original paper

haematologica 2001; 86:368-374
http://www.haematologica.it/2001_04/0368.htm

The value of bone marrow histology in differentiating between early stage polycythemia vera and secondary (reactive) polycythemias

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Background and Objectives. The diagnostic criteria of the Polycythemia Vera Study Group (PVSG), although generally acknowledged as the gold standard for establishing a diagnosis of polycythemia vera (PV), do not consider bone marrow features. It may, therefore, be speculated that initial-early stages of PV are overlooked. In this retrospective study we tried to investigate whether bone marrow morphology of patients with an only borderline to slight increase in hemoglobin/hematocrit not conforming with the postulates of the PVSG enabled a clear-cut differentiation between PV and secondary (reactive) polycythemias (SP).

Design and Methods. From a series of 348 patients with a borderline to pronounced erythrocytosis and representative pre-treatment bone marrow trephine biopsies a cohort of 86 cases was selected showing only a borderline increase in hemoglobin (males < 18.5 g/dL, females < 16.5 g/dL). Biopsies and clinical records were evaluated independently and following histologic and clinical work-up a straightforward consensus was reached. The diagnostic impact of histologic findings was tested by means of discriminate analysis of 20 standardized morphologic features based on histochemical and immunohistochemical staining techniques.

Results. Bone marrow histopathology in 47 patients diagnosed as having SP was characterized by a minimal to slight increase in cellularity with predominance of the erythroid lineage. Neutrophil granulocytopoiesis was prominent and left-shifted and small to medium-sized megakaryocytes without maturation defects were scattered throughout the bone marrow. There was an increased number of eosinophils, marked perivascular plasmacytosis, histiocytic reticular cells with accumulated cell debris and many iron-laden macrophages. Contrasting this appearance in SP our 39 patients with initial-early stage PV revealed a hypercellular bone marrow with trilineage proliferation (pan-myelosis) showing confluent sheets of erythropoiesis and loose clusters of megakaryocytes. Megakaryocytopoiesis was characterized by a pleomorphous appearance, i.e. giant cells were lying adjacent to small ones, but lacked an obvious cytologic abnormality. There was usually no prominent inflammatory reaction of the interstitial compartment. In ten patients lymphoid nodules were found, but no conspicuous iron deposits and in six patients a borderline to minimal increase in reticulin fibers was present. Following stepwise discriminate analysis of histologic features a set of parameters emerged including increase in megakaryocyte size, perivascular plasma cells, overall bone marrow cellularity and cellular debris. This pattern exerted a significant impact on separation (Wilks' lambda statistics = 0.110, $p < 0.0001$) of early stage PV from SP. Most patients with SP had an underlying bronchopulmonary condition, frequently associated with heavy smoking or rarely renal pathology. In addition to the histopathologic features, splenomegaly, thrombocyte count, LDH, LAP and erythropoietin levels proved to be different in the two groups of patients.

Interpretation and Conclusions. Initial-early PV is characterized by a specific pattern of bone marrow histopathology. Clinical features of distinctive impact include splenomegaly, thrombocyte count, LDH, LAP and in particular erythropoietin level. Taking these clinical and histologic findings into consideration, reactive-secondary causes of polycythemia (SP) are clearly distinguishable from autonomous ones (PV).

Key words: polycythemia vera, secondary polycythemia, bone marrow histology, discriminate analysis, clinical findings

In early stage polycythemia rubra vera (PV) when manifest erythrocytosis (hematocrit in males > 51% and in females >48%) is the only pathologic finding, the differential diagnosis includes secondary and reactive polycythemias with a normal red cell mass (RCM).[1,2] The diagnostic criteria of the Polycythemia Vera Study Group (PVSG) are generally acknowledged as the gold standard for establishing PV[3-7] and have been applied in leading clinical trials on this disorder.[2,3,8] However, the diagnostic value and feasibility of determining the red cell mass (RCM) as the primary criterion using radioisotope techniques as routine clinical practice may be challenged. In this regard the inaccuracy of the predicted normal RCM as related to body weight, set as >36 mL/kg for males and >32 mL/kg for females by the PSVG,[1] is a very fraught issue especially in obese patients.[9] Because it seems to be essential that all patients with an absolute erythrocytosis are investigated to exclude PV, three other methods of clinical

