Intracellular Hemoglobin—A Specific Marker for Erythroid Cells in Paraffin Sections

An Immunoperoxidase Study of Normal, Megaloblastic, and Dysplastic Erythropoiesis, Including Erythroleukemia and Other Myeloproliferative Disorders

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Intracellular hemoglobin represents an excellent marker for specific characterization of normal, megaloblastic, or dysplastic erythroid cells in paraffin sections. Using an immunoperoxidase indirect sandwich technique for detection of intracellular hemoglobin, erythroid cells at all stages of maturation were readily identified in bone marrow biopsies (58 specimens total) with *a*) normal erythropoiesis, *b*) megaloblastic erythropoiesis, and *c*) various myeloproliferative disorders, including erythroleukemia. In other tissues (6 spleens, 2 lymph nodes, 1 liver) with extramedullary hematopoiesis, erythroid cells were similarly defined on the basis of this immunohistochemical method. Initial fixation in Zenker's-acetic acid solution (employed for bone marrow biopsies), B5 solution, or formalin, appeared equally effective in preserving the antigenicity of intracellular human hemoglobin. This sensitive and specific immunoperoxidase technique for erythroid cell characterization is particularly applicable to tissues with abnormal erythropoiesis, in which precise cell identification generally presents a diagnostic problem. (Am J Pathol 1971, 102:308–313)

IN DYSMYELOPOIETIC SYNDROMES, myeloproliferative disorders, particularly erythroleukemia, and megaloblastic erythropoiesis, accurate identification of immature and dysplastic hematopoietic cells in paraffin sections often presents a difficult diagnostic problem. Immunoperoxidase techniques for detection of intracellular lysozyme assist in the defining of cells of myeloid and monocytic types.[1,2] However, methods are currently unavailable for precise characterization of cells of erythroid derivation, especially dysplastic forms. Since hemoglobin production begins at early stages of erythropoiesis,[3] a sensitive technique for detection of intracellular hemoglobin should permit identification of erythroid cells. The purpose of this study is to determine whether normal, megaloblastic, and dysplastic cells of erythroid derivation may be specifically identified and characterized in paraffin sections on the basis of intracytoplasmic hemoglobin with the use of an immunoperoxidase indirect sandwich technique.

Materials and Methods

All specimens were retrieved from the surgical pathology files of the Peter Bent Brigham Hospital. Bone marrow biopsies (58 specimens) were initially fixed in Zenker's-acetic acid solution (20:1, vol/vol), usually for 4–6 hours, washed for 1 hour under running water, then processed routinely. Other tissues (6 spleens, 2 lymph nodes, 1 liver biopsy specimen) were fixed in 10% neutral buffered formalin for 8–24 hours, or in B5 solution[4] for 4 hours, washed under running water, then placed in 10% formalin and processed routinely.

Immunoperoxidase studies were performed by a

Supported in part by NIH Grants HL-06370 and 2-S07-RR05489.

Accepted for publication August 29, 1980.

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modification of reported methods,[5-7] as described previously.[2,8] Three-micron paraffin sections were placed on glass slides, then warmed to 45 C for 1–2 hours to insure adherence. Slides were deparaffinized in xylene and placed in absolute alcohol. The slides then were placed in methanolic peroxide (5 parts methanol/1 part 3% hydrogen peroxide) for 30 minutes at room temperature to consume endogenous peroxidase. Following this treatment, the slides were placed in Tris [tris(hydroxymethyl)aminomethane-hydrochloric acid] buffer (0.1 M, pH 7.6) for at least 15 minutes. The sections were then sequentially incubated for 30 minutes with 1) rabbit antihuman hemoglobin antiserum (1:25 and 1:50 dilution, optimal dilutions; in some cases, dilutions of 1:100 or greater were also employed) (Cappel Laboratories, Cochranville, Pa), 2) swine antirabbit serum IgG, and 3) horseradish peroxidase-rabbit antihorseradish peroxidase soluble complexes. The latter two reagents were obtained from Dakopatts A/S, Copenhagen, Denmark (U.S. distributor, Accurate Chemical and Scientific Corp., Hicksville, NY). Following each incubation, slices were washed with Tris-saline (1 part Tris buffer [0.5 M, pH 7.6] to 9 parts normal saline [0.9 g% sodium chloride]), then placed in Tris buffer supplemented with 1% normal horse serum (Flow Laboratories, Rockville, Md). Antibody localization was effected by incubating the slides for 5 minutes with a freshly prepared solution of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemicals, St. Louis, Mo; 6 mg/10 ml Tris buffer), containing 0.1 ml of 3% hydrogen peroxide. This method[9] yields a brown reaction product. Slides were counterstained with hematoxylin or methyl green, dehydrated, and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ).

