Demonstration That Mast Cells, T Cells, and B Cells Bearing the Activating Kit Mutation D816V Occur in Clusters within the Marrow of Patients with Mastocytosis

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Mastocytosis is characterized by focal heterotypic clusters of mast cells and lymphocytes in the bone marrow and by a somatically acquired activating Kit mutation, D816V. The relationship of the occurrence of this mutation to the heterotypic clusters of mast cells and lymphocytes in bone marrow is unknown. We hypothesized that these two unique features of mastocytosis were related. To explore this hypothesis, laser capture microdissected mast cells, B cells, and T cells, from both lesional and non-lesional areas of bone marrow biopsy tissues from patients with mastocytosis, were examined for the D816V mutation in their DNA, using *HinfI* restriction digestion of nested PCR products amplified from extracts of dissected cells. The D816V mutation was detected in mast cells, B cells, and T cells from lesional but not non-lesional areas of bone marrow tissues. B cells obtained from lesional areas of tissue were also assessed for clonality and were found to at least represent an oligoclonal population. Thus, mast cells and lymphocytes within focal aggregates in the bone marrow of those with mastocytosis are more frequently positive for the codon 816 activating mutation. Further, the B cell population is oligoclonal, suggesting that clonal proliferation is unlikely to be the basis of clustering. (*J Mol Diagn* 2004, 6:335–342)

Focal aggregates of mast cells, and T and B lymphocytes are frequently observed within the bone marrow of patients with systemic mastocytosis.¹ Such heterotypic aggregates are considered a major histopathological feature of this disease, and the occurrence of these lesions is one of the diagnostic criteria for mastocytosis. Mast

cells within these aggregates are tryptase positive, and contain stem cell factor.¹ The basis for the formation of these aggregates is unknown.

To further characterize these heterotypic collections of mast cells and lymphocytes and to explore their etiology, we elected to determine whether the cells within these aggregates were more likely to exhibit the codon 816 activating mutation in Kit,^{2–4} which is now documented to occur in multiple hematopoietic lineages in most adult patients with mastocytosis.^{5,6} Not only are cells expressing mutated Kit believed to have enhanced proliferation on exposure to stem cell factor, the ligand of Kit,^{5,7–10} but also to exhibit enhanced chemotaxis to stem cell factor.¹¹ We thus hypothesized that mast cells and lymphocytes within these aggregates would be more likely to express the codon 816 mutation, either collecting in focal areas in response to a stimulus such as stem cell factor, or because of clonal proliferation. To further examine clonality, we sought to determine whether the population of B cells within a heterotypic aggregate was monoclonal or oligoclonal.

As will be shown, mast cells, and T and B lymphocytes obtained by laser capture microdissection of these lesions consistently demonstrated the codon 816 mutation. Mast cells and lymphocytes collected from non-lesional areas did not exhibit a codon 816 mutation within the limit of detection. Further, the clonal characteristics of B cells obtained from a lesional area of tissue were found to be at least oligoclonal. These data are most consistent with the hypothesis that these collections of cells occur by a mechanism other than clonal proliferation.

Materials and Methods

Reagents and Antibodies

AmpliTaQ DNA polymerase and dNTPs were obtained from Perkin Elmer (San Francisco, CA); TaqStart antibody from Clontech (Palo Alto, CA); *HinfI* restriction enzyme

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Table 1. Characteristics of Patients with Mastocytosis

Type of patient*	Patient	Age	Gender	Bone marrow biopsy	Tryptase (ng/ml)
Indolent systemic mastocytosis	1	39	F	normocellular, focal	26
	2	68	M	normocellular, focal	71
	3	53	F	normocellular, focal	35
	4	52	M	normocellular, focal	>200
	5	61	F	hypercellular, focal	198
Smoldering systemic mastocytosis	6	71	M	hypercellular, focal	192
	7	23	F	hypercellular, focal	>200
	8	45	M	hypercellular, focal	>200
Mastocytosis with associated/ hematologic non-mast cell disease	9	75	M	normocellular, focal, MDS	ND [†]
	10	59	F	hypercellular, CMML	110

*All positive for urticaria pigmentosa.

