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Pathogenesis of Herpes Simplex Labialis: Excretion of Virus in the Oral Cavity

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Excretion of herpes simplex virus (HSV) in the oral cavity was studied in eight human subjects with a history of herpes labialis. Serial intraoral specimens were obtained by gargling broth and examined for virus by centrifugal inoculation of primary human amnion cells. Forty-seven of 637 specimens (7.4%) contained HSV. The majority of isolates (62%) were found in clusters, and the rate of excretion was significantly increased during the common cold (21%) and after oral trauma (17%) ($P = 0.001$ and 0.04, respectively). Oral HSV excretion often occurred in parallel with episodes of herpes labialis but could not be attributed to viral contamination from a labial lesion. Each patient excreted only one strain of HSV type 1 as determined by restriction endonuclease analysis with *Kpn*I and *Bam*HI. Unexpectedly, prodromal symptoms of herpes labialis were commonly not followed by development of a lesion (false prodrome). False prodromes were associated with a high rate of oral HSV excretion (60%). Intraoral ulcers on the gingivae and hard palate were frequently associated with oral HSV excretion (31%) and are the most likely source of HSV in the oral cavity.

Individuals with a history of herpes gingivostomatitis or recurrent herpes simplex labialis excrete herpes simplex virus (HSV) in the oral cavity (2, 4, 6, 9, 11). In a previous report on the natural history of herpes labialis, we observed that HSV could be isolated from 43% of lesion specimens and 7% of concurrent samples of oral secretions (20). Douglas and Couch demonstrated HSV in 24% of oral specimens at the time of herpes labialis and showed that parotid gland secretions did not contain the virus (6). Recurrent intraoral HSV lesions on the gingivae and hard palate can explain the elaboration of HSV in at least some instances (24). Under stressful circumstances, such as fever, immunosuppression, or trigeminal ganglion surgery, high incidences of herpes labialis, HSV excretion in the oral cavity, and intraoral HSV ulcerations have been noted (3, 8, 12, 14–16, 23). In the majority of normal subjects, however, the pathogenesis of oral HSV excretion is unclear, and controversy remains over the relationship between intraoral shedding and herpes labialis.

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MATERIALS AND METHODS

Patient population. The study population consisted of eight healthy persons with a clinical history of recurrent herpes simplex labialis. The average age of the study subjects was 35 years (range, 22 to 55 years), and six were women. The subjects had experienced recurrent herpes labialis for an average of 25 years (range, 8 to 50 years), and the average frequency of episodes at the time of the study was 4.4 episodes per year (range, 2.2 to 8 episodes per year). All study subjects signed an institutional review board-approved document of informed consent.

Study design and specimen collection. The study volunteers were requested to rinse their mouth and throat with 5 ml of Trypticase soy broth (BBL Microbiology Systems) every weekday morning for 5 months. The participants gargled, swished the medium throughout their mouths, and then expectorated the fluid into a polypropylene tube (100 by 17 mm) (BD Labware). After each specimen was collected, the fluid was supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1.25 μg/ml). Each specimen was then inoculated onto human amnion cells as described below, and the residual fluid was frozen at −70°C pending further evaluation.

Each patient maintained a daily log to record the occurrence of herpes labialis, intraoral ulceration, upper respiratory infection, sunburn, menstruation, emotional upset, dental procedures, or other forms of stress. Patients were examined in the clinic if an episode of herpes labialis or intraoral ulceration occurred.

Lesion evaluation. Herpes labialis lesions were followed with measures of lesion pain, stage, area, and virus titer as previously described (20). The oral cavity was examined with an angled dentist's mirror and the number, stage, and location of any lesions were recorded.

