ORIGINAL ARTICLE

A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders

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Aims: To evaluate the diagnostic value of bone marrow aspirates, trephine biopsies (BMB), and flow cytometry (FC) in the assessment of bone marrow infiltration in chronic lymphoid disorders.
Methods: Investigations were carried out in 110 diagnostic and follow up specimens from B cell disorders, namely: chronic lymphocytic leukaemia (CLL; 65), non-Hodgkin's lymphoma (NHL; 39), and hairy cell leukaemia (HCL; 6). A selected panel of monoclonal antibodies was used both for FC and immunohistochemistry.
Results: In CLL there was agreement between the three investigations in 71% of samples and in 88% when only FC and BMB were compared. In nine of 65 samples, FC and BMB were positive, although the aspirate was reported as negative. Four BMB negative samples had minimal residual disease (MRD) detected by FC, whereas two samples were positive both on BMB and aspirate but showed no evidence of disease on FC. In NHL, there was agreement between the three investigations in 22 of 39 cases, and in 27 of 39 cases there was agreement between FC and BMB. In eight of 39 NHL cases, FC was negative but the BMB was either positive (five) or uncertain (three), whereas in three of 39, FC was positive but BMB was either negative (one) or uncertain (two). In three of five uncertain BMB, no clonal population was detected by the polymerase chain reaction, whereas in the remaining two cases the nodular aggregates disappeared on further sectioning.
Conclusions: Both BMB and FC are better than bone marrow aspirates for the detection of infiltration in B cell disorders. FC might be slightly more sensitive than BMB to detect MRD in CLL, whereas BMB may be slightly better than FC in NHL.

The histological pattern of lymphoid infiltration in the bone marrow trephine biopsy (BMB) is thought to be a prognostic factor in B cell chronic lymphocytic leukaemia (CLL).[1 2] In patients with non-Hodgkin's lymphoma (NHL), examination of a trephine bone marrow biopsy is an important part of the staging procedure and it is useful for assessing the response to treatment and re-staging when patients relapse after treatment.[3] Recent studies have suggested that a lymphocytic infiltration (LI) in bone marrow aspirates could be more useful than BMB to predict outcome in patients with stage A CLL.[4 5] However, the reticulin deposition that frequently accompanies lymphoma infiltration may prevent successful aspiration of lymphoma cells. This, together with the possibility of peripheral blood contamination in the bone marrow aspirate, may decrease the sensitivity of detection of lymphoma cells in this type of specimen.

> "Flow cytometry is thought to increase the sensitivity of bone marrow involvement by non-Hodgkin's lymphoma over morphological evaluation alone in the bone marrow aspirates"

However, the morphological evaluation of BMB to assess involvement in lymphoid malignancies can be problematical. The histological criteria for distinguishing reactive lymphoid aggregates from leukaemia and/or lymphoma involvement, such as the extent of lymphoid infiltration or the number, shape, and localisation of lymphoid aggregates, in addition to the cytological characteristics of the lymphoid cells,[6] are helpful but not absolute parameters.[7] Immunohistochemistry (IHC) is useful for distinguishing between reactive and neoplastic lymphoid infiltration; however, a full immunophenotype and determination of monoclonality (immunoglobulin (Ig) light chain restriction) may be difficult because of the destruction of some of the antigenic epitopes by the fixation and decalcification process used for BMB specimens.[8] In most instances, one must rely on the fact that lymphoid infiltrates that are composed of an admixture of B and T cells are more likely to be benign, whereas those composed purely of B cells are probably malignant.[9 10]

Immunophenotypical analysis by flow cytometry (FC) appears to be very sensitive in the diagnosis and detection of minimal residual disease (MRD) in CLL.[11 12] The role of FC for staging or re-staging of NHL is not well defined, but it is thought to increase the sensitivity of bone marrow involvement by NHL over morphological evaluation alone in the bone marrow aspirates.[13–15]

In our study, we have reviewed retrospectively 110 bone marrow specimens from a series of patients with CLL, NHL, and hairy cell leukaemia (HCL) at diagnosis and after treatment in whom bone marrow aspirates, trephine biopsies, and flow cytometry data were available. Our main aim was to assess the sensitivity of these three investigations for the detection of leukaemia/lymphoma cells.

