Divergence of reiterated sequences in a series of genital isolates of herpes simplex virus type 1 from individual patients

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Both serotypes of herpes simplex virus (HSV), HSV-1 and HSV-2, are aetiological agents of genital herpes, although genital herpes caused by HSV-1 recurs less frequently. The HSV-1 genome contains a number of short, tandemly repeated sequences, and some reiterated sequences can serve as sensitive markers for the differentiation of HSV-1 strains. In the present study, variation in reiterations (assumed to be due to different copy numbers of tandemly repeated sequences) was examined in HSV-1 isolates from genital lesions from the same individual. Six sets (three primary-recurrence sets and three multiple-recurrence sets) of HSV-1 isolates were analysed: the primary-recurrence set consisted of two isolates (one isolated at a primary episode and the other at a recurrent episode) from the same individual; the multiple-recurrence set consisted of plural isolates from different episodes of recurrence in the same individual. Variations in length of the major DNA fragment, containing reiteration I (within the a sequence) and/or reiteration IV (within introns of genes US1 and US12), were detected between isolates of each multiple-recurrence set, but not of the primary-recurrence set. Thus, HSV-1 isolates of multiple-recurrence sets are assumed to have diverged more widely within each set than those of primary-recurrence sets, probably because of more rounds of virus DNA replication. This divergence of reiterations seems to indicate a forward step in the division of HSV-1 from a common ancestor into different lineages.

INTRODUCTION

Herpes simplex virus (HSV) is a ubiquitous human pathogen which latently infects neural cells of spinal ganglia and is classified into two serotypes, HSV-1 and HSV-2 (Dolan et al., 1998; Everett, 2000; McGeoch et al., 1988; Preston, 2000). HSV-1 is common in general populations and is often acquired non-sexually during childhood years. Although there is evidence that the majority of genital herpes infections are HSV-2-related, an increase in HSV-1 genital infection in at least some countries (e.g. UK, USA and Japan) has been suggested (Hashido et al., 1997, 1998; Kawana et al., 1982; Lafferty et al., 2000; Ribes et al., 2001; Stanberry et al., 1999; Vanderhoof & Kirby, 1992; White & Wardrop, 1997). HSV-1 genital infection is less likely to recur than that caused by HSV-2 (Kingham, 1993; Lafferty et al., 1987; Mertz et al., 1990; Sucato et al., 1998; Taylor et al., 1999).

The genome of HSV-1 is a 152 kb linear duplex DNA molecule (McGeoch et al., 1988) (Fig. 1a). Epidemiologically unrelated HSV-1 strains can usually be differentiated by analysing variations in restriction endonuclease (RE) cleavage patterns (Buchman et al., 1978, 1980; Chaney et al., 1983; Lonsdale et al., 1980). Variations in RE cleavage patterns between HSV-1 strains are divided into two types (Umene et al., 1984; Umene & Yoshida, 1989, 1993, 1994; Umene, 1998a, b). One type of variation, termed ‘restriction fragment length polymorphism’ (RFLP), is due mostly to a gain or loss of an RE cleavage site and causes a simple change in RE cleavage patterns. RFLPs are stable and serve as physical markers of the HSV-1 genome in molecular epidemiological and evolutionary studies. Another type of variation appears as irregularities in RE-cleaved fragments derived from certain regions of the HSV-1 genome. This variation was found in all strains analysed and was termed ‘common type’ (Umene et al., 1984; Umene, 1998a, b). Common type variation is located in fragments containing tandemly repeated sequences and is due to variation in copy number or nucleotide sequence of the reiterations (Davison & Wilkie, 1981; Mocarski & Roizman, 1981; Rixon et al., 1984; Umene, 1985b, 1991, 1998a, b, 1999). However, use of common type variations to distinguish HSV-1 strains has been avoided as they may be too unstable to qualify as markers.

We previously examined the degree of stability of regions containing reiterated sequences of the S component of the HSV-1 genome in order to search for a common type
variation which is sufficiently stable for use as a marker to distinguish HSV-1 strains (Umeene & Yoshida, 1989). It was proposed that reiterations I, IV and VII can serve as sensitive and convenient markers for the

differentiation of HSV-1 strains. Maertzdorf et al. (1999) later used PCR to re-examine the stability of reiterations, and the usefulness of reiterations IV and VII for differentiation of HSV-1 strains was supported. The PCR-based assay has subsequently been used for molecular epidemiological analyses of HSV-1 infections (Maertzdorf et al., 2000; Remeijer et al., 2001, 2002).

