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Inapparent Herpes Simplex Virus Infection in Inoculated Rabbits. (31948)

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A recent study of experimentally induced herpes simplex virus (HSV) ulcerations in the oral mucosa of rabbits showed that the lesion not only spread laterally along the mucosal surface but also extended downward for a considerable distance into the ducts of the minor salivary glands located well below the surface epithelium(1). Histologic signs of the infection persisted in these ducts several days longer than in lesions on the mucosal surface. This observation suggested that an inapparent herpetic infection of short duration was produced in the gland ducts. Since these ducts are only approximately 1 mm in length, the susceptible epithelial cells necessary for HSV replication were rapidly destroyed thus limiting the duration of the infection in this site. As a result, it was conceivable that an inapparent HSV infection involving the longer ducts of a major salivary gland, where more tissue would be available for viral replication, would persist for a longer period of time. Therefore, the present studies were undertaken to investigate this possibility using cultural, serological and histological methods. A local infection involving the ducts of the submaxillary glands was induced by direct inoculation of HSV into the duct orifice tissue. In view of reports that HSV is readily cultivable from the saliva of rabbits with active herpetic infections(1,2,3), tests were performed on specimens of saliva taken after gross oral ulcerations had healed. Similarly, serum specimens were assayed for HSV neutralizing antibody and ductal specimens were examined for histologic signs of HSV infection.

Materials and methods. Solution and media. Dulbecco's(4) phosphate buffered saline (PBS) was prepared in glass-distilled water and sterilized by Seitz filtration. Trypsin solution (0.25%), lactalbumin hydrolysate medium(5), and Eagle's medium(6) were prepared as described in a previous report(7). Antibiotics (penicillin G, 100 units/ml; streptomycin, 100 ug/ml; tetracycline, 10 μg/ml; nystatin, 100 units/ml) and sera as required, were added to these media at the time they were used.

Cell cultures. Kidneys of 8-10-week-old New Zealand white rabbits (NIH strain) were trypsinized(7) and the dispersed cells were suspended in lactalbumin hydrolysate medium supplemented with 8% inactivated calf serum in a ratio of 1 ml of cells per 125 volumes of medium, yielding an average viable cell count of 5.4×10^5 cells per ml. Portions (0.9 ml) of this suspension were dispersed into disposable(8) 16 $\times$ 150 mm roller tubes and incubated in a stationary position at 37°C for 4 to 5 days. The medium was changed (0.9 ml) and the cultures were inoculated when a confluent cell sheet had formed. A substrain of the S-3 clone(9) of HeLa cells was maintained as stock cultures in Eagle's medium supplemented with 10% inactivated horse serum. Tube cultures were prepared using 0.5 ml of cell suspension containing approximately 10^5 viable cells. Following 24-hr incubation at 37°C, the medium was changed and the cultures were inoculated 1 to 2 days later.

Virus. HSV strain 11139(1) was used throughout this study. Virus pools were prepared in bottle cultures of primary rabbit kidney cells and assayed in plaque-forming units according to methods described elsewhere(10).

Animal procedures. (1). *Virus inoculation.* Female New Zealand white rabbits (NIH strain) weighing approximately 2 kg were anesthetized with pentobarbitol sodium and fitted with a special mouth prop(1) to hold the jaws apart. Using a 1 ml syringe fitted with a 30 gauge needle, $10^{5.7}$ PFU of virus contained in 0.2 ml of PBS were injected into the papilla of each submaxillary gland duct orifice. Control animals were inoculated with PBS without HSV. (2). *Collection of saliva specimens.* Each animal was anesthetized and a sterile mouth prop was inserted. The sublingual area was air-dried and 0.3 ml of pilocarpine (1 mg/ml) was injected intravenously. Sterile cotton applicators were immediately placed in contact with the orifices of the submaxillary gland ducts and held there until they became saturated with fresh saliva. The applicators were placed in 1.0 ml of PBS containing 5% inactivated normal horse serum. This is referred to hereafter as "saliva specimen." In some instances cultures were taken from the sublingual area before air drying or after air drying but before the administration of pilocarpine. Specimens were either tested immediately for HSV or were stored at −55°C until tests could be made. (3). *Collection of serum.* Control serum was obtained from each animal by cardiac puncture prior to inoculation. Subsequent serum samples were collected from the central artery of the ear. All sera were heat-inactivated at 56°C for 30 minutes and stored at −55°C until assayed for antibody. Rabbits were maintained in separate cages throughout the experiments.

