Immunophenotypic characterization of human bone marrow mast cells. A flow cytometric study of normal and pathological bone marrow samples*

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Abstract. The goal of the present paper was to define the immunophenotype of bone marrow mast cells (BMMC) from healthy controls and patients with hematologic malignancies (HM) based on the use of multiple stainings with monoclonal antibodies analyzed by flow cytometry. Our results show that BMMC from both groups of individuals display a similar but heterogenous immunophenotype. The overall numbers of BMMC are higher in the HM group of individuals ($p = 0.08$). Three patterns of antigen expression were detected: (1) markers constantly positive in all cases analyzed (CD9, CD29, CD33, CD43, CD44, CD49d, CD49e, CD51, CD71, CD117, and Fc ϵ RI), (2) antigens that were constantly negative (CD1a, CD2, CD3, CD5, CD6, CD11a, CD14, CD15, CD16, CD19, CD20, CD21, CD23, CD25, CD30, CD34, CD38, CD41a, CD42b, CD65, CD66b, HLA-DR, and CD138), and (3) markers that were positive in a variable proportion of cases – CD11b (50%), CD11c (77%), CD13 (40%), CD18 (20%), CD22 (68%), CD35 (27%), CD40 (67%), CD54 (88%) and CD61 (40%). In addition, BMMC from all cases explored were CD45⁺, and this antigen was expressed at an intensity similar to that of mature granulocytes.

In summary, our results show that BMMC from both healthy controls and HM patients display a relatively heterogenous immunophenotype. Interestingly, we have observed clear differences between the immunophenotype of BMMC and MC from other tissues. This could be due either to the heterogeneity of human MC according to their tissue localization or to the sensitivity of the method used for antigen detection.

Keywords: Mast cells, immunophenotype, bone marrow, flow cytometry

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1. Introduction

Mast cells (MC) are long-living cells derived from precursors which originate in the bone marrow [27–29]. MC have been grown *in vitro* from CD34⁺ precursors from bone marrow, cord blood and peripheral blood mononuclear cells [3,10,19,26,28]. Heterogeneity is a characteristic of MC, which may exhibit variable morphological and phenotypical characteristics [4–7,12,17–20,40].

The immunophenotype of human MC has been studied in different organs such as the uterus [21], lung [43,45], skin [34,43], heart [41], ascites [43], and gastrointestinal tract [43]. Due to their low frequency, enrichment of MC has been a prerequisite for their immunophenotypical analysis. For this purpose, both tissue disaggregation using enzymatic methods [41,44] and procedures to purify the cell fraction containing MC, have been extensively used [44]. While the former techniques have been shown to affect the detection of several MC antigens [41,44], the procedures used for MC enrichment such as sedimentation and centrifugation may induce selective cell loss [44]. These studies have shown that among other markers, MC express two highly characteristic cell surface receptors: the high affinity IgE binding sites (FcεRI) [11,15,22–25,33] and the MC growth factor (MGF) receptor or stem cell factor (SCF) receptor (CD117) [14,21,31,41,43]. Information on the immunophenotype of human BMMC is currently scanty and its analysis has only been performed in BM from patients suffering from chronic myeloid leukemia [43,45] or mast cell leukemia [9,38]. No extensive information has been provided so far on the characteristics of MC from normal human bone marrow.

Recently, we have shown that BMMC, although present at very low frequencies in normal BM, can be identified and enumerated using multiparametric flow cytometry [35]. This would allow the phenotypic characterization of human BMMC in a systematic way. The aim of the present paper is to define the immunophenotype of human BMMC from both normal individuals and patients diagnosed from different hematological malignancies (HM), in which MC apparently are not part of the malignant clone, using a large panel of monoclonal antibodies (MoAbs) analyzed at flow cytometry. In addition, results on the reactivity obtained for each of the markers analyzed were expressed as the molecule equivalent of soluble fluorochrome (MESF) which makes them comparable in future studies using the same monoclonal antibody conjugates.

