A rapid and reliable PCR method for detecting clonal T cell populations

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Abstract

Aims—To establish a reverse transcription polymerase chain reaction (RT-PCR) for the detection of clonal T cell populations, and to evaluate the sensitivity and reliability of the technique.

Methods—After reverse transcription of the target RNA with a consensus T cell receptor (TCR) β constant (C) region primer, consensus C, variable (V), diversity (D) and joining (J) region primers were used to amplify across various portions of the TCR β V-D-J-C junction.

Results—In normal T cells the polyclonal rearrangements produce a ladder of PCR bands representing the different sized junction fragments. The presence of a T cell clone leads to over-representation of one junction fragment, hence a disproportionately brighter band in the PCR ladder. In a series of 16 patients the RT-PCR detected nine of nine shown to have a clonal TCR β rearrangement by Southern blotting and for six of seven patients, it confirmed the presence of a clone indicated by histology or immunophenotyping with FACS analysis, but which was undetectable (five patients) or not investigated (two patients) by Southern blotting. Investigations mixing RNA from normal lymphocytes and the Jurkat TCR-V β 8 T cell line suggested that the method was more sensitive than Southern blotting.

Conclusions—All PCR methods are faster and easier than Southern blotting, but RT-PCR also improves detection of clonal T cell populations, is reliable and produces results that are easy to interpret.

(J Clin Pathol: Mol Pathol 1995;48:M101-M104)

Keywords: T cell receptor β chain rearrangement, T lymphocyte clone, polymerase chain reaction.

Unlike B cells, reactive T lymphocytes cannot be readily phenotypically distinguished from a clonal neoplastic proliferation. The distinction for both types of lymphocytes can be made

genotypically by consideration of antigen receptor gene rearrangement.¹ Because malignant cells have a common clonal origin, cells of a lymphoid malignancy will share the same antigen receptor rearrangement.

The established method for detecting a clonal lymphoid proliferation is by Southern blotting but this is slow and technically demanding.¹ Polymerase chain reaction (PCR) amplification can overcome these difficulties. McCarthy *et al*² detected B cell clones by amplification across the junction of the variable (V), diversity (D) and joining (J) regions to highlight their unique rearrangement. Several PCR methods of varying complexity have been documented for detection of clones with T cell receptor (TCR) γ ³⁻⁵ and TCR δ ⁶ rearrangement. However, TCR β rearrangement is a more T cell specific marker.⁷

Several groups⁸⁻¹⁰ have amplified TCR β complementary DNA (cDNA) with a consensus constant (C) region primer and primers specific for each of the different V β families, hoping to see much stronger amplification for the family utilised by the clonal cells. However, in a normal subject up to 20% of T cells can rearrange using the same V β family,¹¹ making it impossible to distinguish the presence of a small clonal cell population. Re-amplifying the V β family PCR products may reveal the clone,^{8,9} but this is time-consuming and expensive.

The apparently straightforward method for amplifying DNA across the TCR β recombination junction and looking for a single band generated from a clonal rearrangement¹² only works with Taq polymerase from one or two manufacturers (McCarthy KP, 1994, personal communication) and it can be difficult to distinguish the clonal band from many other non-specific PCR products of similar size.¹³

It is possible that these multiple bands result from amplification of TCR β pseudogenes. These do not appear to produce a RNA message,¹⁴ but even if they are not the cause of the extra bands, PCR following gene specific reverse transcription will increase specificity. In the simple method reported here we consider the V-D-J-C junctions of TCR β RNA. The results are easy to interpret and in our short series clonality has been confirmed in most cases where it had been suggested by other methods.

Methods

RNA was extracted from 5×10^6 peripheral blood mononuclear cells or approximately 50 mg frozen lymph node tissue using RNazol

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Accepted for publication
26 January 1995

Table 1 Primer sequences and quantities used in cDNA amplification

Primer	Sequence	ng primer/reaction
V	5'-TGTAYCTCTGTGCCAGCAG	200
C*	5'-CGACCTCGGGTGGGAACAC	100
J ₁	5'-ACAGTGAGCCMGGTGCC	400
J ₂	5'-AGCACYGTGAGCCTGGTGCC	200
D ₁	5'-CAAAGCTGTAACATTGTGGGGAC	100
D ₂	5'-TCATGGTGTAACTTGTGGGGAC	100

Y=C or T; M=T or G.

