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Immunophenotypic Characterization of Plasma Cells from Monoclonal Gammopathy of Undetermined Significance Patients

Implications for the Differential Diagnosis between MGUS and Multiple Myeloma

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Although the immunophenotype of plasma cells (PCs) from multiple myeloma (MM) patients has been extensively explored, information on the phenotypic characteristics of PCs in monoclonal gammopathy of undetermined significance (MGUS) patients is scanty and frequently controversial. Thus, the question of whether or not PCs are phenotypically different in the two disorders and whether this criteria could be useful for the differential diagnosis between MGUS and MM remains to be explored. In the present study, the immunophenotypic profile of bone marrow PCs (BMPCs) from a group of 76 MGUS patients has been analyzed by flow cytometry and compared with that of BMPCs present in both MM patients (n = 65) and control subjects (n = 10). For that purpose, a large panel of monoclonal antibodies against PC-related antigens was used together with a sensitive methodology in which a minimum of 10^3 PCs were studied. In all MGUS cases studied, two clearly defined and distinct PC subpopulations could be identified. One PC subpopulation, population A (33 ± 31% of total PCs), constantly displayed a high CD38 expression with low forward light scatter (FSC)/side light scatter (SSC) and was positive for CD19 and negative for CD56 (only a small proportion of these PCs were weakly positive for CD56). The other PC subpopulation, population B (67 ± 31% of total PCs), showed the opposite pattern; the antigen CD56 was strongly positive and CD19 was constantly negative, and it showed a lower CD38 expression and higher FSC/SSC values than population A. Clonality studies (cytoplasmic light chain restriction, DNA content studies, and polymerase chain reaction assessment) confirmed the clonal nature of PCs from population B and the polyclonal origin of PCs from population A. Moreover, the polyclonal PCs from MGUS displayed a phenotypic profile identical to that found in PCs from healthy individuals. By contrast, clonal PCs from all MGUS patients displayed a similar antigenic profile to myelomatous PCs, with clear phenotypic differences with respect to normal PCs: lower intensity of CD38 expression and a variable reactivity for markers that were not expressed in normal PCs, such as CD28, CD117, and sIg. Although the presence of residual polyclonal PCs was a constant finding in MGUS patients, it was a rare event in MM and, when present (only 22% of MM cases), its frequency was significantly lower than that observed in MGUS (0.25% *versus* 32.9%, respectively; $P < 0.0001$). Only 1.5% of patients with MM had more than 3% of normal PCs, whereas 98% of patients with MGUS had more than 3%. Moreover, as shown by multivariate analysis, the number of residual polyclonal PCs was the most powerful single parameter for the discrimination between MGUS and MM patients at diagnosis, even when only stage I MM cases were considered. *(Am J Pathol 1998, 152:1655–1665)*

Although multiple myeloma (MM) represents the prototype of monoclonal gammopathy, the most common plasma cell (PC) disorder is the monoclonal gammoph-

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any of undetermined significance (MGUS). The incidence of MGUS is around 3% in persons older than 70 years.[1–3] The term MGUS indicates the presence of a small M protein (less than 3 g/dl) with no evidence of MM, primary amyloidosis, Waldeström's macroglobulinemia, or other related disorders.[3–6] The differential diagnosis between MGUS and MM is still a challenge in some cases, as there is no single parameter that allows such a distinction between both entities in all cases. As a consequence, at diagnosis, a combination of several clinical and biological criteria are used to differentiate between MGUS and MM, and in a relatively important proportion of cases the patient's follow-up will be the only discriminating factor. Moreover, neither of the two major MM diagnostic systems (Chronic Leukemia-Myeloma Task Force and Southwest Oncology Study Group) are completely satisfactory, as, according to patient follow-up data, a subset of individuals are not correctly categorized. This differentiation is particularly difficult in patients with borderline criteria between MGUS and clinically indolent or smoldering MM.[5]

Although immunophenotypic studies of PCs from MM patients have been performed for more than 10 years,[7–12] its value in the differential diagnosis between MGUS and MM has not been explored. Moreover, the question of whether or not PCs are phenotypically different in the two disorders remains unresolved. This is probably because information on the immunophenotype of PCs in MGUS patients is scanty, and most immunophenotypic studies in MGUS have been usually based on single antigen staining in which PCs were not specifically identified and they were analyzed with low-sensitivity techniques, such as alkaline phosphatase anti-alkaline phosphatase (APAAP) and fluorescence microscopy. In addition, in these studies the total number of PCs evaluated was relatively small, which would not allow the appropriate assessment of weakly expressed antigens or antigens that are present in only a small proportion of total PCs. All of these items may help to explain, at least in part, some of the overt controversies regarding the immunophenotype of PCs in MGUS found in the literature.[12–17] For these reasons, highly sensitive methods in which evaluation of a high number of PCs is performed must be used to specifically determine the immunological characteristics of PCs from MGUS patients. In the present study, we have evaluated the immunophenotypic profile of PCs from MGUS patients by flow cytometry with a large panel of monoclonal antibodies (MAbs), using an approach that allows the analysis of a great number of PCs per test ($>10^3$ PCs per sample). The immunophenotypic profile was compared with that of PCs from both MM patients and normal bone marrow (BM). Our aim was to explore the possible utility of the immunophenotypic analysis of PCs for the differential diagnosis between MGUS and MM. Our results show the presence of two distinct PC subpopulations in MGUS patients, displaying one identical phenotype to that of normal BMPCs and the other being identical to that of PCs from MM patients, whereas in MM almost all PCs display an aberrant phenotype with respect to that of normal PCs. In addition, the number of residual normal BMPCs, identified by immunophenotyping, is the most powerful single criterion for the differential diagnosis between MGUS and MM.

