Primary Myelodysplastic Syndromes: Diagnostic and Prognostic Significance of Immunohistochemical Assessment of Bone Marrow Biopsies

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Material from 63 cases with primary myelodysplastic syndromes (P-MDS) [French-American-British (FAB) types: refractory anemia (RA) = 21; RA with ring sideroblasts (RARS) = 8; RA with excess of blasts (RAEB) = 10; RAEB in transformation (RAEBt) = 6; chronic myelomonocytic leukemia (CMML) = 10 and unclassifiable = 8, ie, bone marrow aspiration was inadequate and stringent FAB criteria were not applicable) was analyzed for bone marrow histologic and immunohistochemical patterns. Standard Giemsa, hematoxylin and eosin (H&E) and reticulin stains were used for morphologic assessment. To identify the cell lineage precisely, chloroacetate esterase staining and an indirect immunoperoxidase technique using mouse monoclonal antibodies CD15, CD68, HLA-DR, and rabbit polyclonal CD3 and UE-1 (lectin) was developed on formalin-fixed paraffin embedded bone marrow biopsies (BMB). The immunohistochemical assessment permitted accurate identification of dysplastic features such as monocuclear and binuclear megakaryocytes, Pelger-Huet neutrophils, and binuclear erythroblasts. Additional bone marrow histologic and immunohistochemical features observed were heterogeneity of immunohistochemical staining in various cell lineages, megakaryocytic emperipolysis, alteration of bone marrow microarchitecture, intravascular clusters of hematopoietic cells, and the types of benign lymphoid aggregates. The nature of abnormally localized immature precursors (ALIP) was discerned. Three types of clusters of immature cells were found that were difficult to distinguish on Giemsa and H&E morphology, these were erythroid aggregates (n = 18); megakaryocytic aggregates (n = 4), and immature granulocytic and mononuclear aggregates (n = 32). The bone marrow histologic and immunohistochemical patterns permitted the identification of four groups of clinical relevance: Group 1, cases with predominant erythroid hyperplasia and without ALIP (n = 15); group 2, cases with prominent myeloid hyperplasia and presence of ALIP (n = 32); group 3, cases with hypoplastic MDS (n = 10); and group 4, cases with hyperfibrinotic MDS (n = 6). Statistical analysis showed a significant difference in survival and leukemic transformation between groups 1, 2, 3, and 4, with cases in group 2 showing the worst prognosis with early death due to increased propensity to leukemic transformation and cytopenia-related complications (P < .0001). We conclude that immunohistochemistry is feasible on routinely processed BMB and the information obtained is of diagnostic and prognostic importance in P-MDS. The phenotype of ALIP varies with the morphologic and histologic subtypes of MDS and the term should be reserved for cases in whom the clusters in the intertrabecular region are of myeloid (granulocytic and monocytic) lineage on immunohistochemistry.

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MATERIALS AND METHODS

BM trephine biopsies were obtained at presentation from 63 patients (male = 34, female = 29, mean age = 65 years) with primary MDS (P-MDS). Patients were classified according to the criteria proposed by the FAB group: 21 patients had refractory anemia (RA), 8 had RA with ring sideroblasts (RARS), 10 had RA with excess of blasts (RAEB), 6 had RAEB in transformation (RAEBt), and 10 had chronic myelomonocytic leukemia (CMML). A further eight patients could not be classified due to inadequate material obtained by bone marrow aspiration. BMB were taken from the posterior superior iliac spine, fixed in formalin-glutaraldehyde, decalcified in neutral EDTA for 24 to 72 hours, and then processed according to the standard protocol. Sections of 3 to 4 μm were cut and mounted on poly-L-lysine (Sigma, St.

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Louis, MO) coated glass slides. Hematoxylin and eosin (H&E), Giemsa, and reticulin stainings were performed according to standard protocols.\textsuperscript{22,23} Chloroacetate esterase (CAE) histochemistry. CAE enzyme histochemistry was performed by a modified procedure of Schaefer.\textsuperscript{24} The incubation medium was prepared by adding 1 mL of substrate solution (naphthol ASD chloracetate esterase) to 100 mL of 0.1 mol/L Tris/HCl buffer to which 0.5 mL of hexazonitized parasaranoline solution was added, and pH was adjusted to 6.5 to 6.8. The incubation medium was filtered directly onto the slides and left for 1 hour at room temperature. Slides were washed in running tap water and nuclei were counterstained with Mayer's hematoxylin.