MATERIALS AND METHODS

Patients

In total, 110 consecutive specimens from 50 patients with CLL, 33 with B cell NHL (B-NHL), and five with HCL referred to or

Abbreviations: BMB, bone marrow biopsy; B-NHL, B cell non-Hodgkin's lymphoma; CI, confidence interval; CLL, chronic lymphocytic leukaemia; FC, flow cytometry; Ig, immunoglobulin; IHC, immunohistochemistry; LI, lymphoid infiltration; MRD, minimal residual disease; NHL, non-Hodgkin's lymphoma; HCL, hairy cell leukaemia; PCR, polymerase chain reaction

followed at the Royal Marsden Hospital NHS Trust between September 2000 and October 2001 were included in our study. We selected patients in whom bone marrow aspiration, BMB, and immunophenotyping by FC in the aspirate were carried out simultaneously. All samples were representative in terms of cellularity and fragments (bone marrow aspirates) and size of BMB (at least 1 cm, often greater than 1.5 cm in length). There were 65 specimens from 50 patients with CLL, 39 from 33 patients with B-NHL, and six samples from five patients with HCL. Of the 65 CLL samples, 14 specimens were analysed at diagnosis and 51 during follow up, after chemotherapy or stem cell transplantation. Eighteen specimens from patients with B-NHL were for staging at diagnosis and 21 for re-staging or during follow up after chemotherapy or radiotherapy. All six specimens from the patients with HCL were investigated during follow up after chemotherapy. The mean age of the patients was 57 years (range, 41–77) for those with CLL, 56 years (range, 31–84) for those with B-NHL, 47.6 years (range, 28–69) for those with HCL. The male to female ratio was 4.1 for CLL, 1.2 for B-NHL, and 1.5 for HCL.

Flow cytometry analysis

This was carried out on bone marrow isolated mononuclear cells. The monoclonal antibodies used were directed against CD5, CD2, FMC7, CD22, CD23, CD79b, CD25, CD11c, CD103, and anti-κ and anti-λ Ig light chains. Controls included substitution of the relevant monoclonal antibody by a mouse Ig of the same isotype. Analysis was carried out on a FAC-Scan flow cytometer on a lymphocyte gate. In specimens from CLL after treatment, minimal residual disease (MRD) was investigated by double immunostaining for CD5/CD19 and results were analysed on a CD19+ lymphocyte gate. A positive result (MRD+) was considered when > 15% of CD19+ lymphocytes coexpressed CD5, on the basis of findings on normal bone marrow and samples from patients with CLL after chemotherapy and transplantation, as described previously.[12] In HCL specimens, analysis was carried out on a "hairy cell" gate, which overlaps with the monocyte gate and is above and to the right of the lymphocyte gate.

Bone marrow aspirates and biopsies

Bone marrow aspirates were obtained from the posterior iliac crest and followed by BMB in each patient. LI was evaluated by examining 200 cells on May-Grunwald Giemsa stained slides. The criteria for positive involvement were as follows: (1) the presence of more than 30% lymphocytes and/or (2) the presence of atypical lymphocytes or blastoid cells, even if the proportion was lower than 30%; the identification of occasional suspicious cells was considered uncertain. In the BMB, morphology was analysed on haematoxylin and eosin stained, formic acid decalcified sections. The infiltration pattern in BMB in CLL was categorised as nodular, interstitial, mixed (nodular + interstitial), and diffuse, as described previously.[1] The BMB pattern in cases of B-NHL was categorised as diffuse, paratrabecular, and intertrabecular. The final morphological bone marrow diagnosis was graded as positive, negative, or uncertain for involvement. Unequivocal involvement by CLL or B-NHL in the BMB was considered a positive result. Small lymphoid aggregates of uncertain importance or mild lymphoid interstitial infiltration in the BMB constituted the uncertain category, and no evidence of lymphoid infiltrates was categorised as negative.[16 17] In addition to morphological evaluation, immunohistochemistry was carried out on the BMB sections with a panel of monoclonal antibodies against B and T cell antigens, namely: CD20, CD5, CD23, CD3, CD79a, and CD10. DBA44 staining was also performed in HCL, cyclin D1 in mantle cell lymphoma, and bcl2 and bcl6 in other lymphomas. The peroxidase labelled streptavidin–biotin complex method was used for immunostaining.[18]

