

Circulating Ki67 positive lymphocytes in multiple myeloma and benign monoclonal gammopathy

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Abstract

Aims—To estimate the proportion and nature of the proliferating (Ki67+) circulating lymphocytes in a series of patients with multiple myeloma and monoclonal gammopathy of unknown significance (MGUS) and to correlate this with other clinical and laboratory parameters, using blood from healthy adults as a control. To investigate the extent to which the B and T lymphoid components are involved in progression and/or control of disease.

Methods—Blood lymphocytes from 15 patients with multiple myeloma, 10 patients with MGUS and 10 healthy adults were analysed using a sequential double immunoenzymatic staining technique. Antibodies directed against Ki67 were used to detect cells in cycle, CD3, CD4, and CD8 to identify T cells, HLA-Dr as a marker for B cells and activated T cells, and CD11b as a marker for natural killer cells. Polyclonal antibodies directed against the κ and λ immunoglobulin light chains were also used to detect B cells.

Results—The proportion of proliferating (Ki67+) lymphocytes was significantly higher in patients with multiple myeloma (6.8 ± 2.6) and MGUS (3.5 ± 1.1) compared with the normal controls (1.69 ± 0.3); this was also true when multiple myeloma and MGUS cases were compared. In multiple myeloma and MGUS over 50% of the Ki67+ cells were activated T lymphocytes (CD3+/HLA-Dr+); a minority (11%) were non-clonal B lymphocytes. In contrast to controls (6.7 ± 1.9), in patients with multiple myeloma and MGUS the proportion of proliferating T cells expressing CD8 (23.6 ± 12.5 and 15.3 ± 7.7 , respectively) and CD11b (13 ± 8.7 and 11.6 ± 3.9 , respectively) was higher. In multiple myeloma there was a positive correlation between the proportion of Ki67+ lymphocytes, β -2-microglobulin concentrations and disease stage.

Conclusions—Although the number of patients investigated is small, this study suggests that Ki67 expression in blood lymphocytes from patients with multiple myeloma may be a good prognostic indicator for aggressive disease and may help to distinguish multiple myeloma from

MGUS. The activated proliferating T cells in these diseases may represent an immunological reaction against the tumour.

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Multiple myeloma is a B cell disorder characterised by the clonal proliferation of B cells at late stages of lymphoid differentiation and has distinct clinical features such as the presence of a serum monoclonal band, variable degree of bone marrow infiltration by plasma cells and osteolytic bone lesions. The clinical course in multiple myeloma varies remarkably from aggressive to slowly progressive or even stable.^{1,2} Benign monoclonal gammopathy of undetermined significance (MGUS) is closely related to multiple myeloma, and is regarded by many authors as a premalignant condition, gradually evolving into multiple myeloma in 10-20% of patients.^{3,4}

A number of clinical and laboratory features are regarded as prognostic factors in multiple myeloma. These include age, sex, immunoglobulin subclass, haemoglobin, serum calcium, lactate dehydrogenase (LDH), creatinine, β -2-microglobulin, plasma cell morphology, and clinical stage.⁵⁻⁹ Other laboratory parameters, such as T cell subset, expression of natural killer and activation markers in blood lymphocytes,^{10,11} the multidrug resistance glycoprotein P-170 (MDR-1), and CD56 on plasma cells, are also of prognostic relevance and may be of value in distinguishing between MGUS and multiple myeloma.¹²

One of the prognostic factors in multiple myeloma is related to the degree of plasma cell proliferation. The plasma cell labelling index (PCLI), measured using either [³H] thymidine or 5-bromo-2-deoxyuridine uptake, has been used to estimate proliferation in multiple myeloma and has been shown to be a useful parameter for distinguishing between progressive multiple myeloma and stable MGUS as well as being an independent predictor of survival.^{9,13-15} Similarly, Lokhorst *et al*¹⁶ investigated the expression of Ki67, a monoclonal antibody which detects cells in cycle,^{17,18} and demonstrated that the percentage of Ki67+ plasma cells in MGUS is significantly lower than that in untreated multiple myeloma.

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In an earlier study¹⁹ we showed that the percentage of circulating Ki67+ clonal B cells in chronic lymphocytic leukaemia is related to disease progression and that in the early stages of this disease the proliferating cells are mainly reactive T cells. This prompted us to investigate, using a double immunoenzymatic staining technique, Ki67 expression in circulating lymphocytes from patients with multiple myeloma and MGUS to estimate the extent to which the B and T cells modulate disease progression and whether Ki67 expression can be used to distinguish between these two conditions.

