Oral Diseases (2005) 11, 13–16

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Experimental Oral Medicine

Herpes simplex virus type 1 shedding in the oral cavity of seropositive patients

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OBJECTIVE: Investigate the frequency of herpes simplex virus type 1 (HSV-1) reactivation in the oral cavity of seropositive patients with previous history of recurrent herpes labialis (recrudescent group) compared with those without any history of recrudescent lesions (asymptomatic HSV-1 infection). In addition, the relation between recrudescence and the presence of the virus in the saliva was assessed.

MATERIALS AND METHODS: Fourteen individuals with previous history of herpes labialis (recrudescent group) and 11 HSV-1 seropositive asymptomatic volunteers were included in the study. Swabs were performed periodically in all subjects and the presence of HSV-1 DNA was identified by nested PCR.

RESULTS: All the 25 subjects enrolled in the study, revealed at least one positive swab for HSV-1. The frequency of HSV-1 positivity in the group with recrudescent herpes labialis was not statistically different from the other group. Ten subjects of the recrudescent group presented with herpes labialis at least once during the study.

CONCLUSIONS: HSV-1 shedding in the oral cavity occurs independently of herpes labialis recrudescence.
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Introduction

Herpes simplex virus type 1 (HSV-1) belongs to the Herpesviridae family, genus simplexvirus and contains a 152,000 bp double-stranded DNA genome (Vestey and Norval, 1992). HSV-1 is usually acquired during childhood and is transmitted trough direct mucocutaneous contact or droplet infection from infected secretions. As HSV is short-lived on external surfaces, the infection depends on intimate contact with an individual who is shedding live virus through secretions (Ajar and Chauvin, 2002). Acute (primary) herpetic gingivostomatitis typically affects children and involves the gingivae, tongue, lips, buccal mucosa, as well as the hard and soft palate. Lymphadenopathy, fever and malaise are frequently reported (Whitley *et al*, 1998). After primary infection, HSV-1 establishes a latent infection in neuronal cells of trigeminal ganglia. Reactivation causes recurrent disease (Barkvoll and Attramadal, 1987; Scully, 1989; Yeung-Yue *et al*, 2002).

Reactivation of HSV-1 in the oral cavity, either symptomatically (recrudescence) or asymptomatically, may contribute to the transmission of HSV-1. HSV-1 shedding into the oral cavity could occur during the prodromal phase of recurrent herpes labialis (Scott *et al*, 1997b). HSV-1 was detected by PCR in 4.7% of 1000 saliva samples from dental outpatients (Tateishi *et al*, 1994). In order to measure the frequencies of HSV reactivation in the oral cavity, a previous study investigated oral swabs of 30 healthy people over a period of 58–161 days by PCR (Knaup *et al*, 2000). 19 of 25 (76%) seropositive people were PCR-positive at least once, six of these 19 (32%) had recrudescence. The frequencies of recurrence were higher in people showing symptomatic reactivation than asymptomatic cases. The presence of HSV-1 in saliva of 9/10 patients with acute herpes labialis and one of five healthy controls was shown using PCR (Youssef *et al*, 2002).

In the present case–control study the frequencies of HSV-1 reactivation in the oral cavity of seropositive patients with previous history of recurrent herpes labialis were compared with seropositive volunteers without any history of recrudescent lesions. In addition we evaluated whether recrudescence correlates with the presence of the virus in the saliva or not.

Material and methods

Sample collection

Twenty-five HSV-1 seropositive volunteers of high socioeconomic status (dentistry students, teachers and their parents) were included in the study. Fourteen individuals (five men and nine women, mean age

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Received 24 February 2004; revised 14 May 2004; accepted 18 May 2004

30.3 years) reported previous history of at least one episode per year of herpes labialis in the last 5 years (recrudescent group). Eleven of the 25 volunteers (four men and seven women, mean age 34.5 years) had no previous history of herpetic lesions (asymptomatic group). At the beginning of the study none of the subjects of the recrudescent group presented oral lesions. Periodically, from 30 to 60 days, swabs were taken from all subjects (buccal mucosa, lower lip and dorsum of the tongue) over a period between 8 and 11.5 months. These sampling sites were chosen on the basis of previous experiments that showed that swabs of these sites result in the largest quantity of DNA. At each day of sample collection, patients were asked for information about the occurrence and time-point of HSV blisters. In addition, the presence of intra-oral ulceration was also recorded. The study protocol was approved by local Ethical Committee and informed consent was obtained from all patients.