Virus isolation. Monolayers of human amnion cells were prepared from human amniotic membrane by trypsinzation (J. A. Green, Ph.D. dissertation, Boston University, 1972). Fresh human amniotic membranes were obtained by dissection under sterile conditions. The membrane was washed three times with 0.01 M phosphate-buffered saline (pH 7.4) and incubated overnight at 20°C in 50 ml of 0.25% trypsin. The resultant cell suspension was filtered through cheese cloth, harvested by centrifugation, and seeded onto six-well plastic plates (Linbro) at a concentration of 10^5 cells per cm^2. The cells were then incubated in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U of pencillin G, 100 μg of streptomycin, and 1.25 μg of amphotericin B per ml at 37°C in a 5% CO_2 atmosphere. Human amnion cell monolayers were examined for their relative sensitivity to HSV infection by a simultaneous plaque assay of a stock preparation of HSV type 1 (HSV-1) E377 on human amnion cells, human foreskin fibroblasts, primary rabbit kidney cells, and human melanoma cells (from Charles Grose, University of Texas, San Antonio).

The titers obtained were, respectively, 1.3×10^8, 4.5×10^7, 8.7×10^7, and 1.1×10^8 $\log_{10}$ PFU/ml.

For the isolation of HSV from oral rinse specimens, 2 ml of specimen was placed in each of two wells of a six-well plastic tissue culture plate containing 80 to 100% confluent human amnion cells. The plate was then centrifuged at 1,400 $\times$ *g* for 60 min in a Sorvall GLC-2B centrifuge at 20°C. The inoculum was removed by aspiration, each well was washed once with 2 ml of phosphate-buffered saline, and then one of the duplicate wells was overlaid with 2 ml of 0.5% agarose in tissue culture medium and the other was overlaid with 2 ml of medium alone. When cytopathic effect was seen in the cells with liquid overlay, the medium was removed by aspiration and frozen at −70°C. After 5 days, the second well was stained with neutral red, and plaques were counted. Centrifugation produced a fivefold increase in the apparent titer of HSV-1 E377 in human amnion cells compared with the same volume of inoculum uncentrifuged, confirming earlier work by other investigators on the effect of centrifugation (5, 22).

As a second means of quantitating the amount of HSV in oral rinse specimens, all frozen virus-positive oral specimens were simultaneously assayed by plaque formation in Vero cells (Flow Laboratories). Serial 10-fold dilutions of each specimen were made in tissue culture medium, and 0.2-ml volumes of each dilution were inoculated in duplicate onto 80 to 100% confluent cell monolayers. After viral adsorption, the cells were overlaid with 0.5% agarose in medium, incubated for 5 days, and stained with neutral red.

Swab specimens from herpes labialis and intraoral lesions were quantitatively evaluated for HSV by plaque formation in Vero cells.

Restriction endonuclease analysis. The frozen supernatant fluids from virus-positive human amnion cell cultures were used to infect Vero cell monolayers. Viral DNA was labeled by growth in the presence of $^{32}P_i$ (New England Nuclear Corp.), partially purified by phenol extraction and ethanol precipitation, digested with *Kpn*I or *Bam*HI (New England Biolabs), and visualized by autoradiography as previously described (19).

Statistics. Proportions were compared by the chi-square procedure, or, for small numbers, by the Fisher exact test. $P \leq 0.05$ was considered significant.

RESULTS

Recovery of HSV from the oral cavity. Over the course of 5 months, eight healthy volunteers with a history of herpes labialis submitted a total of 637 oral rinse specimens. Of the 637 specimens, 47 (7.4%) were positive for HSV. Positive specimens produced typical cytopathic effect in primary human amnion cell cultures, and the supernatant fluids from these cultures contained HSV DNA by restriction endonuclease analysis. All volunteers had positive specimens, ranging in number from three to nine per subject. Virus could be reisolated after frozen storage from 31 of the 47 positive specimens and quantitated by plaque assay in Vero cells: the average titer in these specimens was 2.1, and the range was 0.4 to 3.7 $\log_{10}$ PFU/ml.

