Diagnostic and prognostic value of DNA image cytometry in myelodysplasia

W AUFFERMANN,* INGRID FOHLMEISTER,† A BÖCKING*

*From the Departments of Pathology of the *Technical University of Aachen and the †University of Cologne, Federal Republic of Germany*

SUMMARY The DNA content of erythropoietic cells from 10 patients with refractory anaemia (RA) with megaloblastic changes, who subsequently developed acute non-lymphoblastic leukaemia (ANL), and from seven patients with megaloblastic marrow aspirates due to pernicious anaemia were compared by DNA image cytometry. The DNA distribution, the rate of aneuploid cells exceeding 5c (5cER), and the square deviation index of DNA values from the normal 2c-peak (2cDI) were recorded. Both variables were of diagnostic and prognostic importance for epithelial tumours, malignant lymphomas, and dysplastic lesions. A rate of 5cER > 0 was found in eight of 10 myelodysplastic, but in none of seven control cases. Hypodiploidy was equally pronounced in both groups of patients. The 5cE had the highest discriminative value of all variables calculated. The 2cDI was not significantly different in either group. In pernicious anaemia the 2cDI depended mainly on the percentage of S cells, reflecting the defect of DNA synthesis. In RA with megaloblastosis the 2cDI correlated with the percentage of G₂ cells, reflecting G₂ arrest. In the myelodysplastic group the 2cDI correlated positively with the length of time until ANL developed, indicating the prognostic relevance of 2cDI. Our findings show that in megaloblastic anaemia DNA image cytometry can distinguish myelodysplasia from pernicious anaemia and that it also provides prognostic information.

Myelodysplastic syndromes (MDS) comprise a range of conditions which vary in their degree of risk of transformation to "overt" acute non-lymphoblastic leukaemia (ANL).¹ In clinical practice diagnosis and classification of MDS are mainly based on a wide range of morphological features,²⁻⁶ but the predictive value of morphological features for risk of transformation to ANL is controversial. Moreover, discrimination between MDS and reactive changes is not always possible.¹ While it has been shown with cytogenetic methods that MDS are usually clonal,⁷⁻¹⁰ such methods fail to detect small clones of low mitotic activity.

Another way to define MDS has been tried using DNA flow cytometry,¹¹⁻¹⁴ which detects an abnormal DNA content even in slowly proliferating or resting cell clones, and permits the study of the cytokinetic characteristics of cell populations. DNA image cytometry combines the advantages of flow cytometry with the ability to select individual cells and is regarded as capable of detecting the "rare event" aneuploid cell.¹⁵ To test the value of this method in MDS we applied it to clinical circumstances where problems in discriminating true MDS from secondary

changes can arise. We chose to examine megaloblastic anaemia, which may either show a certain degree of responsiveness to B12 or folate substitution despite being due to MDS, or on the other hand, may be refractory to treatment in secondary cases—for example, in alcoholism.¹

We compared the cytometric data of erythropoietic cells from 10 patients with megaloblastic anaemia who subsequently developed ANL with those of seven patients who exhibited a characteristic B12 response and had pernicious anaemia. For this comparison, we applied a previously described algorithm,¹⁶ the diagnostic and prognostic importance of which has been shown for solid tumours,¹⁷⁻²³ malignant lymphomas,^{24,25} and dysplastic epithelial lesions.²⁰⁻²²

Material and methods

Bone marrow smears of 10 patients with the initial diagnosis of megaloblastic anaemia were selected from haematological material from the Institute of Pathology of the University of Cologne. Subsequent development of ANL was documented in all 10 patients. According to the FAB criteria, nine cases were classified as refractory anaemia (RA), four (cases 8-11) without, and five (cases 12-16) with pan-

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cytopenia. One patient (case 17) showed refractory anaemia with slight excess of blasts (RAEB). Five patients (cases 8–11 and 17) exhibited slight to moderate and three (cases 12–14) severe nuclear abnormalities of erythropoietic cells, such as nuclear budding and fragmentation. Four patients (cases 9, 10, 15, 17) also showed micromegakaryocytes. In three cases (cases 15–17) a partial myeloperoxidase deficiency was also seen.

ANL developed one to 11 months after the initial cytological diagnosis. Leukaemia was classified as M1 in four cases, as M4 in four cases, as M5a in one, and as M6 in one (table). Seven cases with B12 responsive pernicious anaemia served as controls. Four of them (cases 4–7) exhibited slight to moderate nuclear abnormalities of erythropoietic cells. All specimens were analysed by DNA image cytometry without knowledge of the final diagnosis.