Sterile plastic sticks were used to perform the oral swabs. After immediately suspending in 250 μl of Krebs–Ringer solution (in mM): NaCl (118.4), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4{\cdot}7H_2O$ (1.2), $CaCl_2{\cdot}2H_2O$ (2.5), $NaHCO_3$ (25.0) and glucose (11.7), the swabs were stored at 4–10°C for 48 h or at −20°C until DNA extraction. After centrifugation of the samples at 10 000 *g* for 10 min, the sediments were submitted to DNA extraction.

DNA isolation

The DNA extraction was carried out as described by (Boom *et al*, 1990) and modified as below: 450 μl of lyses buffer (GuSCN 6.0 M, Tris-HCl 0.1 M, EDTA 0.2 M, TritonX-100%) and 20 μl of silica (SiO_2, Sigma S5631, washed with H_2O pH 2.5 corrected with HCl) were added in the pellet. The tubes were vortexed for 10 sand incubated for 20 min at 56°C, centrifuged for 1 min and the supernatant was discarded. The pellet obtained was washed twice with 450 μl washing buffer (GuSCN 6.0 M, Tris-HCl 0.1 M), twice with 70% ethanol and once with 450 μl acetone, always centrifuged as described above. The pellet was dried at 56°C for 10 min or until the silica got completely dry. Finally, 100 μl of TE buffer (Tris-HCl 10 mM pH 8.0, EDTA 1.0 mM) was added and incubated at 56°C for a minimum of 10 min and maximum of 24 h. After that, the solution containing the silica and DNA was vortexed for 5 s, centrifuged for 2 min and the supernatant transferred to a new tube.

Nested polymerase chain reaction

The sequence of DNA was obtained from National Centre for Biotechnology Information and the MacVector program was used to construct and analyse the primers as previously described (Gomez *et al*, 2001). Two sets of primers were used for HSV-1 on the nested PCR (Table 1). The PCR was performed by standard techniques (Saiki *et al*, 1988). Two microliters of DNA purified as described above were subjected to PCR using outer primers. PCR was carried out in a 50 μl mixture containing Taq DNA polymerase (1 unit/reaction), PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$) deoxynucleoside triphosphates (0.1 mM/reaction of each dNTP) and primers (10 pmol/reaction). All samples were amplified using a DNA thermal cycler. After PCR, 2 μl of the final product was transferred to the reaction mixture of the second round PCR (nested) and reamplified with the inner pair of primers. Recurrent oral herpes lesion in a bone marrow transplantation patient was used as positive control for HSV-1. Negative controls included PCR without DNA. DNA sequencing reactions were performed on both strands of some cases using the chain termination method of (Sanger *et al*, 1977). All reactions were carried out using the DNA Sequencer ABI PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA) and the BigDye terminator kit. Nucleic acid sequences were analyzed using the MacVector$^{TM5.0}$ program (Oxford Molecular Group PLC, San Diego, CA, USA). After editing, the sequences were compared with sequences in the GenBanK databases (Altschul *et al*, 1990).

Agarose gel eletrophoresis

Ten μl of each reaction product was added to 2 μl of gel loading dye (0.25% bromophenol blue, 30% glycerol, 10 mM EDTA), loaded into the 1.5% agarose gel and electrophoresis carried out using 1X TAE buffer. DNA fragments were visualized after staining with ethidium bromide (0.5 μg ml^{-1}) and using the photo documentation system BIO-RAD GEL-DOC 1000. The molecular weight of the DNA was estimated using λ *Hin*dIII DNA and 100 bp ladder markers.