There were 12 clusters of HSV excretion in which two to five positive oral specimens from an individual occurred in close temporal sequence. Clusters of positive specimens accounted for 29 (62%) of the total isolations. The remaining 18 isolations were solitary. The 12 clusters and 18 solitary isolates are hereafter referred to as episodes of HSV excretion.

Occurrence of herpes labialis and intraoral lesions. Sixteen episodes of herpes labialis occurred during the study among the eight subjects. Each patient had at least one recurrence, and the maximum number was three. Thirteen lesions were sampled for virus, and 10 were positive. HSV was isolated from at least one lesion in seven of the eight subjects. The mean maximum lesion area was 71 (range, 4 to 255) mm^2, and the mean lesion duration was 7.2 (range, 2 to 16) days. The mean maximum titer of the virus-positive lesions was 5.0 (range, 2.0 to 7.1) $\log_{10}$ PFU.

A prodrome consisting of focal itching, burning, or tingling sensations on lips may precede herpes labialis (20). An unexpected finding in the present study was the report from three subjects of six episodes of prodromal symptoms that were not followed by a lesion (false prodrome). Two patients had one episode each of false prodromata, and one patient had four episodes. The duration of symptoms was 7 days in one instance and 1 to 2 days in the remainder. Prodromal symptoms followed by herpes labialis (true prodrome) occurred with only 3 of the 16 lesions that developed among the patients during the study period. Patients reporting false prodromes experienced six episodes of herpes labialis, one of which was preceded by prodromata.

Fifteen episodes of intraoral ulceration occurred among six patients. Among 10 episodes evaluated in the clinic, 5 were located on the gingiva, 1 was on the hard palate, and 4 were on the labial or buccal mucous membranes. Gingival lesions were seen on the interior, exterior, upper, and lower surfaces. The majority of intraoral lesions were single in number and in the ulcer stage. The average duration of lesions was 2.5 (range, 1 to 4) days. Two swab specimens were obtained from lesions on the gums, and both were positive for HSV (1.0 and 4.4 $\log_{10}$ PFU). In contrast, four lesions on the labial or buccal mucous membranes were cultured and were negative.

Occurrence of stressful events. Seventy-four situations of stress were reported. There were 14 common colds (rhinitis), seven cases of sunburn, nine episodes of unusual emotional distress, 19 periods of menstruation, 15 instances of oral trauma (13 dental procedures, 2 self-bite), and 10 miscellaneous illnesses (3 gastrointestinal upset, 3 pharyngitis, 1 sinusitis, 3 fever-myalgia).

Association of stressful events, oral HSV excretion, and herpes labialis. Of the 14 common colds, 10 were associated with one or more manifestations of HSV infection (Table 1). The manifestation was oral HSV excretion in six instances, and four of these times virus excretion was the only evidence of reactivation. The rate of HSV excretion during periods of the common cold (HSV-positive samples/total samples during the period) was 10 of 48 (21%), a rate significantly different ($P = 0.001$) from, and 3.5-fold greater than, the rate in the remaining periods of the study (37 of 589 [6%]).

Sunburn was followed in two of seven instances by herpes labialis, but the rate of oral HSV excretion in the 5-day period after sunburn was not increased (1 of 25 [4%]). Oral trauma was associated with only one episode of herpes labialis, but the rate of HSV excretion in the 5-day period after dental manipulation or other injury was 6 of 35 (17%), a rate significantly different ($P = 0.04$) from, and twofold greater than, the rate in the remaining periods of the study (41 of 602 [7%]). Episodes of oral HSV excretion related to a dental procedure (patient F) and emotional distress (patient H) are illustrated in Fig. 1.

