

Demonstration of changes in plasma cell subsets in multiple myeloma

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ABSTRACT

Increases in free light chain (FLC) production are associated with disease progression in multiple myeloma (MM). Using a double immunofluorescence staining method to produce a differential count of plasma cells in bone marrow, single populations were demonstrated, containing intact monoclonal immunoglobulins (M-Igs) in 74% and FLCs only in 8% of cases. However, 18% contained a mixture of both cell populations. Progression from cells making intact M-Ig to cells restricted to FLC only production occurred in individual cases during the course of their disease. The presence of FLC only cells was associated with shortened survival.

Key words: multiple myeloma, bone marrow, plasma cells, free light chains, monoclonal immunoglobulins.

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Multiple myeloma (MM) is characterized by a malignant proliferation of monoclonal plasma cells in the bone marrow and of their products in serum and/or urine. M-Igs and FLCs are used to monitor disease and treatment. FLCs are found in the urine of 68% of MM cases. So-called Bence-Jones proteinuria (BJP) and increases of FLCs in serum are associated with disease progression.^{1,2} Up to 20% of MM cases have FLC only disease in which light chains in the serum and/or urine is the only immunochemical abnormality found.³ The serum assay for FLCs in MM has been extensively investigated.⁴ A shift to the secretion of light chains has been associated with relapse but little cellular information is available derived from direct visualization of light chain production.

Immunofluorescence of cytocentrifuge preparations of marrow cells is a useful and reproducible approach to define the proportion of plasma cells that are monoclonal.⁵ Double staining is preferable to single staining as each population is enumerated separately. It also has the advantage of an internal control. Since plasma cells only contain Igs of a single heavy chain and light chain type, the sum of the percentages of the

heavy chain figures should approximate to 100%. This is also true of the sum of the light chain percentages. Discrepancies in these figures led to the identification in individual cases of separate populations of cells staining for either intact M-Igs or FLCs only. These dual bone marrow populations form the subject of this brief report.

Design and Methods

We reviewed archive results of immunofluorescence bone marrow counts of 146 MM cases from five local hospitals in South London. Clinical samples were taken to monitor disease and treatment so the results presented are for first testing but not necessarily at clinical presentation. Approval for the study was obtained from the London-Surrey Borders Research Ethics Committee. The distribution by monoclonal immunoglobulin (M-Ig) isotype was: IgG 78/146 (53%); IgA 19/146 (14%); IgD 8/146 (6%); κ light chain only 20/146 (14%); λ light chain only 11/146 (8%); biclonal gammopathies 8/146 (6%); and non-secretory MM 2/146 (1%). The male to female ratio was 2:1 and the median age at presentation was 53

(range 28-79). In addition, 38 cases of Waldenstrom's macroglobulinemia, 28 cases with monoclonal gammopathy of undetermined significance (MGUS), 17 with solitary or extramedullary plasmacytomas, 18 with AL amyloidosis, 10 with nonsecretory MM and 12 with plasma cell leukemia were reviewed for the presence of dual populations.

Cytocentrifuge preparations were made as previously described.⁵ Eight preparations were fixed and stained in separate mixtures of anti-Ig reagents as follows: monospecific antisera to human IgG, IgM, IgA and IgD heavy chains and to κ and λ light chains conjugated to fluorescein isothiocyanate (FITC) were each mixed with polyspecific anti-human Ig serum conjugated to rhodamine isothiocyanate (TRITC), (Dako). Specimens containing dual populations were confirmed by restaining with anti-heavy chain FITC and anti-light chain TRITC reagents of appropriate isotype (Figure 1).

The differential count of plasma cells was made by taking each stained preparation in turn and counting 100 consecutive plasma cells containing cytoplasmic Ig (red staining) and recording how many of these were also stained green using the FITC filter sets. Thus plasma cells of each heavy and light chain type were counted independently in separate preparations.

M-Igs in the serum and BJP in the urine were detected by electrophoresis and identified by immunofixation using monospecific anti-Ig reagents and gold stain. Abnormal bands were quantified by densitometric scanning.