Results

In total, 438 and 309 swabs were examined for the presence of HSV-1 DNA from the group with recrudescent herpes labialis and with asymptomatic HSV-1 infection,

Table 1 Primers and PCR reaction

Virus	*Primer pair*	*PCR program*	*Product size (bp)*
HSV-1	Outer primers F: 5′ TGC TGG AGG ATC ACG AGT TTG 3′ R: 5′ CAT CGT CTT TGT TGG GAA CTT 3′	95°C/30 s; 61°C/45 s; 72°C/30 s; 30 cycles	663
	Inner primers F: 5′ TGCAGAGCAACCCCATGAAG 3′ R: 5′ ATGACCATGTCGGTGACCTTGG 3′	95°C/30 s; 60°C/45 s; 72°C/30 s; 30 cycles	241

F, forward primer; R, reverse primer; Buffer IB, 10 mM Tris.

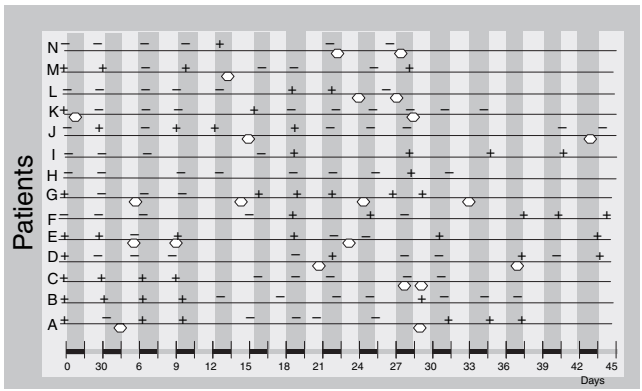

Figure 1 Time course of HSV shedding in the group with recrudescent herpes labialis. +, PCR positive for HSV-1; −, PCR negative for HSV-1, ⌓, occurrence of blister

respectively. The frequencies of positive swabs for HSV-1 on the buccal mucosa (29.5%), lower lip (30.7%) and dorsum of the tongue (33.1%) were not statistically different. As three swabs were performed in each exam, patients with at least one positive swab were considered positive for the presence of HSV-1 on that specific day of sample collection.

Ten of 14 subjects of the recrudescent group presented labial blisters characteristic of herpes labialis at least once during the observed period of time (Figure 1). None of the patients presented intra-oral ulceration suggestive of HSV recrudescence. As it can be observed in the Figure 1, positive swabs for HSV-1 occurred sometimes before or after labial blisters. All the 25 subjects enrolled in the study, revealed at least one positive swab for HSV-1. The frequency of positive exams for HSV-1 in the group with recrudescent herpes labialis ($n = 60$; 41.1%) compared with the group with asymptomatic HSV-1 infection ($n = 54$; 52.4%) was not statistically different.

Discussion

Detection of viral DNA using polymerase chain reaction (PCR) has demonstrated greater sensitivity for identifying HSV-1 than traditional viral culture (Tateishi *et al*, 1994; Scott *et al*, 1997a). In some circumstances HSV-1 is only detected by nested PCR (Knaup *et al*, 2000). Therefore, nested PCR was used in the present study.

One of the aims of the present study was to evaluate whether recrudescence is correlated with the presence of the virus in the saliva or not. HSV-1 detection (Fig 1) on the oral mucosa sometimes has happened before and sometimes after the presence of characteristic blisters of herpes labialis. Similar findings were already reported (Knaup *et al*, 2000; Youssef *et al*, 2002).

Different findings were reported in a previous study evaluating German patients (Knaup *et al*, 2000). The authors reported that seropositive people without a history of blisters showed less reactivation than did people who had symptomatic herpes labialis in the past. Methodological differences such as number of patients, DNA extraction, primers and PCR reaction may partly explain such disparity.

Although a limited number of patients were included in the study, the results indicate that HSV-1 shedding in the oral cavity occurs independently of herpes labialis recrudescence. As this study was only qualitative and not quantitative, it is difficult to evaluate the impact of asymptomatic viral shedding on HSV-1 transmission. The fact that all the patients showed at least one positive swab for the virus may explain the high transmission risk of the disease in dental clinics, kissing, and handling, among other means. Further studies are required to delineate whether there are triggering factors associated with asymptomatic HSV-1 shedding in the oral cavity or whether it is a spontaneous phenomenon.

Acknowledgements

This study was supported in part by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Programa de Excelência (PRONEX) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. Dr R.S. Gomez is research fellow of CNPq.

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