Association of oral HSV excretion and herpes labialis. The association between oral HSV excretion and herpes labialis is shown in Table 2. The rate of oral HSV excretion was not

TABLE 1. Association of stressful events with various manifestations of HSV infections

Event (no.)	No. of episodes associated with an event[a]		
	Oropharyngeal HSV excretion[b]	Herpes labialis	False prodromal symptoms[c]
Common cold (14)	6 (4)	4	2
Sunburn (7)	1	2	1
Emotional upset (9)	1	1	0
Menstruation (19)	2 (1)	1	0
Oral trauma (15)	4 (1)	1	2
Other illness (10)	0	0	0
No known event	16	7	1

[a] Association indicates that the episode began during the time of a common cold, emotional upset, menstruation, or other illness, or in the 5 days after sunburn or oral trauma.

[b] Numbers in parentheses represent the number of episodes in which oropharyngeal virus excretion was the only manifestation.

[c] Focal itching, burning, or tingling on the lips not followed by a clinically apparent episode of herpes labialis.

increased in the week before (2.2%) nor the week after (7.3%) an episode of herpes labialis. During an episode of herpes labialis, the rate of excretion (19%) was threefold greater than the rate in the remaining period of time (P = 0.002). The increased rate of excretion during herpes labialis was due to a high rate of recovery of virus from the oral cavity during the time of a vesicle (57%) and during the first 3 days of the ulcer stage (33%). The highest rate of oral HSV excretion (60%) occurred during false prodromal symptoms of herpes labialis. The mean (± standard deviation) titer of HSV in five oral rinse specimens from the time of the vesicle and early ulcer stages of herpes labialis (2.1 ± 1.4 $\log_{10}$ PFU/ml) was the same as the titer of 24 oropharyngeal specimens obtained in the absence of herpes labialis lesions (2.0 ± 1.0 $\log_{10}$ PFU/ml).

Association of oral HSV excretion and intraoral ulceration. Fifteen episodes of intraoral ulceration occurred during the study period (Table 3). Oral excretion of HSV at the time of intraoral ulceration was highly dependent on the location of the lesion. Five of 16 intraoral rinse specimens were positive when there was a lesion on the gingiva or hard palate,

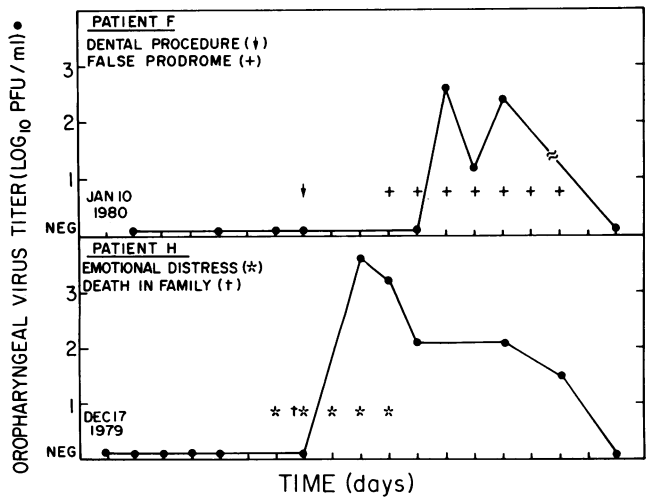

FIG. 1. Episodes of oral HSV excretion associated with oral trauma and emotional stress.

TABLE 2. Occurrence of HSV in oropharyngeal specimens at different times and stages of herpes labialis

Time or stage of herpes labialis	HSV-positive oropharyngeal specimens/total (%)
All time periods	47/637 (7.4)
Week before an episode of herpes labialis	1/45 (2.2)
Week after an episode of herpes labialis	4/55 (7.3)
During herpes labialis	11/57 (19)
Prodrome	0/1
Erythema	0/1
Papule	0/5
Vesicle	4/7 (57)
Early ulcers (1 to 3 days after vesicle)	6/18 (33)
Late ulcer-crust	1/25 (4)
Periods without herpes labialis	36/580 (6.2)
During false prodromal symptoms	6/10 (60)

TABLE 3. Occurrence of HSV in oropharyngeal specimens in association with intraoral lesions

Lesion location (no. of episodes)	HSV-positive oropharyngeal specimens/total (%)
Gingival ulcerations (5)	4/13 (31)
Hard palate lesion (1)	1/3 (33)
Labial or buccal mucous membrane ulceration (4)	0/9
Intraoral lesion, unknown location (5)	0/8

whereas none of 17 specimens were positive in association with labial or buccal mucous membrane ulcerations or intraoral lesions whose location was unknown (P = 0.02). Two patients with positive swab cultures from lesions on the gingiva also had positive cultures from concurrent oral rinse specimens.

