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Technical Advance

Flow Cytometric Analysis of Mast Cells from Normal and Pathological Human Bone Marrow Samples

Identification and Enumeration

Alberto Orfao,* Luis Escribano,† Jesús Villarrubia,† José L. Velasco,† Carlos Cerveró,† Juana Ciudad,* José L. Navarro,† and Jesús F. San Miguel‡

From the Servicio Central de Citometría and Servicio de Hematología,‡ Hospital Clínico Universitario, Salamanca, Spain, and the Servicio de Hematología,† Hospital Ramón y Cajal, Universidad de Alcalá de Henares, Madrid, Spain*

In the present paper we have used a three-color immunofluorescence procedure combined with flow cytometry cell analysis and sorting for the identification and enumeration of human mast cells in both normal and pathological bone marrow samples. Our results show that bone marrow mast cells are clearly identifiable on the basis of their light-scatter properties and strong CD117 expression. These cells were negative for the CD34, CD38, and BB4 antigens. In addition, they were CD33$^+$ and displayed a high reactivity for the anti-IgE monoclonal antibody. The identity of the CD117-strong$^+$ cells (mast cells) was confirmed by both microscopic examination and flow cytometry analysis. The overall frequency of mast cells in the bone marrow samples analyzed in the present study was constantly lower than 1%. The lowest frequencies corresponded to normal human bone marrow samples (0.0080 ± 0.0082%) and the highest to those patients suffering from indolent systemic mast cell disease (0.40 ± 0.13%). In summary, our results show that the identification and enumeration of bone marrow mast cells can be achieved using multiparametric flow cytometry. Moreover, once identified, mast cells are suitable for being characterized from the phenotypic and the functional point of view, facilitating the comparison between normal and abnormal mast cells. (Am J Pathol 1996, 149:1493–1499)

Mast cells (MCs) are present at very low frequencies in normal human bone marrow (BM). In histological sections, MCs are distributed especially near the endosteum, in the periosteum, in association with small blood vessels and at the periphery of lymphoid nodules or aggregates.[1] Since the availability of monoclonal antibodies, the immunophenotype of human MCs has been analyzed in most of the organs where they have been identified.[2–7] Accordingly, these studies have shown that, among other markers, MCs characteristically express two surface receptors: the high affinity IgE receptor (FcεRI)[8,9] and the CD117 antigen (MC growth factor receptor, c-*kit*, or stem cell factor receptor).[2,4,7,10,11] In the past years, an increasing number of reports have been published[3–5,12,13] in which the immunophenotype of MCs was analyzed, although their identification is still based on morphological, cytochemical, and ultrastructural criteria. This is probably related to the fact that the antigens found to be expressed by MCs are also present in other cell

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Address reprint requests to Dr. Alberto Orfao, Servicio de Citometría, Laboratorio de Hematologia, Hospital Clínico Universitario, Paseo de San Vicente, 37007 Salamanca, Spain.

types.[3,13–19] Due to their low frequency, enrichment of MCs has been a prerequisite for their immunophenotypical analysis. For that purpose, both tissue disaggregation using enzymatic methods[7,13] and procedures to purify the cell fraction containing MCs have been extensively used.[13] Although the former techniques have been shown to affect the detection of several MC antigens,[7,13] the procedures used for MC enrichment such as sedimentation and centrifugation may induce selective cell loss.[13] Quantitative evaluation of MCs has been attempted in several tissues and pathological conditions using different techniques.[20–30] In any case, the enumeration of the MCs present in a sample has never been achieved with accuracy, despite the fact that the existence of increased numbers of MCs in different tissues, including BM, may have clinical value in several pathological conditions.[20,31–34]

Flow cytometry is a well suited technology for the analysis of single-cell suspensions, even when such cells are present at very low frequencies[35]; however, to the best of our knowledge, immunophenotypical analysis of MCs by flow cytometry has not been performed in either whole BM or peripheral blood samples.

In the present paper, we have used a three-color immunofluorescence procedure combined with flow cytometry cell analysis and sorting for the identification and enumeration of human BM MCs, which may facilitate future phenotypic and functional studies of these cells.

