens that were confirmed to have APL by cytogenetic, cytochemical, and immunophenotypic studies, including four cases of microgranular variant of APL. By contrast, a coarse globular pattern of fluorescence was observed in 53 other clinical specimens that did not contain APL. When we performed dilution studies using artificial mixtures of APL cells with normal bone marrow cells, we detected as few as 5% APL cells in the mixture. Finally, there was complete concordance between the immunofluorescent assay and a polymerase chain reaction-based assay for the PML-retinoic acid receptor α chimeric gene in 12 other clinical specimens. We conclude that the immunofluorescent assay for PML protein is a rapid, sensitive, and accurate method for determining the presence of APL cells in clinical specimens. This assay therefore should be considered as a cost-effective alternative to other diagnostic tests, such as karyotyping or polymerase chain reaction, for the diagnostic evaluation of APL. (Key words: PML; Retinoic acid; Promyelocytic; Leukemia; Monoclonal antibody) Am J Clin Pathol 1998;109:205-210.

Identification of the PML-RARα rearrangement is essential in APL because it is closely correlated with responsiveness to treatment with all-trans retinoic acid, which substantially improves survival of patients with the disease. Patients without the PML rearrangement who were incorrectly classified as having APL by clinical and morphologic criteria alone have received unsuccessful treatment with retinoic acid, thereby delaying treatment with more appropriate chemotherapy. Likewise, patients given chemotherapy for acute myelogenous leukemia before a positive result for the PML-RARα rearrangement is obtained from RT-PCR or karyotyping might lose the potential benefit of retinoic acid. Consequently, an assay that can reliably detect the presence of APL cells in clinical specimens is needed.

Immunofluorescence studies using a rabbit polyclonal antibody directed against the PML gene product have shown that APL cells contain characteristic, finely speckled structures called PML oncogenic domains (PODs) that are unlike the coarse globular PODs observed in non-APL cells. A murine monoclonal antibody (PG-M3) was recently
developed against the amino-terminal portion of the PML gene product, and its reactivity with various human cell lines and paraffin-embedded tissue specimens has been described. Because the reactivity of the PG-M3 monoclonal antibody with clinical bone marrow aspirates has not yet been established, we performed this study to determine whether the PG-M3 monoclonal antibody could be useful for assessing the presence of APL cells in bone marrow aspirates.

**MATERIALS AND METHODS**

*Clinical Specimens and Controls*

The first part of this study was performed on cytopreparations of 68 cryopreserved or fresh bone marrow aspirates that were referred from various laboratories in the United States to Nichols Institute and Quest Diagnostics for diagnostic evaluation by flow cytometry, morphology, cytochemistry, and cytogenetics. A diagnosis of APL was confirmed in 15 of these specimens by a combination of the results of morphologic examination, myeloperoxidase staining, immunophenotyping by flow cytometry (CD13+, CD33+, CD34−, HLA-DR−), and cytogenetics. Four of these 15 cases were the microgranular variant of APL. Karyotyping was performed on 24- and 48-hour, unstimulated cultures with the use of standard Giemsa-banding analysis on 20 metaphase cells (Fig 1).

The remaining 53 cases were randomly selected to represent a broad spectrum of other bone marrow conditions: 5 cases of morphologically and phenotypically normal bone marrow, 20 cases of acute myelogenous leukemia of non-APL type (14 cases of FAB M0-M2, 6 cases of M4-M6, no cases of M7), 10 cases of chronic lymphocytic leukemia, 3 cases of acute lymphoblastic leukemia, 10 cases of bone marrow infiltrated by various malignant lymphomas, and 5 cases of chronic granulocytic leukemia. The positive control consisted of cytopreparations of the NB4 acute promyelocytic leukemia cell line. The HL-60 cell line was used as the negative control.

*Immunofluorescent Assay*

The immunofluorescent assay was a modified version of a previously described assay that used rabbit polyclonal antiserum. Briefly, EDTA- or heparin-anticoagulated bone marrow aspirates were first separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation. The cells were then cryopreserved in liquid nitrogen with the use of carefully controlled freezing of cells suspended in a solution of 90% fetal calf serum and 10% dimethylsulfoxide. Before the cells were used in this study, they were rapidly thawed, washed, and counted. Cytopreparations of the mononuclear cell layer were then made, using approximately 100,000 cells in 100 μL of phosphate buffered saline (PBS) with 1% bovine serum albumin (Sigma, St Louis, Mo). Immediately after the cytopreparations were made, the slides were fixed in 1:1 acetone-methanol at −10°C for 1 minute and allowed to air dry for up to 24 hours before immunostaining.

