Asymptomatically shed recombinant herpes simplex virus type 1 strains detected in saliva

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a DNA virus belonging to the family Herpesviridae encompassing more than 100 herpesviruses. The clinical spectrum of HSV-1 infection includes rare, severe manifestations such as encephalitis and disseminated neonatal infection, oral and genital clinical lesions, and asymptomatic shedding. Recrudescent disease has been estimated to occur in 32 % of blood donors resident in England (Cowan et al., 1996), and in Sweden, a recent study demonstrated that 26 % of HSV-1-infected individuals presented oral lesions (Lowhagen et al., 2002). Thus, a minority of HSV-1-infected subjects have clinical lesions. It is well-known that HSV-1-infected individuals shed virus in saliva during episodes of herpes labialis as well as during asymptomatic phases, and that asymptomatic oral shedding of HSV-1 is essential for transmission (Scott et al., 1997). The molecular basis for asymptomatic shedding is not well understood, i.e. the difference between subclinical reactivation with asymptomatic shedding and reactivation leading to clinical lesions.

Although most HSV-1-infected individuals have an asymptomatic infection, recent phylogenetic analyses of HSV-1 based on DNA sequencing have been exclusively from HSV-1 isolates derived from clinical lesions. These analyses identified three evolutionary clades (A–C) with a genetic distance between the most distant HSV-1 isolates of approximately 2 %, as well as recombinants (Bowden et al., 2004; Norberg et al., 2004). The datasets on which previous genotyping studies are based and interpretation of the evolutionary history of HSV-1 may therefore be biased, as DNA sequence information from HSV-1 shed asymptomatically has not been included. The lack of such sequences may partly be explained by the fact that asymptomatic HSV-1 is shed intermittently for only a few days per month (Augebnaun et al., 2001; Spruance, 1984; Tateishi et al., 1994).

Homologous recombination is a common feature in the evolution of the subfamily Alphaherpesvirinae (Norberg...
mutation rate has been estimated to be $3.5 \times 10^{-8}$ mutations per site per year (Sakaoka et al., 1994). As nucleotide substitutions are rare events in the HSV-1 genome, homologous recombination is a powerful molecular mechanism to exchange genetic segments between different genomes containing several point mutations by a single recombination event. By such an event, a genome can collect beneficial mutations from several other genomes and increase the rate of adaptation (Felsenstein & Yokoyama, 1976) or exclude randomly introduced harmful mutations (Keightley & Otto, 2006).

In this report, we investigated sequence variation of HSV-1 shed asymptomatically and compared the sequences with previously described HSV-1 sequences derived from clinical lesions (Norberg et al., 2004). For this purpose, saliva samples from individuals with both symptomatic and asymptomatic HSV-1 infection were collected on consecutive days. Recombinant HSV-1 isolates were detected from subjects with no clinical HSV-1 infection. In addition, two evolutionarily separated strains were detected within the same individual. We also estimated the rate and frequency of HSV-1 shedding in saliva.

**METHODS**

**Subjects.** Two female adults (aged 40 and 42), two female teenagers (aged 18) and one male child (aged 7) were included. Sera from these individuals were positive for HSV-1 type-specific anti-glycoprotein G-1 (gG-1) antibodies using HerpeSelect HSV-1 ELISA (Focus) and were negative for HSV-2 antibodies determined by HerpeSelect HSV-2 ELISA (Focus). The individuals were all healthy and did not use medications regularly. One adult (PT) and one teenager (JL) suffered from recurring herpes labialis, whilst the other two subjects (EL and CG) were asymptomatic with no history of clinical HSV-1-induced oral lesions. The child (FT) was also truly asymptomatic. One millilitre of saliva was collected daily before breakfast for 29 days from FT, 79 days from CG, 108 days from PT, 200 days from EL and 232 days from JL. The samples were kept frozen at $-20^\circ$C until analysis.

