Latent Multiple Infections by Herpes Simplex Virus Type 1

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Summary: Viral strains obtained during the same period which were asymptotically shed into the saliva of patients with oral cancer after surgery or isolated from the focus of patients with herpes simplex virus type 1 (HSV-1) infection after first visit to hospital were analyzed using molecular epidemiology. There were 10 subjects in each group. Viral strains used were from 3 to 5 strains (mean: 3.4 strains) in the malignant patients and from 3 to 8 strains (mean: 5 strains) in the herpetic patients. One of 10 patients with oral cancer showed a different DNA cleavage pattern in viral strains shed into saliva at the last day. In 10 patients with HSV-1 infection, all viral strains in each of the cases showed identical DNA cleavage patterns. These results suggested the existence of a latent multiple infection and a simultaneous reactivation of HSV-1.

Key words herpes simplex virus type 1, latent infection, latent multiple infection, shed virus, oral cancer, molecular epidemiology, herpes labialis

INTRODUCTION

Multiple viral infections have been reported in airway viral infections, enterovirus, measles, and varicella [1-6], but there have been few reports of multiple infection with herpes simplex virus (HSV) [7-12], and the actual status has not yet been elucidated.

We have reported results that suggest a multiple latent infection in many subjects who excreted HSV-1 in lacrimal fluid and saliva. Murase [13] performed a molecular epidemiological investigation of isolates asymptomatically excreted in lacrimal fluid and saliva from the same individual during the same period and found that the viral DNA pattern differed between the lacrimal fluid and saliva in 9 of 14 cases, showing a high frequency of 64.8. Later, Terasaki [14] performed a similar investigation of isolates asymptomatically excreted into saliva from an individual during different periods and those from lesions in a separate patient who developed symptoms at different times and showed that in the asymptomatic excretion group, different patterns existed in many cases (9 of 13 subjects) as Murase reported. These findings indicate the presence of a multiple latent infection with HSV-1 and suggests a reinfection with HSV-1 or a simultaneous infection with multiple strains.

Therefore, in this study, we attempted to confirm the presence of a multiple latent infection by successively obtaining isolates during a period of fixed duration from a patients with HSV-1 infection and from patients who asymptomatically excreted the virus. The results were analyzed using molecular epidemiology in order to clarify simultaneous reactivation of multiple strains.

MATERIALS AND METHODS

Subjects and collection of specimens

Subjects were 10 patients with oral malignant tumors and 10 patients with HSV-1 infection (Tables 1 and 2), in whom the HSV-1 was continuously isolated during a period of 3 or more days from saliva or focus. All subjects were treated in the Department of Oral Surgery at Kurume University School of Medicine. The patients with oral malignant tumors were free of herpetic lesions in the oral cavity. All samples were collected from the saliva of patients...
with oral cancer and the oral lesions of patients with HSV-1 infection.

Saliva specimens were collected using a sterile periostial elevator to wipe the floor of the mouth. The samples were then dispersed into a sterile tube containing 2 ml phosphate buffered saline (PBS) supplemented with 4% bovine serum.

Specimens from patients with HSV-1 infection were collected from the erosions or vesicles of oral lesions by wiping with a sterile periostial elevator or by puncturing with a sterile syringe.

All specimens were collected during a period of 7 days after surgery in the patients with oral cancer and after first visit to hospital in herpetic patients. All samples were stored at −70 °C.

Number of viral strains in each of these patients were used from 3 to 5 strains (means: 3.4 strains) in asymptomatic group and from 3 to 8 strains (means: 5 strains) in infections group.

### Cells

Cells derived from an African Green Monkey Kidney (GMK) were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum, and were used for the isolation and assay of the virus and for the antibody titration.

### Isolation of the virus

Specimens stored at −70 °C were thawed and centrifuged at 3,000 rpm for 10 min. One milliliter of the supernatant was inoculated at room temperature in a stationary rack for 2 hs. Fresh MEM supplemented with 2% fetal bovine serum was added after the inoculum was discarded. The cultures were incubated at 37 °C and checked for a cytopathic effect (CPE) every day for 1 week. When a CPE characteristic for HSV was observed, the culture was frozen, and the isolated virus was grown once more in GMK cells and stored at −70 °C [15].

