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Editorial

Bone marrow tissue and proliferation markers: results and general problems

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Introduction

Generally, assessment of cell proliferation may be achieved by a variety of methods amongst which are mitotic count, tritiated thymidine labelling index, bromo-deoxyuridine incorporation, flow cytometry, silver-stained nucleolar organizer regions (AgNORs) and immunohistochemistry. For an evaluation of proliferative activity in human tissues which have been processed routinely, the mitotic count is a relatively simple way to measure proliferation. However, the relationships between numbers of mitotic figures and proliferation are not as close as might be expected, because of the great degree of variation in the duration of the different cell-cycle phases and the well-known difficulties in discriminating cells in mitosis from pyknotic nuclei or other artefacts (Baak 1990). In addition to AgNORs, several nuclear proteins have been recently identified that are believed to characterize cycling or non-quiescent cells (Brown and Gatter 1990; Hall and Levinson 1990; Quinn and Wright 1990; Linden et al. 1992; Yu et al. 1992a; Kreipe et al. 1993a; Schwarting 1993). In the last few years a number of monoclonal antibodies directed against these nuclear proteins have been raised with the aim of determining the proliferative potential of tissue material which has undergone formalin fixation and paraffin-wax embedding, more precisely (Galand and Degraef 1989; Van Dierendonck et al. 1991; Cattoretti et al. 1992; Linden et al. 1992; Sawhney and Hall 1992; Kreipe et al. 1993a; McCormick et al. 1993a). The possibility of using tissue blocks from files for an estimation of the growth fraction (cells in G1-, S-, G2-, M-phase of cell-cycle) in various malignancies has already been proven to be a very informative adjunct to histology. In this context immunohistological methods involving markers that indicate proliferative capacity seem to help considerably in our understanding not only of the complex pathological mechanisms associated with neoplastic transformation, but also in the recognition of their biological behaviour, including their prognostic impact (Hall et al. 1990; Linden et al. 1992; Diebold et al. 1992; Sampson et al. 1992; Shrestha et al. 1992; Aaltomaa et al. 1993; Filipe et al. 1993; Kreipe et al. 1993b). Although there has been a tremendous flood of publications dealing with immunohistochemical demonstration of newly-developed proliferation markers in a great variety of neoplastic conditions and related disorders and their association with clinical features, little attention has been paid to the bone marrow. In contrast to the frequently performed cell culture studies on haemopoietic cells, proliferative activity concerning normal bone marrow "in situ" as well as pathologically altered conditions is largely unexplored (Wilkins et al. 1992; Horny et al. 1993; Kitagawa et al. 1993; Thiele et al. 1993a–d). This lack of knowledge may be the result of several unfavourable circumstances which preclude an easy access to these problems:

1. The complicated nature of bone marrow which is composed of very different cell lineages.
2. The many drawbacks surrounding the application of monoclonal antibodies directed against formalin-resistant epitopes of nuclear proteins (McCormick and Hall 1992; McCormick et al. 1993b).
3. The need for morphometric analysis in order to determine the results of AgNOR-staining and the labelling index of immunohistochemical reactions. Finally, a critical evaluation of immunoreactivity has to consider definitely the results of experimental studies on haemopoietic cells and should always regard the gold standard of cell kinetics; the triatiated thymidine labelling index.

In consideration of the controversial issue of whether proliferation markers are usefully applied to routinely processed bone marrow tissue (trephine biopsies) and whether the labelling index exerts some bearing on the evolution of disease features, several methods and antibodies should be reviewed. These include AgNORs, proliferating cell nuclear antigen (PCNA) with the mono-

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clonal antibody PC10, Ki-67 antigen with the monoclonal antibody MIB1 and Ki-S1.