2. Material and methods

BM samples. BM was obtained after informed consent from 44 subjects: 15 healthy volunteers undergoing either orthopedic surgery or bone marrow harvest and 29 patients diagnosed as chronic lymphoproliferative disorders – 13 B-cell chronic lymphocytic leukemia (B-CLL) cases, 4 B-cell non-Hodgkin lymphomas (B-NHL), 3 Waldenström's macroglobulinemia (WM), 4 multiple myeloma (MM), and 5 monoclonal gammopathy of undetermined significance (MGUS).

All samples were collected in K₃-EDTA anticoagulant and immediately diluted 1/1 (vol/vol) in phosphate-buffered saline (PBS). After collection, the sample was passed several times through a 25 mm gauge needle in order to disaggregate the bone marrow particles and adjusted to a concentration of 7.5×10^9 nucleated cells/l.

Immunological marker analysis. BM samples were analyzed by direct immunofluorescence using triple- and double-staining combinations of monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and either the PE-cyanin 5 (PE-Cy5) fluorochrome tandem or PerCP. In all cases antibodies were used at saturation concentrations. The following monoclonal

antibody conjugates were used: (1) 10 μ l of FITC-conjugated CD2, CD3, CD5, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD33, CD34, CD44, CD71, anti-HLA-DR (purchased from Becton-Dickinson, San José, CA, USA), CD40 (Cymbus Biosciences, Ltd., Southampton, UK), CD38, CD65, (Caltag Laboratories, San Francisco, CA, USA), CD11a, CD11b, CD11c, CD35, CD54, CD66b (CLB, Amsterdam, The Netherlands), CD29 (Coulter Corporation, Miami, FL, USA), CD21, CD30, CD42b, CD61 (Dakopatts, Copenhagen, Denmark), CD41a (Immunotech, Marseille, France), CD18, CD45, CD51 (Menarini Diagnostics, Barcelona, Spain), CD117 (Nichirei Corporation, Tokyo, Japan), CD6 (Centro de Inmunología Molecular, La Habana, Cuba), CD9, CD43, CD49d, CD49e (Serotec, UK) anti-IgE (The Binding Site, Birmingham, UK), and CD138 (Immunoquality Products, Gröningen, The Netherlands); (2) 10 μ l of PE-conjugated CD13, CD16, CD23, CD33, CD38 (purchased from Becton-Dickinson), CD1a (Cymbus Biosciences, Ltd.), and CD117 (Immunotech and Nichirei Corporation); and (3) 5 μ l of PE/Cy5-conjugated CD13, CD14, CD33, CD34, CD38, CD45 (Caltag Laboratories), or 10 μ l of PerCP-conjugated CD45 (Becton-Dickinson)

Briefly, 200- μ l of a sample containing approximately 1.5×10^6 nucleated cells were incubated for 10 min at room temperature with the MoAb mentioned above. Afterwards, erythrocytes were lysed by incubating cells for 10 min (room temperature) with 2 ml/tube of FACS lysing solution (Becton-Dickinson). Isotype-matched mouse non-specific immunoglobulins and a tube stained for CD117-PE were used as negative and positive controls, respectively. For the MC localization, the following panel of MoAb combinations (FITC/PE/PE-Cy5 or PerCP) was constantly used: CD34/CD117/CD45, CD138/CD117/CD38, and anti-IgE/CD117/CD33. Data acquisition was performed on a FACScan flow cytometer (Becton-Dickinson) using the LYSYS II software program (Becton-Dickinson) for at least 10,000 events/tube. In addition, a second acquisition step, through a SSC/CD117 live gate, was performed to increase the sensitivity of the method for the analysis of MC present at a low frequency [35]. For data analysis, the Paint-A-Gate Plus software (Becton-Dickinson) was used. The quantitation of positivity for each of the markers tested was performed using QuickCal beads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico), and results were expressed as the mean number of molecules equivalent of soluble fluorochrome (MESF) obtained specifically for the MC. The threshold for positivity was 5,000 MESF for FITC and 500 MESF for PE.

