Clonality of T cell and phenotypically undefined lymphoid neoplasms: the value of genotypic analyses

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Abstract
The value of genotypic analysis for routine assessment of leukaemia and lymphoma was shown by the findings in a selected series of 30 cases. T cell receptor (TcR) gene rearrangements were observed in six out of nine cases of CD3+ CD8+ lymphocytes and provided clear evidence for clonality in this group. The T cell proliferations in two of the remaining cases masked B cell lymphocytic leukaemia and hairy cell leukaemia, while in the third case no cause was found for the polyclonal proliferation. Heterogeneity of phenotype and genotype were observed in peripheral T cell lymphomas: one out of six cases showed TcR gene rearrangement, one case retained its germline configuration, a further case masked B cell lymphoma and the remainder were polyclonal. Genotypic analysis was helpful in the analysis of a tumour of mixed T cell and myeloid phenotype which was shown to be germline for TcR and immunoglobulin genes, consistent with a myeloid origin. Two histiocytic tumours were found to have clonal rearrangement of TcR genes. Nine out of 11 B cell tumours showed immunoglobulin gene rearrangement.

It is concluded that genetic analyses are useful in the analysis of T cell, histiocytic, and B cell tumours in which an immunoglobulin phenotype cannot be defined.

Phenotypic analyses have been widely used for the analysis of non-Hodgkin's lymphoma (NHL). Current systems of classification relate the morphology of neoplastic cells to normal patterns of lymphoid differentiation, and prediction of immunological type on the basis of histology is moderately successful.1-3 In B cell tumours the surface phenotype can be correlated with the normal maturation pathway of immunoglobulin producing lymphocytes.4 Irregularities occur, however, and a significant number of these tumours often fail to express immunoglobulin. Proportions vary from 3% of centroblastic/centrocytic follicular lymphomas1 to 10-30% of all B cell tumours derived from follicular B cells.5,6 The nature of these tumours can usually be resolved by the presence of other B lineage markers and immunoglobulin gene rearrangement.

In T cell tumours the situation is less clear. Chronic proliferative disorders of CD3+ CD8+ T cells, often represented by proliferations of large granular lymphocytes (LGLs), have been reported to exhibit chromosomal abnormalities and clonal rearrangement of TcR genes.7,8 In some circumstances, however, these proliferations may represent benign populations in the absence of underlying neoplastic disease9 which can be resolved on the completion of TcR gene rearrangement studies. Furthermore, lymphomas of T cell origin display great phenotypic heterogeneity10 and often represent a diagnostic difficulty, which in the absence of a readily available immunochromical marker of monoclonality, requires analysis of TcR gene rearrangements.11,12

In routine analyses gene rearrangement studies maintain a small but important role in tumour identification and classification. In this report we identify useful areas of investigation in NHL and CD3+ CD8+ blood lymphocytes.

Methods
The tissues studied comprised fresh tissue biopsy specimens, blood, and bone marrow aspirates taken at the time of diagnosis or staging from patients presenting at the Southampton University hospitals. Biopsy tissue was divided into three portions for routine histology, immunocytochemistry, and preparation of cell suspensions. Full details of these methods and preparation of cell suspensions from blood and marrow have been given elsewhere.13

Formalin fixed, paraffin wax embedded 5 µm sections were stained with haematoxylin and eosin, Giemsa, periodic acid Schiff and for reticulin. The tumours were classified according to the Kiel classification.14

Phenotypic studies
Phenotypic studies were carried out on tissue sections and cell preparations using a panel of antibodies. Immunoglobulin was shown using rabbit anti-Ig heavy and light chain reagents (Dako) or with monoclonal antibodies to κ and λ light chains. Lineage and subset markers were identified using the appropriate monoclonal antibodies identified by their workshop cluster numbers.15 Cell suspensions were stained by indirect immunofluorescence, and cytocentrifuge preparations were stained by
indirect immunoperoxidase using methods described previously.\(^1\)

Immunohistochemical staining of frozen tissue was performed using a modification of Stein et al.\(^2\) Briefly, 6 μm cryostat sections were dried at room temperature and stored at −20°C over desiccant until stained. On the day of staining sections were fixed in dry acetone for 15 minutes at room temperature and washed in TRIS buffered saline (TBS), pH 7.6. Peroxidase conjugated rabbit anti-mouse Ig (Dako) (diluted 1/80 in TBS) was then applied for a further 30 minutes and the sections again washed.