For negative control of CAE, naphthol-AS-D chloroacetate was omitted from the incubation medium.

Lectin and immunohistochemistry. An indirect two-stage immunoperoxidase technique was used for mouse monoclonal antibodies (MoAbs) CD15 (Leu-M1) (Becton Dickinson, Mountain View, CA), CD68 (KPI) (Dako Ltd, Wycombe, UK), HLADR (Dako) and lectin UEAI (Dako). Three-stage immunoperoxidase technique was used for rabbit polyclonal CD3 (Dako). Mouse MoAbs recognizing CD15, CD68, and HLADR were chosen for staining of granulocytic, monocytic, and macrophage cells.\textsuperscript{25-27} Lectin UEAI was used to stain erythroid and megakaryocytic progenitors.\textsuperscript{28} The choice of the antisera used was based on the fact that the mouse MoAbs, CD13 and CD33, do not work on paraffin-embedded material and anti–glycophorin-A fails to stain proerythroblasts.\textsuperscript{27} The technique used is a modification of the method described by Sternberger.\textsuperscript{26} Briefly, sections were dewaxed in xylene and brought to tris buffer saline (TBS) through graded alcohol.

Endogenous peroxidase was blocked by incubation for 30 minutes in methanol with 0.5% H2O2. Sections were washed in running tap water for 5 minutes and then in TBS. The optimally diluted primary monoclonal or polyclonal antibody or UEAI was overlaid on the section for 1 hour. After washing in TBS, a peroxidase-conjugated rabbit antirabbit Ig or peroxidase-conjugated rabbit anti-UEAI was applied at optimal dilution and incubated for 30 minutes. (For polyclonal antisera [CD3], before applying rabbit antirabbit Ig peroxidase conjugate, goat antirabbit Ig was applied for 30 minutes.) The color was developed by using 3,3′ diaminobenzidine in 0.1 mol/L tris buffer, pH 7.6 and nuclei were counterstained with Mayer's Hematoxylin.

Sections of BMB or cytospin preparations of known monoclonal/ polyclonal antibody and lectin reactivity were included as positive controls in each batch of staining. For negative control of immunohistochemistry the primary antibody layer was replaced by TBS.

Methods of assessment. BMB cellularity was assessed by visual examination and graded into three groups\textsuperscript{29} as follows: normocellular (30% to 50% of intertrabecular space occupied by hematopoietic tissue), hypercellular (>50% of intertrabecular space occupied by hematopoietic tissue), and hypocellular (<30% of intertrabecular space occupied by hematopoietic tissue). Abnormal localization of immature precursors were defined according to Tricot et al\textsuperscript{29} as aggregates of three to five immature myeloid precursors (myeloblasts and promyelocytes) in the intertrabecular region. The reticulin content was graded from 0 through 4.\textsuperscript{30} The strength of reactivity for CAE, lectin, and immunohistochemistry was assessed after examining the whole BMB. Counting of the percentage of positively stained cells permitted the following arbitrary groups: <5% positive cells; +, 5 to 25 positive cells; ++, 25 to 50 positive cells; +++, >50%. A comparison of routinely stained Giemsa and H&E sections was made with the information obtained from immunohistochemistry.

Computer-assisted survival analysis was performed by the log-rank test using the Biomedical data package (BMDP) program.\textsuperscript{31,32} RESULTS

BM was normocellular in 18 cases, hypercellular in 35 cases, and hypocellular in 10 cases. The reticulin content was not increased (grade 0) in 19 cases, grade 1 in 13 cases, grade 2 in 25 cases, grade 3 in 5 cases, and grade 4 in 1 case. Immunohistochemistry showed CD3 stained T cells; CD15 stained granulocytic cells; CD68 stained immature granulocytic, monocytic, and macrophage cells; HLA-DR stained myeloblasts, monocytic, macrophage and B cells; and UEAI stained erythroid, megakaryocytic, and endothelial cells.

