Human herpes virus 6 and endogenous biotin in salivary glands

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Abstract

Aims: To detect the presence of human herpes virus 6 (HHV6) and endogenous biotin in paraffin wax embedded and frozen salivary glands.

Methods: Two stage indirect and streptavidin-biotin immunoperoxidase techniques were used to visualise the antigens.

Results: HHV6 could not be shown in any of the tissues. However, considerable endogenous biotin antigenicity was detected in the glandular elements of the paraffin wax embedded material.

Conclusions: Results obtained with avidin-biotin detection systems should be interpreted with caution, especially when glandular epithelium is being stained. This may apply to both immunoperoxidase and in situ hybridisation techniques. The use of an anti-biotin antibody as a standard control should be considered.

HHV6 was first described in 1986, and since then studies have attempted to discover the sites of active or latent infection. Salivary glands have been implicated because of the high isolation rate of HHV6 from saliva.¹ Two studies found virus at this site using both immunocytochemical and nucleic acid hybridisation techniques, and an avidin-biotin detection system.^{2,3}

The increasing use of biotin labelled detection systems in recent years has led to improved sensitivity in many instances. However, the interpretation of results produced by this method relies on knowledge of the distribution and localisation of endogenous biotin. Kidney, liver, and pancreas contain sufficiently large amounts of endogenous biotin to pre-

clude the use of avidin-biotin detection systems, unless an avidin-binding blocking procedure is used.⁴

This paper describes an attempt to show the presence of HHV6 in paraffin wax embedded archival, and fresh frozen salivary gland tissues.

Methods

A total of 30 salivary gland paraffin wax blocks were examined, 24 of which were stained for HHV6 and 20 for biotin. A further 10 blocks of thyroid, parathyroid, breast, adrenal, pancreas and prostate tissue were examined for the occurrence of glandular cytoplasmic biotin localisation.

These blocks were used to: (1) investigate the effects of proteolytic digestion; (2) compare the results obtained by two and three stage immunoperoxidase techniques; (3) observe any staining due solely to the detection system used; and (4) compare detection sensitivities of frozen and paraffin wax tissues (table).

The 30 archival (post 1988) blocks of mainly serous salivary gland contained histologically normal tissue. All had been fixed in formal-saline, post-fixed in formol-sublimate and processed to paraffin wax. The 15 frozen blocks had been embedded in OCT (Miles), frozen in carbon dioxide, and stored at -70°C until use. Frozen sections ($4\text{ }\mu\text{m}$) were air dried overnight at room temperature.

Cells from the J-Jhan cell line, infected with the AJ strain of HHV6, were either fixed in formalin and processed to paraffin wax, or embedded in OCT and snap-frozen in liquid nitrogen, to produce positive control material. The staining controls comprised: (a) normal mouse serum (NMS) to replace the primary

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Protocols used for archival salivary gland material

Digestion	Primary antibody	Detection system	Number of cases	Objective
None	HHV6 (1 in 20)	Three stage sABC	14	Detection of HHV6 and
None	HHV6	Two stage ShamPo	24	comparison of detection systems
None	None	sABC only	14	Demonstration of endogenous
None	None	sAPo only	14	avidin binding
None	Biotin	Two-stage	30	Detection of endogenous
0.1% trypsin 10 minutes, 37°C	HHV6	Three stage	6	biotin in 20 salivary glands
0.1% pepsin in 0.1M HCl 10 minutes, 37°C	HHV6	Three stage	3	and 10 other tissue blocks
				Investigation of effects of
				proteolytic digestion and
				comparison of enzymes

sABC: streptavidin-biotin-peroxidase complex* (1 (A) + 1 (B) in 100); ShamPo: sheep-anti-mouse peroxidase conjugate (1 in 25); sAPo: streptavidin-peroxidase conjugate (1 in 50); biotin: mouse-anti-biotin (1 in 50).

*sABC is a mixture of A: streptavidin and B: biotinylated horseradish peroxidase, prepared 30 minutes before use.

Figure 1 Nuclear positivity in peripheral serous acinar cells stained for HHV6 and detected with sABC.

Figure 2 Cytoplasmic biotin in serous acini and ducts.

Figure 3 Cytoplasmic biotin in mucous acinar cells.

antibody; (b) application of the peroxidase labelled stage only; and (c) non-infected J-Jhan cells stained for HHV6.

HHV6 was detected using a mixture of three mouse monoclonal antibodies, designated 6.1, 6.6, and 16.1, kindly supplied by Dr J D Fox, Department of Medical Microbiology, University College and Middlesex School of Medicine. Each monoclonal was used at a dilution of 1 in 20.

The rabbit-anti-mouse biotin conjugate, streptavidin-biotin-peroxidase complex (sABC), streptavidin-peroxidase (sAPo), and mouse-anti-biotin-reagents were purchased from Dako Ltd. The sheep-anti-mouse peroxidase conjugate (ShaMPo) was supplied by Amersham.

Before immunostaining, the paraffin wax tissue sections were treated with 0.5% hydrogen peroxide in absolute methanol to inhibit endogenous peroxidase, digested with either (a) 0.1% porcine pancreatic trypsin (Sigma), 0.1% calcium chloride in 0.05M TRIS buffered saline (TBS), pH 7.8, or (b) 0.1% pepsin (Sigma) in 0.1M HCl. They were then incubated with 5% normal lamb serum (NLS) (Seralab) in TBS, pH 7.6, for 10 minutes to block tissue Fc receptors.

