

Multiparameter Immunophenotyping by Flow Cytometry in Multiple Myeloma: The Diagnostic Utility of Defining Ranges of Normal Antigenic Expression in Comparison to Histology

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Background: Numerous studies have reported on the immunophenotype of plasma cells (PCs) in monoclonal gammopathy of undetermined significance (MGUS) and in plasma cell myeloma (PCM), but very few have examined the immunophenotype of normal PCs. In this study, an objective definition of normal range of expression for each antigen was found on normal control PCs. Using these new ranges of normal expression (new method) is different from using a static 20% of PCs cut-off for all antigens as described in the literature (traditional method). These newly calculated normal ranges for each antigen were applied to our data, and compared to histologic and immunohistochemical findings.

Methods: Bone marrow samples from 46 patients with PC neoplasms and 15 normal controls were studied. A minimum of 100 PC were analyzed for each patient and control sample. An 8-color staining method was applied to study the immunophenotype of PCs, using a BD FACSCanto II.

Results: By the new ranges of normality calculated in this study it was determined that different antigens have different level of expression on polyclonal PCs. CD19 correlated with histology by both the traditional and new methods, but had superior correlation by the new method.

Conclusions: This report is the first 8-color immunophenotypic study of PCM in which a “range of normal expression” for each antigen is defined. This is a critical step to help distinguish between a normal and neoplastic PC immunophenotype and discern which antigens are of diagnostic importance. © 2010 Clinical Cytometry Society

Key terms: multiple myeloma; flow cytometry; ranges of normal antigenic expression; multiparameter flow cytometry; polyclonal plasma cells' immunophenotype

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Plasma cell myeloma (PCM) is a clonal B-cell neoplasm in which malignant plasma cells (PCs) accumulate in the bone marrow and produce excessive amounts of monoclonal protein that accumulates in the serum and/or urine, ultimately leading to end-organ damage (hypercalcemia, renal insufficiency, anemia and/or lytic bone lesions). Evaluation of disease in PCM is based on a vari-

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ety of laboratory techniques, including bone marrow morphology and immunophenotyping, analysis of serum or urine M-component and free light chains, hematological and biochemical parameters, cytogenetics, DNA ploidy, and labeling index/proliferative activity of PCs. These laboratory investigations have several goals: to support the diagnosis of a plasma cell neoplasm, help guide therapy, provide prognostic information, and monitor treatment efficacy (1).

Immunophenotypic studies of PCM have been performed for more than 15 years. Flow cytometry (FC) is a potentially useful diagnostic tool for clinical practice. Advantages of FC include its ability to distinguish between normal, reactive, and malignant PCs (2-9), to evaluate the risk of progression from MGUS to PCM (10-12), to provide prognostic information (5,13-21), to evaluate for the presence of minimal residual disease (MRD) (2,19-22), and to identify new therapeutic targets (5,16,23-37). However the literature contains discrepancies in results regarding the exact immunophenotype of normal and neoplastic PCs and their associated clinical significance. These controversies could be due to methodological problems, such as different gating strategies, the use of different monoclonal antibody clones, different fluorochromes, and the use of single vs multiparametric antigen staining procedures. It is also important to appreciate that FC can be an imperfect technique to obtain quantitative information in evaluating PCs in bone marrow because of the frequent physical loss of these cells and/or hemodilution with peripheral blood.

To study PCM using FC it is necessary to distinguish between the normal and neoplastic PCs' immunophenotype. Numerous studies have reported on the immunophenotype of PCs in MGUS and in MM (2,10-12) and have examined the immunophenotype of polyclonal PCs (7-9,15). In most existing reports, 20% of cells expressing a marker of interest is considered the threshold value to define an antigen as "positive" or "negative" and discriminate if the PCs are normal or neoplastic (13,14,38-40). By looking for the optimal distribution curve and the 95% confidence intervals for the mean, we developed an objective definition of the range of expression for various antigens on normal PCs.

After establishing these new ranges of normal expression, they were compared with the previous threshold of 20% to determine which method showed better correlation with histologic and immunohistochemical findings in a series of patients with plasma cell neoplasms.

PATIENTS AND METHODS

Patients and Controls

Control specimens consisted of 15 bone marrow samples from patients seen by the orthopedic service of our hospital for elective hip replacement. These patients had no history of PCM, MGUS or other hematological diseases and the PCs identified in these samples were polyclonal and were CD19+CD56-. These control speci-

mens were used to define ranges of normal expression for each antigen.

