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Plasma Cell DNA Content in Multiple Myeloma and Related Paraproteinemic Disorders. Relationship with Clinical and Cytokinetic Features*

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Abstract—*In* 62 *patients with multiple myeloma (MM) and related disorders, the nuclear DNA content distribution of bone marrow plasma cells was assessed by flow and conventional cytofluorometry. Abnormal distributions, suggesting the presence of aneuploid populations, were observed in* 53% *of MM at diagnosis, in* 50% *of benign monoclonal gammopathies and in* 12% *of Waldenström's macroglobulinemias. Eighty-six percent of aneuploid cases had DNA stem-lines falling between the diploid and triploid value. In advanced and relapsing MM, abnormal distributions were found in* 75% *of cases. In* 4 *out of* 14 *patients with MM serially studied during the course of disease, emergence of new abnormal clones was documented. The abnormal DNA content of bone marrow plasma cells was not correlated with any clinical and laboratory characteristic and it affected neither response to therapy nor survival in patients studied at diagnosis. In advanced phases of MM, the presence of abnormal clones was correlated with high plasma cell proliferation rates (studied by tritiated thymidine incorporation) and poor response to chemotherapy. Seven out of* 8 *patients in acute terminal phase of MM had abnormal clones. Among these, five had DNA stem-lines over triploid value.*

INTRODUCTION

CYTOGENETIC studies in multiple myeloma (MM) are limited by the low mitotic rate of neoplastic cells in most patients at diagnosis [1]. At relapse [2] and during the 'acute phase' [3] of the disease, the proliferative activity is, on the contrary, greatly increased and thus plasma cell metaphases become easily available for cytogenetic analysis. This may explain why abnormal karyotypes are more frequently observed in patients with advanced disease, although it cannot be excluded that aneuploid clones may develop late in the course of MM [1].

Abnormalities in DNA content distribution suggesting aneuploidy may be detected, by means of quantitative cytochemistry, independently from the proliferation rate of the abnormal clone. Thus, the analysis can be successfully carried out even in very slowly proliferating populations. Cytometric ploidy measurements showed abnormal values in more than half of the patients with MM studied at diagnosis [4–8]. Conflicting results, however, have been reported on the evolution of plasma cell ploidy during the course of disease, as well as on the prognostic relevance of ploidy abnormalities [7, 8].

This paper deals with the measurements of the nuclear DNA content carried out by flow and/or conventional cytofluorometry on bone marrow cells of 62 patients with MM and related disorders followed during their clinical course. In many patients the *in vitro* tritiated thymidine ([^{3}H]-Tdr) labeling index of neoplastic plasma cells was also determined. The data obtained show that aneuploid clones, not detectable at diagnosis, may become detectable following chemotherapy, at an

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advanced phase of disease. These clones are usually highly proliferating and resistant to conventional chemotherapy.

PATIENTS AND METHODS

Patients

Sixty-two patients with monoclonal gammopathy were studied. Eight had monoclonal gammopathy of undetermined significance (MGUS) [9], 8 had Waldenström's macroglobulinemia (WM) and 46 were diagnosed to have MM according to the criteria of the Chronic Leukemia Myeloma Task Force [10]. All patients with MGUS were followed for a period of 3–12 yr (median 6 yr). Since these patients neither showed changes in paraproteinemia level nor developed neoplasias, they were classified as having benign monoclonal gammopathies (BMG). All patients with WM were studied at diagnosis before any treatment was started.

Thirty-six patients with MM, including 2 patients with primary plasma cell leukemia, were studied at diagnosis and the remaining 10 were studied first at relapse. Fourteen of the patients studied at diagnosis were examined at relapse too. Among the 24 relapsing patients, 8 were also followed during the so-called acute terminal phase of the disease [3]. Patients with acute terminal phase had advanced disease characterized by fever of unknown origin, and peripheral cytopenia with cellular marrow. All of these patients were resistant to chemotherapy and survived less than 4 months.