Materials and Methods

Patients and Controls

A total of 141 patients with monoclonal gammopathies (76 MGUS and 65 MM) and 10 healthy subjects were included in the study. The following diagnostic criteria for MGUS were used: presence of a serum M-component lower than 3 g/dl, or a small amount of urine light chain protein excretion; ≤10% BMPCs, in the absence of clinical symptoms; lytic bone lesions; anemia; hypercalcemia; and renal function impairment. Patients with MM were diagnosed according to the criteria of the Chronic Leukemia-Myeloma Task Force (1973).[18] The mean age of MGUS patients (41 males and 35 females) was 66 ± 11 years (range, 23 to 84 years; median, 68 years). The isotype of the monoclonal component in the MGUS group was as follows: IgG, 68%; IgA, 28%; Bence-Jones, 1.3%; IgM, 1.3%; and biclonal, IgG and IgA, 1.3%. The monoclonal Ig light chain was kappa in 56% of the cases and lambda in the remaining 44%. The reference group of MM patients included 65 cases (39 males and 26 females), with a mean age of 61 ± 12 years (range, 40 to 89 years). The M-component observed in MM patients was IgG in 44%, IgA in 38%, light chains only in 12%, IgD in 2%, and biclonal (IgG and IgA) in 4% of the cases, with the light chain kappa in 58% and lambda in 42% of the cases. According to the Durie and Salmon's clinical staging system (1975), MM patients were distributed as follows: stage IA, 12%; IIA, 34%; IIB, 2%; IIIA, 38%; and IIIB, 14%. The 10 healthy subjects (5 males and 5 females) used as normal controls underwent orthopedic surgery and did not have any systemic disorders; their mean age was 48 ± 25 years (range, 20 to 88 years). In all cases, BM samples were obtained after informed consent according to the University Hospital Ethical Committee.

Regarding the follow-up of the MGUS patients, 7 cases had been diagnosed 5 years or more before the present investigation was performed, 20 had a follow-up ranging from 1 to 5 years, and the remaining 49 cases were studied at diagnosis or within the first year after diagnosis.

Bone Marrow Samples and Immunophenotypic Studies

BM samples obtained by aspiration were collected in EDTA anticoagulant and immediately diluted 1/1 (v/v) in phosphate-buffered saline (PBS). Whole BM samples (approximately 2×10^6 cells in 100 μl/test) were stained using direct immunofluorescence and simultaneous triple labeling with the following MAbs: CD38 (Caltag Laboratories, San Francisco, CA); CD9, CD10, CD13, CD19, CD20, CD22, CD23, CD28, CD33, CD34, CD45, CD56, and CD80 (Becton Dickinson, San Jose, CA); CD40, CD49e, and CD138 (Serotec, Oxford, UK); FMC7 and CD117 (IMICO, Madrid, Spain); and sIg (kappa and lambda) antigens and HLA-DR (Becton Dickinson).

MAbs were conjugated with one of the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), and the PE/cyanin 5 (PE/Cy5) fluorochrome tandem. Briefly, BM samples were incubated for 15 minutes at room temperature in the dark in the presence of 5 to 20 μl of each of the above mentioned MAbs, according to the recommendations of the manufacturers. Afterwards, 2 ml of FACS lysing solution (Becton Dickinson) diluted 1/10 (v/v) in distilled water were added, and the samples were incubated for another 10 minutes under the same conditions mentioned above, to lyse the nonnucleated red cells. Then, cells were centrifuged (5 minutes at 540 × *g*), and the cell pellet was washed with 4 ml of PBS. Finally, cells were resuspended in 0.5 ml of PBS until analyzed in the flow cytometer. As PCs usually display a higher level of autofluorescence with respect to other nucleated BM cells, in all cases an FL1/FL2 isotype-matched negative control (Becton Dickinson) plus CD38-PE/Cy5 was used, to specifically evaluate the level of PC autofluorescence. Data acquisition was performed on a FACSort flow cytometer (Becton Dickinson) equipped with an argon ion laser tuned at 488 nm and 15 mW. To increase the sensitivity and accuracy of the analysis, in all MGUS and control subjects as well as in those MM patients displaying a low proportion of bone marrow PCs (<15%), acquisition was performed in two consecutive steps. First, a total of 15,000 events/tube were acquired; in the second step, acquisition through a "live-gate" drawn on SSC/CD38 strongly positive cells (where PCs are located) was performed. In this latter step, a minimum of 300,000 events were measured. For data analysis, the Paint-A-Gate PRO software (Becton Dickinson) was used. PCs were identified according to their positivity for CD138, their strong reactivity for CD38, and their typical light-scatter distribution.

The quantitation of the mean fluorescence intensity for FITC- and PE-conjugated MAbs was performed using Quickcal beads and Quickcal software (Flow Cytometry Standards Corp., San Juan, PR), and results were expressed as the mean number of molecules equivalent of soluble fluorochrome (MESF) specifically obtained for PCs upon subtracting the mean PC fluorescence intensity obtained for the isotype-matched negative control.

Cytoplasmic Ig Light Chains

For the analysis of light chain restriction, a double-staining technique for the membrane antigen CD38 and cytoplasmic Ig light chains was performed on BM samples. For that purpose, the Fix & Perm reagent (Caltag Laboratories) was used following the manufacturer's recommendations. Briefly, the samples were incubated for 15 minutes in the dark at room temperature with 100 μl of solution A from the Fix & Perm kit and incubated for another 15 minutes with 100 μl of solution B and both anti-kappa and anti-lambda Ig light chain MAbs (Becton Dickinson). Once this incubation was finished, cells were washed once with 5 ml of PBS, and after removing the supernatant, they were resuspended in 0.5 ml of PBS until analyzed in a FACSort flow cytometer. In all cases, an FL1/FL2 isotype-matched negative control (Becton Dickinson) was used as previously described. Data acquisition was performed in two steps, as described above.