Silver-stained nucleolar organizer regions (AgNORs)

In normal human cells the secondary constrictions or satellite stalks on the short-arms of the D- (No. 13–15) and G-group (No. 21, 22) acrocentric chromosomes, represent the genes coding for 18s and 28s ribosomal RNA (Henderson et al. 1972; Evans et al. 1974). These sites of ribosomal RNA gene clusters are involved in forming the nucleolus immediately following mitosis and for this reason, were called nucleolar organizer regions (NORs). For a number of years silver impregnation techniques have been available (Goodpasture and Bloom 1975; Howell et al. 1975) which are able to distinguish NORs that were transcriptionally active in the preceding interphase, so-called silver-stained nucleolar organizer regions or AgNORs. However, a serious drawback of the AgNOR-staining technique is its sensitivity even to minor alterations of temperature and incubation time. Changes in these variables may generate a confluence of silver-stained dots and thus may cause large cluster-like areas in the nuclei with a drastic reduction of the corresponding counts (Rüschoff et al. 1989). Moreover, methods regarding the assessment of the nuclear AgNOR-content per cell are debatable and range from simple counting of dots to image analysis (Crocker et al. 1988; Rüschoff et al. 1989). These technical difficulties are probably the reason for the conflicting reports which exist about the presence of AgNORs in bone marrow cells derived from smear preparations (Mamaev et al. 1985, 1987; Sato et al. 1986; Arden et al. 1989; Grotto et al. 1991, 1993; Nakamura et al. 1992) or very rarely, from haemopoietic tissue (Matolscy et al. 1992; Pich et al. 1992). Considering metaphases of blood or marrow cells with one or more silver-stained areas as positive, the AgNOR-activity was found to be significantly lower in stable phase chronic myeloid leukaemia (CML) and in acute myeloid leukaemia (AML) cases than in the acute lymphoblastic leukaemia (ALL) group, and similar to the normal bone marrow (Arden et al. 1989). These results from leukaemic cell populations were unsatisfactory since overlaps occurred and the data should therefore be interpreted with caution. However, recently published studies regarding the method of AgNOR-evaluation emphasize the need for an exact quantification of the silver-stained areas into clusters of dots within a light nucleolar matrix, that is to say nucleoli of cells in proliferation, small dots dispersed throughout the nucleoplasm and not associated with the nucleolus, and condensed argyrophilic structures consistent with nucleoli of resting cells (Crocker et al. 1988). Following this discrimination between different components of the AgNOR-staining pattern, a distinctive arrangement of clusters and dots was detectable for each haemopoietic cell type (Grotto et al. 1991, 1993; Metze and Lorand-Metze 1993). An elaborate statistical analysis also revealed that the presence of clusters correlated significantly with cells in proliferation, that is to say, the most immature precursors of erythro- and granulopoiesis showed the highest counts of clusters (Grotto et al. 1991). For this reason, separate counting of clusters and dots following silver impregnation technique is clearly warranted and using this method it has been established that in AML myeloblasts showed a significantly lower dot and cluster formation in comparison with the myeloblasts of normal bone marrow (Grotto et al. 1993). This finding correlated very well with results of PCNA-labelling, flow cytometric analysis and bromo-deoxyuridine assay in peripheral blood and marrow cells derived from patients with AML (Giordano et al. 1993).

In malignant myeloma assessment of the mean AgNOR-number per plasma cell in trephine biopsies of the bone marrow revealed striking relationships to histological features (Pich et al. 1992). The AgNOR-count was directly correlated with the degree of plasma cell differentiation, with the percentage of bone marrow plasma cells and finally with the pattern of medullary involvement and occurrence of fibrosis. In the summary, these results indicate an association between AgNORs and plasma cell proliferation and emphasize the diagnostic value of this method (Pich et al. 1992). In this context another publication which deals exclusively with bone marrow tissue derived from trephine biopsies should be mentioned. In chronic myeloproliferative disorders (CMPDs) most subtypes (polycythaemia rubra vera, osteomyelofibrosis primary thrombocythaemia) showed a significant increase in the mean number of clustered AgNORs determined in mature megakaryocytes, whereas in CML and in the control group as well, silver-stained areas were remarkedly reduced (Matolscy et al. 1992). These preliminary investigations support the assumption that an increased amount of silver-stained dots and clusters is consistent with a stimulation of cell activation and proliferation. Persuasive evidence has been produced to link the number and extent of AgNORs per nucleus with the histological grading in a variety of tumour cells as well as with the percentage of Ki-67- and PCNA-immunostained nuclei, the c-*myc*-expression, the bromo-deoxyuridine labelling index and the prognostic impact (Hall et al. 1988; Trere et al. 1991; Yu et al. 1992b; Cohen et al. 1993; Delahunt et al.; Korkolopoulou et al. 1993).

