Detection of tumour necrosis factor $\alpha$ in sarcoidosis and tuberculosis granulomas using in situ hybridisation

N Myatt, G Coghil, K Morrison, D Jones, I A Cree

Abstract

**Aims**—To determine the site of tumour necrosis factor $\alpha$ (TNFa) product and mRNA in granulomas.

**Method**—In situ hybridisation with digoxigenin labelled or biotinylated oligonucleotide probes was used to demonstrate the presence of total mRNA, and then the presence of TNFa mRNA in the biopsy specimens of 37 granulomas (31 sarcoidosis, six tuberculosis).

**Results**—TNFa mRNA was detected in epithelioid cells, giant cells, and lymphocytes in the granulomas. Some sarcoidosis specimens did not contain detectable mRNA for TNF, but did contain TNF peptide in the epithelioid or giant cells on immunostaining. This may have been due to stored TNF present in cells in which mRNA for TNF is no longer being produced.

**Conclusion**—The results suggest that giant cells should not be regarded as effete cells, as they contain large amounts of mRNA and seem to be actively producing TNFa.

Granulomatous diseases such as tuberculosis, leprosy, schistosomiasis, and sarcoidosis pose serious health problems throughout the world. To prevent the development and clinical consequences of granulomatous diseases, either the tissue insult must be removed or the processes underlying granuloma formation need to be interrupted before tissue damage becomes clinically relevant. Sarcoidosis is characterised by the formation of simple (non-caseating) granulomas in many tissues, primarily lung and lymph node. Its aetiology is unknown, but it is clear that granuloma formation is a major cause of the fibrosis which eventually leads to respiratory failure and death in a substantial number of patients.

The pivotal role of tumour necrosis factor $\alpha$ (TNFa) in granuloma formation and maintenance was elegantly shown in a series of experiments by Kindler et al. in which injection of antibodies against TNFa at various stages following inoculation with Bacille Calmette Guérin (BCG) in mice prevented or reversed granuloma formation. This stimulated us to look at TNFa using in situ hybridisation (ISH) to determine which cells in the granuloma were expressing messenger RNA (mRNA) for this cytokine.

Although ISH was first described over 20 years ago, it has only recently become widely used in histopathology. In common with other nucleic acid hybridisation techniques, ISH is based on the re-annealing of complementary sequences of nucleic acid bases. ISH for messenger RNA (mRNA) is unique in that the technique is carried out without first removing the target sequence from its cellular surroundings, thereby allowing the exact position of the mRNA in a group of cells to be determined. Furthermore, the use of synthetic oligonucleotide probes permits easy penetration of routinely fixed, paraffin wax processed tissue. In this study we used two non-isotopic methods to demonstrate the presence of TNFa mRNA in biopsy specimens of sarcoidosis and tuberculosis that had been stored for varying periods as paraffin wax blocks.

**Methods**

Two methods were used: digoxigenin labelled oligonucleotide probes adapted from Farquharson et al.; the other used biotin labelled probes.

The biopsy specimens were drawn from the pathology files for 1986 to 1989 at Ninewells Hospital and Medical School and from the pathology files for 1976 to 1990 at Southampton General Hospital. The biopsy specimens of sarcoidosis were from lymph node or skin (21 from Dundee, 10 from Southampton), while the six biopsy specimens of tuberculosis (Southampton) were all lymph node specimens.

Twenty base length poly dT and poly dA oligonucleotide probes were synthesised by the Biochemistry Department at Dundee University on a 394 Applied Biosystems DNA synthesiser using $\beta$ thioctanoethyl phosphoramidite chemistry. Similar probes, 28 bases long, were purchased from Pharmacia Ltd (Milton Keynes, Bucks, England), and used in Southampton with their biopsy tissue.

The complete cDNA sequence for TNFa has been published. Sense and anti-sense TNFa probes, 25 bases long, were synthesised in Southampton also using an Applied Biosystems DNA synthesiser. Two probes were prepared from the open reading frame at the 5' end of the TNFa mRNA and two sequences from the coding region of the mature TNFa polypeptide. Sequences were chosen for their high GC content and low homology with lymphotoxin mRNA to ensure no probe cross-reaction. Both the sense and
anti-sense probes were used as mixtures of four probes to give increased sensitivity. Sequences for the antisense probes are given below: corresponding sense probes were also synthesised for use as controls.