Bone marrow samples of 55 patients with plasma cell neoplasms, or suspected to have, submitted to our laboratory for routine analysis from April to November 2008 were evaluated by FC, histology and immunohistochemistry. Excluded were nine patients with fewer than 100 PCs for flow cytometric analysis, which was deemed insufficient (2). Among the remaining 46 patients, nine had a new diagnosis of PCM, 16 were evaluated post-chemotherapy, 15 were status post autologous stem cell transplant, and six carried a diagnosis of MGUS. These patient specimens were used to evaluate the ranges of antigen expression found in this study with those described in literature. The study was approved by the Institutional Review Board of Partners Healthcare.

Flow Cytometry

Analysis was performed on bone marrow aspirates of patients with plasma cell neoplasms, or suspected to have, that were submitted for routine diagnostic analysis in our laboratory, and on hip marrow samples of controls. Specimens were run on a 3-laser FACSCanto (BD Biosciences, San Jose, CA) running DiVa 6.1.1 software; instrument calibration was performed and tested weekly as previously detailed (41). Whole bone marrow and hip marrow samples collected in EDTA anticoagulant were washed three times in phosphate-buffered saline (PBS) and resuspended with 1% bovine serum albumin in PBS. Totally, 100 μ l of washed cells were incubated with each of the following titrated monoclonal antibodies and corresponding volumes for 15 min at room temperature in the dark: 7 μ l CD138-PeCy7 (BD Biosciences), 5 μ l CD81-allophycocyanin (APC) (BD Biosciences), 3 μ l CD200-phycoerythrin (PE) (BD Pharmingen), 3 μ l CD221-PE (BD Pharmingen), 3 μ l CD45 Amcyan (BD Biosciences), 8 μ l CD38-fluorescein isothiocyanate (FITC) (BD Biosciences), 3 μ l CD28-PE (BD Biosciences), 8 μ l CD19-peridinin chlorophyll protein (PerCP) (BD Biosciences), 5 μ l CD27-APC, 5 μ l CD117-APC (BD Biosciences), 5 μ l CD38-APC (BD Biosciences), 7 μ l CD33-PacBlue (BD Biosciences), 7 μ l CD20-APC-Cy7 (BD Biosciences); 7 μ l CD56-PacBlue (BD Biosciences), 7 μ l CD10-PacBlue (BD Biosciences), 10 μ l of each immunoglobulin κ and λ light chains (DAKO, Carpinteria, CA) PE and FITC respectively. The antibodies were combined as tabulated in Table 1.

Erythrocytes were lysed by incubating with 2 mL of 1:9 diluted (10 ml of BD FACS lysing solution in 90 ml of H₂O). BD FACS lysing solution for 10 min. After incubation, cells were pelleted by centrifugation (416g for 5 min at room temperature), the supernatant was aspirated and the cells washed once in PBS. After the final wash step, cells were resuspended in 0.5 ml of 1% of paraformaldehyde in PBS.

To detect cytoplasmic κ and λ light chain expression, the Fix and Perm (Caltag, Burlingame CA) cytoplasmic staining kit was used as follows; after incubating specimen with anti-surface antibodies for 15 min, the sample

Table 1
Seven and Eight Color Monoclonal Antibody Staining Panels

AmCyan	FITC	PE	PerCP	APC	PeCy7	PacificBlue	APCCy7
CD45	CD38	CD221	CD19	CD27	CD138	CD56	
CD45	CD38	CD200	CD19	CD81	CD138	CD10	
CD45	CD38	CD28	CD19	CD117	CD138	CD33	
CD45	cyto λ	cyto κ	CD19	CD38	CD138	CD56	CD20

was washed with 1X PBS and added to 100 μ l of Fix and Perm reagent A solution. After 15 min of incubation the cells were washed again and added to 100 μ l of Fix and Perm reagent B solution, together with anti- κ and λ light chain antibodies and incubated for 15 min. The supernatant was discarded and cells resuspended into 0.5 ml of PBS. All reagents were kept cold and protected from light and exposure to the air; all stained samples were run promptly after processing to minimize any effect of degradation of tandem-dyes after fixation (42).