Proliferating cell nuclear antigen (PCNA)

PCNA is a 36 kD acidic non-histone nuclear protein which functions as an auxiliary protein for DNA polymerase delta and is expressed in virtually all phases of the cell cycle (Keim and Hanash 1992; McCormick and Hall 1992). PCNA-activity increases through G1, peaks at the G1/S-interphase, decreases in G2, and reaches very low levels in M-phase. Before the introduction of the new Ki-67 homologous antibody (MIB1- see later), determination of PCNA-labelling by the most widely used monoclonal antibody PC10 was hailed with much enthusiasm. The reason for this singular interest in PCNA/PC10-staining was that it can be performed on routinely processed and archival material stored for

many years. However, following a flood of studies which, directly and without much criticism, related PCNA-immunostaining to the G1/S-phase of the cell-cycle, it has now become clear that the nuclear protein detected by PC10 may be also expressed in non-cycling cells. For this reason, it must be emphazised that PCNA-activity is not necessarily associated with DNA-synthesis, but may be involved in unscheduled DNA repair (following ultraviolet irradiation) at all phases of the cell-cycle (Nishida et al. 1988; Toschi and Bravo 1988; Shivji et al. 1992). Moreover, the reactivity of PCNA is also very sensitive to the concentration and duration of formalin-fixation and incubation time, and thus PC10-staining results may be very variable (Golick and Rice 1992; Wolf and Dittrich 1992; McCormick et al. 1993b). Another major drawback of PC10-immunostaining is the long half-life of PCNA which obviously varies with the kind of tissue involved (Scott et al. 1991). The biological half-life of PCNA has been estimated to be about 20 h, which in some rapidly cycling tumours could exceed the length of the late G1 and S-phases resulting positive staining in the G2-phase and beyond (Bravo and MacDonald-Bravo 1987; Scott et al. 1991). Finally, to further complicate any assessment of PCNA-expression, it is well-known that synthesis of this nuclear protein is regulated at both the transcriptional and post-transcriptional level and stability of mRNA for this "cell-cycle marker" may be influenced by certain growth factors (Jaskulski et al. 1988; Hall et al. 1990; Baserga 1991; Harrison et al. 1993). Considering all these complexities of PCNA-reactivity there may be situations where positive immunolabelling does not correlate with the other indices of cell proliferation and PCNA may be detected in quiescent cells (McCormick and Hall 1992).

Thus positivity for PCNA and PC10 should be treated with much caution and the exclusion of immunostaining which does not reflect cell proliferation found by another cell-cycle marker (i.e. Ki-67) is prudent.

However, despite these restrictions surrounding the interpretation of PCNA-activity, the first studies on bone marrow tissue revealed promising results. In comparison with healthy individuals striking increases in the number of PCNA-positive cells were described in trephine biopsies derived from various pathological conditions. These included pernicious anaemia, myelodysplastic syndromes (MDS), acute leukaemias (AML, ALL) and CMPDs. Following immunolabelling of the bone marrow, it has been concluded that in MDS the overall ratio of the PCNA-positive cell fraction was of predictive value for the transition into manifest leukaemia and that in contrast with the latter disorder, in aplastic anaemia only infrequent staining occurred (Kitagawa et al. 1993). Furthermore, cases with pernicious anaemia due to vitamin B12/folate deficiency demonstrated a greatly increased erythroid PCNA-expression and there was also a frequent labelling of megakaryocytes in CMPDs (Wilkins et al. 1992). A serious handicap should not be overlooked in these early reports on bone marrow cells and PCNA-reactivity; with the exception of the erythroid cell line (Wilkins et al. 1992) there is an apparent failure to discriminate each cellular component of the marrow by application of double-immunostaining techniques.