Statistical methods. Mean values and their standard deviations were calculated for all variables in each group of samples. The Mann-Whitney *U*-test was used in order to assess the statistical significance of the differences observed between groups of individuals.

3. Results

In all BM samples analyzed, MC were clearly identified based on their strong CD117 expression and in spite of their low frequency. BMMC displayed a typical FSC/SSC pattern similar to that of normal mature monocytes. The mean number of BMMC was $0.029 \pm 0.038\%$ and it was significantly ($p = 0.08$) higher in patients with HM as compared to normal controls ($0.018 \pm 0.023\%$) versus $0.034 \pm 0.043\%$).

The immunophenotypical characteristics of BMMC are shown in Fig. 1 and Table 1. As may be seen, three patterns of antigen expression were detected: (1) markers constantly positive in all cases analyzed (CD9, CD29, CD33, CD43, CD44, CD45, CD49d, CD49e, CD51, CD71, CD117, and Fc ϵ RI), (2) antigens that were constantly negative (CD1a, CD2, CD3, CD5, CD6, CD11a, CD14, CD15, CD16, CD19, CD20, CD21, CD23, CD25, CD30, CD34, CD38, CD41a, CD42b, CD65, CD66b, HLA-DR,

Fig. 1. Representative dot plots (A–L) of the immunophenotypic analysis of bone marrow mast cells (black dots) once selected on the basis of a high expression of CD117. MESF values ($\times 10^3$) were as follows: IgE, 40.0 (B); CD9, 134.4 (C); CD22, 9.9 (D); CD13, 4.5 (E); CD35, 2.7 (F); CD54, 6.3 (G); CD49d, 19.1 (H); CD51, 8.3 (I); CD44, 163.3 (J); CD43, 12.5 (K); CD29, 39.7 (L).

and CD138), and (3) markers that were positive in a variable proportion of cases – CD11b (50%), CD11c (77%), CD13 (40%), CD18 (20%), CD22 (68%), CD35 (27%), CD40 (67%), CD54 (88%) and CD61 (40%). In addition, among those antigens that were constantly positive three groups could be identified according to their intensity of antigen expression: (1) strong positive ($> 12,000$ and $> 50,000$ MESF for FITC and PE, respectively) – CD9, CD11c, CD29, CD44, CD49d, CD117 and Fc ϵ RI, (2) dim positive (from 5,000 to 19,000 MESF of FITC) – CD49e, CD51, CD54, and CD71, and (3) antigens showing a variable intensity of expression (from 5,000 to 51,000 MESF of FITC) – CD33 and CD43. Those antigens showing a variable expression for the different cases analyzed such as CD11b, CD13, CD18, CD22, CD35, CD40, and CD61, were expressed at a low intensity ($< 12,000$ MESF) when positive; the relative distribution of positive and negative cases as well as the range of antigen expression are shown in Table 1. In addition, BMMC from all cases explored were CD45 $^{+}$, this antigen was expressed at an intensity similar to that of mature granulocytes.

Table 1
Immunophenotypic characteristics of bone marrow mast cells in healthy controls and patients with hematological malignancies

