Virchows Archiv A Pathol Anat (1988) 414:39–43

Virchows Archiv A
Pathological Anatomy and Histopathology

Comparison of in situ DNA hybridization (ISH) and immunocytochemistry for diagnosis of herpes simplex virus (HSV) encephalitis in tissue*

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Summary. Formol-fixed and paraffin-embedded brain tissue of 33 cases of human necrotizing encephalitis was investigated for Herpes simplex virus (HSV) by immunocytochemistry with a polyclonal antiserum, and by in situ hybridization (ISH) with a biotinylated cDNA probe. HSV antigens (VA) were found in various types of cells in the cytoplasm, cellular processes and nuclei. Labelling by ISH was mostly restricted to nuclei and intranuclear inclusions but otherwise matched the distribution of VA. Eighteen of 25 acute cases had HSV antigen detectable by immunocytochemistry, and 18 of the acute cases contained HSV DNA detectable by our ISH technique. However, results differed somewhat between the techniques: three brains negative for VA showed hybridization, and other 3 VA-positive cases remained negative by ISH. Thus 21 brains with acute necrotizing encephalitis were labelled with one or both techniques. In 8 cases with a subacute course (duration of disease was longer than 1 month), HSV antigens were never detectable although 4 brains showed hybridization. All brains labelled by one or both techniques contained nuclear inclusions bodies. Only one case, of subacute course, with inclusion bodies remained unlabelled. Brain tissue of 11 controls, including cytomegalic inclusion body disease, was never labelled.

These results demonstrate that immunocytochemistry and ISH are techniques of comparable sensitivity (72%) for detection of HSV in paraffin sections of acute necrotizing encephalitis brains; their combined use enhanced sensitivity, in our hands, to 84%. In cases with a disease course longer than one month, ISH seems to be the method of choice to demonstrate HSV in situ.

* Supported by the Medical-Scientific Fund of the Lord Mayor of the Federal Capital of Vienna, Austria

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Key words: Herpes simplex virus (HSV) – Encephalitis – Immunocytochemistry – DNA hybridization – In situ hybridization

Introduction

Human acute necrotizing encephalitis due to Herpes simplex virus (HSV) is the most frequent sporadic acute encephalitis in temperate parts of the world (Baringer 1978). Early diagnosis and subsequent initiation of antiviral therapy are key factors in reducing the very high mortality and morbidity of HSV encephalitis (HSE). The high antiviral effectivness and low toxicity of modern antiviral agents such as acyclovir (Furman et al. 1986) have been considered to justify therapeutic attempts without a biopsy proven diagnosis of HSE (Schroth et al. 1987; Schuchardt and Buchner 1987). However, mutation to drug resistence occurs at a fairly high rate (Coen et al. 1986) in human HSV strains. So the question arises in appropriately treated but fatal cases, whether drug resistance was responsible for ineffectiveness of therapy or whether the encephalitogenic agent was different from HSV. In this situation, some authors still advocate brain biopsy for establishing a diagnosis of HSE (Hanley et al. 1987). Therefore methods for a reliable diagnosis of HSE in tissue have remained important.

This retrospective study compares the diagnostic potential of immunocytochemistry for HSV with that of an in situ hybridization (ISH) technique in routinely processed autopsy material. ISH is shown to augment slightly the diagnostic sensitivity in acute cases when used in combination with immunocytochemistry, and to be the diagnostic method of choice in cases with protracted course.