DNA IMAGE CYTOMETRY

DNA image cytometry was performed with an automatic microscope using a computer controlled television image analysis system (TAS plus, Leitz, West Germany), which includes a video memory for automatic relocation of cells as described previously.²⁶ After photographic mapping of 150 erythropoietic cells in bone marrow smears stained by the Pappenheim method the slides were subjected to hydrolysis (4N hydrochloric acid at 28°C for 45 minutes) and then stained with Schiff's reagent according to the Feulgen method. Erythropoietic nuclei were relocated and marked manually on the screen using a light pen. The integrated optical density and the total area of a mask adapted individually to the shape and size of each individual nucleus were measured. Masks of touching nuclei were automatically separated before measurement. The diploid (2c) standard for erythropoietic cells was defined as the mean of 20

granulocytes, determined in identical specimens. DNA measurements of 100 to 150 erythropoietic nuclei were processed by the computer using the following algorithm.

ALGORITHM

The algorithm used in this study has been reported previously.^{16,17} This algorithm describes the prospective development of cell populations by computing two variables from the DNA histogram: the 5cER and the 2cDI. The 5cE represents the percentage of cells with a DNA content of more than 5c but less than 8c or $16c \pm 12.5\%$ and is taken as an index for aneuploidy. It can also be taken as a hyperdiploidy index. 2cDI represents the square deviation from the 2c value and is increased by all influences which deviate the cellular DNA content from the normal 2c value: increase of the fraction of proliferating cells, shortening of the G₁ phase, prolongation of the S or G₂ phase, hypodiploidy and hyperdiploidy.

The algorithm is only applicable to tissues not previously treated with radiation or cytostatic agents and only to cells without cytologically detectable virus infection.^{16,17} Furthermore, in all patients the fraction of cells with 2n, 4n, 2n–4n and (2n–4n)/4n DNA content was calculated according to Silvestrini *et al* and Riccardi *et al*.^{27,28} Cells were taken as 2n or 4n, respectively, when their DNA content was within an interval of $\pm 12.5\%$ of the respective value. The percentage of hypodiploid cells was determined in each case.

The significance of differences was calculated using the Wilcoxon rank test (continuous variables). For comparison of frequency distributions an exact contingency table test (dichotomous or categorised variables) was applied. Correlations between the various categories of values were evaluated using the Spearman rank correlation coefficient.

Table Clinical, cytological, and DNA-cytometric findings in 10 patients with RA and megaloblastosis, and in seven controls with pernicious anaemia

Case No	Clinicomorphological diagnosis	5cER	2cDI	2n–4n%	4n%	(2n–4n)/4n	Hypodiploidy	Nuclear abnormalities	Follow up
1	Pernicious anaemia	0	1.70	18.0	12	1.50	0	0	Response to B-12
2	Pernicious anaemia	0	0.64	51.0	6	8.5	0	0	Response to B-12
3	Pernicious anaemia	0	0.96	13.0	13	1	30	0	Response to B-12
4	Pernicious anaemia	0	2.12	4.0	15	0.27	2	+	Response to B-12
5	Pernicious anaemia	0	1.74	1.0	17	0.06	5	+	Response to B-12
6	Pernicious anaemia	0	1.82	14.5	17.5	0.82	1	+	Response to B-12
7	Pernicious anaemia	0	1.09	29	17	1.7	12	+	Response to B-12
8	RA	0.93	1.08	14	7	2	18	+	M1 after 8 months
9	RA	1.67	0.82	15.5	4.5	3.4	36	+	M4 after 1 month
10	RA	0	1.59	19	18	1.1	4	+	M1 after 5 months
11	RA	2	1.72	10	2	6	6	+	M5a after 8 months
12	RA with pancytopenia	4.4	7.79	15.5	17.5	0.9	5	++	M1 after 11 months
13	RA with pancytopenia	4.3	1.58	19.0	8	2.4	32	++	M4 after 6 months
14	RA with pancytopenia	2.67	3.07	20	15	1.3	7	++	M6 after 2 months
15	RA with pancytopenia	0	0.76	35.5	8.5	4.2	5	0	M4 after 1 month
16	RA with pancytopenia	1	0.63	14.7	4	3.7	56	0	M1 after 2.5 months
17	RAEB	2.15	2.63	20	14	1.4	0	+	M4 after 7 months

Results

Results of DNA image cytometry and morphological evaluation are given in the table. 5cER was greater than 0 in eight of 10 cases of MDS and in none of seven control cases. The difference was significant ($p < 0.0023$). 5cER is therefore a possible diagnostic indicator of hyperdiploid aneuploidy, which was found only in MDS. Hypodiploidy, on the other hand, which is not included in this index, was also present in pernicious anaemia (cases 3 and 7). Hypodiploidy did not discriminate MDS from control cases. A diagnostic value of other variables calculated from the DNA histograms, as 2cDI, 4n%, (2n-4n)%, (2n-4n)% + 4n%, and (2n-4n)/4n was not evident. There was also no correlation of morphological nuclear abnormalities, either to the final diagnosis ($p < 0.5$) or to 5cER ($p < 0.5$).