A more elaborate evaluation of haemopoietic cell proliferation was based on a standardized procedure including a very low concentration (about 1.5%) and short duration of buffered formalin fixation (12–48 h) as well as an optimal antibody-PC10-dilution (1:100) to avoid the well-known drawbacks of tissue processing (Golick and Rice 1992; McCormick et al. 1993b). Sequential double-immunostaining was then used for a proper identification of the different cell lineages also suggested by Horny and co-workers (1993). Finally, a comparative assessment of the Ki-67-labelling index on corresponding frozen material was carried out (Thiele et al. 1993a, b). In comparison with normal bone marrow, in reactive (secondary) polycythaemia and predominantly in polycythaemia rubra vera (P. vera) the PCNA-labelling index was significantly enhanced (Thiele et al. 1993a). According to relevant cell culture studies in P. vera this increase in PC10-positivity is probably due to stimulated DNA-synthesis, that is cell proliferation with reduction of G1/G2-phases (Adamson et al. 1980; Eridani et al. 1983, 1987; Lemoine et al. 1986; Partanen et al. 1989). However, in MDS, primary (idiopathic) osteomyelofibrosis (OMF) and particularly in pernicious anaemia, an undue over-expression of PCNA was recognizable in erythroid precursor cells (Wilkins et al. 1992; Thiele et al. 1993b, 1994a). Comparative morphometric analysis of MIB1 (Ki-67)-immunostaining (Thiele et al. 1993c) and previous results of experimental studies lend support to the assumption of an arrest in the S-phase of the cell-cycle generated by vitamin B12/folate acid (haematinic) deficiency (Hoffbrand et al. 1968; Wickramasinghe 1968a, b; Geary 1985). Contrasting P. vera and OMF, in CML the PCNA-labelling index of the erythroid lineage was shown to be in the normal range (Thiele et al. 1993d). This result is not only in keeping with the determination of the AgNOR-count (Mamaev et al. 1985; Sato et al. 1986; Arden et al. 1989), but far more importantly, with the former cell kinetic studies in this disorder. These studies reported a not-significantly altered proliferation capacity in CML in comparison with the control group (Ogawa et al. 1970; Dörmer et al. 1980; Goto et al. 1982; Andreeff 1986; Strife and Clarkson 1988) and the other subtypes of CMPDs (Adams et al. 1988).

Interpretation of proliferation marker and particularly PCNA-expression in megakaryocytes has, in addition to the aforementioned restrictions, to regard three salient points: Firstly, a clear-cut identification of this cell lineage should include immunohistochemistry with suitable monoclonal antibodies like Y2/51 (CD61). Following conventional staining procedures, small immature megakaryocytic elements (pro- and megakaryoblasts) are easily missed; this means about 40–50% of megakaryopoiesis (Thiele and Fischer 1991). Secondly, there is a significant relationship between cell and nuclear size, nuclear lobulation and maturity with stages of ploidy (Levine et al. 1982; Williams and Levine 1982). Therefore, the sizes of megakaryocytes, determined by morphometry, have to be considered in context with their staining ability by any so-called proliferation marker. Finally, it is generally accepted that megakaryopoiesis proceeds initially through a phase characterized by mitotic division of committed stem and progenitor cells followed