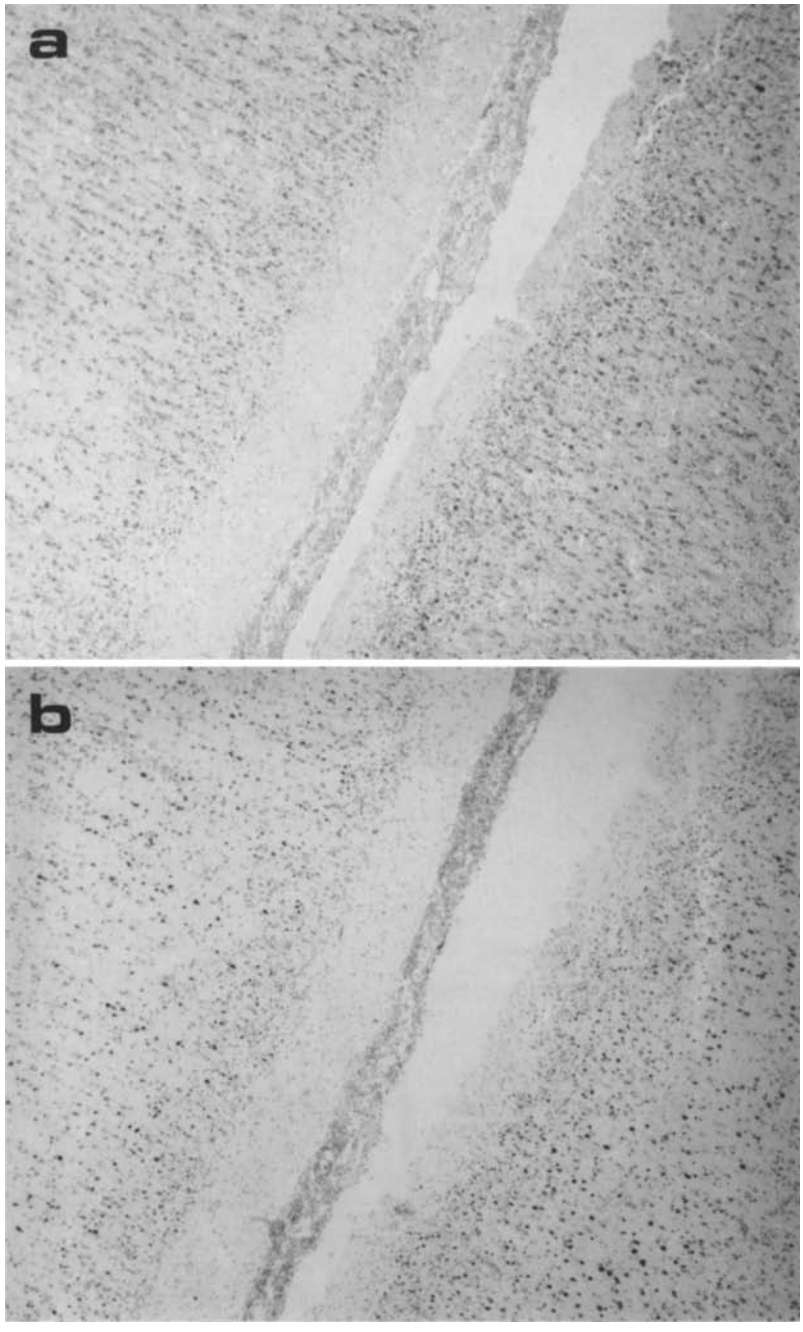

Fig. 1. Serial sections of acute HSE brain demonstrate, at low magnification, similar numbers of cells labeled by immunocytochemistry (**a**) and by ISH (**b**). Slight haemalum counterstain. Magnification ×63

Materials and methods

Thirty-three human autopsy cases with a histopathological diagnosis of necrotizing encephalitis were investigated. In 28 cases, material from a previous immunocytochemical study on HSE brains (Budka and Popow-Kraupp 1981) was used. Five more recent cases were included. After formalin fixation, the material has been stored in paraffin blocks up to 31 years. Twenty-five cases with a clinical course shorter than 4 weeks showed features of acute necrotizing encephalitis; eight subacute cases had a clinical course longer than 4 weeks. Identically treated brain tissue of 11 controls (age range 1 week to 76 years) included one case each of connatal cytomegalic inclusion body disease, Alzheimer's disease, Parkinson's disease, cerebral infarction, cerebral haemorrhage, sudden death in infancy, influenza, myocardial infarction, nephritis, sudden death with brain oedema, and herpes zoster.