The air-dried slides were first rehydrated in PBS and then blocked for 20 minutes in PBS–10% normal goat serum. The cells were then stained at room temperature for 60 minutes with a solution containing 10 μg/mL of PG-M3 monoclonal antibody (Santa Cruz Biologics, Santa Cruz, Calif) in PBS–1% fetal calf serum. After three washes with cold PBS, the slides were incubated for 45 minutes with the secondary antibody (1:20 goat anti-mouse IgG conjugated to fluorescein and obtained from Santa Cruz Biologics). After the cells were rinsed in PBS, they were counterstained for 1 minute in a solution of Evan’s blue (Sigma), prepared at a concentration of 50 μL of 2% Evan’s blue dye per 50 mL of PBS, rinsed again, and then mounted in a medium containing 90% glycerol–10% water.

Fig 1. Confirmation of t(15;17) in a case of acute promyelocytic leukemia.
Preparations were examined and photographed under blue-light epifluorescence microscopy, using a Nikon Optiphot microscope (Garden City, NY). The cytospins were examined without prior knowledge of the clinical and pathologic diagnoses. Cells stained by this procedure do not display cytoplasmic granules, cellular morphology, or other cytologic features that might otherwise indicate a diagnosis of APL. A negative control slide for each case also was included and was stained with an equal concentration of isotype-matched, mouse IgG (Santa Cruz Biologies) instead of the PG-M3 monoclonal antibody. For comparison, archived, air-dried bone marrow aspirate slides from three of the APL cases were also immunostained as described above with the PG-M3 antibody.

**Mixing Studies**

To determine the sensitivity of this assay for detecting APL cells among normal bone marrow cells, we prepared artificial mixtures of APL cells into normal bone marrow cells. In brief, a nearly pure population of APL cells was obtained by Ficoll-Hypaque separation of a cryopreserved clinical bone marrow specimen that consisted almost entirely (> 95%) of malignant promyelocytes and blasts that were confirmed to be of M3 type as described above. These purified cells were counted and then serially diluted into equal volumes of the same concentration of cryopreserved normal bone marrow cells, which were also purified by Ficoll-Hypaque centrifugation to yield final APL concentrations of 50%, 25%, 10%, 5%, and 1%. Duplicate slides were examined, and at least 500 cells per slide were evaluated. A slide was considered positive if at least two cells with the immunofluorescent pattern characteristic of APL could be definitively identified.

**Reverse Transcription–Polymerase Chain Reaction Study**

We also compared the results of our immunofluorescent assay with the results of a PCR-based assay for PML-RARα chimeric gene in eight additional specimens from patients with newly diagnosed APL. The RT-PCR assay was performed as previously described. In addition, we compared four other bone marrow specimens from patients who previously received treatment for APL and whose bone marrow now contained abundant promyelocytes of uncertain derivation (leukemic vs regenerating).

**RESULTS**

**Immunofluorescent Assay**

In all 15 cases of APL that were initially studied (including the 4 cases of microgranular variant) and in the NB4 cell line, there was a distinctive, finely...
Fig. 4. Cytopreparation of a fresh bone marrow specimen from a patient who previously received treatment for acute promyelocytic leukemia. The regenerating promyelocytes had fewer than eight fluorescent PML oncogenic domains per cell; the result of the reverse transcription–polymerase chain reaction test was also negative.

Fig. 5. Reverse transcription–polymerase chain reaction assay for PML-RARα fusion gene. Lane A, the 100-base pair ladder. Lanes B–D, skin, bone marrow, and blood specimens from a patient with acute promyelocytic leukemia (APL). The 314-base pair band is characteristic of a "short" fusion variant of APL. Lanes E and F, bone marrow cells from the same patient after treatment with retinoic acid. No amplification products are evident, and the results of the immunofluorescent studies of these specimens were also interpreted to be negative. Lanes G and H, NB4 messenger RNA controls at 1:100 and 1:1,000 dilutions. Characteristic multiple bands are present, indicating a type-B "long" fusion variant of APL. Lane I, water (negative) control.

Mixing Studies

In the duplicate mixing studies, dilutions of APL cells as low as 5% could be reliably identified. The cells could not be reliably distinguished at a 1.0% dilution.