To obtain a minimum of 100 PCs in the analyzed region, the number of total bone marrow cells acquired for each sample tube ranged from 160,000 to 1,000,000 (2). In order to detect MRD at 0.01%, the minimum number of total events required is thus $\sim 10^6$. To optimally detect PCs, we analyzed a combination of CD138, CD38, and CD45 together with light scatter (Fig. 1), as previously described (2). Briefly, the initial analysis gate was devised using CD38 versus CD138 expression, the second one using CD38 brightly positive versus CD45 positive and negative cells and the last one using light scatter characteristics (high FSC and low SSC). These gates were intersected to obtain a defined PC population in multidimensional space. Comingling gated patient and control specimen lymphocytes served as an internal control for studying the associated PC population; thus the expression of antigens expressed on PCs was compared directly to that of gated lymphocytes in the patient and control specimen.

Histologic and Immunohistochemical Examination

Tissue examination of patient samples was performed to determine the presence of monoclonal or polyclonal PCs and disease status. Two-micron thick B+fixed, paraffin-embedded bone marrow core biopsy sections were prepared, deparaffinized and rehydrated according to standard laboratory protocols. A combination of ethylenediaminetetraacetic acid and boric acid in Tris buffer (CC1 reagent, Ventana) was used for antigen retrieval before primary antibody incubation. Immunohistochemistry for CD138 (Serotec, Raleigh, NC; 1/30 dilution) was performed using avidin-biotin peroxidase complex with a peroxidase-labeled detection system on a Benchmark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ) using validated staining protocols. In situ hybridization for kappa and lambda immunoglobulin light chains was performed using HX Automatic System Benchmark (Ventana) according to the manufacturer's instructions. Signals were detected

using the Alkaline Phosphatase Enhanced Detection Kit (Ventana).

Cases with plasma cell neoplasms were confirmed to have a clonal PC population as determined by one of the following: (1) >10% morphologically abnormal PCs on the bone marrow aspirate and fulfilling WHO criteria for a diagnosis of PCM or (2) A monotypic kappa or lambda positive PC population identified in the bone marrow biopsy sample by CD138 immunohistochemistry and in situ hybridization for kappa and lambda light chains.

Comparison Between Histologic Results and Flow Cytometric Analysis

After establishing the new normal ranges of antigen expression using control samples, these normal ranges were then applied to patient samples to define the normal or abnormal expression for each antigen. In most published reports, 20% of cells expressing an antigen of interest is considered the threshold value to define an antigen as "positive" or "negative" and discriminate if the PCs are normal or neoplastic, while a κ/λ ratio ranging between 0.5 and 3 defines polyclonal (normal) PCs (13,14,25,38-40). Therefore, this traditional method described by literature was also applied to our patient samples to define the normal or abnormal expression for each antigen. In this article, the term "new method" specifies the flow cytometric analysis using the calculated ranges of normal expression described herein; the term "traditional method" refers to the "20% threshold" method described by the literature. The detection of abnormal PCs by the new method vs. the traditional method was correlated with the presence or absence of neoplastic PCs in bone marrow samples evaluated by histology. Correlation with clinical diagnosis, stage of disease, and the presence or absence of minimal residual disease (MRD) were not the goal of this study and as such were not here explored.

Statistical Analysis

Mean, standard deviation, curve of optimal distribution, and 95% confidence intervals of the mean were calculated on control samples for each antigen studied. The normality of distributions was preliminary tested by means of Shapiro-Wilk test. The modified Z score test was used to identify outliers. The modified Z score was determined by the median of absolute deviation (MAD):