Restriction endonuclease analysis. Restriction endonuclease analysis of 57 isolates from oral rinse, herpes labialis, and intraoral lesions with *Kpn*I and *Bam*HI identified all as HSV-1. Each of the patients excreted a unique strain of virus (Fig. 2). All of the oral rinse isolates from any one subject

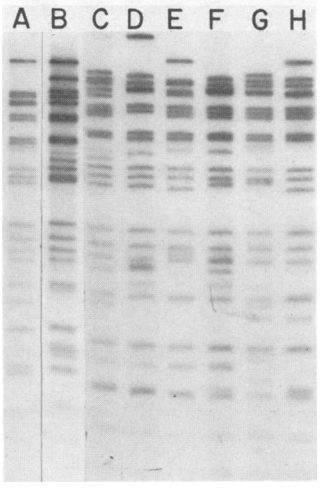

FIG. 2. Autoradiogram of [^{32}P]DNA from the HSV isolates from the eight study subjects (A to H) after digestion with *Kpn*I restriction endonuclease and electrophoresis in a 1% agarose gel.

were of the same strain. In addition, 10 isolates from intraoral and herpes labialis lesions in seven patients matched the oral rinse isolates in each case (Fig. 3).

DISCUSSION

When eight healthy volunteers with a history of recurrent herpes labialis were examined on weekdays for 5 months to detect excretion of HSV in the oral cavity, HSV was found on 7.4% of the patient-days tested. In an earlier study, Douglas and Couch found HSV in oral cavity specimens on 3.6% of patient-days (6). The difference between the two studies can be attributed to the selection of patients and the methods used for virus isolation. If only patients with recurrent herpes labialis are considered, the isolation rate for this subgroup in the study of Douglas and Couch is 4.7%. All of the isolates described by Douglas and Couch could be reisolated from frozen specimens. In the present study, 16 specimens were positive only with the primary isolation procedure on centrifuged human amnion cell monolayers, and the frequency of isolates that could be recovered from frozen specimens was 31 of 637 (4.9%).

The majority of viral isolates in this study (29 of 47 [62%]) were found in temporal clusters of two to five isolates. In addition, the rate of HSV excretion was significantly increased during periods of the common cold (21%) and after oral trauma (17%). These data indicate that oral HSV excretion is episodic and can be provoked by some of the same stressful events that are associated with precipitation of herpes labialis.

The present study identified a significantly increased rate of HSV excretion during the time of an episode of herpes labialis (11 of 57 [19%]). This rate is comparable to that described by Douglas and Couch (24.1%). Lower values of 8 and 7% obtained in two additional studies can probably be attributed to the use of swab samples of saliva for virus isolation (1, 20). Virus excretion was uncommon in the week before an episode of herpes labialis (1 of 45 [2.2%]). These data showed that oral HSV excretion was not antecedent and a source of virus leading to the development of labial lesions. Because of the frequency of specimens taken in this study, we were able to associate oral virus excretion with the vesicle and early ulcer stages of herpes labialis, at which times 57 and 33%, respectively, of specimens were positive. A high rate of virus excretion (60%) also occurred during periods of false prodromal symptoms. Since virus excretion in the oral cavity is independent of whether the herpes labialis lesion is closed (vesicle stage, false prodrome) or open (ulcer stage), contamination by the external lesion is an unlikely explanation for the presence of virus in the oral cavity. In conclusion, when oral HSV excretion concurs with herpes labialis, they do not appear to be causally related to one another and are most likely separate, parallel events.

