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+ lymphocytoses 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 lymphomas³ 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 presence or 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 immunochemical 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 lymphocytoses.

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

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indirect immunoperoxidase using methods described previously.³

Immunohistochemical staining of frozen tissue was performed using a modification of Stein *et al.*¹⁶ 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 methods¹⁷ 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.¹⁸ These filters were prehybridised in 6 × SSC (saline sodium citrate), 0.5% sodium dodecyl sulphate (SDS), 5 × Denhats solution, and 6% poly-ethyleneglycol 8000 containing heterologous fragmented salmon sperm DNA at 65°C overnight, followed by hybridisation at 65°C overnight in the same solution containing added ³²P-oligolabelled gene probe. Filters were subsequently washed under stringent conditions¹⁷ and hybridising bands visualised by autoradiography for three to 10 days at -70°C using Fuji-RX film. After autoradiography, probe was removed from filters by washing

with 0.4M NaOH at 45°C for 30 minutes, followed by 0.1 × SSC, 0.1% SDS, 0.2M TRIS-HCl, pH 7.5, at 45°C for a further 30 minutes. Filters were then subsequently re-hybridised with further gene probes.

The probes used in this study were an immunoglobulin heavy chain joining region J_H probe (C76 R51A),¹⁹ an Igκ chain constant region probe (PUCR17),²⁰ an Igλ constant region probe (CHR22λS8c),²¹ a TcRβ chain gene probe (Jurkat 2),²² a TcRγ gene probe²³ and TcRδ gene probes for Jδ1, JδS16²⁴ and Jδ2, R21XH.²⁵

Each DNA preparation was digested with three different restriction enzymes—*Bam* HI, *Eco* RI, and *Hind* III. All digests were analysed with the TcRβ probe. The TcRδ probes were used with *Bam* HI and *Hind* III digests. The TcRγ and J_H probes were used to investigate at least two restriction digests in each preparation, and the Cκ probe was used in conjunction with *Bam* HI digests and Cλ with *Eco* RI digests. In addition, DNA from seven cases was digested with the restriction enzyme *Kpn* I and probed with the TcRγ gene probe.

DNA probes were prepared by plasmid amplification in bacterial host strains. Probe gene inserts were excised from low gelling temperature agarose gels after appropriate endonuclease restriction and radiolabelled with α-³²P-dCTP by the random hexanucleotide primer method.²⁶

Results

CD3 + CD8 + PERIPHERAL BLOOD

LYMPHOCYTOSES

Data from patients investigated with CD3 + CD8 + 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 V γ 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 co-existent B cell chronic lymphocytic leukaemia.

Table 1 CD3 + CD8 + lymphocytoses

Case No	Leucocytes (10 ⁹ /l)	Lymphocytes (10 ⁹ /l)	Phenotype							Genotype						
			CD2	CD3	CD4	CD8	CD16	CD57	HLA			TcR		Ig		
									Class II	Igκ	Igλ	Tγ	Tβ	J _H	Cκ	Cλ
1	9.5	6.8	90	96	3	92	1	9	5	1	1	R	R	G	G	G
2	9.8	7.7		63	5	78	0	55	2	3	1	R	R	G	G	G
3	7.6	4.6	85	90	10	81	24	30	4	1	1	R	G	G	G	G
4	10.8	7.1	90	95	8	64	1	6	13	1	1	G	G	G	G	G
5	11.8	9.5	81	54	22	68	1	36	16	3	3	P	G	G	G	G
6	3.4	2.4		75		69		10	34	10	8	R	R	G	G	G
7	2.6	2.2	95	90	9	86	86	0	90	0	0	R	R	G	G	G
8	13.9	4.7	90	95	18	90		80	11	1	1		R	G	G	G
9	14.3	10.1	50	70	10	80				1	1	G	G	R	R	G
10*	23.6	9.4	25	23					70	30	30	R	R	G	G	

*Represents a case for which there was no clear neoplastic phenotype.

Figure 1 Cases 1 and 3: 32 P-labelled TcR γ (A) and TcR β (B) probes. Rearranged bands are arrowed. Lanes marked C represent control DNA digests with germline bands indicated by kilobase pair size. Clonally rearranged TcR γ bands are present in case 1 (V γ 8) and case 2 (V γ 8 and 9). Case 1 is clonally rearranged for TcR β : + indicates a partial digest and * represents a plasmid contaminant.

(This case has been reported in detail.⁹)

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 TcR β 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 TcR γ and β chain genes. Three cases were germline for TcR β and immunoglobulin genes but exhibited polyclonal rearrangements of TcR γ 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 TcR δ gene rearrangement: case 12 was rearranged for J δ , but cases 11, 13, and 15 were germline for TcR, J δ 1, and J δ 2. The remaining cases showed regressing atypical histiocytosis affecting the skin (table 2). One of these (case 18) exhibited clonal TcR γ

Table 2 T cell or histiocytic NHL

Case No	Histology	Tissue	Phenotype								Genotype				
			CD2	CD3	CD4	CD5	CD7	CD8	HLA Class II		TcR		Ig		
											T γ	T β	J δ	C κ	C λ
11c	Lymphoblastic	Lymph node	+	(+)	-	+	+	-	-		G	G	G	G	G
		Bone marrow	+	-	-	-	+	-	-		G	G	G	G	G
12	Pleomorphic	Lymph node	+	-	-	-	+	-	+		R	R	G	G	G
13	Pleomorphic	Lymph node	-	-	-	-	(+)	-	+		G	G	G	G	G
14	Pleomorphic	Skin	-	+	-	-	-	+			P	G	G	G	G
15	Lennert's	Lymph node	+	+	-	-	+	-			P	G	G	G	G
16b	T zone	Lymph node									P	G	G	G	G
17a	T zone	Lymph node		-	-	-	-	+			G	G	R	R	G
18	Histiocytic	Skin	(+)	+	(+)	-	-	-			R	R	G	G	G
19	Histiocytic	Skin	-	-	-	-	-	-	+		R	G	G	G	G

Tumour population was positive +, variably positive (+) or negative -.

a Atypical cells were also CD37 positive.

b Phenotype was not assessed.

c Lymph node cells were positive for CD11 and CD33, bone marrow cells were positive for CD11, CD13, and CD33. Cells from both preparations were negative for TdT.

