In situ hybridisation in herpetic lesions using a biotinylated DNA probe

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Abstract

In situ hybridisation was performed with a biotinylated DNA probe for herpes simplex virus (HSV) using high temperature denaturation on formalin fixed, paraffin wax sections of lung, brain, ganglion and keratinising and non-keratinising squamous epithelia. Eosinophilic viral nuclear inclusions or characteristically moulded multiple nuclei with altered chromatin, which were present in two cases of HSV encephalitis and one case of viral pneumonitis, all showed complete hybridisation visualised by an alkaline phosphatase/nitroblue tetrazolium detector system. HSV encephalitis and trigeminal ganglionitis, which were confirmed serologically or clinicopathologically but lacked nuclear changes, also gave positive dense nuclear signal in neurons, glia and satellite cells. No staining was present in the ganglion cells in trigeminal zoster, the glia in progressive multifocal leucoencephalopathy, or in a variety of cells in a lung coinfecting with cytomegalovirus. In 10 herpetic blisters of squamous epithelia, infected cells hybridised strongly, while morphologically similar herpes zoster lesions remained negative. In neural tissues non-hybridisation staining was most obtrusive in corpora amylacea and seemed to reflect non-specific probe adherence. In squamous epithelium, major non-hybridisation staining was caused by probe and antibody possibly adhering to intracellular keratin.

The HSV probe permits specific detection of virus in the absence of characteristic nuclear changes and allows varicella zoster virus to be differentiated from HSV, provided that the aforementioned problems with non-hybridisation staining are borne in mind.

Several reviews of in situ hybridisation using biotinylated nucleic acids have recently been published which critically describe the use¹⁻⁴ and interpretation⁵ of such probes in the study of pathological specimens routinely processed to paraffin wax. In the absence of confirmative virus isolation, in particular, in situ hybridisation using biotinylated probes should be able to identify with high specificity infected cells lacking pathognomonic inclusions or at least be able to confirm the nature of inclusions which are equivocal, as claimed by some authors.⁶

In this study we used a commercially available biotinylated DNA viral probe to confirm the presence of herpes simplex type I/II (HSV) in herpetic lesions from lung, brain, ganglion and squamous epithelia. A modification of a widely used protocol was used which requires a high denaturing temperature to optimise target hybridisation.¹ Cases were selected to detect virus both in the presence and absence of inclusion bodies. The ability of the HSV probe to distinguish specifically morphologically identical herpetic nuclear changes in squamous epithelium (HSV *v* varicella/herpes zoster virus, HVZ) was also studied. We also addressed the problem of non-hybridisation staining, which may lead to errors in interpretation.

Methods

Tissue specimens were fixed in 10% neutral formalin for several hours (skin specimens), up to several days (most necropsy specimens), or at least two weeks (whole brains). One case with pulmonary cytomegalovirus (CMV) and HSV was fixed for six hours in paraformaldehyde-lysine-periodate (PLP).⁷ Cases were divided into two main groups (table 1): group A, which showed characteristic nuclear changes in haematoxylin and eosin tissue sections (three cases) indicating HSV,⁸ and group B, which lacked inclusions (three cases) but which had serological or clinical signs indicating specific infection. We then applied probe for HSV to the appropriate sections to determine sensitivity relative to the number of virally altered cells in group A. Sensitivity was judged in group A on the basis of whether more, fewer, or the same number of nuclei with inclusions hybridised in the same counterstained section. In group B, sensitivity was either positive or negative relative to laboratory test results or clinical diagnosis. In a separate squamous epithelial group, one case each of HSV stomatitis and vulvitis showing herpetic giant cells with characteristically moulded multiple nuclei were hybridised, and 10 cutaneous herpetic infections with similar changes, clinically typical of varicella/zoster (two cases confirmed by serology, all zoster cases with typical dermatomal distribution), served as specificity controls (table 2).

