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Bone Marrow Lymphoid Aggregates in Malignant Lymphomas

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Aim To examine the usefulness of molecular analysis of IgH gene rearrangement in assessment of clonality in bone marrow biopsies with lymphoid aggregates (LA) and/or nodular lymphoid hyperplasia (NLH) in patients with different subtypes of malignant lymphomas.

Method Five hundred and twenty nine samples of bone marrow biopsies, taken in a staging procedure at the time of the initial presentation of illness, were processed routinely. Results were grouped in positive, negative, and cases with LA or NLH. In 43 samples with present LA/NLH, polymerase chain reaction (PCR) analysis of the CDR3 region of immunoglobulin heavy chain gene (IgH) for B-cell clonality was performed.

Results Bone marrow malignant lymphoma infiltrates were present in 33.8% of lymphoma cases. The incidence of LA/NLH in bone marrow was 8.1%. LA/NLH were more frequently found in patients with extranodal disease and aggressive subtypes of B cell non-Hodgkin lymphoma (B-NHL), but there was no significant difference among the incidence according to the biological behavior of malignant lymphoma ($P = 0.232$). Results of IgH-CDR3 region PCR analysis showed a monoclonal pattern in 1 case of Hodgkin lymphoma and in 1 control case, an oligoclonal pattern in 2 cases of extra nodal B-NHL, whereas all other had polyclonal.

Conclusion The results support our initial hypothesis that LA/NLH could be differentiated from malignant infiltrates in bone marrow staging procedure of malignant lymphoma by topographic pattern and histocytomorphology of LA/NLH. Surprisingly, our patients with aggressive B-NHL, nodal, as well as extranodal, had LA/NLH in bone marrow biopsies more often than patients with indolent B-NHL.

Bone marrow, an organ of lymphopoiesis, normally contains up to 20-35% lymphocytes. They are either diffusely dispersed or formed as nodular lymphoid aggregates. Reactive lymphoid lesions include lymphocyte aggregates, benign follicles with germinal centers, or reactive nodular polymorphous lymphohistiocytic lesions, ie nodular lymphoid hyperplasia (NLH), and polyclonal immunoblastic proliferations (1).

Lymphocyte aggregates have been reported to occur in between 1% and 60% of normal bone marrow specimens (2). They are common and considered as incidental findings in patients older than 70 years and in women. However, lymphoid aggregates could be associated with certain non-hematological diseases, such as collagen vascular diseases, immune disorders, drug therapy, and viral infections (2).

Morphological features of benign lymphoid aggregates (Fig. 1) are well defined (2). They are circumscribed, usually few in number, average 0.4 mm in size, and polymorphous in cell population (lymphocytes, plasma cells, mast cells, occasional histiocytes, and capillaries) within a delicate network of reticular fibers. They are distributed at random in the marrow, rarely paratra-

becular. LA are composed of B and T lymphocytes, although T lymphocytes usually predominate.

Germinal centers (Fig. 2) with a mantle zone of small lymphocytes are present in a small proportion (about 5%) of lymphoid aggregates, and are usually benign in nature. They are more often found in female patients with underlying immune disorders. However, they are not specific for benign infiltrates. Rarely, non-Hodgkin lymphomas, such as splenic marginal zone lymphoma, may exhibit reactive germinal centers as part of lymphomatous infiltrate in the bone marrow (2).

The term nodular lymphoid hyperplasia (NLH) is used when more than four lymphoid nodules or aggregates are seen in a low power field, or if the nodules are greater than 0.6 mm in their largest diameter (Fig. 3) (2). Lymphocytic nodules of various size and nodular lymphocytic hyperplasia are suspicious of lymphoma infiltration, especially if they are located paratrabecularly, around large sinuses, or if they contain fat cells.

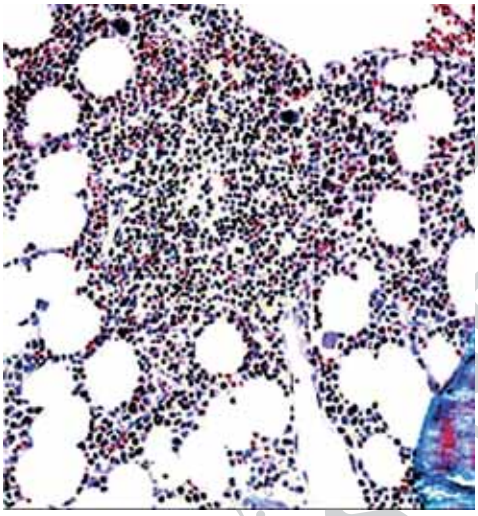

Figure 1. Lymphoid aggregate in the bone marrow, hematoxylin-eosin staining (×10 magnification).

