LABORATORY TECHNIQUES

Use of immunocytochemistry and biotinylated in situ hybridisation for detecting measles virus in central nervous system tissue

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Abstract

Optimised immunocytochemical (ICC) and in situ hybridisation (ISH) protocols for long term, formalin fixed, central nervous system tissue infected with measles virus were developed. The effectiveness of 10 proteases for the enzymatic unmasking of formalin fixed antigen and nucleic acid was investigated. Protease VIII gave maximal signal generation with optimal tissue preservation and no background staining for both techniques. The use of a microwave oven as an additional pre-hybridisation step for RNA-RNA in situ hybridisation produced a significant increase in the number of cells labelled for genomic RNA. The ability to show the presence of antigen and nucleic acid in long term, formalin fixed tissue facilitates the use of stored necropsy material available in pathology departments for ICC and ISH investigations.

Immunocytochemical techniques (ICC) for localising antigens and in situ hybridisation (ISH) for detecting nucleic acids allow specialised studies to be made on routine necropsy material. Formalin fixed, paraffin wax embedded tissue is the most readily available source of material in pathology departments and gives good morphological preservation. Therefore, optimised protocols for both ICC and ISH have the potential to provide valuable information for both diagnosis and research. Formalin fixation, however, is known to diminish virus antigenicity¹ and crosslinks viral nucleic acid to host protein² decreasing the sensitivity of ICC and ISH. The observation that some proteolytic enzymes can enhance the reactivity of formalin fixed antigens and nucleic acids and reduce non-specific background staining has led to their use in ICC and ISH procedures.³⁻⁵ Various enzymes have been used successfully for this purpose, but results have been variable and proteolytic digestion is a potential source of error.⁶ These problems may arise because each different combination—that is, antigen-tissue or nucleic acid-tissue—has different specific characteristics which must be taken into account if the antigen or nucleic acid is to be shown successfully in the particular tissue.

We previously compared the sensitivity of nick translated double stranded DNA and single stranded RNA probes and showed the former to be at least 100-fold lower in their level of detection (unpublished data). The

results of a comparative study between radioactive and biotinylated probes⁷ showed that if detection systems for biotinylated probes are optimised, then sensitivity can be greater than that achieved with ³⁵S labelled probes. In this study, therefore, measles specific antisera and biotinylated single stranded RNA probes specific for measles nucleic acid sequences were used to investigate the suitability of using different proteolytic enzymes for ICC and ISH procedures on long term formalin fixed tissue from a well characterised case of subacute sclerosing panencephalitis (SSPE), a persistent measles virus infection in man.⁸ The use of a microwave as an additional denaturing step in the ISH protocol was also investigated.

Methods

Blocks of brain tissue (frontal cortex and temporal) from a case of SSPE which had been stored in formalin for 23 years were processed to paraffin wax in an automatic tissue processor. Sections (4 µm) were cut on to 3-amino-propyltriethoxysilane (APES) treated slides⁹ and after drying at 60°C for 30 minutes were stored at room temperature before use. Tissue from normal brain was processed similarly to serve as a negative control.

Ten proteolytic enzymes were used in this study. Proteinase K (Boehringer, Mannheim, West Germany), protease III (prolase), protease VIII (subtilopeptidase A), protease IX, protease XIII (aspergillopeptidase molsin), protease XIV (pronase E), protease XVII, protease XIX, protease XXIII and protease XXIV (all available from Sigma). Initially all enzymes were diluted to the same specific activity of 4 units/mg in 10 mM phosphate buffered saline (PBS), pH 7.2, and incubated on tissue sections for five minutes at room temperature. Each enzyme was used on two serial sections, one for ICC and one for ISH. From the results obtained from this experiment selected enzymes were applied at room temperature to serial sections at concentrations and times as shown in table 1. After this incubation alternate sections were used for ICC and ISH. Sections for microwaving were incubated at an optimal enzyme concentration and time for ISH, as determined above, and then subjected to microwave irradiation.

The hyperimmune SSPE serum used in this study was obtained from a patient with SSPE, confirmed by biopsy, and was absorbed through normal human brain and liver before use. The control serum was obtained from a

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Accepted for publication
19 October 1989

Table 1 Effect of enzymatic titration of proteinase K and protease VIII on tissue preservation and numbers of labelled cells

	Time (minutes)	Tissue* preservation I/S	No off labelled cells I/S
<i>Proteinase K</i>			
1.0 mg/ml	5	1/2	8/9
1.0 mg/ml	10	1/2	9/9
1.0 mg/ml	20	2/2	8/9
2.0 mg/ml	5	2/2	8/9
4.0 mg/ml	5	2/2	8/9
<i>Protease VIII</i>			
0.5 mg/ml	5	0/0	12/10
0.5 mg/ml	10	1/0	11/9
0.5 mg/ml	20	2/2	11/9
1.0 mg/ml	5	1/1	11/10
2.0 mg/ml	5	2/2	11/9
0.5 mg/ml (MW)†	5	ND/2	ND/13

*Tissue preservation on a scale of 0–4 (0, well preserved; 4, tissue destruction).

