The rapid diagnosis of herpes encephalitis

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VIRUS infections can be rapidly diagnosed either by isolating the virus, if it is one which grows quickly; or by finding morphologically identifiable virus particles in tissue by electron microscopy; or by identifying virus-specified proteins in tissue by immunofluorescence.

All these methods are applicable to the diagnosis of herpes encephalitis and this paper attempts to assess their relative merits on the basis of experience of their use in the Birmingham region from 1966 to the present. Biopsies from the temporal lobe in nineteen patients suspected of herpes encephalitis have been submitted, mostly from the Midland Centre for Neurosurgery and Neurology. Of these, twelve patients have been confirmed as cases of herpes encephalitis by isolation of virus from the biopsy and from the ventricular cerebrospinal fluid. Ten out of twelve were found positive by immunofluorescence. I expect no apology, because none is due, from the neurosurgeons for the seven negatives, who had other forms of encephalitis. As far as I know, none of these seven patients appears to have suffered any permanent damage as a result of the biopsy. It seems already clear, from everybody's published results and the papers already given at this meeting, that if we wait to do the biopsy until the clinical indications are unmistakable we have waited so long that the patient, if he survives, will be left a severe neurological cripple.

Methods

Tissue culture

Herpesvirus hominis is, fortunately, easy to cultivate in a wide variety of tissue cultures. The most sensitive cells are probably human amnion and human embryo kidney, but human embryo lung fibroblasts are almost as good and are usually available 'off the shelf' in our laboratory. A cytopathic effect in cultures inoculated with ventricular cerebrospinal fluid or suspension of triturated brain fragment is often visible the next day, usually after 3 days; and if no cytopathic effect is visible after 7 days it is unlikely that any live virus is present. We have never isolated herpes febrilis virus from lumbar CSF. Virus is usually present in ventricular CSF, though CSF often takes 1–2 days longer than brain suspension to cause a cytopathic effect in tissue culture. We have not made comparative titrations of virus in brain and CSF.

Human embryo kidney and embryo lung cultures are propagated in Eagle's medium ('basal' mixture) plus 10% foetal calf serum, and are maintained in Eagle's medium + 2% foetal calf serum.

Cytology

In impression preparations one may be able to find intranuclear inclusions by conventional staining (Fig. 1). This, of course, affords a presumptive diagnosis only. Herpes virus is not the only one which may cause intranuclear inclusions—measles virus in subacute sclerosing leuco-encephalitis, and a polyoma-like virus in progressive multifocal leuco-encephalopathy cause somewhat similar nuclear changes.

FIG. 1. A small nucleus filled by a dense inclusion body. Impression preparation from brain biopsy. Weigerts iron haematoxylin and eosin ($\times 400$).

Immunofluorescence

Method. The surface of the biopsy is pressed gently several times (depending on its size) on to the surface of one or more microscope slides. When dry, the impression preparations are fixed for 1 min exactly in acetone chilled to -20°C . When we first started looking for herpes in brain biopsies we used to fix for 10 min; fluorescence was weak and results disappointing. A trial of different fixatives and fixation times on impression preparations from the same brain suggested that fluorescence was not as bright after 5 min fixation, weak after 10 min and almost absent after 20 min and was better after acetone than ethanol or methanol fixation. This experience does not seem to be shared by everybody,

PLATE 1. Fluorescent nuclei. Brain biopsy. $\times 400$ approx.

PLATE 2. Phase contrast fluorescence. $\times 400$ approx.

PLATE 3. One olfactory tube is necrotic. Part of the temporal lobe surface on the same side appears hyperaemic.

but Dr C. A. C. Ross has also had good results after 1 min fixation. My colleague, Mr B. J. Young, finds that immunofluorescence with tissue cultures infected with some enteroviruses is best with no fixation at all, the cells just being allowed to dry before staining. Unfortunately, brain impressions are easily washed off the slide if unfixed.

I see no advantage in cutting frozen sections of unfixed brain. I should not care to cut my finger on a knife used for sectioning encephalitic brain, and feel that frozen sections constitute an unnecessary hazard.

Staining. The slides are allowed to dry thoroughly after fixation (residual acetone precipitates antibody and gives much trouble) and are then overlaid with hyper-immune rabbit anti-herpes serum, which is left to react at 37°C in a wet-chamber for $\frac{1}{2}$ hr. A control preparation is put up with normal rabbit serum. After washing, fluorescein-labelled anti-rabbit globulin serum is applied for $\frac{1}{2}$ hr; after washing again, the preparations are mounted in glycerol and examined.