Immunoperoxidase staining of paraffin wax embedded material was performed on 4 μm sections using peroxidase-antiperoxidase or avidin-biotin complexes as described previously.\(^3\) Endogenous peroxidase activity was inhibited using 0.5% H₂O₂ in methanol for 10 minutes. The sections were predigested where necessary using 0.1% trypsin in 0.1% calcium chloride solution at 37°C. All first layer staining reactions were incubated overnight at 4°C and appropriate controls performed for antibody specificity and titre. For both paraffin wax and frozen section peroxidase methods the final reaction product was developed using 3,3 diaminobenzidine tetrahydrochloride (DAB).

**GENOTYPIC STUDIES**

High molecular weight DNA was extracted from frozen biopsy specimens or cell suspensions by routine methods\(^4\) and 10 μg aliquots digested with 50 units of appropriate restriction endonucleases under conditions specified by the manufacturers. DNA fragments were separated according to size in 0.7% agarose gels and transferred to nylon (Hybond-N) filters, according to the method of Southern.\(^5\) These filters were prehybridised in 6 x SSC (saline sodium citrate), 0.5% sodium dodecyl sulphate (SDS), 5 x Denhats solution, and 6% polyethylene glycol 8000 containing heterologous fragmented salmon sperm DNA at 65°C overnight, followed by hybridisation at 65°C overnight in the same solution containing added \(\alpha\)32P-dCTP by the random hexanucleotide primer method.\(^6\)

**Results**

**CD3 + CD6 + PERIPHERAL BLOOD LYMPHOCYTOSIS**

Data from patients investigated with CD3 + CD6 + lymphocytoses are summarised in table 1. Five of these cases were rearranged for TcRβ chain, and except for case 8 (not tested); four were rearranged for TcRγ chain. Monoclonal TcRγ gene rearrangements were associated with the use of particular Vγ gene segments. Case 3 had rearranged TcRγ in the presence of germline TcRβ, and case 5 exhibited rearrangement of TcRγ chain consistent with polyclonal T cell populations. The remaining two cases (cases 4 and 9) had germline TcRγ and β chains. Case 4 had coexistent B cell hairy cell leukaemia confirmed on examination of the splenic biopsy specimen and case 9 had coexistent B cell chronic lymphocytic leukaemia.

| Case No | Leucocytes (10⁹/l) | Lymphocytes (10⁹/l) | CD2 | CD3 | CD4 | CD8 | CD16 | CD57 | HLA | C1aii | Igκ | Igλ | Tγ | Tβ | Jυ | Cx | Cl |
|---------|--------------------|--------------------|-----|-----|-----|-----|------|------|-----|-------|-----|-----|----|----|----|----|
| 1       | 9-5                | 6-8                | 90  | 96  | 3   | 92  | 1    | 9    | 5   | 1    | 1   | 1   | R  | R  | G  | G  | G  |
| 2       | 9-8                | 7-7                | 63  | 95  | 8   | 78  | 0    | 95   | 2   | 3    | 1   | 1   | R  | R  | G  | G  | G  |
| 3       | 7-6                | 6-6                | 85  | 90  | 10  | 81  | 24   | 30   | 4   | 1    | 1   | 1   | G  | G  | G  | G  | G  |
| 4       | 10-8               | 7-1                | 90  | 95  | 8   | 64  | 1    | 6    | 13  | 1    | 1   | 1   | G  | G  | G  | G  | G  |
| 5       | 11-8               | 9-7                | 81  | 54  | 22  | 68  | 1    | 1    | 16  | 3    | 1   | 1   | G  | G  | G  | G  | G  |
| 6       | 14-8               | 1-8                | 75  | 75  | 69  | 10  | 1    | 34   | 10  | 8    | 1   | 1   | G  | G  | G  | G  | G  |
| 7       | 2-8                | 1-2                | 95  | 90  | 9   | 86  | 86   | 0    | 90  | 0    | 0   | 1   | G  | G  | G  | G  | G  |
| 8       | 13-9               | 4-7                | 90  | 95  | 18  | 90  | 1    | 80   | 11  | 1    | 1   | 1   | G  | G  | R  | R  | G  |
| 9       | 14-3               | 10-1               | 50  | 70  | 10  | 80  | 70   | 30   | 1   | 1    | 1   | 1   | G  | G  | R  | R  | G  |

*Represents a case for which there was no clear neoplastic phenotype.
This case has been reported in detail.  

Case 10 was found to have a lymphocytosis with no clear evidence for B or T cell predominance. At the time of study CD4+ and CD8+ cells were not evaluated. Genotypic analysis showed this case to have clonally rearranged TcRβ and γ genes, consistent with T cell lymphocytic disease.

**NON-HODGKIN'S LYMPHOMA**

Biopsy specimens from patients referred for investigation were initially analysed for phenotype. Where the biopsy phenotype was not diagnostic or was at variance with the histology, DNA was isolated from the biopsy specimen and investigated for immunoglobulin and TcR gene rearrangements.