### Preparation of viral DNA

Cell monolayers in 2-oz bottles (20 cm²) were infected with virus, and incubated in MEM supplemented with 2% fetal bovine serum at 37 °C. When CPE was generalized the culture was harvested by freezing at −70 °C. After thawing, the culture was centrifuged at 3,000 rpm for 10 min to remove the cellular debris. The supernatant was centrifuged at 30,000 rpm for 30 min at 4 °C and the pellet was resuspended in 225 ml of TE buffer (1 mM Tris, pH 7.6, and 0.1 mM EDTA). Then, 25 ml of 10% SDS and RNase (1 mg/ml) were added and the mixture was incubated at 37 °C for 1 h. After 70 ml of 5 M sodium per chlorate was added, followed by two cycles of extraction with chloroform-isooamil alcohol (24:1). DNA was precipitated from the aqueous phase by adding 1 ml of cold ethanol. After standing overnight at −20 °C, the DNA was pelleted by centrifugation, washed once with 70% ethanol and dissolved in 40 ml of TE buffer.

### Restriction endonuclease digestion and gel electrophoresis

BamHI was purchased from Takara Company Ltd. Digestion of the DNA (5-10 ml of DNA sample in 50 ml of reaction mixture) was carried out at 37 °C in 6 mM Tris (pH 8.0, with 6 mM Mgcl2 and 150 mM Nacl). Electrophoresis was performed in 1% agarose gels containing 36 mM Tris, 30 mM Na2HPO4, and 1 mM EDTA at 40 V for 15 to 18 hs. After electrophoresis, the gels were stained with 0.5

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**TABLE 1.** Subjects with asymptomatically shed virus

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>No. of viral strains</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>81</td>
<td>3</td>
<td>Tongue cancer</td>
</tr>
<tr>
<td>B</td>
<td>M</td>
<td>52</td>
<td>3</td>
<td>Gingival cancer</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>65</td>
<td>3</td>
<td>Gingival cancer</td>
</tr>
<tr>
<td>D</td>
<td>F</td>
<td>43</td>
<td>3</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>E</td>
<td>F</td>
<td>81</td>
<td>3</td>
<td>Buccal mucosa cancer</td>
</tr>
<tr>
<td>F</td>
<td>M</td>
<td>52</td>
<td>4</td>
<td>Tongue cancer</td>
</tr>
<tr>
<td>G</td>
<td>F</td>
<td>81</td>
<td>5</td>
<td>Lymph node metastasis</td>
</tr>
<tr>
<td>H</td>
<td>M</td>
<td>67</td>
<td>4</td>
<td>Tongue cancer</td>
</tr>
<tr>
<td>I</td>
<td>F</td>
<td>93</td>
<td>3</td>
<td>Buccal mucosa cancer</td>
</tr>
<tr>
<td>J</td>
<td>F</td>
<td>64</td>
<td>3</td>
<td>Oral floor cancer</td>
</tr>
</tbody>
</table>

**TABLE 2.** Subjects with HSV-1 infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>No. of viral strains</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>M</td>
<td>75</td>
<td>6</td>
<td>Recurrent herpes labialis</td>
</tr>
<tr>
<td>L</td>
<td>F</td>
<td>26</td>
<td>3</td>
<td>Recurrent herpes labialis</td>
</tr>
<tr>
<td>M</td>
<td>M</td>
<td>78</td>
<td>4</td>
<td>Recurrent herpes labialis</td>
</tr>
<tr>
<td>N</td>
<td>F</td>
<td>63</td>
<td>7</td>
<td>Recurrent herpes lingualis</td>
</tr>
<tr>
<td>O</td>
<td>M</td>
<td>64</td>
<td>5</td>
<td>Recurrent herpes lingualis</td>
</tr>
<tr>
<td>P</td>
<td>M</td>
<td>21</td>
<td>3</td>
<td>Primary gingivostomatitis</td>
</tr>
<tr>
<td>Q</td>
<td>M</td>
<td>20</td>
<td>5</td>
<td>Primary gingivostomatitis</td>
</tr>
<tr>
<td>R</td>
<td>M</td>
<td>78</td>
<td>6</td>
<td>Recurrent herpes labialis</td>
</tr>
<tr>
<td>S</td>
<td>M</td>
<td>77</td>
<td>8</td>
<td>Recurrent herpes labialis</td>
</tr>
<tr>
<td>T</td>
<td>F</td>
<td>62</td>
<td>3</td>
<td>Recurrent herpes labialis</td>
</tr>
</tbody>
</table>
The bands of fragments 1 and 2 from HSV DNA (case F) (Fig. 2) were cut out under UV light, and the DNA fragments were extracted by Qiaex II Gel Extraction kit (Qiagen, Hilden, Germany). The fragments were ligated into the BamH I site of pUC118 plasmid. The resulting plasmid amplified in E. coli (DH5) was purified using Qiagen Plasmid Kit. The construct was sequenced using the ABI PRISM Dye terminator Cycle Sequencing Kit and M13 primers, then by automated sequencing an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA).