We performed control studies by substituting normal rabbit serum or Tris buffer for immune serum. Specificity of staining with rabbit antihuman hemoglobin antiserum was verified in parallel studies using antiserum which had previously been absorbed with purified human hemoglobin (two separate sources evaluated: ICN Laboratories, Cleveland, Ohio, and Sigma Chemicals, St. Louis, Mo). Specificity of the absorption studies was confirmed further by cross-absorption studies using bovine hemoglobin (ICN Laboratories, Cleveland, Ohio), which revealed staining patterns similar to those of the immune serum.

In most cases, studies for intracellular lysozyme were also performed as previously described,[2] in order to evaluate thoroughly in sequential paraffin sections the possible origin of immature cells and to confirm the specificity of the results using the antihemoglobin serum.

Results

Sixty-seven specimens were evaluated, including bone marrow biopsy specimens (58 specimens) of patients with *a*) no evidence of myeloproliferative or lymphoproliferative disorders (10 cases), *b*) anemia associated with erythroid hyperplasia (5 cases), *c*) megaloblastic anemia (6 cases), *d*) myeloproliferative disorders (34 cases), and *e*) acute lymphocytic leukemia (3 cases). Other tissues (6 spleens, 2 lymph nodes, and 1 liver biopsy specimen) with extramedullary hematopoiesis obtained from patients with various hematologic disorders were also assessed.

Bone Marrow Biopsy Specimens—Normal and with Erythroid Hyperplasia

Strong staining for intracellular hemoglobin was observed in erythroid cells at all stages of maturation (Figure 1). Reactivity appeared localized to the cyto-

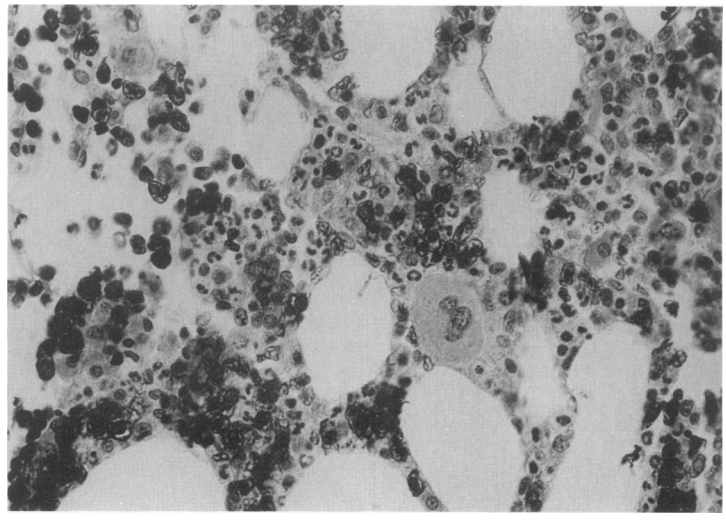

Figure 1—Normal erythropoiesis. Bone marrow biopsy (Zenker's-acetic acid fixation, paraffin section). Immunoperoxidase studies demonstrate cytoplasmic staining for hemoglobin (*black*) in erythroid cells at all stages of maturation. Myeloid cells, megakaryocytes, and plasma cells are negative. (Hematoxylin counterstain, × 600)

plasm, though faint staining overlying the nucleus was noted in some erythroid precursors, possibly representing a fixation artifact. Mature erythrocytes present in these and all other sections were positive and served as an internal control for the method. Specificity of staining was verified in parallel sections in which normal rabbit serum, Tris buffer, or rabbit antihuman hemoglobin antiserum adsorbed with human hemoglobin was substituted for immune serum. In all of the latter, no reactivity was noted, except for focal faint staining of mature erythrocytes. Similar results were obtained for all other specimens assessed with the control serums.

Other hematopoietic elements, including myeloid cells and megakaryocytes, showed no staining. Plasma cells and lymphocytes were similarly negative. Serial sections evaluated for intracellular lysozyme revealed strong staining in myeloid elements and no staining in cells of erythroid type.