An HSV-1 strain (ancestor) is presumed to go through variations and consequently diverge into distinguishable strains (descendants) (McGeoch & Cook 1994, McGeoch et al., 1995; Sakaoka et al., 1994; Umeene & Sakaoka, 1997, 1999). Shortly after the generation of descendant viruses (the ‘relatively earlier stage’ of divergence), a significant difference between descendant isolates is not usually detectable as either RFLP or common type variation (Fig. 2a, b). Long after the generation of descendant viruses (the ‘relatively later stage’ of divergence), significant difference is detectable as both RFLP and common type variation (Fig. 2a, b). A ‘state of transition’ from the ‘relatively earlier stage’ to the ‘relatively later stage’ must have been present during divergence. Common type variation occurs at a faster pace than nucleotide substitution detectable by RFLP patterns, thereby allowing a finer level of resolution of the diversification process. Thus, the presence of a ‘state of transition’ was considered to be identifiable if a significant difference could be detected as common type variation between HSV-1 isolates from the same patient that could not be distinguished in terms of RFLP (Fig. 2). In the present study, variation was analysed between HSV-1 isolates which were isolated from genital lesions of the same individual at different episodes of recurrence. Significant differences between these HSV-1 isolates were detected as common type variation but not as RFLP. Thus, the ‘state of

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**Fig. 1.** Maps of the HSV-1 genome (McGeoch et al., 1988; Umeene & Yoshida, 1989; Umeene, 1998a, 1999). (a) Structure of the HSV-1 genome. HSV-1 DNA consists of two components, L and S, each composed of unique sequences (UL and US) bracketed by inverted repeat sequences (TR_L, IR_L, IR_S, and TR_S). The 5' sequence is present at both termini and at the L–S junction. (b) An expansion of the S component. Locations and 5'→3' orientations of mRNA species are indicated by horizontal arrows. Introns of genes US1 and US12 are shown as V-shaped indents on the horizontal arrows. Protein-coding regions are shown as open boxes. (c) Reiterations. Locations of tandemly reiterated sequences are indicated by vertical arrows. Roman numerals under the vertical arrows denote the reiterations as defined by Davison & Wilkie (1981) and Rixon et al. (1984).

**Fig. 2.** Hypothetical scheme of HSV-1 diversification and detection of differences between HSV-1 isolates. (a) Stage of divergence. HSV-1 isolates in the ‘relatively earlier stage’ are assumed to have passed through fewer rounds of virus DNA replication, while those in the ‘relatively later stage’ have passed through more rounds. (b) Detection of differences between HSV-1 isolates. Differences are assumed to be usually detectable in both RFLP and common type variation in the ‘relatively later stage’ of divergence but not in either in the ‘relatively earlier stage’. Significant differences in common type variation are presumed to be often detectable in the ‘state of transition’ because common type variation occurs at a faster pace than nucleotide substitution detectable by RFLP, allowing a finer level of resolution of the diversification process. (c) Clinically observed situations between progeny viruses. HSV-1 isolates from the same individual are counted as epidemiologically related isolates, which are assumed not to be in the ‘relatively later stage’ of divergence. HSV-1 isolates which were isolated independently of each other, from an epidemiological viewpoint, are counted as epidemiologically unrelated and are assumed to be in the ‘relatively later stage’ of divergence.
transition’ from ‘relatively earlier stage’ to ‘relatively later stage’ was shown to exist.

METHODS

HSV-1 clinical isolates. Six sets of HSV-1 clinical isolates, which were isolated from genital lesions of immunocompetent Japanese female patients at hospitals in Tokyo (Japan) and were previously reported, were analysed (Table 1) (Umene & Kawana, 2000). HSV-1 isolates of each set of sets 1–3 were isolated from recurrent genital lesions of the same patient at different episodes of the recurrence (multiple-recurrence set). Two isolates of each set of sets 4–6 were isolated from genital lesions of the same patient at different episodes of the primary infection and recurrence, respectively (primary-recurrence set).

