Technical report

Analysis of T cell receptor β chain CDR3 size using RNA extracted from formalin fixed paraffin wax embedded tissue

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Accepted for publication
12 June 1997

Abstract
***Aims*—To isolate RNA and DNA simultaneously from formalin fixed paraffin wax embedded tissue to assess the clonality of enteropathy associated T cell lymphomas and to analyse it in detail by a non-radioactive method of T cell receptor complementarity determining region 3 (CDR3) spectratyping.**
***Methods*—DNA and RNA were isolated simultaneously from formalin fixed paraffin wax embedded tissue blocks and subjected to the polymerase chain reaction (PCR) and semi-nested reverse transcription PCR (RT-PCR), respectively. The RT-PCR T cell receptor Vβ products were analysed by CDR3 spectratyping using a denaturing polyacrylamide gel and silver staining.**
***Results*—Usable DNA and RNA were isolated simultaneously from formalin fixed paraffin wax embedded tissue. The specific clonality of the tissue was successfully analysed by a non-radioactive method of T cell receptor CDR3 spectratyping of the RT-PCR products. CDR3 spectratying of the RT-PCR products demonstrated the precise clonal nature of the tumour and non-tumour tissue showing that the non-tumour tissue comprised an oligoclonal population of a number of different T cell receptor Vβ families. The tumour tissue comprised two T cell subtypes of the one family, T cell receptor Vβ9.**
***Conclusions*—RNA and DNA were isolated from formalin fixed paraffin wax embedded enteropathy associated T cell lymphoma tissue. Detailed analysis of clonality can be carried out by a non-radioactive method of CDR3 spectratyping.**
(*J Clin Pathol* 1997;**50**:811–814)

Keywords: T cell receptor; CDR3; polymerase chain reaction; reverse transcription-polymerase chain reaction

Most tissues available for pathological investigation are in the form of formalin fixed paraffin wax embedded specimens. Techniques that allow detailed molecular analysis of these sources are important. This is particularly true for uncommon disorders such as enteropathy associated T cell lymphomas where, realistically, a comprehensive study of the disease can only be performed using archival material. A number of studies has been carried out to investigate the clonality of lymphomas using DNA extracted from formalin fixed paraffin wax embedded tissue.[1–3] Recently, DNA was used to study clonality in patients with enteropathy associated T cell lymphoma.[4] Clonality was demonstrated by detecting rearrangements of the T cell receptor β and T cell receptor γ chain genes. These studies only used DNA, and without extensive sequencing of the polymerase chain reaction (PCR) products, the precise nature of the clonal cells present could not be determined.

Isolation of RNA from paraffin wax embedded tissue was described in 1987.[5] Recently, we isolated RNA from enteropathy associated T cell lymphoma formalin fixed paraffin wax embedded tissue blocks.[6] Using a radioactive reverse transcription-polymerase chain reaction (RT-PCR) method we demonstrated the oligoclonality of the T cells in the tumour.[6] Use of RNA rather than DNA gives a more comprehensive indication of the clonal nature of enteropathy associated T cell lymphoma as it is possible to identify the T cell receptor families present. Very detailed analysis of T cell clonality can be assessed by a method known as T cell receptor complementarity determining region 3 (CDR3) spectratyping. It is based on the fact that random nucleotides are inserted between the V-D and D-J regions of the T cell receptor β chain during DNA rearrangement resulting in different sized PCR products.[7]

In this paper we demonstrate that it is possible: simultaneously to isolate usable RNA and DNA from formalin fixed paraffin wax embedded tissue; to use them to assess clonality; and to do detailed analysis of enteropathy associated T cell lymphomas by a non-radioactive method of CDR3 spectratyping.

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Methods

TISSUE SAMPLES

Formalin fixed paraffin wax embedded tissue blocks were obtained from patients with enteropathy associated T cell lymphoma and from non-enteropathy associated T cell lymphoma patients with normal histology, at St James's University Hospital, Leeds. All tissue blocks were examined initially by staining with haematoxylin and eosin and assessed by a pathologist.

EXTRACTION OF RNA AND DNA FROM FORMALIN FIXED PARAFFIN WAX EMBEDDED TISSUE

RNA was extracted from 10 × 10 μm dewaxed sections by the previously described method[6] using 25 mM EDTA, 100 mM NaCl, 10 mM Tris (pH 8.0 containing 0.25% wt/vol sodium dodecyl sulphate) and 2 mg/ml proteinase K. RNA was extracted using 300 μl of phenol (pH 4.5–5.5) and chloroform (1:1). The top RNA containing layer was removed and the bottom layer used for DNA extraction.