investigations have gained increasing acceptance in recent years. These are autonomous growth of erythroid colonies in semisolid cultures,[10-13] the establishment of a clonal population by the presence of an acquired karyotypic abnormality[14-16] and in particular the nowadays very popular determination of the erythropoietin (EPO) level.[17-20] In this concert of diagnostic criteria and various techniques to distinguish reactive causes of a raised hematocrit/hemoglobin level from a myeloproliferative disorder (Ph[1]-MPD),[21] the sensitivity of bone marrow histopathology is still a conflicting or, at least, an ambiguous issue. As was pointed out, characteristic morphologic features[22-26] were usually described in patients with PV already diagnosed by the well-known clinical criteria[2,5] and not explicitly in early stages of this disorder not completely conforming with the stringent diagnostic postulates of the PVSG.[2-7]

For this reason, the purpose of the present study was to investigate whether initial PV can be differentiated from secondary polycythemias (SP) by its specific histologic pattern, especially in patients with an only borderline to slight increase in the hemoglobin/hematocrit level.

Design and Methods

Patients

This retrospective study included clinical data and representative pre-treatment bone marrow biopsies from 86 patients selected from a total series of 348 consecutively recruited cases (years 1980 to 1999) with a borderline to marked erythrocytosis. To focus specifically on early stage PV the cohort of patients we chose to study presented with a borderline hemoglobin, <18.5 g/dL in males (range 16.0 to 18.1 g/dL) and <16.5 g/dL in females (range 15.0 to 16.5 g/dL). This value corresponded to a hematocrit slightly higher than 51% (upper limit 53%) in 44 males and marginally exceeding 48% (upper limit 51%) in 15 females. For practical reasons the RCM was determined in only a few patients as a routine method. Bone marrow histopathology and clinical data were evaluated independently and in a blind fashion, i.e. the pathologists were only aware of the age, gender and raised hemoglobin/hematocrit level. On the other hand, the clinicians made their diagnosis on the basis of laboratory findings. Following histologic work-up and clinical assessment a straightforward consensus was reached concerning final diagnosis by the two pathologists (J.Th., H.M.K) and the two clinicians (R.Z.,V.D.) involved in this investigation. Moreover, follow-up studies confirmed the diagnosis of PV, because subsequently all patients developed manifest symptoms and signs fullfilling the PVSG criteria.[3-7]

Bone marrow biopsies

Large (length >15 mm) bone marrow trephine biopsies were performed from the posterior iliac crest in all patients with a raised hemoglobin-hematocrit level on admission. These specimens were fixed in a low-concentrate aldehyde solution for 12-48 hours (2 mL 25% glutaraldehyde, 3 mL 37% formaldehyde, 1.58 g anhydrous calcium acetate, per 100 mL of distilled water). Further processing included decalcification for 3-4 days in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.4, paraffin embedding, and employment of several routine staining techniques, involving Giemsa, PAS (periodic acid Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perls' reaction for iron and the silver impregnation method, following Gomori's technique. For specific staining of marrow cells, two monoclonal antibodies were selected: CD61 (anti-platelet glycoprotein IIIa) for the identification of megakaryocytes including precursor cells (pro-megakaryoblasts and megakaryoblasts) and Ret40f (anti-glycophorin C) to selectively stain for erythrocytopoiesis.[27] Monoclonal antibodies and other reagents were purchased from Dako-Diagnostica GmbH (Hamburg, Germany). Details of staining procedures (APAAP-method) and proper identification of corresponding cell populations were reported in previous communications. [28,29]