**Real-time PCR.** PCR was performed directly from saliva samples to exclude artefacts and selection of HSV-1 variants in cell culture. Viral DNA was extracted from 200 µl saliva using a Magnapure LC Robot (Roche). Subsequent amplification of a segment of the gB gene was performed using TaqMan probes labelled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine and an ABI Prism 7000 PCR instrument (Applied Biosystems). This assay offered high specificity and similar sensitivity to a nested PCR (Namvar et al., 2005). A plasmid (pUC57) containing the target sequence was constructed (GenScript) and amplified in *Escherichia coli* XL-1 Blue, purified using a HiSpeed Plasmid Maxi kit (Qiagen) and quantified by spectrophotometer analysis. A standard curve was included in each run, based on six fivefold dilutions of the plasmid using an initial concentration of $1 \times 10^6$ HSV-1 copies per reaction. All samples were tested in duplicate and expressed as mean values. The saliva samples were analysed by seven TaqMan runs. The mean of the slope for the standard curves was $-3.43$ with a SD of 0.075 and $r^2$ was 0.99 for all runs. As saliva may contain substances that are inhibitory for PCR, selected positive samples from all individuals were tested using twofold dilutions. No inhibition was detected for any of the saliva samples. The detection limit was 50 HSV-1 genome copies (ml saliva)$^{-1}$.

**DNA sequencing.** Direct sequencing was performed for all real-time PCR-positive samples using primers specific for the gene encoding gI as described previously (Norberg et al., 2004). This target was selected for two reasons. Firstly, the gI gene has been sequenced recently for clinical HSV-1 isolates and for laboratory HSV-1 strains (Norberg et al., 2004), so data was available for comparison. Secondly, the gI gene contains more nucleotide substitutions than the gE gene. In addition, clinical HSV-1 isolates harbour a polymorphic tandem-repeated (TR) region in the gI gene composed of two to eight blocks, each encompassing 21 nt, which can be used for further classification (Norberg et al., 2007). The gI gene was amplified and sequenced where possible from a single PCR product (1173 nt, strain 17). To gain more information on the putative recombinant strains detected for EL and FT a single PCR product of 2882 nt was amplified covering almost the complete gI gene (nt 76–1173, strain 17) and most of the gE gene (nt 1–1358, strain 17) located adjacent to the gI gene in the HSV-1 genome. DNA sequencing was performed from single PCR fragments to exclude the possibility that the sequences originated from mixed HSV-1 variants in the saliva samples.

**Phylogenetic analysis.** The sequences were aligned manually and compared with previously published HSV-1 sequences (Norberg et al., 2004) and classified by constructing phylogenetic trees using the maximum-likelihood method included in the *phylog* package. A limitation with phylogenetic trees is their inability to present correctly the evolutionary history of organisms or species when hybridization, horizontal gene transfer or recombination has been involved. In order to visualize such events, a more complex graph or phylogenetic network is essential. In contrast to trees, which are a subset of a network, where an organism or species only has one adjacent parental node, several parental nodes may be present in a network to illustrate recombination events. Thus, in order to evaluate the presence of recombination, the strains were further analysed by constructing phylogenetic networks with and without the putative recombinants included. The networks were constructed using the SplitsTree program (Huson, 1998), and the Φ test for statistical significance for recombination (Bruen et al., 2006) was applied on each sequence set. The bootscan method included in the SimPlot program was then applied to each recombinant candidate to evaluate further the presence of recombination.

**RESULTS**

**Quantification of HSV-1 by real-time PCR**

In total, 232 saliva samples were collected from JL who suffered from a clinical HSV-1 infection. HSV-1 was detected in 21/232 samples (9.1%) distributed over eight episodes with durations of between 1 and 4 days. Concurrent symptomatic labial lesions were apparent during five of these episodes (Fig. 1). The amount of HSV-1 produced varied between 100 and 32,900 genome copies (ml saliva)$^{-1}$ (mean copy number 5400) (Table 1). The level of shedding in saliva was similar during episodes involving clinical lesions (mean copy number 5300) compared with episodes without lesions (mean copy number 5600). The asymptotically infected teenager
(EL) was HSV-1 PCR positive in 10/200 samples (5%) from three episodes with durations of 3–4 days. The HSV-1 genome copies varied from 100 to 2,814,150 ml⁻¹ (mean copy number 313,000). The clinically infected adult (PT) presented three episodes of asymptomatic shedding, two with durations of 1–2 days and one episode lasting 3 days. The amount of HSV-1 varied from 900 to 1,108,300 genome copies ml⁻¹ (mean copy number 441,000). Clinical oral lesions were noticed during the last episode and the genome copies in saliva varied from 11,200 to 660,250 (mean copy number 414,000). In total, HSV-1 DNA was detected in 8/108 samples (7.4%) for PT. The asymptomatically infected woman (CG) had no detectable shedding during the study period (79 days). Finally, 29 samples were collected from the child (FT) and HSV-1 was detected from two episodes covering in total 10 days (34%). The difference in duration of shedding compared with JL, EL and PT was highly significant \((P<0.001, \text{ Fisher's exact test})\). The amount of HSV-1 DNA varied from 150 to 1,170,900 genome copies ml⁻¹ (mean copy number 368,000). In summary, for all individuals, 648 saliva samples were processed and the overall shedding of HSV-1 DNA was 7.6 % (49/648).