For immunocytochemistry for viral-antigens (VA) sections were stained by the PAP technique, using an anti-HSV type I rabbit serum (Wellcome Labratories, London) as detailed previously, including appropriate controls (Budka and Popow-Kraupp 1981). Serial sections were used for ISH.

In situ hybridization (ISH) was performed with a biotinylated cDNA probe for HSV I & II (ENZO BIOCHEM, New York) prepared from a mixture of three sequences, each cloned into the Bam HI site of pBR322. These include two HSV I

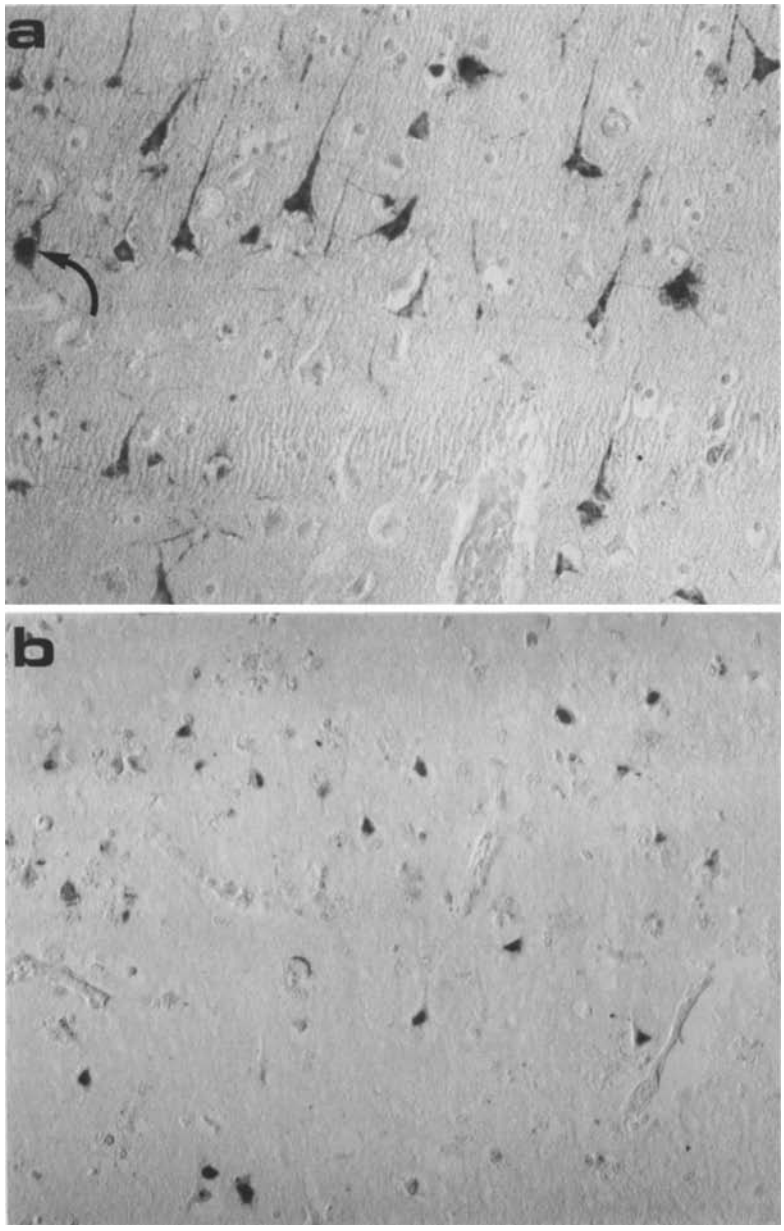

Fig. 2. Acute HSE. Immunocytochemistry stains neuronal perikarya, processes, and some (*curved arrow*) but not all nuclei (**a**) whereas hybridization is limited to nuclei (**b**). Slight haemalum counterstain, Nomarski optics. Magnification ×250

fragments (Locker and Frenkel 1979) and one HSV II fragment (Swain and Galloway 1983). Our visualization system as described below gave stronger signals than the HSV Patho-Gene kit purchased from ENZO BIOCHEM which was tested for comparison, using the producer's protocol. Paraffin sections of about 3–5 µm thickness were picked up with glass slides coated with 0,1% poly-D-lysine.