CD	Controls	Hematological malignancies
9	945.8 ± 165.8 780.0–1111.7 (100%)	1837.1 ± 373.7 523.9–2727.5 (100%)
11b	< 5.0 (0%)	5.6 ± 0.2 < 5.0–6.0 (76%)
11c	11.6 ± 2.4 < 5.0–14.0 (66.6%)	17.7 ± 5.8 5.1–22.5 (87.5%)
13	7.5 ± 1.8 < 5.0–9.3 (67%)	6.5 ± 0.7 < 5.0–7.9 (34%)
18	5.6 ± 0.5 < 5.0–6.1 (33%)	< 5.0 (0%)
22	6.4 ± 0.5 < 5.0–7.9 (50%)	7.0 ± 0.5 < 5.0–9.9 (78.5%)
29	39.7 ± 5.7 34.0–45.5 (100%)	34.3 ± 4.3 15.1–62.7 (100%)
33	22.7 ± 6.2 7.8–51.7 (100%)	19.4 ± 2.6 5.8–33.8 (100%)
35	< 5.0 (0%)	7.0 ± 0.7 < 5.0–9.0 (33.3%)
40	NA	9.0 ± 3.3 < 5.0–12.3 (67%)
43	38.7 ± 22.4 11.4–83.2 (100%)	17.7 ± 3.1 7.9–30.5 (100%)
44	185.9 ± 43.8 82.1–339.2 (100%)	177.2 ± 33.0 56.9–296.6 (100%)
45*	(100%)	(100%)
49d	18.4 ± 0.7 16.9–19.2 (100%)	18.1 ± 2.8 12.1–25.9 (100%)
49e	7.1 ± 1.1 5.0–8.9 (100%)	7.6 ± 1.3 5.0–14.5 (100%)
51	8.6 ± 2.3 5.6–13.2 (100%)	8.4 ± 0.8 5.4–12.8 (100%)

Table 1
(Continued)

CD	Controls	Hematological malignancies
54	14.2 ± 2.4 < 5.0–18.7 (75%)	9.3 ± 1.7 5.0–18.6 (100%)
61	11.3 ± 0.5 < 5.0–11.8 (66%)	7.8 ± 1.6 < 5.0–7801 (50%)
71	5.7 ± 0.4 5.0–6.5 (100%)	6.4 ± 1.3 5.1–7.9 (100%)
117**	303.9 ± 52.7 87.8–860.1 (100%)	293.5 ± 124.6 94.8–645.6 (100%)
FCεRI	428.3 ± 140.1 288.1–568.5 (100%)	171.7 ± 39.2 41.2–463.0 (100%)

Results expressed as mean + SD range of MESF ($\times 10^3$). In brackets, percentage of positive cases. *PE/Cy5 or PerCP and **PE-conjugated monoclonal antibodies. NA, not analyzed.

After comparing the immunophenotype of BMMC from healthy subjects and patients suffering from HM, no significant differences were observed between both groups of individuals for any of the markers explored, regarding both the incidence of positivity and their fluorescence intensity. Nevertheless, it could be noted that a slightly higher mean expression of CD43, CD54, CD61, and FcεRI was evident among normal BMMC controls. In contrast, expression of both CD9 and CD11c was slightly higher for the BMMC from patients with HM.

4. Discussion

The immunophenotype of human MC has long been hampered by their low frequency and the need to use MC enrichment procedures prior to their phenotypic analysis. This could contribute to the increased variability of the results obtained. We have recently shown [35] that multiparametric flow cytometry is a useful tool for the identification and enumeration of human MC on whole BM erythrocyte-lysed samples which obviates sample manipulation prior to the analysis of the immunophenotype of BMMC. Accordingly, BMMC could be easily identified on the basis of their strong reactivity to the CD117 and FcεRI antigens [2,8,37,39] in the absence of expression for the CD138, CD34 and CD38 markers [35].

At present, it is well established that MC belong to the myeloid lineage [1,26,27,29,30]. Thus, MC are expected to express myeloid-associated antigens. In the present study, it is shown that BMMC do constantly express CD33 in variable amounts. Moreover, in most of the individuals analyzed BMMC were CD13⁺, although the expression of this antigen was weaker than that observed for CD33. In contrast, the CD14, CD15, CD16, CD65 and CD66b myeloid-associated markers were found to be constantly negative in the BMMC from all the individuals studied. These findings are, to a large extent, in agreement with previous reports on the immunophenotypical characteristics of human MC from other tissues different from BM [21,41–45]. However, interestingly, CD13 has been reported to be negative in MC from all tissues analyzed [41–44]. The weak intensity of CD13 expression may

account for this discrepancy. The selection of sensitive fluorochromes, such as phycoerythrin, is of particular relevance for its detection. In this sense, it should be noted that this is the first study in which the phenotype of human BMMC is extensively analyzed in a large group of individuals using flow cytometry, and this technique has shown to be a much more sensitive approach for the detection of surface antigens than conventional immunocytochemistry [36]. In relation to myeloid antigens, another finding of this study that has not been previously reported, is the lack of expression of the CD66b antigen on BMMC.