Materials and Methods

Samples

BM samples were obtained after informed consent from 12 healthy volunteers undergoing either orthopedic surgery or BM harvest, 15 patients diagnosed with chronic lymphoproliferative disorders (8 B-cell chronic lymphocytic leukemia cases, 2 Waldenström macroglobulinemia, 4 multiple myeloma, and 1 benign monoclonal gammopathy), 4 cases of urticaria pigmentosa, 5 individuals suffering from indolent systemic MC disease, 1 patient with acute myeloid leukemia after a myelodysplastic syndrome, 1 case of megaloblastic anemia, and 1 case of idiopathic thrombocytopenic purpura. BM samples (3 ml) were collected in EDTA anticoagulant and immediately diluted 1/1 (v/v) in phosphate-buffered saline (PBS). After collection, the sample was passed several times through a 25-gauge needle to disaggregate the BM particles.

Immunological Marker Analysis

BM samples were analyzed by direct immunofluorescence using triple-staining combinations of monoclonal antibodies (MAbs) directly conjugated with fluorescein isothiocyanate, phycoerythrin (PE), and the PE-cyanin 5 fluorochrome tandem. The following panel of MAb combinations (fluorescein isothiocyanate/PE/PE-cyanin 5) was used: CD34/CD117/CD45, BB4/CD117/CD38, and anti-IgE/CD117/CD33. CD117 (100 μg/ml) MAb was purchased from Immunotech (Marseille, France), CD34 (50 μg/ml) from Becton Dickinson (San Jose, CA), BB4 (100 μg/ml) from Immunoquality Products (Gröningen, The Netherlands), and anti-IgE (10 μg/L) from The Binding Site (Birmingham, UK). CD33 (200 μg/ml), CD38 (200 μg/ml), and CD45 (200 μg/ml) were purchased from Caltag Laboratories (San Francisco, CA). Staining of MCs was performed by incubating (10 minutes at room temperature) 200 μl of the diluted BM sample containing 10^6 nucleated cells with each of the MAb combinations mentioned above. The dilution used for each MAb were as follows: CD34, 1/10; CD117, 1/20; CD45, 1/40; BB4, 1/20; CD38, 1/40; IgE, 1/250; CD33, 1/40. Once this incubation was finished, the stained cells were incubated for another 10 minutes (room temperature) with 2 ml of FACS lysing solution (Becton/Dickinson) per tube to lyse erythrocytes. Afterward, cells were centrifuged (5 minutes at 1800 rpm) and washed once in PBS containing 0.2% bovine albumin (Sigma Chemical Co., St. Louis, MO) and 0.1% sodium azide. Fluorochrome-conjugated isotype-matched mouse nonspecific immunoglobulins were used as negative control at similar concentrations as specific antibodies.

Data acquisition was performed on a FACSort flow cytometer (Becton Dickinson) using the LYSYS II software program (Becton Dickinson) and a double-step procedure. First, 15,000 events per tube, corresponding to the lysed whole BM sample, were collected and information on them stored. Immediately after, a second acquisition of the cells present in the same tube was performed for at least 300,000 events/tube. From these events, only those included in a pre-established SSC/CD117 gate were stored to increase the sensitivity of the method (Figure 1). For data analysis, the Paint-A-Gate Plus software program (Becton Dickinson) was used. Enumeration of the MCs present in each sample was performed by means of calculating the proportion of $CD117^+$ MCs stored in the second acquisition step from the total number of events acquired upon excluding cell debris.

Flow Cytometry Sorting of Mast Cells

The cell-sorting experiments were performed on a FACSort flow cytometer. Cells were sorted into PBS containing 1% bovine albumin. The sorted cells were centrifuged for 5 minutes at 200 × *g* and resus-