Dewaxed slides were treated by 1% H_2O_2 in methanol for 30 min. After rinsing in phosphate buffered saline (PBS) for three times, slides were incubated with 0,5% protease K XI (Sigma, P-0390) in PBS at 37° C for 20 min. Blocking of digestion was performed by 3 changes of PBS on ice. Slides were dried at room temperature. To prepare the hybridization mix, the following components were used: deionized formamide, pH 6,8–7,2 50 µl, 50% dextrane sulphate 20 µl, 3.0 M sodium chloride +0.3 sodium citrate (20 × SSC) 10 µl, probe/carrier mix (20 µl/4 µl carrier DNA) 20 µl. The final 100 µl were sufficient for hybridization to 5 samples, each covering an area on a slide of 20 mm × 20 mm. Sections were exposed to 20 µl of the hybridization mix and covered by coverslips. Slides were then put on a heating block at 92°–95° C for 3 min for DNA denaturation. Hybridization was performed at room temperature for 30 min. Finally coverslips were removed in 2 × SSC. Sections were rinsed in three changes of 2 × SSC.

For washing, sections were exposed to 20% deionized formamide in 1 × SSC at room temperature for 10 min, and then rinsed in three changes of 2 × SSC. For visualization, avidin-peroxidase complex (Sigma) 1:280 in swine serum/PBS (1:10) was applied for 30 min. After three rinses in PBS, the label was developed in 0,05% DAB in PBS with 32 µl H_2O_2 (for 100 ml). Finally, slides were washed with water and counterstained with haemalum.

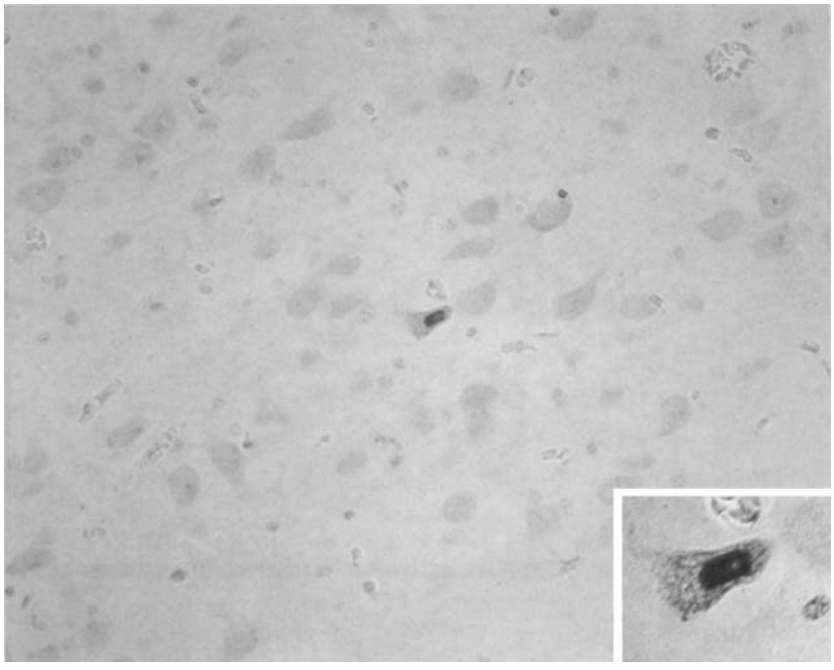

Fig. 3. In one case of acute HSE, ISH labels single scattered neurons outside of inflammatory lesions; the nuclear hybridization spares the nucleolus (*inset*). Slight haemalum counterstain. Magnification ×250, *inset* ×630

In addition to treatment of control tissue material, specificity was checked on brain sections of 2 HSE cases which were strongly positive by HSV-ISH, by ISH using a biotinylated cytomegalovirus (CMV) cDNA probe which strongly hybridized to the CMV-infected brain but did not hybridize to HSE inclusion bodies.