TNFa ANTISENSE (5'-3')
(1) 346A CCG CCT GGA GCC CTG GGG CCC CCC C216
(2) 346 GGG GAA CTC TTC CCT CTG GGG GCC G250
(3) 733 GGC CTC AGC CCC CTC TGG GGT CTC C460
(4) 106 C TCT CTC CAG CTG GAA GAC CCC TCC41

DIGOXIGENIN PROBE LABELLING
Unlabelled TNFa probes were sent to Dundee in 100 μg quantities. On arrival, the probes were stored at -20°C overnight and then spun down in a microcentrifuge for 10 minutes at 12,000 × g. They were then freeze-dried overnight and re-precipitated in 50 μl 0.1M TRIS + 0.01M EDTA buffer to a concentration of 2 μg/ml. The probes were labelled at the 3' end using terminal deoxynucleotide transferase, as detailed by Farquharson et al.® Labelling of the probes was confirmed by spotting the labelled probes on to nitrocellulose filters and detecting their presence by incubating them first with anti-digoxigenin alkaline phosphatase conjugated Fab, fragments (Boehinger Mannheim), washing, and then incubating the filters with NBT/BCIP reagent, as detailed by Farquharson et al.® All reagents were prepared to be free of RNAse by autoclaving solutions and glassware, and by using DEPC-treated water.

The ISH method developed by Farquharson et al® was used without modification with probes for TNFa and poly dT. Controls included no probe, sense probe, and ribonuclease A (Sigma, Poole, Dorset, England) treated sections.

Probes were 3' labelled with biotin-11-dUTP (Sigma) using terminal deoxynucleotidyl transferase (Gibco BRL). Labelling was confirmed by development of dot blots of labelled probe by avidin-biotin.

Tissue sections were cut to 4 μm and mounted, by drying at 37°C overnight on aminopropyltriethoxysilane (Sigma) coated slides.12 Hybridisation was carried out at 37°C overnight in 50% formamide using probe at a concentration of 2 μg/ml. Unbound and partially bound probe was washed off by stringent high temperature low salt washes.13 Biotinylated probes were detected using direct application of an avidin-biotin complex alkaline phosphatase kit (Dako Ltd, High Wycombe, Bucks, England). Hybrids were visualised by naphthol ASBi Fast Red salt entrapment (Sigma).

Biotinylated and digoxigenin labelled poly dT and poly dA (sense control) probes were used to evaluate the mRNA content of each biopsy specimen before extensive evaluation using TNFa mRNA specific probes.14 Parallel immunocytochemical tests were performed for each biopsy specimen assayed. These were tested with an antibody panel consisting of Mac387 (calgranulin in macrophages; Dako), KP1 (CD68, activated macrophages; Dako), and 52B83 (anti-TNFα; Celltech, Slough, England). After application, bound primary antibody was detected indirectly using the avidin-biotin complex peroxidase method (Dako).

Results
In the Dundee study, using digoxigenin ISH, eight out of 21 sarcoidosis biopsy specimens gave a strong to medium signal with the poly dT probe. Signal was located in the cytoplasm of most cells in the tissue. Of these specimens, six out of eight showed hybridisation of the TNFa anti-sense probe in the cytoplasm of epithelioid cells, macrophages, giant cells, and possibly lymphocytes, identified morphologically. A representative high power view of one granuloma and the control (sense probe) is shown in fig 1. Control sections with antisense of ribonuclease A showed no specific staining.
In the Southampton study, using biotinylated probes, six out of 10 sarcoidosis biopsy specimens contained hybridisable mRNA and of these, four were positive for TNFα mRNA. However, all six tuberculosis biopsy specimens tested contained intact mRNA (fig 2A), and all six were also positive for TNFα mRNA (fig 2B). Immunoperoxidase staining (avidin-biotin complex method) of the same biopsy specimens with a monoclonal antibody (32B83, Celltech) against the TNFα mature peptide showed positivity in both giant cells and epithelioid cells (fig 3). A pattern of staining was frequently seen in which a negative giant cell was surrounded by positive epithelioid cells or a positive giant cell appeared within a group of negative epithelioid cells (fig 3). Two cases of sarcoidosis with no TNFα mRNA were positive for TNFα peptide by immunostaining.