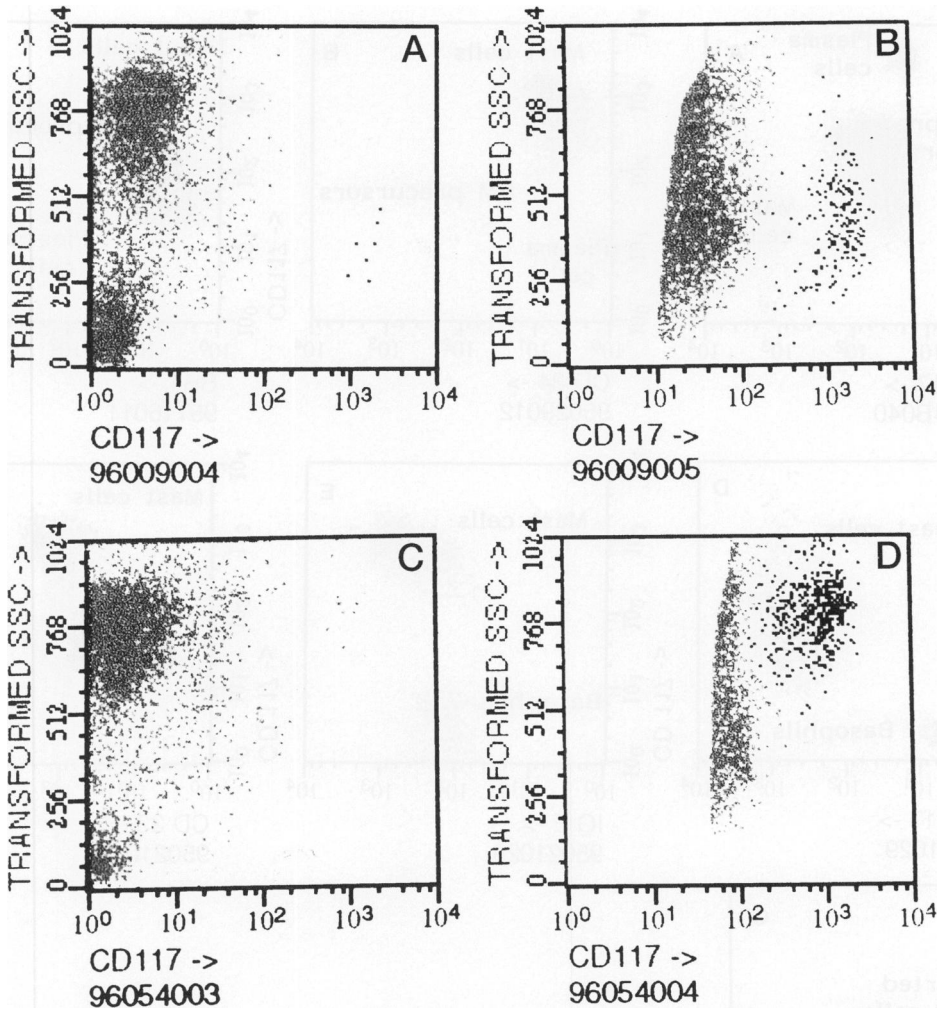

Figure 1. *Transformed SSC/CD117$^+$ gated human BM MCs* (B *and* D) *from erythrocyte-lysed whole BM* (A *and* C). A *and* B *correspond to a B-cell chronic lymphocytic leukemia and* C *and* D *to the BM of a patient suffering from indolent systemic MC disease.*

pended at a cell concentration of 1×10^3/ml in RPMI 1640 containing 10% fetal calf serum. Cytospin preparations were made by means of a Shandon cytocentrifuge (Shandon, Southern Products, Sewickly, UK). For each slide, 100 μl of RPMI 1640 containing 10,000 sorted cells were used. Slides were stained with either Giemsa or toluidine blue and examined under a light microscope.

Results

Flow Cytometric Identification of Bone Marrow Mast Cells

MCs have been shown to have two characteristic surface markers: the IgE receptor and the CD117 antigen. Figure 2 shows two-dimensional projections of CD117$^+$ BM cells. As it is seen there, regarding CD117 expression, two different cell subpopulations were identified: minor subset that displays a strong reactivity for CD117 and a major CD117-dim$^+$ subpopulation. The former cell subset became evident only upon applying an electronic gate on the intermediate-low SSC/CD117$^+$ cells as shown in Figure 1. As may be seen in Figure 1, differences in the light-scatter properties of MCs were observed between individuals, SSC ranging from values similar to those of BM plasma cells (Figure 1B) to those observed for mature granulocytes (Figure 1D). Further immunophenotypical characterization of these cell populations (Figure 2A) showed that almost all CD117-dim$^+$ cells were CD38$^+$ whereas the CD117-strong$^+$ cell subpopulation was CD38$^-$. In addition, among the CD117-dim$^+$ cells, two subsets were identified based on the expression of CD38: the CD38$^{+/++}$ and the CD38^{+++} cells (Figure 2A); from these two subsets, the former expressed CD34 in the absence of BB4 whereas the CD38^{+++} cells were