†Average number of positive cells in eight corresponding fields.

‡Section microwaved before hybridisation.

I = Immunocytochemistry; S = In situ hybridisation.

subject with a measles titre of less than 10 as determined by immunocytochemical titration.

IMMUNOCYTOCHEMICAL TECHNIQUES

After the sections had been dewaxed and rehydrated through graded alcohols endogenous peroxidase was blocked by a 10 minute incubation in 3% H₂O₂ in methanol followed by a five minute wash in running tap water. After proteolytic enzyme treatment sections were immersed in phosphate buffered saline (PBS) for five minutes, followed by incubation in 5% normal goat serum for 20 minutes, and then in 100 µl of the hyperimmune SSPE serum (1/1000 in 10 mM PBS) overnight at 4°C. After two five minute washes in PBS the sections were incubated in biotinylated goat anti-human IgG at a 1/500 dilution in PBS (BRL, Gibco) for 30 minutes at room temperature. After a further PBS wash sections were incubated in streptavidin peroxidase (Zymed Laboratories, California USA) for 10 minutes, washed in PBS, and incubated in the substrate 3-amino-9-diethylcarbazole (Zymed) for 10 minutes. Sections were then washed in tap water, counterstained in haematoxylin, and mounted in glycerine jelly.

PROBES

Biotinylated single stranded RNA probes to the N gene were prepared by subcloning the N gene sequence of MV into a gemini in vitro transcription vector (Promega, Biotec). A sequence derived from this vector was used as a control probe. The derivation and preparation of the probes used in this study have been previously described.¹⁰

IN SITU HYBRIDISATION

After dewaxing, rehydration, endogenous peroxidase blocking, and enzyme treatment, sections were air dried. Hybridisation was carried out as described previously.¹⁰ Briefly, tissue sections were covered with 100 µl of hybridisation buffer containing 200 ng of biotinylated probe and the slides placed in a humidified atmosphere and hybridised at 37°C

overnight. After hybridisation washes were carried out as follows: (1) in washing buffer (0.6% NaCl, 10 mM TRIS-HCl, pH 7.0, 1 mM EDTA) at room temperature for five minutes; (2) in 45% v/v formamide in washing buffer for 30 minutes at room temperature; (3) in 1× SSC for five minutes at room temperature; (4) in 0.1× SSC at 40°C for 30 minutes; (5) in 10 mM PBS twice for five minutes at room temperature.

MICROWAVE PROCEDURE

After covering the sections with 200 ng of the measles probe in 100 µl of hybridisation buffer the sections were covered with a glass coverslip and subjected to microwave denaturation, as described previously.⁷ Briefly, sections were placed in a sealed microwave dish containing 25 ml of 2× SSC and incubated in a 650 watt microwave oven (Toshiba) for two minutes at a 50% power setting, followed by a further seven minute incubation at a 10% power setting to maintain the temperature. After overnight hybridisation at 37°C the coverslip was removed by a brief wash in 2× SSC at room temperature, followed by the higher stringency washes as described.

IMMUNODETECTION OF BIOTINYLATED HYBRIDS

Biotinylated hybrids were subsequently detected using a five step detection protocol, as described elsewhere.⁷ Briefly, monoclonal anti-biotin (Dakopatts) at a 1/30 dilution in PBS was applied at 37°C for 30 minutes, and after two washes in PBS the biotinylated anti-mouse immunoglobulin from the Zymed Histostain kit for mouse primary antibody (Zymed Laboratories, USA) was applied for 10 minutes at room temperature. The sections were washed again and the monoclonal anti-biotin reapplied at a 1/100 dilution for 30 minutes at 37°C. After a further two washes in PBS the biotinylated anti-mouse immunoglobulin was reapplied for 10 minutes at room temperature, and after two washes in PBS at room temperature a 1/20 dilution of streptavidin peroxidase conjugate (Zymed) was applied for a further five minutes at room temperature. The detection protocol was completed by addition of the substrate 3-amino-9-diethylcarbazole for 10 minutes, washing in running tap water for five minutes, and blueing in haematoxylin before mounting in glycerine jelly.

Evaluation of specific staining, tissue morphology, and background staining was scored on a scale of 0–4. When cell counts were necessary, eight corresponding fields were counted in each section and the average number of positive cells recorded.