[†]ND equals not determined.

from New England Biolabs (Beverly, MA); and proteinase K from Roche Diagnostics (Indianapolis, IN).

Patient Characteristics

The clinical profile of the patient population is summarized in Table 1. A total of 10 patients (five females and five males, age range 23 to 75) comprising various categories of mastocytosis were studied. Five of the patients had indolent systemic mastocytosis with normocellular focal increases of mast cells. Three of the patients were diagnosed with smoldering systemic mastocytosis with hypercellular focal distribution of mast cells. Two patients had associated hematological non-mast cell disease, more specifically, myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML). The patient with MDS had a normocellular focal bone marrow involvement while the CMML patient had a hypercellular focal involvement. In all these cases, mast cells were accompanied by lymphoid aggregates. All patients exhibited urticaria pigmentosa and had tryptase levels in plasma that ranged from 26 to >200 ng/ml. The study was reviewed and approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (National Institutes of Health) and informed consent was obtained from all patients.

Processing and Immunohistochemistry of Bone Marrow Biopsies from Patients with Mastocytosis and Normal Human Appendix

Trephine core bone marrow biopsies were obtained from the posterior iliac crest. Biopsy tissues were decalcified in 0.07% (w/v) EDTA, placed in B5 fixative overnight and then embedded in paraffin. Eight- μ m-thick sections were cut on charged slides and immunophenotyping was performed with antibodies against tryptase (Dako Corp., Carpinteria, CA; 1:50 dilution), CD20 (L26, Dako, 1:200 dilution) and CD3 (Dako, 1:100 dilution) using an automated immunostainer (Ventana Medical Systems, Inc, Tucson, AZ) according to the manufacturer's paraffin-embedded tissue staining protocols. Heat-induced antigen retrieval was performed with a microwave pressure

cooker as described.¹² All primary antibodies were applied to the sections for 32 minutes.

Frozen normal human appendix tissue from a 4-year-old child preserved in OCT was obtained from Childrens National Medical Center, Washington, D.C. Eight- μ m-thick sections were cut on charged slides and immunophenotyping was performed with antibodies against CD79a (Pharmingen, San Diego, CA, 1:50 dilution). The primary antibody was applied for 30 minutes followed by incubation with the secondary antibody, goat-anti-mouse IgG biotin (Southern Biotechnology, Birmingham, AL, 1:50 dilution) for 20 minutes.

Laser Capture Microdissection

Laser capture microdissection (LCM) of cells from paraffin-embedded bone marrow biopsy tissue sections was performed using a PixCell laser capture microscope with an infrared diode laser (Arcturus Engineering, Santa Clara, CA). Briefly, the stained, dehydrated tissue section was overlaid with a transfer polymer film mounted on an optically transparent cap. Once the cells of interest (400 to 500 cells) were located, low energy laser pulses were applied leading to focal expansion, melting of the polymer, and resultant fusion of the microdissected tissue to the cap. The captured cells on the cap were digested with 40 μ l of 0.5 mg/ml DNase-free proteinase K in 1X PCR buffer for 3 hours at 50°C. Following digestion, the extract was centrifuged at 2000 rpm for 2 minutes. The proteinase K enzyme was heat-inactivated and the entire extract was used directly for PCR.

Detection of the D816V Mutation in Bone Marrow Biopsies

The D816V mutation was amplified by nested polymerase chain reaction (PCR). The primers Kit-1-F (TCCTCAACCTAATAGTGTATTACAG) and Kit-1-R (TTTGAGGACTGTCAAGCAGAGAATG) were used for the first round and Kit-2-F (TATCCTCCTTACTCATGGTCGG) Kit-2-R (AGAGAATGGGTACTCACGTTTCC) for the second round of amplification as described.¹¹ The PCR was performed in a final volume of 50 μ l containing 50 mmol/L KCl, 10 mmol/L-Tris-

Figure 1. Representative laser capture microdissection. Mast cells, lymphocytes, B cells, and T cells were obtained from lesional and non-lesional areas of bone marrow biopsy tissue from a patient with mastocytosis. Micrographs shown before and after microdissection and cells captured on cap.