Discriminate analysis of histologic features

The histologic slides were reviewed independently by two observers; there were no cases of discordant diagnosis regarding SP versus PV. The following histologic parameters were considered for morphologic evaluation (Table 1): 1) overall bone marrow cellularity compared to an age-matched control; 2) quantity of megakaryocytes in respect to the reported normal values;[30] 3) clustering of megakaryocytes implying aggregates of more than three cells lying adjacent to each other; 4) size of megakaryocytes with differentiation into dwarf (small) and giant forms according to relevant classifications;[24-26,30] 5) extent of nuclear segmentation of megakaryocytes; 6) maturation defects implying abnormalities of nuclear-cytoplasmic development, i.e. dysplasia - clumsy (hyperchromatic) nuclei; 7) presence of denuded (naked) nuclei of megakaryocytopoiesis; 8) quantity of neutrophil granulocytopoiesis; 9) normal or abnormal maturational development (left-shifting) of the myeloid lineage from the bone trabeculae to the central marrow space; 10) presence of eosinophils; 11) quantity of erythrocytopoiesis with regard to normal values;[29] 12) normal or abnormal maturation (left shifting) of erythroid precursors; 13) increase and thickening of reticulin fibers exceeding the normal content by more than three-fold (early reticulin fibrosis);[30] 14) stainable iron deposits in the macrophages; 15) prominence of perivascular plasma cells; 16) occurrence of cell debris in the interstitial compartment - macrophages; 17) presence of lymphoid nodules (aggregates); 18) dilated and prominent sinusoidal vessels.

Statistical analysis

The diagnostic importance of these standardized bone marrow features was tested by stepwise discriminate analysis. The aim of this method is to obtain a small set of relevant discriminatory variables which provide a reliable prediction of group classification, i.e. PV or SP. In

Table 1. Descriptive analysis of histologic features (semiquantitative evaluation with reference to age-related normal values) in 86 patients presenting with a borderline to slight erythrocytosis.

Parameter	Breakdown (number) of patients: Increase: normal or absent (0)	slight (+1)	moderate (+2)	overt (+3)
Cellularity	24	27	21	14
Megakaryocytopoiesis				
quantity	30	27	22	7
clusters	51	21	10	4
Size				
small	35	47	4	–
giant	48	18	16	4
nuclear lobulation	46	12	28	–
maturation defects	–	–	–	–
clumsy nuclei	–	–	–	–
naked nuclei	19	45	20	2
Granulocytopoiesis				
quantity	19	45	20	2
left shift	30	55	1	–
eosinophils	21	36	23	6
Erythrocytopoiesis				
quantity	2	41	37	6
left shift	14	71	1	–
Myeloid stroma				
reticulin fibers	80	4	2	–
iron–laden macrophages	38	23	19	6
perivascular plasma cells	37	22	24	3
cell debris	42	22	22	–
lymphoid nodules	72	12	12	–
prominent (dilated) sinuses	63	18	5	

this context it should be explicitly mentioned that to enhance sensitivity of this calculation, evaluation of the histologic parameters was based on certain histochemical and immunohistochemical staining techniques: neutrophil granulocytopoiesis - naphthol-AS-D-chloroacetate reaction, erythrocytopoiesis - Ret40f and megakaryocytes - CD61.[30]