Virus isolation. Duplicate roller tube cultures of rabbit kidney or HeLa cells were inoculated with 0.2 ml of a saliva specimen and incubated at 37°C in a stationary position for 5 to 7 days. These cultures were examined daily for evidence of herpetic cytopathic effect (CPE) and when the CPE was at its peak or at the end of the 7-day incubation period, the supernatant fluid was harvested and centrifuged at 700 × *g* for 10 minutes. Fresh rabbit kidney or HeLa cell cultures were inoculated with 0.2 ml of this supernate. If the CPE was not observed after 3 such passages, a specimen was considered negative for HSV. Controls consisted of rabbit kidney or HeLa cell cultures which were inoculated with saliva specimens taken from rabbits which had not been inoculated with HSV. Confirmation of an isolate as HSV was based upon its neutralization by antiserum from rabbits which had been immunized with strain 11139 of HSV.

Neutralization test. Serial 2-fold dilutions of serum prepared in PBS were incubated for 1 hour at 37°C with 500 $TCID_{50}$ of HSV, also diluted in PBS. Duplicate tube cultures of rabbit kidney cells were inoculated with 0.2 ml of a serum-virus mixture and incubated at 37°C for 4 to 5 days. The antibody titer of a serum was taken as the reciprocal of the highest dilution which completely inhibited the herpetic CPE. Each serum was tested at least twice in experiments performed on different days.

Histologic techniques. Jaw specimens were fixed overnight in Zenker-acetic acid fixing fluid and then further fixed in neutral buffered formalin. Sublingual tissue containing the orifice and approximately 1 cm of the length of the submaxillary gland duct was dissected from the bone and trimmed so that longitudinal sections could be made of the duct. Specimens were embedded in paraffin and 6 μ sections cut serially and stained with hematoxylin and eosin.

Results. Oral lesions. The gross ulcerations which formed at the inoculation sites were observed to include the duct orifices within 4 days. Within 7 days the ulcerated areas appeared to have re-epithelialized and no grossly evident lesions remained. Histologically, the submaxillary ducts of one animal sacrificed 8 days after inoculation showed definite signs of HSV infection in the terminal 1 mm. In contrast, serial sections of 12 ducts from 6 animals sacrificed at various times from 19 to 165 days after inoculation revealed no signs of HSV infection in the terminal 1 cm segment. Recurrent herpetic lesions were never observed during this study. No gross or his-

TABLE I. Results of Tests for HSV in Saliva of Inoculated Rabbits.

Periods after inoculation (days)*	No. rabbits positive/ No. rabbits tested
5– 8	5/ 8
12–14	7/11
15–19	2/ 7
21–26	4/ 4
27–34	2/ 5
36–43	1/ 4
7 wk–6 mo	1/ 6
7–21 mo	0/ 4

* Time is expressed in days except where otherwise indicated.

tologic signs of HSV infection were seen in control rabbits.

Cultural data. The results of culturing saliva for HSV after inoculation are summarized in Table I. HSV was most readily demonstrable in the saliva of the majority of the animals when they were tested during the 4-week period immediately following oral inoculation. However, in some instances detectable levels of virus were found in saliva specimens taken after much longer intervals.

