Immunofluorescence staining for the diagnosis of herpes encephalitis

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SYNOPSIS Direct immunofluorescence staining for herpes simplex virus was applied to cryostat sections of 43 specimens removed at brain biopsy. Herpes antigen was found in 10 specimens and virus was isolated from them. Antigen was found in one specimen from which virus was not isolated. Two specimens from which virus was isolated gave equivocal fluorescence. Thirty specimens gave no fluorescence or live virus. Immunofluorescence provided a diagnosis in three hr compared with 24-42 hr for virus isolation.

Indirect immunofluorescence staining was applied to sections of brain removed at necropsy and fixed in formalin. Herpes antigen was found in sections of six of the 12 brains examined.

Over the past 10 years there has been a growing awareness of the encephalitis caused by herpes simplex virus, and the most recent survey (Illis and Gostling, 1972) shows that the mortality of untreated cases may be as high as 70% and that survivors are frequently left with serious physical and mental disabilities.

Dodge and Cure (1956) made the first attempt, unsuccessfully, to culture herpes virus from brain tissue removed at biopsy as a diagnostic procedure. The isolation of virus from biopsy brain tissue of four patients was described by MacCallum, Potter, and Edwards (1964) and similar results have been reported by many other groups (Miller, Hesser, and Tompkins, 1966; Rawls, Dyck, Klass, Greer, and Herrman, 1966; Breeden, Hall, and Tyler, 1966; Evans, Gray, Miller, Jones, Weeks, and Wells, 1967; Miller and Ross, 1968; Rappel, Dubois-Dalcq, Sprecher, Thiry, Lowenthal, Pelc, and Thys, 1971). The need for rapid diagnosis became pressing when chemotherapy of herpes encephalitis was introduced, first iodo-deoxyuridine (Breeden, Tyler, and Hall, 1966) and later cytosine arabinoside (Juel-Jensen and MacCallum, 1972).

In 1966 we began to apply immunofluorescence staining for herpes simplex virus to all tissue from brain biopsies sent to the laboratory and we reported preliminary results (Tomlinson and MacCallum, 1969). Flewett (1973) described the examination of tissue from the temporal lobes of 19 patients, 12 of which yielded herpes virus and 10 were rapidly diagnosed by immunofluorescence staining.

We now describe our experience with 43 biopsy specimens submitted for virological examination. We have also used the technique of Sabin and Messoré (1961) for immunofluorescent staining of herpes virus antigen in paraffin sections of formalin-fixed brain from 12 patients.

Materials and Methods

TISSUE

Fresh brain tissue removed at craniotomy or burr-hole exploration was received from 40 patients in the Radcliffe Infirmary, from one patient in Bristol, and from two patients in London. Two of these specimens from other hospitals were received four hr, and the third 20 hr, after operation. Many of the biopsies had been performed to differentiate between tumour, abscess, or viral encephalitis and some, when the clinical indications were strong, to confirm a suspected encephalitis. Each specimen was divided into three for histological examination, for virus culture, and for immunofluorescence. Formalin-fixed brain, from the Neuropathology Laboratory, Radcliffe Infirmary, or referred from other hospitals, was received as 6 μ m sections, still in paraffin, on slides.

CULTURE FOR VIRUSES

The tissue was ground in maintenance medium and 0.2 ml volumes of the suspension were inoculated onto three to six tubes of primary human amnion cells and two tubes of secondary monkey kidney cells. After centrifuging off the debris the fluid was used to inoculate further cultures and suckling mice.

PREPARATION OF SECTIONS

Biopsy tissue, usually a 2-3 mm cube, was blocked in 10% gelatin, snap frozen, and 6 μ m sections were cut in a cryostat, dried in air, and fixed in acetone for 10 minutes.

Sections of formalin-fixed tissue, also 6 μ m, were dewaxed in xylene, taken through alcohol to water, and washed in several changes of buffered saline for four hours.

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Serum

The antigen for immunizing rabbits was made by growing type 1 herpes virus in 6 oz bottles of RK 13 cells for 48 hours. The medium was discarded and the cells were suspended in 0.5 ml distilled water/bottle, frozen, thawed, and disrupted with ultrasonic energy. Antigen used for the first injection was incubated at 37°C for 18 hr with 0.017% formaldehyde to inactivate the virus. Intramuscular injections were given: day 0, 0.25 ml inactivated antigen plus incomplete Freund's adjuvant; day 56, 0.25 ml 'live' antigen with adjuvant; day 84, 0.5 ml antigen; day 112, 0.5 ml, and the animals bled 10 days later. The serum had a complement-fixation titre of 1:800 and for indirect immunofluorescence-stained, acetone-fixed herpes virus at 1:80 or 1:160.