HCl, 200 $\mu\text{mol/L}$ of each dNTP, 5 units AmpliTaq DNA polymerase and 0.5 $\mu\text{mol/L}$ of each primer. One μl of the PCR product from the first round was used as the template for the second round of PCR. The rest of the components in the reaction mixture were the same as described above for the first round. The same temperature conditions were used for the first and second round touchdown PCR. There was an initial 95°C 1-minute denaturation step, followed by denaturation at 94°C for 1 minute and an extension at 72°C for 2 minutes; for the first cycle, annealing was at 68°C for 30 seconds which dropped down to 58°C at the end of the fifth cycle at the rate of 2°C per cycle. The annealing temperature for the remaining 23 cycles was 58°C. The PCR ended with a 5-minute extension at 72°C. The PCR was performed

on a PTC-100 programmable thermal cycler with a hot bonnet (MJ Research, Inc., Watertown, MA).

To detect the D816V mutation which creates an additional *HinfI* site, the second round PCR product was digested with the restriction enzyme *HinfI* for 2 hours at 37°C. The digested product was electrophoresed on a 20% TBE polyacrylamide gel (Novex, San Diego, CA). Predicted fragment sizes for the homozygous wild-type *c-kit* sequence were 68 and 43 bp and for the heterozygous mutated sequence were 68, 54, 43, and 14 bp. As the mutation is found on only one of the two alleles, cells with mutations also have the wild-type fragment of 68 and 43 bp generated from the wild-type copy of the *c-kit* gene. Control cell lines, HMC-1.1 (homozygous for the

Figure 2. Detection of the D816V mutation in mast cells, lymphocytes, B cells, and T cells in patients with different categories of mastocytosis. **A:** Indolent mastocytosis. **B:** Smoldering mastocytosis. **C:** Mastocytosis with associated hematological non-mast cell disease. Analysis of T cells and B cells in lesional areas only. A non-lesional area is defined as an area where there are a sparse number of mast cells. L, denotes lesional area; N, denotes non-lesional area.

absence of the Kit D816V mutation), and HMC-1.2 (heterozygous for the Kit D816V mutation) were analyzed in parallel.

Determination of the Sensitivity of Detection of the D816V Mutation

To determine the sensitivity of detection of the D816V mutation in the assay, increasing numbers of mutant HMC1.2 cells (0,5,10,50,100) were mixed with wild-type HMC1.1 cells (100,95,90,50,0). The cells were then digested with proteinase K, followed by nested PCR and *HinfI* restriction digestion to detect the D816V mutation as described above. The level of detection was quantified by gel fluorescence imaging.¹¹

Amplification, Cloning, and Sequencing of Rearranged Immunoglobulin from B Cells in Bone Marrow and Control Human Appendix Sections

The complementarity determining-region 3 (CDR3) of the rearranged immunoglobulin heavy chains in CD20-stained B cells from a lesional area of bone marrow from a patient with indolent mastocytosis and in CD79a-stained control normal appendix was amplified using previously published primers directed at conserved sequences present in all JH region genes (JH) and the framework region 3 (FR3).^{13,14} Hotstart PCRs were per-

formed using TaqStart antibody. Forty cycles of amplification consisting of an initial denaturation at 95°C for 2 minutes, followed by denaturation at 94°C for 1 minute, elongation at 72°C for 1 minute, and annealing at 56°C for 30 seconds were performed with DNA from microdissected tissue (the total 40 μ l of crude extract from laser capture microdissected cells was added as template). The PCR product was electrophoresed on a 20% TBE polyacrylamide gel (Novex).

