ISOLATION AND CHARACTERISATION OF TWO STRAINS OF *HERPESVIRUS HOMINIS* TYPE 1 FROM FALLOPIAN TUBES

I. G. BARTON, S. N. NAJEM, M. J. WALKER
AND C. W. POTTER

*Department of Virology, The University of Sheffield Medical School,
Beech Hill Road, Sheffield S10 2RX*

SUMMARY. *Herpesvirus hominis* type 1 (HSV-1) was isolated from fallopian-tube biopsies of two women who were using an intra-uterine contraceptive device; the strains were isolated on different dates and at different hospitals. Both the isolates were shown to be HSV-1 by restriction-enzyme analysis, polypeptide profiles and indirect immunofluorescence; the two isolates could not be distinguished by these tests, but the tests distinguished them from HSV-1 strain 71-15 and HSV-2 strain 333. The viruses were again isolated from samples of fallopian-tube tissue stored in liquid nitrogen, and their polypeptide and restriction-enzyme profiles were the same as those of the initial isolates. Virus was not isolated from fallopian-tube samples obtained from other IUD patients undergoing surgery at the same two hospitals at the same time.

INTRODUCTION

Herpesvirus hominis (HSV) is divided into two serotypes, type 1 (HSV-1) and type 2 (HSV-2), which differ clinically in their sites of infection. HSV-1 usually infects the head and neck whilst HSV-2 infects the skin below the waist and usually causes genital lesions. However, the difference in site of infection is not absolute and 22% of genital HSV lesions were reported to be associated with type-1 serotype (Smith, Peutherer and Robertson, 1976). The two serotypes are genetically very similar (Kieff *et al.*, 1972), but can be distinguished by several methods. Although they can be differentiated by serological techniques, these are often complicated by the considerable degree of antigenic cross reactivity so that biochemical methods are more suitable for typing the virus. The virus serotypes are best differentiated by an analysis of their nucleic acid; the DNAs of type 1 and type 2 exhibit clearly distinguishable DNA-fragment patterns after restriction-enzyme digestion (Skare, Summers and Summers, 1975; Lonsdale, 1979); in addition, many of the virus polypeptides produced in infected cells by the two types can be distinguished by polyacrylamide-gel electrophoresis (Courtney and Powell, 1975). These methods are very discriminating, and can be used to show differences between isolates of a single serotype (Lonsdale *et al.*, 1979).

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HSV-1 and HSV-2 can become latent in nerve ganglia after a primary infection. HSV-1 has been isolated from the trigeminal ganglia in 60% of cadavers by co-cultivation with susceptible cells (Warren *et al.*, 1977), and virus has been demonstrated in the superior cervical and vagus ganglia (Warren *et al.*, 1978). Latent HSV-2 has been isolated from the sacral ganglia (Baringer, 1974; Linneman *et al.*, 1978). This report describes the isolation and characterisation of two strains of *Herpesvirus hominis* isolated from fallopian tubes. Restriction-enzyme studies showed the isolates to be identical, but distinct from another strain of HSV-1.

MATERIALS AND METHODS

Tissue culture. BHK-21 (C13) cells (Flow Laboratories, PO box 17, Irvine, Scotland) were grown as monolayers in Glasgow-modified Eagle's medium containing tryptose-phosphate broth 10% (w/v) and newborn-calf serum 10% (v/v). Cells were subcultured at regular intervals on a split-ratio basis.

Preparation of virus stocks. *Herpesvirus hominis* HSV-1 strain 71-15 and HSV-2 strain 333 were obtained from Dr F. Rapp, Milton S. Hershey Medical Center, Pennsylvania, USA. Pools of these viruses were prepared by inoculating confluent cultures of BHK-21 cells, grown in 570-ml glass screw-capped bottles, with virus at a concentration of 0.01–0.1 plaque forming units (pfu)/cell in 1 ml of Eagle's minimal essential medium (EMM) containing 2% (v/v) newborn-calf serum. After adsorption at 37°C for 1 h, 50 ml of EMM were added to each bottle and incubation was continued for 36–48 h. The infected cells were then scraped from the glass surface into the medium, and the cell suspension was sonicated, to release intracellular virus, for 30 s at full power in an MSE 150-W ultrasonic disintegrator. After cell disruption, the suspension was centrifuged for 10 min at 200 *g*, and the supernatant fluid containing the released virus was stored in 1–2 ml portions at –80°C.

Titration of virus. For determination of the infectivity titre of the viruses, confluent monolayers of cells in 5-cm plastic petri dishes (Sterilin, 43 Broad Street, Teddington, Middlesex) were inoculated with 0.1 ml of an appropriate dilution of virus. The virus was allowed to adsorb for 1 h at 37°C and then 5 ml of EMM containing 0.8% carboxymethylcellulose was added to each dish. The dishes were further incubated at 37°C in an atmosphere of 5% CO₂ in air for 3–4 days; the cells were then fixed with 10% formol saline, stained with Leishman's stain and the plaques were counted.