Conjugated antibody

A crude globulin fraction was separated from the serum by dialysis against 1.56 M ammonium sulphate, labelled with fluorescein isothiocyanate and freed from unreacted fluorescein on a column of Sephadex G-25. Before use it was absorbed three times with an homogenate of guinea pig liver.

Staining

For the fresh tissue the absorbed conjugate was diluted 1:10 in normal rabbit serum as test reagent, and 1:10 in unlabelled anti-herpes rabbit serum for the blocking control. Test and control reagents were applied, to at least three sections each, for 30 min at room temperature. After washing the sections were mounted in buffered glycerol.

Sections of formalin-fixed tissue were treated with a 1:20 dilution of either unlabelled antiherpes serum (test) or of normal rabbit serum (control) for 60 min at room temperature. After washing, the sections were overlaid with a 1:40 dilution of fluorescein-conjugate swine antirabbit globulin (Nordic Diagnostics) for 20 min, re-washed and mounted.

As a final check on specificity, some specimens of formalin-fixed tissue were stained with absorbed antiherpes serum thus: 0.2 ml antiserum was diluted to 2.0 ml and absorbed with BHK-21 cells,

from a Roux bottle, for two hr at 37°. Another 0.2 ml was absorbed with herpes-infected BHK-21 cells. The cells were centrifuged off, the absorbed sera were heated at 56° for 30 min and diluted two-fold for indirect staining as above.

Microscopy

Preparations were examined under a Vickers Patholette microscope with halogen quartz illumination and an interference filter (Barr and Stroud). The Vickers $\times$ 20 panchromatic objective, NA 0.65, combined adequate magnification with a bright image.

Results

TISSUE REMOVED AT BIOPSY

The results on the 43 specimens are summarized in table I. Immunofluorescence staining of the sections from 10 cases showed unequivocal evidence of herpes simplex virus antigen. Sections treated with the test reagent showed brightly fluorescent neurones and glial cells, many per section of some specimens (fig 1 and table II), whereas the control staining produced either no fluorescence at all or a just discernible coloration (fig 2). Cultures of the specimens from these 10 cases all yielded herpes simplex virus.

Despite the convincing demonstration by immunofluorescence of viral antigen in the specimens from case 9, virus was not grown from that specimen. The patient had been only mildly ill for 16 days before the biopsy was performed and at that date the titre of complement-fixing antibody was already 1:512 in serum and 1:64 in cerebrospinal fluid. The histopathological diagnosis from the biopsy tissue was necrotizing encephalitis. It appears that immunofluorescence detected remaining antigen from an infection already terminated by the host's defences, at the time of biopsy.

The two cases listed in table I as 'culture positive immunofluorescence doubtful' illustrate the limitations of the technique. The sections from case

Results of Tests	No. of Cases	Final Diagnosis
Immunofluorescence positive	10	HSV encephalitis
Virus isolation positive		
Immunofluorescence positive	1	HSV encephalitis
Virus isolation negative		
Immunofluorescence doubtful	2	HSV encephalitis
Virus isolation positive		
Immunofluorescence negative	30	Other than HSV encephalitis
Virus isolation negative		

Table I Correlation of immunofluorescence tests and virus isolation with final diagnosis

Fig 1

Fig 2

Fig 1 Test staining of biopsy tissue from case 11. $\times 160$ Green areas: antigen containing cells. Yellow flecks: autofluorescence of lipofuchsin.

Fig 2 Control staining of biopsy tissue from case 11. $\times 160$

Case No.	Site of Biopsy	No. of Fluorescent Cells/Section	Herpes Virus	
			Isolated	Time to Isolation (hr)
1	R. temporal	2, 1 (equivocal)	+	42
2	R. frontal	16, 22	+	24
3	R. frontal	7, 4	+	42
	L. frontal	10, 12	+	42
	L. temporal	Many, many, many	+	42
4	L. temporal	24, many, many	+	42
5	L. temporal	Many, many, many	+	42
6	temporal	28, 32, 33	+	42
7	Not known	1, 1, 1	+	42
8	R. temporal	2, 6, 3, 4	+	18
9	L. temporal	3, 10, 2	-	
10	R. temporal	Many, many, many	+	24
11	R. temporal deep	Many, many, many	+	24
	superficial	Many, many, many	+	24
12	L. temporal	Many, many, many	+	96 ¹
13	frontal	Many, many, many	+	42