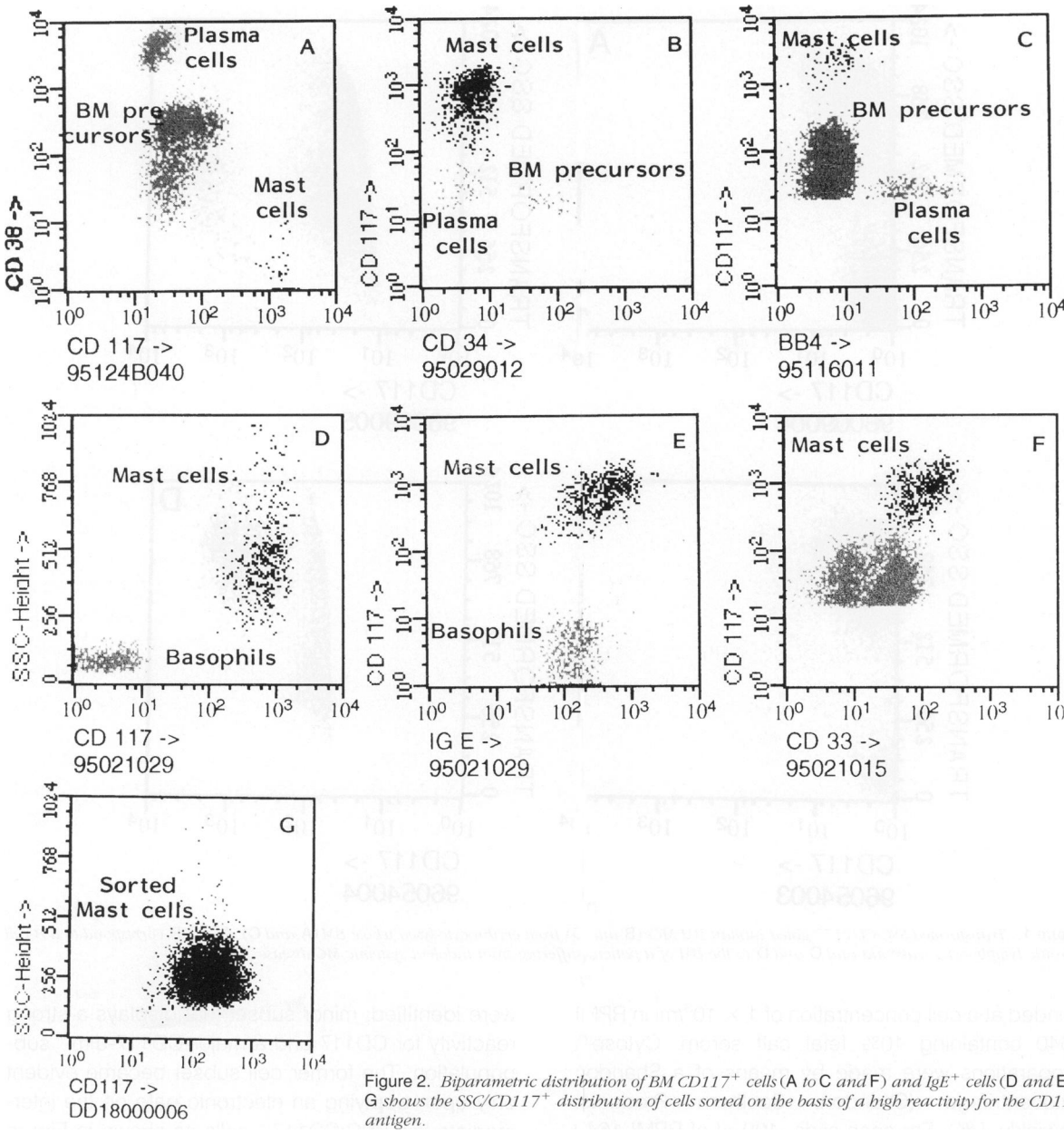

Figure 2. *Biparametric distribution of BM CD117$^+$ cells* (A *to* C *and* F) *and IgE$^+$ cells* (D *and* E). G *shows the SSC/CD117$^+$ distribution of cells sorted on the basis of a high reactivity for the CD117 antigen.*