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**Table 1.** HSV-1 copy number ml⁻¹ detected in saliva by real-time PCR from four individuals

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*Fig. 1.* Time scale and pattern of HSV-1 shedding from saliva from five individuals detected by real-time PCR. Each black box represents an episode of shedding. Concurrent symptomatic lesions are marked with a cross.
**Phylogenetic analysis**

Due to lower sensitivity (approx. 3 logs) of the PCR targeting the gI and gE genes compared with real-time PCR based on the gB gene, it was possible to generate sequence data from 29/49 real-time PCR-positive saliva samples.

Twelve saliva samples collected from JL from episodes 1 and 2 and from episodes 4 to 7 were sequenced. The gI gene was sequenced for these 12 samples and all sequences were identical and classified into clade B using the maximum-likelihood method (Fig. 2). The TR region within the gI gene contained four repeats. Identical HSV-1 sequences were detected from three symptomatic labial lesions (from episodes 1, 2 and 6).

Two saliva samples collected from EL from episode 1 and three saliva samples from episode 2 were sequenced. For these five samples, the gI gene and most of the gE gene were sequenced. These sequences were identical and the TR region contained six repeats. This strain was designated EL_040127 and did not cluster to clade A, B or C in the phylogenetic tree, a feature common for recombinants (Fig. 2).

Two saliva samples collected from EL from episode 3 were also sequenced. The HSV-1 sequences were classified into clade A and the TR region displayed two repeats (designated EL_040229). Thus, two different strains were shed in different episodes. The two strains EL_040127 and EL_040229 differed by six positions out of 1035 nt analysed (nt 76–1173 excluding the TR region) and by four repeats in the TR region in the gI gene. The results were confirmed by new preparations of the saliva samples, and DNA sequencing gave identical sequences from both strands.

For PT, the gI gene of HSV-1 was sequenced for two saliva samples collected from episode 2 and for three saliva samples collected from episode 4. These five sequences were identical and were classified into clade A. The TR region contained five repeats. HSV-1 collected from clinical lesions (derived from episode 4 and from 1 year earlier) was identical with the strain shed asymptomatically in saliva.

Five saliva samples collected from FT from episode 2 were sequenced. For these samples, the gI gene and most of the
Fig. 3. Analyses of the putative recombinant strains FT and E_040127 based on gI and gE gene sequences. The Φ test for statistical significance of recombination (a) was applied to the sequences included in the three networks presented in (b), (d) and (f). A phylogenetic network based solely on non-recombinant HSV-1 isolates as described previously (Norberg et al., 2004) was first constructed (b). Bootscan analyses were performed to illustrate recombination crossovers, and phylogenetic networks were created for FT and E_040127 (c and d, and e and f, respectively). GenBank accession numbers are in parentheses.
gE gene were sequenced. All five sequences were identical and the strain did not cluster distinctly to phylogenetic clade A, B or C (Fig. 2). The TR region contained two repeats.

The two putative recombinant strains E_040127 and FT were further analysed by constructing phylogenetic networks, applying the Φ test for statistical significance for recombination, and by performing bootscan analyses. The network excluding recombinant candidates presented a tree-like topology (Fig. 3b), whilst the two networks with the recombinant candidates included presented reticulate topologies consistent with recombination (Fig. 3d, f). The Φ test based on the sequence set excluding the possible recombinants presented a P-value of 1.00, whilst the P-value decreased to 0.13 and 0.04, respectively, when E_040127 and FT were included (Fig. 3a). In addition, the bootscan plots presented distinct alterations between different tree topologies, a feature that further supports recombination (Fig. 3c, e).

**DISCUSSION**

A major finding presented here is that one of the two truly asymptomatic individuals shed different HSV-1 strains. Based on the gI gene sequences, the recently described HSV-1 clades A, B and C differ by 0.6–2% (Norberg et al., 2004). The genetic distance between the HSV-1 strain shed during episodes 1 and 2 (EL_040127) and the HSV-1 strain shed during episode 3 (EL_040229) was 0.58%. The TR region in the gI gene also displayed different lengths. Thus, we concluded that EL shed two different viral strains, i.e. the genetic distance was too great to be explained by gradually introduced point mutations in the same individual. Our findings imply that EL had been infected by at least two strains on the same occasion or that she had been reinfected on different occasions. The possibility that the same person can shed different viral strains implies that epidemiological relationships between HSV-1 isolates are difficult to establish.