Results

SCREENING OF PROTEOLYTIC ENZYMES

Table 2 summarises the effect of 10 different proteolytic enzymes on serial sections for both ICC and ISH. Positive cells with both techniques had dark red deposits and staining was predominantly cytoplasmic (figs 1 and 2). Positive cells were identified morphologically as neurons and glial cells (predominantly astrocytes). Sections of control normal tissue

Table 2 Effect of proteolytic enzymes on tissue preservation, background staining, and numbers of positive cells

	Tissue* preservation	Background† staining	No of‡ positive cells
Preferred result	I/S 0/0	I/S 0/0	I/S 4/4
Proteinase K (1.0 mg/ml)	1/2	1/1	2/3
Protease III (10.0 mg/ml)	3/3	3/3	4/1
Protease VIII (0.5 mg/ml)	0/0	0/0	4/4
Protease IX (4.0 mg/ml)	2/2	2/1	3/0
Protease XIII (1.0 mg/ml)	1/1	2/2	3/0
Protease XIV (1.0 mg/ml)	2/3	0/2	3/1
Protease XVII (0.1 mg/ml)	1/1	3/1	3/0
Protease XIX (10.0 mg/ml)	1/1	2/0	3/0
Protease XXIII (1.0 mg/ml)	2/2	2/0	3/0
Protease XXIV (0.5 mg/ml)	0/2	1/1	3/2

*Tissue preservation on a scale of 0–4 (0, well preserved; 4, tissue destruction).

†Background staining levels (0, no background; 4, heavy background).

‡Numbers of positive cells (0, no positive cells; 4, maximum number of positive cells observed).

I = Immunocytochemistry; S = In situ hybridisation.

were consistently negative with both the anti-serum and the measles virus probe. Sections of SSPE brain, stained with the control human serum or hybridised with the gemini vector control probe, were also negative. Tissue preservation was variable from enzyme to enzyme, ranging from almost complete tissue destruction with protease III (fig 1b) to very little tissue damage with proteases VIII and XXIV. The use of APES coated slides completely eliminated detachment of tissue sections, even when tissue degradation was considerable. Morphological preservation of the

Figure 1 Formalin fixed sections of central nervous system tissue infected with measles virus and immunocytochemically labelled with a measles specific antiserum after (a) pretreatment with protease VIII; (b) pretreatment with protease III. Note that both enzymes show large numbers of labelled cells but the degree of tissue destruction and background is much higher with protease III.

Figure 2 Formalin fixed section of central nervous system tissue probe infected with measles virus using a biotinylated probe to genomic RNA. (a) after pretreatment with protease VIII; (b) adjacent section after pretreatment with protease XVII. Note positive cells with protease VIII but the lack of positive cells with protease XVII.

tissue was better in the sections used for ICC than in the sections used for ISH. Endogenous peroxidase was effectively blocked with hydrogen peroxidase in methanol. Background staining, however, was still a problem with proteases III and XVII. Good signal (number of positive cells) for antigen was observed after pretreatment with all the enzymes, but a number were less effective in the unmasking of the genomic RNA; five of the enzymes generated no signal in the ISH procedure. Proteinase K and protease VIII showed maximal signal with a minimum of tissue degradation for both techniques. For both these enzymes similar numbers of labelled cells were observed following both antigen and genomic RNA detection.

ENZYMATIC TITRATION

The results of the experiment titrating the selected enzymes protease type VIII and proteinase K are shown in table 1. For both enzymes, either increasing the concentration at a set time or increasing the time of incubation at a set enzyme concentration produced no increase in signal for either ICC or ISH. A noticeable decrease in morphological preservation, however, was noted. Background staining, although never a problem in interpretation for either ICC or ISH, was observed to rise with increasing enzyme concentration or time of incubation. Incubation for five minutes at room temperature with 0.5 mg/ml protease VIII resulted in optimised signal generation for both techniques with no tissue damage and no background staining and was used in the subsequent microwave experiments.

MICROWAVE PROCEDURE

Figure 3 shows the result of microwaving the

Figure 3 Formalin fixed section of central nervous system tissue probe infected with measles virus using a biotinylated probe to genomic RNA, after pretreatment with protease VIII and microwaving. Note large number of intensely labelled cells generated after microwaving, while the tissue morphology is only slightly decreased.

section before hybridisation. The morphological preservation of the tissue was only slightly decreased by the microwave procedure, but specific signal was easily interpretable. The number of labelled cells per field was increased from an average of 10 to 13 (table 1) and signal generation within individual cells was more intense than in sections processed with no microwave pre-step. Increasing the power setting or time of microwaving resulted in considerable tissue damage, making the signal uninterpretable.