Megaloblastic Erythropoiesis

Strong staining for intracellular hemoglobin was present in megaloblastic erythroid elements at all stages of maturation, in cases of anemia associated with vitamin B12 or folate deficiency, or in megaloblastic erythropoiesis observed in a treated case of acute lymphocytic leukemia (Figure 2). Other hematopoietic elements were negative.

Myeloproliferative Disorders

Polycythemia Vera (2 Cases)

Strong staining for intracellular hemoglobin in erythroid elements at all stages of maturation was observed. Other marrow elements revealed no reactivity.

Erythroleukemia (9 Cases)

Bone marrow morphology varied considerably in these cases, particularly with regard to the number of blast forms. Dysplastic and/or megaloblastic erythroid elements were identified in varying numbers in all specimens and were characterized on the basis of intracellular hemoglobin (Figures 3–5). Using intracellular lysozyme and hemoglobin, respectively, as cell markers, immunoperoxidase studies demonstrated that erythroid elements ranged from 10% to 80% (mean, 42%), myeloid elements from 5% to 80% (mean, 24%), and blast forms lacking both markers from 0% to 85% (mean, 33%). Dysplastic forms showing staining for intracellular hemoglobin revealed no reactivity for intracytoplasmic lysozyme. Specificity of staining was further verified as described previously. In bone marrow biopsy specimens with increased reticulum, nonspecific staining of reticulum fibers was observed in sections incubated with rabbit antihuman hemoglobin antiserum. This staining, however, did not interfere with interpretation.

Acute Myelogenous Leukemia (4 Cases)

In bone marrow biopsies of two treated and two untreated patients, islands of erythroid elements were strongly positive for intracellular hemoglobin. Myeloblasts (the predominant cell type present) were negative.

Acute Leukemia, Indeterminate Type (5 Cases)

Erythroid elements, including macronormoblastic forms, revealed strong staining for intracellular hemoglobin. In most cases, blasts were negative for both intracellular hemoglobin and lysozyme, though small numbers were positive for the latter.

Chronic Myelogenous Leukemia in Blast Crisis (6 Cases)

Scattered islands of erythroid elements, including macronormoblastic forms, were identified on the basis of intracellular hemoglobin and constituted varia-

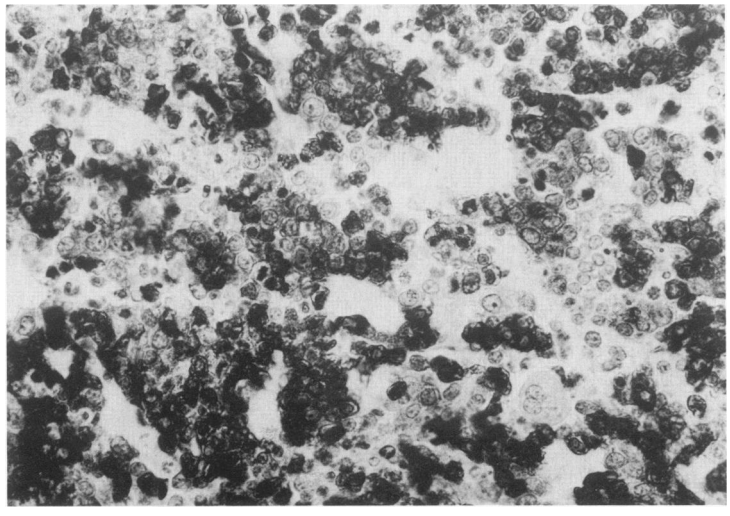

Figure 2—Megaloblastic erythropoiesis. Bone marrow biopsy, acute lymphocytic leukemia, treated (Zenker's-acetic acid fixation, paraffin section). Clusters of megaloblastic erythroid cells reveal strong cytoplasmic staining for hemoglobin (*black*). There is no cytoplasmic staining in lymphoblasts, myeloid cells, and a megakaryocyte. (Hematoxylin counterstain, ×600)

