Technical report

Use of proteinase K for RT-PCR of cytokine mRNA in formalin fixed tissue

Graham N Davies, Ian S Bevan, Jytte B Lundemose, Harry Smith, Clive Sweet

Abstract
Fresh tissue from cases of sudden infant death syndrome is becoming increasingly scarce and therefore researchers interesting in studying the aetiology of this syndrome have had to resort to archival tissue, usually in the form of paraffin wax sections. A simple method for isolating mRNA from formalin fixed, paraffin wax embedded material of sufficient purity for reverse transcription (RT)-PCR is described. Proteinase K treatment of formalin fixed, wax embedded tissue followed by RNA STAT-60 extraction was successful in isolating mRNA suitable for RT-PCR. Interleukin (IL)-1α, IL-6 and tumour necrosis factor (TNF) transcripts were amplified successfully from heart, but not thyroid, kidney or liver tissue, of a patient who died following rejection of a transplanted heart, and IL-1α, but not IL-6 or TNF, transcripts from lung tissue of a six month old baby who died of viral pneumonia. Transcripts of a housekeeping gene were detected in all tissues. This method should be useful for examining expression in archival material.

Methods
Lung tissue from a six month old baby with viral pneumonia was collected within 36 hours of death in 1988 in Denmark. Thyroid, heart, liver, and kidney tissue was collected from a patient who died following rejection of a heart transplant in Birmingham in 1994. The tissues in each case were fixed in formal saline and embedded in paraffin wax following standard histological procedures.

Primers were designed from sequence data published previously to amplify specifically sequences from the human IL-1α, IL-6, TNFα, and PBGD genes. The sequences of the oligonucleotide primers (synthesised by Alta Biosciences, Birmingham University, Birmingham, UK) were as follows: IL-1α forward 5'-GGCAAAGTTCCAGACATG-3' and reverse 5'-GATGATCAATGGAGGAACTG-3'; IL-6 forward 5'-CTGAACCTTCCAAAGATGGC-3' and reverse 5'-GCCAAAGTTCCAGACATG-3'; TNFα forward 5'-GCCAAAGTTCCAGACATG-3' and reverse 5'-TGTACTCATCTGCACAGCC-3'; PBGD forward 5'-AGAGGACTCTGGGAGTAGATG-3' and reverse 5'-TGTACTCATCTGCACAGCC-3'; and reverse 5'-GTACGCAATGGAGAAGACTC-3'.

In children dying of sudden infant death syndrome (SIDS) there is often a history of upper respiratory tract infection and inflammatory changes can sometimes be seen at necropsy. The inflammatory response probably results from the production of inflammatory mediators such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF). We wished to examine tissue from children who died of SIDS for the presence of cytokines but fresh tissue is becoming increasingly scarce in the UK and elsewhere because of a notable reduction in such cases following recommendations to place babies in the supine rather than the prone position. Consequently, to obtain evidence for the production of cytokines, it is necessary to resort to formalin fixed, paraffin wax embedded archival material. Because of its sensitivity we wished to use reverse transcription (RT)-PCR to study cytokine gene expression in such tissues. While amplification of DNA isolated from archival material is well established, few studies have examined gene expression in such tissue using PCR; and, to our knowledge, no such studies on the detection of cytokine mRNA have been published. In the present paper we describe a method which has been used to identify successfully cytokine expression in lung tissue obtained from a six month old baby who died of viral pneumonia and in heart tissue from a patient who died following rejection of the transplanted heart but not in the thyroid, kidney or liver tissue of the same patient. Messenger RNA from the constitutively expressed porphobilinogen deaminase (PBGD) gene was detected in all tissues tested.

Keywords: RT-PCR, proteinase K, cytokine mRNA in fixed tissue.
from mRNA obtained using sample using same extractions of IL-1α and markers; of Figure unfixed respectively; K for (A) six month old RT-PCR IL-1α amplification v a E 4, Photoshop. DNA primers. extracted from 1, baby and primers for IL-1α, 3 and using who 6, product of 1. The kilobase DNA size between exons 174 (79, 127, 158 bp, respectively). This was because the primers spanned an intronic sequence between exons 2 and 3 for IL-1α and TNFα, and between exons 3 and 4 for IL-6. The product amplified from PBGD mRNA was 127 bp. The sequences of the probes used to confirm the identity of the amplified products were as follows: IL-1α 5'-TTCCAGACATGTGGAGACCT GAAGAAGCT-3'; IL-6 5'-AATCATCACCTGG TCTTTTGGAGTTTAGGTT-3'; TNFα 5'-AG CTGGGGGCAGCTCGTGATCGTGAAC CG-3'.

A lymphoblastoid cell line (U937) known to express these cytokines on stimulation was used to establish that the primers were suitable for amplification of DNA and RNA of the required specificity. U937 cells at a concentration of 1 × 10⁶ cells/ml were incubated with 50 ng/ml PMA (phorbol myristic acetate) at 37°C for 24 hours. Total DNA was extracted from the cells using the single tube method (Biotec Laboratories, Oxford, UK) while total RNA was extracted using RNA STAT-60 (Biogenesis Ltd, Poole, Dorset, UK) based upon a conventional guanidinium hydrochloride/phenol/CHCl₃ single-step extraction method.