DNA was extracted using a modification of the DNA isolation instructions on the TRIZOL (Life Technologies, Paisley, Scotland) protocol sheet. Briefly, 0.3 ml of ethanol was added, incubated for three minutes at room temperature and centrifuged. The DNA pellet was washed in 1 ml 0.1 M sodium citrate in 10% ethanol for 30 minutes at room temperature, resuspended in 1 ml 75% ethanol, incubated at room temperature for 20 minutes and centrifuged. The DNA was dried at room temperature for two to three hours, dissolved in 60 μl of 8 M NaOH, and 4 μl of 0.1 M HEPES buffer was added; the DNA was then measured and stored at 4°C until use.

REVERSE TRANSCRIPTION AND SEMI-NESTED PCR OF RNA

Reverse transcription of total RNA (2 μg) was performed (37°C for 1.5 hours) using a T cell receptor Cβ primer, Cβ-RT (5'-GCTCTACCCCAGGCCTCGGCGC-3'). Semi-nested PCR was performed using Cβ1 (5'-CCTTTTGGGTGTGGGAGATC-3') and T cell receptor Vβ family primers, Vβ1, 2, 3, 4, 5.1, 5.2, 6, 7, 8, 9, 10, 11, 12, 13.1, 13.2, 14, 16, 18, and 20[8] and T cell receptor Vβ15, 17, and 19.[9] T cell receptor Vβ21 (5'-AGCCAGCAGAGCTTGGGG-3'), 22 (5'-CTGAACATGAGCTCCTTGG-3'), 23 (5'-GCTGGAGGACTCAGCCAT-3'), and 24 (5'-CATCCGCTCACCAGGCCTG-3') primers were also used and were designed inhouse. Controls were performed using a pair of T cell receptor constant β primers, Cβ1 and Cβ2 (5'-AACACCTTGTTCAGGTCCTC-3'). Hot start PCR was performed (94°C for five minutes, then 40 cycles of 57°C for 45 seconds, 72°C for one minute, 94°C for 45 seconds, and 57°C for 45 seconds, followed by a final incubation of 72°C for 10 minutes). Each PCR product was diluted 1/10 and 1 μl added to the nested PCR. The second 30 cycle PCR was performed using the same T cell receptor Vβ primers and a different T cell receptor Cβ primer, Cβ3.[8] The PCR products were analysed on a 2% agarose gel containing ethidium bromide.

PCR OF DNA

PCR was performed on DNA (1 μg) using T cell receptor Vβ, Dβ1, Dβ2, Jβ1, Jβ2, Vγ and Jγ primers, and the immunoglobulin (Ig) V_H and J_H primers.[1,3] The conditions were as follows: 94°C for five minutes, 40 cycles of 94°C for one minute, 55°C for one minute, and 72°C for one minute, followed by a final extension at 72°C for five minutes. The PCR products (20 μl) were run on a 10% non-denaturing polyacrylamide gel (250 V for 1.5 hours) and stained with ethidium bromide.[4]

T CELL RECEPTOR CDR3 SPECTRATYPE ANALYSIS

A 6% denaturing polyacrylamide gel was cast in a sequencing gel rig and heated to 55°C. T cell receptor Vβ PCR products (3 μl) were added to 2 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), and heated to 94°C. The samples (1 μl) were run until the xylene cyanol reached the bottom.

SILVER STAINING

The gels were stained using the protocol for silver sequencing detailed by Promega. Briefly, the gels were fixed in 10% acetic acid, stained in silver solution (1 g silver nitrate, 1.4 ml 40% formaldehyde in H_2O) and developed in developing solution (30 g sodium carbonate and 1.4 ml of 40% formaldehyde in H_2O containing 200 μl of fresh sodium thiosulphate (10 mg/ml)).

Results

DNA PCR RESULTS

DNA PCR results from a non-enteropathy associated T cell lymphoma control patient were consistent with a polyclonal or oligoclonal cell population (fig 1A). A smear of bands containing a strong band was observed with the T cell receptor primers. A smear of bands was detected with T cell receptor Dβ1+2 and Jβ2 primers and the Ig primers. Nothing was observed for the Dβ and the Jβ1 primers (lanes 3 and 5). This had been documented previously.[1] Two bands of 80 and 90 base pairs were observed with the T cell receptor Vβ and Jβ2 (lane 2) and two fainter bands of similar size detected with T cell receptor Vβ and Jβ1 (lane 1).

