

Reactive and neoplastic lymphocytes in human bone marrow: morphological, immunohistological, and molecular biological investigations on biopsy specimens

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Abstract

Background—Slight, diffuse or focal lymphocyte proliferation is relatively common in bone marrow biopsy specimens. It may be impossible to determine whether this represents a reactive lymphocytosis or low grade non-Hodgkin lymphoma (NHL) on the basis of routine investigations alone.

Aim—To investigate the supplementary use of molecular biological techniques in this situation.

Methods—529 formalin fixed, paraffin embedded bone marrow biopsy specimens from the iliac crest were subjected to histological and immunohistochemical staining to determine the number and nature of the lymphocytes present. The cases were divided into three groups according to the lymphocyte count: normal (< 10% of nucleated bone marrow cells), slightly increased (10–30%), and markedly increased (> 30%). All of the last group could be diagnosed as NHL from the morphological findings alone. The clonality of rearrangements of the IgH and TCR γ genes was investigated by polymerase chain reaction (PCR).

Results—Monoclonality was observed in 7.5% of the 372 cases with a normal lymphocyte count, in 50% of the cases with a modest increase in lymphocyte numbers (suggesting a diagnosis of low grade NHL not detected by immunostaining), and in 77% of the cases with markedly increased lymphocyte numbers.

Conclusions—If PCR is used in addition to the immunohistochemical investigation of bone marrow biopsies, considerably more cases of NHL can be identified, making this of particular use in staging and detection of recurrences.

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Keywords: non-Hodgkin lymphoma; reactive lymphocytosis; polymerase chain reaction; bone marrow

An increase in the number of lymphocytes in the bone marrow is a relatively common finding in routine diagnostic pathology. Focal infiltration is much more common than diffuse infiltration (70% as opposed to 30%) in patients with a history of extramedullary non-Hodgkin lymphoma (NHL).¹ So called benign lymphocytic aggregates² are found,

depending on age and sex, in certain non-neoplastic diseases, for example, rheumatic disorders.³ It is often impossible to determine whether focal lymphocytic infiltrates are malignant or benign on the basis of morphological and immunohistochemical findings alone, although peritrabecular infiltration is considered to be a fairly reliable criterion for the diagnosis of follicular lymphoma.^{1 4 5} Because, by definition, the finding of even minimal bone marrow infiltration by a malignant NHL is indicative of generalisation of the disease and therefore carries therapeutic and prognostic implications, it is of the utmost importance to identify the nature of even a slight diffuse or focal increase in bone marrow lymphocytes.

The following three situations may cause particular problems, and both immunohistochemical and molecular biological analysis are nearly always necessary for a clear diagnosis to be made:

- (1) Disseminated, mainly small, non-peritrabecular lymphocyte foci are found in the bone marrow of a patient with a history of extramedullary NHL, but these lymphocytes account for less than 10% of nucleated bone marrow cells.
- (2) The bone marrow shows a small, diffuse or focal increase in lymphocyte numbers of up to 10–30% of nucleated bone marrow cells in a patient with no history of lymphoid neoplasia.
- (3) Technical problems with the trephine biopsy specimen make it inadequate for diagnostic purposes, or it may be too small for the number of lymphoid cells to be assessed accurately.

Up to now there has been no report of a systematic investigation into the proportion of cases in which monoclonal lymphocyte proliferation is responsible for a modest increase in lymphocytes in patients without evidence of extramedullary NHL. We have undertaken such a study using molecular biological techniques. Unlike Southern blot hybridisation, the polymerase chain reaction can be used to investigate very small specimens of formalin fixed, paraffin embedded tissue. This method was therefore chosen to characterise the lymphoid cell population in bone marrow biopsy specimens by investigating the incidence of monoclonal rearrangements of the immunoglobulin heavy chain (IgH) gene and the T cell antigen receptor (TCR) gene.