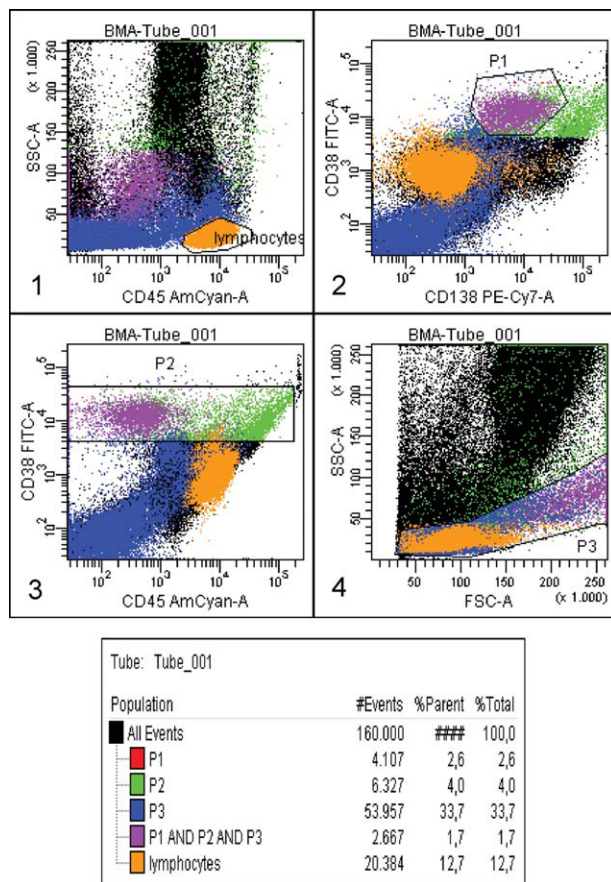


Fig. 1. Gating strategy. (1) Comingling gated patient and control specimen lymphocytes (orange) served as an internal control for studying the associated PC population (purple). (2) The initial analysis gate was devised using CD38 versus CD138 expression (P1); (3) the second gate used CD38 brightly positive versus CD45 positive and negative cells (P2); (4) the last gate used light scatter characteristics (high FSC and low SSC) (P3). These gates were intersected to obtain a defined PC population (purple) in multidimensional space.

$$\text{MAD} = \text{median}\{|x_i - \bar{x}|\}$$

An observation with a modified Z-score greater than three and a half was labeled as an outlier.

The evaluation of the concordance between the traditional method and histologic results, and the new method described in this article and the histological results, was performed by the Cohen's κ statistic. Analyses, except the Cohen's κ statistic, were performed with the software STATGRAPHICS XV (Statgraphics, Warrington, VA). The Cohen's κ statistic was performed with the software STATA (Stata, College Station, TX: StataCorp LP).

RESULTS

Determination of the Ranges of Normal Expression

Control samples PCs were showed to be normal by their relatively elevated expression of CD19 and polyclonal cytoplasmic light chain expression, defined as a κ/λ ratio ranging between 0.5 and 3 (43).

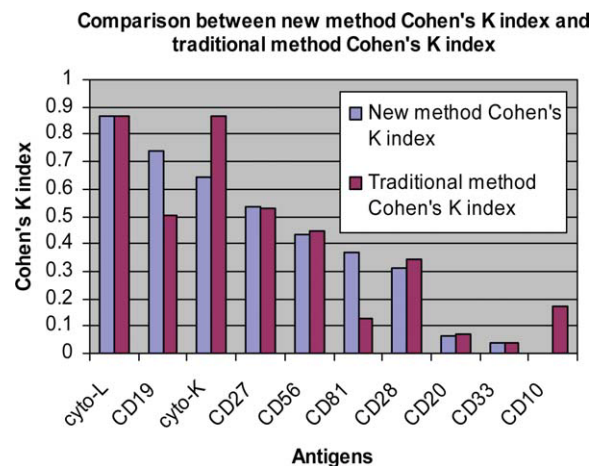


Fig. 2. Cohen's κ index calculated for each antigen comparing the new versus the traditional flow cytometric methods.