Results

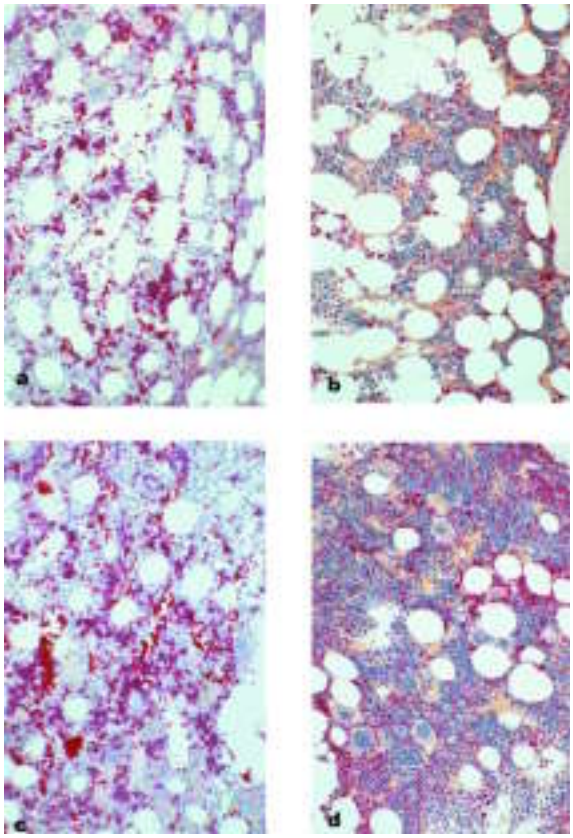
Bone marrow histopathology

Following evaluation of representative bone marrow biopsies including discriminate analysis of prominent histologic features (Table 1) in our series of 86 patients with a borderline to slight rise in hemoglobin/hematocrit, two histologic patterns emerged.

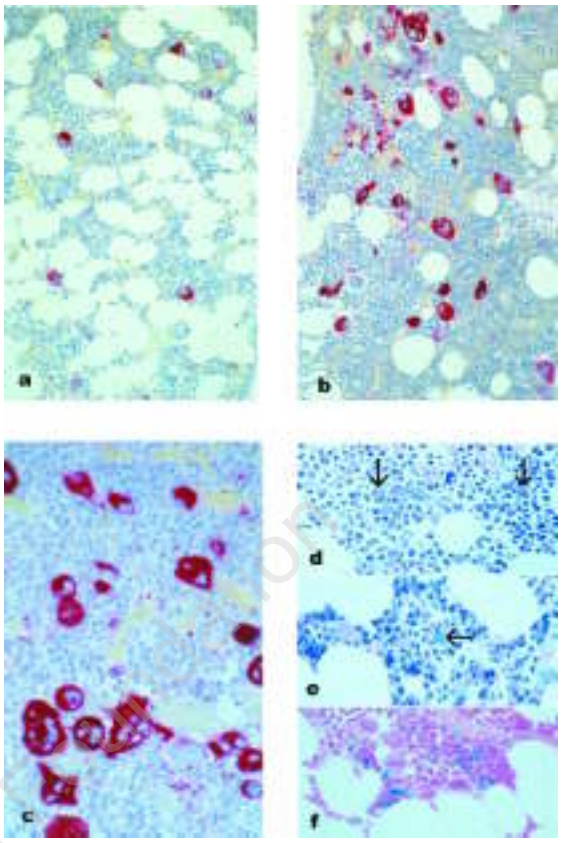
Group I patients (47 patients) had no or only minimal to moderate increase in cellularity. With the exception of two cases there was a predominance of the erythroid lineage (Figure 1a) while neutrophil granulocytopoiesis was only marginally left-shifted and hyperplastic (Figure 1b). Megakaryocytes were small to medium-sized and dispersed throughout the bone marrow space, while cluster formation, giant cells or cytological abnormalities were not encountered (Figure 2a). Loose groupings of many eosinophils of all maturation stages could frequently be detected close to sinusoidal vessels. In most patients the interstitial compartment of the bone marrow was characterized by a prominent mesenchymal reaction consistent with remarkable perivascular plasmacytosis (Figure 2d), histiocytic reticular cells with large accumulations of cell debris - nucleo-phagocytosis (Figure 2e) and a lot of coarsely iron-laden macrophages (Figure 2f). In keeping with these histologic features, bone marrow changes were consistent with reactive-secondary polycythemia (SP).