Both digoxigenin and biotin labelled probes proved suitable for study of TNFα mRNA in paraffin wax processed tissue. The major technical problems encountered with digoxigenin were high background staining (overcome by optimising the final incubation in NBT/BCIP substrate) and contamination of reagents with RNAases. The latter was removed by sterilisation of bottles and water used to make up reagents.

**Discussion**

In this study both digoxigenin and biotin labelling of probes for non-isotopic in situ hybridisation have been used to good effect, with few differences between the results of both methods. The results show that mRNA for TNFα is present in both sarcoidosis and tuberculosis granulomas. The signal localised to the cytoplasm of the epithelioid cells, giant cells, and possibly lymphocytes in these granulomas. These cells have also been shown by immunohistochemistry to contain TNFα peptide. Our results challenge the traditional opinion of giant cells as degenerate, and suggest that they are in fact active cytokine producing cells which may have an important role in maintenance of granulomas.

Although the results from ISH are not quantitative, there does not seem to be any difference between sarcoidosis and tuberculosis in terms of the TNFα mRNA signal. In this study TNFα mRNA was undetectable by ISH in some cases of sarcoidosis in which total mRNA was identified, reflecting either low or absent TNFα mRNA values within the cells. However, immunostaining shows that in such cases, TNFα peptide may still be present within epithelioid and giant cells. As TNFα secretion depends on cleavage of membrane bound peptide, one explanation could be that epithelioid and other cells maintain an internal and surface complement of TNFα ready for release, even after transcription has ceased and mRNA for TNFα has become undetectable. Rook et al have suggested that macrophages can be primed for the release of TNFα by interferon γ (IFNγ). A trigger, such as lipopolysaccharide or mycobacterial
infection, is then required for release of the mature peptide.\(^7\)

Giant cells were found to contain considerable amounts of hybridisable mRNA even when TNFα mRNA was not demonstrated. They should not therefore be regarded as non-functioning cells,\(^9\) but are likely to be actively synthesising many products. In this study giant cells have been shown to contain both TNFα mRNA and product: they may also be important sources of other inflammatory mediators.

Although patients with sarcoidosis are treated with corticosteroids and these drugs are known to inhibit TNFα translation in vitro, they do not interfere with mRNA synthesis and would be unlikely to have direct influence on the values of TNFα mRNA.\(^8\) The lack of TNFα mRNA in some sarcoidosis biopsy specimens could be explained by selective destruction. This may occur as a result of the UA rich regulatory element which the mRNA of TNFα shares in common with many oncogene products and which renders mRNA very susceptible to breakdown by UA nuclease activity.\(^9\) Thus TNFα mRNA values may be normally low and suppression of UA nuclease as a result of cell activation may be necessary to allow concentrations of TNFα mRNA to rise and be detected by ISH.

Granulomas constantly evolve: they form from loose cell aggregates into highly organised structures and have considerable cell turnover within them.\(^10\) TNFα seems to be essential for granuloma maintenance,\(^1\) but its role in the formative stages of granulomas and in regression is less certain.\(^1,2\) It should therefore come as no surprise that TNFα mRNA is not always found in the temporal snapshots of granulomas represented by histological sections.

We thank Mr S Macpherson and Mrs S Gibbs for expert photographic assistance.

9. Kretschmer C, Morrison K, Jones DB, Schuler C, Ulmer AJ, Diamanstein T, et al. Tumour necrosis factor (TNF) mRNA to UA rich structures could be explained by selective destruction. This may occur as a result of the UA rich regulatory element which the mRNA of TNFα shares in common with many oncogene products and which renders mRNA very susceptible to breakdown by UA nuclease activity. Thus TNFα mRNA values may be normally low and suppression of UA nuclease as a result of cell activation may be necessary to allow concentrations of TNFα mRNA to rise and be detected by ISH.
Detection of tumour necrosis factor alpha in sarcoidosis and tuberculosis granulomas using in situ hybridisation.

N Myatt, G Coghill, K Morrison, D Jones and I A Cree

doi: 10.1136/jcp.47.5.423