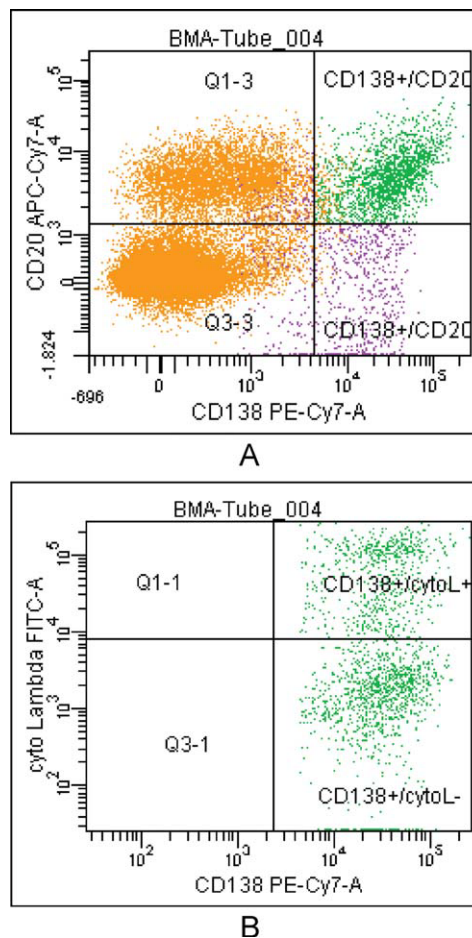


Fig. 3. **A:** PCs (violet and green) and lymphocytes (orange) distributions on CD138/CD20 dot-plots. A specimen's comingling lymphocyte population served as an internal control in assessing PCs relative expression of CD20. CD20+ PCs are green. **B:** CD20+ PCs are polyclonal on a dot-plot of CD138 versus cytoplasmic λ ; the κ/λ ratio was 1.5/1.

Table 2
Mean, Standard Deviation, Outliers, Curve of Optimal Distribution, and 95% Confidence Intervals for the Mean for Each Antigen Studied in Control Samples

Antigens	Unit measured	Min ^a	Max ^b	$\bar{x}$ ^d	σ ^e	CV% ^f	Distribution	P-Value ^g	Log likelihood ^h	n ⁱ	Outliers ^j
CD19	%	61	96	86.6	8.9	10.3	Beta (4-Parameter)	0.023	33.6	15	0
CD56	%	2	45	23.7	11.1	46.8	Gaussian	0.207	–	15	0
CD27	%	66	98	93	6.8	7.3	Beta (4-Parameter)	0.000	90.5	15	2
CD28	%	0	27	13.1	8.3	63.4	Gaussian	0.313	–	15	0
CD200	%	NE ^c	NE	NE	NE	NE	NE	NE	NE	15	NE
CD221	%	NE	NE	NE	NE	NE	NE	NE	NE	15	NE
CD117	%	NE	NE	NE	NE	NE	NE	NE	NE	15	NE
CD33	%	2	34	9.3	6.9	73.7	Gamma (3-Parameter)	0.013	30.9	11	1
CD10	%	0	55	24.5	18.6	75.7	Gaussian	0.051	–	15	0
CD20	%	17	95	56	19.8	35.4	Gaussian	0.751	–	15	0
CD81	%	87	100	97.4	3	3.05	Beta (4-Parameter)	0.011	98.4	15	1
cyto-K	%	28	68	48.1	10.4	21.6	Gaussian	0.518	–	15	0
cyto-L	%	16	63	39.6	11.8	29.8	Gaussian	0.980	–	15	0

^aMinimum value: probability of fitted distribution below this value is equal to 2.5%.

^bMaximum value: probability of fitted distribution above this value is equal to 2.5%.

^cNot evaluable - antigen not expressed on normal plasma cells.

^dAverage.

^eStandard deviation.

^fCV% = $\sigma/\bar{x}$.

^gTo accept the Gaussian curve the *P*-value must be >0.05% by Shapiro-Wilk W test.

^hIn the absence of a Gaussian distribution (for CD19, CD27, CD33 and CD81) a best fit distribution was determined based on the Log Likelihood statistic.

ⁱCount, number of observations.

^jNumber of outliers detected. Outliers were determined by the /MAD Z-score/ >3.5.

The results from 15 normal control samples are shown in Table 2. The mean age $\pm$ standard deviation in the control group was 72 ± 10.10 . The combination of CD45, CD38, and CD138 expression was used to identify the PCs. All antigens tabulated were measured as a percentage of total PCs based upon their initial identification using CD45, CD38, and CD138 expression. For 148 total valid observations (sum of column 2), four outliers (2.6%) for three antigens (CD27, CD33, and CD81) were identified. CD33 expression could not be determined on four patients for technical reasons, reducing the total number measured for CD33 from 15 to 11.

The distribution of expression for the majority of antigens (CD56, CD28, CD10, CD20, cyto-K, cyto-L) (6/10; 60%) followed a Gaussian distribution, as tested by the Shapiro Wilk W test (*P*-value > 0.05). CD19, CD27, CD33, and CD81 expression did not follow a Gaussian distribution but rather a Gamma or Beta distribution, using the Log Likelihood statistic. There was no expression of CD200, CD221, or CD117 on normal PCs.