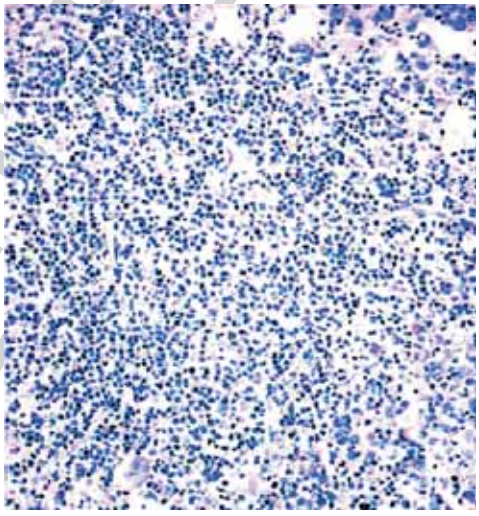

Figure 3. Nodular lymphoid hyperplasia in the bone marrow, Giemsa staining (×10 magnification).

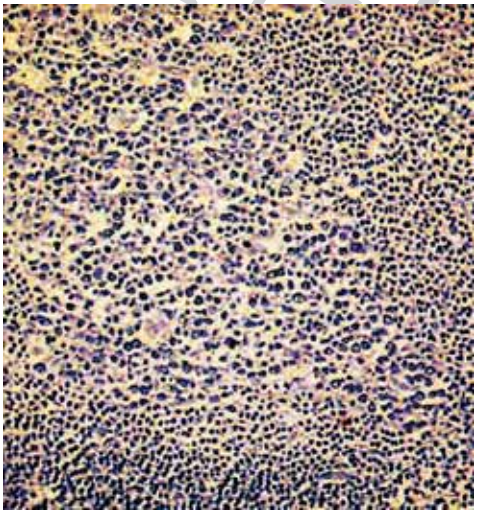

Figure 2. Lymphoid aggregate germinal center in the bone marrow, Giemsa staining (×40 magnification).

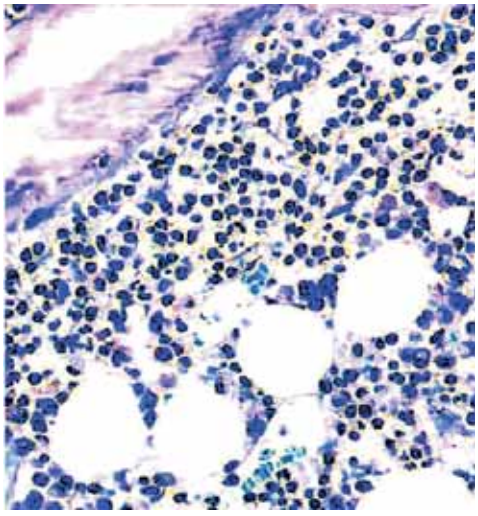

Figure 4. Paratrabecular lymphoid aggregate in the bone marrow, Giemsa staining (×60 magnification).

There are five major patterns of bone marrow infiltration in patients with non-Hodgkin lymphoma: focal nonparatrabecular, focal paratrabecular (Fig. 4), intrasinusoidal, diffuse interstitial, and diffuse solid (1,3-9). The follicular pattern of bone marrow involvement by follicular lymphoma is defined as the presence of malignant lymphocytes arranged in follicles in the intertrabecular position, with less than 10% of the tumor in the paratrabecular location. This is important to recognize, because it can be misinterpreted as a benign lymphoid aggregates (3). Lymphoma cells vary with the subtype of lymphoma and generally exhibit a uniform cellular population.

Long term studies revealed that younger patients with numerous lymphocytic aggregates may eventually develop a clonal disease, usually a low grade non-Hodgkin lymphoma. This occurs especially in patients with nodular lymphocytic hyperplasia (10).

Lymphoid aggregates in patients with AIDS are distinctive. The lymphoid aggregates are large and ill defined, and the cells exhibit nuclear atypia with an additional mixture of immunoblasts. They can be misinterpreted as non-Hodgkin or Hodgkin lymphoma (11).

Furthermore, patients with known lymphoproliferative diseases may have lymphoid aggregates in the bone marrow. These are considered to be normal or physiological findings, with no association with the underlying disease if they are small and single. However, when they are larger or numerous, ie if they are in the form of NLH, findings are controversial and it is believed that they are neoplastic from the beginning (12).