Reverse Transcription–Polymerase Chain Reaction Study

There was complete concordance between the results of the immunofluorescent assay (Fig 4) and the results of the RT-PCR assay (Fig 5) in the 12 specimens that were tested by both methods. In the four specimens from patients with APL who received treatment with retinoic acid consolidated with cytotoxic drugs (Fig 4), the results of the immunofluorescent and RT-PCR assays were both negative, thus correctly identifying the presence of regenerating, nonleukemic promyelocytes in the bone marrow and ruling out the diagnosis of relapse.

DISCUSSION

The PG-M3 monoclonal antibody is useful in the diagnostic evaluation of bone marrow aspirates from patients suspected of having APL or APL in relapse. Our findings in 27 clinical specimens from patients with APL were similar to the findings reported when rabbit polyclonal antiserum directed against PML was used in 17 cases of APL.8 The results confirm that the appearance of PODs is distinctly different in APL cells compared with...
non-APL cells. Thus, PG-M3 should be valuable in the initial diagnostic evaluation in cases of suspected APL, as well as in the follow-up of patients who received treatment for APL, particularly those patients in whom regenerating promyelocytes must be distinguished from neoplastic promyelocytes.

Compared with other diagnostic tests for APL, such as RT-PCR, morphology, or karyotyping, the immunofluorescent assay that we have described has certain major advantages for routine clinical use. Although conventional morphologic and cytochemical studies can correctly identify most APL cases, there are some situations in which additional confirmatory studies may be needed. For example, the evaluation of follow-up bone marrow aspirates from patients who have recently received treatment for APL is sometimes difficult. Many of these bone marrow specimens contain numerous promyelocytes that may represent either benign regeneration of myelopoiesis or recurrence of the APL, thus posing interpretive difficulties for the hematopathologist. In such cases, we propose that the immunofluorescent assay could be of considerable value in distinguishing between benign and malignant promyelocytes.

Another example involves the initial evaluation of suspected APL specimens in which the malignant promyelocytes either have an unusual microgranular morphology or resemble FAB M2 or M5b acute leukemia. In such cases, a positive confirmatory immunofluorescent result could help establish a diagnosis of APL.

Another advantage of the immunofluorescent assay is its low cost (approximately $50 for materials and labor vs average costs of approximately $180 for PCR and $200 for karyotyping). Because the immunofluorescent assay does not require access to a molecular biology or cytogentic facility, it also can be easily performed in most hospital laboratories, thereby reducing the need to send APL specimens to an external laboratory for PCR testing or cytogetic studies. Finally, intact messenger RNA suitable for use in the RT-PCR assay cannot always be obtained from every APL specimen, and the immunofluorescent assay could be useful as a back-up or confirmatory test for APL in such cases.

With regard to turnaround time, the immunofluorescent assay is clearly the fastest confirmatory test for APL (<3 hours for immunofluorescent results vs 2 days for RT-PCR results and 3-7 days for karyotyping results). There is sometimes a clinical urgency to establish a diagnosis of APL, especially when the patient is potentially at risk for life-threatening coagulopathy. In such cases, the immunofluorescent assay could be especially valuable.

At this time, however, it is still unclear whether a rapid diagnosis of APL is also needed to facilitate initial induction therapy with all-trans retinoic acid. Although it is crucial to establish a correct diagnosis of APL in a timely fashion, this is primarily to alert clinicians to the need to incorporate retinoic acid therapy at some point during the treatment but not necessarily as the first drug. To date, randomized trials have failed to show a reduction in morbidity or mortality rates in APL patients who receive treatment with retinoic acid induction compared with those who receive chemotherapy. Although treatment with all-trans retinoic acid and chemotherapy does improve the overall survival of patients with APL, compared with chemotherapy alone, retinoic acid does not have to be given first. The improved survival occurs as long as the patients receive all-trans retinoic acid at some point during their antileukemic treatment.11,12

In summary, the main advantages of this immunofluorescent assay are its simplicity, speed, accuracy, and low cost—all of which make it a cost-effective alternative to karyotypic analysis or RT-PCR and a complement to conventional morphologic studies. In experienced hands, the results of the assay are relatively straightforward and easy to interpret and correlate extremely well with the current “gold standards” of PCR and karyotyping. For initial diagnostic purposes, therefore, we propose that the assay may now be considered a reliable substitute for those methods. Because the threshold of reliable detection of APL cells in bone marrow aspirates is approximately 5%, however, the assay should not be used to assess minimal residual disease in patients receiving treatment for APL. For such patients, the RT-PCR assay remains a more appropriate approach for assessing disease status.

REFERENCES