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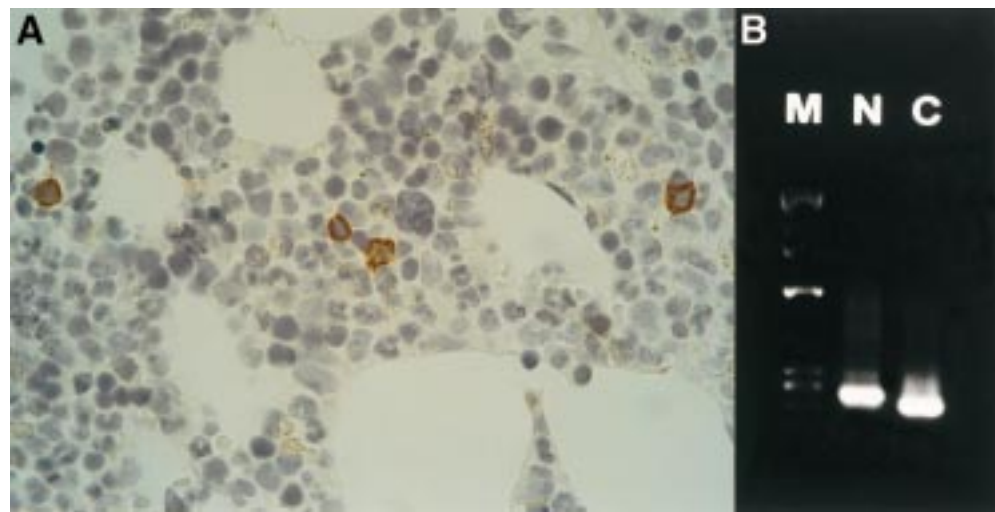


Figure 1 Bone marrow with normal lymphocyte numbers. (A) Immunohistochemical identification of B cells with the antibody L26 (anti-CD20). The B cells are diffusely distributed and account for no more than 5% of nucleated cells. (B) Gel electrophoresis of the polymerase chain reaction products for the region CDR2 of the immunoglobulin heavy chain gene, which reveals a monoclonal gene rearrangement. Lane M, length marker; lane N, DNA extracted from bone marrow with normal lymphocyte numbers; lane C, control DNA extracted from a lymph node infiltrated by CLL.

Methods

We collected 529 consecutive bone marrow biopsy specimens taken from the iliac crest because of suspected infiltration by malignant NHL over a period of 2.5 years. There was a history of malignant NHL in 474 (89%) of the cases. The specimens were subjected to the routine processing procedure, that is, fixation in 5% buffered formalin, decalcification overnight in EDTA, and embedding in paraffin. Sections were stained with Giemsa and immunostained by the avidin–biotin–peroxidase complex method⁶ with antibodies against CD3 (Novocastra) for T cells, CD20 (Dako) for B cells, and the Ki-67 antigen (MIB-1, Dianova) to assess the proliferation rate.

The cases were divided into three groups according to the approximate percentage of nucleated bone marrow cells that were lymphocytes (figs 1–3):

- (1) Cases with a normal lymphocyte count (< 10%), the ratio CD3+ lymphocytes to CD20+ lymphocytes usually being about 4:1.
- (2) Cases with a modest increase in lymphocytes to 10–30% of nucleated bone marrow cells, usually with a shift in the CD3+ to CD20+ ratio towards CD20+, that is, B cells.
- (3) Cases with a marked increase in lymphocytes to more than 30% of nucleated bone marrow cells, usually with a clear predominance of CD20+ B cells.