DNA Studies

Simultaneous staining for BMPCs and DNA cell content was performed according to previously described techniques[19] using the Cycloscope reagent (Cytognos, Salamanca, Spain). In all cases, measurements were made within 1 hour after the sample preparation was finished using a FACSort flow cytometer (Becton Dickinson). Data acquisition was performed in two consecutive steps; in the first step, a total of 10,000 events from the global cellularity were acquired, and in a second step, cells were acquired through a live-gate drawn on PCs. In this step, PCs from a total of at least 50,000 events were acquired. For data analysis, the ModFit software program (Verity House, Topsham, ME) was used. Adjustment of the electronics of the instrument and calculation of the DNA index of PCs were performed according to previously reported methods.[19]

Fluorescence-Activated Cell Sorting of BMPCs

Cell-sorting experiments were performed on a FACStar flow cytometer (Becton Dickinson). PCs from MGUS patients were sorted into PBS containing 1% bovine albumin based on the expression of CD38, CD19, and CD56. Two PC subpopulations were sorted according to their different reactivity for these antigens: 1) $CD38^+CD56^+CD19^-$ and 2) $CD38^+CD56^-CD19^+$.

Rapid Extraction of DNA and Detection of Clonal Populations by the PCR Technique

FACSorted PCs were centrifuged for 5 minutes, resuspended in 50 μl of K buffer (100 μg/ml proteinase K in 15 mmol/L Tris HCl, pH 8.3, 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, and 0.5% Tween 20), and sequentially incubated at 56°C for 45 minutes and at 95°C for 10 minutes.[20] Five microliters of the preparation was used for amplification of IgH V-D-J_H junctional regions. Amplifications were performed by single-round polymerase chain reaction (PCR) in an automated thermocycler (model 9600, Perkin Elmer, Cetus, Emeryville, CA) using a 5′ V_H consensus primer that hybridizes at the FR3 region (5′-CTGTCGACACGGCCGTGTATTACTG) and a 3′ J_H consensus primer (5′-AACTGCAGAGGAGACGGTGACC).[21] Samples containing 5 μl of the extracted DNA, 200 nmol/L oligonucleotide primers, 200 μmol/L each deoxynucleotide triphosphate, 2 mmol/L MgCl, and 1.5 U of *Taq* DNA polymerase (Promega, Madison, WI) in a final volume of 50 μl of PCR buffer (50 mmol/L Kcl, 10 mmol/L TrisHCl, 1% Triton) were amplified for 35 cycles (cycle conditions: denaturation step at 94°C for 45 seconds, annealing step at 55° for 45 seconds, and extension step at 72°C for 45 seconds). The first denaturing step and the final extension