The following controls were used: (1) for probe specificity, paraffin wax sections of formalin-fixed cultured fibroblasts infected with CMV and cytospin preparations of cultured monkey kidney cells infected with HSV of either type 1 or 2 and briefly fixed in cold 10% formalin; (2) sections of the tissue with HSV inclusions were run in parallel with test sec-

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Table 1 In situ hybridisation of biotinylated HSV DNA probe in histological sections of tissue (A) showing characteristic nuclear changes and (B) with histologically normal nuclei but with positive serology (specific IgG and IgM titres), culture, or characteristic clinicopathological features

Case No	Tissue	Estimated fixation time	Clinico-pathological features	Relative sensitivity*
Group A:				
1	Lung	6 hours, PLP	25 year old woman; Hodgkin's disease, CMV HSV pneumonitis	>
2	Brain (biopsy)	> 24 hours, 10% neutral formalin	41 year old man; HSV encephalitis	>
3†	Brain	14 days, 10% neutral formalin	8 month old female; necrotising encephalitis	=
Group B:				
4	Trigeminal ganglion	> 24 hours, 10% neutral formalin	68 year old woman; zoster ophthalmicus (HVZ), ganglionitis, subarachnoid haemorrhage	—
5†	Trigeminal ganglion	> 24 hours, 10% neutral formalin	55 year old man; necrotising HSV encephalitis	++
6†	Whole brain	14 days, 10% neutral formalin	81 year old woman; HSV encephalitis with left temporal lobe necrosis	+++

* > number of positive cells exceed those with unequivocal inclusions.

= positive cells all with inclusions; — no reaction; + to +++ relative number of infected cells.

† positive cerebrospinal fluid serology:

Case 3—serum HSV:IgG titre from 60 to 360 in six days, HSV isolated from brain and shown to be type 1.

Case 5—cerebrospinal fluid:serum HSV:IgG titre ratio increased to 0.8 and after four month interval 0.9.

Case 6—HSV:IgM positive at 300; HSV:IgG cerebrospinal fluid serum titre ratio increased to 1.5 and after eight days to 2.1.

tions (table 2) as a positive control; (3) hybridisation mixture without probe was applied to adjacent sections and processed in parallel with test sections as a negative control; (4) viral probes irrelevant to the section under study (CMV and JC virus, JCV) provided specificity controls for the test sections; and (5) HSV probe applied to six cases of progressive multifocal leucoencephalopathy, a demyelinating viral inclusion disease of brain caused by JCV, served as a further probe specificity control.

A commercially available (Enzo) HSV probe, labelled with bio-11-dUTP, consisted of fragment lengths of 200–2000 base pairs derived from clones of the plasmid pBR322 containing two cloned viral genomic segments of 8.0 and 16.0 kilobase pairs which had been inserted in the *Bam*HI site of the vector. Biotinylated probes for CMV and JCV (Enzo) were similarly produced, and all probes were freed of vector sequences by restriction enzyme treatment and electrophoretic gel purification. A probe concentration of 0.5 µg/ml was used.

The method of Unger *et al* was used with several modifications for hybridisation protocol (table 3). Loosening of sections on slides coated with poly-D-lysine was prevented by

Table 2 HSV probe applied to epithelial biopsy specimens of classic HSV and HVZ bullae (fixation in most cases < 24 hours in 10% formalin)

Case No	Clinicopathological diagnosis	Hybridisation results
7	HSV stomatitis	+++
8	HSV vulvitis	+++
9, 10	Zoster*	—
11–17†	Zoster	—

*Positive serum HVZ titre.

†Age range 20–80 years, two men and six women, grouped vesicles in all seven cases of zoster and diffuse rash in one case of varicella in a 20 year old man; two patients immunosuppressed before rash.

Hybridisation results: + to +++ relative number of infected cells; — no reaction.

switching to 3-aminopropyl-triethoxysilane (Sigma),⁹ a covalently binding adhesive applied at a concentration of 2% in acetone.

In an attempt to increase the intensity of hybrid formation by increasing target accessibility, we added a predigestion step of 0.2 N HCl for 15–25 minutes and also tried different proteases (proteinase K [Sigma], 100 µg/ml for 30 minutes, or 0.4% pepsin in 10 mM HCl for one hour at 37°C).¹⁰

Sources of non-hybridisation staining which detracted from the specificity of the technique were determined by sequential omissions in the procedure. Attempts were made to reduce such staining by subjecting sections treated with proteinase to 5 ml acetic anhydride in 100 ml triethanolamine (pH 8.0) before hybridisation.¹¹

Results

Hybridisation results were reproducible when several runs were made. Positive hybridisation produced a dark, coarsely granular blue-brown reaction product. This was sharply localised to the nucleus unless cell lysis had occurred during the pathological process. All controls stained appropriately.