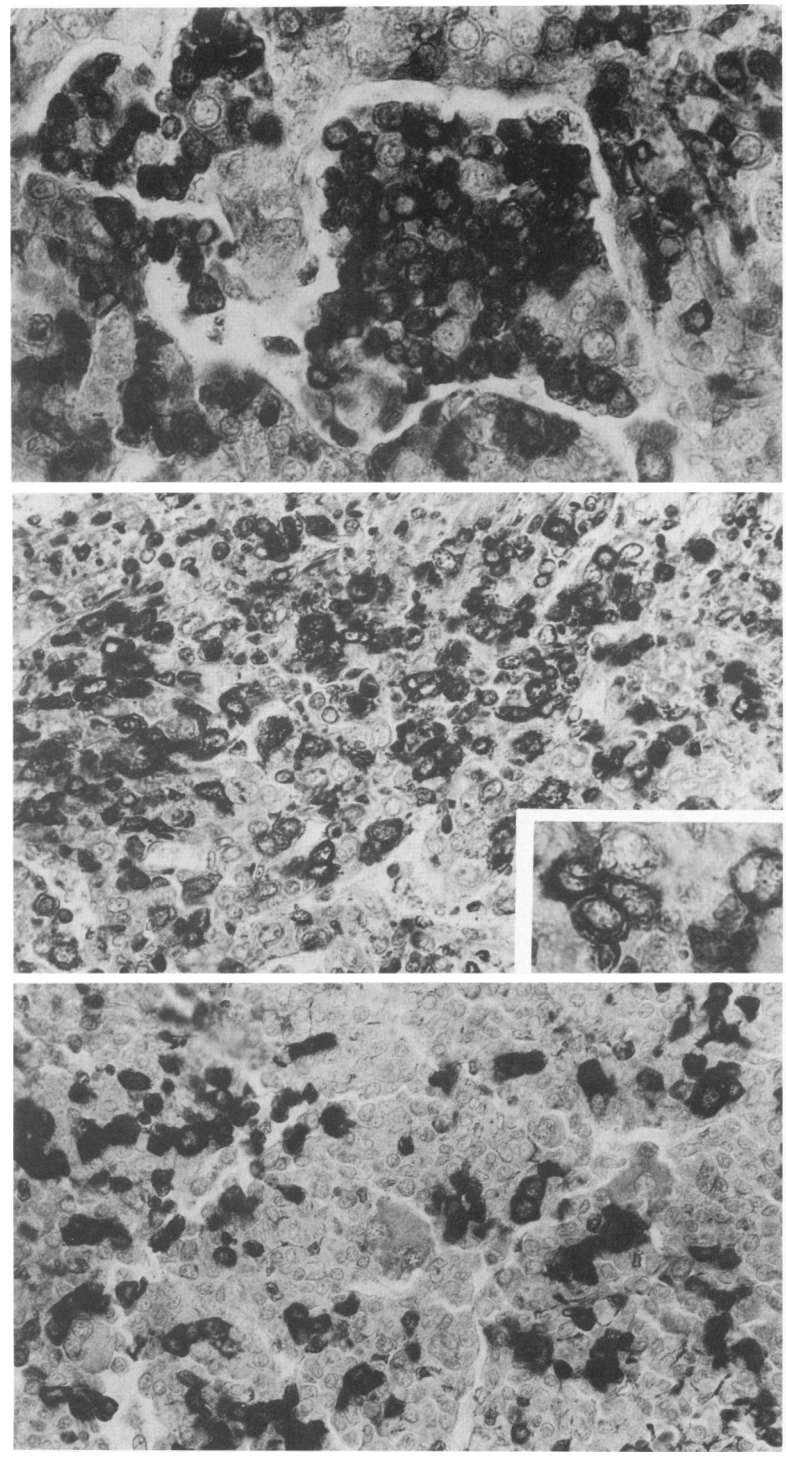

Figure 3—Erythroleukemia. Bone marrow biopsy (Zenker's-acetic acid fixation, paraffin section). Prominent clusters of erythroblasts reveal positive cytoplasmic staining for hemoglobin (*black*). A minority of blasts failed to reveal cytoplasmic staining for either hemoglobin or lysozyme (as assessed in sequential section). (Hematoxylin counterstain, ×500) **Figure 4**—Erythroleukemia. Bone marrow biopsy (Zenker's acetic acid fixation, paraffin section). The majority of the blasts, including large dysplastic forms, reveal cytoplasmic staining for hemoglobin (*black*). The **inset** shows a higher magnification of dysplastic and binucleate erythroblasts with strong cytoplasmic staining for hemoglobin. (Hematoxylin counterstain, ×400; **inset**, ×800) **Figure 5**—Erythroleukemia. Bone marrow biopsy (Zenker's-acetic acid fixation, paraffin section). Blasts with prominent cytoplasmic staining for hemoglobin (*black*) occur singly and in small clusters. The majority of the blast forms in this case failed to show cytoplasmic staining for hemoglobin or lysozyme. (Methyl green counterstain, ×400)

ble percentages of the marrow population. Blast forms were negative for hemoglobin and revealed little or no staining for intracellular lysozyme.