Table 1 Flow cytometry, bone marrow aspirate, and trephine biopsy in CLL

	Group	Number
1	FC+ BMB+ BMA+	46/65
2	FC+ BMB+ BMA–	9/65
3	FC+ BMB– BMA–	4/65
4	FC– BMB– BMA–	2/65
5	FC– BMB+ BMA+	2/65
6	FC+ BMBu BMA+	1/65
7	FCu BMB+ BMA–	1/65
	Total	65/65

BMA, bone marrow aspirate; BMB, bone marrow trephine biopsy; CLL, chronic lymphocytic leukaemia; FC, flow cytometry; +, positive for involvement by CLL; –, negative for involvement by CLL; u, uncertain for involvement by CLL.

Statistical analysis

Exact 95% confidence intervals (CI) are quoted as a measure of the uncertainty in the results.

RESULTS

In 46 of 65 (71%; 95% CI, 58% to 81%) CLL specimens, all three parameters (FC, BMB, and bone marrow aspirates) were positive, and in two of 65, all three parameters were negative (table 1). Thus, the agreement level of all three parameters in CLL was 74% (95% CI, 60% to 84%). Discrepancies between FC, BMB, and aspirate were found in 17 of 65 (26%; 95% CI, 16% to 38%). In nine of these (14%; 95% CI, 7% to 25%), FC and BMB were positive, whereas morphological analysis in the bone marrow aspirate was regarded as negative. In all these samples, LI on the aspirates was below 20% (range, 2–16%). Eight of these nine specimens had MRD by FC and nodular partial remission or residual disease on BMB. In four specimens only, FC alone was positive for CLL (MRD+) although the BMB and the aspirate (< 15% lymphocytes) were negative (table 1). In two specimens, FC did not detect CLL although the BMB and aspirate were positive. There was a single case in which both FC and aspirate were positive for MRD whereas the BMB was reported as uncertain. In this case, there were small clusters of mature lymphocytes in the original section, but these clusters were not found consistently in further sections stained immunohistochemically; thus, a definite conclusion could not be made. There was only one case in which the aspirate and BMB were positive but the FC was uncertain. The agreement level rose to 88% (57 of 65; 95% CI, 77% to 94%) when only FC and BMB were compared and to 83% (54 of 65; 95% CI, 72% to 91%) when BMB and aspirates were compared.

The 33 B-NHL cases comprised 10 diffuse large cell lymphomas, eight follicular centre cell lymphomas, seven mantle cell lymphomas, two Burkitt's lymphomas, one each of splenic lymphoma with villous lymphocytes and lymphoplasmacytic lymphoma, and four not otherwise specified.

In 13 of the 39 (33%; 95% CI, 19% to 50%) specimens of NHL, all the three parameters (FC, BMB, and aspirates) were positive, and in nine of 39 (23%; 95% CI, 11% to 39%) all the three parameters were negative (table 2). Thus, the agreement level in B-NHL was 56% and discrepancies between FC, BMB, and aspirate were found in the remainder. The agreement level rose to 69% (95% CI, 50% to 83%) when FC and BMB were compared and to 72% (95% CI, 55% to 85%) when FC and aspirate were compared. In eight of 39 (20%; 95% CI, 9% to 36%) specimens, BMB alone was positive (five) or uncertain (three), although FC was negative. In two of the three uncertain specimens, a small cluster of CD20 positive cells admixed with T cells was seen in one of the patients and in another a small nodular collection of lymphocytes disappeared on

Table 2 Flow cytometry, bone marrow aspirate, and trephine biopsy in B cell NHL

	Group	Number
1	FC+ BMB+ BMA+	13/39
2	FC– BMB– BMA–	9/39
3	FC+ BMB+ BMA–	3/39
4	FC– BMBu BMA–	3/39
5	FC– BMB+ BMA–	2/39
6	FC– BMB– BMAu	2/39
7	FC+ BMBu BMA–	2/39
8	FC+ BMB– BMA+	1/39
9	FC– BMB+ BMA+	1/39
10	FC– BMB+ BMAu	1/39
11	FCu BMB+ BMA–	1/39
12	FCu BMB– BMA–	1/39
	Total	39

BMA, bone marrow aspirate; BMB, bone marrow trephine biopsy; FC, flow cytometry; NHL, non-Hodgkin's lymphoma; +, positive for involvement by NHL; –, negative for involvement by NHL; u, uncertain for involvement by NHL.