The antibodies were diluted in 10% NLS in TBS, pH 7.6. Sections were incubated in the primary antibody for 45–60 minutes, and the secondary and tertiary antibodies for 30 minutes at room temperature. The sections were washed for 10 minutes between stages in 0.1% NLS in TBS, pH 7.6.

The reaction was visualised with a solution of 50 mg% 3,3'-diaminobenzidine (BDH), 0.068% imidazole (Sigma), and 0.01% hydrogen peroxide in TBS, pH 7.6. A Mayer's haemalum counterstain was then applied.

For the frozen sections the antibody diluent was 10% NLS in PBS, pH 7.1, and the wash buffer was PBS with the addition of 0.1% NLS. The whole technique was carried out at room temperature except where indicated. Buffer washes of 10 minutes were carried out between each stage.

The air dried, unfixed sections were incubated successively in 10% NLS in PBS for 10 minutes, primary antibody for 60 minutes, fresh 10% formaldehyde in PBS for 20 minutes, 0.1% phenylhydrazine (Sigma) in PBS for 60 minutes at 37°C (to block endogenous peroxidase), sheep-anti-mouse peroxidase conjugate at 1 in 25 for 30 minutes, diaminobenzidine, then haemalum as for the paraffin wax sections.

Results

In all cases stained by the three-stage technique the reaction product was mainly localised to the cytoplasm of the duct lining cells. However, the same result was obtained from both the test sections and the NMS negative controls. Nuclear positivity was seen in two cases in cells at the edge of the section. However, this seemed to be non-specific and may have been a fixation artefact, (fig 1).

The positive control material stained well for

HHV6, independent of the detection system; non-infected cells incubated with HHV6 antisera and infected cells stained with NMS were uniformly negative.

Focal, cytoplasmic positivity was also seen when only the avidin-peroxidase (sABC or sAPo) reagents were applied. No staining of any kind was seen using the two-stage ShaMPo system.

Biotin was detected in all the 20 salivary glands stained. Staining was intense in the cytoplasm of the duct lining cells, scattered serous acini and mucous acinar epithelial cells (figs 2 and 3). In the six further non-salivary gland tissues biotin was detected in the gland/duct/follicle lining cells and in interstitial macrophages and plasma cells.

The use of trypsin eliminated the spurious nuclear staining but had no effect on the salivary duct positivity. Pepsin seemed to increase the level of avidin binding. However, pepsin greatly reduced the positivity of the HHV6 stained J-Jhan cells. Neither above digestion protocol, nor that suggested by Fox *et al*² (0.4% pepsin for 20–30 minutes), produced any unequivocal staining for HHV6.

The 15 assorted frozen submandibular, parotid, and minor salivary glands were uniformly negative for HHV6, although the control cells were well stained by the technique used.

Discussion

Fox *et al* found HHV6 by in situ hybridisation and immunocytochemistry in the nuclei of serous cells and in both the nuclei and cytoplasm of mucous and duct columnar cells in a high proportion of the salivary glands studied.² We were not able to reproduce these results in either paraffin wax or frozen sections. However, we have shown avidin-binding material and non-specific nuclear staining in most of the above sites. Contrary to the findings of Fox *et al* was the almost total loss of HHV6 staining after pepsin digestion of sections of infected J-Jhan cells. This would indicate that pepsin destroys the HHV6 antigen in infected cell culture material and presumably in tissue sections also. Methanol-peroxide and phenylhydrazine inhibition of endogenous peroxidase had no effect on the demonstration of HHV6.

These discrepancies may be due to the use of different antibodies, tissue fixation regimens,

or staining protocol. Other work in our laboratory on mouse kidney (F H Carpenter, personal communication) suggests that tissue fixation has an important role in subsequent reactivity with anti-biotin. Primary fixation in formol-sublimate resulted in no demonstrable endogenous biotin; with Carnoy's and Bouin's fixatives tissue contained large amounts. With formalin the endogenous biotin was intermediate but reduced further as the time of fixation increased. Thus studies describing results derived from the use of avidin-biotin complex (ABC) detection systems should state the type and duration of tissue fixation.

In 1981 Hsu *et al* claimed that "no unwanted staining is derived from intrinsic biotin" in their test tissues, including thyroid, liver and breast.⁵ Most workers now agree that of those tissues, liver contains sufficient endogenous biotin to cause problems with ABC techniques, and we have further shown the presence of intrinsic avidin binding material not only in thyroid and breast but also a range of glandular tissues.

Although the intensity of non-specific staining produced by the avidin-biotin technique was less than that seen in the positive controls or the biotin stained sections, it was still above background levels and could be mistaken for positivity. This must place constraints on the interpretation of results obtained using biotinylated antibodies or cDNA probes, and emphasises the need for strict controls.

The use of the anti-biotin antibody showed the extent of endogenous biotin in the tissues tested, and this antibody would provide an ideal staining control for avidin-biotin detection systems, especially when results are weak or equivocal.

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