Apparent stability of herpes simplex virus genomes isolated from primary and recurrent infections and dorsal root ganglia in the mouse

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Summary. Following natural or experimental primary infection, herpes simplex virus (HSV) becomes latent in sensory ganglia. Reactivation of latent virus may lead to recurrent disease. If HSV DNA remains stable during primary, recurrent and latent infections, that stability would enable us to trace the transmission of HSV from one individual to another. We inoculated mice in the ear pinna with HSV and collected virus at various intervals during primary infection. In mice surviving primary infections, recurrent disease was induced from which virus was isolated. Virus was also recovered from explanted dorsal root ganglia. Virus isolates were characterised by restriction endonuclease digestion and compared with the original inoculate(s). The data indicate that in all cases except two, the isolates from primary and recurrent infections remained identical to the original inoculates.

Introduction

Herpes simplex virus (HSV), type 1 or type 2, becomes latent in its host after primary infection (Klein, 1982, 1985). It is believed that during primary infection, HSV is taken up by peripheral nerve endings and migrates to the sensory ganglia, there becoming latent. Reactivations of the latent virus can either occur spontaneously or be caused by reactivation stimuli at the periphery and lead to recurrent disease at or near the site of the primary infection.

Latency, although an accepted phenomenon, remains a subject of controversy. "Dynamic state" supporters believe that the virus replicates at a low level during latency while "static state" supporters argue that the virus exists in a nonreplicating form (Blyth and Hill, 1984). Additionally, controversies concerning the molecular state of the HSV genome remain unresolved. Is the latent genome integrated into the host DNA or does the genome remain

autonomous in the cytoplasm of the latently infected cell (Klein, 1982)?

Of considerable importance is the genome stability of HSV DNA during primary, latent and recurrent infections. Is the virus that originally infects its host identical to the virus that causes the recurrent disease? This question has been and can be studied by use of restriction endonuclease digest analysis. A report by Smith *et al.* (1981) suggested that during primary infection with HSV and following several replication cycles *in vivo*, the infecting virus adapts to the new host and that, during this adaptation period, variations in the virus genome may occur. However, Roizman and Tognon (1982) disagreed with the conclusion of this report. Centifanto-Fitzgerald *et al.* (1982) found that prior infection with HSV type 1 in the rabbit cornea, leading to the establishment of latency in the related ganglia, prevented the subsequent superinfection of the same ganglia with a second, heterotypic, introduced strain of HSV.

To study HSV DNA stability *in vivo*, we employed the mouse ear model (Hill *et al.*, 1975) and the techniques of restriction endonuclease digest analysis (McFarlane and James, 1984) of the genomes of HSV, recovered from the ears of mice during either primary or recurrent disease, or from cervical dorsal root ganglia, or from both sites.

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Materials and methods

Cell culture

Human neonatal foreskin fibroblast cell lines, established in our laboratory (Embil and Faulkner, 1964), were used exclusively throughout this study.

Virus

Four strains of HSV were used in this study: two prototypes from the American Type Culture Collection—ATCC VR No. 539 HSV type 1 (HSV-539) and ATCC VR No. 540 HSV type 2 (HSV-540)—and two clinical isolates—HS76-HSV type 1 (HSV-76) and HS106-HSV type 2 (HSV-106)—obtained in our laboratory in Halifax, Nova Scotia. Before inoculations, all virus suspensions were titrated by standard plaque assay. The titres of the suspensions were as follows: HSV-539 (type 1), 5.5×10^5 pfu/ml; HSV-540 (type 2), 1.65×10^4 pfu/ml; HSV-76 (type 1), 1.9×10^4 pfu/ml and HSV-106 (type 2), 7.3×10^4 pfu/ml.

Transport medium for virus cultures

Basic virus transport medium was prepared by supplementing Eagles Diploid Medium (EDM) (Gibco Canada, Burlington, Ontario, Canada) with Garamycin Reagent Solution (GRS, 10 mg/ml) (USP Schering Corporation, Kenilworth, NJ, USA) 2%. This mixture was then divided in 1.5-ml amounts and stored at -20°C until used.