Although morphologic and phenotypic clues are helpful in distinguishing benign from malignant lymphoid aggregates in bone marrow biopsies, in some cases it is not possible to make a definitive diagnosis (13). We examined the frequency of lymphoid aggregates or NLH in bone marrow biopsy specimens in patients with different subtypes of malignant lymphomas. However, since there is no definitive numerical and morphological "cutoff" between lymphoid aggregates/nodular lymphoid hyperplasia (NLH), and malignant lymphoid aggregates in malignant lymphoma, we examined the usefulness of molecular analysis of IgH gene rearrangement in the assessment of clonality in bone marrow biopsies with lymphoid aggregates and/or NLH in patients with different subtypes of malignant lymphomas.

Patients and Methods

Bone marrow biopsies of 529 patients with malignant lymphoma, classified according to the classification of the World Health Organization (14), were evaluated during 1997-2001 period.

All biopsies were done at the time of initial diagnosis, as a routine hematological staging procedure which also included cytomorphology, flow cytometry, polymerase chain reaction (PCR), and other relevant clinical and laboratory procedures.

The bone marrow biopsies were processed routinely with hematoxylin-eosin, Giemsa, PAS, Prussian blue, Goldner trichrom stain for collagen and Gomori silver reaction for reticular fibers. The immunohistochemistry was not done because all samples were embedded in methylmethacrylate and therefore unsuitable for immunohistochemistry. According to the revisited analysis of bone marrow pathology in malignant lymphomas, the results were grouped into positive cases (histologically evident infiltration of malignant lymphoma), negative cases (histologically without infiltration of malignant lymphoma), and cases with lymphoid aggregates or NLH.

In bone marrow samples with present lymphoid aggregates or NLH, DNA was isolated from all 43 resin embedded block sections and used for PCR analysis of CDR3 region of IgH gene rearrangement (15,16). Six bone marrow biopsies with lymphoid aggregates or NLH, 5 patients with non-hematological diseases, and 1 patient with the diagnosis of malignant epithelial tumor were used as control samples.

DNA Isolation from Resin Blocks

Two formalin fixed, resin-embedded, 10 μm sections were deplastinized with acetone and washed with absolute ethanol. Samples were then incubated overnight at 37 °C in 200 μL of digestion buffer (50 mmol/L Tris-HCl (pH 8.5), 1mM EDTA, 0.5% Tween (Sigma, Deisenhofen, Germany) with proteinase K (0.3 mg/mL). Proteinase K activity was stopped the next day by boiling samples at 95 °C for 8 minutes (15).

Analysis of CDR3 Region of Immunoglobulin Heavy Chain Gene Rearrangement

Polymerase chain reaction (PCR) strategy for the amplification of CDR3 region of heavy chain immunoglobulin gene uses one pair of primers: sense primer Fr3 and antisense primer JHcon. PCR reaction was performed in 25 μL volume with 1μg of paraffin extracted tissue DNA, 10 pmol of primer Fr3 and 10 pmol of primer JHcon in buffer containing 10 mM Tris (pH 9.1), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L of each dNTP and 0.5 U Taq polymerase (AmpliTaq DNA Polymerase, 5 U/μL, Roche Diagnostics GmbH, Indianapolis, IN, USA). Conditions for amplification were: 95 °C for 3 minutes, 45 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and elongation (72 °C for 30 s), followed by 72 °C for 7 minutes. The PCR products were loaded on Spredex EL 300 high resolution gel (Elchrom Scientific AG, Cham, Switzerland) and stained with Sybr Gold (Sybr Gold Nucleic Acid Gel Stain, Molecular Probes, Eugene, OR, USA). Sizes of the products were between 80 and 150 bp (16).

Statistical Analysis

Non-parametric Fisher exact test was used to compare the groups, using STATISTICA software, version 5.0 (StatSoft Inc., Tulsa, OK, USA). The level of statistical significance was set at $P<0.05$.