Immunohistochemistry assisted in unravelling some special features in MDS. These were Auer rod-positive blasts (3%); decreased immunostaining in the granulocytic cells (84%) and megakaryocytic cells (82.5%). Megakaryocytic emperiplois was observed in 44% of cases (Fig 1) and 95% of cases showed broad-based nuclear divisions and intranuclear bridges in erythroblasts. Erythropagocytosis was seen in 31% of cases (Fig 2) and 19% of cases showed necrobiosis. Intravascular collection of granulocytic cells (16%); monocytic cells (6%); erythroid cells (9.5%) and megakaryocytic cells (22%) (Fig 3) was also observed. The phenotype of benign lymphoid aggregates was discerned, six cases (9.5%) showed B-cell type and four cases (6.3%) showed T-cell type benign lymphoid aggregates.

Abnormal localization of immature precursors. ALIP were present in routinely processed BMB in 14/29 (48%) cases of RA/RARS and in all cases of RAEB (n = 10), RAEBt (n = 6), and CMML (n = 10). Immunohistochemistry showed that 6/14 ALIP-positive cases with RA stained for mouse MoAbs CD68 and HLA-DR. These and all cases with RAEB, RAEBt, and CMML also stained for these antibodies and are therefore referred to as “true ALIP,” i.e., immature granulocytic/ monocytic aggregates (Figs 4 and 5). Other immature cell aggregates that were morphologically similar to true ALIP but stained for UEAI-1 were referred as pseudo ALIP. In erythroid aggregates (pseudo ALIP) (n = 18/63) UEAI-1 gave membranous and dotlike cytoplasmic staining for proerythroblasts (Fig 6), while megakaryocytic aggregates (pseudo ALIP) (n = 4/63) deep granular cytoplasmic staining was observed (Fig 7).

On the basis of the BM cellularity, reticulin content, and presence or absence of ALIP, four distinct subgroups of clinical relevance were recognized.

Group 1 (RA = 7, RARS = 8). These cases showed normocellular or hypercellular BM with predominant erythroid hyperplasia, moderate dysmegakaryopoiesis, minimal dysmyeloipoiesis, and absence of ALIP. Immunostaining showed strong positivity (+++) of UEAI-1–labeled erythroid precursors, which were morphologically indistinguishable from myeloid precursors on H&E and Giemsa stains (see Fig 6). CD15 and CAE reactivity was variable, i.e., mild/moderate (++), but HLA-DR and CD68 labeling was generally negative except for few CD68 labeled macrophages and HLA-DR–positive lymphoid cells.

Group 2 (RA = 6, RAEB = 10, RAEBt = 6, CMML = 10). These cases showed increased cellularity with predominant myeloid hyperplasia, minimal dyserythropoiesis, moderate dysmegakaryopoiesis, and presence of ALIP (see Figs 4 and 5). Immunostaining demonstrated paucity of UEAI-1–
Fig 1. UEA-1 showing megakaryocytic emperipolesis of granulocytic cells (arrow) (original magnification ×1,000). Fig 2. CD68 (KP1) showing staining for macrophage and myeloid cells. A. Erythrophagocytosis of binuclear red blood cells; B. Pelger-Huet form (original magnification ×1,000).

Fig 3. UEA-1 showing intravascular clusters of megakaryocytic cells in hyperfibrotic MDS (arrow). A, Non-nucleated megakaryocyte; B, binuclear megakaryocyte; C, mononuclear megakaryocyte; D, mononuclear megakaryocyte with decreased staining for UEA-1 (original magnification ×1,000).

Fig 4. True ALIP: CD68 (KP1) showing staining for clusters of myeloblasts in P-MDS (arrow). Note morphologically similar unstained erythroblasts (arrowhead).

Fig 5. True ALIP: HLA-DR showing staining for clusters of monoblasts in P-MDS (arrow). Note morphologically similar unstained erythroblasts (arrowhead).