Transport medium for ganglia

Basic transport medium for ganglia was as described for virus cultures with the addition of 3% of a Fetal Bovine Serum: Calf Serum (1:1) mixture.

Mice

Swiss white mice (Charles River Canada Inc., St-Constant, Quebec, Canada) were housed in our animal quarters. Their offspring were used exclusively throughout this study. Three weeks after birth, mice were separated by sex, pooled and randomly distributed, 6–8 per cage. They were maintained according to the guidelines for laboratory animals set by the Canadian Council for Experimental Animals.

Inoculation of mice with HSV

Mice were anaesthetised with 1.2 mg/kg of sodium pentobarbital (Abbott Laboratories, Montreal, Quebec, Canada) injected intraperitoneally. They were then inoculated in the right ear with 15–30 μl of undiluted virus suspension with a 50- μl or 100- μl Hamilton microlitre syringe and a 30-gauge bevel needle. Control

mice were inoculated with phosphate-buffered saline (PBS). Each animal was identified by a marking on its left ear.

Ear stripping of mice

At intervals after inoculation, mice were anaesthetised as above or by ether inhalation. The right ear was then stripped six times with cellophane tape (Hill *et al.*, 1978) (Scotch Transparent Tape, 3M Canada Inc., London, Ontario, Canada).

Swabs and ear biopsy specimens

Mice from which swabs or ear biopsies were to be obtained were anaesthetised by ether inhalation. Swabs of infected areas were placed in transport medium (as described above) which was kept in an ice bath until transported to the laboratory. Specimens were either assayed immediately or after storage at -70°C . Ear biopsies were cut from the region of the ear corresponding to infection. The tissue was placed in transport medium and transferred to the laboratory in an ice bath. The tissues were frozen and thawed twice, minced into small pieces, frozen and thawed twice more, and then assayed for infectious virus. Specimens not assayed immediately were stored at -70°C .

Isolation of radioactive DNA and restriction endonuclease digestion

The technique for isolation of radiolabelled DNA and band separation following restriction endonuclease digestion was extensively described by McFarlane and James (1984). Briefly, virus isolates to be analysed were inoculated into 150-cm² Falcon flasks containing monolayers of neonatal foreskin fibroblast cells; 6 h later, 2–10 $\mu\text{Ci/ml}$ of ³H-thymidine was added and when 80% or more of the cell monolayer was infected, the spent medium was centrifuged and the pellet and infected cell monolayer suspended in Tris containing sodium dodecyl sulphate and EDTA (Hirt, 1967). We then added NaCl to the cell suspension and left it at 4°C overnight. The next day, after centrifugation of the cell-virus lysing solution, we treated the supernatate (containing the labelled DNA) with phenol, centrifuged it, extracted the material twice with ether and dialysed it overnight against Tris containing NaCl and EDTA. A sample of the radioactive DNA was placed in scintillation buffer to determine its radioactivity and then it was subjected to restriction endonucleases. Restriction endonucleases used in this study were: *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sst*I, *Xba*I and *Xho*I. For consistency, the same lots of restriction enzymes were used throughout this study. Due to differences in radioactive band intensities, photographic reproduction can lead to some lighter bands being absent from the photographs.

Differences were seen between the HSV 1 and HSV 2 isolates and between the two HSV type-I strains when digested with *Kpn*I. *Sal*I digestion also showed a

difference between the type 1 and type 2 HSVs and this enzyme detected a significant banding difference between the two HSV type-2 isolates. Thus, we could identify each of our virus inoculates.

Results

We wished to test the genome stability of HSV DNA isolated during primary and recurrent disease as well as that of virus isolated from the cervical dorsal root ganglia. We compared virus isolated during primary disease and virus isolated from successfully induced recurrent disease with the original virus inoculated. Where multiple isolates were recovered from the ganglia of mice (each cultured separately), the isolates were compared with each other and with the original inoculate(s).

