RESTRICTION ENDONUCLEASE DIGESTION ANALYSIS OF DNA FROM VIRUSES ISOLATED FROM DIFFERENT SITES OF TWO FATAL CASES OF HERPES SIMPLEX VIRUS TYPE-1 INFECTION

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SUMMARY. Herpes simplex virus (HSV) type-1 was isolated from a fatal case of herpes simplex encephalitis (case 1) and from a fatal case of disseminated herpes simplex (case 2). The virus was isolated from the lip lesion, the frontal lobe and the temporal lobe of the brain in case 1 and from a mesenteric node, myocardium and salivary gland in case 2. Restriction endonuclease digestion analysis showed that each case was infected with different substrains of HSV. The changes in band pattern in isolates from case 1 occurred in the "variable" region of the genome, showing that viruses with such variations can be isolated simultaneously from different tissues. The changes in band patterns in isolates from case 2 indicated the presence of two virus substrains, one in the mesenteric node and salivary gland and a second in the myocardium.

INTRODUCTION

The technique of restriction endonuclease digestion analysis is based upon the fact that DNA of different nucleotide sequences, i.e., those which have a different genetic origin, will give a different band pattern in gel electrophoresis after enzyme hydrolysis. Several workers have used this technique to study the epidemiology of herpes simplex virus (HSV) infections. Recurrent infections are frequently caused by the same virus (Buchman *et al.*, 1979; Maitland *et al.*, 1982; Ueno *et al.*, 1982; Kit *et al.*, 1983) as are concurrent infections at different sites (Embil *et al.*, 1981; Gerdes *et al.*, 1981). Genital infections have been caused by two substrains of the same type-1 virus and by concurrent infection with both virus types (Fife *et al.*, 1983). This technique has also been used to study nosocomial outbreaks of herpes simplex (Buchman *et al.*, 1978; Linnemann *et al.*, 1978; Halperin *et al.*, 1980; Adams *et al.*, 1981).

We used restriction endonuclease digestion analysis to identify the viruses isolated from samples of tissue taken at necropsy from two children who died of HSV

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infections. While this work was in progress, Whitley *et al.* (1982) showed that HSV type 1 (HSV-1) isolated from the brain biopsy and oral lesions of the same person could be of different substrains whereas Dix *et al.* (1983) showed that HSV-1 isolated from the cerebrospinal fluid (CSF) and brain biopsy of a patient were of the same viral substrain.

PATIENTS AND METHODS

Case 1. A three and a half year old girl, with no previous serious illnesses, developed repeated convulsions 3 days after the onset of a flu-like illness and was admitted to hospital. On physical examination, her temperature was 40°C; she was semicomatose, but showed no signs of meningism. Although she had lesions resembling *herpes labialis* on the upper and lower lips, she did not have a skin rash. Her chest examination was normal. A spinal tap showed increased CSF pressure, with 30 cells/mm³, 80% lymphocytes. A CAT (computerised axial tomography) scan showed a diffuse lesion in the left temporal region. A diagnosis of herpes simplex encephalitis was thought probable, and she was treated with adenine arabinoside. Her condition deteriorated rapidly; within 2 days, she was deeply comatose, with shallow and intermittent breathing, and a decerebrate posture. She died 8 days after admission to hospital. Culture of CSF obtained the day before she died was negative for herpes simplex virus. A complement fixation test showed a four-fold rise in serum antibody titre to herpes simplex virus from 32 to 128.

Permission was obtained for post-mortem examination of the brain only. The brain was markedly oedematous and there was cerebellar tonsillar herniation. Microscopically, there was a diffuse encephalitis, with perivascular infiltration by lymphocytes and plasma cells, and areas of softening, with microglial proliferation, in temporal, frontal, and parietal lobes. Occasional intranuclear eosinophilic inclusions, surrounded by a halo, were seen in neurones.

Examination of the face revealed a focal ulcerated lesion, resembling *herpes labialis* on the upper lip. Tissue from the frontal and temporal lobe was submitted for virological study.

Case 2. A 20-month-old girl was in cardiac arrest and had fixed, dilated pupils when her father brought her to the Emergency Department, where resuscitation measures were unsuccessful. The previous day, she had been irritable and refused her food. She had a previous admission to hospital for pneumonia at 11 months of age but had suffered no other illnesses.

The autopsy revealed acute myocarditis consistent with a viral infection. There was non-specific hyperplasia of lymphoid tissue in the spleen, lymph nodes, gastrointestinal tract and tonsils. The tonsillar epithelium was ulcerated and rare intranuclear inclusions resembling herpes virus inclusions were seen around the area of ulceration. The brain showed no significant pathological changes. The cause of death was considered to be acute myocarditis. Tissue from the salivary gland, mesenteric lymph nodes and myocardium were submitted for virological study.

Virological examination. The lip lesions of case 1 were cultured, on the day of admission to the hospital, with sterile swabs that were placed in 1.5 ml of Eagle's diploid minimal essential medium containing antibiotics. Between 0.1 and 0.3 ml of this suspension was inoculated into cell cultures of human neonatal foreskin fibroblasts prepared according to the technique of Embil and Faulkner (1964).

Post-mortem specimens from cases 1 and 2 were prepared as 20% suspensions of the tissues in Eagle's diploid minimal essential medium containing antibiotics and, after centrifugation, the supernate was inoculated into human neonatal foreskin fibroblasts (Embil and Faulkner, 1964).

These inoculated cell cultures were examined daily for CPE. A virus resembling HSV was isolated from each sample and identified as HSV-1 by rate neutralisation and biological marker tests. The viruses were further characterised by restriction endonuclease digestion analysis. Virus was isolated from three sites in each case. The viruses isolated from case 1 were used at passage 2, and the isolates from case 2 were used at passage 3 for restriction endonuclease digestion analysis as follows.

