

Mass-like lesion in the bone marrow of a dog with formalin-sensitive granules

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Questions:

1. What are reasonable diagnoses for this neoplasm based on aspirate, core, and flow cytometry data?

The bone marrow core contained two morphologically distinct subpopulations forming mass-like aggregates. Despite the size variation, the two populations shared similar nuclear features, which suggested a relationship between the two and the possibility of maturation within a single neoplastic lineage. The initial differential diagnoses included acute leukemia (acute myeloid leukemia [AML], acute lymphoblastic leukemia, or acute unclassifiable leukemia) with an atypical mass-like pattern, granulated lymphoma originating within the bone marrow, leukemic phase of a granular lymphoma, or (less likely) acute leukemia with a concurrent metastatic neoplasm (i.e., mast cell neoplasia).

Routine immunophenotyping by flow cytometry was performed on the bone marrow aspirate at the CSU Clinical Hematopathology Lab (Figure 5). The sample was processed, as previously described^{1,2} and analyzed on a Sony ID7000 spectral flow cytometer (Sony Biotechnology, San Jose, CA). This demonstrated an expanded population of small-sized CD34+ cells.

Approximately half of the CD34+ cells co-expressed CD18. There was a separate population of intermediate to large-sized cells that expressed CD18 and the monocyte antigen CD14; these cells also lacked expression of CD34 and had low class II major histocompatibility complex (MHC). There was also a minor population of larger cells that expressed CD18 but did not express CD34, nor mature neutrophil (CD4) or monocyte (CD14) antigens. The cross-reactive

anti-human CD18 antibody used by flow cytometry binds primarily to myeloid cells and binds minimally to lymphoid cells.³ Taken together, the immunophenotyping results were most consistent with AML.⁴

Alkaline phosphatase (ALP) activity also supported acute leukemia characterization, consistent with prior reports describing its utility as an adjunctive diagnostic tool in canine leukemias.^{5,6} The ALP positivity was seen within both smaller and larger cell morphologies.