It is well known that the same individual can harbour and/or excrete non-identical HSV-1 strains (Bower et al., 1999; Lewis et al., 1984; Roest et al., 2004; Umene et al., 2007; Whitley et al., 1982). In the reinfection model, the central issue is how the second strain can infect and replicate in an already HSV-1-infected cell and create recombinants. There are alternative explanations. The simplest explanation may be that the same neuron can be superinfected by different strains. Conflicting data has been reported from animal models, where some authors have shown that the same ganglia can be superinfected by a new HSV-1 strain (Meignier et al., 1983), whilst others have shown the preclusion of a superinfecting strain (Centifanto-Fitzgerald et al., 1982; Mador et al., 2002; Thomas et al., 1985). Data from humans are sparse, but single human trigeminal neurons can be dually infected by HSV-1 and varicella-zoster virus (Theil et al., 2003) and, although rare, multiple HSV-1 strains have been recovered from the same human trigeminal ganglia (Lewis et al., 1984). Other possibilities facilitating recombination within the same individual are that different HSV-1 strains reactivate from different neurons in the same ganglion and co-infect and replicate in the same peripheral epithelial cell, or that viruses are spread from different neurons within the same ganglion via the central nervous system ‘back-door route’ and the dorsal root entry zone (Shimeld et al., 1995; Tullo et al., 1982).

An intriguing question is whether truly asymptomatic HSV-1-infected subjects more frequently reactivate multiple HSV-1 viruses compared with HSV-1 collected from clinical lesions. An earlier study using RFLP analyses showed that 9/13 asymptomatically HSV-1-infected subjects with malignant tumours in the throat region shed multiple HSV-1 strains in saliva on different occasions, whilst 23 strains collected from 10 subjects with primary or clinical reactivations were all identical (Terasaki, 1996). Another interesting finding in the present study is that two of the three strains from the truly asymptomatic individuals (EL and FT) represented HSV-1 recombinants. Recently, we detected 11 recombinants out of 28 clinical HSV-1 isolates based on sequence analyses of the same gene segments (Norberg et al., 2004). Further studies are warranted to establish whether multiple HSV-1 strains and recombinants are more common in truly asymptomatic HSV-1-infected individuals.

Homologous recombination is dependent on replicating viruses and has been shown to increase when the virus dose is high (Thiry et al., 2005). The estimated copy number of HSV-1 detected in saliva in this study varied considerably from person to person, as well as within the same individual. It is notable that the mean copy numbers of HSV-1 for both individuals with a truly asymptomatic HSV-1 infection (EL and FT), as well as for PT with a clinical HSV-1 infection, were more than $3 \times 10^5$ with peak values $>1 \times 10^6$. Similarly, for at least a few individuals, more than $1 \times 10^7$ HSV-1 genome copies have been detected in saliva after swabbing the buccal mucosa (Kaufman et al., 2005). Considering that $>1000$ ml saliva usually is produced per day, the total amount of HSV-1 viral particles produced may reach high levels. Data from HSV-1 shedding among children are sparse. The increased rate of shedding for children younger than 10 years, here documented for child FT, has been described previously (Tateishi et al., 1994) and may facilitate the transmission of HSV-1 during childhood and adolescence (Smith & Robinson, 2002). HSV-1 is now a common cause of the first episode of genital herpes infection in several Western countries (Lafferty et al., 2000) where orogenital sex is a risk factor for acquisition of genital HSV-1 infection (Lowhagen et al., 2000). Although the number of HSV-1 particles necessary for transmission is not defined (Sacks et al., 2004), it is reasonable to assume that orogenital transmission is facilitated by high copy numbers of HSV-1 in saliva.
We conclude that the truly asymptomatic HSV-1-infected individuals shed two different HSV-1 strains as well as recombinants. From the data presented in the current study, further exploration of sequence variation of HSV-1 might include infected individuals with no clinical symptoms. More data on such strains, preferably based on longer genomic regions, may reveal new HSV-1 variants and possibly new clades, as well as genetic markers for asymptomatic shedding.

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