Results

Eighteen of 25 acute cases have HSV antigens (VA) detectable by immunocytochemistry. Eighteen of the acute cases also contain HSV DNA detectable by our ISH technique. However, results differ somewhat between the techniques: three brains negative for VA show hybridization, and other 3 VA-positive cases remain negative by ISH. Thus 21 brains with acute necrotizing encephalitis are labelled with one or both techniques. In 8 cases with a subacute course (duration of disease longer than 1 month), VA are never detectable although 4 brains show hybridization. All brains labelled by one or both techniques contain nuclear inclusion bodies. Only one case, of subacute course, with inclusion bodies remains unlabelled. On serial sections of brains which are reactive both for VA and HSV DNA, immunocytochemistry and ISH show a comparable number of labelled cells (Fig. 1).

VA are prominent in perikarya and processes of neurons (Fig. 2a). In many cases, nuclear involvement is less prominent than that of the cytoplasm and is frequent as reactivity at the nuclear membrane; staining of intranuclear inclusion bodies is variable (Fig. 2a). In glial cells and a few macrophages, both nuclei and cytoplasm are stained. VA are not found in areas outside of the inflammatory process.

Labelling by ISH is mostly restricted to nuclei of neurons and glial cells and is strongest in intranuclear inclusions (Fig. 2b). In addition, equivocal inclusions or nuclei without inclusions are labelled by ISH, including some small dots of hybridization. A few neurons within structurally intact parenchyma outside of the inflammatory process are labelled by ISH only in one case (Fig. 3).

Discussion

This is the first study correlating immunocytochemical and ISH investigations for HSV in a series of paraffin embedded human brain tissue with acute and subacute necrotizing encephalitis. Both techniques prove to be of comparable sensitivity (72%) for in situ detection of HSV in acute necrotizing encephalitis; however, discordant results obtained by our techniques in a minority of cases demonstrate some of the technical limitations of these methods on our material. Similar sensitivities were previously found in a comparison of ISH and direct immunofluorescent (IF) staining on smear preparations of fresh autopsy or biopsy brain tissue (Forghani et al. 1985). It is questionable if our ISH technique is sensitive enough to detect latent infection. A strong ISH signal in a few nuclei of intact neurons remote from the inflammatory nec-

rotizing process of productive infection, as seen in one case, might represent latent infection; but it might also reflect early DNA replication. The distribution of VA as found in our cases is in accordance with previous reports (Boos and Kim 1984; Budka and Popow-Kraupp 1981; Esiri 1982; Charpin et al. 1985).

The advantage of ISH for an in situ diagnosis of HSE becomes most evident in subacute cases of necrotizing encephalitis, with a clinical course protracted longer than 4 weeks, which consistently lack HSV antigens: half of these subacute cases are diagnosable by ISH. In acute cases, the diagnostic sensitivity is enhanced from 72% to 84% by use of ISH in addition to immunocytochemistry.

We conclude that the combined use of immunocytochemistry and ISH enhances the diagnostic potential and helps to confirm an HSV aetiology in necrotizing encephalitis tissue; ISH seems to be the method of choice to demonstrate HSV in cases with protracted course.

Acknowledgements. The authors are grateful to Mrs. H. Flicker for excellent technical assistance, and to Mag. O. Schlögl for bibliographic assistance.

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Received June 30, 1988 / Accepted September 12, 1988