Myelofibrosis (6 Cases)

Strong reactivity for intracellular hemoglobin was observed in aggregates of erythroid elements. No staining was observed in myeloid cells. Faint nonspecific staining was apparent in some megakaryocytes. Reticulum fibers, abundant in all cases, also showed nonspecific staining.

Myeloproliferative Disorders, Unclassified (2 Cases)

Erythroid elements at all stages of maturation revealed staining for intracellular hemoglobin.

Extramedullary Hematopoiesis (10 Specimens)

A total of 6 spleens with extramedullary hematopoiesis was obtained from patients with myelofibrosis (3 cases), chronic myelogenous leukemia (1 case), hairy cell leukemia (1 case), and idiopathic thrombocytopenic purpura (1 case). In all cases, splenic tissue fixed in formalin and in B5 solution was evaluated. With either fixative, erythropoietic elements were identified in all cases and were characterized by strong staining for intracellular hemoglobin (Figures 6 and 7). Similar staining of erythroid elements was observed in lymph nodes of patients with myelofibrosis (1 case) or chronic myelogenous leukemia (1 case) and in a liver biopsy specimen from a patient with myelofibrosis (Figure 8). Other cell types in these tissues, eg, myeloid cells, megakaryocytes, plasma cells, lymphoid cells, hairy cells, and histiocytes, did not stain. The results obtained for this group of tissues indicate that all fixatives employed in this study (Zenker's-acetic acid solution, formalin, and B5 solution) provide sufficient preservation of antigenicity of human hemoglobin to permit detection by the immunoperoxidase technique.

Acute Lymphocytic Leukemia (3 Cases)

In bone marrow biopsy specimens from 1 treated and 2 untreated patients, erythroid elements at cell stages of maturation, including megaloblastic forms apparent in the treated case (Figure 2), showed strong staining for intracellular hemoglobin. Lymphoblasts were negative.

Discussion

Immunoperoxidase techniques employing immunoglobulin enzyme bridges for identification and characterization of various tissue antigens have been extremely helpful in evaluation of hematologic disorders. Detection of intracellular immunoglobulin in B lymphoid cells and plasma cells permits distinctions between neoplastic (monoclonal) and reactive (poly-