Discussion

Formaldehyde fixation of tissues is the accepted standard for morphological preservation and such material forms the largest single source of human tissues available for research. Its efficacy as a preservative for antigens is, however, suboptimal.¹¹ Formalin is also not a good fixative for nucleic acid and nucleoproteins, with up to 30% of nucleic acids being lost during fixation.¹² This is because native RNA does not react to any extent with formaldehyde at the temperatures normally used for fixation (20–22°C) and therefore RNA denaturation probably takes place at this stage. Only at the increased temperatures used, when tissues are infiltrated with paraffin wax or resin, can a reaction with any remaining fixative take place. The ability to make optimal use of formaldehyde fixed tissue is, however, of major importance in research and diagnosis.

In this study we used several proteolytic enzymes on long term formalin fixed, SSPE tissue and compared their suitability for use in ICC and ISH procedures. The mode of action of proteolytic enzyme digestion in enhancing immunoreactivity in formalin fixed tissues is not clear. It has been suggested that the effect could be due to the exposure of antigenic

epitopes, to an increase in cell and tissue permeability, or to the breaking of formaldehyde induced intermolecular cross-links.^{4,13} For nucleic acid detection, enzyme pretreatment is a preferential step for showing the target sequences in formalin fixed tissue.¹⁴ This is supported by the finding that repeated proteinase K digestion is required to extract DNA from formalin fixed pathology specimens for Southern blot analysis.¹⁵

Our results, which compare the pretreatment of formalin fixed tissue with 10 proteolytic enzymes at a fixed specific activity, illustrate the effect on the number of positive cells (for both ICC and ISH) and the degree of tissue preservation. The initial enzyme concentration, treatment time, and temperature of incubation were chosen from a previous report describing the immunocytochemical staining for a morbillivirus.¹⁶ All enzymes generated strong signals for the detection of antigen, but only four showed a sizeable signal generation for genomic RNA detection. Further titration of all 10 proteases was impractical because of the limited availability of tissue from this rare central nervous system disease. As illustrated in table 1, increased levels of enzyme digestion with protease VIII or proteinase K (the two enzymes chosen for further study) caused no increase in signal. Protease type VIII at a concentration of 0.5 mg/ml for five minutes at room temperature was chosen as the enzyme giving maximum signal with best tissue preservation and no background staining. It is important to emphasise, however, that this finding may not relate to other virus infections or to tissues other than brain.

The ability to show the presence of measles virus antigen and genome simultaneously in terminal SSPE brain agrees with the findings of previous reports.^{17,18} The similar number of labelled cells for both antigen and nucleic acid detection is indicative of the accumulation of stable antigens such as the nucleocapsid protein seen at this late stage of SSPE. Examination of short term, formalin fixed, biopsy material from a patient with suspected SSPE using our optimised protocol for long term fixed tissue showed that genomic RNA was detectable in a large population of cells which were antigen negative (unpublished observation). This agrees with the findings of previous studies of frozen tissues from preterminal SSPE brain using ISH and ICC techniques.¹⁷ We are currently examining tissue from terminal SSPE cases and from SSPE biopsy material for mRNA expression using single stranded probes to measles virus mRNA to investigate further the relation between gene expression and antigen synthesis.

Microwave irradiation has been shown to be an extremely efficient method for denaturing nucleic acid in previous ISH studies using double stranded DNA probes.^{7,19} In this study the microwave irradiation produced a considerable increase in the number of cells in which signal for genomic RNA can be generated. This may be due to the microwave procedure exposing the target sequences by denaturing the protein matrix which is causing

physical entrapment of the nucleic acid.²⁰ Alternatively, the microwave irradiation could be having a direct effect on the conformation of the target RNA, giving more efficient hybridisation.

This study shows that measles virus antigens and nucleic acid have remained stable in formalin over an extremely long fixation period. This should encourage more detailed molecular pathogenetic studies of SSPE and possibly other central nervous system diseases. We have shown the need for optimisation of the pre-detection steps for both ICC and ISH for any antigen or nucleic acid system under investigation. This seems to be especially relevant for nucleic acid detection where it was found that a combination of enzyme treatment and microwave irradiation was required to fully optimise the number of positive cells. The use of monoclonal anti-biotin and the five step detection protocol is also of particular importance in maximising numbers of positive cells.⁷ The use of one enzyme as an unmasking agent for both ICC and ISH should also allow double labelling for antigen and genome to be optimised within one tissue section. The methods of investigation described should, therefore, allow pathology departments to make optimal use of their large supply of stored necropsy material for ICC and ISH investigations.

We thank Mrs K Kennedy for technical assistance. This work was supported by the Multiple Sclerosis Society of Great Britain and Northern Ireland.

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