Isolation of viruses from fallopian tubes. All tissue samples were processed on the day of collection. The tissue was finely minced with scissors, homogenised in a Dounce homogeniser and then centrifuged at 5000 *g* for 10 min. The pellet was collected and resuspended in EMM then sonicated at full power for 30 s. The sonicate was centrifuged at 1500 *g* for 15 min and inoculated directly onto confluent cultures of BHK-21 cells in 60-ml screw-capped bottles. Cultures were examined daily for cytopathic effect (CPE) and were maintained, with changes of medium every 3–5 days, until the cells degenerated. Bottles of cells with CPE were harvested when 75–100% of the cells were affected. Samples of tissue were also placed in Hams F10 medium containing 10% dimethylsulphoxide, and stored in the vapour phase above liquid nitrogen (–140°C). The samples were subsequently thawed rapidly at 37°C, sonicated at full power for 30 s, and inoculated without clarification onto confluent monolayers of BHK-21 cells grown in 120-ml bottles. The cultures were incubated at 37°C and examined daily for CPE; the viruses were harvested when the cells showed 75–100% CPE and were passed once more in BHK-21 cells before being used for restriction-enzyme and polypeptide analysis.

Labelling of virus and cellular proteins. The labelling of virus proteins with ³⁵S-methionine (³⁵S-met) was performed in the wells of Linbro trays (Flow Laboratories; catalogue number 76-033-05). The wells were each seeded with 5 × 10⁵ BHK-21 cells and incubated overnight at 37°C. The cells were then infected with virus at a concentration of 20–50 pfu/cell in 0.1 ml of

EMM, and allowed to adsorb for 1 h at 37°C. After adsorption, the cells were washed once with 1 ml of EMM containing one-fifth of the normal concentration of methionine and 2% (v/v) newborn-calf serum, and then 0.5 ml of the same medium was added to each well and the trays incubated for 2 h at 37°C. The infected cells were then labelled with ³⁵S-met (specific activity 1080 Ci/mmol) at 10 µCi/ml for 21 h.

After incubation in the presence of ³⁵S-met, the medium was removed and replaced by 150 µl of buffer of 0.05M Tris-HCl, pH 6.8, containing sodium dodecyl sulphate (SDS) 2%, 2-mercaptoethanol 5%, glycerol 10% and bromophenol blue. The trays were heated at 80°C for 10 min and the contents of the wells were transferred to vials. The samples were then boiled for 2 min, and samples were taken for the determination of trichloroacetic acid (TCA)-precipitable radioactivity. The samples were either assayed immediately or stored at -70°C and when subsequently thawed were placed in a boiling-water bath for 1 min before electrophoresis.

Polyacrylamide-gel electrophoresis was performed on 18 × 14-cm slab gels consisting either of 8.5% acrylamide or of a linear 5.5–12.5% gradient of acrylamide with a 5% stacking gel; the preparation of these gels has been described elsewhere (Lonsdale *et al.*, 1979). The ratio of acrylamide:bisacrylamide was determined from the relationship, bisacrylamide (mg/100 ml) = 1300/acrylamide (g/100 ml). An upper buffer solution of 0.05M HCl (pH 9.8) containing 0.55M glycine and 0.1% SDS, and a lower buffer of 0.01M Tris-HCl (pH 8.7) were used. Thyroglobulin (mol. wt 330 × 10⁶), bovine serum albumin (mol. wt 67 × 10⁶), lactate dehydrogenase (mol. wt 36 × 10⁶) and ferritin (mol. wt 18.5 × 10⁶) were all obtained from Pharmacia Ltd, Prince Regent Road, Hounslow, Middlesex, and used as markers of migration. Electrophoresis of samples was done at 200 V for 5–7 h or 50 V overnight at room temperature; after this time, the gels were fixed and stained in methanol:water:acetic acid (50:50:7, v/v/v) containing Kentacid blue 0.2% (BDH, Fancy Road, Poole, Dorset), and then destained in several changes of methanol:water:acetic acid (50:880:70, v/v/v). For autoradiography, gels were dried under vacuum, as described by Fairbanks, Levinthal and Reeder (1965), and exposed to Kodak NS 2T film for varying lengths of time.

Preparation of ³²P-labelled virus DNA. For restriction-enzyme studies, analysis was made of virus nucleic acid; the DNA was labelled with ³²P, as described by Lonsdale (1979). The number of TCA-precipitable counts was determined on 10-µl samples of labelled DNA.