The clinical stage at diagnosis was evaluated in all MM patients according to Merlini *et al.* [11]. Two patients with stage I asymptomatic MM did not receive any cytostatic treatment. The remaining patients received one of the following regimens for induction therapy: melphalan (MPH) and prednisone (P) [12] (in 9 cases), peptichemio (PTC) and P [13, 14] (in 12 cases), and PTC, vincristine (VCR) and P [15] (in 13 cases). Maintenance therapy consisted of cyclic MPH (or cyclophosphamide) and P in all patients for at least 12 months or until relapse occurred. At relapse, all patients were given PTC in association with VCR and P. In patients resistant to this treatment, the M2-protocol [16] was tried. Criteria for response were a $\geqslant 50\%$ decrease in serum M protein levels and disappearance of Bence Jones protein. Furthermore, the achievement of more than one-half of the following conditions was requested: (a) decrease of the bone marrow plasma cell percentage (BMPC%) by at least 20 points or a return to $<10\%$; (b) a 2 g/dl rise in Hb concentration in anemic patients (Hb <11 g/dl) lasting more than 4 weeks; (c) return of serum calcium and BUN to normal values; (d) elevation of serum albumin value up to 3 g/dl in the absence of other causes of hypoalbuminemia and (e) absence of progression of the osteolytic lesions. Just for the relapsing patients the decrease up to or $>25\%$ in M protein plus at least half of the above conditions were considered as a clinical improvement. Two patients treated at diagnosis and two additional patients at relapse were not evaluable owing to their early death in the course of treatment. Survival was calculated from the onset of treatment. In untreated patients it was calculated from diagnosis. Unless otherwise specified, both cytometric and autoradiographic studies were carried out before treatment at diagnosis, and after a treatment-free period of at least 30 days at relapse.

Conventional cytofluorometry

Conventional fluorometric determination of nuclear DNA content on fixed bone marrow smears was carried out in all cases. Plasma cells were identified and mapped photographically on Giemsa stained smears which were subsequently decolored [17] and processed for the Feulgen reaction. The nuclear DNA content of 100–200 plasma cells was evaluated by using the Leitz microscope-photometers MPV 2 and MPV Compact. The staining procedure and technical characteristics of the instrumentations are detailed by Prenna *et al.* [18]. The ploidy level of plasma cells was estimated on the basis of the nuclear DNA content of 25–50 neutrophil granulocytes, as diploid value. For the comparison of the DNA values in various experiments, they were transformed as a function of the mean value of diploid reference standard set equal to 10 [19]. A plasma cell population was considered diploid ($2n$) if their modal DNA content fell within the range defined by 10 ± 2 S.D. where S.D. is the standard deviation of the control neutrophils. Accordingly, hypodiploid ($<2n$), hyperdiploid ($2n$–$3n$), triploid ($3n$), hypotetraploid ($3n$–$4n$) and tetraploid ($4n$) populations were recognized [19]. The coefficient of variation (CV) [20] for the control neutrophils ranged from 2.59 to 4.85% (median 3.34%).

Flow cytofluorometry

Flow cytometric analysis has been carried out since 1977 in 39 patients. Nuclear DNA content distribution of the overall bone marrow cell population was determined using Krishan's method [21], which has been slightly modified [22]. Propidium iodide (Calbiochem, San Diego, CA) stained suspensions of cell nuclei were measured by a cytofluorograph Bio/Physics Systems (Mahopac, NY) 4800 A. Peripheral blood of healthy subjects was chosen as diploid reference

standard. The aneuploid distributions were recognized according to the criteria of Barlogie *et al.* [23]. The shift along the abscissa of the whole population was defined by the evaluation of the DNA index (DI = modal channel of bone marrow cell sample: modal channel of diploid reference standard ratio). Normal range of DI was assumed to be the interval between mean ± 2 S.D. (0.99 ± 0.056) value of DI from 25 bone marrow samples of control subjects without hematologic disorders. The ploidy level of extra peaks in the DNA profile was assessed by an internal DI, using the modal value of the diploid subpopulation as a reference standard. The control samples CV ranged from 2.87 to 6.33% (median 4.98%). No significant difference was found between CV values obtained in studying diploid patients and those obtained in aneuploid patients. Control sample CV values were also similar in MM patients studied at diagnosis (median 4.7; range 3.4–6.1) and in those studied at relapse (median 4.9; range 2.9–6.3).