Table 3 B cell NHL

Case No	Histology	Tissue	Phenotype					Genotype				
			CD3	CD37	Ig	HLA Class II		TcR		Ig J δ	C κ	C λ
								T γ	T β			
20	Centroblastic/centrocytic follicular	Lymph node	-	+	-	+		G	G	R	R	G
21	Centroblastic/centrocytic follicular		b	b	b	b		G	G	R	R	R
22	Centroblastic/centrocytic follicular		b	b	b	b		G	G	R	R	G
23	Centroblastic/centrocytic follicular diffuse		b	b	b	b		R	R	G	G	G
24	Centroblastic/centrocytic diffuse		b	b	b	b		G	G	G	G	G
25	Centroblastic diffuse		-	(+)	-	+		G	G	R	D	R
26	Centroblastic diffuse		-	+	+b	+		G	G	R	R	G
27	Centroblastic diffuse		(-)	+	-	+		G	G	R	R	G
28	Centroblastic diffuse		-	+	-	+		G	G	R	R	G
29	Centroblastic diffuse	Blood	-	+	-	+		G	G	R	R	G
30	a		b	b	b	b		G	G	R	R	R

Tumour population was positive +, variably positive (+), or negative -.

a Lymph node biopsy specimen histology was consistent with centroblastic/centrocytic follicular lymphoma.

b Phenotype could not be ascribed to tumour population.

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 32 P labelled TcR γ (A) and TcR β (B) probes. Rearranged bands are arrowed. Lanes marked C represent control DNA digests with germline bands indicated by kilobase pair size. Clonally rearranged TcR γ bands representing V γ 3 V γ 9 and V γ 2 gene use are present in cases 18 and 12 respectively. Cases 14–16 exhibit polyclonal V γ bands corresponding to V γ 11, 3, 8, 9, 5, 2/4 in case 15, V γ 11, 3, 8, 9, 5 in case 15, and V γ 2 + 4, 9, 5, 8 in case 14. RH clonal TcR β gene rearrangements are represented in B. *represents a plasmid contaminant.

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

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 Ig κ and λ 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 TcR γ using V γ gene segments 8 and 9 in the absence of TcR β gene rearrangement, but this case was not tested for TcR δ rearrangement and we were unable to exclude the possibility that this case may have represented a population of cells expressing TcR $\gamma\delta$. This possibility is supported by genotypic data because V γ 8 and 9 gene segments are frequently used by cells expressing TcR $\gamma\delta$.²⁸

Three cases of CD3+ CD8+ lymphocytoses failed to show monoclonal patterns of TcR β gene rearrangement. One case exhibited multiple TcR γ 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+ lymphocytoses masked underlying B cell disease. In case 9 the emerging B cell tumour had immunoglobulin clonal

Figure 3 DNA was isolated from lymph node biopsy specimens from cases 28 and 23 and from blood lymphocytes from case 30. DNA was digested with restriction enzymes *Bam* HI, *Eco* RI, and *Hind* III and probed with 32 P labelled Ig C κ (A), Ig C λ (B), Ig J μ (C) and TcR β (D) probes. Rearranged bands are arrowed. Lanes marked C represent control DNA digests with germline bands indicated by kilobase pair size. Clonally rearranged bands are present in all DNA samples analysed. □ indicates the λ pseudogene.

rearrangements identical with those identified in the peripheral blood at the time of the CD3+ CD8+ lymphocytosis.⁹ 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 IgM λ 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.*³⁰ 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 origin³¹ and is a similar to a series of cases reported recently by Kurtzberg *et al.*³² 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 TcR γ gene rearrangements consistent with polyclonal T cell proliferations. In a third case there was increased intensity of one TcR γ rearranged band (V γ 8) among multiple rearranged bands. In the absence of TcR β clonal rearrangement it was difficult to decide whether this represented a clonal TcR γ gene rearrangement or preferential V γ gene segment use in the polyclonal population. Unfortunately, insufficient DNA was available from this case for probing with the TcR δ 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 J $_H$ and κ 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 poly-

clonal 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,³⁴ 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 TcR γ but not TcR β . Insufficient DNA was available for TcR δ probing and we are unable to conclude whether the cells expressed TcR $\gamma\delta$. RH was rearranged for TcR γ 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.*³⁵

The B cell lymphomas included in this study were referred 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%.^{5 6} In our experience immunoglobulin negative tumours may comprise 5% of B cell follicular lymphomas.³ We were able to show that all the immunoglobulin negative tumours were both CD37 positive and were rearranged for Ig J $_H$ 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 cytogenetic 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 TcR γ and β genes consistent with a pseudofollicular T cell lymphoma.³⁷ The tumour cells from the remaining case were shown to be clonally rearranged for immunoglobulin J $_H$ and κ 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 fact that the phenotypic analysis was not diagnostic for NHL, isolated DNA showed IgJ $_H$ and λ 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.³⁸

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