In control subjects, the normal ranges of expression for CD19, CD27, CD33, and CD56 were observed to be close to the extreme values of 0% and 100%. According to previous publications, the majority of normal PCs were found to express CD19 and CD27 but not CD33 nor CD56 (2,5,13,20,21). It is possible that due to the relatively small numbers of control samples the extreme values of antigen expression were not actually observed. However, because subjects with 100% of expression of CD19 and CD27 and with 0% of expression of CD33 and CD56 are considered to be normal in clinical routine, we assumed that the ranges of normal expression

for CD19 and CD27 are 61–100% and 66–100% respectively, while for CD56 and CD33 are 0–45% and 0–34%, respectively.

Comparison Between Histologic Results and Flow Cytometric Analysis for Plasma Cell Neoplasms

For each patient it was determined if an antigen was expressed normally or not, using the new and traditional methods. Then, the corresponding histology was checked for each case as either correlating with (i.e., positive by FC and positive by histology or negative by FC and negative by histology) or not correlating (discrepant result; Table 3). Table 4 shows histological results for each group of patients.

Referring to Table 5 for the Cohen's κ index interpretation, Figure 2 shows that: cyto- λ and cyto- κ resulted in the best agreement with histology by both methods. CD19 has a substantial agreement with histology by the new method, better than the traditional one. CD27 and CD56 have a moderate agreement with histology by both methods. CD81, CD28, CD20, CD33 have a fair or slight agreement with histology by both methods. CD10 expression did not correlate with histology by the new method, with a slight agreement by the traditional one.

DISCUSSION

In addition to the immunoglobulin light chain clonality of PCs, the study of PCM by FC is improved by the capacity to distinguish other immunophenotypic differences between normal and neoplastic PCs. This information can be very useful to help discriminate neoplastic PCs from normal or reactive PCs when clonality results

Table 3
Number (n) of Patients (pt) Which Express Each Antigen Normally (–) or Not (+) Using the New Method and the Traditional One and Which Resulted With Disease (+) or Without Disease (–) by Histology; Number Of Patients Resulted +/- at the New Method/Histology and at the Traditional Method/Histology

	New method		Traditional method		Histology		New method/Histology	Traditional method/Histology
	n pt +	n pt –	n pt +	n pt –	n pt +	n pt –	n pt +/-	n pt +/-
CD19	22	24	14	32	26	20	21	14
cyto-κ	26	20	27	19	26	20	22	25
cyto-λ	25	21	27	19	26	20	24	25
CD27	17	29	10	36	26	20	16	10
CD56	23	23	34	12	26	20	18	24
CD81	17	29	6	40	26	20	14	5
CD28	9	37	12	34	26	20	9	11
CD20	3	43	43	3	26	20	2	25
CD33	1	41	1	41	23	19	1	1
CD10	0	46	5	41	26	20	0	5

are unclear (2–9). This information may also be useful to help define potential prognostic markers in PCM and MGUS progression to PCM as shown in (5,13–21). The combination of immunophenotype together with clonality results is important in evaluation of MRD (2,43–45) and also to identify new targets for myeloma therapy (5,15,23–37). Because of the need to define the normal PC immunophenotype, bone marrow aspirate samples obtained from patients without PCM, MGUS or other PC neoplasms were studied here for their expression of normal PC antigens. These samples included as “normal” were those obtained from patients undergoing elective hip implantation. While these samples were not formally obtained from “normal” volunteer samples, they were proven by to be disease free (see Patients and Methods section). Since the median age at diagnosis of MM is ~70 years (46), it is felt the control population used provides a reasonable comparison.

In most existing reports, 20% of cells expressing an antigen of interest is considered the threshold value to define an antigen as “positive” or “negative” and discriminate if the PCs are normal or neoplastic (13,14,38–40). However, rather than a strict 20% cut-off, Rawstron et al. (2) suggested variable levels of expression of CD19, CD56, CD20, CD117, CD28, CD27, CD81, and CD200 on polyclonal PCs. In this study, no normal PCs expressed CD200, CD221, or CD117. Other antigens like CD56 and CD20 were abnormally expressed on normal PCs (expressed at a value greater than 20%).