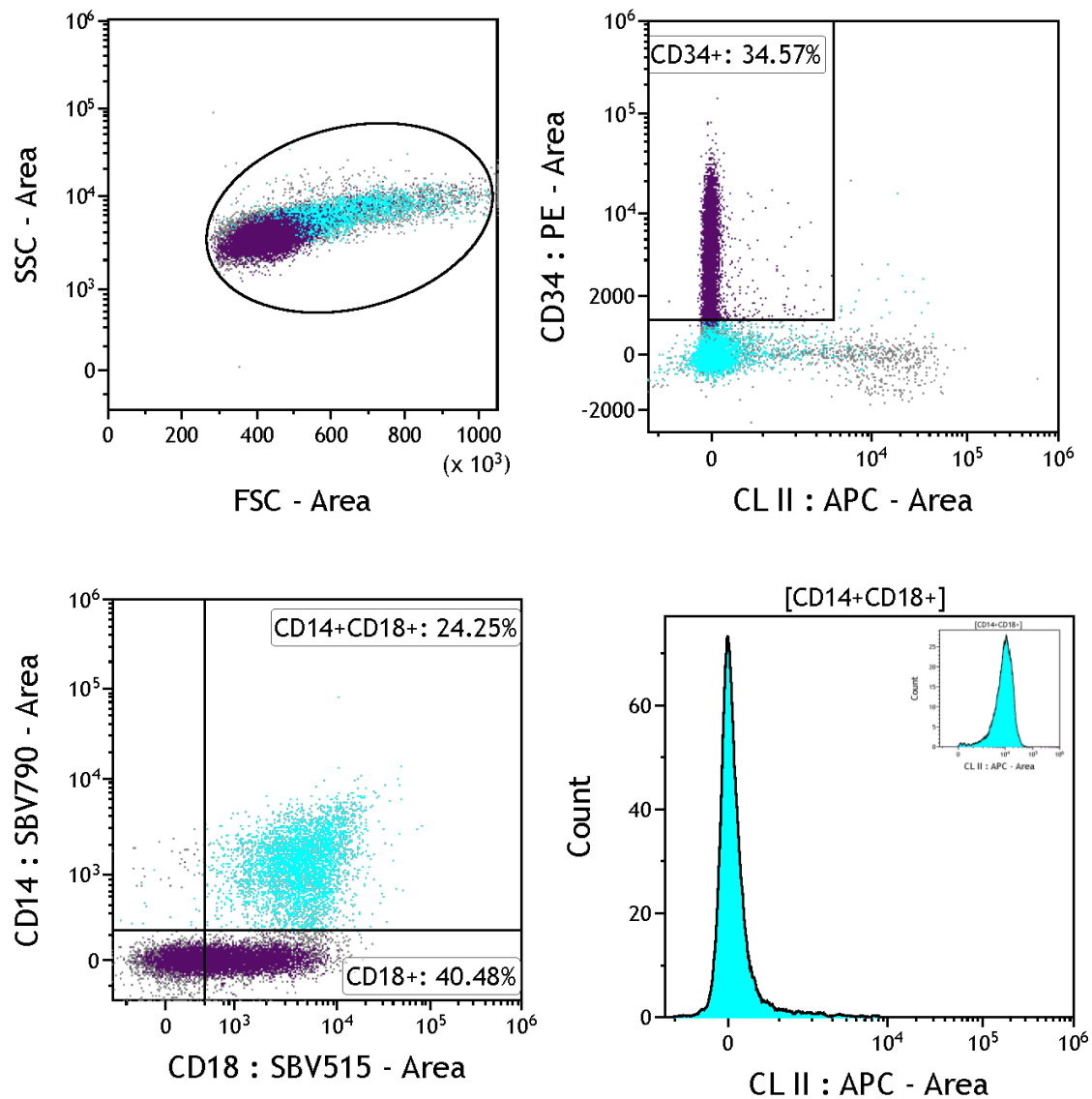


Figure 5. Flow cytometry immunophenotyping from the bone marrow aspirate. CD34+ cells (purple) have low forward scatter and a subset express CD18. CD14+CD18+ cells (teal) have higher forward scatter. In the histograms on the bottom right, CD14+CD18+ cells in the patient

sample express low levels of class II MHC (CLII), as compared to monocytes from a normal canine peripheral blood sample (inset). FSC, forward scatter. SSC, side scatter.

2. What additional immunohistochemical or special staining would be most helpful to better characterize the two cell populations described?

Because of the atypical aggregates within the bone marrow core, immunohistochemical stains for CD34, anti-canine CD18, Myeloperoxidase (MPO), Tryptase, MUM1, IBA1, CD3, CD79a, and PAX5 were performed, as previously described (Figure 6),^{7,8} in attempt to further characterize these two cell populations. Approximately 50-60% of the smaller cells noted along the periphery of the aggregates were CD34 positive, 30-40% were CD18 positive, 10-20% were IBA1 positive, and <3% were MPO positive; this was concordant with the cytologic and flow cytometric findings and supported a diagnosis of AML. These cells were also negative for Tryptase, MUM1, CD3, CD79a, and PAX5, which effectively excluded mast cell, plasma cell, and lymphoid differentiation.⁹

The larger cells forming aggregates were diffusely CD18 positive, supporting leukocytic differentiation. However, these cells were negative for CD34, MPO, Tryptase, MUM1, IBA1, CD3, CD79a, and PAX5, limiting further characterization. The shared nuclear morphology with the dominant AML population, combined with CD18 expression and expanded cytoplasm, suggested a maturing leukocyte population rather than a separate neoplasm. However, the exact lineage of the focal aggregates could not be definitively established.

3. How would you best explain the discrepancy in granulation between the aspirate and core samples?

Additional staining was performed according to the kit manufacturer's instructions to characterize the granules found in high numbers on the aspirate preparations, but which were not readily observed in the histologic sections. All positive and negative controls were considered appropriate prior to analysis. Approximately 20-30% of the granules observed on Wright-Giemsa-stained aspirates were also positive with safranin, which typically binds to heparin-containing granules¹⁰ (Figure 7). In contrast, granules were not detected in the histologic sections with Giemsa, toluidine blue, Luna, and safranin stains.