Methods

Samples, obtained at diagnosis, from 15 patients with multiple myeloma, 10 patients with MGUS and 10 healthy adult volunteers were studied. Diagnosis and clinical staging of multiple myeloma and MGUS were based on the criteria of the Committee of the Chronic Leukemia/Myeloma Task Force.²⁰ The median age of the patients was 65 years (range 42–80 years) and there was a male predominance (M/F 2 in multiple myeloma and 1.2 in MGUS). Clinical staging of multiple myeloma according to the Durie–Salmon system⁷ was as follows: IA, 3; IIA, 5; IIB, 2; IIIA, 4; IIIB, 1. A serum monoclonal band was present in 22 patients: IgG in 20 and IgA in two, and κ in 18 and λ in four. The remaining three patients had Bence–Jones proteinuria only.

Haematological parameters in patients with multiple myeloma were as follows: haemoglobin concentration, 10.3 g/dl (range 6–14.5 g/dl); white blood cell count, $6.9 \times 10^9/l$ (range 3.1 – $13.3 \times 10^9/l$); lymphocyte count, $2.2 \times 10^9/l$ (range 1.08 – $3.3 \times 10^9/l$); and platelet count, $257 \times 10^9/l$ (range 158 – $456 \times 10^9/l$). The corresponding parameters in patients with MGUS were: haemoglobin concentration, 11.7 g/dl (range 9.9–15.6 g/dl); white blood cell count, $6.09 \times 10^9/l$ (range 3.6 – $10.8 \times 10^9/l$); lymphocyte count, $2.02 \times 10^9/l$ (range 1.2 – $2.8 \times 10^9/l$); and platelet count $219 \times 10^9/l$ (range 143 – $312 \times 10^9/l$).

Mononuclear cells were isolated from heparinised peripheral blood by Ficoll–Hypaque density gradient centrifugation. Cytospins were then made, dried overnight and stored at -20°C until immunostaining was performed. The monoclonal antibodies used were as follows: Ki67 (Dako, Glostrup, Denmark) to detect cells in cycle; CD3, CD4 and CD8 (Dako) to identify T cells; HLA-Dr (Becton Dickinson) as a marker for B cells and activated T cells; and CD11b (Becton Dickinson) as a marker for natural killer cells. Polyclonal anti-

bodies directed against the κ and λ immunoglobulin light chains (Dako) were also used to detect B cells.

Sequential double immunoenzymatic staining was applied as described previously²¹ using an immunoperoxidase sandwich technique with Ki67 and the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method to detect cytoplasmic expression of lymphoid antigens. Second and third layer reagents used included peroxidase conjugated goat antimouse immunoglobulin and monoclonal mouse peroxidase/antiperoxidase (PAP) complexes, a rabbit antimouse immunoglobulin, monoclonal mouse APAAP complexes, and swine antirabbit APAAP conjugate (all from Dako). For the negative control, the monoclonal antibody was replaced by a mixture of mouse immunoglobulins with differing isotypes.

The proportion of Ki67+ cells was evaluated by examining 2000 mononuclear cells under light microscopy using an oil immersion lens ($\times 100$). The proportion of double stained cells was estimated by examining 200 Ki67+ cells. Double positive cells were easily identified as they contained a dark brown deposit in the nucleus (Ki67+), while the cytoplasm was stained red (APAAP).

Statistical analysis was performed using the Student's *t* test. A univariate correlation was established between the percentage of Ki67+ cells and the serum concentration of LDH, creatinine, β -2-microglobulin, calcium, and haemoglobin, monoclonal protein value, percentage of plasma cells, and clinical stage.

Results

A small number of lymphocytes from patients with multiple myeloma and MGUS and from controls were Ki67+ (table 1). The proportion of Ki67+ cells was significantly higher in multiple myeloma and MGUS compared with the normal samples and was higher in multiple myeloma than in MGUS. Analysis of the expression of lymphoid markers within the Ki67+ cell population showed that over half of Ki67+ cells were CD3+ in all three groups (fig 1). Thus, most of the Ki67+ cells were T lymphocytes; only a minority expressed the immunoglobulin κ and λ light chains (B cells) (table 1). There were no significant differences in the proportion of CD3+ Ki67+ cells among the three groups, whereas the proportion of Ki67+ B lymphocytes (κ or λ positive) in patients with multiple myeloma and MGUS was significantly lower than that in normal controls (table 1).