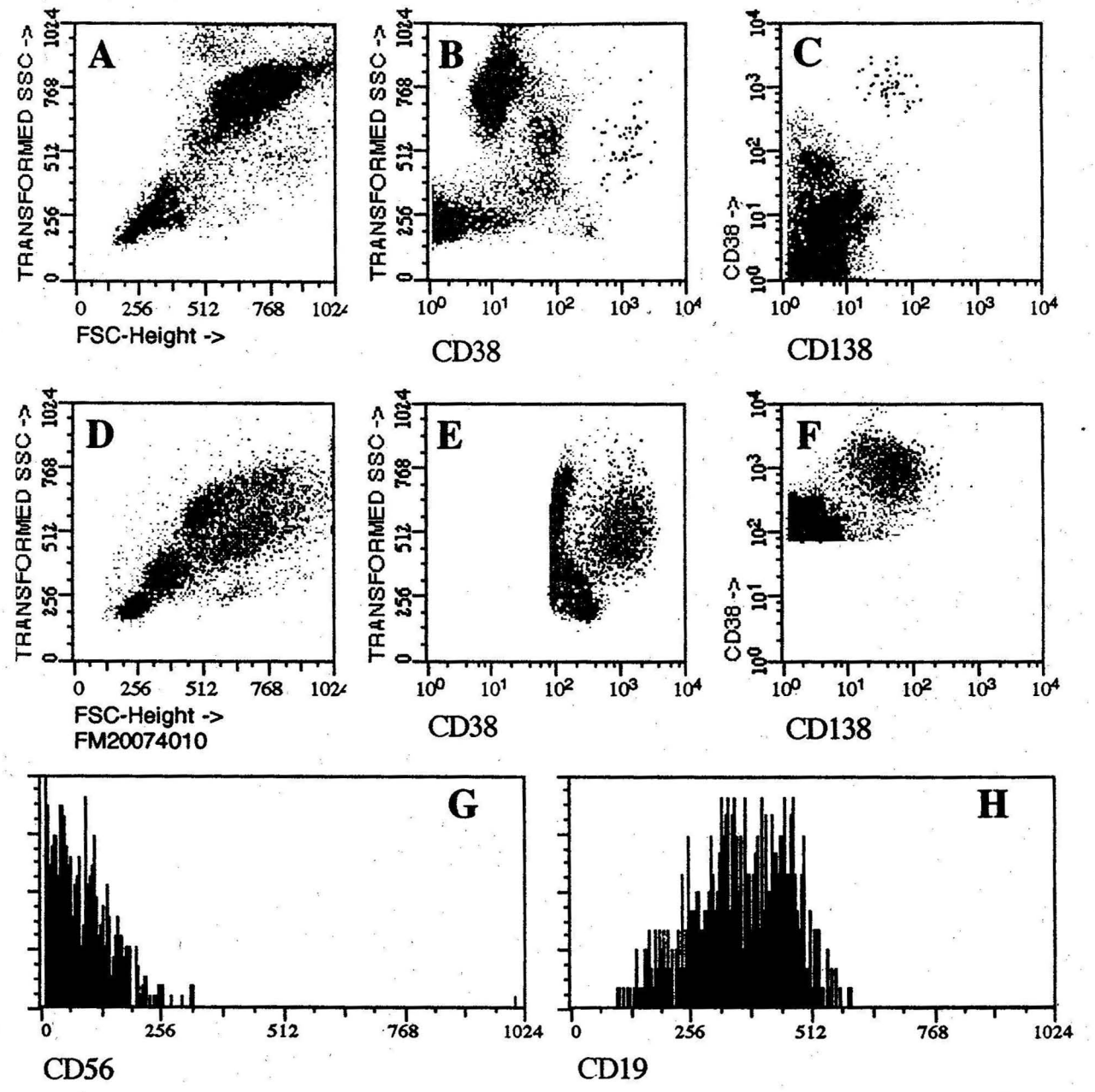

Figure 1. Identification of plasma cells in normal bone marrow. Identification of BMPCs in normal BM on the basis of CD38, CD138, and light scatter characteristics in the whole sample (dot plots A, B, and C) and in BMPC gated files (dot plots D, E, and F). Dot plots G and H display two representative single-parameter histograms of CD56 and CD19 expression in normal BMPCs, respectively.

step were extended to 5 and 7 minutes, respectively. To confirm the monoclonality or polyclonality of the V-D-J_H amplified products, heteroduplex formation and high-resolution polyacrylamide electrophoresis were carried out according to the method described by Bottaro et al.[22] To facilitate heteroduplex formation, 15 μl of each amplified product was heated at 95°C for 5 minutes and incubated at 40°C for 1 hour. Samples were run overnight on a 6% nondenaturing polyacrylamide gel (29:1, acrylamide: bisacrylamide) in 0.5X Tris/borate/EDTA buffer (TBE) at 20 V (SE 600 series apparatus, Hoefer, San Francisco, CA). Heteroduplex/homoduplex patterns were shown by ethidium bromide staining.

Statistical Analysis

The different clinical and biological parameters of MGUS and MM patients were considered individually by univariate tests; differences in the means of continuous measurements were tested by two-tailed Student's *t*-test, and differences in qualitative variables between groups were tested by the χ^2 test. For the assessment of the discriminant power of the parameters used in the differential diagnosis between MGUS and MM, a multivariant analysis was performed, using a logistic regression model with the forward stepwise option and a probability comparison test (SPSS 5.0,

Chicago, IL). *P* values of less than 0.05 were considered to be statistically significant.

Results

Immunophenotypic Analysis of Bone Marrow Plasma Cells

Immunophenotype of Normal Bone Marrow Plasma Cells

BMPCs from all 10 healthy subjects were clearly identified by their expression of CD138, their strong reactivity for CD38, and their distribution according to light scatter characteristics (Figure 1). The mean percentage of total BMPCs identified by flow cytometry was 0.25 ± 0.09% (ranging from 0.14% to 0.43%; median of 0.23%). In all cases, PCs displayed a very strong positivity for CD38 using either a FITC- or a PE/Cy5-conjugated MAb (mean fluorescence intensity for FITC of 1,299,650 ± 396,251 MESF). CD19 was expressed in the majority of BMPCs (76 ± 9%; range, 66 to 92%) from all individuals. In contrast, most BMPCs were negative for CD56; in fact, only a minor proportion of PCs (10.8 ± 3.7%; range, 6.2 to 16; median, 9.4) expressed this antigen, and the mean fluorescence intensity of CD56 expression in these PCs was very low (mean value of 25,274 ± 21,644 MESF for PE; Figure 1).