The absence of granules on histologic preparations raised the concern that they were not preserved in formalin-fixed tissue sections. To assess whether the granules were sensitive to formalin fixation, one Wright-Giemsa-stained aspirate slide was soaked in 10% neutral buffered formalin at room temperature for 18 hours, rinsed briefly with distilled water, and then re-stained with Wright-Giemsa. The granulation was markedly reduced after formalin exposure, supporting formalin sensitivity of the cytoplasmic granules (Figure 8). Based on assessment of all data, the final diagnosis was AML with formalin sensitive granulation. The larger cells forming mass-like aggregates could not be definitively classified.

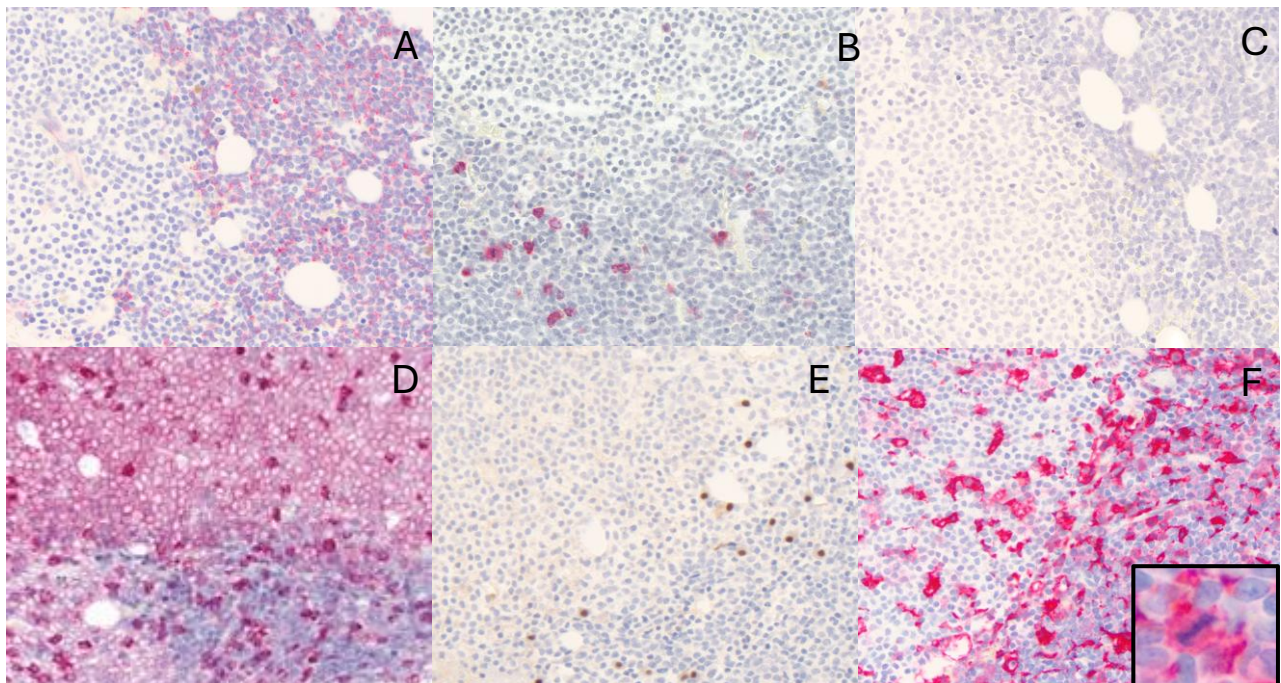


Figure 6. Bone marrow core. A, 50-60% of the surrounding, smaller cell population exhibited membranous CD34 positivity, whereas the aggregates of larger cells were CD34 negative (H&E, 50x, oil immersion). B, <3% of the surrounding smaller cells and none of the larger cell aggregates displayed cytoplasmic MPO positivity (50x objective, oil immersion). C, All cells were diffusely negative for Tryptase (50x objective, oil immersion). D, 30-40% of the peripheral smaller cells and nearly all of the larger cell aggregates exhibited membranous CD18 positivity (50x objective, oil immersion). E, Rare, intermixed cells exhibited nuclear MUM1 positivity, consistent with resident non-neoplastic plasma cells; however, all neoplastic cells were MUM1-negative (50x objective, oil immersion). F, Numerous intermixed cells exhibiting strong

cytoplasmic IBA-1 positivity were interpreted as tumor associated macrophages. Approximately 10-20% of the neoplastic cells at the periphery of the aggregates exhibited moderately strong IBA-1 positivity. The lower right inset shows a higher-magnification image of a mitotic neoplastic cell with cytoplasmic IBA-1 positivity (50x objective, oil immersion).