Table 3 Flow cytometry, bone marrow aspirate, and trephine biopsy in HCL

	Group	Number
1	FC– BMB– BMA–	4/6
2	FC+ BMB+ BMA–	1/6
3	FC– BMB+ BMA–	1/6
	Total	6/6

BMA, bone marrow aspirate; BMB, bone marrow trephine biopsy; FC, flow cytometry; HCL, hairy cell leukaemia; +, positive for involvement by HCL; –, negative for involvement by HCL.

further sections. In three of 39, FC was positive whereas BMB was negative (one) or uncertain (two), and only one of these three was positive in the aspirate. In one of these samples, a small cluster of CD20 positive cells was seen along with T cells, and in the other sample there was a small nodular collection of small lymphoid cells not present on further sections for immunohistochemistry. In only three of 39 samples, the aspirate was positive (one) or uncertain (two), with the presence of abnormal cells, although the BMB was negative.

The patterns of infiltration in the bone marrow for 26 specimens classified as positive and uncertain for NHL were as follows: paratrabecular, 12; interstitial, five; nodular, four; mixed, three; and diffuse, two. Four of 12 specimens with a paratrabecular pattern also showed an interstitial pattern. The patterns of infiltration in 12 BMB specimens of B-NHL showing discrepancies with FC were as follows: paratrabecular, six; moderate interstitial, five; and nodular, one.

Overall, the concordance between FC and BMB was higher both for CLL and B-NHL in diagnostic specimens than in follow up specimens. In CLL, there was agreement between FC and BMB in 13 of the 15 diagnostic specimens and in 44 of the 51 follow up specimens. In B-NHL, there was concordance between FC and BMB in 15 of the 18 diagnostic specimens but in only 11 of the 21 follow up specimens.

DISCUSSION

Immunophenotypic analysis by FC is an extremely useful adjunct in the diagnosis of patients with CLL and also in the detection of residual malignant cells in these patients.[11 12] Although the pattern of infiltration in the BMB in CLL has been documented to be a major prognostic factor,[1 2] other studies have shown no major impact on survival.[19 20] Moreover, it has been suggested that a lymphoid infiltration $\geqslant$ 80% in bone marrow aspirates could be more useful than a diffuse bone marrow infiltration to predict outcome in patients with early stage CLL.[5] Nevertheless, BMB is regarded to be more reliable and reproducible than bone marrow aspirates for disease progression in all stages of CLL.[4]

Our study in CLL comparing the results of FC, BMB and aspirates showed a good correlation between these three parameters in over two thirds of samples (table 1) and up to 88% when only FC and BMB were compared. There were very few discrepancies between FC and BMB and these were found mainly in cases with MRD. In such cases, FC proved to be more sensitive than BMB in detecting residual numbers of CLL. In contrast, in two of the 65 specimens, FC failed to detect CLL although BMB and the aspirate were positive. It seems that BMB and FC are the best methods to assess MRD in CLL.

BMB is important for staging at the time of initial diagnosis of NHL and for re-staging when patients relapse after treatment. The adjunct role of FC is not well defined in this setting. A handful of previous studies investigating the role of FC in detecting bone marrow involvement by NHL (T and B cell) has not provided evidence of much benefit beyond morphological examination alone in the biopsy.[8 14 15] Fineberg *et al* found a good correlation (85%) between morphology on BMB and FC,[8] which can be explained in part by the inclusion of a large number of CLL cases, where overt bone marrow involvement is expected. Similarly, Dunphy[14] documented a high correlation (81%) between these two investigations in 188 cases, with only 2.6% of BMB negative cases having evidence of disease by FC. This study also included a large number of CLL cases. Naughton *et al* reviewed 273 bone marrows from patients known to have NHL.[13] FC failed to detect NHL in 25 of 62 positive BMB and detected disease in only three of 211 (1.5%) negative or equivocal BMB. Recently, Duggan *et al* reviewed 227 cases of NHL and found a correlation between FC and BMB in 78% of cases.[15] Cases with discrepancies included 7% BMB positive with no evidence of disease by FC, whereas 12% were positive by FC and had no morphological involvement on BMB. Of the 162 negative or suspicious BMB, 27 were shown to be involved by FC, resulting in a false negative detection rate of 17% for BMB.