Lung tissue from case 1, containing numerous CMV inclusions, showed a few scattered cells with multiple nuclei and washed-out chromatin suggestive of a herpetic coinfection. Hybridisation yielded a surprisingly large number of strongly reactive nuclei throughout the section.

Case 2, with herpes encephalitis, showed only one eosinophilic inclusion in a stained section, while four hybridising cells were detected in an adjacent section. In case 3 with pronounced HSV inclusion body encephalitis, all inclusions hybridised with a strong sharply localised signal in neurons and glia (fig 1).

Table 3 Hybridisation protocol based on Unger *et al*¹²

1	Dewaxing in xylene and dehydration in 95% ethanol
2	Enzymatic predigestion with nuclease-free pronase, 0.3 mg/ml in 0.05M TRIS-HCl with 5 mM EDTA (pH 7.4), 15 minutes at 37°C, then two washes in glycine, 2 mg/ml, 0.1M NaCl and 0.1M TRIS-HCl, pH 7.5
3	Probe cocktail (0.5 µg/ml probe + 1 × Denhardt's solution + 25 mM sodium phosphate + 250 µg/ml sheared herring sperm + 10% dextran sulfate, 5 × SSC. Solution heated to 90°C, then cooled rapidly to 0°C; * 20 µl applied to section and covered with polypropylene slip)
4	Denaturation in warm air oven* at 100°C for 10 minutes
5	Hybridisation at 37°C for two hours
6	Washes in 2 ×, 0.2 ×, and 0.16 × SSC for three minutes each
7	3% BSA in 0.1M TRIS-HCl (pH 7.5), for five minutes, then 20 minutes* in 10% normal serum diluted in 2 × SSC
8*	Amplification with goat anti-biotin AB 1:5000 overnight at 4°C, then biotinylated rabbit anti-goat 1:200 one hour at 37°C
9	Three washes in TRIS (pH 7.5)
10	30 minutes in avidin-alkaline phosphatase conjugate* (Dako) diluted 1/300 in normal serum
11	Three minute wash in 0.1M TRIS-HCl (pH 7.5) 0.1 N NaCl, 5 mM MgCl ₂ , 1% BSA, and 0.01% triton X-100; repeat with first three ingredients after changing pH to 9.5 and MgCl ₂ to 50 mM
12	Slides up to two hours at 37°C in fresh solution of 0.1 ml of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide + 0.2 ml of nitroblue tetrazolium chloride, 50 mg/ml in 50% DMF, both added to 30 ml TRIS buffered saline (pH 9.5)
13	Wash in distilled water, counterstain, with metanil yellow, and mount

*Modifications to method.

Figure 1 Confirmatory hybridisation with HSV probe in herpes encephalitis with widespread nuclear inclusions.

Relatively clean background was achieved in the lipid rich brain, whereas fibrous components displayed varying degrees of finely granular or dusky coloration. Erythrocyte membranes were often heavily coloured by the detector reagents rather than by probes. Background was not reduced by previous treatment with acetic anhydride.

In group B, the trigeminal ganglion removed at necropsy (case 5) with resolving herpes simplex encephalitis contained a mild lymphocytic infiltrate. The HSV probe hybridised with about one third of the nuclei of ganglion cells and surrounding satellite cells (fig 2), while no hybridisation was noted in the nerve cell body. Case 4, with zoster ophthalmicus and trigeminal mononuclear cell infiltrates, showed no reaction with HSV or CMV probes. Non-hybridisation staining was minimal and confined to collagenous structures.

Both cases 7 and 8, with mucosal herpes

Figure 2 Densely stippled HSV signal in nuclei of trigeminal ganglion cells associated with herpes encephalitis. Note also staining of satellite cell nuclei (arrow). Lipofuscin granules are seen in a ganglion cell in the upper left corner.

simplex, produced strong specific nuclear hybridisation in the infected blister cells (fig 3a). None of the 10 HVZ sections hybridised convincingly (fig 3b), although interpretation was sometimes made difficult by non-hybridisation cytoplasmic staining in keratinocytes adjoining the viral bullae and in follicular epithelium. Occasional ring-like staining of degenerated cells containing characteristic nuclear alterations was evident in HVZ, but true hybridisation was conspicuously absent from the nuclei. Addition of RNase (100 mcg/ml for 30 minutes at 37°C) after step 2 in table 3 did not decrease background staining, which was diminished but not abolished in controls without probe.