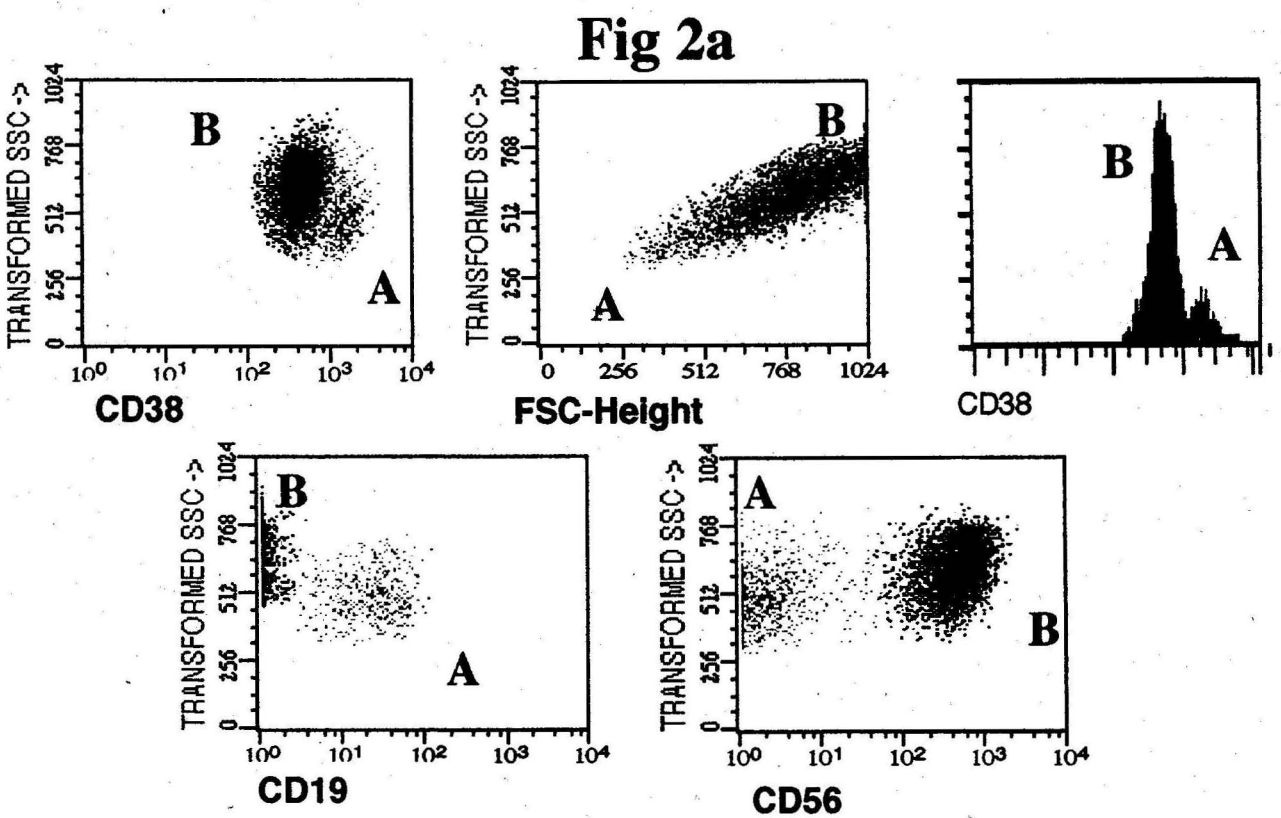

Figure 2. Phenotypic characteristics of the subpopulations of plasma cells in MGUS. Immunophenotypic identification of BMPC populations A (painted gray) and B (painted black) in an MGUS patient. Differences between both PC subpopulations with respect to CD38, CD56, CD19 (a) and CD28, CD117, and sIg (b) are shown.

Table 1. Intensity of CD38, CD19, and CD56 Antigen Expression in Both BMPC Subpopulations from MGUS Patients and PCs from MM Patients

Antigen	MGUS		MM, Myelomatous PCs
	Population A	Population B	
CD38-FITC	1,299,650 ± 396,251*	694.067 ± 367,668*	467,801 ± 158,652
CD19-PE	50,985 ± 35,224†	2002 ± 2906†	3457 ± 5222
CD56-PE	14,316 ± 16,243‡	55.792 ± 73,808‡	67,679 ± 62,478

Results are expressed as mean ± SD in MESF units. There were no significant differences between clonal BMPCs and myelomatous PCs. For the CD56-PE antigen, for polyclonal BMPCs, MESF values refer exclusively to $CD56^+$ cells.
*$P = 0.001$.
†$P = 0.007$.
‡$P = 0.008$.

Regarding the other markers analyzed, all normal BMPCs were consistently positive for the CD9, CD40, and CD45 antigens; HLA-DR was present in 90 ± 10% of the PCs in all cases. A variable expression of myeloid antigens was also observed in normal PCs: CD13 (dim expression was found for all PCs in 60% of the cases) and CD33 (dim expression was observed for all PCs in 20% of the cases). In one case, 42% of the PCs displayed a weak reactivity for the CD28 antigen, although this antigen was negative in all other cases. PCs from normal individuals were consistently negative for the rest of the antigens analyzed (CD10, CD20, CD22, CD23, CD34, CD49e, CD80, CD117, FMC7, and sIg).

Immunophenotype of MM Bone Marrow Plasma Cells

CD38 was positive in all MM patients whereas CD19 was negative in 97% of the patients and CD56 was strongly positive in 67% of cases. Other antigens frequently present in myelomatous PCs were CD138, CD40 (both in 100% of MM cases), CD9 (70% of MM cases), CD45 (44%), and HLA-DR (40%). The CD117 and CD28 antigens were positive in 27% and 41% of the cases, respectively. The CD49e antigen was positive in 36% of MM BM samples. By contrast, the incidence of the reactivity for the CD19, CD20, and CD22 pan-B antigens as well as for CD10 and CD23 was low (3%, 23%, 4%, 7%, and 20%, respectively). Approximately one-fourth of the patients were positive for the myeloid-associated antigens CD13 and CD33 (28% and 24% of the cases, respectively). Interestingly, the expression of sIg was observed in approximately one-third of MM patients. Finally, CD34, CD80, and FMC7 were found to be consistently negative.

Immunophenotype of MGUS Bone Marrow Plasma Cells

The mean percentage of BMPCs identified by flow cytometry was 1.76 ± 1.2% (range, 0.23% to 6%). Interestingly, in all MGUS cases studied, two clearly defined and distinct PC subpopulations could be identified according to the fluorescence intensity of the CD38 antigen and their FSC/SSC distribution pattern (Figure 2a). Accordingly, one PC subpopulation (population A), representing 32.9 ± 31.4% (range, 1.7 to 98%) of total PCs, consistently displayed a higher CD38 expression with lower FSC/SSC values than the other (population B), which was predominant in most cases (mean of 67.1 ± 31.3% of total PCs; range, 2% to 98.3%). Moreover, population A was positive for CD19 in almost all PCs (86.9 ± 16.2% of PCs; range, 54% to 100%; median, 100%) from all individuals analyzed, whereas only a small proportion of these PCs (11.7 ± 7.4% PCs; range, 2% to 35%) showed weak CD56 expression. The opposite pattern was observed in population B, in which CD56 was strongly positive in almost all PCs in 70% of the MGUS cases studied (Figure 2a; Table 1), whereas CD19 was consistently negative. No differences between the two PC subpopulations were observed regarding CD138 expression.

The CD28 antigen was consistently negative in population A, whereas in 47% of the MGUS patients, PCs from population B showed reactivity for this antigen. Moreover, in the positive cases, the intensity of expression for CD28 was relatively high (mean of 30,146 ± 48,032 MESF;

Table 2. Immunophenotypic Characteristics of Both Plasma Cell Subpopulations (Population A and Population B) from MGUS Patients

Antigen	MGUS Population A PCs	MGUS Population B PCs	*P* value
CD9	100	100	NS
CD10	0	14	<0.05
CD13	17	22	NS
CD19	100	6	<0.001
CD20	4	24	<0.01
CD22	11	10	NS
CD23	0	14	<0.05
CD28	0	47	<0.001
CD33	3	6	NS
CD34	0	0	NS
CD38	100	100	NS
CD40	100	100	NS
CD45	72	45	0.05
CD49e	29	24	NS
CD56*	0	69	<0.001
CD80	0	3	NS
CD117	0	23	<0.01
CD138	100	100	NS
FMC7	0	0	NS
HLA-DR	80	83	NS
sIg	0	28	<0.01

Results are expressed as percentage of cases with more than 15% PCs positive for the antigen tested. NS, statistically nonsignificant.
*Percentage of cases displaying strong reactivity for the CD56 antigen.