RESULTS

When DNA fragments are separated by electrophoresis through agarose gels, the fragments with the smaller molecular weights migrate faster than those with relatively large molecular weights. These smaller DNA fragments are arranged in the lower portions of the figures. The DNA fragments were designated as a, b, and c in the order of decreasing molecular weights, in accordance with previous reports [16-18]. The electrophoresis bands of the isolated viral strains were not HSV-2, therefore the isolated viruses must be HSV-1.

The BamH I DNA cleavage patterns of asymptotically shed viral strains which were isolated from 8 patients, A-E, H, I and J are shown in Fig. 1. Viral strains of each of these patients had the same patterns with BamH I, therefore an attempt was made to digest them with another restriction enzyme, Sal I.

Sequencing

mg/ml ethidium bromide solution and photographed using an UV transilluminator.

The bands of fragments 1 and 2 from HSV DNA

Fig. 1. Restriction digest patterns with BamH I of the viral strains shed in asymptomatic patients, A-E, H, I and J.

Fig. 2. BamH I restriction digest patterns of the virus strains shed in asymptomatic patients F and G. The difference of patterns in virus strain shed at the last day is indicated by arrows.
Fig. 3. Restriction digest patterns with BamH I of the viral strains isolated from patients with primary and recurrent herpetic infection.

The restriction mapping of these strains with Sal I also showed identical patterns.

However, the viral strains isolated from patients, F and G could be differentiated by restriction mapping with BamH I (Fig. 2). As shown by the arrow in Fig. 2, in patient G the restriction endonuclease cleavage pattern of the viral strain isolated at the last day differed from that of the other four strains. Also, in patient F, the cleavage pattern of the last strain differed from that of the other three strains.

However, when the DNA sequences of the fragment 1 and 2 (patient F) were analyzed, it was found that the fragment 1 and 2 were restriction fragment BamH I-k and s. Therefore it was the all same strains.

The results of analysis by BamH I digestion of the viral DNA isolated from 10 patients with HSV-1 infections are shown in Fig. 3. All viral strains in each of these patients had the same cleavage patterns. Analysis by Sal I also produced identical cleavage patterns in all viral strains of each patients.

These findings indicate that the HSV-1 lesions were all caused by reactivation of the same strain of virus.

DISCUSSION

In recent years, investigations of restriction enzyme cleavage patterns of viral DNA have made viral differentiation relatively easy. The nucleic acid carrying the genetic information of HSV is approximately 150 kbp linear double-stranded DNA with a molecular weight of $100\times10^6$ and consists of an L region comprising 82% of the genome and an S region comprising the remaining 18%. There are sequences consisting of these regions in different directions, exhibiting four DNA isomers. Furthermore, at both ends of these two regions, there are nucleotide sequences in opposite directions to each other (inverted repetitive sequences). These inverted repetitive sequences vary on restriction enzyme cleavage, but the L- and S-specific regions are very stable in each strain. Therefore, restriction enzyme cleavage patterns specific to viral strains are not altered after replication in a culture system or in vivo, and thus, are considered useful in differentiation of HSV strains and typing [7,19-21].

As the above molecular epidemiological studies have progressed, multiple latent infections including reinfections of the primary HSV infection site with other HSV strains have been suggested in several reports. Buchmans et al. [9] collected at least two isolates from each of 8 patients with HSV-2 type herpes progenitalis, and the isolates were the same strain in 6 patients. In the remaining two patients, a mixed infection with a different HSV-2 type was
detected in the second isolation in one patient in addition to the strain obtained in the first isolation. In the other patient, the isolates were the same strain in both the first and second isolations, but a different strain was obtained in the third isolation. Maitland et al. [10] also reported that they similarly collected at least two isolates from 30 patients with HSV-2 type herpes progenitalis HSV-1. Whitley et al. [11] investigated whether isolates from a brain biopsy were of the same strains as those isolated from their labial herpes in 8 encephalitis patients. Different strains were isolated in 3 patients. Lewis et al. [12] cultured in vitro the trigeminal nerve and nerve roots from 20 cadavers to reactivate latent HSV, and multiple HSV-1 strains were isolated from two cadavers.