Lymphoid-associated markers have been reported as absent in normal human MC [21,41–45], although the CD2 expression, alone or together with CD22, has been found in a case of mast cell leukemia [9] and in a patient suffering from SMCD [13], respectively. In the present study, BMMC from a high proportion of the individuals analyzed showed reactivity for the B-cell-associated CD22 antigen. Interestingly, BMMC from B-CLL patients constantly displayed reactivity for this antigen which was found in variable proportions in normal samples, suggesting predominance of this subset of BMMC in these individuals. The absence of CD2 in all samples analyzed indicates that the finding of BMMC expressing the CD2 antigen should be considered as an aberrant phenotype [13]. In our experience, other lymphoid related markers were constantly negative.

In the present study, we have also explored the expression of activation-related markers as well as cell adhesion molecules. Regarding activation markers, CD25, CD30 and the HLA-DR antigens were found to be absent in BMMC in all the cases studied. In contrast, reactivity to the transferrin-receptor (CD71) was constantly present. Interestingly, CD71 has been reported to be negative in human MC from different tissues [43–45], while controversial results have been obtained regarding the CD25 expression which has been described as positive or negative in lung and intestinal MC [32], and constantly negative in the uterus, skin and ascitic fluid [42,43,45]. Once again, the reactivity found in the present study for the CD71 antigen was relatively dim, suggesting that the use of sensitive techniques, such as flow cytometry immunophenotyping and PE-conjugates, is essential for its detection. On the other hand, CD25 was constantly negative, indicating that expression of this antigen could be either specific for MC from tissues other than the BM or related to the MC activation state. CD35 expression, which has been previously described as negative in several tissues [16,41, 42,44,45], was weakly expressed in BMMC from 27% of the cases studied. Regarding cell adhesion molecules, all the cases studied were positive for CD11c, CD29, CD43, CD44, CD49d, CD49e, CD51, and CD54. In addition, CD11b, CD18, and CD61 expression was variable, while CD11a, CD41a, and CD42b were constantly negative. In general, these results confirm previous findings on the immunophenotype of human MC except for CD11c which in almost all tissues analyzed has been reported as negative [41–45]. Interestingly, we have recently shown that BMMC from a patient suffering from an aggressive form of systemic mast cell disease were CD41a negative while this antigen was clearly positive on the peripheral blood MC from the same patient [38]. Regarding CD18 expression, discrepant results have been reported since MC from the lung, ascitic fluid, skin, and intestinal mucosa [42,43,45] have been described as CD18 negative, while MC from the uterus have been reported to be CD18 positive [21]. In the present study, both CD18⁺ and CD18[−] cases were observed, suggesting that CD18 expression by BMMC may vary from one individual to another.

Upon comparing the BMMC from healthy subjects and patients suffering from HM, it was observed that the mean number of BMMC was significantly higher in the latter group as compared to normal subjects. However, from the immunophenotypical point of view, no significant differences were detected between both groups of individuals for any of the markers explored. These results indicate that, although increased numbers of BMMC can be detected in patients diagnosed with different

haematological malignancies, these cells are reactive since, from the phenotypical point of view, they were identical to normal human BMNC.

In summary, our results show that BMNC from both healthy controls and HM patients display a relatively heterogeneous immunophenotype. Interestingly, we have observed clear differences between the immunophenotype of BMNC and MC from other tissues. This could be due either to the heterogeneity of human MC according to their tissue localization or to the sensitivity of the method used for antigen detection.

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