Results

In 529 patients, the following histological diagnoses on tumor tissue were established: 240 cases of nodal B cell non-Hodgkin lymphoma (B-NHL), 34 cases of T cell non-Hodgkin lymphoma (T-NHL), 49 cases of anaplastic large cell lymphoma (ALCL), 93 cases of extranodal B-NHL (EXTRA) – all localizations, and 113 cases of Hodgkin lymphoma (MH). The results of bone marrow pathology in 529 cases of malignant lymphomas are summarized in Figure 5. The malignant infiltrates were present in 179 (33.8%) of bone marrow samples, in 134 (55.8%) in nodal B-NHL, and in 27 (29.0%) extranodal B-NHL. Infiltrates of T-NHL and ALCL were found in 11 (32.4%) and 3 (6.1%) samples of these tumors, respectively. The malignant infiltrates of MH were less common than infiltrates of NHL. All together, lymphoid aggregates/NLH in bone marrow were present in 43 (8.1%) samples. Surprisingly, they

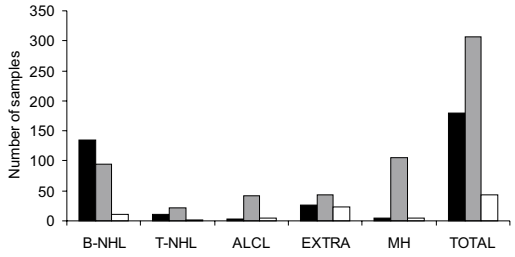

Figure 5. Frequency of lymphoid infiltrates in bone marrow in malignant lymphomas. B-NHL – nodal B cell non-Hodgkin lymphoma; T-NHL – T cell non-Hodgkin lymphoma; ALCL – anaplastic large cell lymphoma; EXTRA – extranodal B-NHL, MH – Hodgkin lymphoma. Closed bars – positive cases (infiltration of malignant lymphoma), gray bars – negative cases (without infiltration of malignant lymphoma), open bars – cases with lymphoid aggregates/nodular lymphoid hyperplasia.

were present in only 11 of 240 patients with nodal B-NHL, but in 23 of 93 patients with extranodal disease presentation. In patients with T-NHL, ALCL, and MH, lymphoid aggregates/NLH were present in 1 (2.9%), 4 (8.2%), and 4 (3.5%) of all bone marrow samples, respectively.

Table 1 summarizes the results of frequencies of lymphoid aggregates/NLH in B-NHL according to biological behavior of malignant lymphoma subtype. Regarding biological characteristics of B-NHL, lymphoid aggregates/NLH were more frequently found in aggressive types than in indolent subtypes. In patients with extranodal disease and aggressive subtypes of B-NHL, lymphoid aggregates/NLH were found in 18 (78.3%) samples, but there was no significant difference among the groups according to biological behavior of malignant lymphoma (Fisher exact test, $P=0.232$).

Results of IgH-CDR3 PCR analysis of bone marrow samples with lymphoid aggregates/NLH are summarized in Table 2. One case of MH and 1 control case had a monoclonal pattern (one band on the gel), two cases of extranodal

Table 1. Frequency of lymphoid aggregates/nodular lymphoid hyperplasia in 34 samples of bone marrow biopsies in patients with nodal and extranodal B cell non-Hodgkin lymphoma (B-NHL) according to biological behavior of malignant lymphoma

Lymphoma behavior	No. of bone marrow samples with lymphoid aggregates/nodular lymphoid hyperplasia*		
	nodal	extranodal	total
Indolent	5	5	10
Aggressive	6	18	24
Total	11	23	34

*P=0.232, Fisher exact test.

Table 2. Results of PCR analysis of IgH gene rearrangement of 43 bone marrow samples with lymphoid aggregates/nodular lymphoid hyperplasia in patients with malignant lymphomas*

Clonality	B-NHL (n=11)	ALCL (n=4)	MH (n=4)	Extranodal (n=23)	Control (n=6)
Polyclonal	10	4	3	16	5
Monoclonal			1		1
Oligoclonal				2	
Not done	1			5	

*Abbreviations: B-NHL – nodal B cell non-Hodgkin lymphoma; ALCL – anaplastic large cell lymphoma; MH – Hodgkin lymphoma; extra nodal – extra nodal B cell non-Hodgkin lymphoma.

B-NHL were oligoclonal (2 to 5 bands on the gel), whereas B-NHL, ALCL, and the rest of extranodal B-NHL cases were polyclonal (more than 5 bands on the gel).

Discussion

We found infiltration in 41.9% of bone marrow biopsies from patients with malignant lymphoma. Bone marrow malignant infiltrates were present in 33.8% of the samples, more common in NHL than MH cases. In contrast, the incidence of lymphoid aggregates/NLH in the bone marrow was 8.1%, more frequently found in aggressive, particularly extranodal, than in indolent B-NHL subtypes.