[^{3}H]-TdR autoradiography

In vitro [^{3}H]-TdR autoradiography of bone marrow cells was carried out as previously described [19] in 22 patients with MM at diagnosis and in 17 patients with MM at an advanced phase of disease. The LI was performed on at least 1000 morphologically identifiable plasma cells. Only cells with more than 6 grains per 100 μm^2 of cell area were considered labeled. In some cases, the differential Feulgen-DNA content of unlabeled and labeled plasma cells was performed after removing silver grains and emulsion from photographically mapped autoradiographic smears [19].

Statistical considerations

Statistical analysis was performed using χ^2 and Student's *t* test. Simple linear regression was used to correlate the DI values of aneuploid populations found by Feulgen reaction with those found by flow cytometry in the same patient. Differences in survival between patients with diploid and aneuploid myeloma cells were assessed by the Log-Rank Test [24].

RESULTS

Analysis of DNA content distribution

All samples showing abnormal population at flow cytometric analysis proved to have evidence of a plasma cell population with aneuploid DNA content at Feulgen analysis. The correlation between DI of the abnormal peak at flow and conventional analysis was excellent ($r = 0.967$). Figure 1 shows two typical DNA histograms of the overall bone marrow population obtained by

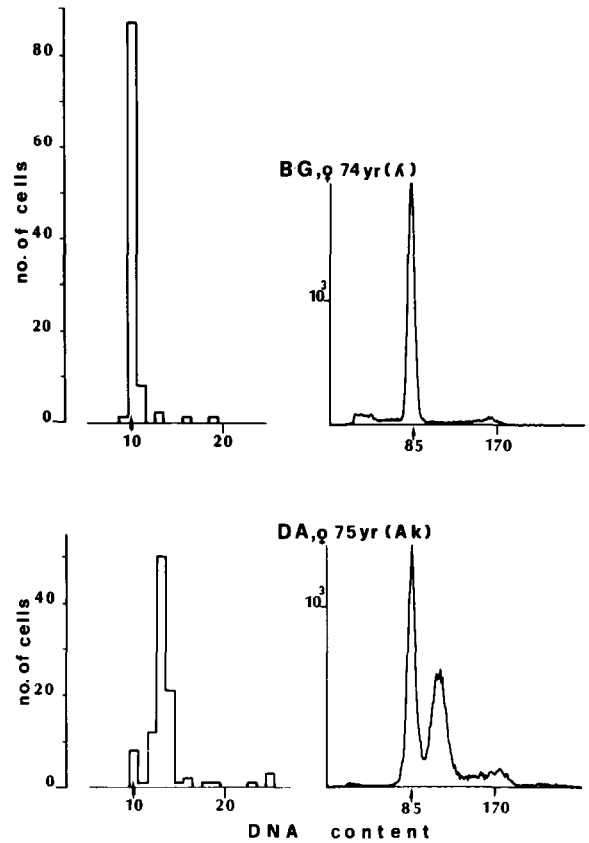

Fig. 1. Cytofluorometric DNA content distribution in 2 patients with multiple myeloma studied at diagnosis. Histograms on the left were obtained by measuring selectively bone marrow plasma cells by Feulgen reaction. Histograms on the right refer to the overall bone marrow cell population as measured by propidium iodide flow cytometry. Arrows indicate modal values of the diploid reference standards. Patient BG showed normal diploid distribution, while patient DA had plasma cell population with hyperdiploid stem-line.

flow cytometry compared to those of the plasma cell population selectively examined by conventional Feulgen cytofluorometry in the same patients.

In no patients with hyperdiploid DNA distribution and available autoradiographic data, did the value of LI account for the increased $2n$–$4n$ cell percentage.