Cells and viruses. Passage of virus stock in cell culture was kept to a minimum (one or two passages) after establishment of the original virus stock derived from clinical material. Working stocks of HSV-1 isolates were made on Vero cells in Eagle’s MEM supplemented with 2% (v/v) foetal bovine serum at a low m.o.i. A Vero cell monolayer infected with an HSV-1 stock was collected by low speed centrifugation and viral DNA was extracted by the method of Hirt, as described by Umene (1985a).

Gel electrophoresis and Southern blot hybridization. DNA digested with an RE was separated in a 0.8% (w/v) agarose gel and in a 5% (w/v) acrylamide gel, as described by Umene (1985b). Southern blot hybridization was carried out on a Biodyne B membrane (Pall Biosupport), according to the manufacturer’s instructions. DNA fragments used as probes were prepared from hybrid phages and plasmids and were labelled with \( ^{32}P \)-dCTP, as described by Umene & Yoshida (1989).

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<th>Table 1. Analysed HSV-1 isolates from genital lesions</th>
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*HSV-1 isolates within each set were isolated from the same individual at different times (Umene & Kawana, 2000).
†HSV-1 isolates were isolated from primary (p) or recurrent (r) genital lesions.
‡Day of virus isolation after the first isolation in each set.

RESULTS

RFLP analyses of six sets of HSV-1 isolates from the same individual

Previously, we analysed RFLP variation in 16 isolates in the six sets analysed in the present study, using three REs recognizing 6 bp sites (6-bp REs) of BamHI, KpnI and SalI (Umene & Kawana, 2000). The RFLP patterns (using three 6-bp REs) of HSV-1 isolates of each set, which were isolated from the same patient, were identical. Analyses using REs recognizing 4 bp sites (4-bp REs) can reveal more RFLPs than is the case with 6-bp REs, which made feasible the identification of RFLPs not detectable using 6-bp REs (Umene, 1987; Umene & Sakaoka, 1991; Umene & Yoshida, 1994).

In the present study, we analysed the RFLPs of 16 HSV-1 isolates in six sets (Table 1) using three 4-bp REs (HaeIII, Hhol and Mbol) in the same manner as in previous studies (Umene, 1987; Umene & Sakaoka, 1991; Umene & Yoshida, 1994). The RFLP patterns (using three 4-bp REs) of HSV-1 strains from each set were the same. Thus, HSV-1 isolates belonging to each set were not differentiated by RFLPs using 6-bp and 4-bp REs.

Analyses of variation of reiterated sequences in each of six sets of HSV-1 isolates

Variation of reiterations I, IV, and VII was analysed in six sets of HSV-1 isolates.

Reiteration VII. Southern blot hybridization profiles of reiteration VII are shown in Fig. 3. Regions containing reiteration VII in isolates of sets 1–4 and 6 were detected as a 0·17 kb fragment (Fig. 3a, lanes 1–10 and Fig. 3b, lanes 1, 2, 5 and 6) and those of set 5 as a 0·15 kb fragment (Fig. 3b, lanes 3 and 4). Therefore, HSV-1 isolates in each set were not differentiated by reiteration VII.