by nuclear endoreduplication (endomitosis) taking place in a non-dividing cell (promegakaryoblast through mature polyploid megakaryocyte). Taking into account these cell biological features of megakaryopoiesis, proliferation markers enable the identification of endoreduplicative activity associated with maturation and polyploidization. When regarding these criteria PCNA-staining of megakaryocytes showed a insignificantly altered labelling index in many reactive lesions, but strikingly different values in CMPDs (Thiele et al. 1994b). Megakaryocytes of patients with CML were not only characterized by a predominance of microforms, but also by a relevant reduction of PCNA-reactivity comparable with findings on erythroid precursor cells (Thiele et al. 1993d), Ag-NORs (Matolscy et al. 1992) and cell culture studies (Andreeff 1986; Juvonen 1988). Correlations between PCNA-labelling and size of megakaryocytes revealed a remarkable aberration of the index reflecting endoreduplicative activity in pernicious anaemia and OMF and, additionally a significant relationship between PCNA-positivity and size (Thiele et al. 1993b, 1994b). The conspicuously increased PCNA-staining capacity of smaller-sized megakaryocytes in reactive and neoplastic lesions (MDS, CMPDs) of the bone marrow is in support of a former hypothesis explaining the dynamics of megakaryocytic endoreduplication and polyploidization (Queisser et al. 1971). Following this assumption, differences in the duration of single cell-cycle phases at successive ploidy levels (corresponding with size as well as maturity) were proposed, resulting in a prolongation of the G1/G2-phases. The finding of a relative reduction of PCNA-staining in larger megakaryocytes is therefore compatible with the expression of this nuclear protein, particularly in the S-phase (Thiele et al. 1993b, 1994b). However, it remains possible that a number of giant (hyperpolyploid) megakaryocytes could have reached their endstage of polyploidization (endoreduplicative activity) and have entered into G0-phase of the cell-cycle. The discrepancy between PCNA-labelling and megakaryocyte size in pernicious anaemia (Wilkins et al. 1992, Thiele et al. 1993b) and OMF (Thiele et al. 1994b) may be related to a block in the S-phase of the cell-cycle, since cytokinetic analysis revealed an arrest in DNA synthesis due to vitamin B12/folate deficiency similar to erythropoiesis (Wickramasinghe et al. 1968b; Queisser et al. 1971). In conclusion, an increase in PCNA and also Ki-67 (MIB1)-immunostaining of megakaryocytes is not necessarily associated with a stimulation of endomitotic activity leading to enforced endoreduplication, but may be indicative for disturbances in cell kinetics.

Studies on the PCNA-labelling index of human mast cells in trephine biopsies of the bone marrow derived from patients with systemic and malignant mastocytosis revealed significant differences which were associated with survival in these disorders (Horny et al. 1993). Following identification of PC10-positive mast cells by double-staining (mast cell tryptase) a relevant increase in labelling was recognizable in malignant mastocytosis, contrasting corresponding measurements in reactive mast cell hyperplasia, benign cutaneous form of mastocytosis and also systemic mastocytosis with lymph node involvement (Horny et al. 1993). In chronic lymphocytic leukaemia, the leukaemic cell population which was isolated from peripheral blood and marrow aspirates revealed a PCNA-labelling index that correlated significantly with proliferation as measured by tritiated thymidine incorporation, clinical stage of disease and the lymphocyte doubling time (Del Giglio et al. 1992, 1993). Similar results were obtained in AML, where cell proliferation was determined by different methods (PCNA-expression, bromo-deoxyuridine assay and flow cytometry). The data gathered from this study reinforce the prognostic weight that pretreatment cell kinetic features have, particularly regarding response to chemotherapy (Giordano et al. 1993).

Finally, a linear relationship between PCNA and Ki-67 labelling has been shown to exist in a variety of solid tumours (Dervan et al. 1992; Cohen et al. 1993) and also in malignant lymphomas (Hall et al. 1990) as well as in cultured human haemopoietic and different lymphoma cell lines (Landberg and Ross 1991). A most recently published study (Sabattini et al. 1993) reported a similar ratio of immunostained neoplastic cells in only 70% of Hodgkin's and non-Hodgkin's lymphomas and thus is not entirely in agreement with these previous studies or with relevant findings on bone marrow and splenic tissue with myeloid metaplasia (Thiele et al. 1993a, c). However, we may speculate that these discrepancies could be due to methodology, since tissue processing has a significant bearing on staining results of both proliferation markers (Golick and Rice 1992; McCormick et al. 1993b; Munakata and Hendricks 1993).

Ki-67 antigen

Amongst the monoclonal antibodies raised against proliferation-associated antigens, Ki-67 has been most widely applied. The nuclear antigen which is detected by this antibody was recently characterized as a bimolecular complex of polypeptides of 345 and 395 kD (Gerdes et al. 1991). The antigen is expressed exclusively in the nuclei of cycling cells in the G1, S, G2 and M-phases of the cell-cycle, and is definitely absent in G0. The diagnostic and prognostic impact of Ki-67-positivity is generally accepted and extensively documented in a multitude of papers as reviewed in several articles (Brown and Gatter 1990; Hall and Levison 1990; Quinn and Wright 1990; Linden et al. 1992; Yu et al. 1992a; Schwarting 1993). Until recently, an inherent impairment of this antibody was the need for frozen material owing to the lability of this antigen. However, in the last year new monoclonal antibodies raised against recombinant parts of the Ki-67 antigen have become available and for this reason, particular interest has been focused on an antibody termed MIB1 (Cattoretti et al. 1992; Sawhney and Hall 1992; Key et al. 1993; Schwarting 1993). MIB1 facilitates the recognition of the Ki-67 antigen in routinely processed, formalin-fixed tissue material (Cattoretti et al. 1992; Key et al. 1993) following microwave oven heating (Shi et al. 1991). There is an effect of fixation time and duration of microwave oven heating and the