Results and Discussion

The sums of the counts of cells staining for heavy chains and those for light chains formed a control for the reliability of the counting procedure. In 236 counts from MM cases the mean sum was 99.8% (S.D=2.95) giving a reference range of 94-106 (mean +/- 2SDs).

Intact M-Igs were detected in the sera of 113/146 (78%) of these MM cases. The staining of the predominant marrow plasma cells corresponded with the heavy and light chain of the M-Igs detected in the serum in all but one case. This patient's cells stained as a biclonal IgG λ and IgA λ but only monoclonal IgA λ was detected in the serum. In 7/113 (6%), the sum of the heavy chain staining cells was substantially lower than that of the light chain staining cells, indicating a subpopulation restricted to light chain staining only. BJP was detected in the urine of 6/7 (86%) of these M-Ig⁺ dual population cases.

FLC only disease was diagnosed in 31/146 (22%) of cases. A subpopulation of marrow cells containing monoclonal heavy chains was detected in 11/31 (35%) of these cases (Table 1). Many dual population samples gave a characteristic pattern of dim and bright staining

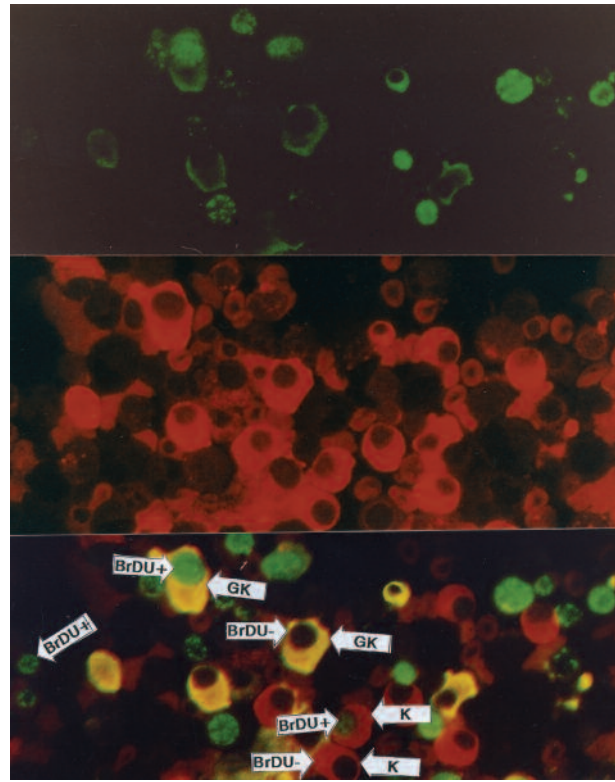


Figure 1. The same microscope field of an IgG κ bone marrow sample showing dual populations stained with anti-IgG FITC and anti- κ TRITC. In addition, anti-BrDU staining of nuclei has been performed to show cells in S-phase of the cell cycle. Upper panel shows anti-IgG FITC cytoplasmic and anti-BrDU FITC nuclear staining, middle panel shows the same field with anti- κ TRITC staining and lower panel shows a double exposure of the two upper plates superimposed to demonstrate double stained IgG κ cells (yellow), κ only cells (red) and S-phase cells with green nuclei. Arrows indicate BrDU⁺ and BrDU⁻ M-Ig⁺ cells, BrDU⁺ and BrDU⁻ κ only cells together with non-plasma cells in S-phase.

with the anti-Ig TRITC reagent. This may be due to the M-Ig⁺ cells binding to two components (anti-heavy and anti-light chain) of the pentavalent antiserum while the FLC restricted cells bind to only one.

Full bone marrow, serum and urine immunochemistry and clinical records, were available for 95/146 (66%) of cases tested. They were divided into four groups according to the population(s) of cells found in the marrow and by the presence or absence of FLCs in the urine. Findings for each group are presented in Figure 2.

In 91 cases, multiple bone marrow aspirates were presented at different time points during the course of their disease. Progression from one cellular category to another was seen in 11/91 (12%) cases tested during the period of observation (1 month to 6 years). All 11 cases had BJP in the urine. Four cases progressed from having only a single population of M-Ig⁺ cells to a dual population, by the acquisition of light chain only staining cells. Two cases progressed from dual population to a single population of FLC only cells, by the loss of the

Table 1. Eighteen cases in which there was a marked discrepancy in the marrow either between the sum of cells staining for Ig heavy chains and the sum of cells staining for light chains, indicating a sub-population of cells restricted to light chain production, or between the cellular results and the serum immunochemistry.