In our study, there was an excellent correlation between FC, BMB, and aspirate in more than half of the cases of B-NHL (table 2), and the agreement level rose to 69% when only FC and BMB were compared. Cases that were FC negative were positive (five) or uncertain (three) on BMB, whereas the FC positive cases scored negative (one) or uncertain (two) on BMB.

In the absence of cytologically definite lymphoma cells or diagnostic patterns of infiltration, the diagnosis of NHL in the bone marrow is often complicated by the presence of benign lymphoid aggregates. These lymphoid aggregates are common in normal bone marrow[7] and lack specific features to allow their unequivocal distinction from NHL.[6 7] In our study, BMB was uncertain in five cases of NHL, but two of these were positive by FC. Small nodular clusters of B cells admixed with T cells were seen in three of five cases. No definite clonal population was detected by a polymerase chain reaction (PCR) study carried out on dewaxed slides, and finally the lymphoid aggregates seen in BMB were considered "non-specific" in these three samples that were FC negative. Neither immunohistochemistry nor PCR could be performed on the remaining two samples that were FC positive and thus the FC findings remain uncertain. The fact that 13% of positive BMB were negative by FC in B-NHL might be explained by a poor yield of the aspirate compared with the BMB, which may show paratrabecular infiltration. It is possible that no disruption of these aggregates takes place during aspiration and an intact paratrabecular aggregate is removed only by en block resection during biopsy.[21] The patchy intertrabecular nature of many

Take home messages

- Both bone marrow biopsy (BMB) histology and flow cytometry (FC) are better than bone marrow aspirates for detecting lymphocyte infiltration in B cell disorders
- FC might be slightly more sensitive than bone marrow biopsy for detecting minimal residual disease in chronic lymphocytic leukaemia
- BMB may be slightly better than FC for detecting lymphocyte infiltration in non-Hodgkin's lymphoma
- In most cases BMB and FC seem to complement one another

lymphomas involving the bone marrow may produce aspirate samples free of disease, although by chance the biopsy is involved. This is supported by the improved sensitivity of bilateral over unilateral BMB.[22] Furthermore, dilution of the aspirate samples with peripheral blood during the procedure may decrease the proportion of neoplastic cells to below the threshold of detection by FC.

> "There were very few discrepancies between flow cytometry and bone marrow biopsy and these were found mainly in cases with minimal residual disease"

Alternatively, the fact that three of the 39 NHL specimens were positive by FC but negative or uncertain on BMB might partly result from a low sensitivity in detecting minimal bone marrow involvement. The three specimens were MRD positive by FC and contained only a small collection of lymphoid cells on haematoxylin and eosin stained sections in the two uncertain samples. No clonal population was detected by PCR in one and the lymphoid collection disappeared on further sectioning for immunohistochemistry and PCR in another. Recently, Duggan *et al* suggested that FC is more sensitive for detecting minimal involvement by lymphoma in the bone marrow, whereas BMB will detect most cases in which involvement is $> 5\%$.[15] In that study, only 13 of 35 cases of minimal disease NHL (B and T cell) by FC were detectable by BMB. The high rate of detection of NHL by FC in Duggan's study may be partly related to the use of more sensitive and specific three or four colour multiparameter analysis.

Because of the low number of specimens in our study, we cannot make firm conclusions about the efficacy of three procedures to assess residual HCL. Recently, Pittaluga and colleagues[21] compared morphology, immunohistochemistry, and Ig heavy chain gene rearrangement by PCR in BMB specimens from 71 HCL and 53 mantle cell lymphoma cases. PCR analysis was not superior in negative or positive cases to BMB but was helpful in cases diagnosed as inconclusive (positive in six of eight). Nevertheless, they found one false positive and eight false negative PCR results and suggested that were the result of sampling error or DNA degeneration of the fixed tissues.

In conclusion, we have shown that bone marrow aspirates, BMB, and FC are useful in the diagnosis of chronic lymphoid disorders; in particular, FC and BMB are better predictors of involvement than bone marrow aspirate. FC seems to be slightly more sensitive than BMB in detecting MRD in CLL, whereas biopsy is slightly superior to FC in NHL, although both methods seem to complement each other in most cases.

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