To test HSV genome stability during primary infection in the mouse, we compared isolates recovered early or late in primary infection with the original inoculate. Fig. 1 shows a comparison of isolates recovered at two different times during primary infection in a mouse inoculated with HSV-106 (type 2). No significant differences were detected between those isolates when compared with each other or with HSV-106 (not shown). This observation was consistent for all isolates collected during the primary infections, and was also consist-

ent when the isolates were digested with the remainder of the enzymes listed in *Materials and methods*.

To test whether the HSV genome remained stable *in vivo* over long periods, we analysed virus isolates recovered from mice 4, 7 and 11 months after inoculation. A representative autoradiograph is shown in fig. 2. When the isolates were digested with the restriction enzymes and the patterns compared with those of the original inoculates, no detectable differences were noted between the isolates and original inoculates.

To determine the genome stability of isolates recovered from cervical dorsal root ganglia of mice inoculated with HSV-106 we digested the isolates from mouse C4-2 and compared them with each other and with HSV-106. The banding profiles for these isolates are shown in fig. 3. No differences were detected between the isolates when they were compared with each other and with HSV-106.

To test the hypothesis that latently infected mouse ganglia could not be superinfected with a second heterotypic strain of HSV, selected mice suspected of harbouring latent HSV were inoculated approximately 30 weeks after primary inoculation with a heterotypic strain of HSV at the same site as the initial inoculation. Viruses recovered from the cervical dorsal root ganglia of doubly inoculated

Fig. 1. Autoradiographs of the fragments produced following digestion of ^3H -thymidine-labelled DNAs from HSV isolates obtained from mouse C4-1 (a) 3 days and (b) 7 days after it was inoculated with HSV-106.

Fig. 2. Autoradiographs of the fragments produced following digestion of ^3H -thymidine-labelled DNAs from HSV-106 (x) and from an isolate (a) recovered from mouse C6-1 approximately 7 months after it had been inoculated with HSV-106.

Fig. 3. Autoradiographs of the fragments produced following digestion of ^3H -thymidine-labelled DNAs from HSV-106 (x) and from isolates recovered from the second cervical dorsal root ganglion (2) and from the third cervical dorsal root ganglion (3) of mouse C4-2, which had been inoculated with HSV-106.

Fig. 4. Autoradiographs of the fragments produced following digestion of ^3H -thymidine-labelled DNAs from HSV-540 (x) and from isolates recovered from the second cervical dorsal root ganglion (2) and from the fourth cervical dorsal root ganglion (4) of mouse C12-2, which had been inoculated first with HSV-540 and, approximately 30 weeks later and at the same site, with HSV-76.

animals were analysed by restriction endonucleases and compared with the original inoculates. Fig. 4 shows a representative response which occurred in all but one set of mouse isolates. In all cases, the restriction enzyme digestion revealed that the banding patterns of the isolates were those of the first virus inoculated and no patterns of the second virus inoculated were found. It did not matter in what order the virus inoculations were given (i.e., HSV 1 followed by HSV 2 or *vice versa*); the isolates were always the same as the first virus inoculated.

Comparison of the isolates and original inoculates revealed that in all but two cases examined, the isolates were identical to the first original inoculate. However, in one mouse, two out of three ganglia isolates showed banding profiles which significantly differed from the original inoculate while the third isolate was identical to the original inoculate. This banding alteration was only detected with *KpnI* (fig. 5).

Fig. 5. Autoradiographs of the fragments produced following digestion of ^3H -thymidine-labelled DNAs from HSV-540 (x) and from isolates recovered from the second, third and fourth cervical dorsal root ganglia (2, 3 and 4, respectively) of mouse C12-1, which had been inoculated first with HSV-540 and, approximately 30 weeks later and at the same site, with HSV-76. There was a significant banding difference between the controls (x) and the isolates from the second and fourth ganglia when they were digested with *KpnI* (analyses were done on different days, but the protocol was unaltered and the same lots of restriction enzymes were used); one band (▲) disappeared and two bands (←) emerged. No such alteration occurred when the isolate from the third cervical dorsal root ganglion was digested with *KpnI* or when any of the three isolates was digested with any other enzyme.