M-Ig in serum	BJP in urine	Plasma cells in marrow	Sum of heavy chain positive cells	Sum of light chain positive cells	Proportion of M-Ig+ plasma cells	Proportion of light chain only plasma cells
IgGK 4g/L κ		28%	90%	98%	IgG K 82%	κ 16%
IgGK 9g/L κ		11%	23%	99%	IgG K 20%	κ 77%
IgGL 16g/L λ		15%	92%	100%	IgG L 90%	λ 9%
IgGL 14g/L λ		7%	36%	92%	IgG L 20%	λ 56%
IgAK 44g/L λ		16%	61%	100%	IgA K 61%	κ 39%
IgDL 5g/L λ		27%	29%	98%	IgD L 26%	λ 70%
IgAL 64g/L ¹ λ		16%	89%	100%	IgG L 19%	λ 13%
					IgA L 67%	
None κ		98%	20%	100%	IgG K 20%	κ 80%
κ 21g/L κ		67%	3%	100%	IgG K 3%	κ 97%
None ² κ		40%	99%	99%	IgG K 99%	None
None κ		23%	26%	99%	IgG K 24%	κ 73%
None κ		25%	19%	101%	IgG K 16%	κ 83%
None λ		7%	82%	97%	IgG L 44%	λ 18%
None λ		19%	20%	97%	IgG L 11%	λ 80%
None λ		90%	20%	102%	IgG L 15%	λ 81%
None λ		81%	11%	100%	IgG L 11%	λ 89%
None λ		58%	9%	98%	IgG L 8%	λ 89%
None λ		44%	13%	101%	IgG L 10%	λ 88%

¹An IgG λ M-Ig was not detected in this patient's serum by immunofixation.

²An IgG κ M-Ig was detected in this patient's serum on only 1 of 6 occasions and that 10 months after this initial result.

intact M-Ig⁺ population. One case progressed from a single M-Ig population to a single FLC only population. On first testing, 2 cases had FLC only cells alone, while on second testing dual populations were detected. Finally both reverted to FLC only cells alone. In contrast, 2 cases showed the reverse process, from FLC only cells alone to dual populations, but both were in complete remission after treatment. These changes could indicate the competitive selection process involved.

Similar results were found in other plasma cell dyscrasias. Dual populations were found in 1/28 (4%) of MGUS cases and in 3/18 (17%) of cases with AL amyloidosis but none in 38 cases of Waldenstrom's macroglobulinemia. Monoclonal plasma cell populations were found in 7/19 (37%) of investigations of solitary or extramedullary plasmacytomas and one of these had a dual population. No dual populations were seen in 10 cases with non-secretory MM. All had a monoclonal plasma cell population and 5 of them showed single populations of FLC only cells. In 12 cases of plasma cell leukaemia there were no dual populations but 5/12 (42%) had a single population of FLC only cells.

The double immuno-staining technique employed here allowed the direct demonstration of the emergence of dual plasma cell subsets in MM that have been suggested for many years by the association of disease

Cellular Type	Single population M-Ig + BJP Neg	Single population M-Ig + BJP +	Dual population M-Ig + & FLCOnly BJP+	Single population FLCOnly BJP+
Marrow plasma cells				
& extra-cellular products	M-Igs with or without free light chains	M-Igs with free light chains	M-Igs with free light chains	Free light chains only
Number of cases in group (%)	30 (32%)	40 (42%)	17 (18%)	8 (8%)
M-Ig in Serum	+	+	+ in 7/17 (42%) Neg in 10/17(56%)	Negative
FLCs in urine	Negative	+	+	+
Median survival since diagnosis	86 months	38 months	22 months	22 months

Figure 2. Classification of 95 cases according to the presence of FLCs in the urine (BJP + or Neg) and cell populations demonstrated in the marrow (M-Ig and/or FLC only) together with their incidence, immunochemical findings and survival since diagnosis.

progression originally with BJP and more recently increases in serum FLCs. Single-staining or the κ and λ staining which are frequently used to establish monoclonality would not detect these differences. The presence of dual populations in a proportion of both intact M-Ig⁺ and FLC only cases and the 12% who changed their cellular protein profile suggest that such changes in subsets are not uncommon. Although VL sequences were not available to prove that the two populations were clonally related in individual cases this is highly likely since intraclonal heterogeneity is not known in MM. Loss of heavy chains is likely to be the result of acquired genetic aberrations in the functional VDJH alleles at the genomic DNA level. However, mechanisms acting at the mRNA level cannot be excluded.