Reiteration IV. Southern blot hybridization profiles of reiteration IV are shown in Fig. 4. Regions containing reiteration IV in sets 3–6 were detected as fragments of the same length within each set: set 3, 0·19 kb (Fig. 4a, lanes 5–10); set 4, 0·25 and 0·27 kb (Fig. 4b, lanes 1 and 2); set 5, 0·19 kb (Fig. 4b, lanes 3 and 4); set 6, 0·23 kb (Fig. 4b, lanes 5 and 6). However, regions containing reiteration IV in sets 1 and 2 were not detected as fragments of the same length within each set: the C1 isolate fragment was 0·23 and 0·27 kb, and the C3 isolate fragment was 0·23 and 0·25 kb in set 1 (Fig. 4a, lanes 1 and 2); the C2 isolate was 0·23 kb, and C4 isolate was 0·19 and 0·21 kb in set 2 (Fig. 4a, lanes 3 and 4). Differences in reiteration IV were detected for the first time in the present study between HSV-1 isolates which were isolated from the same patient and could not be distinguished in terms of RFLP variation (Umene & Yoshida, 1989, 1993; Umene & Sakaoka, 1991).
Reiteration I. Southern blot hybridization profiles of reiteration I are shown in Fig. 5. Regions containing reiteration I in sets 2, 4 and 6 were detected as fragments of the same length within each set: set 2, 0–24 and 0–265 kb (Fig. 5b, lanes 1 and 2); set 4, 0–205 kb (Fig. 5b, lanes 3 and 4); and set 6, 0–26 kb (Fig. 5b, lanes 5 and 6). Regions containing reiteration I in sets 1, 3 and 5 were detected as a ladder-like set of fragments (Fig. 5a). Reiteration I (corresponding to the DR2 array) consists of a number of DR2 elements, with the length of the DR2 element being 11 or 12 bp (Davison & Wilkie, 1981; Mocarski & Roizman, 1981; Mocarski et al., 1985; Umene, 1991, 1998a, 2001; Varmuza & Smiley, 1985). Ladder-like fragments of reiteration I are assumed to be due to a different copy number of DR2 elements and hence intervals of ladder-like fragments are multiples of 11 or 12 nucleotides (Davison & Wilkie, 1981; Mocarski & Roizman, 1981; Umene, 1991, 1998a, 2001; Varmuza & Smiley, 1985). Ladder-like fragments of reiteration I are assumed to be due to a different copy number of DR2 elements and hence intervals of ladder-like fragments are multiples of 11 or 12 nucleotides (Davison & Wilkie, 1981; Mocarski & Roizman, 1981; Umene, 1991). Major fragments containing reiteration I in isolates of set 1 differed in length (Fig. 5a, lanes 1 and 2): C1, 0–37 kb; C3, 0–38 kb. Major fragments containing reiteration I in isolates of set 3 were between 0–41 and 0–48 kb (Fig. 5a, lanes 3–8): C8, 0–42 kb; C10, 0–41 and 0–42 kb; C11, 0–43 kb; C12, 0–42 and 0–43 kb; C17, 0–43 kb; C19, 0–48 kb. Thus, the length of the major fragment containing reiteration I differed between isolates of set 3, and major and minor fragments common to plural strains of set 3 were present. The major fragments containing reiteration I in isolates C73 and C75 of set 5 were 0–42 kb (Fig. 5a, lanes 9 and 10). Therefore, the length of the major fragment containing reiteration I was the same in two strains of set 5 but additional minor fragments were present.

DISCUSSION

Detection of significant differences in common type variation between HSV-1 isolates from the same patient in the multiple-recurrence set

HSV-1 isolates of each set analysed were from the same patient; hence, they were considered to be epidemiologically

Fig. 3. Southern blot hybridization profiles of regions containing reiteration VII. The HSV-1 DNAs were digested with BamHI, electrophoresed in 5% (w/v) polyacrylamide gels, and hybridized with a 0–20 kb BamHI fragment (prepared from hybrid λ phage Dec36) as probe (Umene & Enquist, 1981; Umene & Yoshida, 1989). Panel (a) lanes 1–10 and panel (b) lanes 1–6 are from HSV-1 isolates listed in Table 1. Lane M indicates the positions of relevant size markers of φX174 phage DNA digested with Haelll. Sizes of markers are shown in kb.

Fig. 4. Southern blot hybridization profiles of regions containing reiteration IV. The HSV-1 DNAs were digested with MspI, electrophoresed in 5% (w/v) polyacrylamide gels, and hybridized with a 0–18 kb MspI fragment (prepared from hybrid phage λDD-1) as probe (Umene, 1985b; Umene & Yoshida, 1989). Panel (a) lanes 1–10 and panel (b) lanes 1–6 are of HSV-1 isolates listed in Table 1. Lane M indicates the positions of relevant size markers of φX174 phage DNA digested with Haelll. Sizes of markers are shown in kb.
Divergence of reiterated HSV-1 sequences

In our previous study, differences in reiteration IV were not detected between single-plaque isolates and between clinical isolates from the same patient (Umene & Yoshida, 1989). However, significant differences in reiteration IV were detected in the present study between clinical isolates, which were isolated from the same patient and could not be distinguished in terms of RFLP variation (sets 1 and 2) (Fig. 4a, lanes 1–4). Differences in reiteration IV were detected in two of the three multiple-recurrence sets but in none of the primary-recurrence sets; hence, the probability of detection of events relating to differences in reiteration IV seemed to be larger in multiple-recurrence sets than in primary-recurrence sets.