The relative incidence of patients with evidence of aneuploid plasma cells, related to the clinical diagnosis, is reported in Table 1.

Plasma cell ploidy during the course of MM

Table 1 shows that patients with MM at relapse tend to have more (75%) aneuploid plasma cell populations with respect to the patients studied at diagnosis (53%) ($P < 0.1$). The most relevant difference between the two groups of patients seems, however, to be the presence of plasma cell populations with high decree aneuploidy ($\geq 3n$), which were found in 3% of newly diagnosed

patients, in 25% of all relapsing patients, and in 63% of patients observed during the acute terminal phase ($P < 0.01$). All but two patients with high degree aneuploid clones showed the concomitant persistence of diploid or hyperdiploid (or both in one case) plasma cell populations.

Constancy of the DNA stem-line was found in 11 out of 14 patients studied both at diagnosis and at first relapse. The other 3 patients showed the emergence of a stem-line in the hyperdiploid (1 case) or hypotetraploid (2 cases) range. An additional patient, who had a hyperdiploid stem-line both at diagnosis and first relapse, showed a shift in cell ploidy toward the triploid range 10 months later. This change was associated with the appearance of large chemoresistant plasmoblasts completely infiltrating the bone marrow. Figure 2 shows changes in plasma cell ploidy found during the course of the disease in 3 patients in which both flow and conventional cytofluorometric analysis were done.

Relationship of plasma cell ploidy with clinical and cytokinetic features in MM

The presence of aneuploid plasma cell populations at time of diagnosis did not correlate with any of the clinical parameters tested, including clinical stage and survival (Table 2). Plasma cell LI values were, on the contrary, sharply related to patient survival both at diagnosis and at relapse. Seven patients with values higher than 3.5% at diagnosis had median survival of 7 months (range 1–27), while 15 patients with lower values had median survival of 50.5 months (range 2–96) ($P < 0.01$). At relapse, median survival was 4 months (range 1–17) in

Table 1. Plasma cell ploidy in benign monoclonal gammopathies (BMG), Waldenström's macroglobulinemia (WM) and multiple myeloma (MM) at various stages of the disease

Clinical diagnosis	No. of patients	DNA values*			
		$<2n$	$2n$	$2n$–$3n$	$\geq 3n$
BMG	8	0	4	4	0
WM	8	0	7	1	0
MM at diagnosis	36	2	17	16	1
MM at relapse	24†	1	6	11	6
MM terminal phase	8†	0	1	2	5

*Only the modal value of the most abnormal population is considered in patients with two or more plasma cell clones with different DNA content.
†Most of these patients had also been studied in a previous phase of the disease.

Table 2. Clinical data of 36 patients with multiple myeloma studied at diagnosis

Parameter		DNA values	
		Diploid (17 patients)	Aneuploid (19 patients)
Sex	M/F	10/7	9/10
Median age, yr	(range)	64 (50–74)	60 (35–75)
Median BMPC%	(range)	56 (5–100)	53 (8–96)
M-component	G	10	11
	A	4	6
	BJ	3	2
	k/λ	8/9	9/10
Clinical stage	I	3	4
	II	5	8
	III	9	7
Median BMPC-LI%	(range)	2.7 (0.9–16.2)†	2.2 (0.01–6.0)†
Induction therapy	MPH/PTC	5/11	4/14
Median survival	(range)	44 months (0–58+)	30 months (3–96+)*
4-yr survival	% of patients	31	42*

BMPC = bone marrow plasma cells; LI = labeling index; MPH = melphalan; PTC = peptichemio.
*One patient who had myeloma complicating a carcinoma of the larynx was excluded.
†The data refer to 11 patients in each group.