Table 3. Immunophenotypic Features of Both PC Subpopulations from MGUS Patients and Control Subjects and Clonal PCs from MGUS and MM Patients

Antigen	MGUS population A	Control BMPCs	MGUS population B	MM BMPCs
CD38*	100	100	100	100
CD19	100	100	6	3
CD56†	0	0	69	67
CD28	0	0	47	41
CD117	0	0	23	27
sIg	0	0	28	31

Results expressed are as percentage of cases displaying more than 15% positive PCs. Differences between MGUS population A PCs normal BMPCs and MGUS population B PCs/MM PCs were not statistically significant.

*Although CD38 was positive in both MGUS PC subpopulations from MGUS patients, population B displayed a lower intensity of CD38 expression.

†Strong reactivity for the CD56 antigen.

range, 1,563 to 217,895 MESF for PE). In a similar way, CD117 was expressed by PCs from population B (23% of cases) but not by those of population A. The intensity of CD117 expression in population B varied between cases displaying a weak reactivity to others for which CD117 was strongly positive (mean of 15,157 ± 15,058 MESF; range, 2,141 to 35,618 MESF). Regarding sIg, a weak reactivity was found in 30% of the cases (12% for kappa light chains and 18% for lambda light chains) in PCs from population B, whereas PCs from population A were consistently negative. Representative examples of the reactivity found for CD28, CD117, and sIg are shown in Figure 2b. The remaining antigens explored (CD9, CD10, CD20, CD22, CD23, CD34, CD40, CD45, CD49e, CD80, CD138, FMC7, and HLA-DR) displayed a very similar pattern of expression in both PC subpopulations (Table 2). Special attention was paid to the expression of the myeloid-associated antigens CD13 and CD33; they were present in only a minority of MGUS patients, but no significant differences were found regarding their expression in the two PC populations (Table 2).

Upon comparing the immunophenotypic characteristics of PCs from MGUS patients with those of PCs from both healthy individuals and MM patients (Table 3), we found that the antigenic profile of population A from MGUS patients was identical to that observed in PCs from healthy controls whereas population B from MGUS patients displayed a similar phenotype to that of PCs from MM patients (Table 3).

Clonality Studies

Assessment of Cytoplasmic Ig Light Chains

Staining for cytoplasmic Ig light chains showed that, both in MGUS patients and in MM cases, population B constantly displayed light chain restriction, whereas population A was polyclonal with a mean kappa/lambda ratio of 1.9 ± 0.46 (Figure 3a).

Flow Cytometric DNA Studies

Upon analyzing the DNA content of BMPCs from MGUS patients, two clearly different cells subsets could also be discriminated in 73% of the cases. Thus, population A showed a normal DNA content, whereas population B displayed DNA aneuploidy (mean DNA index of 1.14 ± 0.07; range, 1.05 to 1.33; Figure 3b). In contrast, based on the DNA content of BMPCs, only one population could be clearly observed in all MM cases, this being aneuploid in 62% of the cases (mean DNA index of 1.2 ± 0.09; range, 1.05 to 1.44). Although in some of these cases a second PC population seemed to be present, it was in such a low proportion that it did not permit an accurate analysis of its DNA content.

PCR Studies

To confirm the differences in clonality between the two PC populations found in MGUS patients, PCs from populations A and B present in MGUS patients were individually sorted according to their differential antigenic expression. PCR analysis was then performed on the sorted cell fractions. As expected, PCR studies confirmed the existence of clonality in population B, with the presence of a homoduplex corresponding to the V-D-J_H region containing the CDR3 segment, but not in population A, where heteroduplex formation produced a typical polyclonal pattern (smear; Figure 4).

Differential Diagnosis between MM and MGUS

In contrast to what was found in MGUS, in MM patients, the presence of population A was a rare event, being clearly identified in only 22% of cases. Moreover, the number of PCs within this cell population (0.25 ± 0.76%; range, 0% to 5%) in MM cases was significantly lower than that found in MGUS patients ($P < 0.0001$). Accordingly, MGUS and MM patients displayed statistically significant differences in the proportion of the two PC populations identified (A and B). Focusing on the relative number of residual immunophenotypically polyclonal PCs (population A), the percentage of immunophenotypically normal BMPCs (INPCs), using a cut-off value of 3%, we found that, whereas 98% of MGUS cases displayed values above this threshold, only 1.5% of MM patients showed a percentage of INPCs higher than 3%. Other relevant clinical and biological disease characteristics for differential diagnosis between MM and MGUS patients were age, the overall percentage of BMPCs as assessed by both microscopy and flow cytometry, the presence of osteolytic lesions, the amount of serum M-component, and hemoglobin, serum calcium, and β2-microglobulin levels (Table 4). On the other hand, the labeling index as assessed by flow cytometry, LDH, and C-reactive protein did not show significant differences. When a multivariate analysis was carried out, only the percentage of INPCs and the presence of osteolytic lesions retained their independent value. Upon comparing the impact of this new criterion with the classical criteria used in the differential