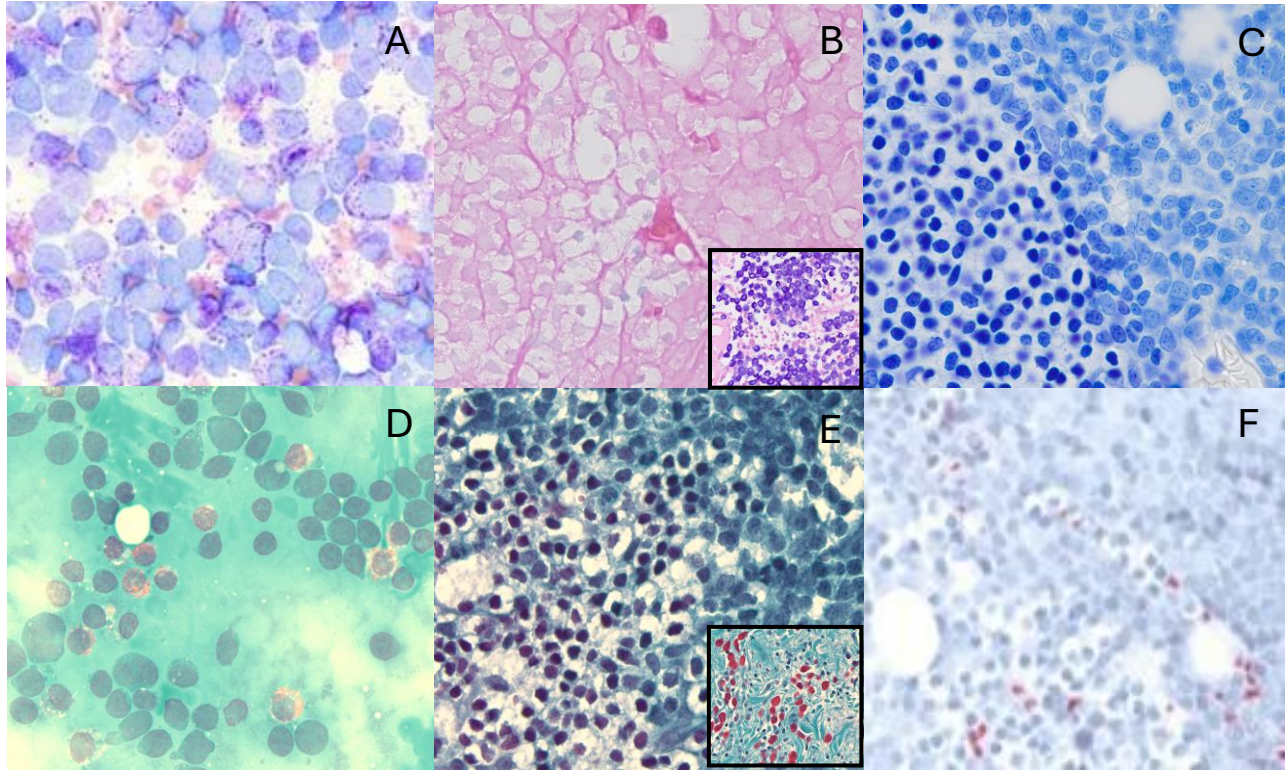


Figure 7. Bone marrow core and aspirate. A, Bone marrow aspirate demonstrating a prominent granulated cell population (Wright-Giemsa stain, 100x, oil immersion). B, Bone marrow core demonstrating the absence of granulated cells. The right lower inset represents a positive control mast cell population (Wright-Giemsa stain, 100x, oil immersion). C, All cells within the bone marrow core exhibited diffuse negativity for Toluidine Blue stain (50x, oil immersion). D, Approximately 20-30% of the cells in the bone marrow aspirate demonstrate safranin positive granules (100x, oil immersion). E, Bone marrow core in which all cells exhibited diffuse safranin negativity. The right lower inset represents a positive control (50x, oil immersion). F, Few erythrocytes exhibited strong cytoplasmic staining for Luna stain but the remaining cells were negative (50x, oil immersion).