Group II patients (39 cases) showed prominent hypercellularity, provided that age-related expansions of the adipose tissue, particular into the subcortical-superficial bone marrow spaces were taken into consideration. A trilineage proliferation (so-called pan-myelosis) was always present and consisted mainly of extensive clusters and sheets of nucleated erythroid precursors without maturation defects (Figure 1c). Neutrophil granulocytopoiesis was left-shifted, i.e. an endosteal broad seam of promyelocytes and metamyelocytes was recognizable with continuous maturation to polymorphonuclear cells towards the marrow centers (Figure 1d). Most conspicuous was the megakaryocytic lineage, especially in patients with accompanying thrombocytosis. There was a loose grouping of giant to small megakaryocytes exhibiting no gross deviations of nuclear-cytoplasmic differentiation, thus creating a pleomorphous appearance (Figure 2b). This peculiar feature implied that even the giant megakaryocytes contained staghorn-like lobulated and correspondingly large nuclei with slender segments (Figure 2c). In contrast to the prominent lesions in SP, no conspicuous inflammatory reaction of the bone marrow stromal compartment and only minimal deposits of iron (seven patients) were observable. Moreover, in six patients a perisinusoidal increase in thin reticulin fibers was present as a first sign of initial (reticulin) myelofibrosis. On the other hand, almost two-thirds of patients showed dilated sinusoidal vessels packed by erythrocytes. In ten patients small well-marginated lymphoid aggregates were recognizable, often localized arround a vascular structure in the marrow centers. Consequently this group of patients had histopathologic findings that were compatible with early-initial PV.

A stepwise discriminate analysis of 20 histologic features (Table 1) revealed a relevant pattern of parameters (Wilks' lambda statistics = 0.110, $p < 0.0001$, predicted group membership 100%) regarding the distinction between PV and SP. These variables included increase in megakaryocyte size (giant megakaryocytes), prominent perivascular plasma cells, overall cellularity and cell debris in the interstitial compartment.



Figures 1a-d. Patients with SP (group I) show almost normal cellularity with prominent erythrocytopoiesis (a) while neutrophil granulocytopoiesis is marginally hyperplastic (b). Group II patients (PV) show hypercellularity with significant proliferation of erythroid precursors (c), but also of the other lineages, i.e. so-called pan-myelosis (d) a and c - Ret 40f immunostaining, b and d - naphthol AS-D-chloroacetate esterase stain; a-d × 170.



Figures 2a-f. Patients with SP (group I) show an inconspicuous megakaryocytopoiesis (a) whereas in group II patients (PV), but especially in those cases with accompanying thrombocythemia, there is pronounced proliferation and clustering of megakaryocytes (b) giving a pleomorphous appearance with giant to small cells mingled together and containing deeply lobulated (staghorn-like) nuclei, but no gross cytological atypias (c). In SP there is frequently a prominent perivascular plasmacytosis (d), accumulation of debris (e) and iron-laden macrophages are often found (f) a-c - CD61 immunostaining, d and e - Giemsa, f - Perls' reaction; a and b × 170, c × 570, d-e × 380.

Clinical findings

In accordance with laboratory findings our series of 86 patients could be separated into two groups presenting with signs and symptoms of either SP or PV exactly matching with bone marrow morphology (Table 2). Concerning the extent of erythrocytosis, 19 of the 23 male patients with PV had a hematocrit level at or slightly exceeding 51 % compared to 10 of the 16 female patients with a hematocrit exceeding 48 %. The corresponding values in SP were 25 of 39 patients in the male cohort and 5 of 8 patients in the female group. Clinical data of distinctive value included splenomegaly, thrombocyte count, LDH, LAP and EPO level (Table 3). Moreover, in the majority of patients (more than 80 %) with SP further work-up showed an underlying bronchopulmonary disorder due to heavy smoking (so-called smoker's polycythemia) often associated with a decreased oxygen saturation (≤ 90%). In a few patients a renal pathology (cysts, tumors, or chronic nephritis) was diagnosed. On the other hand, in many patients (between 40 to 60%) with early PV there was a history of recurrent headaches, dizziness, fatigue, night sweats, itching, but rarely conspicuous conjunctival plethora or preceeding hemorhage and thrombosis (3 patients). Patients with SP mostly presented with ill-defined complaints (malaise, dizziness, weight loss) some of which obviously arose from respiratory tract pathology such as frequent coughing, dyspnea, recurrent infections and weakness. Depending on the selection criteria, the (low) level of hemoglobin/hematocrit was not significantly different in the two groups of patients. Except for a history of smoking and symptoms related to bronchopulmonary disorders in SP and evidence of thrombosis in PV, no other clinical finding proved to have discriminatory value.