For the molecular biological investigations, sections were cut at 8 μ m, dewaxed, and digested with proteinase K at 60°C for at least two hours. DNA was extracted with phenol, chloroform, isoamyl alcohol, and alcohol precipitation, and tested for structural integrity and amplifiability with primers for the β globin

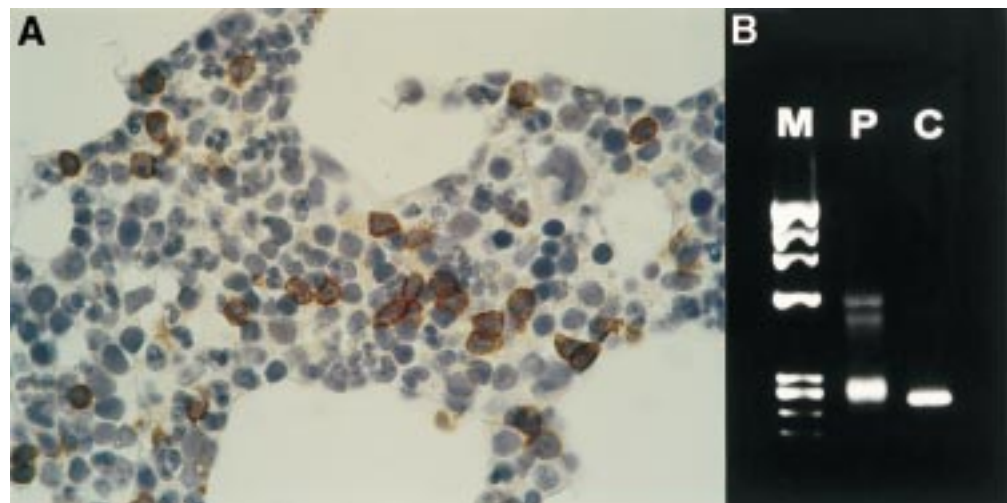


Figure 2 Bone marrow with a small increase in lymphocyte numbers. (A) Immunostaining for B cells with the antibody L26 (anti-CD20). About 15–20% of the nucleated cells (focal and diffuse) show membrane associated reactivity. (B) Examination of the polymerase chain reaction products for the CDR2 region of the immunoglobulin heavy chain gene reveals polyclonality (lane P). Lane M, length marker; lane C, control DNA extracted from a lymph node infiltrated by CLL.

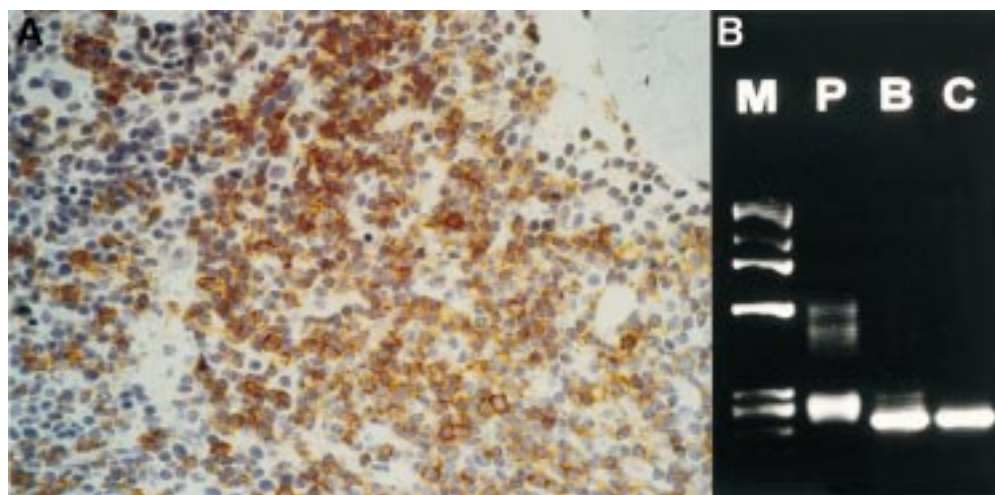


Figure 3 Bone marrow with a marked increase in lymphocyte numbers owing to infiltration by malignant lymphoma. (A) Immunostaining of the tumour cells with an antibody against CD20. The diagnosis is one of small cell B-NHL. (B) Gel electrophoresis of the polymerase chain reaction products from the investigation of rearrangements of the CDR2 region of the immunoglobulin heavy chain gene. Lane B, DNA extracted from the bone marrow in this case; the presence of a dominant band indicates monoclonality. Lane M, length marker; lane C, control DNA extracted from a lymph node infiltrated by CLL; lane P, polyclonal DNA extracted from a lymph node with lymphadenitis.