Table II Numbers of fluorescent cells in and time to isolation of virus from biopsy specimens

¹Specimen received 20 hr after operation

Fig 3 Test staining of formalin-fixed brain. $\times 160$

Fig 4 Control staining of formalin-fixed brain. $\times 160$

were examined before our technique had been standardized; a less potent antiserum was used and an inefficient fluorescence microscope employed so that a firm diagnosis had to await the isolation of virus. The sections from case 7 each contained only one fluorescent cell in the test staining, and although no fluorescence was seen in the controls, this was deemed inadequate for a firm diagnosis. As cultures from this specimen revealed only a single focus of infection in one of the five tubes inoculated it was apparent that the sample had come from the edge of the infected area.

From 30 cases the specimens showed no evidence of herpes virus antigen and virus was not isolated. The final diagnoses of these patients was disease other than herpes encephalitis.

The time elapsing between operation and the first appearance of cytopathic change in tissue cultures (table II), not including time for a neutralization test, ranged from 18 to 72 hr, so that the immunofluorescence technique materially speeded diagnosis.

POSTMORTEM TISSUE FIXED IN FORMALIN

Eleven of the specimens examined were from cases suspected to be herpes simplex encephalitis on clinical or histopathological grounds, but the brain had not been cultured for virus. Immunofluorescence staining revealed antigen containing neurones (fig 3) in five of these specimens, with no staining of such cells in the controls (fig 4). With formalin-fixed tissue the control staining was particularly important as non-specific staining of meninges and the walls of blood vessels was often prominent. When supposedly specific staining was found it was essential to determine, under a low-power objective, its exact location in the section, and then check the same area of the control section for absence of fluorescence.

A typical example of the use of this technique was in the case of an 11-yr boy with a life-long history of a neurological disorder, who died after an acute febrile illness lasting 14 days. Herpes encephalitis had not been suspected in life but the histopathology was found to be that of necrotizing encephalitis. Immunofluorescence staining revealed herpes antigen in cells of the hippocampus.

The twelfth specimen was from a brain, the temporal lobes of which had been sampled at necropsy and shown by culture to contain virus. After the brain had been fixed in formalin the appearance of stained sections suggested the presence of infection in the midbrain and immunofluorescence provided confirmation.

Discussion

As indicated in the introduction, brain biopsy is

widely used for the diagnosis of herpes encephalitis and when brain tissue has been removed the use of immunofluorescence provides a diagnosis in two to three hr compared with 24 to 72 hr for virus isolation. In our hands it gave no false positives with 30 specimens. The only equivocal fluorescence of a virus-containing specimen was due to the poor serum first used. The small amount of fluorescence, and of virus, in one specimen was a consequence of unfortunate sampling of the brain so that immunofluorescence gave no false negatives.

The cutting of frozen sections is more time consuming than the preparation of smears or imprints and, as Flewett (1973) has pointed out, there is some risk to the operator from an infected knife. Despite these considerations we believe that sections are preferable because, being thin and uniform and because infected cells occur at the same sites, test and control staining can be carefully compared, a matter of some importance when there are only few infected cells. The risk of accidental infection is no greater than that incurred by a surgeon or histopathologist handling herpes-infected brain.

The direct staining technique was chosen for biopsy tissue because it is quicker and gives less non-specific staining than the indirect technique, but for formalin-fixed tissue the greater sensitivity of the indirect method was necessary.

Although formalin-fixed tissue exhibits more non-specific fluorescence than fresh tissue the absorption-with-antigen procedure provides a certain identification of specific staining. If brain cells in a section fluoresce when treated with BHK-cell absorbed serum and similar cells, in the same area of the adjacent section, do not fluoresce after treatment with serum absorbed with herpes-infected BHK cells, the fluorescence must be due to herpes antigen in the brain cells.

When applied to sections of formalin-fixed tissue immunofluorescence brings immunological specificity to a diagnosis otherwise resting on histology and the morphology of particles under the electron microscope. The tissues reported here had been embedded for only weeks or a few months, but the technique will detect herpes antigen in tissue years after embedding (Sabin and Messoré, 1961; Tomlinson, 1970).

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