CD34$^-$/BB4$^+$, indicating that they would correspond to BM CD34$^+$ precursor cells and to plasma cells, respectively (Figure 2, B and C). The CD117-strong$^+$ cells were negative for both the CD34 and BB4 antigens (Figure 2, B and C) and presumably would correspond to the MCs. In a similar way, two subsets of cells that displayed high amounts of IgE bound to their surface were observed (Figure 2, D and E); nevertheless, only one of them was CD117$^+$, corresponding to those cells that displayed a strong reactivity for the CD117 MAb. The CD117$^-$/IgEεR$^+$ cells showed a CD45/SSC distribution identical to that of human basophils, whereas the CD117 cells displayed a more heterogeneous and higher SSC (Figure 2D); both cell populations were CD33$^+$ (Figure 2F).

Confirmation of the Flow Cytometric Identification of Mast Cells

The CD117-strong$^+$ cells, shown in Figure 2, A to F, were sorted based on their high CD117 expression. Afterward, they were either placed on microscopic slides and stained with either Giemsa or toluidine blue or reanalyzed in the flow cytometer. As shown in

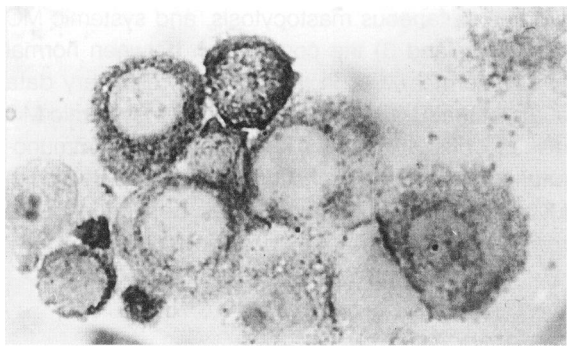

Figure 3. *Giemsa stain of sorted MCs from a patient suffering from indolent systemic MC disease. MCs display round nucleus and sparsely granulated cytoplasm.*

Figure 2G, the flow cytometric analysis of the sorted cells revealed the existence of a homogeneous cell population; these cells displayed a characteristic morphology of MCs (Figure 3) and stained metachromatically with toluidine blue. The purity of the sorted cell fraction was greater than 90%.

Enumeration of Human Bone Marrow Mast Cells

The overall frequency of MCs in the BM samples analyzed in the present study was low, constantly representing less than 1% of all nucleated cells. The mean coefficient of variation observed for the number of CD117-strong$^+$ cells obtained in the different tubes analyzed per individual was relatively low as shown in Table 1. According to the different groups of individuals analyzed, it was observed that the lowest mean frequencies corresponded to normal human BM samples (0.0080 ± 0.0082%) and the highest to those patients suffering from indolent systemic MC disease (0.40 ± 0.13%; Table 1). From the remaining groups of patients showing intermediate numbers of BM MCs, the frequency of these cells was slightly higher in those cases diagnosed with Waldenström macroglobulinemia (0.25 ± 0.06%) as compared with that of patients with urticaria pigmentosa (0.05 ± 0.04%), B-cell chronic lymphocytic leukemia (0.05 ± 0.05%), and monoclonal gammopathies (0.025 ± 0.03; Table 1).

Discussion

The low frequency at which MCs are usually present in human BM together with the lack of MC-specific antigens have been important limitations for the accurate identification of BM MCs. In recent years it has been shown that the combined use of multiple staining and flow cytometry allows the sensitive detection of rare cells.[35–37] In this sense, our group has shown[35] that the use of a double-acquisition procedure allows the detection of small populations of leukemic cells in complete remission human BM samples even when their frequency is as low as that of 1 leukemic cell in 10^6 normal cells. Thus, based on this method and the selection of bone marrow CD117$^+$ cells, which represent only a small fraction of all BM cells,[37] we expected to overcome the two major limitations for the identification of mast cells in BM samples mentioned above. Previous reports have shown that MCs display two highly characteristic surface markers: the CD117 antigen[2,4,7,11] and the high-affinity IgE receptor.[8,9] However, it has been shown that both markers can also be detected on the surface of other cell types present in normal human BM. Accordingly, although significant amounts of the high-affinity IgE receptor have been found to be present in human basophils,[3,13,18] CD117 expression has been reported in both BM precursors, including multipotent and erythroid- and myeloid-committed progenitor cells,[14–16,19] and in normal (Orfao, unpublished observations) and myelomatous plasma cells.[17] Almost all cells within