gene (268 bp) or the glycerine aldehyde phosphate dehydrogenase gene (GAPDH; cDNA: 248 bp and 325 bp), or both. IgH gene rearrangements were investigated with semi-nested polymerase chain reaction (PCR) using sense primers for framework II and III and antisense primers for the joining region.⁷ Rearrangements of the TCR γ gene (V γ I and V γ III/IV) were analysed in a two step multiplex PCR with antisense primers as described.⁸ The PCR products were transferred to a 3% or 8% special agarose gel (NuSieve GTG Agarose, FMC Bioproducts) and separated electrophoretically. When monoclonal rearrangement was found, the PCR investigation was repeated. If the result was again monoclonal, the PCR products were purified and subjected to direct sequencing (373 DNA Sequencer Stretch, Applied Biosystems). DNA extracts from the blood of a patient with T cell acute lymphoblastic leukaemia (T-ALL) and a cervical lymph node from a patient with B cell chronic lymphatic leukaemia (B-CLL) served as positive controls.

The sensitivity of the PCR was tested with a DNA extract from a bone marrow biopsy specimen in which the cells of a B cell NHL represented 10% of the nucleated cells. This was mixed at various dilutions (1:2, 1:3, 1:5, 1:10, 1:20, 1:30, 1:50, 1:100) with a DNA

extract from a bone marrow biopsy specimen with a normal lymphocyte count. Aliquots of 300 ng DNA were then used for seminested PCR to investigate IgH gene rearrangements. A clear sharp band was observed for dilutions of up to 1:20 (5%), implying a sensitivity of 0.5%, as the monoclonal fraction represented 10% of the mononuclear cells in the original biopsy specimen.

Results

Amplifiable DNA could be obtained from all 529 specimens, as reflected in a positive PCR for the β globin or GAPDH gene. Only 28 (7.5%) of the 371 cases with normal lymphocyte numbers (< 10% of nucleated bone marrow cells) showed a monoclonal rearrangement of the IgH gene (combined in one case with a monoclonal rearrangement of the TCR γ gene; table 1). The distribution of the lymphocytes was focal in 14 cases, diffuse and focal in five cases, and diffuse in nine cases. In four of the nine cases with diffuse distribution, the lymphocytes represented less than 1% of nucleated bone marrow cells. Of the 28 cases with monoclonality, 11 were known to have an extramedullary low grade NHL that was usually nodal, including five cases of B-CLL and one hairy cell leukaemia. Five further cases had lymphadenopathy without evidence of leukaemic spread, including three patients with a high grade B cell NHL.

Lymphocyte proliferation was found to be monoclonal in 77.4% of the 106 cases in which it was marked (> 30%; 78 IgH, four TCR γ), supporting the diagnosis of malignant NHL (tables 1 and 2).

Of the 52 cases with mild lymphocyte proliferation (10–30% of nucleated bone marrow cells), 26 (50%) showed monoclonal rearrangement of the IgH gene (24) or TCR γ gene (2) (table 1). Lymphomatous bone marrow infiltration was suspected on the basis of immunohistochemical findings in only 21 of

Table 1 Monoclonal rearrangements of the IgH and TCR γ genes in bone marrow biopsy specimens showing normal lymphocyte numbers (< 10%), mild lymphocyte proliferation (10–30%), and marked lymphocyte proliferation (> 30%)

Group	No of cases	Histological/immunohistological diagnosis (bone marrow)	Monoclonal gene rearrangements	
Lymphocytes < 10%	372	Reactive	28 (27×IgH, 2×TCR γ)	7.5%
Lymphocytes 10–30%	31	Lymphocytosis, not NHL	11 (10×IgH, 1×TCR γ)	
	21	Lymphocytosis, compatible with NHL	15 (14×IgH, 1×TCR γ)	50%
Lymphocytes > 30%	106	NHL	82 (78×IgH, 4×TCR γ)	77.4%

NHL, non-Hodgkin lymphoma.