Discussion

Analysis of HSV DNA by restriction endonuclease digestion showed that, in all cases except two, the genomes of HSV isolated during primary and recurrent infection and from cervical dorsal root ganglia were identical to the original inoculates. The stability of HSV type 1 and HSV type 2 genomes has been discussed in the past, Roizman and Tognon (1982) arguing in favour of stability with minor variations and Smith *et al.* (1981) citing evidence of genome instability. However, in spite of these conflicting reports, the accepted opinion, at the present time, is that the genomes of HSV are stable both *in vivo* and *in vitro* and that variability not restricted to the variable regions of the genome is a rare event in nature (Roizman and Buchman, 1979).

Therefore, the observation that the genomes of HSV recovered from primary and recurrent lesions and cervical dorsal root ganglia were identical to the original inoculated virus was not surprising. It has been reported that genome stability was observed in HSV isolates 12 years apart in a human patient (Buchman *et al.*, 1979; Roizman and Buchman, 1979). Kit *et al.* (1983) reported stability of HSV isolates from an individual sequentially infected with HSV type 1 and HSV type 2. HSV genome stability was maintained in isolates recovered over a 4–9-month period. Concurrent isolates from an individual infected at both the genital and oral sites were identical, showing genome stability at different sites in the same host (Embil *et al.*, 1981).

The one exception, noted earlier, where there was a banding alteration between isolates from the ganglia of mouse C12-1 and the original inoculated virus is shown in fig. 5. As is indicated in the autoradiograph, the banding difference occurs when the isolates are digested with the enzyme *KpnI*, but banding differences do not occur when the isolates are digested with any other of several enzymes. Furthermore, the banding alteration appears in isolates C12-1-2 and C12-1-4 but not in isolate C12-1-3. As is not readily obvious in the autoradiograph, the appearance of the two additional bands occurs in the absence of a band (of higher molecular weight) present in the original inoculate. Enzyme digestion maps of *KpnI*-digested HSV type 2 (Buchman *et al.*, 1979; Kit *et al.*, 1983) indicate that the banding alteration occurs in a band in the unique (L) region of the genome, an area thought to be stable.

Interpretation of these results can be considered by two possible mechanisms. First is the fact that

mouse C12-1 was inoculated with HSV-540 type 2 initially and, secondly, with HSV-76 type 1. A recombination event occurring between the two inoculated viruses could produce alterations in the banding patterns. Recombinants between HSV-1 and HSV-2 have been studied *in vitro* (Morse *et al.*, 1977). Although recombinants are possible, that this event occurred *in vivo* in our system is unlikely. The observation that latently infected mice were not latently reinfected (McKendall, 1977) with a second strain of HSV makes it improbable that the two viruses would be present in the same infected cell. Recombination might be more probable if the two virus populations were mixed and inoculated together. Since the viruses were inoculated at different times, recombination within two ganglia seems unlikely. The fact that the alteration in banding patterns was detected only with the enzyme *KpnI* also argues against a recombination. A recombinant would more than likely affect more than one enzyme recognition site and, therefore, be detected with more than one enzyme.

A more probable explanation for the band alteration is that the band affected underwent a point mutation that affected the enzyme digestion recognition site for *KpnI*. A point mutation in this band may not be detected with other enzymes since the recognition sites for those enzymes would not be altered. Although a point mutation is a rare event (Roizman and Buchman, 1979), it appears to be more probable than a recombination.

The observation that two of the ganglion isolates (C12-1-2 and C12-1-4) had the banding alteration while the remaining one (C12-1-3) did not, is confusing to us. Multiple analysis of the samples showing band alterations (and the one that did not) consistently gave the same results. While it is conceivable that a point mutation could occur in one virus in a ganglion body, that the same mutation could occur in a second virus in a different ganglion body is unlikely. In addition, the observation that the C12-1-3 isolate remained identical to the original inoculates confounds the possibility that the mutation occurred before latency, since one would then expect the alteration to be present in all three ganglion isolates.

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