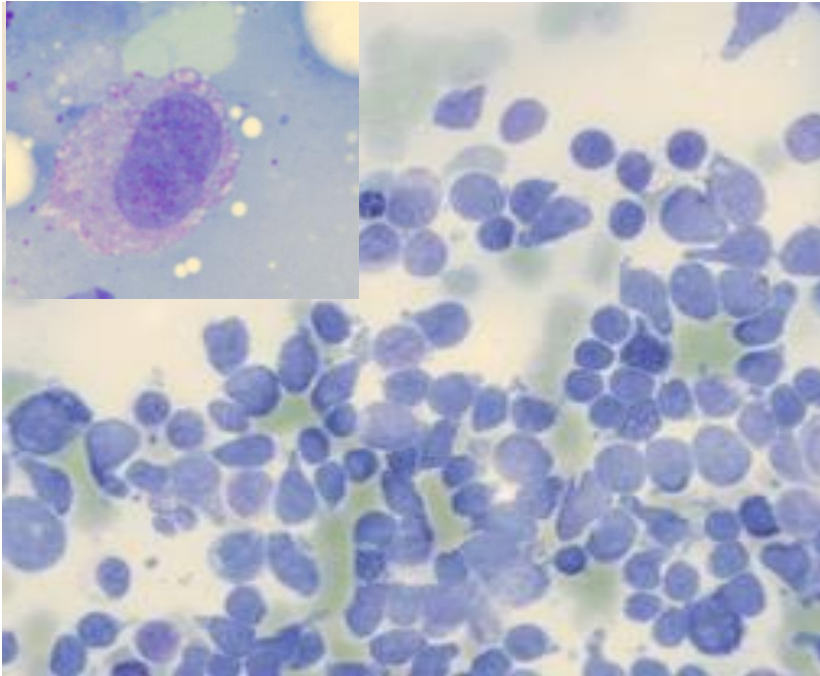


Figure 8. Bone marrow aspirate. After staining with Wright Giemsa, the same sample was soaked in neutral buffered formalin for 18 hours and re-evaluated. The remaining cells exhibited markedly decreased to no granulation (50x oil immersion) compared to the pre-formalin exposure morphology. The proportion of cells with granulation was markedly reduced, and in those cells with granulation, the intensity of the staining was diminished. The inset on the top left of the image displays higher power magnification at 100x oil immersion.

Clinical outcome

The patient was hospitalized for supportive care, including dexamethasone, immunoglobulin G, cyclosporine, and gastrointestinal support. The pancytopenia persisted, but the dog's clinical status improved sufficiently for discharge. Approximately 36 hours after discharge, the patient re-presented with melena and pyrexia (40.6°C), and thoracic radiographs revealed changes consistent with aspiration pneumonia. The patient became tachycardic and hypotensive, despite resuscitation efforts. Due to the concern for septic shock, the patient was euthanized.

Discussion

AML is an uncommon but rapidly progressive hematopoietic neoplasm in dogs and represents the most frequently reported form of acute leukemia in this species.^{5,11} It is a clonal proliferation of immature myeloid precursors within the bone marrow. Traditional veterinary diagnostic

criteria include >20% circulating blasts or >30% blasts within the marrow.^{12,13} However, many veterinary pathologists now apply the World Health Organization (WHO) human cutoff value of 20% myeloid blasts in either the peripheral blood or bone marrow for a diagnosis of AML.^{14,15} In the present case, both the high proportion of unclassified cells in the peripheral blood (60%) and the proportion of total cells in the bone marrow expressing CD34 (34.6%) exceeded the diagnostic thresholds, supporting a diagnosis of acute leukemia. Reported prognoses for dogs with AML are poor, with a median survival of 2-3 weeks.^{14,16,17}

One unusual feature of this case was the morphologic heterogeneity within the neoplastic population. Although the marrow was diffusely effaced, as is typical of AML, neoplastic cells within aggregates were morphologically distinct from the surrounding cells. This presentation initially broadened the differential diagnosis to include metastatic neoplasia, granular lymphoma, or mast cell disease in addition to the apparent AML.