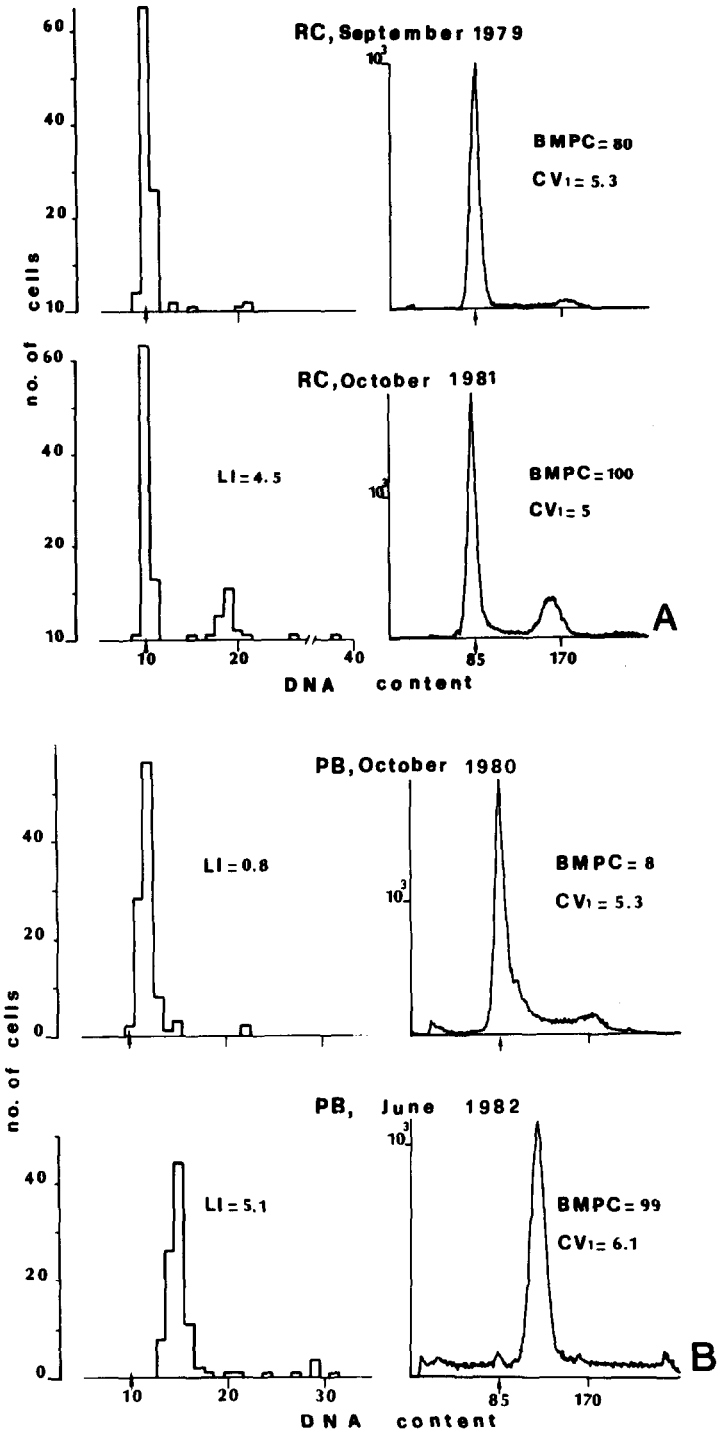

Fig. 2. (Continued overleaf.)