In contrast, in the DNA PCR products from an enteropathy associated T cell lymphoma patient (fig 1B), a single 66 base pair band was detected using T cell receptor primers, indicating a clonal expansion (lane 7). However, a single band over a fainter smear of bands was observed using Dβ1 and Jβ2 primers (lane 4). Two bands of 80 and 90 base pairs were observed with the T cell receptor Vβ and Jβ2 primers (lane 2), as well as number of bands using the Ig primers, indicating the presence of polyclonal B cells.

RNA PCR RESULTS

CDR3 spectratype analysis of RT-PCR products from the control tissue demonstrated that

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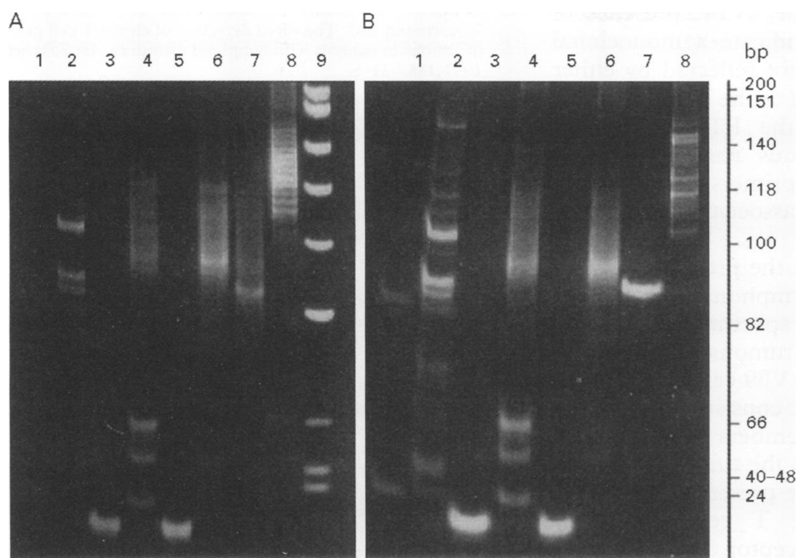

Figure 1 PCR results of DNA extracted from (A) normal small intestine and (B) enteropathy associated T cell lymphoma tumour tissue. Primers tested were: lane 1, T cell receptor (TCR) Vβ + TCR Jβ1; lane 2, TCR Vβ + TCR Jβ2; lane 3, TCR Dβ1 + TCR Jβ1; lane 4, TCR Dβ1 + TCR Jβ2; lane 5, TCR Dβ2 + TCR Jβ1; lane 6, TCR Dβ2 + TCR Jβ2; lane 7, TCR γ, and lane 8, immunoglobulin. Lane 9 is a base pair ladder.

a number, but not all, of the families were expressed (fig 2A). Some of the TCR Vβ families expressed comprised one band, such as TCR Vβ13.1, while others comprised a number of bands, such as TCR Vβ1 (fig 2A). In contrast, in the enteropathy associated T cell lymphoma only one TCR Vβ family, TCR Vβ9, was observed. This consisted of two distinct bands indicating the presence of two clones (fig 2B). This result was also observed in repeat experiments where the RNA was extracted again from the same block and from another block taken from elsewhere in the tumour (data not shown).

Discussion

Extensive studies on rare disorders require the use of archival formalin fixed tissue. Most work to date on enteropathy associated T cell lymphomas has either been on individual cases where fresh tissue was available, or has used DNA extracted from formalin fixed tissue.[4,10] In this study, we have simultaneously isolated DNA and RNA suitable for PCR based experiments from the same tissue blocks. This allows a more extensive investigation of the clonality of the tissues. Significantly, the RT-PCR products were suitable for analysis by CDR3 spectratyping. CDR3 spectratyping is most commonly carried out using radioactive labels, but in this paper we demonstrate the use of silver staining, which has not been used before for T cell receptor Vβ analysis of formalin fixed tissue. This technique has many advantages as it is fast, sensitive, informative, and economical.

DNA extracted from formalin fixed paraffin embedded tissue can be used to analyse T cell clonality by amplifying across the TCR Vβ junctional region. A PCR product of unique size for each T cell depending on gene usage and randomly inserted nucleotides is generated. Without sequencing PCR products, analysing DNA does not give information regarding which T cell receptor Vβ families are being expressed in the tissue. Furthermore, rearrangement of DNA does not necessarily indicate expression of a particular allele. The γ and δ genes are rearranged early in development and non-productive rearrangements of these loci allow for rearrangement of the α and β loci.[11,12] Therefore, it is possible to have a rearranged gene without expressing the T cell receptor on the cell surface. Furthermore, rearrangement of the T cell receptor β chain genes is in two stages. If the first V-N'-D-N-J rearrangement of the T cell receptor β allele does not produce a functional peptide, the second allele then undergoes rearrangement.[1,13] Therefore, T cell receptor β chain DJ rearrangement, which is only a partial rearrangement of the allele, does not give information on the T cell receptor β chain expression.