Primer sequences after McCarthy *et al*¹² and *Bahler *et al*.⁹

Table 2 Primer combinations used and the expected size range of the products

Primer combination	Approximate product size (bp)
V/C	100–120
V/J ₁	70–90
V/J ₂	70–90
D ₁ /J ₁	60–80
D ₁ /J ₂	60–80
D ₂ /J ₂	60–80

bp = base pairs.

B (Biogenesis, Poole, UK) according to the manufacturer's instructions, and resuspended in 25 µl water. One third to one half (approximately 5 µg) of this RNA was used to prepare a TCRβ specific cDNA by reverse transcription with M-MLV reverse transcriptase (Gibco, Paisley, Scotland, UK) using a consensus Cβ primer (5'-TCTGCCAGAAG-GTGGCCGAG¹⁵). The cDNA was diluted with 160 µl water and 4 µl used as template in the PCR reactions.

PCR reactions were performed in a 50 µl volume containing the cDNA, 200 µM nucleotides, 1 × the Taq manufacturer's reaction buffer, the primer combinations and amounts (which take account of the number of degenerate bases in each primer) shown in table 1 and 0.5 µl Taq polymerase. We routinely used SuperTaq (HT Biotechnology, Cambridge, UK), when 0.5 µl of a 1 in 10 dilution of enzyme in 10 × reaction buffer was sufficient. The enzyme was added only after two minutes of initial denaturation at 94°C and samples were then subjected to 30 cycles of 94°C, 55°C and 72°C for one minute each in a Perkin-Elmer 480 thermocycler.

The PCR product (20 µl) was separated at 200 volts on a 12% mini polyacrylamide gel. The larger V/C PCR products (table 2) were electrophoresed for 1 3/4 hours whereas one hour was sufficient for all other products.

The sensitivity of the method was established by mixing cDNA obtained from normal blood with that from the T cell line Jurkat J6 (from the European Collection of Animal Cell Cultures) in various proportions (on the basis of absorbance at 260 nm) and subjecting the mixtures to PCR with the V/C primers.

For Southern blotting, 10 µg genomic DNA was digested with *Eco*RI, *Bam*HI or *Hind*III, separated on a 0.8% agarose gel and transferred to Hybond N (Amersham, Little Chalfont, UK). The TCRβ constant region probe M13IB10BBI¹⁶ and TCRγ joining region probe M13H60¹⁷ were labelled with DIG-dUTP (Boehringer Mannheim UK, Lewes, UK) by

PCR.¹⁸ Hybridisation and detection with Lumi-Phos 530 were carried out broadly according to Boehringer Mannheim protocols. T cell disease was suggested in the peripheral blood mononuclear cells of some patients by abnormalities in leucocyte immunophenotyping and in lymph node sections by an antibody panel including UCLH1 (DPC Ltd, Llanberis, Caernarvan, Wales, UK) which detects the T cell CD45RO antigen.

Results

The RT-PCR product from normal peripheral blood mononuclear cells amplified with all primer combinations appeared as a ladder, with seven or eight bands usually clearly distinguishable (fig 1, lane 1). The sizes of the ladder fragments were normally distributed with the greatest concentration (brightest bands) close to the mean of the known size ranges for the different fragments amplified (table 2).

Of nine patients previously shown to have a clonal T cell population by Southern blotting and seven where the diagnosis was made on the basis of histology or immunophenotype (table 3), all but one (patient 5) produced PCR products with at least one of the primer combinations that were distinctly different from normal individuals. Some patients (fig 1, lane 8) produced only a single band for some or all of the primer combinations tested. In others (fig 1, lane 2) ladders of bands were still visible, but for at least one primer combination one of the bands was over-represented and assumed to be derived from the clonal cells. Experiments diluting Jurkat cDNA with that from a normal patient showed that as little as 2% Jurkat cDNA gave a sufficiently bright PCR band within the normal polyclonal ladder to permit unequivocal detection of its presence (data not shown).

Patients with lymphocyte proliferations known to be because of a range of other factors were also investigated (patients 17–27, table 3). All of these showed a normal ladder pattern (fig 1, lanes 4–7) with no indication of over-expression of one particular size junction fragment.

We were able to repeat a random selection of these results using Taq polymerase from several other manufacturers. Samples of mononuclear cells isolated from some patients (1, 4, 13, and 17) on the same and different occasions produced consistent results.