Reiteration I was considered to be capable of serving as a marker for differentiating HSV-1 strains, since the major (or single) fragment containing reiteration I was the same size in single-plaque isolates (Umene, 1991; Umene & Yoshida, 1989). Differences in the major fragment of reiteration I between clinical isolates from the same patient were detected in multiple-recurrence sets 1 and 3, but not in primary-recurrence sets (Fig. 5). Maertzdorf et al. (1999) did not adopt reiteration I to differentiate HSV-1 strains, because PCR-amplified fragments containing reiteration I were not identical in size between single-plaque isolates. Reiteration I is presumed to be unsuitable for PCR amplification since it has a high G+C content, a strong bias towards purines on one strand and pyrimidines on the other, and adopts unusual DNA structures (Martin & Weber, 1998; Wells et al., 1987). However, reiteration I can serve as marker if HSV-1 DNA is analysed without PCR amplification, although it appears to be less stable than reiterations IV and VII.

Development of HSV-1 lineages within the body of an individual

The probability of detecting differences between progeny viruses is assumed to increase in proportion to the number of rounds of virus DNA replication. Isolates of the multiple-recurrence sets are presumed to have passed through more rounds of DNA replication than strains of the primary-recurrence sets. Thus, differences between isolates from the same patient would be expected to be more frequent in multiple-recurrence sets than in primary-recurrence sets. As expected, differences were detected in each multiple-recurrence set but not in primary-recurrence sets. This supports the view that a significantly larger number of replicative cycles separates the viruses shed in different recurrences of the same primary infection (multiple-recurrence set) than occurs between primary infection and the first recurrence (primary-recurrence set). Through a significantly larger number of replicative cycles occurring during long term infection, HSV-1 is assumed to pass into a 'state of transition' from a 'relatively earlier stage' (Fig. 2b). Isolates of set 3 are possibly less differentiated than isolates of sets 1 and 2 because of a common sharing of ladder-like fragments of reiteration I and lack of differences in reiteration IV (Fig. 4a, lanes 5–10 and Fig. 5a, lanes 3–8).

**Fig. 5.** Southern blot hybridization profiles of regions containing reiteration I. The HSV-1 DNAs were digested with SmaI, electrophoresed in 5% (w/v) polyacrylamide gels, and hybridized with 0.2-0.5 kb SmaI fragment (prepared from hybrid phage λDD-1) as probe DNA (Umene, 1985b; Umene & Yoshida, 1989). Panel (a) lanes 1–10 and panel (b) lanes 1–6 are of HSV-1 isolates listed in Table 1. Lane M indicates the positions of relevant size markers of φX174 phage DNA digested with HaeIII. Sizes of markers are shown in kb.

Differences in the degree of stability between reiterations in the HSV-1 genome

Differences in reiteration VII were not detected between HSV-1 isolates from the same patient yet differences were evident in reiterations I and IV (Figs 3–5). Thus, reiteration VII is assumed to be the most stable (Umene & Yoshida, 1989). Related (Fig. 2c). Isolates of each set were not distinguishable from each other using RFLP; thereby proving a close relationship between isolates of each set in terms of RFLP variation and indicating that isolates of each set were not in the ‘relatively later stage’ of divergence (Fig. 2a). HSV-1 isolates of the six sets were analysed with regard to common type variation (Fig. 3–5). Significant differences were not detected between isolates in the primary-recurrence sets, whereas differences were evident between isolates in the multiple-recurrence sets in reiteration I of sets 1 and 3 (Fig. 5) and in reiteration IV of sets 1 and 2 (Fig. 4). Thus, HSV-1 isolates of a multiple-recurrence set could be differentiated in terms of common type variation but not in terms of RFLP variation (Fig. 2b).
Reiterated sequences are useful for study of the epidemiology and evolution of HSV-1.

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