No difference was found in the intensity of staining in positive cases predigested with one or the other of the proteinases, in contrast to the experience of others who found pepsin digestion to be superior,¹⁰ and no case gave a positive result with an irrelevant probe.

Discussion

Sensitivity in terms of the number of cells with diagnostic viral inclusions which hybridise was 100% for HSV in all five cases studied. The probe we used also specifically identified productive viral infection in the absence of pathognomonic histological signs of the disease in both group B cases with HSV related disease. Although formalin is not usually considered to be the optimal fixative for in situ hybridisation detection of DNA due to cross-linking of proteins to nucleic acid, the signal is nevertheless obtainable in tissues with numerous viral inclusions subjected to pro-

Figure 3 (a) All nuclei with herpetic changes in gingival biopsy specimen show strong hybridisation with HSV probe; moderate non-hybridisation background staining. (b) Negative reaction in cutaneous zoster control. Note non-hybridisation cytoplasmic staining in keratinocytes which flank the blister and slight perinuclear background in degenerating blister cells.

longed fixation as was evidenced by strong reactivity for HSV in brains fixed for at least two weeks in this study. Generally, several hundred virus copies in a cell are needed before biotinylated probes give a visible reaction, and it has been shown that viral culture remains more sensitive than in situ hybridisation.⁵

The frequent absence of HSV inclusions in cases of suspected HSV encephalitis suggests that in situ hybridisation could be a useful tool in confirming infection, especially if the sensitivity and specificity could be shown to surpass that of immunohistochemical detection methods using virus isolation as the standard (70% true v 9% false positive results in one large series).¹² Interestingly, the results in the case of trigeminal HSV ganglionitis indicate that active viral replication occurs not only in nuclei of neurons but also in nuclei of their supporting satellite cells, as shown experimentally in mice.¹³

A strong signal was achieved in HSV giant cells in mucosal herpes simplex vesicles, whereas zoster cases remained negative. These results suggest that in situ hybridisation might be applied to cases in which clinical differentiation of HSV from HVZ is difficult, and further trials, perhaps using serotype specific probes, may show whether the method is superior to immunofluorescence on smears of blister material.

Specificity of hybridisation reactions in tissue sections requires the definitive presence of granular compact product and its differentiation from opaque, sharply contoured colour precipitates and non-hybridisation staining, often due to endogenous biotin. A minimal amount of finely stippled signal product was produced in some nuclei when suitable probe was applied, but in our experience this effect can be seen even with irrelevant probes and this would by itself be considered insufficient for a positive interpretation.

Background reactions in squamous epithelia were not dependent on predigestion or denaturation, and sequential omission of steps in the protocol incriminated both biotinylated probe and antibiotin antibody (monoclonal as well as polyclonal) as ultimate sources that would be more important than endogenous biotin. As non-keratinising mucosal epithelium did not show a similar degree of background, it is possible that DNA adheres to intracellular keratin, a well recognised phenomenon in regard to antibodies.

Increased background staining due to endogenous biotin in tissues such as liver and kidney has been noted,⁴ which makes correct interpretation of cytoplasmic staining essential. Sections of spuriously stained cytoplasm, as may occur in acantholytic blister cells, for example, can be misleading unless appropriate localisation of solid hybridisation staining in all pathologically altered cells is demanded. True cytoplasmic hybridisation is not likely to be a problem with HSV when biotinylated DNA probes are used because intact cells contain too few cytoplasmic viral particles for detection. Viral mRNA may be detectable in latently

infected cell bodies of trigeminal ganglion, which has been reported for a radioactive HSV probe,¹⁴ but it remains to be shown whether the double stranded probe and hybridisation conditions we used would allow mRNA to be detected in latent infection. RNase pretreatment of skin sections did not, in any case, decrease the cytoplasmic background flanking the viral blisters.

We agree with Myerson,⁵ who found that corpora amylacea of the brain often produced spurious staining. This could generally be distinguished from true hybridisation by its typical location, and confirmed by examining adjacent sections stained preferably with periodic acid Schiff. This type of staining could also be related to non-specific probe adherence, but we noted that it could affect only one out of several slides run simultaneously but with different probes.

In summary, the findings of this study indicate that the diagnosis of herpes simplex infection may be expedited by hybridisation using biotinylated DNA in a protocol requiring one working day compared with several days for virus isolation. Careful attention must be paid to avoiding the pitfalls in interpretation due to non-specific staining.

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