Amplification from DNA followed a standard procedure. Briefly, 1 µg DNA in 100 µl reaction volume (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 1 mM each primer, 200 mM each dNTP, and 2.5 units of Taq DNA polymerase (Gibco-BRL, Paisley, UK) was amplified in a Techne PHC-2 temperature cycler under the following conditions: denaturation for three minutes at 94°C followed by 30 cycles of incubation at 94°C for 30 seconds, 60°C for 30 seconds for primer annealing and at 72°C for 60 seconds for strand elongation. This was followed by incubation at 72°C for nine minutes before amplified products were electrophoresed through agarose or acrylamide for analysis. Amplification of RNA was similar except that 500 ng of total RNA was reverse transcribed in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.7 mM MgCl₂, 0.005% gelatin, 1 mM each primer, 200 µM each dNTP, and 5 units of avian myeloblastosis virus reverse transcriptase (Pharmacia Biotech, St Albans, UK) for one hour at 42°C in the presence of 0.5 units RNAGuard (Pharmacia Biotech). The resultant cDNA was then denatured, 2.5 units Taq polymerase added and amplified as described earlier.

Oligonucleotide probes were 5'-end labelled with γ-[³²P] ATP using the T4 bacteriophage enzyme polynucleotide kinase.³ PCR products were electrophoresed through an 8% polyacrylamide gel and electroblotted onto a nylon membrane (Amersham, Aylesbury, UK) following the protocol supplied by the manufacturer. Membranes were prehybridised for two hours at 68°C in a solution containing 6x SSC buffer (1x SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 0.5% (w/v) sodium dodecyl sulphate (SDS), 5x Denhardt's solution (1x Denhardt's solution = 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin) and 100 µg/ml denatured calf thymus DNA. Hybridisation was carried out overnight at 5–10°C below the Tm of each individual probe in prehybridisation solution containing 2–5 × 10⁷ cpm/ml of radioactive probe (see earlier). The membrane was then progressivly washed
in SSC, varying the concentration of SSC and SDS and the temperature depending upon the probe. Membranes were then dried and exposed against Hyperfilm MP (Amersham) overnight at -70°C.

Results and Discussion

IL-1α, IL-6 and TNFα DNA and mRNA were detected in PMA stimulated U937 cells (fig 1A). Both the size of the amplified products and Southern blotting (data not shown) confirmed their identity. Other bands in lanes 5 and 7 represent non-specific priming as they did not hybridise with the oligonucleotide probes. IL-1α DNA could be detected readily in deparaffinised, formalin fixed tissue (fig 1B) but extraction of RNA from such tissue proved more problematical. The RNA STAT-60 method alone failed to yield suitable RNA as did disruption of tissue by one to three 30 second bursts of sonication in a water bath or three rapid freeze-thaw cycles in liquid nitrogen and thawing at 65°C followed by RNA STAT-60 extraction. However, RNA was extracted successfully by incubating four 10 μm sections of deparaffinised lung tissue from a six month old baby in 100 μl of deionised water containing 20 mg/ml proteinase K, either alone or with 1% SDS, followed by incubation at 37°C for 48 hours and extraction with RNA STAT-60. This RNA was satisfactory for amplification of PBGD and IL-1α gene transcripts (fig 1B).

This procedure was limited neither to lung nor infant tissue. The PBGD gene transcript was amplified successfully from liver, kidney, heart, and thyroid tissue of an adult (data not shown) and transcripts of the three cytokine genes were evident in heart tissue but not thyroid, kidney or liver tissue of this patient (fig 2A). The identity of the amplified products was confirmed by Southern blotting (fig 2B).

As in our studies, others have failed to detect mRNA (from the house keeping gene hypoxanthine phosphoribosyltransferase) in formalin fixed tissue although it was detected in tissue fixed in Carnoy’s fixative or Omnifix II. Using proteinase K digestion followed by RNA STAT-60 extraction, we have identified transcripts from the house keeping gene PBGD in several tissues (heart, lung, liver, kidney, and thyroid) and cytokine gene expression in tissues undergoing an inflammatory response: in lung as a result of pneumonia and in heart because of immune rejection, but not in other organs which were pathologically normal. Proteinase K digestion is also suitable for extraction of viral RNA.*

DNA contamination of RNA samples is a potential problem in RT-PCR but this was overcome by the use of RNA STAT-60 and primer pairs spanning intronic sequences which gave unambiguous evidence of RNA amplification as RNA and DNA amplified products were of different sizes.

This method is robust, easy to use, has been repeated by two different workers in several tissues and, in a blind trial, identified tissue undergoing inflammatory changes. We now intend to use the method to examine cytokine gene expression in tissues from cases of SIDS.

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