Seven cases of NHL of lymphoblastic and peripheral T cell lymphoma histologies are included in table 2. The lymphoblastic lymphoma in case 11 was of mixed T cell/myeloid phenotype, but the lymph node and marrow DNA from this case were not clonally rearranged for TcR or immunoglobulin genes. Case 12 from six cases of peripheral T cell lymphomas was clonally rearranged for TcRγ and β chain genes. Three cases were germline for TcRβ and immunoglobulin genes but exhibited polyclonal rearrangements of TcRγ genes, indicated by multiple rearranged bands. Case 13 was germline for both TcR and immunoglobulin genes while case 17 exhibited immunoglobulin gene rearrangement. Four cases were investigated for TcRγ gene rearrangement: case 12 was rearranged for Jγ, but cases 11, 13, and 15 were germline for TcR, J61, and J52. The remaining cases showed regressing atypical histiocytosis affecting the skin (table 2). One of these (case 18) exhibited clonal TcRγ rearrangements.

**Table 2  T Cell or Histiocytic NHL**

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<th>Case No</th>
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<th>Tissue</th>
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**Table 3  B Cell NHL**

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Clonality of T cell and phenotypically undefined lymphoid neoplasms

Figure 2 DNA isolated from lymph node biopsy specimens from cases 12, 14, 15, 16, 18 and digested with restriction enzymes Eco RI, Bam HI and Hind III and probed with 32P labelled TcRγ (A) and TcRβ (B) probes. Rearranged bands are arrowed. Lanes marked C represent control DNA digests with germline bands indicated by kilobase pair size. Clonally rearranged TcRγ bands representing \( V_{\gamma}3 \) \( V_{\gamma}9 \) and \( V_{\gamma}2 \) gene use are present in cases 18 and 12 respectively. Cases 14–16 exhibit polyclonal \( V_{\gamma} \) bands corresponding to \( V_{\gamma}11, 3, 8, 9, 5, 2/4 \) in case 15, \( V_{\gamma}11, 3, 8, 9, 5 \) in case 15, and \( V_{\gamma}2 + 4, 9, 5, 8 \) in case 14. RH clonal TcRβ gene rearrangements are represented in B. *represents a plasmid contaminant.

and β rearrangements and the other (case 19) was clonally rearranged for TcRγ.

Eleven B cell tumours are included in table 3. In four cases no B or T cell phenotype could be ascribed to the tumour population while one case was phenotypically normal. Tumour cells from five cases failed to express immunoglobulin and in the remaining case (case 26) tumour cells expressed both Igk and \( \lambda \) light chains. In nine cases DNA was clonally rearranged for immunoglobulin genes. Of the remaining cases, case 23 exhibited TcR gene rearrangement in the presence of germline immunoglobulin genes and case 24 was germline for both.

Discussion

Chronic CD3+ CD8+ lymphoproliferative disorders often exhibit TcR clonal rearrangements.27 Six of the nine cases reported in this study were consistent with this finding, and included two out of six cases tested that were positive for CD16 expression. CD57 expression was investigated in eight cases and was associated (more than 10% of cells positive) with clonally rearranged T cell populations in all cases except one. HLA class II expression was associated with CD3+ CD8+ clonally rearranged populations in case 7. Case 3 was clonally rearranged for TcRγ using \( V_{\gamma} \) gene segments 8 and 9 in the absence of TcRβ gene rearrangement, but this case was not tested for TcRβ rearrangement and we were unable to exclude the possibility that this case may have represented a population of cells expressing TcRγ\( \delta \). This possibility is supported by genotypic data because \( V_{\gamma}8 \) and 9 gene segments are frequently used by cells expressing TcRγ\( \delta \).

Three cases of CD3+ CD8+ lymphocytes failed to show monoclonal patterns of TcRβ gene rearrangement. One case exhibited multiple TcRγ gene rearrangements, consistent with polyclonal T cell populations.28 29 Clinically this patient was well at the time of writing and the CD3+ CD8+ lymphocytosis has since resolved. In the remaining two cases the CD3+ CD8+ lymphocytes masked underlying B cell disease. In case 9 the emerging B cell tumour had immunoglobulin clonal
rearrangements identical with those identified in the peripheral blood at the time of the CD3+ CD8+ lymphocytosis. Blood from case 4 showed no TcR or immunoglobulin rearrangements; the spleen was removed from this patient one year before the blood picture was assessed and the immunohistology of the spleen was consistent with hairy cell leukaemia of IgMα phenotype. The T cells in the blood seem to represent a reactive population of polyclonal CD3+ CD8+ T cells. In this case populations of T cells have been described in the presence of B cell disease by Bassan et al. 39 In case 10 there was no clear evidence for B or T cell predominance but TcR genes were rearranged, consistent with T cell disease.