Later, Murase in our laboratory analyzed DNA cleavage patterns of viruses simultaneously excreted asymptomatically from different sites in the same individuals and showed the presence of different strains in many individuals. HSV-1 was isolated from left and right lachrymal fluid and saliva from patients who underwent ophthalmological and oral cavity surgeries, and was compared with 32 strains from 14 patients who asymmetrically excreted the virus in lachrymal fluid and saliva during a similar period. As a result, strains were found to be differed in 9 of 14 patients (64.3%). A similar comparison was performed by Terasaki among 32 strains from 13 individuals, which were asymmetrically excreted in saliva in the same individuals during different periods, and among 55 strains isolated from 10 patients with HSV-1 infection. The same strains were detected in all patients with an HSV-1 infection, while strains differed in 9 of 13 individuals (69%) who asymmetrically excreted the viruses, showing more clearly the presence of a multiple HSV-1 latent infection. Moreover, regarding the frequency of a multiple viral infection, the frequency of excretion of different strains was very high compared to the 2 in 20 reported by Lewis et al., 3 in 8 reported by Whitley et al., 2 in 8 reported by Buchman et al., and 1 in 30 reported by Maitland et al. The difference between this finding and those from the previous reports was that all viruses were asymmetrically excreted. Therefore, as Tani [22] noted, some specific strains may dominate in the development of symptoms by reactivating.

Furthermore, Murase suggested the possibility of localization of latent viruses because of differences among sites, and this was supported by the experimental results obtained by Sekizawa [23]. In the study reported by Sekizawa et al., the reactivating rate by corneal stimulation was 48% in mice inoculated with HSV in the cornea, while that by labial stimulation was low, at 11%. In contrast, in mice with labial inoculation, the reactivation rates by labial stimulation and corneal stimulation were 90% and 0%, respectively, showing stimulation site-specificity in the localization and reactivation of HSV.

Therefore, in this study, in an attempt to further clarify the presence of a multiple latent infection, strains successively obtained from the same individual in the asymptomatic virus excretion group or from the patient group with HSV-1 infections were subjected to DNA analysis. The DNA cleavage patterns were compared and the latent infection with multiple strains was investigated.

In the asymptomatic virus excretion group, a different cleavage pattern was detected in only one individual on the final day. This finding suggested the presence of a multiple latent infection and simultaneous reactivation of multiple strains as suggested by Murase and Terasaki. However, regarding the simultaneous reactivation, because a different strain was detected on the final day, reactivation on different days may also be possible, and further investigation is necessary. In the patients with HSV-1 infection, the presence of different strains was not detected. No patients were detected with a primary infection with multiple strains. Furthermore, a similar strain was detected in all recurrent lesions, and simultaneous reactivation was not observed.

It is not clear why such a multiple latent infection should exist. It has been reported that when one type of virus strain latently infects a nerve root, latent infection with other viruses is inhibited [22-24]. This is contradictory to the results of the present study. In fact, it has been shown that a simultaneous latent infection with multiple strains in the same nerve root can be established, and that a latent infection of the right and left nerve roots with a different strain, each can also be established [23]. On investigating the properties of viruses from patients with an HSV-1 infection and those of asymmetrically excreted viruses, the sensitivity to asymmetrically excreted virus strains was rapidly decreased by defrosting, and a comparison of the replication ability by growth curve also showed a clear difference between the two groups [26]. Furthermore, on isolation sample solution of an asymmetrically excreted virus was directly inoculated, no viral clone was recovered, showing that the decrease in sensitivity is very rapid. Considering these findings together, asymmetrically excreted viruses latently infect local sites in an
asymptomatic state upon infection, and many different strains latently infect a different site at a different time. To further clarify the presence of a multiple latent infection, we are planning to experimentally cause and investigate multiple latent infections. In addition, we will attempt to clarify the reactivation of multiple strains in a lesion by comparing simultaneously collected specimens from many recurrent sites in the oral cavity in immunosuppressed patients.

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