Detection of tumour necrosis factor α in sarcoidosis and tuberculosis granulomas using in situ hybridisation

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Abstract

Aims—To determine the site of tumour necrosis factor α (TNFα) 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 TNFα mRNA in the biopsy specimens of 37 granulomas (31 sarcoidosis, six tuberculosis).

Results—TNFα mRNA was detected in epithelioid cells, giant cells, and lymphocytes in the granulomas. Some sarcoidosis specimens did not contain detectable mRNA for TNFα, 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 TNFα 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 TNFα.

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-annailing 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 TNFα 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 al9; the other used biotin labelled probes.9

The biopsy specimens used 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 β thiocyanatoethyl 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 TNFα has been published.10,11 Sense and anti-sense TNFα 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 TNFα mRNA and two sequences from the coding region of the mature TNFα 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 Anti-sense (5'-3')
(1) 348A CCG CCT GGA GCC CTG GGG CCC CCC61
(2) 346GGG GAA CTC TTC CCT CTG GGG GCC G300
(3) 715GGC CTC AGC CCC CTC TGG GGT CTC C669
(4) 106C TCT CTC CAG CTG GAA GAC CCC TCC84

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/µl. The probes were labelled at the 3' end using terminal deoxy-

nucleotide transferase, as detailed by Farquharson et al.8 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 (Boehringer Mannheim), washing, and then incubating the filters with NBT/BCIP reagent, as detailed by Farquharson et al.8 All reagents were prepared to be free of RNAase by autoclaving solutions and glassware; and by using DEPC-treated water.8

The ISH method developed by Farquharson et al.8 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.8,13 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 ASB 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 anti-sense 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 TNFa mRNA. However, all six tuberculosis biopsy specimens tested contained intact mRNA (fig 2A), and all six were also positive for TNFa mRNA (fig 2B). Immunoperoxidase staining (avidin-biotin complex method) of the same biopsy specimens with a monoclonal antibody (32B83, Celltech) against the TNFa 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 TNFa mRNA were positive for TNFa peptide by immunostaining.

Both digoxigenin and biotin labelled probes proved suitable for study of TNFa 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 TNFa 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 TNFa 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 TNFa mRNA signal. In this study TNFa mRNA was undetectable by ISH in some cases of sarcoidosis in which total mRNA was identified, reflecting either low or absent TNFa mRNA values within the cells. However, immunostaining shows that in such cases, TNFa peptide might still be present within epithelioid and giant cells. As TNFa secretion depends on cleavage of membrane bound peptide, one explanation could be that epithelioid and other cells maintain an internal and surface complement of TNFa ready for release, even after transcription has ceased and mRNA for TNFa has become undetectable. Rook et al. have suggested that macrophages can be primed for the release of TNFa by interferon γ (IFNγ). A trigger, such as lipopolysaccharide or mycobacterial
infection, is then required for release of the mature peptide.17

Giant cells were found to contain considerable amounts of hybridisable mRNA even when TNFa mRNA was not demonstrated. They should not therefore be regarded as non-functioning cells,19 but are likely to be actively synthesising many products. In this study giant cells have been shown to contain both TNFa 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 TNFa translation in vitro, they do not interfere with mRNA synthesis and would be unlikely to have direct influence on the values of TNFa mRNA.18 The lack of TNFa 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 TNFa shares in common with many oncogene products and which renders mRNA very susceptible to breakdown by UA nuclease activity.10 Thus TNFa mRNA values may be normally low and suppression of UA nuclease as a result of cell activation may be necessary to allow concentrations of TNFa 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 TNFa seems to be essential for granuloma maintenance,1 but its role in the formative stages of granulomas and in regression is less certain.12 It should therefore come as no surprise that TNFa mRNA is not always found in the temporal snapshots of granulomas represented by histological sections.

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