The purified PCR products were cloned into the pCR4-TOPO vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and transformed into One Shot TOP 10 chemically competent *E. coli* (Invitrogen) according to the manufacturer's instructions. Plasmid DNA recovered from white (recombinant) colonies was digested with *EcoRI* to confirm the presence of the insert. The digested product was electrophoresed on a 20% TBE polyacrylamide gel (Novex). The cloned inserts were sequenced using T3 primers (5'-ATTAACCCTCACTAAAGGGA-3') and the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA); and run on the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Sequence analysis was performed by MacVector Software version 7.0 (Accelrys, San Diego, CA). HCDR3 lengths were defined as the number of amino acids after the generally conserved VH amino acid Arg 94 and before the conserved JH amino acid Trp 103. The DH segment used was identified as having a stretch of at least seven nucleotides of uninterrupted identity with a germline gene sequence or eight or more identical nucleotides interrupted by one mismatch.

Figure 3. Sensitivity of detection of the D816V mutation by nested PCR. Results of *HinfI* restriction digestion of mixtures of mutant HMC1.2 (0,5,10,50,100) and wild-type HMC1.1 cells (100,95,90,50,0) with the corresponding gel fluorescence graph. Data are representative of three experiments.

Results

Laser Capture Microdissection

To verify that laser capture microdissection could be applied to the study of mastocytosis lesions, mast cells, lymphocytes, B cells, and T cells were obtained by LCM from dehydrated and immunohistochemically stained bone marrow biopsy sections from patients with mastocytosis and examined (Figure 1). The upper panel in Figure 1 shows a representative picture of mast cells obtained from both lesional and non-lesional areas of tryptase-stained bone marrow biopsy tissue from a patient with mastocytosis. An extensive accumulation of tryptase-stained mast cells is shown in the lesional areas of the biopsy tissue compared to sparse numbers seen in the non-lesional area. Lymphocytes were usually found clustered adjacent to the mast cell infiltrates in lesional areas of tryptase-stained bone marrow biopsy tissues from patients with mastocytosis (Figure 1, middle panel). To confirm the results obtained in lymphocytes, tissues were immunostained with either anti-CD 20 or anti-CD3 mAb. As can be seen in the lower panel of Figure 1, both B cell and T cell aggregates were found in the lesional areas of bone marrow biopsy tissues with B cells generally found in greater numbers than T cells.

Detection of the D816V Mutation

To assess the role of the activating Kit mutation D816V in the pathology of mastocytosis, DNA obtained from discrete populations of cells from both lesional and non-lesional areas of bone marrow tissues by LCM were examined for the mutation. As can be seen in Figure 2A, the D816V mutation was detected in mast cells, lymphocytes, B cells, and T cells from lesional areas of bone marrow biopsy sections in all four patients with indolent mastocytosis. In contrast, the D816V mutation was not detected in non-lesional bone marrow cells in these patients. Similarly, the D816V mutation was detected in mast cells, lymphocytes, B cells, and T cells in the lesional but not in the non-lesional areas of bone marrow biopsies from patients with smoldering mastocytosis (Figure 2B) and in two patients with mastocytosis with associated hematological non-mast cell disease (Figure 2C).

Sensitivity of Detection of the D816V Mutation

To determine the minimum number of mutant cells that can be detected by nested PCR, increasing numbers of mutant HMC 1.2 cells (0,5,10,50,100) were mixed with wild-type HMC 1.1 cells (100,95,90,50,0). As shown in Figure 3, the D816V mutation was detectable when at least 10 HMC 1.2 cells/per 100 total cells were present as revealed by three bands at 68, 54, and 43 bp (the 14-bp band is not shown in this figure). These results were also quantified by gel fluorescence (Figure 3). There was a fluorescence of 0.01 with 5 cells (band was not visible), 0.1 with 10 cells, 0.5 with 50 cells, and 0.55 with 100 cells. Therefore, as a general interpretation, the absence of detection of the D816V mutation in cells obtained from non-lesional marrow means that the mutational frequency is less than 1 in 10 cells.