Discrete nodular infiltrates of small cell malignant lymphomas simulating benign hyperplasia can be found in chronic lymphocytic leukemia, follicular lymphoma, and lymphoplasmacytic lymphomas (1,17). Also, a frequent problem is discordant morphology between the lymph node and bone marrow involvement by NHL, causing diagnostic difficulties (18-20). The PCR for the IgH gene locus may help distinguish benign from malignant bone marrow lymphoid aggregates, although the presence of false-negative samples may be related to the lack of lymphocytes in bone marrow samples or the failure of PCR strategy amplifying only the selected immunoglobulin heavy chain gene region (13,21-23).

A number of histological parameters have emerged with a distinctive significance for distinguishing reactive focal lymphoid aggregates and malignant lymphomas. Histotopography, content of reticulin fibers, cytology, and immunohistochemistry are the most important among them (24). Our experience and the literature reports show that each subtype of lymphoma can be recognized by the distinct combination of a growth pattern and a particular cytological composition (5-9). Conlan et al (25) found abnormalities in 41% of bone marrow samples in patients with NHL, 32% had lymphoma infiltrates in their bone marrow samples, and 9% had benign LA. The infiltrates were more common in low grade than high grade lymphomas and equally common in B- and T-NHL (25). As reported previously and in our work, the finding of positive bone marrow infiltrates were more common in NHL than in MH (26,27). The location within the marrow space is thought to be a crucial factor in distinguishing between benign and low-grade malignant lymphoid infiltrates (8,28). Deposits of low-grade NHL involving the bone marrow mostly assume a paratrabecular pattern of infiltration at some point. This is in direct contrast with the pattern of bone marrow infiltration shown by benign lymphoid aggregates (28). The frequency of the lymphoid aggregates or NLH found in bone marrow biopsies in our study is similar to findings of other studies (25,29).

Additional methods, such as histochemistry, immunohistochemistry, flow cytometry, and PCR are used for detection and evaluation of lymphoid aggregates in bone marrow biopsy specimens (1,13,29-33). Immunohistochemistry on bone marrow samples, in our opinion, should be considered with caution. Very few monoclonal antibodies (ie epithelial but not lymphoid and granulocytic markers) give satisfactory results on resin embeded samples. Bone marrow samples, decalcified with an HCl-based reagent, are not suitable for immunohistochemistry. The EDTA-based reagents are the best decalcifying solutions so far (24). The focal pattern of bone marrow lymphoid infiltration may be related to the lack of lymphocytes in bone marrow aspirates and false negative samples, therefore bone marrow biopsy, flow cytometry, and/or PCR are better in the detection of B cell infiltration than cytomorphology alone (13,29).

Finding of the same IgH gene rearrangement pattern in cases with discordant morphology in the bone marrow as in the lymph node may serve as a proof of bone marrow involvement by the same clone of malignant cells (18,20). Monoclonality was detected in a single control case: a patient with renal cell carcinoma and morphologically benign lymphoid aggregates. The monoclonal pattern was also detected in a bone marrow specimen in one case of MH and the oligoclonal pattern in two cases of extranodal B-NHL. In a sub-

sequent follow up, no evident development of malignant lymphoma was seen in these cases.

Adequate analysis should also involve lymph node biopsies to avoid possible problems in analyzing bone marrow samples of patients with malignant lymphomas. The good reproducibility of histological parameters for distinguishing reactive focal lymphoid aggregates and malignant lymphomas shown in our study could be evaluated by analyzing the clonality of malignant lymphoma infiltrates. In our opinion, the algorithm for evaluating lymphoid aggregates/NLH in the bone marrow of patients with malignant lymphomas should be a precise analysis of cytomorphology, as well as topographic localization pattern of infiltrates, and in rare indistinct cases, flow cytometry or molecular testing. The finding of clonal proliferation should be interpreted with caution, because monoclonality does not necessary mean malignant behavior of the disease.

We found that the group with extranodal disease was the most interesting because 23 of 93 patients had lymphoid aggregates/NLH in the bone marrow as well as a polyclonal pattern. The selected CDR3 region of the immunoglobulin heavy chain gene is a hypervariable region with the greatest number of mutations, considered a good target for a clonality test. However, as rearrangements can occur in other gene segments, one has to be aware of the theoretical limitations of the test. This opens questions of the adequacy of the techniques; in our experience, there is a fair correlation of the CDR3 region results with immunophenotyping methods of B-cell clonality determination.

In conclusion, by analyzing the characteristics of histotopography and cytology, it is possible to differentiate lymphoid aggregates and NLH in bone marrow staging procedure, particularly in patients with low grade NHL. The molecular analysis of the CDR3 region of IgH gene rearrangement in the assessment of clonality correlates with the morphologic clues for distinguishing benign from malignant lymphoid aggregates in bone marrow biopsies.

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