The 2cDI, as well as the percentages of (2n-4n) cells and 4n cells, were highly variable in pernicious anaemia and in MDS. In the group with pernicious anaemia 2cDI was negatively correlated with (2n-4n)/4n ($p < 0.01$). (2n-4n)/4n showed a positive correlation with (2n-4n) ($p < 0.001$) and (2n-4n)/4n ($p < 0.005$). There was no correlation between 2cDI and the percentage of hypodiploid cells. In MDS 2cDI was negatively correlated with (2n-4n)/4n ($p < 0.001$). In MDS 2cDI was positively ($p < 0.05$), and (2n-4n)/4n negatively ($p < 0.005$) correlated with 4n%. 2cDI was also positively correlated with 5cER ($p < 0.001$) and negatively with hypodiploidy ($p < 0.05$).

To find out whether there was a prognostic implication to be derived from any DNA variable, data were correlated with the time interval between initial cytological diagnosis and the date of transformation to ANL (transformation time). As a result, 2cDI was the only variable that correlated with transformation time ($p < 0.05$); all other DNA variables, morphological nuclear abnormalities, as well as FAB criteria showed no correlation with prognosis. Representative histograms of two cases of MDS with different transformation time intervals are shown in the figure.

Discussion

Among the myelodysplastic syndromes the risk of developing ANL is particularly indeterminate for those classified as RA or acquired idiopathic sideroblastic anaemia (AISA). This is partly due to the difficulty in discriminating them from secondary changes. Since MDS has been shown to be of a clonal nature,⁷⁻¹⁰ an early indicator of developing clonality would be helpful. Aneuploidy of the pathological clone may be used as a marker, but small aneuploid clones of low mitotic activity with minor

chromosomal aberrations may escape detection by cytogenetic methods or DNA flow cytometry.^{13,29}

In our study we used DNA image cytometry to discriminate RA with megaloblastosis from pernicious anaemia. Only erythropoietic cells were measured and only values exceeding 5c were accepted as abnormal (5cER > 0). Using this algorithm, eight of 10 RA cases with megaloblastosis and subsequent development of ANL exhibited an abnormal DNA distribution pattern, which was not seen in any of the seven control cases with pernicious anaemia.

Compared with 40-50% of MDS cases with cytogenetic abnormalities,³⁰ the sensitivity of this method in discriminating clonal disease from deficiency states seems to be high. This is due to the fact that even small numbers of aneuploid cells are detected, irrespective of their mitotic activity. It might

Figure Typical DNA histograms of erythropoietic cells in RA with megaloblastosis. (a) Case 12: slight hyperdiploid aneuploidy (5cER = 4.4). Strongly increased variation around the normal 2c value (2cDI = 7.79), partly due to G₂-arrest (4N = 17.5%); long transformation time of 11 months. Cytologically severe nuclear abnormalities. (b) Case 15: no hyperdiploid aneuploidy (5cER = 0). Little variation around normal 2c value (2cDI = 0.76) and low fraction of G₂ cells (4N = 8.5%). Short transformation time of one month. Cytologically no nuclear abnormalities.

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be worth while knowing if computing the 5cER in granulopoietic and megakaryopoietic cells would result in a further increase of the sensitivity of DNA image cytometry.

Surprisingly, hypodiploidy, which was recorded separately, was of no diagnostic relevance. Hypodiploidy was also present in pernicious anaemia, probably due to the defect of DNA synthesis in these cases. 5cER, which only recognises hyperdiploid but not hypodiploid aneuploidy, can therefore be regarded as the most adequate diagnostic DNA variable.

Our second algorithmic variable, 2cDI, was of no discriminative value for the diagnosis of MDS because the values varied within the same range in MDS and in pernicious anaemia. Once the diagnosis of MDS is established, however, 2cDI might help define the prognosis. In our study the time span to development of ANL (transformation time) was positively correlated with 2cDI. Low 2cDI values indicated imminent leukaemic transformation; high 2cDI values were associated with a longer stable course. For diagnostic purposes we chose only cases which were retrospectively verified as MDS by the later development of ANL. As patients with true MDS may succumb to infection or bleeding without manifestation of leukaemia our results, which indicate the prognostic relevance of 2cDI, may be biased. The prognostic implication of this index therefore needs confirmation in a prospective study.

2cDI was influenced by cell cycle variables. In pernicious anaemia the index was indirectly dependent on the fraction of S cells (2n-4n%), reflecting a variable prolongation of the S phase due to the defect of DNA synthesis. In MDS, however, 2cDI was positively correlated with the fraction of G₂ cells (4n%) but not with the fraction of S cells, though both variables were increased. As has been shown earlier,¹³ an increase of the G₂ fraction of erythropoietic cells is mainly found in the RA and AISA subtypes of MDS, and is due to a large amount of G₂ arrested cells.

In conclusion, in patients with megaloblastic anaemia DNA image cytometry distinguishes MDS from secondary changes due to pernicious anaemia and also provides valuable prognostic information in MDS. The algorithm reported here may be of importance for clinical routine diagnosis.

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Reprint requests to: W Auffermann, Department of Radiology, University of California San Francisco, San Francisco, CA 94143, USA.