Table 2 Diagnosis and numbers of cases with monoclonal gene rearrangements in 106 cases with marked lymphocyte proliferation in the bone marrow

Lymphocytes > 30% (n = 106)		
No of cases	Diagnosis	Monoclonal gene rearrangement (77.3%, n = 82)
30	B-CLL	29
4	Hairy cell leukaemia	3
44	Low grade B cell lymphoma	32
15	Diffuse large B cell lymphoma	10
1	Anaplastic large B cell lymphoma	1
1	T-ALL	1
4	Peripheral T cell lymphoma, unspecified	2
1	Anaplastic large T cell lymphoma	1
6	Non-classifiable NHL	3 (IgH)

B-CLL, B cell chronic lymphatic leukaemia; NHL, non-Hodgkin leukaemia; T-ALL, T cell acute lymphoblastic leukaemia.

the 52 cases. Among the 26 monoclonal cases, the lymphoid infiltrates were peritrabecular, at least in part, in 11, and immunohistochemical investigation revealed immunoglobulin light chain restriction in two. Clinically manifest B-CLL was present in two cases, low grade B cell lymphoma has since been diagnosed in two cases, and one patient had hairy cell leukaemia. In three cases each of B cell lymphoma and T cell lymphoma, infiltrates that appeared to be of low grade malignancy were found in the bone marrow, although the primary peripheral tumour was known to be of high grade malignancy. In the remaining two cases, biopsy had been performed because of haematological abnormalities, such as haemolysis, splenomegaly, leucocytosis, and blasts in the peripheral blood.

The following diagnoses were made on the basis of histological and immunohistological findings: 94 B cell NHL (75 monoclonal for IgH gene rearrangement), six T cell NHL (four monoclonal for TCR γ gene rearrangement), and six NHL of undefined immunophenotype (three monoclonal for IgH gene rearrangement).

Discussion

In this study, immunohistochemical stains and molecular biological techniques (to detect monoclonal rearrangements of the IgH or TCR γ genes) were used to determine whether slight diffuse or focal proliferation of lymphocytes in the bone marrow was reactive or neoplastic.

Until recently, unfixed bone marrow tissue has usually been used to investigate possible infiltration by NHL.⁹ However, PCR based analysis of gene rearrangements using not only fresh tissue and native body fluids but also paraffin embedded material has become established as a diagnostic method for lymphoproliferative diseases.^{8 10-12} Amplifiable DNA can be obtained even after decalcification, as has also been demonstrated for formalin fixed bone marrow biopsy specimens taken from the iliac crest.¹³ Using the mild method of decalcification in EDTA, we were able to extract structurally intact and amplifiable DNA from all 529 specimens. Other studies, using formic

acid¹³ and trichloroacetate or EDTA¹⁴ for decalcification, reported DNA extraction rates of 6/10 and 35/36, respectively.

The sampling error is a factor that needs to be taken into account when PCR is used as a diagnostic technique. In our case, the question arises as to whether the tumour cells detected were intravascular, that is, circulating lymphoma cells, or true infiltrating cells. Because circulating lymphoma cells are rarely detectable in the absence of true bone marrow infiltration, it can be assumed that the discrete monoclonal collections of lymphocytes we detected really did represent the infiltrating cells of a low grade malignant NHL.^{1 15-18}

The PCR assay we used investigated gene rearrangements of the CDR2 and CDR3 regions of IgH using seminested primers, and gene rearrangements of the γ chain of the TCR using multiplex primers. This appears to be a reliable method of differentiating between monoclonal and reactive lymphocyte proliferation in bone marrow biopsies.