A previous study of the clonality of a series of enteropathy associated T cell lymphomas, using DNA isolated from fixed tissue, produced some ambiguous results.[4] In nine of 14 enteropathy associated T cell lymphoma patients no reactivity with two sets of VJ primers was detected, either in the tumour or uninvolved enteropathic bowel, and 11 of 14 showed no reactivity in uninvolved bowel, where a polyclonal smear of bands would have been expected. More significantly, of the five patient samples where a result was obtained with the VJ primers, one was shown to be monoclonal with the VJγ primers but polyclonal with the VJβ primers. Most rearrangements were observed in the T cell receptor γ genes and the DJ regions of the T cell receptor β genes and these may represent non-functional rearrangements.

In this paper we are able to demonstrate by spectratyping of RT-PCR T cell receptor Vβ products the oligoclonal nature of the normal small intestine, which is consistent with previously published results.[14] From the DNA results it was deduced that the cells present are polyclonal in nature owing to the smears obtained with the T cell receptor Vβ DJ, T cell receptor γ, and Ig primers. The T cell receptor

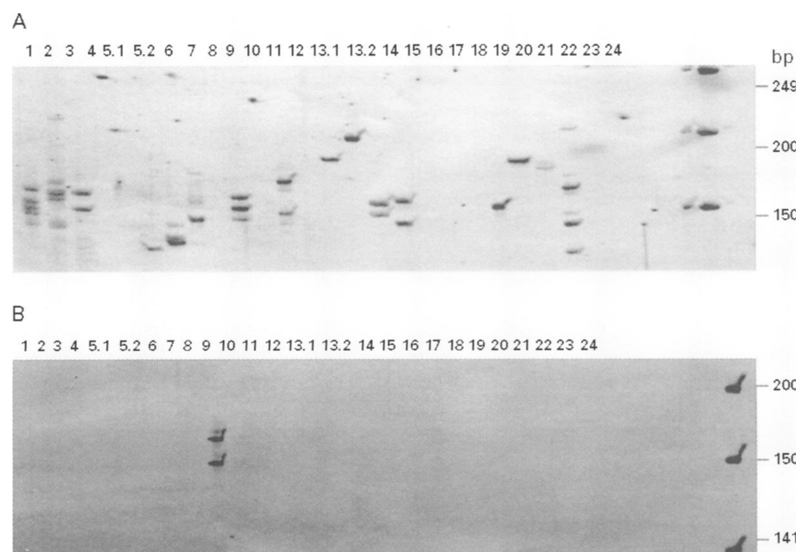

Figure 2 CDR spectratype analysis of (A) normal small intestine and (B) enteropathy associated T cell lymphoma using primers TCR Vβ1–24.

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Vβ VJ results are unclear, as the presence of isolated bands would indicate a monoclonal population, which was not reflected by either the T cell receptor Vβ DJ or the T cell receptor γ results, nor indeed the RT-PCR results. Given these and previous results,[4] perhaps these are not suitable primers for assessing clonality of enteropathy associated T cell lymphomas.

In the tumour sample the precise nature of the cells present in the lymphoma was demonstrated by the CDR3 spectratyping of the RT-PCR products. The tumour was found to comprise T cell receptor Vβ9 cells only. It is of interest that the tumour consists of cells that express two different members of this family. From the DNA analysis, the tumour is shown to be monoclonal by the presence of a single specific band with the T cell receptor γ primers. The T cell receptor Vβ DJ results show the presence of a single band over a lighter smear of bands. Perhaps the dominant band represents the monoclonal expressed allele over a number of non-functional alleles.

This paper demonstrates a useful method to analyse in detail the precise molecular nature of enteropathy associated T cell lymphoma. Usable RNA and DNA were isolated simultaneously from formalin fixed paraffin embedded tissue. The RNA was used successfully in RT-PCR and the products analysed in detail by a non-radioactive method of CDR3 spectratyping. The procedure allowed us to identify precisely the T cell receptor Vβ families being expressed in the tumour and normal tissue. This allows for easy comparison between tumour and non-involved tissue from the same patient and tumours from different patients. We are performing ongoing studies in such situations.

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