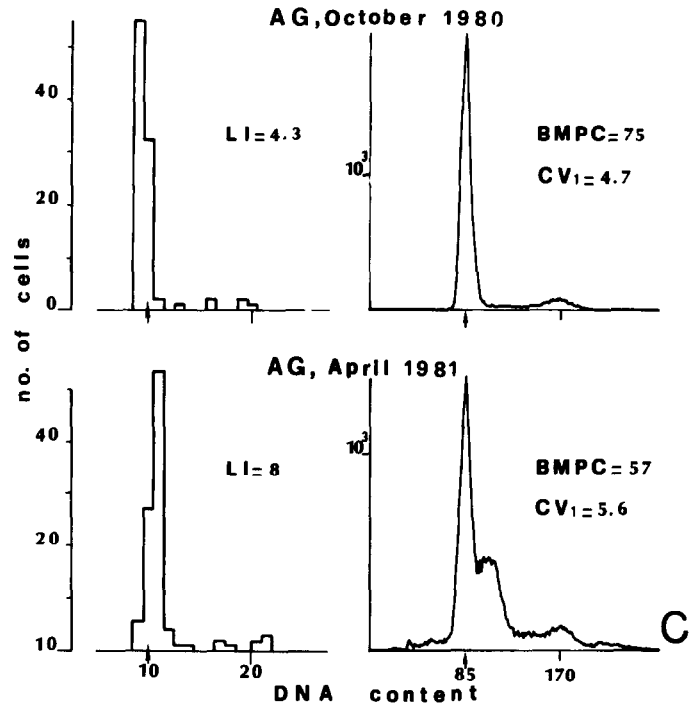

Fig. 2. Changes in plasma cell DNA stem-line in 3 patients with MM during the course of the disease. (A) Patient RC had only a diploid stem-line at diagnosis and both a diploid and hypotetraploid population at first relapse; (B) patient PB showed a slightly hyperdiploid stem-line at diagnosis and a triploid myeloma cell population during the acute terminal phase; (C) patient AG, who showed only a diploid stem-line at diagnosis, had evidence of a hyperdiploid plasma cell population at first relapse. (BMPC = % of bone marrow plasma cells; LI = plasma cell labeling index; CV_1 = coefficient of variation of the control sample; further explanations in Fig. 1.)

patients with high LI, while it was 16 months (range 1–38) in patients with low LI ($P < 0.05$).

Diploid and aneuploid plasma cells showed identical proliferative activity at diagnosis. However at an advanced phase of disease, patients with aneuploid clones showed a plasma cell LI higher than that observed at diagnosis ($P < 0.05$), while the values of diploid populations remained nearly unchanged (Fig. 3). Nine patients were studied serially. Increase in LI at time of relapse was observed in all 3 patients who developed new aneuploid clones and in 2 out of 6 patients who showed constancy of the DNA stem-line.

Relationship of plasma cell ploidy with response to therapy in MM

Response to chemotherapy was similar in patients with diploid and aneuploid plasma cells at diagnosis. However, the presence of aneuploid clones was significantly related to a poor response to chemotherapy at relapse (Table 3).

Among relapsing patients, those who had aneuploid plasma cells with low (LI < 3.5%) proliferative activity responded well to therapy (3 responses, 1 no response) as did those with diploid plasma cell population (Table 3). On the contrary, 5 out of 7 patients with highly proliferating aneuploid clones proved to be

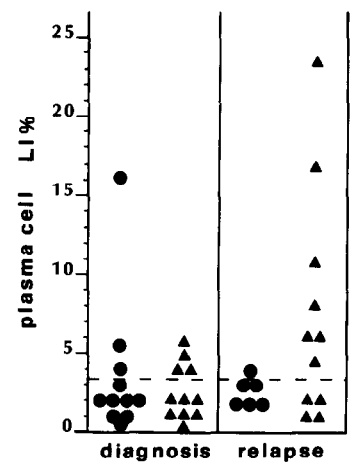

Fig. 3. Tritiated thymidine labeling index (LI) of bone marrow plasma cells in 22 patients with MM studied at diagnosis and 17 patients studied at relapse. ●, Diploid cases; ▲, aneuploid cases.

completely resistant, even to aggressive combination chemotherapy. Of these, one had a single hyperdiploid population, one had both diploid and hyperdiploid plasma cells, and three had evidence of high degree aneuploid clones. Between the remaining patients, one had a hyperdiploid population which rapidly disappeared from the bone marrow simultaneously

Table 3. Difference in response rate according to the plasma cell ploidy in patients with multiple myeloma at different phases of disease

Time of therapy		DNA values		χ^2
		Diploid	Aneuploid	
1st treatment (32 patients)	Reponse	9	12	NS
	No response	5	6	
Relapse (22 patients)	Response	4	4	$P < 0.02$
	Clinical improvement	2	3	
	No response	0	9	

with the disappearance of Serum M component. The second patient had a clinical improvement followed, 4 months later, by a chemoresistant phase. This patient (not shown in Fig. 2) had, at relapse, both a diploid population and a hypotetraploid clone not detected at diagnosis. Different patterns of response to chemotherapy were found in the two clones. New aneuploid subpopulation was less affected by treatment than a diploid one and was also rapidly recruited to proliferate after chemotherapy (Fig. 4).