Restriction-enzyme digests, agarose-gel electrophoresis and autoradiography. The restriction endonuclease SmaI was obtained from Boehringer-Mannheim, Bell Lane, Lewes, Sussex. Digests of virus DNA, containing 1–2 × 10⁴ Cerenkov counts/min in 50 µl of 0.006M tris-HCl (pH 7.5) containing 0.006M MgCl₂, and 0.006M 2-mercaptoethanol, were incubated with 1 unit of restriction enzyme for 3 h at 37°C. Reactions were stopped by the addition of 5 µl of 60% sucrose containing 0.2M ethylenediaminetetra-acetate (EDTA) (pH 7.5) and bromophenol blue. The samples were electrophoresed in 0.7% or 1.0% vertical-slab gels at 1.5–2 V/cm overnight, with 0.03M NaH₂PO₄ and 0.001M EDTA in 0.036M Tris solution as buffer. After electrophoresis, the gels were dried overnight at 37°C, and exposed to Ilford Rapid R type 5 film (Ilford Ltd, Essex) for various lengths of time.

Antisera. Purified immunoglobulin fractions of antisera to HSV-1 (strain MacIntyre VR3) and HSV-2 (strain MS) were obtained from Dako Laboratories, Denmark. The optimal dilution of the sera for use in immunofluorescence tests was determined by titration on virus-infected cells. FITC-conjugated swine anti-rabbit serum was obtained from Nordic Immunologicals (London).

Indirect immunofluorescence. Confluent monolayers of BHK-21 cells grown on coverslips in 10-cm glass petri dishes were infected with virus at a concentration of 5 pfu/cell; after adsorption for 1 h at 37°C, 10 ml of EMM was added to each petri dish and incubation was continued for 24 h. The coverslips were then washed twice in ice-cold phosphate-buffered saline (PBS) and once in acetone at -20°C, and stored in acetone at -20°C.

For immunofluorescence tests, the coverslips were air-dried and covered with either rabbit anti-HSV-1 serum at a dilution of 1 in 500 or rabbit anti-HSV-2 serum at a dilution of 1 in 200; the coverslips were then incubated in a moist chamber at 37°C for 30 min, washed in PBS, and incubated for a further 30 min with FITC swine anti-rabbit serum diluted 1 in 20. After these treatments, the coverslips were again washed twice in PBS, then mounted in glycerol:saline (9:1) and examined under a Zeiss Universal microscope with epifluorescent illumination.

RESULTS

Patients

Biopsies of fallopian tubes were obtained from the two patients. One patient (MW-2) had had histological evidence of pelvic infection, but at the time of sterilisation the pelvic organs were macroscopically normal and there was no evidence of inflammation in the tubal biopsies. The second patient (MW-1) had had a hysterectomy for dysfunctional uterine bleeding. The only abnormality found at hysterectomy was a small intramural fibroid. There was no histological evidence of inflammation in the tubal biopsies. The patient had no history of pelvic inflammatory disease. Neither patient had a history of venereal disease and both had negative cervical smears.

Characterisation of viruses

Restriction-enzyme analysis. The DNA from the two viruses isolated from the fallopian tubes of patients MW-1 and MW-2 and the DNA of HSV-1 strain 71-15, and HSV-2 strain 333 were each digested with the restriction endonuclease SmaI. The resulting fragments were then separated by electrophoresis in either 0.7% or 1% agarose gels, and fragment migration was revealed by autoradiography. The results are shown in figs. 1 and 2. The autoradiographs show that the DNAs of strains MW-1 and MW-2 gave identical patterns, but that they could be differentiated from those of HSV-1 strain 71-15 and HSV-2 strain 333. Previous studies have shown that restriction-enzyme digests of HSV-1 and HSV-2 are quite distinct; a comparison of the patterns from strains MW-1 and MW-2 with those from three other HSV-1 strains, 71-15, KOS and Patton, showed all these to be similar, but unlike that obtained with HSV-2 strain 333. Strains MW-1 and MW-2 are therefore both HSV-1 because the patterns obtained from all five strains contained fragments with mobilities similar to those of strain 71-15. Fragments a, c, g and l from HSV-2 strain 333 did not have the same mobility as those from HSV-1 strains (fig. 2). Additionally, HSV-1 strains KOS and Patton had fragments with mobility similar to that of fragment b from strain MW-1. Only the mobility of strain 333 fragment j was found to be similar to that of HSV-1 strains MW-1 and MW-2 fragment i. HSV-2 strain 333 fragment g was found in strains MW-1 and MW-2, HSV-1 strain Patton and HSV-1 strain 71-15. When the restriction-enzyme profiles from the two original clinical isolates were compared with those obtained from virus from tissue stored in liquid nitrogen, the appearance was identical.