Virus-infected monolayers of neonatal human foreskin cells were incubated for 24–48 h in Eagle's diploid minimal medium containing fetal calf serum 2% to which had been added ³H-thymidine 10 µCi/ml. When 80% or more of the cells were infected, the viral DNA was

isolated by the method of McFarlane and James (1984). After phenol extraction of the cell lysate, the aqueous layer was removed, extracted twice with ether and dialysed overnight against 0.05 M Tris, pH 8.0, containing 0.05 M NaCl and 0.001 M EDTA. This supernate contained the ^3H -labelled DNA. The restriction endonucleases used in this study (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I and *Xho*I) were purchased from Bethesda Research Laboratories, Gaithersburg, MD, USA and were incubated in their recommended conditions. The agarose gel electrophoresis and the time of exposure of the gel to Kodak X-Omat AR film were as described earlier (Embil *et al.*, 1981; McFarlane and James, 1984).

RESULTS

HSV-1 was isolated from the necropsy material from both children. In each case, the virus was isolated from three sites: in case 1, from the lip, and from the frontal and middle lobes of the brain, and in case 2, from a mesenteric node, the myocardium, and a salivary gland. The isolates from case 1 were shown to be different from those from case 2 by the results of treatment with restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I (fig.), *Pst*I, *Sal*I, and *Xho*I. After digestion with enzyme *Kpn*I, isolates from patient 1 had fragment *L* (unique region) whereas those from patient 2 did not (fig.).

The three viral isolates from case 1 gave identical patterns of fragments when digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III and *Xho*I, but the isolate from the lip (1-1) from case 1 gave a pattern of fragments different from those of the isolates from the frontal lobe (1-2) and the middle lobe (1-3) of the brain with restriction endonucleases *Kpn*I (fig.), *Sal*I and *Pst*I. However, these isolates had changes in the variable region of the genome (arrows, fig.).

The three isolates from case 2 gave identical results when digested with restriction enzymes *Eco*RI, *Hind*III and *Xho*I. Differences were seen between the isolate from the myocardium (2-2) and the isolates from the mesenteric node (2-1) and salivary gland (2-3) after digestion with restriction endonucleases *Kpn*I (fig.), *Bam*HI, *Sal*I and *Pst*I. The changes in digestion pattern in the isolates 2-1, 2-2 and 2-3 from case 2 occurred in the unique region. After digestion with the enzyme *Kpn*I, the fragment *m* was lacking from isolates 2-1 and 2-3 and an additional fragment was seen in the *v* region of the digestion pattern indicating the presence of two substrains; isolates 2-1 and 2-3 were identical substrains and were different from isolate 2-2. Similar changes were observed in the unique region of the genome with restriction endonucleases *Bam*HI and *Sal*I.

DISCUSSION

Restriction endonuclease digestion analysis is the most sensitive technique available now for determining the genetic relatedness of HSV isolates and has been used to study clinical and epidemiological problems. The use of restriction endonucleases depends upon two factors: (1) the mutation rate of the virus is low and, at most, only a small fraction of the virus in an individual represents the possibly mutated virus; and (2) only a small fraction of the nucleotides are hydrolysed by the restriction endonucleases and the statistical chance that the mutation would occur in these bases in epidemiologically-related virus isolates is very small (Roizman and Tognon, 1982).

However, recently a controversy has arisen between two groups of workers (Smith *et al.*, 1981; Roizman and Tognon, 1982) about the interpretation of changes in

FIG.—Fluorogram of restriction endonuclease digestion analysis pattern of isolates from case 1 (1-1, 1-2, 1-3) and from case 2 (2-1, 2-2, 2-3) digested with enzyme *Kpn*I. The letters L, m, v, wx, yz, indicate changes in fragments produced by *Kpn*I digestion in the unique area of the HSV genome; the arrows (→) indicate changes in the variable region of the HSV genome.

restriction endonuclease digestion patterns relating to infection with different substrains of HSV in the same person. Roizman and Tognon (1982) have indicated that there are specific regions of the viral genome in which variation can occur, and these are not used to subtype the virus. Changes in the digestion patterns can result from changes in base sequences in either the unique or variable regions of the HSV genome (Smith *et al.*, 1981; Roizman and Tognon, 1982). In view of this, the digestion patterns we obtained were compared with the digestion patterns of prototype viruses Justin and F (Locker and Frenkel, 1979) and ANG (Kaerner *et al.*, 1983). After digestion with enzyme *Kpn*I, all three isolates from case 1 (1-1, 1-2, 1-3) had band *L* (unique region) whereas all three isolates from case 2 (2-1, 2-2, 2-3) lacked this band (fig.). Moreover, the three isolates from case 1 had changes in the variable region of the genome (arrows, fig.) and, therefore, represent only one substrain of HSV in this patient. The changes in the digestion patterns in the isolates 2-1, 2-2 and 2-3 from case 2 after digestion with *Kpn*I were in several fragments, *m* and *v*, found in the unique region of the genome and indicate the presence of two substrains of virus.

Recent reports by Chaney *et al.* (1983*a* and *b*) and Kaerner *et al.* (1983) indicate that certain areas of the viral genome may be important in the pathogenicity of HSV. In view of this report and those about genital lesions (Buchman *et al.*, 1979; Maitland *et al.*, 1982; Ueno *et al.*, 1982; Kit *et al.*, 1983), encephalitis (Whitley *et al.*, 1982) and meningitis (Heller *et al.*, 1982), from which two substrains of HSV have been isolated, it is important that more information about substrains and type of virus, and site and time of isolation of substrain with particular reference to pathogenicity and latency of multiple strains, be obtained before the further development of HSV vaccines.

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