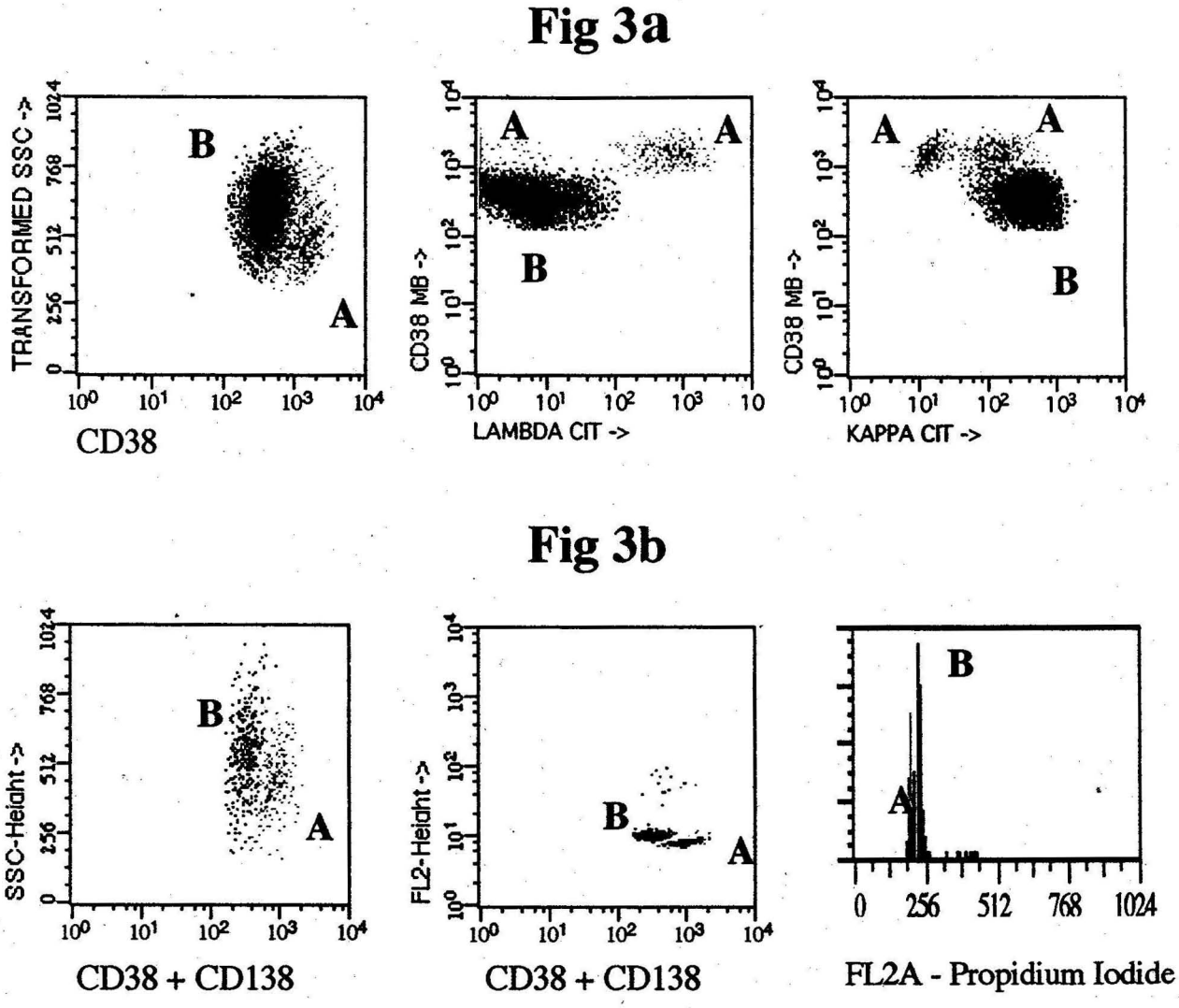

Figure 3. Clonality Studies of the Subpopulations of BMPCs Detected in an MGUS case. a: Light chain restriction cIg-kappa$^+$ in population B from an MGUS patient (painted black); population A shows both cIg-kappa$^+$ and cIg-lambda$^+$ cells. b: Two clearly different BMPC populations were identified by the analysis of DNA cell content at flow cytometry, one corresponding to normal diploid BMPCs (population A, painted gray) and the other to DNA aneuploid BMPCs (population B, painted black).

diagnosis between MM and MGUS we found that the percentage of INPCs with a 3% cut-off value was the most powerful single criterion, as overall it failed to correctly classify only 2% of the patients, which is significantly better than conventional criteria. Moreover, upon analyzing even the 3 of the 141 patients who were incorrectly classified according to the percentage of INPCs, it was observed that two of them had values within the limit of currently used diagnostic criteria, corresponding to borderline cases. Finally, when only stage IA MM patients were considered, the percentage of INPCs led to the correct classification of all of these patients (Table 5).

Discussion

In contrast to MM, information on the immunophenotypic features of PCs from MGUS patients is still scanty and most currently available data are based on single-antigen staining in which PCs were not specifically identified; they were analyzed with low-sensitivity methods, and a relatively low number of PCs was usually evaluated. Moreover, results on the immunophenotypic features of PCs from MGUS patients[13,14,23–27] are frequently controversial. Accordingly, the reported incidence of CD19 expression ranges between 77% and 100%,[14,26] and reactivity for CD56 ranges from 0% to 62%.[15,16,28,29] In the present paper, we report the existence of two different PC subpopulations in the BM from all MGUS patients analyzed. This is concordant with the observation by Harada et al[26] on the coexistence of both CD56$^+$CD19$^-$ and CD56$^-$CD19$^+$ PCs in MGUS patients. The distinction between these two PC populations was possible due to the highly sensitive method used for the analysis of BMPCs, based on a two-step acquisition procedure. In the second step, a "live gate" was performed that allowed storage of information on a high number of BMPCs even when they were present at very low frequencies. One of these PC subpopulations (population A) showed pheno-