Table 1. *Enumeration of Human Bone Marrow Mast Cells*

Group	% mast cells*	CV of replicates
Controls (n = 12)	0.0080 ± 0.0082 (102.5%)	17.9 ± 3.3%
B-CLL (n = 8)	0.0513 ± 0.0506 (98.7%)	12.2 ± 6.9%
WM (n = 2)	0.2485 ± 0.0655 (26.4%)	18.6 ± 1.4%
MG (n = 5)	0.0250 ± 0.0390 (156.0%)	12.3 ± 6.8%
UP (n = 4)	0.0577 ± 0.0419 (119.7%)	14.5 ± 9.5%
SMCD (n = 5)	0.4040 ± 0.1324 (43.9%)	9.2 ± 5.0%
Others (n = 3)*	0.0195 ± 0.0120 (61.4%)	21.7 ± 9.5%

The percentage of mast cells was obtained based on the number of events displaying a strong CD117 expression among BM nucleated cells. Results are expressed as mean ± SD (coefficient of variation in parentheses). The coefficient of variation (CV) of replicates was calculated from the percentage of MCs CD117 strong + obtained within each patient with the different monoclonal antibody combinations used. B-CLL, B-cell chronic lymphocytic leukemia; WM, Waldenström macroglobulinemia; MG, monoclonal gammopathy; UP, urticaria pigmentosa; SMCD, indolent systemic MC disease.

*One case of megaloblastic anemia, one case of idiopathic thrombocytopenic purpura, and one case of acute leukemia after a myelodysplastic syndrome.

these three cell types have been shown to be $CD38^+$,[37,38] the extraordinarily high expression of this marker being characteristic of human plasma cells.[39,40] In the present paper, it is shown that, upon gating on the $CD117^+$ cells, three different $CD117^+$ cell subsets were identified in the BM samples analyzed. These three $CD117^+$ cell subsets could be clearly distinguished from each other based on their reactivity for the CD117 antigen and the other markers that have been explored: 1) CD117-strong$^+$, $CD34^-$, $CD38^-$, and $BB4^-$; 2) CD117-dim$^+$, $CD34^-$, CD38-strong$^+$, and $BB4^+$; and 3) CD117-dim$^+$, $CD34^+$, $CD38^+$, $BB4^-$. In addition, it was shown that the former subpopulation also expressed high amounts of the high-affinity IgE receptor; in this sense, it was assumed that MCs were included within the CD117-strong$^+$ cell compartment whereas the other two cell subsets would correspond to $CD117^+$ BM precursors and plasma cells, respectively. Sorting experiments confirmed this notion.

The mean frequency of MCs in the BM samples analyzed was quite low according to previous morphological observations.[1] Interestingly, although the number of BM MCs was very low, the precision of the method, as measured by the coefficient of variation obtained for the number of CD117-strong$^+$ cells in the different tubes analyzed per patient, was high, especially when compared with that of widely represented cells such as the peripheral blood $CD4^+$ T cells measured by flow cytometry.[41] The lowest values were observed within healthy individuals, suggesting that this technique could be used for the detection of abnormally high numbers of BM MCs. Accordingly, our results show that patients with B-cell chronic lymphoproliferative disorders, urticaria pigmentosa, and indolent systemic MC diseases with a relatively high frequency show increased numbers of BM MCs, a relatively high degree of variability being detected between patients. Although this variability may be related to a direct regulation of MC numbers, it may also be associated to either an increase or decrease of other cell types. In any case, the clinical significance of such findings remains to be elucidated.

Our results show that the procedure proposed in the present paper will allow 1) the specific identification of BM MCs even when present at very low frequencies, 2) their accurate enumeration, and 3) their phenotypic and functional characterization without a need to use preparative steps. This will contribute to establish 1) the normal ranges of BM MCs according to age and sex, 2) the significance of increased numbers of BM MCs in several pathological conditions (ie, chronic lymphoproliferative and myeloproliferative disorders, myelodysplastic syndromes, cutaneous mastocytosis, and systemic MC diseases), and 3) the comparison between normal and abnormal MCs. In this sense, preliminary data suggest that BM MCs from aggressive systemic MC disease display distinct light-scatter and immunophenotypical characteristics[42] as compared with MCs from normal human BM as defined by CD2 reactivity (Escribano et al, data not published).

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