Figure 1 Acrylamide gel analysis of RT-PCR products detects clonal T cell populations. Lane 1, normal patient with V/C primers; lane 2, patient with monoclonal lymphocytosis of unknown significance (MLUS) (16) with V/C primers; lane 3, patient with T cell chronic lymphocytic leukaemia (2) with V/C primers; reactive node (24), lane 4 with V/J₁ primers, and lane 5 with V/J₂ primers; reactive node (22), lane 6 with D₁/J₂ primers, and lane 7 with D₂/J₂ primers; patient with MLUS (13), lane 8 with V/J₁ primers, and lane 9 with V/J₂ primers; patient with T cell non-Hodgkin's lymphoma (8), lane 10 with V/J₂ primers, and lane 11 with D₂/J₂ primers. Size markers are a *Hinf* I digest of ΦX 174 DNA. bp, base pairs.

Table 3 Pathological details of the cases studies, and PCR and Southern blotting results

Case No.	Diagnosis	Tissue	Primer					Southern blotting
			V/C	V/J ₁	V/J ₂	D ₁ /J ₂	D ₂ /J ₂	
1	T-CLL (01) ^a	PB	+	+	+	+	NT	+
2	T-CLL (01)	PB	+	+	+	0 ^b	0	+
3	T-CLL (01)	PB	+	+	—	NT	NT	+
4	T-NHL (07)	PB	+	+	+	NT	NT	+
	CD3 ⁺ CD5 ⁺ CD4 ⁺ CD8 [—]							
5	T-NHL (10)	Node	—	—	—	—	—	—
6	T-NHL (15)	Node	+	—	+	+	+	+
7	T-NHL (17)	Node	+	—	+	—	NT	—
8	T-NHL (18)	Node	—	—	—	+	+	+
9	T-NHL (18)	Node	—	—	—	+	+	+
10	T-NHL (18)	PB	+	+	—	NT	NT	NT
11	T-NHL (19)	Node	—	+	+	—	—	—
12	T-NHL (19)	Node	+	+	+	NT	NT	NT
13	MLUS	PB	+	+	+	NT	NT	+
	CD3 ⁺ CD8 ⁺ CD4 [—]							
14	MLUS	PB	+	—	+	NT	NT	+
	CD2 ⁺ CD8 ⁺ CD4 [—]							
15	MLUS	PB	+	+	—	0	0	—
	CD5 ⁺ CD8 ⁺ CD4 [—]							
16	MLUS	PB	+	+	+	+	NT	—
	CD2 ⁺ CD8 ⁺ CD4 ⁺							
17	Autoimmune neutropenia	PB	—	—	—	—	—	—
18	NK leukaemia	PB	—	—	—	0	0	NT
	CD4 ⁺ CD8 ⁺ CD56 ⁺							
19	Glandular fever	PB	—	—	—	—	—	NT
20	Reactive lymphocytosis ^c	PB	—	—	—	—	—	NT
21	Reactive lymphocytosis ^c	PB	—	—	—	—	—	NT
22	Reactive lymphadenopathy	Node	—	—	—	—	—	NT
23	Reactive lymphadenopathy	Node	—	—	—	—	—	NT
24	Reactive lymphadenopathy	Node	—	—	—	—	—	NT
25	Follicular hyperplasia	Node	—	—	—	—	—	NT
26	B-NHL (09)	PB	—	—	—	—	—	NT
27	B-NHL (14)	Node	—	—	—	—	—	—
28	Normal	PB	—	—	—	—	—	NT
29	Jurkat cell line		+	+	+	NT	NT	NT

+, T cell clone detected; —, T cell clone not detected; PB, peripheral blood; MLUS, monoclonal lymphocytosis of unknown significance; NHL, non-Hodgkin's lymphoma; NK, natural killer; T-CLL, T cell chronic lymphocytic leukaemia.

^aPathological classification of tumours using the modified Kiel classification: 01 = chronic lymphocytic leukaemia, 07 = mycosis fungoides; 09 = low grade unclassified, 10 = peripheral T zone lymphoma; 14 = lymphoblastic; 15 = immunoblastic; 17 = large cell anaplastic; 18 = pleomorphic T cell; 19 = high grade unclassified.

^b0 = no amplified product detectable.

^cThese patients had a normal CD4/CD8 ratio.

Discussion

The RT-PCR described here improves detection of clonal T cell populations, being much faster than Southern blotting (receipt of sample to result is less than 1½ days) and more lineage specific than most other PCR methods. It is easy to perform, using the same "RNA to PCR" technology as is standard for the detection of a variety of chromosome translocations—for example, t(9;22),¹⁹ and the results are clear cut with no problems of interpretation.