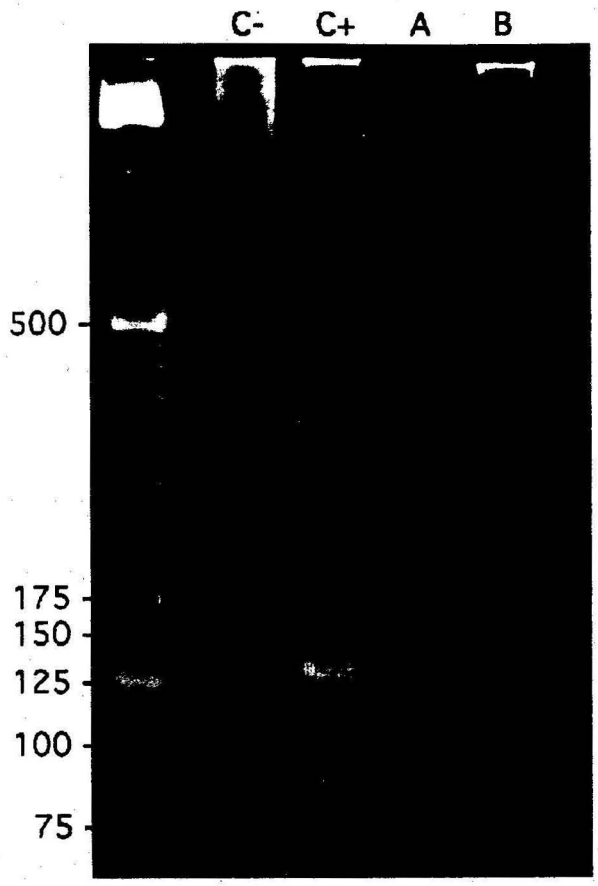

Figure 4. PCR analysis of FACSorted BMPCs in MGUS. PAGE analysis of the PCR products from V-D-J_H after heteroduplex formation. C−, negative control; C+, positive control. A: Amplified products obtained using the DNA from $CD38^+/CD56^-/CD19^+$ sorted BMPCs. B: Amplified products obtained using the DNA from $CD38^+/CD56^+/CD19^-$ sorted BMPCs. A typical polyclonal pattern (smear) is documented in the negative control and population A, whereas a homoduplex was obtained in the positive control and population B.

typic characteristics identical to those of normal PCs, and they would correspond to residual immunophenotypically normal PCs (INPCs). Accordingly, they displayed very strong reactivity for the CD38 antigen, low FSC/SSC, and positivity for CD19, although they were generally negative for the CD56 antigen. Actually, only a minor fraction of this PC population displayed weak reactivity for CD56, but the low intensity of expression observed was completely different from that detected in the other PC subpopulation (Table 1). The polyclonal origin of these PCs is further supported by the observation that in all cases explored this subpopulation had a normal cell DNA content by flow cytometry, displayed polyclonal clg. and had a polyclonal IgH rearrangement by PCR. In contrast, the second, and predominant, PC subpopulation (population B) showed a completely different immunophenotype, which was typical of myelomatous PCs, displaying a slightly lower reactivity for CD38 and strong CD56 expression and an absence of positivity for CD19. It should be noted that, although circulating $CD19^+$ clonotypic B lymphocytes can be detected in MM,[30] PCs are usually $CD19^-$. In addition, these PCs showed a higher FSC/SSC. This subpopulation would represent the clonal PC counterpart, which is supported by their abnormal DNA content, the light chain restriction, and the presence of clonal V-D-J_H segments. The clonal PC population is responsible for the monoclonal component present in serum and/or urine, whereas the persistence of normal PCs in the BM of MGUS would explain the maintenance of normal levels of polyclonal immunoglobulins. In our opinion, the variation in the relative distribution of normal and clonal PC populations within MGUS patients may help to explain the reported discrepancies about the immunophenotype of PCs from MGUS patients.

Table 5. Stage IA MM Patients: Individual Distribution According to Conventional Diagnostic Criteria

Patient	% BMPCs by morphology	Amount/type of MC	% INPCs
1	27	2700/IgA	0
2	21	1350/IgG	0
3	25	3680/IgG	0
4	15	3100/IgA	0
5	11	3710/IgA	1.7
6	12	4500/IgA	0

% INPCs, percentage of immunophenotypically normal BMPCs; MC, monoclonal component.

Table 4. Clinical and Biological Features in MGUS and MM Patients

Parameter	MM	MGUS	*P* value
Age (years)	61 ± 12	66 ± 11	0.04
Osteolytic bone lesions	66%	0%	0.0001
Hemoglobin (g/dl)	10.5 ± 3.0	13.7 ± 1.6	0.0001
Total protein (g/L)	9.1 ± 2.1	7.5 ± 0.	0.0001
Amount of MC (g/dl)	4658 ± 2526	1541 ± 550	0.0001
Ca^{2+} (mg/dl)	9.7 ± 1.3	9.5 ± 0.6	NS
β2-microglobulin (mg/L)	4.8 ± 5.3	2.3 ± 1.0	0.005
LDH (IU/L)	311 ± 103	342 ± 88	NS
CRP (mg/dl)	3.1 ± 3.6	2.1 ± 4.0	NS
% BMPCs by morphology	43.3 ± 26.1	5.4 ± 4.2	0.0001
% BMPCs by immunophenotype	14.5 ± 14.1	1.8 ± 1.2	0.0001
% S-phase BMPCs	1.8 ± 2.2	1.4 ± 2.0	NS
% INPCs	0.25 ± 0.76	32.9 ± 31.4	0.0001
% Cases with >3% INPCs	1.5	98	0.0001

% INPCs, percentage of immunophenotypically normal BMPCs; CRP, C-reactive protein; LDH, lactic dehydrogenase; MC, monoclonal component; NS, statistically nonsignificant.