We showed BrDU labeling of both populations (Figure 1) but only 1 case was associated with morphological differences.^{6,7} Tumor escape mutants may be expected to increase with modern treatment and longer patient survival and testing for M-Ig and FLC will remain imperative.⁸ These population changes may contribute to a cellular basis for some disease progression, for changes in serum FLC concentrations, for so-called *Bence-Jones escape*⁹ and the *FLC breakthrough* that has been recently reported.^{10,11} The proliferative advantage, and the molecular, genetic and drug sensitivity cellular mechanisms influencing the natural history of MM could be further investigated if the two component populations were separated by flow cytometry.

Authors' Contributions

MA conceived and designed the study, acquired, analysed and interpreted the data and was involved in the drafting, revision and decision to submit the paper for publication; FD interpreted data, provided clinical input and was involved in the drafting, revision and the decision to submit the paper for publication; DG interpreted data, provided genomic input and was involved in the drafting,

revision and the decision to submit the paper for publication; GM interpreted data, provided clinical input and was involved in the drafting, revision and the decision to submit the paper for publication.

Conflicts of interest

The authors reported no potential conflicts of interest.

References

1. Mead GP, Carr-Smith HD, Drayson MT, Morgan GJ, Child JA, Bradwell AR. Serum free light chains for monitoring multiple myeloma. *Br J Haematol* 2004;126:348-54.
2. Rajkumar SV, Kyle RA, Therneau TM, Clark RJ, Bradwell AR, Melton LJ, et al. Presence of monoclonal free light chains in the serum predicts risk of progression in monoclonal gammopathy of undetermined significance. *Br J Haematol* 2004;127:308-10.
3. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 cases with newly diagnosed multiple myeloma. *Mayo Clin Proc* 2003;78:21-33.
4. Bradwell AR, Carr-Smith HD, Mead GP, Harvey TC, Drayson MT. Serum test for assessment of cases with Bence Jones MM. *Lancet* 2003;361:489-91.
5. Ayliffe MJ. Double staining immunofluorescence procedure for enumeration of cells containing cytoplasmic immunoglobulin in human bone marrow. 40 selected cases. *J Clin Pathol* 1985;38:989-94.
6. Greipp PR, Lust JA, O'Fallon WM, Katzmann JA, Witzig TE, Kyle RA. Plasma cell labelling index and β 2-microglobulin predict survival independent of thymidine kinase and C-reactive protein in multiple MM. *Blood* 1993;81:3382-7.
7. Rajkumar SV, Fonseca R, Lacy MQ, Witzig TE, Therneau TM, Kyle RA, et al. Plasmablastic morphology is an independent predictor of poor survival after autologous stem-cell transplantation for multiple MM. *J Clin Oncol* 1999;17:1551-7.
8. Durie BGM, Harousseau J-L, Miguel JS, Blade J, Barlogie B, Anderson K, et al. International uniform response criteria for multiple MM. *Leukaemia* 2006;9:1467-73.
9. Hobbs JR. Growth rates and responses to treatment in human myelomatosis. *Br J Haematol* 1969; 16:607-17.
10. Kunnemund A, Liebisch P, Bauchmuller K, Haas P, Kieber M, Bisse E, et al. Secondary light chain multiple MM with decreasing IgA paraprotein levels correlating with renal insufficiency and progressive disease: clinical course of two cases and review of the literature. *Onkologie* 2005;28 Suppl 3:165.
11. Dawson MA, Patil S, Spencer A. Extramedullary relapse of multiple myeloma associated with a shift in secretion from intact immunoglobulin to light chains. *Haematologica* 2007;92:143-4.