Molecular Analysis of Clonality of B Cells

To explain how lymphocytes cluster in bone marrow tissue, assessment of IgH VDJ gene rearrangements in B cell clones obtained from lesional areas of a bone marrow biopsy from a patient with mastocytosis was performed. Approximately 400 to 500 B cells obtained by LCM from a lesional area of a bone marrow biopsy from one patient with indolent mastocytosis were examined (Figure 4A). Similar numbers of cells were obtained from normal human appendix. Analyses of the IgH VDJ gene rearrangements in B cells from this patient and from normal human appendix were then performed. According to PCR-based criteria established from a previous study,¹⁵ the occurrence of one or two distinct bands is indicative of B cell monoclonality. However, B cell monoclonality was not observed in this patient. Oligoclonality, defined as three or more bands, was demonstrated both in B cells from the patient with mastocytosis and in normal human appendix (Figure 4B). These results were further confirmed by sequencing. Forty-five sequences from this patient with varying HCDR3 lengths fell into five groups. Twenty-four sequences obtained from normal human appendix

Figure 4. Molecular analysis of clonality of B cells from bone marrow biopsies from a patient with mastocytosis. **A:** B cells obtained from lesional areas of a bone marrow biopsy from a patient with mastocytosis and from normal human appendix by LCM. **B:** Starting PCR product of B cells (400 to 500 cells) as well as cloned CDR3 obtained from microdissected B cells from a patient with mastocytosis and from normal human appendix. **C:** Corresponding IgH CDR3 VDJ sequences from the patient. The nucleotide and deduced amino acid sequences are shown with the segments encoded by D genes (**regular font**) and n nucleotides (**bold**) indicated. GenBank Accession Numbers for these and control human appendix cell sequences are given in Table 2.

fell into four groups (Table 2). D and J gene usage in the cells from the patient with mastocytosis and in cells from normal human appendix was comparable except that within cells from groups 1 and 5 from the patient with mastocytosis, the rarely expressed JH1 gene was rearranged. Group 3 identified in normal human appendix used the infrequently expressed JH2 gene. The IgH VDJ sequences found in B cells from the patient with mastocytosis are shown in Figure 4C.

Discussion

Bone marrow biopsies in mastocytosis are characterized by focal or diffuse accumulations of mast cells in paratrabecular and perivascular regions often associated with lymphoid aggregates.^{16,17} However, neither a comprehensive assessment of the activating Kit mutation D816V in mast cells and lymphocytes in bone marrow from various categories of these patients, nor the explanation for the associated lymphocyte clustering found in lesions of tissues in this disease has previously been determined. This study did not examine other cell types not consistently found in these lesions including adjacent myeloid cells.

In the present study, the D816V mutation was detected in mast cells, B cells, and T cell specifically in the lesional but not in the non-lesional areas of bone marrow biopsies from different categories of patients with mastocytosis. Our mutation sensitivity study indicates that mutation detection requires that at least 10% of the cell population contain the D816V mutation (Figure 3). While we cannot rule out the possibility that there may be a few mast cells present in the microdissected lymphoid aggregates, it is highly unlikely that these approach 10% of the total cells because adjacent tryptase staining of microdissected slides indicates few, if any, mast cells present in the selected areas. Thus, as may be seen in the upper panel in Figure 1, there is an extensive infiltration of tryptase-positive granulated mast cells clearly stained and with no evidence of other cell types in the lesional area of tryptase-stained bone marrow biopsies. This cluster of mast cells surrounds a lymphocyte population that was subsequently microdissected. We believe it is clear from this tryptase-stained slide that the lymphocyte cluster contained a negligible number of mast cells (at most less than 10, nowhere near the 50 cells required to detect the mutation). Also of note is that mast cells are mainly located in the periphery, which makes it easier to avoid microdissecting these cells with other cells. On closer examination, we believe one can also observe that most if not all of these mast cells were, in fact, not included in the microdissected sample as they remain in the tissue. Application of this technique has as a primary advantage, the ability to directly observe cell types for microdissection. It is our conclusion that between direct observation and the actual dissected materials provided for visualization that we avoided significant mast cell contamination in the lymphoid population that was microdissected and that the results presented are valid. Finally, these results are in agreement with a previous report where mast cells in a patient with mastocytosis associated with CMML were positive for a codon 816 mutation.¹⁸