DISCUSSION

The analysis of both DNA content distribution, by means of Feulgen reaction, and proliferation rate, by [^{3}H]-TdR autoradiography, showed that abnormalities in the DNA histograms obtained by flow cytometry in MM patients [5, 7, 8] are to be ascribed to plasma cell populations with abnormal DNA stem-line. Although a reduced number of chromosomes is frequently found in myeloma cells [25], hypodiploid cells were only occasionally detected in our study as well as in other series [4, 5, 7]. Aneuploid plasma cells, in fact, had nearly always hyperdiploid DNA content. Such a discrepancy had been previously pointed out in neoplastic cells, due to the presence of marker chromosomes with high content of DNA [26].

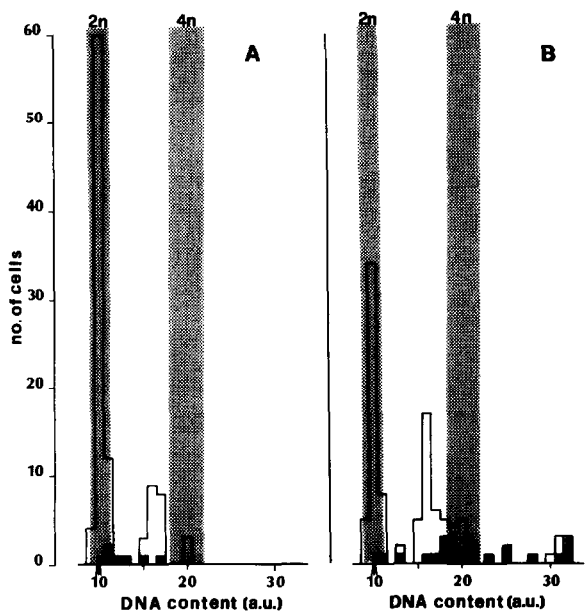

Fig. 4. Distribution of the nuclear DNA content of [^{3}H]-TdR unlabeled (□) and labeled (■) bone marrow plasma cells in patient CI at relapse. (A) Before therapy (LI = 6.5%), (B) 5 days after chemotherapy was stopped (LI = 15.7%). A marked increase in both the relative size and the proliferation rate of the hypotetraploid clone was evident following chemotherapy. 2n = Diploid range; 4n = tetraploid range; ● = mitosis.

In our series, the percentage of DNA abnormalities in MM at diagnosis (53%) is close to that (57%) found by Bunn *et al.* [8], who used similar techniques and instrumentations as far as flow cytometric analysis is concerned. On the contrary, it is distinctly lower than that (70–80%) found by Latreille *et al.* [5, 7]. The higher frequency of aneuploidy at diagnosis reported by these authors may be due to the higher sensitivity of their instrumentations detecting both a lower degree of aneuploidy and a smaller aneuploid subpopulation. However, these considerations do not appear to be relevant at relapse, when the percentage of aneuploid patients is the same (70–80%) in Latreille's, Bunn's and our series. This suggests that, when DNA histogram resolution is suboptimal, abnormal DNA distributions not detectable at diagnosis may become so at relapse. Possible explanations are the improvement in flow cytometric analysis and/or increase in tumor cell infiltrate over the time of investigation. However, in our studies carried out at diagnosis and at relapse, differences in DNA histogram resolution, as inferred by CV values, did not account for the increased rate of aneuploidy during the course of the disease. Furthermore, selective examination of plasma cell population, by Feulgen rection, allowed us to avoid mistakes due to a low percentage of tumor cells in bone marrow samples.