It is most important to make sure that the final wash is at about pH 7·8 and that the conjugate dilution used is at a pH well above 7·0; 7·6 has been found satisfactory. Glycerol for mounting is buffered (90% glycerol, 10% phosphate-buffered saline, pH 8). Nearly all our work has been done by the indirect staining method, which has been found sensitive and reliable. My erstwhile colleague Dr El Ganzoury (unpublished observations) tried the complement fluorescence staining method; this works perfectly well, but takes a little longer; and although it has been found the most sensitive method for detecting antigen in carefully controlled experimental systems, in diagnostic practice it did not seem to have any decided advantage. Results are shown in Plates 1 and 2.

Site of biopsy. Two early biopsies from the frontal area, taken before the importance of the correct site was realized, gave negative results (they are not included in our series). Although the infection eventually may spread throughout the brain so that the virus can be isolated and inclusions found anywhere in the cortex, this is a terminal stage of the disease, when therapy of any kind would be useless. It is important that the biopsy should be taken low down on the side of the temporal lobe where lesions occur early. The other parts of the limbic system which are affected early are not suitable for biopsy.

False-negatives. One patient gave a biopsy with doubtful result; one observer thought the immunofluorescence test was positive, another (myself) could not be certain; this was before we fixed for 1 min only. This patient was diagnosed by virus isolation, was treated with idoxuridine and made a fair recovery, but lost his speech centre. He is one of the two out of twelve we recorded as negative.

False-positives. In one patient small areas of rather feeble fluorescence, not present in the controls, were seen within nuclei. The clinical history of this patient was very short and the appearance was taken as consistent with early infection. No herpes virus was ever isolated from the biopsy or ventricular CSF, and a rising antibody titre to mumps eventually established the probable true diagnosis. She was treated with idoxuridine, developed purpura, but rapidly made a complete recovery despite biopsy and treatment. We have had more experience of the characteristic appearance of true positive biopsies since then and would, I hope, now suspect the appearance in this patient's biopsy as being false-positive.

Electron microscopy

Method 1. Small pieces of tissue were rubbed up in a drop of distilled water, and after the larger pieces had settled, formvar-coated electron microscope grids were touched to the surface, allowed almost to dry, washed thrice in distilled water and negatively stained with potassium phosphotungstate. This method, most sensitive and reliable in the diagnosis of skin lesions due to viruses of herpes febrilis or varicella-zoster, was disappointing for brain biopsies; in only two of five consecutive biopsies, all strongly positive by immunofluorescence, could characteristic herpes particles be found. We have not looked at centrifuged deposits, using negative staining, from ventricular cerebrospinal fluid; this could be done within an hour and might well be rewarding. We have found plentiful particles in lumbar CSF from a patient with herpes zoster and encephalitic symptoms.

Method 2. Embedding in plastic and cutting thin sections. This is slow and of use only for establishing a retrospective diagnosis. Even long afterwards, recognizable particles can be found in brains preserved in museum jars. It also serves to demonstrate the process of virus synthesis within cells (Figs. 2 and 3).

Serological diagnosis

A rise in titre of complement-fixing antibodies can usually be demonstrated in patients who live long enough. But the antibody rise in primary herpes in adults is sometimes quite slow.

Course of infection

Further work is needed to confirm the route by which infection reaches the brain. Plate 3 illustrates the olfactory lobes—one necrotic, from which virus was isolated—from a case of herpes virus encephalitis. Virus was also isolated from the olfactory mucosa. This sort of observation has, of course, previously been made, but the infection in olfactory

FIG. 2. Virus capsids within the nucleus. Uranium acetate and lead citrate ($\times 10,500$).

lobes and mucosa has not always been looked for or its frequency established. Nor has it been established whether it occurs before or after the temporal lobe lesions.

Conclusions

The most sensitive method for diagnosis of herpes virus encephalitis is virus culture from temporal lobe biopsy and ventricular cerebrospinal fluid, using tissue cultures of human amnion or human embryo lung fibroblasts. The most rapid and very sensitive method is immunofluorescence by the indirect ('sandwich') technique on impression preparations from brain biopsies. A short fixation time

FIG. 3. Within the nucleus is a capsid whose capsomeres are visible (arrow). In a tube apparently derived from the nuclear membrane (double arrow) a capsid is in process of extrusion into the cytoplasm ($\times 75,000$).

and careful attention to pH of reagents is necessary. Direct electron microscopy by negative staining is much less sensitive. Electron microscopy of thin sections is valuable in making a retrospective

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