In all cases, Ki67/ κ and Ki67/ λ double stained lymphocytes were observed (non-clonal B lymphocytes). HLA-Dr determinants were expressed in a high proportion of cells in cycle in all cases (table 1) (fig 2). Although triple labelling analysis with Ki67, HLA-Dr and CD3 was not performed, the data shown here, when the percentage of Ki67+ cells coexpressing CD3 and immunoglobulins is taken into consideration, suggest that a significant proportion of proliferating T cells (CD3+ Ki67+) were activated T lymphocytes (HLA-Dr+) (table 1).

Table 1 Percentage of Ki67+ T and B lymphocytes in blood from patients with multiple myeloma, MGUS and controls[‡]

Subject group	Ki67	Ki67/CD3	Ki67 κ and λ	Ki67/HLA-Dr
Multiple myeloma	6.8 \pm 2.6* [†]	51.0 \pm 11.5	10.1 \pm 3.2*	73.6 \pm 11.4*
MGUS	3.5 \pm 1.1* [†]	51.3 \pm 11.2	11.3 \pm 1.2*	64.5 \pm 11.1
Controls	1.69 \pm 0.3	55.5 \pm 6.8	18.6 \pm 3.1	62.8 \pm 8.9

[‡] Results of double labelling are shown as the proportion of cells positive for one other marker and for Ki67. * $p < 0.05$ between multiple myeloma or MGUS and controls. [†] $p < 0.05$ between multiple myeloma and MGUS.

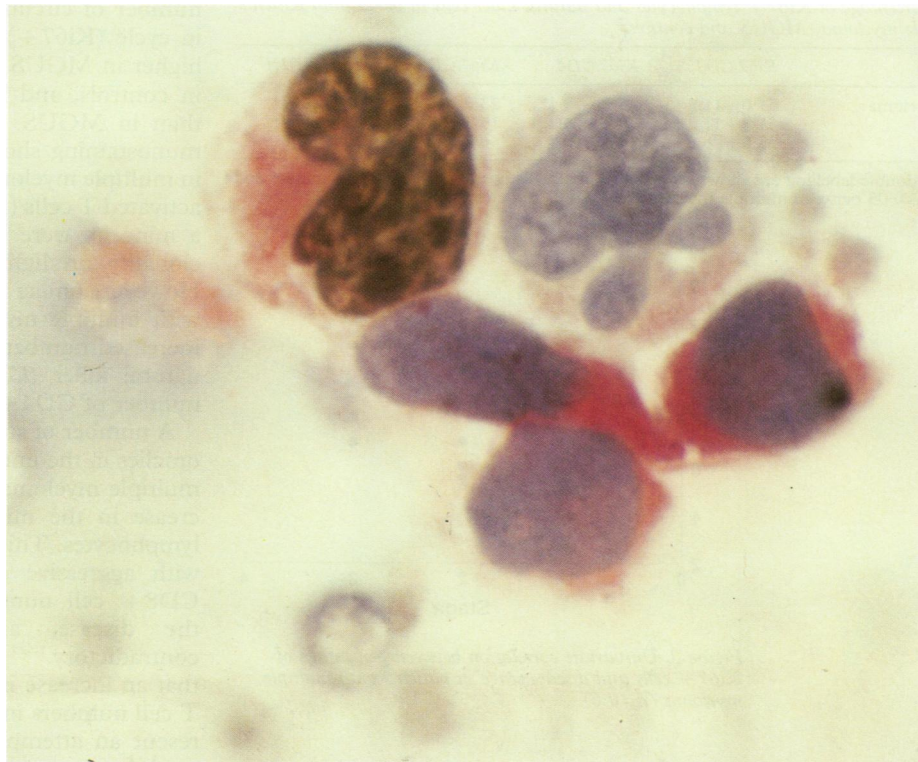


Figure 1 Lymphocytes from a patient with multiple myeloma, four of which were CD3+ (red stained cytoplasm (APAAP)). One was also Ki67+ as indicated by the brown stained nucleus.