Polypeptide analysis. Analysis of the virus polypeptides produced in cells infected with HSV-1 strains MW-1, MW-2 and 71-15 and HSV-2 strain 333 on 8.5% single-concentration polyacrylamide gels showed that the profile of HSV-1 strains 71-15, MW-1 and MW-2 were very similar but could be differentiated from that of HSV-2 strain 333; thus, polypeptide analysis clearly indicated that the clinical isolates were strains of HSV-1 virus (fig. 3). Analysis of HSV-1 strains 71-15 and MW-2 on 5–12.5% linear-gradient polyacrylamide

FIG. 1.—Autoradiograph showing *Sma*I digest of HSV DNA electrophoresed overnight in 0.7% agarose at 2 V/cm. Fragments of mol. wt $c 5 \times 10^5$ have migrated off the bottom of the gel. MW-1 and MW-2 = viruses from fallopian-tube tissue of patients; 333 = HSV-2 strain 333; 71-15 = HSV-1 strain 71-15.

FIG. 2.—Diagram constructed from autoradiographs to show the relative migration of *Sma*I-digested HSV DNA fragments in 1% agarose gels.

gels gave better resolution of some polypeptides (fig. 4), and showed that whilst strains MW-1 and MW-2 could not be differentiated there were some minor differences between the two clinical isolates and HSV-1 strain 71-15. Additionally, the polypeptide profiles of the two original clinical isolates and the viruses isolated from tissue stored in liquid nitrogen were indistinguishable.

Immunofluorescence tests. The original isolates and the viruses re-isolated from tissue stored in liquid nitrogen gave fluorescence with HSV-1 antisera diluted 1 in 500 and HSV-2 antisera diluted 1 in 200; control coverslips infected with HSV-1 strain 71-15 and stained with HSV-1 antisera also gave fluorescence, but coverslips infected with HSV-2 strain 333 fluoresced only with HSV-2 antisera. This result further indicated that the two clinical isolates were HSV-1 strains.

FIG. 3.—Autoradiograph of dried gel to show polypeptides produced in HSV-infected cells labelled 2–21 h after infection with ³⁵S-methionine. Gel was 8.5% single-concentration polyacrylamide, electrophoresed overnight at 50 V. Strain identification as in fig. 1. Positions of molecular-weight ($\times 10^6$) markers determined from stained gel are shown.

FIG. 4.—Autoradiograph of infected-cell polypeptides produced as in fig. 3. Gel was 5–12.5% linear gradient of polyacrylamide. Mock = mock-infected cells; strain identification as in fig. 1. Positions of molecular-weight ($\times 10^6$) markers determined from stained gel are shown.

DISCUSSION

Epidemiological studies have shown that the isolation of HSV-1 from sites below the waist is unusual but not rare (Smith *et al.*, 1976). We report the isolation of HSV-1 from fallopian-tube biopsy of two patients. The viruses were isolated from surgically removed tissue taken by different operators, collected at different times in different hospitals, and processed and tested in the laboratory at different times. In addition, the viruses were re-isolated from samples of tissue that had been stored in liquid nitrogen, but virus was not isolated from other similar samples taken from other patients and stored and

treated in the same manner. We are confident, therefore, that these are genuine isolates from the patients and not contaminants. The viruses were studied by three techniques to determine the serotype. The results of all three tests clearly indicated that both isolates were strains of HSV-1. Thus, restriction-enzyme analysis of virus DNA and polypeptide profiles indicated that the two viruses were very similar, but distinct from HSV-1 strain 71-15 and from HSV-2 strain 333. Additionally, the restriction-enzyme and polypeptide profiles of the viruses re-isolated from tissue stored in liquid nitrogen were identical to those of the initial isolates.

Previous reports have indicated that HSV-1 isolates from different individuals could be distinguished from each other on the basis of their restriction-enzyme or polypeptide profiles (Lonsdale *et al.*, 1979; Buchman *et al.*, 1978; Buchman, Roizman and Nahmias, 1979). However, Lonsdale *et al.* (1979) reported that three of their isolates from 17 individuals gave identical profiles. Whilst laboratory contamination cannot be completely excluded from the present results, it is an unlikely explanation of the present findings, because the original tissue was obtained from different sources and processed at different times; in addition, virus was re-isolated from samples of tissue stored in liquid nitrogen. Because HSV-1 strains can be further subtyped by restriction-enzyme analysis of virus DNA (Lonsdale *et al.*, 1979) it is of interest that two isolates from the fallopian tubes of two patients should be identical. This result could indicate that some strains are associated with specific types or sites of infection, and the possibility that specific types are implicated in diseases such as carcinoma of the cervix invites urgent investigation.

However, the technique that we used does not label virus DNA to a very high specific activity, and small or lightly labelled fragments would not have been detected in our studies. Thus, although both the clinical isolates appeared very similar as judged by polypeptide and restriction-enzyme analysis, some minor differences may exist between them.

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