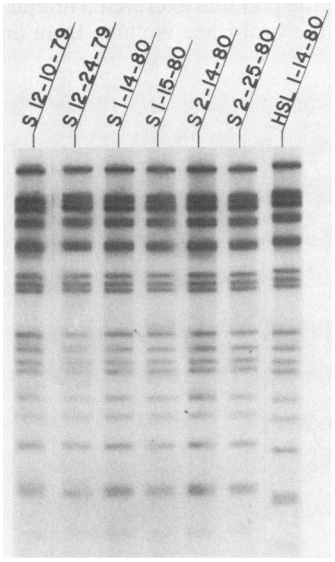

FIG. 3. Autoradiogram of [^{32}P]DNA from six oral rinse isolates (S) and one herpes labialis isolate (HSL) from patient A after digestion with *Kpn*I restriction endonuclease and electrophoresis in a 1% agarose gel. Numbers refer to the date of each isolation.

The results of the present study confirm earlier observations that the gingivae and hard palate are specific sites of recurrent intraoral HSV disease (24). Localization of intraoral HSV disease to the gingivae and hard palate may be related to the frequency and severity of primary herpes gingivostomatitis at these sites (17). Since HSV may frequently bathe the interior of the oral cavity in subjects with herpes labialis, and diffuse involvement of the intraoral cavity can be seen in primary disease, it is puzzling that recurrent HSV lesions are not commonly identified on the buccal or labial mucous membranes. Heineman and Greenberg (10) have reported that saliva contains a substance that reduces the susceptibility of cells to HSV infection. The mucosa of the gums and hard palate are keratinized, and the buccal and labial mucosa are not (13). Inhibitory substances in saliva may be able to penetrate and block or limit epidermal cell infection of the buccal and labial mucosa, but not the keratinized mucosa of the gingivae and hard palate. Small, aborted lesions of buccal and labial mucosa could be a source of intraoral HSV in the absence of clinically detectable lesions.

Restriction endonuclease analysis of viral isolates with *Kpn*I and *Bam*HI indicated that all isolates from any one patient were of the same strain whether obtained from oral rinse, an intraoral ulcer, or herpes labialis. Accordingly, this study provided no evidence for reinfection or infection with more than one strain of HSV in the pathogenesis of recurrent herpes labialis. In contrast, infection with more than one virus type and strain has been documented in herpes genitalis (7).

Sixteen episodes of herpes labialis occurred during the study and were followed by clinical and virological measurements. The values obtained for mean maximum lesion area, mean maximum virus titer, and mean lesion duration were similar to those described in a previous report on the natural history of herpes labialis (20). However, in the previous study, 60% of patients experienced prodromal symptoms with their presenting lesion, and prodromal symptoms were judged by history to be false (not followed by a lesion) only once in 10 instances. In the present prospective investigation, a true prodrome preceding herpes labialis was noted only in 3 of 16 (19%) instances, and six episodes of false prodromal symptoms occurred. These differences are likely due to the differences in study design.

Low frequency of true prodrome before herpes labialis and a high proportion of false prodromal symptoms are new observations that have major implications for treatment studies. Based on these findings, one might expect to have difficulty starting treatment in the early stages of herpes labialis. The results of treatment studies at the University of Utah and Boston University would seem to reflect this point. In two trials of antiviral therapy for herpes labialis (18, 21),

only 5 of a total of 441 subjects studied were in the prodromal or erythema stage at the time of their first clinic visit.

The present study provides evidence that oral HSV excretion is an episodic event that may be provoked by stress and may occur alone or in parallel with herpes labialis, but is not the cause or the consequence of herpes labialis. Small, short-lived lesions on the gingivae and hard palate are the source of intraoral HSV in at least some instances. It is likely, but unproven, that small, clinically inapparent epithelial lesions in the intraoral cavity account for intraoral HSV the remainder of the time.

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