Fig 6. Pseudo ALIP: Erythroid clusters with membranous and dotlike cytoplasmic stain surrounded by unstained mature granulocytic cells.

Fig 7. Pseudo ALIP: Megakaryocytic clusters with deep granular cytoplasmic stain for UEA-1 (closed arrow) surrounded by mature granulocytic cells. Note accompanying small mononuclear megakaryocyte (arrowhead) and erythroblast in mitosis (open arrow).

Fig 8. Hypoplastic MDS. Dysplastic megakaryocyte (arrow) (original magnification ×400).

Fig 9. Hyperfibrotic MDS with predominant dysmegakaryopoiesis (original magnification ×100).
positive (+) erythroid progenitors. CD68 and HLA-DR—positive cells were striking (+ + +), but CD15 and CAE reactivity was variable.

**Group 3 (RA = 8, unclassifiable = 2).** Hypoplastic cases showed BM hypocellularity (<30%). All three cell lines showed dysplastic features with variable staining for UEA-1, HLA-DR, CD68, and CD15 (Fig 8). None of the cases showed presence of true ALIP; however, immature erythroid aggregates (pseudo ALIP) were seen in three cases.

**Group 4 (N = 6, unclassifiable on BM aspirate).** Hyperfibrotic cases showed increased reticulin fibrosis (grades 3 and 4). Dysmegakaryopoiesis was predominant, and dysmyelopoiesis and dyserythropoiesis was variable (Fig 9). UEA-1 staining showed all abnormal forms of megakaryocytes (+ + +) along with some megakaryocytic aggregates (n = 4). None of the cases showed presence of true ALIP.

**Prognosis of various histologic groups.** All patients were observed for a median period of 5 years (2 to 9 years) for progression of disease. Twelve of 32 cases in group 2 died due to cytopenia-related complications, and 17 of 32 cases in group 2 progressed and died as a consequence of leukemic transformation. In contrast the patients in groups 1, 3, and 4 showed all abnormal forms of megakaryocytes (+ + +) along with some megakaryocytic aggregates (n = 4). None of the cases showed presence of true ALIP.

**DISCUSSION**

Paraffin-embedded BM trephine biopsies provide a means of assessing intact tissue for cellularity and architectural features in MDS. They did not yield their full diagnostic potential because of the difficulties encountered in identifying individual cells. Previously immunophenotypic studies in MDS have been based exclusively on cell suspensions, while immunohistochemical assessment of routinely processed BM tissue sections has been applied mainly to lymphoid disorders and only rarely to myeloproliferative diseases. It is suggested that plastic-embedding techniques reduce the need for special stains and are consequently cost-effective. When actual cost of consumables for plastic-embedding and paraffin-embedding with special stains was evaluated, the results were comparable. The expenditure for each paraffin-section with special stain was $15.00 and for a methacrylate-embedded section was $14.50. These expenses are still in agreement with the earlier findings of Germain, who reported reagent cost $8.00 and $5.50 for each plastic and paraffin section, respectively. In a routine pathology unit other deterring factors for plastic-embedding techniques are the capital expenditure for new equipments, the lack of automation, difficulty in using immunohistochemical techniques, and the inability to use the archival material.

The application of immunohistochemical techniques on paraffin-embedded BMB, as demonstrated in this study, enhanced the diagnostic accuracy of BM trephines by clearly identifying the dysplastic features such as mononuclear and binuclear megakaryocytes, Pelger-Huet neutrophils, and binuclear and trinuclear erythroblasts.

Two types of blasts have been described in cases with MDS. These are relatively easier to distinguish on BM aspirate. In contrast, standard stains on BMB do not allow the distinction of these blast cells. Although immunohistochemical stains facilitated this distinction, it should be stressed that stringent cytologic criteria were not applicable as some of the granules may have been destroyed during conventional processing of the trephines.

Auer rod-positive blasts are difficult to identify on the BMB. Immunohistochemical assessment assisted in their identification. Two of 63 cases (3%) demonstrated presence of Auer rods. Both cases were young and behaved clinically like the ones reported byMichels et al.