Table 2. Clinical findings (mean ± SD) in 86 patients with a borderline to slight increase in hemoglobin / hematocrit level.

	Group I (SP)		Group II (PV)	
	Men	Women	Men	Women
No. of patients	39	8	23	16
Age (years)	48±14	54±14	61±14	67±11
Hemoglobin (g/dL)	17.4±0.8	16.1±0.4	17.2±0.6	15.6±0.5
Hematocrit (%)	51.3±4.9	49.2±2.9	53.0±3.4	51.0±5.7
Erythrocytes ($\times 10^{12}$/L)	5.8±0.8	5.1±0.4	6.5±1.0	6.3±0.8
Thrombocytes ($\times 10^{9}$/L)	246±102	296±100	514±261	694±303
Leukocytes ($\times 10^{9}$/L)	11.8±1.3	10.2±3.8	13.3±5.8	18.8±10.6
LDH (U/L)	191±64	194±63	222±88	315±158
LAP (score)*	79±42	47±24	196±108	180±107
Erythropoietin (U/L)	> 8	> 8	< 8	< 6
Spleen size (cm)°	0.1±0.4	0	3.0±2.9	3.8±2.6

**Leukocyte alkaline phosphatase - normal range 20-80; °spleen size - cm below costal margin.*

Table 3. Constellation of certain clinical findings (%) that have a distinctive relevance for diagnosis in our 86 patients with a borderline to slight increase in hemoglobin / hematocrit.

Parameter	Group I (SP)	Group II (PV)
Spleen size > 1.5 cm below costal margin	3	52
Leukocytes > 10.5×10^{9}/L	44	78
Thrombocytes > 400×10^{9}/L	7	70
LDH > 290 U/L	6	26
LAP > 80 (score)	28	86
EPO > 8 U/L	97	5

Discussion

The diagnostic criteria of the PVSG[2-7] are generally held to represent rather robust guidelines for establishing a diagnosis of PV, because they do not explicitly regard initial-early stages of this disorder[9,21,31] and more importantly, totally neglect histopathology.[31,32] For this reason, recently modified and updated criteria were postulated that also incorporate distinctive bone marrow morphology as a major diagnostic requirement.[32] Keeping in mind that there is no single diagnostic marker for PV, the original PVSG postulates used a combination of major and minor clinical criteria.[1] Concerning the increase in RCM, it is well known that the use of mL per kg is a rather inaccurate measurement in overweight patients.[9] Consequently standards calculating height and weight should be applied. However, even when taking 25% above the mean normal values as the upper cut-off points of the reference ranges, 0.5% to 1.0% of normal males and females, respectively will show up with a falsely raised RCM.[33] Splenomegaly, as detected by palpation, was another major diagnostic criterion of the PVSG.[2-7] Again, as with the exact determination of the RCM, results on spleen size obtained by scanning techniques must be interpreted with some caution. This shortcoming is due not only to inter- and intra-observer differences, but also to the variations in normal spleen sizes depending on age and size of patients.[34] Concerning the so-called minor criteria, such as increases in the platelet and leukocyte counts, there are numerous causes for thrombocytosis including a variety of reactive etiologies as well as all the other subtypes of Ph[1]-MPDs.[35,36] Moreover, a borderline to slightly raised neutrophil granulocytosis may often be encountered in heavy smokers as in many of our patients with SP. The determination of EPO level has now become very popular, since definitively raised values are consistent with SP and low values are believed to be a strong indicator of PV, even at normal hemoglobin levels following hemorrhage or phlebotomy.[18-21] For this reason, together with specific features of bone marrow morphology, reduced serum EPO-levels should join the characteristic cell culture findings (spontaneous erythroid colony formation)[10-13] as a reliable criterion for diagnosing initial-early stage PV. Finally, it is noteworthy that our patients resemble, to a certain extent, the formerly described cases presenting with idiopathic (pure) erythrocytosis, the majority of whom subsequently developed overt PV.[37,38]