Case 11 with lymphoblastic lymphoma posed a diagnostic difficulty in that the affected marrow was more consistent with myeloid rather than with lymphoid tumour infiltration. This histological diagnosis was further compounded by the immunophenotype which suggested a tumour of mixed T cell/myeloid lineage, tumour cells from both lymph node and marrow expressing CD11, CD33, CD2 and CD7. In this respect this case reflects the lineage promiscuity of early stem cell leukaemias of myeloid origin 34 and is similar to a series of cases reported recently by Kutzberg et al.35 The absence of TcR and Ig gene rearrangements in DNA isolated from lymph node and marrow was more consistent with a tumour of myeloid not lymphoid origin. Furthermore, this patient responded to treatment for acute myeloid leukaemia and remained well 12 months after diagnosis.

Six cases of NHL with the histology of peripheral T cell lymphoma were investigated. The peripheral T cell lymphoma group form a heterogeneous phenotypic and genotypic entity and often fail to exhibit clonal TcR gene rearrangements.11 12 One of the cases was clonally rearranged for TcR genes but the remainder failed to exhibit clonal TcR gene rearrangements. Two cases showed multiple TcRγ gene rearrangements consistent with polyclonal T cell proliferations. In a third case there was increased intensity of one TcRγ rearranged band (Vγ8) among multiple rearranged bands. In the absence of TcRβ clonal rearrangement it was difficult to decide whether this represented a clonal TcRγ gene rearrangement or preferential Vγ gene segment use in the polyclonal population. Unfortunately, insufficient DNA was available from this case for probing with the TcRδ probes. In case 17 the initial histology suggested T zone NHL but on close examination a minor population of large atypical cells was found which failed to stain for T cell markers or Ig but which were positive for CD37. On analysis this case was rearranged for immunoglobulin JH and k light chain genes: this was consistent with the presence of an underlying B cell lymphoma, masked by the presence of a pleomorphic but reactive T cell population, similar to that described by Arnold et al.33 The failure to find clonal gene rearrangements in the remaining cases of peripheral T cell lymphoma suggests that some of these tumours may represent expansions of polyclonal cell populations.11 12

Nevertheless, these lymphomas often manifest phenotypes not representative of normal T cell populations, suggestive of abnormal or aberrant gene expression in some of these malignancies,24 and they require further investigation. Two cases of regressing atypical histiocytosis affecting the skin were shown to be clonally rearranged for TcR genes (table 2). Case 19 was clonally rearranged for TcRβ but not TcRα. Insufficient DNA was available for TcRδ probing and we are unable to conclude whether the cells expressed TcRδ. RH was rearranged for TcRγ and β genes. The detection of clonal TcR gene rearrangements in two cases of this entity suggest that it may be justified to include it among the spectrum of peripheral T cell lymphoma, as suggested by the previous findings of Headington et al.33

The B cell lymphomas included in this study were diagnosed for investigation because the neoplastic cells were either immunoglobulin negative or because an immunological phenotype could not be ascribed to the tumour population (table 3). Immunoglobulin negative tumour populations of all B cell histologies have been reported to vary from 10–20%.36 In our experience immunoglobulin negative tumours may comprise 5% of B cell follicular lymphomas. We were able to show that all the immunoglobulin negative tumours were both CD57 positive and were rearranged for Ig JH and light chain genes. The failure of immunoglobulin negative tumours to express or secrete immunoglobulin may be related to chromosomal translocations associated with these lymphomas.36 As cytogentic data were not available for the lymphomas in this study we are unable to comment on this published finding.

Table 3 also includes three cases in which the phenotype could not be ascribed to the tumour cells because of the presence of reactive polyclonal normal lymphoid populations. Two of these cases exhibited immunoglobulin gene rearrangements consistent with B cell origin and the third case was clonally rearranged for TcRγ and β genes consistent with a pseudofollicular T cell lymphoma. The tumour cells from the remaining case were shown to be clonally rearranged for immunoglobulin JH and k light chain genes. The phenotype of this case could not be established because of the presence of extrinsic immunoglobulin on the tumour cells.

Case 30 deserves comment. The histological assessment of the lymph node was consistent with a centroblastic/centrocytic follicular lymphoma and the neoplastic cell population was shown to be phenotypically CD37 positive and immunoglobulin negative. As insufficient DNA was available from the lymph node biopsy specimen, a blood sample was analysed. Despite the restricted immunophenotypic analysis was not diagnostic for NHL, isolated DNA showed IgJH and k light chain gene rearrangements which provided evidence for B cell NHL (table 3). The value of this technique for analysis of blood from patients with NHL has recently been reported.34
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