Hypogranulations and hypergranulations of granulocytes are well-recognized features of MDS. These are better seen in the bone marrow and peripheral blood cytologic preparations. The immunohistochemical assessment of BM trephines showed reduced staining for CD15, CD68, and CAE showed comparable abnormal granulation. Decreased staining for these MoAbs was also observed in myeloid cells undergoing mitosis.

In megakaryocytic series decreased staining for UEA-1 was observed in 82% of cases. This is in agreement with other reports of morphologic and functional abnormalities of megakaryocytic series in cases with MDS. Dysmegakaryopoiesis was demonstrated by finding increased number of mononuclear, binuclear, trinuclear (Pawn ball), and multinuclear megakaryocytes. In addition, megakaryocyte emperipolesis, which has been reported in myeloproliferative disorders (MPD), was present in 44% of cases. It should be stressed that this morphologic abnormality is not confined to MDS and has been reported in 4% of normal marrow biopsies. It is suggested that it may reflect the increased number of megakaryocytes.

The occurrence of internuclear bridges (INB) and broad-based nuclear division (BBND) in the erythroid cells was confirmed. These cells were easily defined by UEA-1
staining. The importance of INB and BBND as diagnostic features of cases with MDS has been highlighted by Head et al. However, it has to be emphasized that the findings of INB and BBND were easily overlooked on H&E- and Giemsa-stained preparations of BMB. Furthermore, it was difficult to distinguish between binuclear megakaryocytes, Pelger-Huet granulocytes, and BBND in the erythroid series.

The lymphoid aggregates observed in MDS were like the benign lymphoid aggregates reported by others. Although, the association of MDS with lymphoproliferative disorders is reported, none of the cases showed concurrent lymphoid neoplasia or progressed to lymphoid leukemia during the observation period.

BM histology and immunohistochemistry provided the facility to study the changes in BM vasculature. Dilated sinuses and intravascular clusters of hematopoietic cells were observed in up to 22% of cases. The occurrence of intravascular clusters of hematopoietic cells in myelofibrosis or agnogenic myeloid metaplasia is well recognized, but the diagnostic and pathophysiologic importance of these findings in MDS is unclear.

CD68 staining provided an opportunity to assess the BM macrophage lineage in MDS. The finding of erythrophagocytosis and necrobiosis in MDS, which are commonly reported in viral infections, warrant further investigation.

The occurrence of ALIP in MDS is of considerable interest and some debate. The appreciation of ALIP has been difficult in the paraffin-embedded BMB. Rios et al reported 38% of cases with RAEB and 33% of cases with RAEBt without ALIP in a series of 120 patients analyzed on paraffin-embedded BMB. Technical differences could well be responsible for these discrepancies. Moreover, their results do not allow the precise immunophenotyping of cells within the BM biopsies.

Recently, Verhoef et al have reported four histologic subtypes of MDS based on the proliferative cells accompanying ALIP. The values of this classification cannot be ascertained because proliferative cell specific markers were not used. Our preliminary study using proliferative cell nuclear antigen (PCNA) showed increased expression of PCNA in erythroid aggregates rather than the granulocytic/monocytic aggregates. The mechanisms underlying the cluster formation of primitive cells in MDS are unclear.

Recent reports suggest that there may be a role for the autocrine or paracrine secretion of hematopoietic colony-stimulating factors at these sites in the bone marrow. This concept is supported further by the observation of ALIP-like clusters during the BM regenerative phase after chemotherapy or BM transplantation in acute myeloid leukemia (AML), chronic granulocytic leukemia (CGL), and aplastic anemia. However, as yet, no direct evidence has been provided for this hypothesis.

**BM histology and FAB subtypes.** Attempts to classify MDS into the five FAB subtypes on histologic grounds have led to conflicting results. While Delacretaz et al were able to apply FAB proposals on methacrylate-embedded BMB sections, others were unsuccessful in applying FAB criteria on BM trephines. Frisch and Bart have taken their own initiative to classify MDS in seven histologic types, ie, sideroblastic, megaloblastic, proliferative, blastic, inflammatory, hypoplastic, and fibrotic types of MDS. In contrast, four types of BM histologic patterns were recognized in our study, ie, group 1, erythroid hyperplasia without ALIP; group 2, myeloid hyperplasia with ALIP; group 3 hypoplastic MDS; group 4, hyperfibrotic MDS.