Differences between multiple myeloma, MGUS and control samples became apparent when the two T cell subsets, CD4+ and CD8+, were considered separately (table 2). In multiple myeloma the proportion of CD4+ T cells in cycle was significantly lower than that in the controls whereas the numbers of CD8+ Ki67+ cells were higher in multiple

myeloma and MGUS samples, as was expression of CD11b in Ki67+ cells (table 2).

Statistical analysis revealed a positive correlation between the percentage of Ki67+ cells and clinical stage and β -2-microglobulin concentrations ($r=0.6$) (figs 3 and 4). No correlation was found between the percentage of Ki67+ cells and the other parameters studied.

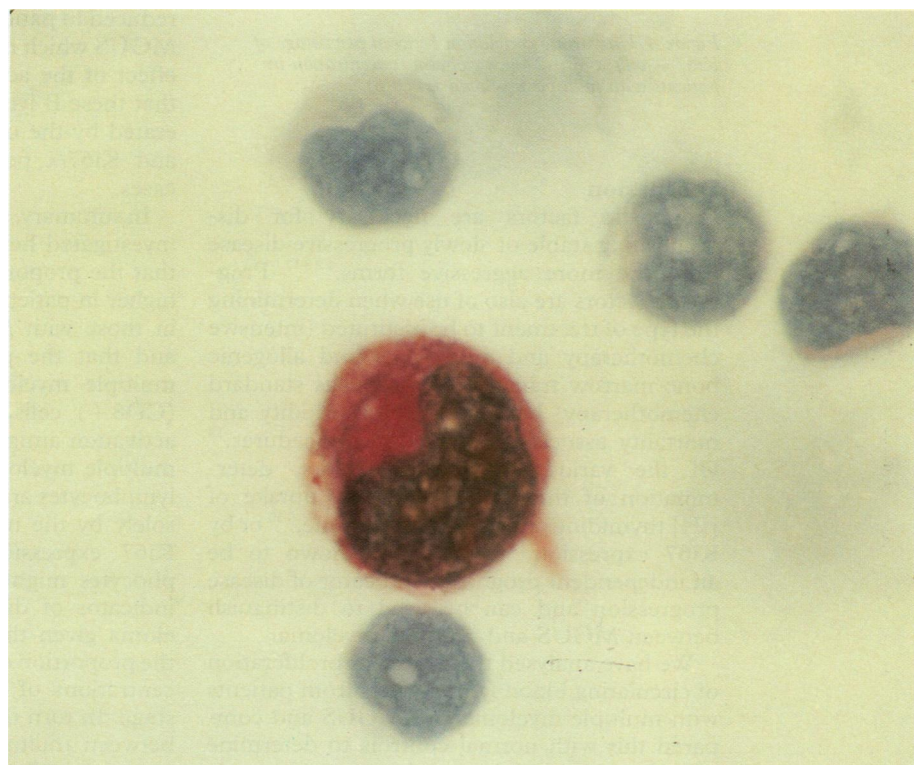


Figure 2 Lymphocytes from a patient with MGUS, one of which was both Ki67+ (brown stained nucleus) and HLA-Dr+ (red stained cytoplasm (APAAP)).

Table 2 Percentage of Ki67+ lymphocytes and natural killer cells in blood from patients with multiple myeloma, MGUS and controls†

Subject group	Ki67/CD3	Ki67/CD4	Ki67/CD8	Ki67/CD11b
Multiple myeloma	51.0 ± 11.5	25.8 ± 8.1*	23.6 ± 12.5*	13.0 ± 8.7*
MGUS	51.3 ± 11.2	30.3 ± 6.4	15.3 ± 7.7*	11.6 ± 3.9*
Controls	55.5 ± 6.8	38.4 ± 10.5	6.7 ± 1.9	5.3 ± 2.8

† Results of double labelling are shown as proportion of cells positive for one other marker and for Ki67. *p<0.05 between multiple myeloma or MGUS and controls.

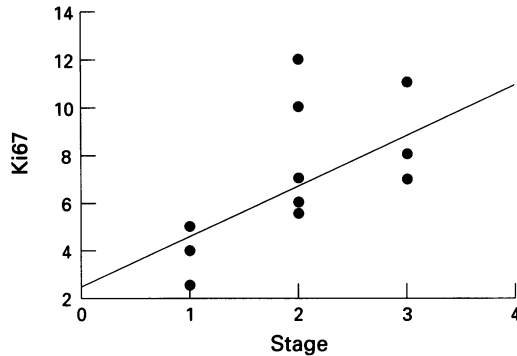


Figure 3 Univariate correlation between percentage of Ki67+ cells and disease stage in patients with multiple myeloma ($r=0.6$).