Ultimately, cytochemical and immunophenotypic findings supported an immature myeloid lineage. ALP activity in the bone marrow aspirate aided in acute leukemia characterization, consistent with prior reports describing its utility as an adjunctive diagnostic tool in canine leukemias.^{5,6} Flow cytometry and immunohistochemistry demonstrated an expanded CD34+ population, consistent with the presence of immature hematopoietic cells. CD14 and CD18 expression provided further evidence of myeloid differentiation and, together with the morphologic findings, confirmed the diagnosis of AML. A subset of the smaller cell population expressed CD34 and a subset expressed IBA1 on immunohistochemistry, which is suggestive of myelomonocytic or monocytic differentiation.¹⁸

The larger cells forming the aggregates could not be fully characterized. These cells lacked tryptase reactivity, arguing against mast cell differentiation.⁹ The absence of lymphoid marker expression (CD3, MUM1, PAX5, and CD79a) also made lymphoid differentiation unlikely.^{7,8} The lack of CD34 expression within this population alongside concurrent positive CD18 labeling suggested a leukocyte differentiation. One possibility is that the neoplasm represents a myelomonocytic process with the morphologic differences reflecting distinct cellular components—namely, immature myeloid and differentiating monocytic cells. However, IBA1 immunohistochemistry did not support this hypothesis within the larger cell aggregates. Alternatively, the larger aggregating cells may represent a focal area of maturation from the AML neoplasm accompanied by aberrant loss of lineage markers. Whole genome sequencing

coupled with cell sorting may have provided additional characterization of cell lineage and confirmed the relationship of the two morphologically distinct populations on histology, but this was not available.¹⁹

In addition, in both flow cytometry and histology/immunohistochemistry, the CD34+ precursors were smaller in size than the larger, CD34- cells. Immature hematopoietic cells can be smaller in size than their mature counterparts²⁰ and cell size does not reliably define maturation or lineage in acute leukemia. These findings emphasize the importance of immunophenotyping, rather than morphology alone, when diagnosing and classifying acute leukemia.

Hematologic abnormalities in this case were also consistent with previous reports for acute leukemia. Bicytopenia and pancytopenia have been documented in approximately 44% of dogs with AML, of which, pancytopenia was ultimately identified in this patient.²¹ These findings are attributable to the marrow effacement by the neoplastic cell population, resulting in ineffective erythropoiesis and megakaryopoiesis.

An unusual and diagnostically relevant finding in this case was the discrepancy in cytoplasmic granule preservation between the bone marrow aspirate and the formalin-fixed core biopsy. Prominent magenta to purple granules were readily observed in the populations of interest (both small and large sized cells) on Wright-Giemsa stained cytologic preparations and 20-30% of these cells retained weak safranin positivity. The origin and composition of these granules could not be further characterized. Although mast-cell granules were initially considered, stronger and more uniform safranin positivity would be anticipated if the granules contained abundant heparin-rich mast-cell proteoglycans.

In contrast, granules were not evident in the corresponding formalin-fixed tissue sections and markedly diminished after formalin exposure of the stained smear. Formalin-induced dissolution of cytoplasmic granules has been well described in mast cells of dogs, with Kube et al. documenting significant reduction in granule visibility after formalin fixation.²² Similarly, Marshall et al. demonstrated reduced mast cell detection in formalin-fixed human dermis using toluidine blue and safranin stains, with only approximately 50% of mast cells remaining identifiable.²³ The present case suggests that a similar artifact can occur in at least some myeloid neoplasms. To the authors' knowledge, this phenomenon has not been previously reported in cases of AML. If the granules in these neoplastic cells behave similarly to those of mast cells, alternative fixation methods such as Carnoy's and Mota's fixatives may better preserve granules

and might have helped determine which cellular population was granulated on histopathologic evaluation. While it is tempting to presume that the non-labeling cells had expanded cytoplasm due to dissolved granules, this cannot be confirmed by histology alone and some of the granulated cells on the aspirate were small with a high N:C ratio.

In summary, this case documents an atypical presentation of canine AML with atypical marrow aggregates and formalin-sensitive cytoplasmic granules. Recognition of this potential artifact is important, as reliance on formalin-fixed tissue alone may obscure diagnostically relevant cytoplasmic features and underscores the value of integrating cytologic, histologic, immunophenotypic, and histo/cytochemical findings when evaluating acute leukemia.

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