These findings of the detection of D816V mutation in cells within clusters in bone marrow are consistent with the hypothesis that this mutation occurs in a precursor which gives rise to mast cells as well as other cell lineages.⁶ It may be speculated that precursors in the lesional area of bone marrow tissue express the D816V mutation, thereby contributing to the aberrant proliferation of these cells into mast cells and lymphocytes.^{3,5} Alternatively, mutant precursor cells may preferentially migrate to stem

Table 2. Summary of IgH VDJ and HCDR3 Sequencing Results from a Patient with Mastocytosis and Normal Human Appendix

Group	No. cloned	HCDR3 length*	D-gene	J-gene	No. basepairs D-gene differs from germline	Accession Number†
Patient bone marrow						
Group 1 (AC100)	28	17	D2 –8	J1	0/24	AY177274
Group 2 (AC103)	9	14	D3 –10	J6	5/28	AY177275
Group 3 (AC151)	3	9	D6 –19	J4	2/15	AY177277
Group 4 (AC148)	3	7	D3 –16	J4	3/14	AY177278
Group 5 (AC146)	2	12	D3 –22	J1	0/27	AY177276
Human appendix						
Group 1 (H3-001)	4	10	D2 –15	J4	3/15	AY177279
Group 2 (H3-002)	1	14	D3 –16	J4	2/18	AY177280
Group 3 (H3-008)	6	14	D1 –26	J2	0/10	AY177281
Group 4 (H3-003)	13	8	Unknown Only 6 bp long	J6		AY177282

*Length of heavy chain third complementarity determining region (HCDR3) in amino acids.

†The sequences were submitted to GenBank and assigned the Accession Numbers shown.

cell factor producing cells in tissue.¹¹ A side benefit of this observation is the ability to target analysis for codon 816 mutations to cell clusters in archived paraffin blocks, as the identification of such mutations is a diagnostic criterion for mastocytosis.

The explanation for lymphocyte clustering within lesions in tissue is unknown. To address this question, clonality of B cells from lesional areas of a bone marrow biopsy from a patient with mastocytosis was examined. IgH VDJ gene rearrangements in B cell clones from a patient with indolent mastocytosis showed at least an oligoclonal population of B cells in lesional areas of bone marrow. Similar results were also obtained in B cells from normal human appendix. Taken together, these results suggest that clonal proliferation may not be the only explanation for B cell clustering observed in lesional areas of tissues in this disease.

The mechanism(s) by which B cells are preferentially being attracted to lesional areas in the bone marrow are currently unresolved. It is plausible to assume that early B cells which have been shown to constitutively express Kit on their surface¹⁹ migrate to areas in which stem cell factor-producing mast cells reside. Indeed, it has recently been demonstrated that mast cells produce stem cell factor in bone marrow from patients with mastocytosis.¹ Another possibility is that B lymphocytes may also be attracted to lesional areas by some other mast cell-derived mediators such as cytokines and chemokines. These issues are currently being investigated in our laboratory.

The presence of lymphoid aggregates in association with mast cells may also suggest that a lymphoproliferative process is occurring. Although patients with mastocytosis do not characteristically develop lymphoid malignancies, in rare cases, mastocytosis has been associated with lymphomas,²⁰ acute lymphocytic leukemias,^{5,21} hairy cell leukemia,²² and Castleman's disease.²³

Thus, data in this paper demonstrate that cells within heterotypic clusters of mast cells and lymphocytes exhibit a codon 816 activating mutation. One explanation of the clustering is the preferential chemotaxis of mast cells and early lymphocytes to sites of stem cell factor production, possibly by aggregates of stem cell factor-producing

ing mast cells. Finally, this ability to identify codon 816 mutations in mast cell-lymphocyte clusters provides a useful diagnostic adjunct.

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