Several aspects of the histologic classification of MDS should be considered. The reproducibility and reliability with which the clinical outcome can be evaluated are two important issues. The controversies surrounding histologic classification as proposed by Frisch and Bart could be due to imprecise definitions and difficulties in achieving diagnostic reproducibility of their classification. Furthermore, it has to be emphasized that no evidence was found to support the occurrence of inflammatory MDS as a separate histomorphologic entity. Indeed, the occurrence of some inflammatory cells (ie, perivascular plasma cells and slight increase in mast cells and eosinophils) is a well-recognized
feature of many myeloproliferative disorders\textsuperscript{72,77} and is also reported in reactive conditions.\textsuperscript{74} It has to be stressed that the perivascular plasma cells have also been observed in normal human BMs.\textsuperscript{77} Therefore, it is suggested that this may be an epiphenomenon observed in MDS and other myeloproliferative disorders and should not be misclassified as an inflammatory MDS.

The histologic classification adopted in the present work is simple, reproducible, and precise as immunohistochemical assessment provided a rationale for such a classification. Furthermore, cases in groups 1 and 2 showed some correlation with the FAB classification. The inclusion of RAEB, RAEBt, and CMML in group 2 is based on the presence of true ALIP in the BMB. Although various investigators have reported different predictive value for these FAB subtypes,\textsuperscript{5,6,76} BMB immunohistochemical examination alone does not allow the distinction of these subtypes. The studies for the assessment of early hematopoietic markers, cell proliferation markers, and in situ staining for hematopoietic growth factors are underway that may assist in the subdivision of this group of patients.

In most cases of groups 3 and 4, MDS was suspected but BM aspirate was either inadequate or it yielded "dry tap," consequently it was not possible to apply the FAB criteria stringently. BM trephine histology and immunohistochemistry ruled out other conditions and established the diagnosis of MDS according to the comparable criteria.

Distinction between hypocellular MDS, hypocellular AML, and aplastic anemia can be difficult.\textsuperscript{7,9,80} Likewise, the distinction between hyperfibrotic MDS and myelofibrosis can be difficult.\textsuperscript{81,92} Careful BM morphologic, histologic, and immunohistochemical examination of the dysplastic features in the megakaryocytic, erythroid, and granulocytic lineage can assist in the diagnosis in these cases. However, until biologic differences between these disorders are known, the morphologic criteria alone will continue to create contention among investigators.\textsuperscript{83}

**Prognosis of histologic groups.** This study confirmed previous reports that BM histology complements the prognostic information obtained from the BM cytology.\textsuperscript{4,11} This was demonstrated in six patients with the FAB-type RA, who had no excess of myeloblasts in the BM smear, but demonstrated true ALIP and myeloid hyperplasia (group 2). These patients had a shorter survival, and four of the six patients died as a consequence of leukemic transformation. The remaining two patients also showed shorter survival than group 1 and succumbed from sepsis and bleeding. Bournemouth score\textsuperscript{7} provided useful information in most cases; however, in this group of cases BMB examination illustrated more predictive value than Bournemouth score (personal observations, May 1991).

Attempts to correlate BM hypoplasia and BM fibrosis with prognosis have led to conflicting results. While in accordance with Riccardi et al,\textsuperscript{10} Nand and Godwin,\textsuperscript{12} and Pagliuca et al\textsuperscript{11} we observed a relatively good prognosis in cases with hypoplastic MDS and hyperfibrotic MDS, others\textsuperscript{4,11,12} reported hypoplastic MDS and hyperfibrotic MDS with poor prognosis and an increased risk of leukemic transformation. Future multivariate analysis by various scoring systems,\textsuperscript{5,6,76} karyotype,\textsuperscript{84} BM immunohistochemical patterns, and molecular genetic analysis on a larger number of patients would provide comprehensive knowledge of the natural history of MDS.

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