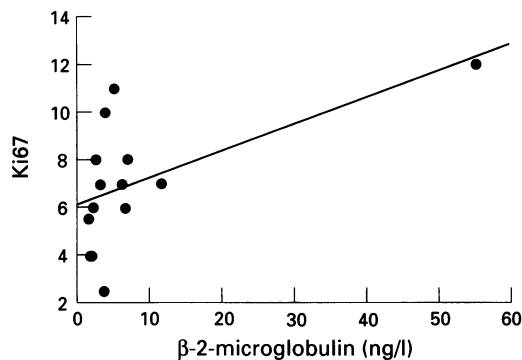


Figure 4 Univariate correlation between percentage of Ki67+ cells and β -2-microglobulin concentration in patients with multiple myeloma ($r=0.6$).

Discussion

Prognostic factors are necessary for distinguishing stable or slowly progressive disease from the more aggressive forms.^{12,8,12} Prognostic factors are also of use when determining the type of treatment to be instituted (intensive chemotherapy and autologous and allogeneic bone marrow transplantation versus standard chemotherapy) because of the morbidity and mortality associated with some procedures.¹³ Of the various prognostic factors, determination of the PCLI, either by uptake of [³H] thymidine or bromodeoxyuridine,^{9,15} or by Ki67 expression,^{16,22} has been shown to be an independent prognostic indicator of disease progression and can be used to distinguish between MGUS and multiple myeloma.

We have analysed the degree of proliferation of circulating blood lymphocytes from patients with multiple myeloma and MGUS and compared this with normal controls to determine whether this can be used as a prognostic indicator. Our findings demonstrate that, in multiple myeloma and MGUS, only a small

number of circulating blood lymphocytes are in cycle (Ki67+), with this proportion being higher in MGUS and multiple myeloma than in controls, and higher in multiple myeloma than in MGUS. As in controls, double immunostaining showed that most Ki67+ cells in multiple myeloma and MGUS samples were activated T cells (HLA-Dr+ CD3+) and only a minority were B cells with no evidence of clonality on light chain restriction analysis. However, unlike healthy individuals, patients with multiple myeloma and MGUS have an increased number of suppressor (CD8+) and natural killer (CD11b+), but only a small number of CD4+, cells in cycle.

A number of studies have demonstrated anomalies in the immune system in patients with multiple myeloma, mainly presenting as a decrease in the number of circulating CD4+ lymphocytes. This has been found to correlate with aggressive disease and an increase in CD8+ cell numbers in the early stages of the disease, although the reports are contradictory.^{10,23} Gonzalez *et al*¹¹ postulated that an increase in natural killer and activated T cell numbers in multiple myeloma may represent an attempt by the immune system to modulate tumour cell growth. These and other data support the hypothesis of an imbalance in the immune system, more marked in patients with multiple myeloma than in those with MGUS.

B cell immunological abnormalities have also been reported in multiple myeloma. Joshua *et al*²⁴ demonstrated a potential host mediated modulation of tumour cell proliferation in multiple myeloma as suggested by the presence of light chain isotype suppression in the stable phases of the disease. In the present study we found that the number of B cells in cycle was reduced in patients with multiple myeloma and MGUS which may be related to the suppressor effect of the activated T cells. It is also clear that these B lymphocytes in cycle are not generated by the malignant clone as both Ki67/ κ and Ki67/ λ positive cells were found in all cases.

In summary, although the number of samples investigated here is small, our results suggest that the proportion of lymphocytes in cycle is higher in patients with multiple myeloma than in those with MGUS or in normal controls, and that the proliferating (Ki67+) cells in multiple myeloma are mainly suppressor T (CD8+) cells, some of which also express activation antigens (HLA-Dr) and CD11b. In multiple myeloma, only a small number of B lymphocytes are in cycle and are not generated solely by the neoplastic clone. Assessment of Ki67 expression in peripheral blood lymphocytes might constitute a good prognostic indicator of disease activity in multiple myeloma given the positive correlation between the proportion of Ki67+ lymphocytes and concentrations of β -2-microglobulin and disease stage. In turn this could be used to distinguish between multiple myeloma and MGUS. Sequential studies in MGUS may predict whether a particular subgroup of patients is at greater risk of developing multiple myeloma.

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