The primer sets used amplify across all or part of the V-D-J-C junction producing a ladder of fragments differing in size largely because of the random nucleotides (N) inserted at individual joins. The normal distribution of fragment size is as predicted.²⁰ Where there is a T cell clone present, a particular band amplified from the identical junction of the clonal cells is over-represented. The intensity of this band in relation to the rest of the ladder depends on the proportion of T cells constituted by the clone, and these results suggest that as little as 2% of clonal cells produce a band sufficiently bright to be distinguished.

The primers are based largely on those used by McCarthy *et al*¹² and thus will not involve further expense for those who have already tried the DNA method. However, we considered it advisable to include a V to C amplification, which becomes possible in RT-PCR because the C segment is brought into contact with the V-D-J rearrangement during RNA transcription. A positive result with the V/C primers in addition to at least one of the V/J combinations increases confidence in the result.

Because both J primers cross-react with some members of the other J family,¹² both V/J combinations may produce a positive result. Theoretically, clones detected with the V/C primers should always be positive with the V/J primers and vice versa. This was the case in this study.

Using RT-PCR, we have successfully confirmed the presence of a clonal T cell population demonstrated by Southern blotting in nine of nine patients and in six of seven patients diagnosed on the basis of histology or immunophenotype, who were negative or not tested by Southern blotting. The most usual reason for Southern blotting failing to confirm the presence of a clonal rearrangement is that digests of the rearranged DNA produce fragments indistinguishable in size from the germ-line pattern. The apparent negative results on Southern blotting here may also be because of the lower sensitivity of the technique (5% of clones detectable¹) compared with the RT-PCR.

Conversely, two patients (8 and 9) shown by Southern blotting to have a clonal T cell population could not be detected by PCR using the V/C or V/J primer pairs. Such a failure might have been because of insufficient homology between the clonal rearrangement and the consensus V region primer which will not detect Vβ 2, 4, 8.3, 18, or 26 and we are currently investigating extra primers that would permit amplification from these Vβ families. However, in these cases the clones were not detected because they had a partial D to J rearrangement. Such rearrangements are detected by Southern blotting and have been transcribed¹⁶ so would

be detectable by PCR, but not using the V region primer. Amplifying across the D/J junction did reveal the T cell clones in these two patients (fig 1, lanes 10 and 11). Although complementarity to the D primers is likely to be destroyed by complete rearrangement of the TCR β gene, the D/J primers did indicate the presence of a clone in some patients (for example, patient 6) where it was also detectable with the V primer (that is, complete rearrangement). The most likely explanation is that the clone had rearranged at both alleles, but at one the rearrangement was incomplete.¹⁶

In one case (patient 11) there was no clonal band on amplification with the V/C primers although a band was visible using the V/J primers, probably because the percentage of clone was below the detection limit with the V/C primer combination, but on slight restriction of the rearrangements, which can be amplified with each of the J primers, the clone assumed sufficient significance to be detectable.

One patient (case 5) with a diagnosis of peripheral T cell lymphoma did not show TCR β rearrangement by Southern blotting or RT-PCR. This is perhaps not surprising as Weiss *et al*²¹ suggest that some patients with this lymphoma lack any rearrangement of their TCR genes.

In one patient (case 13) two clonal bands in the V/C and V/J₂ amplifications were observed but only one with the V/J₁ primers (fig 1, lanes 8 and 9). We attribute this to the particular J regions used in the clone, both of which could be detected by the J₂ but only one by the J₁ primer. The two bands may represent the presence of two clones or the rearrangement of both chromosomes within a single clone.

The RT-PCR specifically detects clonal T cell proliferations—no patient with any other lymphoproliferative condition gave a positive result (table 3, patients 17–27; fig 1, lanes 4–7). But, like Southern blotting, positive results indicate a T cell clone but not necessarily malignancy. Patients 13–16 presented with neutropenia and/or lymphocytosis but had no other clinical indicators of chronic malignant disease. Results of molecular biology investigations need to be interpreted with caution.

We suggest that V/C, V/J₁ and V/J₂ primer combinations are used first and if negative, then the D/J combinations. If all amplifications prove negative, Southern blotting might detect non-productive clonal rearrangements but, in our limited series, PCR failure because of an unproductive rearrangement does not seem to be a problem, indeed, the level of detection by RT-PCR was greatly superior to that achieved by Southern blotting. The method reported here forms the basis of a greatly improved system for detection of clonal T cell populations.

We thank Drs Demaine and Hibberd for helpful discussion and gift of the reverse transcription and V β family specific PCR primers. DH is supported by the Musgrove Leukaemia Group, Somerset.

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