It is interesting that monoclonal gene rearrangements were noted in 28 cases (7.5%) in which bone marrow lymphocyte counts were normal. How should this finding be interpreted? In 11 (39%) of these cases it is probable that these were infiltrates of malignant NHL because there was a history of peripheral NHL of low grade malignancy. The small numbers of bone marrow lymphocytes detected by histological and immunohistochemical techniques were insufficient to confirm infiltration by NHL. In three further cases there was probably so called minimal residual disease, a state of post-treatment NHL in which the tumour cells in the bone marrow are detectable only by individual specific molecular biological methods.¹⁹⁻²¹ In minimal residual disease, investigation of the bone marrow is of greater diagnostic and prognostic value than investigation of the peripheral blood,^{22 23} so our findings in these cases were of particular clinical relevance. It is difficult to evaluate the significance of the findings in the cases in which the PCR revealed monoclonality, although the proportion of lymphocytes in the bone marrow was less than 1% and there was no history of extramedullary NHL (eight cases). In such cases we suggest that the patient should be observed, as the possibility that an NHL may become manifest with time cannot be excluded.²⁴ Other possibilities are so called benign monoclonal B cell lymphocytosis, corresponding to CLL stage 0, or monoclonal lymphoproliferative disease of unknown significance (MLDUS).²⁵⁻²⁷

We were surprised to find that 50% (26) of the cases with only a modest increase in lymphocyte numbers showed monoclonal gene rearrangements, making the diagnosis of low grade malignant NHL at least probable. The 26 cases included 13 in which reliable morphological criteria for the diagnosis of malignant NHL were fulfilled. A further five cases were known to have extramedullary low grade B cell NHL. In the six cases in this group with bone marrow infiltration by NHL of low grade malignancy but a known peripheral primary

high grade B or T cell NHL, the bone marrow infiltrates were probably caused by a separate, coexisting low grade NHL.²⁸ In the two remaining cases the bone marrow was biopsied because of haematological abnormalities, and these have still not been explained by further clinical and haematopathological surveillance. At the moment it is not possible to say in what percentage of these cases the monoclonality reflects a clinically relevant malignant NHL. However, the relatively common finding of monoclonality in the presence of only modest lymphocyte proliferation (to 10–30% of nucleated cells) suggests that the inclusion of molecular biological techniques in routine diagnostics would be of value.

Monoclonal rearrangements were detected in 77% of cases in which there was morphologically obvious infiltration by lymphoma. This is consistent with the figures quoted in published reports of 60–80% for soft tissue and blood.^{8 29–31} In the light of these published figures, it is not surprising that not all cases of malignant NHL in our study were identified on the basis of monoclonal rearrangements. The false negative results in 23% of the cases with definite morphological evidence of NHL may have been caused by atypical rearrangements, such as inversion of the immunoglobulin genes and rearrangements that, untypically, do not join a V segment to the DJ sequence.³² Eighteen of these cases were high grade NHL, and another an ALL, both diseases in which relatively frequent false negative PCR findings have been reported because of unusual DNA alterations.^{33 34} The sensitivity of the PCR method we used was 0.5%—that is, a lymphoma cell clone in the bone marrow accounting for as few as one in 200 nucleated cells could be differentiated from the surrounding lymphocyte background.

The detection of monoclonality of immunoglobulin and TCR rearrangements by molecular biological techniques has been cited by many investigators as hard evidence of malignancy.^{8 10 11 30 35–39} However, monoclonality does not necessarily mean malignancy in all cases. Collins⁴⁰ has suggested a *modus operandi*: if clonal rearrangement is found in an extranodal lymphoproliferative disorder of limited extent in which there is a predominance of small lymphocytes, a diagnosis of clonal disorder of uncertain malignant potential should be made. Treatment to reduce lymphocyte stimulation may lead to regression of the clone. However, persistent clones may, after a latent period, transform into malignant NHL.⁴⁰

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