Table 4
Histological Results of Patients

Time of study	Positive (patients with disease by histology)	Negative (patients without disease by histology)	Total patients
New diagnosis	7	2	9
Post-chemotherapy	12	4	16
Post-BMT	5	10	15
MGUS in follow-up	2	4	6

By looking for the optimal distribution curve and then the 95.0% confidence intervals for the mean, an objective definition of range of expression for each antigen was found (Table 3).

In this way, a variable range of expression was found on normal PCs among the antigens studied.

Then it was determined if the established normal range of expression as a criterion for presence/absence of disease, correlated better with immunohistochemistry than using the standard 20% cutoff.

With respect to those values generated in this report, it was observed that CD19 and CD81 are better correlated with histologic results by the new method than the traditional one. The two methods show almost the same correlation with histology for CD27, CD56, CD28, CD20, CD33, and cyto-λ. The traditional method seems better correlated with histology for cyto-κ and CD10.

With respect to the new method, cyto-λ, CD19 and cyto-κ show the best concordance with histology; after these, CD27 and CD56 show a similar moderate concordance.

Immunoglobulin light chains have been confirmed to be the main antigens to detect the absence or presence of disease in PCM. The κ/λ ratio ranging between 0.5

Table 5
Cohen's κ Index Interpretation and Results For Each Antigen Comparing the New to the Traditional Method

κ	Interpretation	New method	Traditional method
<0	No agreement	CD10	
0.0–0.20	Slight agreement	CD20, CD33	CD81, CD20, CD33, CD10
0.21–0.40	Fair agreement	CD81, CD28	CD28
0.41–0.60	Moderate agreement	CD27, CD56	CD19, CD27, CD56
0.61–0.80	Substantial agreement	cyto-κ, CD19	
0.81–1.00	Almost perfect agreement	cyto-λ	cyto-κ, cyto-λ

and 3 is shown to be the best way to define normal, i.e. polyclonal, PCs and this remains true for both the new and traditional methods. This finding could be explained by the fact that histology uses the same criteria to define the presence or absence of clonal PCs.

It is well known that lack of CD19 expression is useful to detect the presence of neoplastic PCs. CD19 is an antigen gained in early stage of B differentiation and always present in the majority of normal PCs. On the other hand, only a small percentage (<5%) of patients with PCM express this antigen (13). This antigen shows better concordance with histological results by the new method than the traditional one.

It is interesting to observe that CD27 plays the same role as CD56 in detecting the presence of disease and so could be considered an additional helpful antigen in this regard.

The range of normal expression obtained for CD56 is relatively wide (0–47%; Table 3). CD56 expression was reported in 70–80% of MM patients (39,47). Rawstron et al. (2) showed that CD56 expression is present throughout the course of disease. Other studies showed that CD56 is present on PCs in PCM, but not in MGUS (48,49), extra-medullary plasmacytoma or PC leukemia (50). This probably occurs because CD56 is an adhesion molecule involved in the anchorage of the PCM PCs to the medullary stroma and its absence is associated with extramedullary PC infiltrates, more aggressive disease and a poor prognosis. Rawstron et al. (2) considered that a CD56 value above 15% is abnormal on PCs. In this study, 47% was found to be the maximum value of expression on normal PCs in control samples. This is not surprising if CD56 is considered a protective antigen in its role as an anchor for PCs in bone marrow stroma, since PCs are not normally found in the circulation.

CD20 expression was constant in control samples and is expressed within a large normal range (19–91%; Table 3). It was also demonstrated that normal PCs can be CD20+ (Fig. 3). In contrast to a previous report (2), our findings suggest that CD20 is a poor antigen in distinguishing between the presence and absence of disease, as also shown by its low Cohen's κ value (0.0648 by the new method and 0.0682 by the traditional method, Fig. 2).

In conclusion, the new normal range of expression method was not found to be superior than the traditional 20% threshold to detect the presence of disease in PCM. However, CD19 showed a good correlation with the presence of disease by the new method, where a cut-off of 61% should be used to distinguish between normal and neoplastic PCs. In this way FC analysis could be considered as a good screening test to detect the presence of plasma cell neoplasms. It is important to consider that due to the relatively small control samples size, the ranges of normal expression found in this study may not be fully sustained after many more normal samples are tested, but certainly do reflect significant results of the samples available to study. It is also reasonable to consider that this study evaluated the role of each antigen separately in detecting the presence of disease. The

contribution of different antigens evaluated together could improve the correlation with histological results, and is an ongoing endeavor in this laboratory.

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