retrieval of the Ki-67 antigen; tonsils fixed for 24 or 48 h showed a significant increase in the percentage of positive nuclei following microwave times of 14 or 21 min and MIB1-staining (Munakata and Hendricks 1993). Decalcification with chelating agents at neutral pH does not interfere with the immunoreactivity of MIB1 on microwave-processed paraffin sections (Cattoretti et al. 1992). In consideration of these major advantages in comparison with AgNORs and particularly PCNA, Ki-67 is thought to hold on to the promise of avoiding the drawbacks already discussed with the other proliferation markers (Schwarting 1993). Nevertheless, this overall positive impression of the reliability of Ki-67 (MIB1)-immunostaining has to be modified for its practicability on haemopoietic tissue. In contrast with solid tumours, the fine structure of the bone marrow and its delicate cell components are apparently not well-preserved following the required microwave oven heating (Shi et al. 1991). Small changes in formalin fixation (the addition of glutaraldehyde) impairs the staining reaction and prevents good reproducibility (Thiele et al. 1993c). The most serious handicap associated with antigen retrieval is the limitation on the application of a second monoclonal antibody (say, Y2/51-CD61). Unequivocal identification of the different cell lines is a basic requirement to assess proliferation as well as the endoreduplicative activity of haemopoiesis (Thiele et al. 1993a–d). Although the positive impact of this new Ki-67 antibody is unquestionable, bone marrow tissue may present an exception to the rule, unless other more suitable methods of antigen retrieval are introduced.

Ki-S1

The new proliferation-associated antigen which is detected by the most recently characterized monoclonal antibody Ki-S1 is resistant to formalin-fixation and reveals a distinctive reactivity in all cycling cells (Kreipe et al. 1993a). Flow cytometric analysis of the DNA content and antigen expression showed a positivity for G1, S, G2/M-phases, but G0-cells were negative (Sampson et al. 1992; Kreipe et al. 1993a). The nuclear antigen exhibits a molecular mass of 160 kD and for this reason differs significantly from the Ki-67 antigen and PCNA (see corresponding data reported before). First studies evaluating the relationship between Ki-S1-labelling and prognosis in mammary carcinomas demonstrated significant correlations between immunostained nuclei and disease-free interval, overall survival and post-relapse survival (Sampson et al. 1992; Kreipe et al. 1993b). Ki-S1 staining of routinely processed and formalin-fixed bone marrow specimens does not require microwave oven heating for antigen retrieval. Some of the drawbacks of MIB1 (Ki-67) already mentioned are thus avoided and sequential immunostaining procedures become possible. Preliminary unpublished data suggest that Ki-S1 is a most promising proliferation marker with a particular value in determinating the growth fraction in haemopoietic tissue.

In conclusion, proliferation markers should be more widely applied to haemopoietic tissue. They are valuable in a great variety of pathological conditions and can be used to assess possible relationships between clinical findings and prognosis. They are valuable in evaluating timing and effect of cytoreductive therapy, which is not practicable using cell-culture studies. There is no firm recommendation which of the currently available staining reactions or antibodies is most suitable for the determination of the growth fraction. The AgNOR-technique does not permit discrimination of various cell lines in tissue sections and is difficult to handle with respect to the different modalities of evaluation and reliability of staining procedures. In routinely processed trephine biopsies, the well-known drawbacks of PCNA-labelling are not entirely avoided by MIB1-immunostaining and experiences with Ki-S1 are limited, due to its most recent introduction. For this reason, further studies are thought to be warranted in search for a modification of antigen retrieval which allows an unimpaired use of Ki-67 (MIB1) on haemopoietic tissue.

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