Alternatively, we can assume that either an evolution from a near-diploid stem-line towards a greater modal DNA content, or a selection of

small aneuploid clones, present at diagnosis as a minority of the tumor cell population, may occur during the course of disease in some patients. This may explain the equal incidence of aneuploid distributions reported by Latreille *et al.* [5, 7] at diagnosis and at relapse as a result of higher resolution measurements allowing both near-diploid stem-lines and small aneuploid clones to be detected. Moreover, it is in agreement with the detection of new DNA stem-lines in 4 patients and the percentage increase of high degree aneuploidy during the course of the disease found in our study. It is important to stress that all new aneuploid clones had an increased modal DNA content with respect to the previous clones in our patients. Changes in myeloma cell stem-line had been reported by Bunn *et al.* [8] and, more recently, by Barlogie *et al.* [27]. Shift of the modal DNA content towards high degree aneuploidy had also been reported in patients with angioimmunoblastic lymphoadenopathy [23, 28] during terminal transformation into immunoblastic lymphoma.

The clinical and prognostic significance of pretreatment plasma cell ploidy is still debated. Bunn *et al.* [8] found that patients with evidence of aneuploidy in bone marrow cells had shorter survival rate as compared to those with only diploid cells. In our series, on the contrary, the presence of aneuploid plasma cells at diagnosis was neither correlated with clinical and biochemical characteristics, nor seemed to influence survival and response rate. On this regard, our findings are similar to those obtained by Latreille et al. [7]. Nearly one third (5 out of 16) of the patients with aneuploidy, but none of those in the diploid group in Bunn's series [8], showed any sign of renal failure. This fact could partially account for the difference in patient survival reported by these authors. The lack of prognostic relevance of the DNA content analysis at diagnosis is furthermore strengthened by the results obtained in our patients with BMG. The percentage of cases with aneuploidy in these patients did not differ from that found in MM at diagnosis. We stress the fact that one of our patients, who constantly showed hyperdiploid plasma cells at repeated examinations, has been carrying asymptomatic gammopathy for 12 yr, while several patients with normal DNA content distribution of plasma cells had stage III MM. We can conclude that BMG cannot be distinguished from MM on the basis of the DNA content of bone marrow plasma cells.

Hyperdiploidy was also detected in one case of WM, which is usually considered a diploid neoplasm as far as the DNA content analysis is concerned [7, 8].

Plasma cell proliferation pattern is a very important prognostic factor in MM. Low LI of bone marrow plasma cells is associated with long survival, while high LIs were found in more aggressive diseases, independent from response to chemotherapy [7, 29]. In our patients studied at diagnosis, the plasma cell proliferative activity was independent from the presence of abnormal ploidy levels. At relapse, on the contrary, increased values of LI were more often observed in patients with aneuploid cells. This may be ascribed in part to the development of clones characterized by increased modal DNA content and high proliferative activity. Clonal evolution and/or clonal selection were also suggested to account for changes in cell kinetics and ploidy occurring in malignant lymphomas [30], which are similar to those seen in our patients. In MM, these clones are frequently chemoresistant. They may be, in some patients, highly anaplastic [31] and responsible for the acute terminal phase of the disease [3].

It can be concluded that the analysis of DNA content on myeloma cells may allow detection of aneuploid (often highly aneuploid) clones growing during the course of disease following treatment. Since clinical and cytokinetic studies suggest that these clones are often aggressive and resistant to conventional chemotherapy, monitoring of cell kinetics and ploidy may give additional insights into managing patients with advanced myeloma. For example, the use of cycle active agents sounds reasonable in relapsing patients with evidence of highly aneuploid (often highly proliferating) clones. Furthermore, early detection of changes in proliferation rate and ploidy, at remission, might allow use of cycle active agents before clinical relapse becomes evident, i.e. before a high tumor mass is reached. Dual parameter flow cytometry will be very useful to this end, allowing plasma cells to be differentiated from other bone marrow cells on the basis of intracytoplasmic proteins [32] and/or RNA staining [27].

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