Although previous studies have produced persuasive evidence that specific bone marrow features exist in PV,[25,26,30,32,37-45] most clinicians are very reluctant to accept histopathology as a major diagnostic criterion and are challenging its sensitivity. One of the main arguments is that standardized objective criteria of histologic assessment have not yet been established for diagnostic use, since so far the morphologic features described were usually those in patients with PV already diagnosed by clinical criteria.[2,5] In this regard pioneering work was carried out by the Thrombocythaemia Vera Study Group that proposed the so-called Rotterdam criteria including bone marrow morphology as a major point for diagnosis.[31] In contrast to reactive causes of polycythemia, PV is always characterized by conspicuous hyercellularity, provided age– and topographic (subcortical marrow spaces) -related changes are taken into account[30,42,45] which, however, were obviously overlooked in previous communications.[23,37] Furthermore, proliferation of all three major cell lineages with predominance of erythroid precursors was not only established by semiquantitative gradings,[23,25,39,42] but also by morphometric measurements, including histochemistry and various markers of cell proliferation.[29,30,40,46] In this context megakaryocytopoiesis is an invaluable clue to diagnosis, because changes in histotopography including loose clusters, an arrangement along sinusoidal walls and a dislocation towards the paratrabecular area, are frequently encountered.[30,41-45] Subsequently, cytology showing giant and small megakaryocytes grouped together

(pleomorphous appearance), but without apparent maturation defects has emerged as a characteristic hallmark for distinguishing PV from SP and the other Ph[1]-MPDs.[30,39,42-45] In contrast to SP there are usually no or only very few iron-laden macrophages or a prominent inflammatory reaction of the interstitial bone marrow compartment observable.[24,32,42,43] Moreover, in comparison with the other Ph[1-]- MPDs and SP, patients with PV frequently (about 20%) display small lymphoid aggregates with distinctive borders[45] composed of a mixture of T- and B-lymphocytes according to immunohistochemical analysis.[47] Discrimination analysis of morphologic features has rarely been applied to hematopathology and to the best of our knowledge there is only one equivalent evaluation in patients presenting with essential thrombocythemia[48] in addition to our center study.[49] Although the generally pursued objectives of a histologic review are different, the clear-cut differentiation of two groups emerging from this calculation performed in an independent and blind fashion, substantiates and extends previous findings on bone marrow morphology in PV in a significant way. For this reason, the histologic parameters under consideration (Table 1) provide valuable clues for distinguishing early-stage PV from reactive causes.

In conclusion, together with erythrocytosis (expressed as an increase in hematocrit/hemoglobin levels) and EPO value,[18-20] characteristic features of bone marrow histopathology should be entered as major points into the set of diagnostic criteria for PV. In comparison with diagnostic cell culture studies[10-13] the latter methods aimed at discriminating PV, even at initial stages, from SP are easy to handle in clinical practice and not time-consuming.

Potential implications for clinical practice

Serum erythropoietin level and bone marrow histopathology should be included as major diagnostic criteria for polycythemia vera.

Contributions and Acknowledgments

JT designed the study, reviewed the bone marrow biopsies, contributed to the interpretation of data and drafted the article. HMK reviewed the bone marrow biopsies, revised the article and performed the statistical analysis and contributed to the interpretation of the data. RZ and VD were involved in the collection and interpretation of clinical-laboratory data including follow-up examinations of patients. The authors are listed according to the importance of their contributions to the work. The last name is that of the principal clinician involved.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Tiziano Barbui, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Prof. Barbui and the Editors. Manuscript received November 17, 2000; accepted March 19, 2001.

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