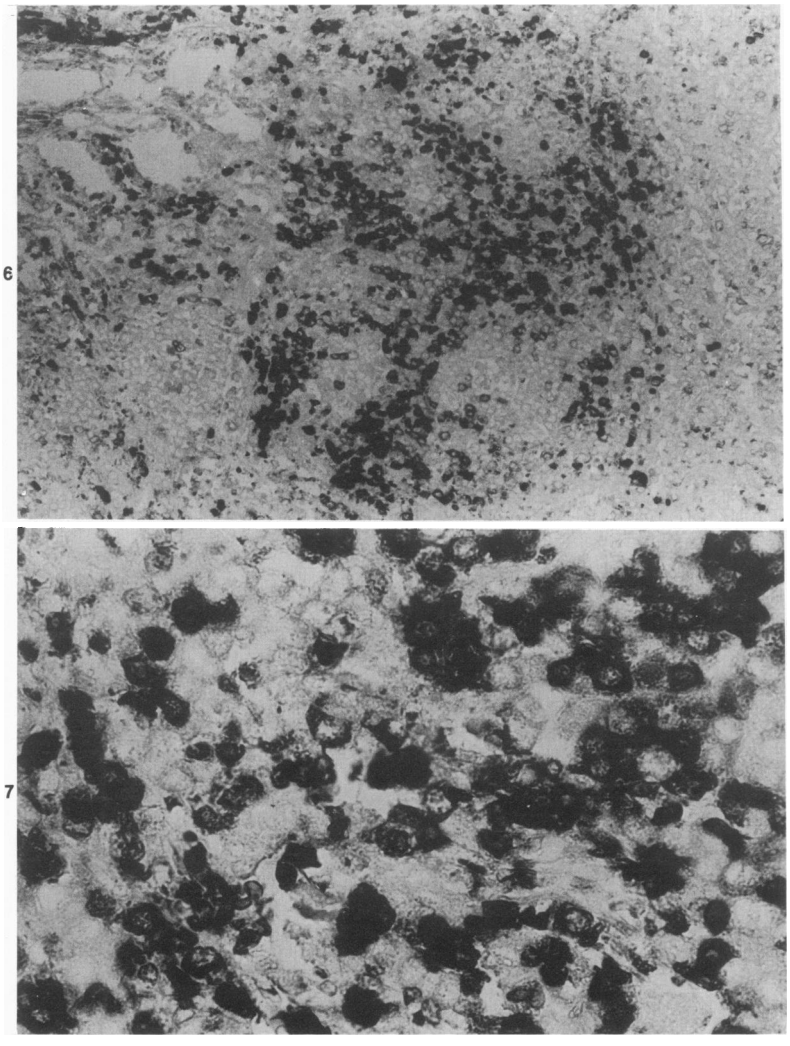

Figure 6—Spleen with extramedullary hematopoiesis (B5 fixation, paraffin section). Erythroid elements stain positively for cytoplasmic hemoglobin (*black*). Other hematopoietic and splenic parenchymal cells are negative. (Methyl green counterstain, × 130) **Figure 7**—Spleen with extramedullary hematopoiesis (B5 fixation, paraffin section). High power shows erythroid cells at various stages of maturation with strong staining for cytoplasmic hemoglobin (*black*). (Methyl green counterstain, × 600)

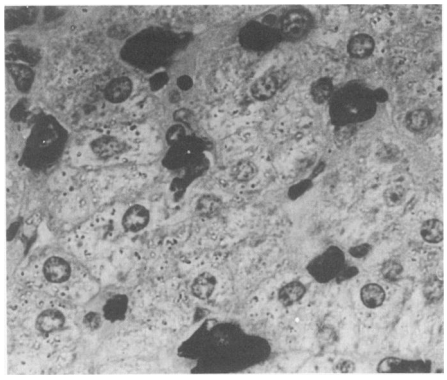

Figure 8—Liver with extramedullary hematopoiesis (B5 fixation, paraffin section). Erythroid cells in sinusoids reveal strong cytoplasmic staining for hemoglobin (*black*). Hepatocytes are negative. (Hematoxylin counterstain, ×700)

clonal) proliferations and further assists in the classification of lymphoid and plasmacytic malignancies.[8,10,11] Cells of myeloid, monocytic, or histiocytic derivation may be identified on the basis of intracellular lysozyme.[1,2] The results of our current study demonstrate that erythroid elements, including dysplastic and megaloblastic forms, also may be accurately detected in tissue sections with the use of an immunoperoxidase technique.

Studies of hemoglobin production during erythropoiesis readily explain the reason intracellular hemoglobin represents an excellent marker for erythroid cells. Hemoglobin synthesis begins at the stage of the proerythroblast, the earliest cell of erythrocytic maturation, which has a hemoglobin content of 0–14.4 μμg per cell.[3] This cell undergoes a series of divisions and maturation stages which include basophilic normoblasts, polychromatophilic normoblasts, "orthochromatic" normoblasts, reticulocytes, and finally the erythrocytes.[12] During the maturation sequence, erythroid precursors continue to synthesize hemoglobin. The most rapid rate of hemoglobin synthesis occurs at the stages of proerythroblast and basophilic erythroblast (0.5 μμg per cell/per hour) and progressively decreases at subsequent stages of maturation to a rate of 0.1–0.2 μμg/cell/hour in the reticulocyte.[3] At the stage of a mature erythrocyte, the hemoglobin content reaches 30–34 μμg/cell. In megaloblastic erythropoiesis, although nuclear-cytoplasmic maturation is dyssynchronous, hemoglobin synthesis still occurs.[12]

The immunoperoxidase technique for identification of intracellular hemoglobin, as described in this report, represents a sensitive and specific method for detection of erythroid elements, including early forms with small quantities of hemoglobin and dysplastic or megaloblastic forms. This immunohistochemical technique is readily applicable to routinely processed tissues fixed in Zenker's-acetic acid solution, B5 solution, or formalin, and provides permanent preparations with optimal cytologic detail. Intracellular hemoglobin is of particular diagnostic assistance as a tissue marker for erythroid cells in cases of myeloproliferative disorders, notably erythroleukemia and megaloblastic erythropoiesis.

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Acknowledgments

The authors are grateful to Ms. Janet A. McLeod, Amy Robson, and Susan Nagy for expert technical assistance.