Specific data on HSV isolation from the saliva of individual animals are contained in Table II. In 10 of 13 animals studied, HSV was cultured from the saliva one or more times during the 35-day interval between 8 and 43 days after inoculation. The saliva of 3 experimental animals and all control animals were uniformly negative for HSV. A positive culture for HSV was obtained from one of the 10 positive rabbits (H-143) 169 days after inoculation. Cultures of saliva taken from all other rabbits after periods longer than 6 weeks did not show detectable levels of virus. Variations in culture technique did not seem to affect the results. Cultures taken either before or after specific salivary gland stimulation with pilocarpine tended to follow the same pattern. In almost all instances either both types were positive or both were negative.

Variability was observed in the cultural results obtained from different rabbits and also in the results from individual rabbits at different times. For example, HSV was recovered from the saliva of H-147 and H-149 on each successive attempt up to 21 days. In attempts made subsequently, however, no recovery was achieved. In other rabbits, the pattern of recovery was less consistent. For example, 8-day specimens taken from H-141 and H-146 were negative for virus, but specimens taken from these animals one week later were positive for HSV. A still different pattern of virus recovery was observed when the saliva of H-153 was tested. The saliva of this animal did not show virus in 4 tests made over a 36-day period; yet virus was recovered from its saliva on the 43rd day.

On several occasions the saliva of 2 rabbits which had been inoculated intraperitoneally with HSV for an earlier study(10) was cultured for the virus. HSV was recovered from one of these animals 747 days after inoculation and from the other 996 days after inoculation. All other attempts to isolate the virus were unsuccessful. As far as could be determined, these 2 animals never experienced an oral ulceration or exhibited any other gross signs of a herpetic infection.

Serological data. These data are contained in Table II. HSV neutralizing antibody was not manifest in the pre-inoculation sera or in the serum of infected animals tested within 5 days after inoculation. Antibody was, however, present in the serum of animals tested within 9 days after injection of the virus. In 4 rabbits from which positive saliva cultures were obtained during the first 4 weeks after inoculation, serum antibody titers rapidly reached peak levels of 128 to 256 during this period. Contrariwise, one rabbit (H-153), in which salivary levels of HSV were not detectable until 6 weeks after inoculation, showed a gradual rise in serum antibody titer. Animals from which the virus was not isolated (*e.g.,* H-140 and H-154) also responded immunologically to the single injection of virus. HSV neutralizing antibody was manifest though in relatively low titer, in the serum of infected animals which were tested over a period of 3 to 21 months. During this time, the titers of several of these animals appeared to fluctuate.

The 2 rabbits which had been inoculated intraperitoneally were tested for neutralizing antibody over a period of 43 months. Fluctuations in titer also occurred in these animals. On several occasions their antibody

TABLE II. Relationship Between HSV Isolation and Neutralizing Antibody Titer.

Rabbit No.	Time after inoculation*	Virus isolation	Time after inoculation*	Antibody titer†
H-145	5	+	5	<4‡
	8§	+	8	8
H-148	13	+	12	128
	21	+	21	128
H-147	5	+	5	<4
	13	+	14	128
	21	+		
	27	—	27	128
	3–5½ mo§	—	3–5½ mo	32
H-149	5	+		
	13	+	12	128
	21	+		
	27	—	27	128
	3–15 mo		3–15 mo	16–32
H-146	5	+	5	<4
	8	—		
	15	+	15	128
	26	+	26	256
	34§	+		
H-151			4	<4
	12	+	11	32
	19	—	20	128
	27	+	29	256
	36	—	39	128
	43	—	46§	128
H-143	14	+	9	8
			135	32
	169	+		
	6–15 mo	—	6–15 mo	16–32
	16–21 mo	—	16–21 mo	32–64
H-141	8	—		
	13	+		
	15	+		
	5 mo	—	5 mo	64
	8–19 mo	—	8–19 mo	32–16
H-144			9	8
	14	+		
H-153			4	<4
	12	—	14	8
	19	—	20	32
	27	—	29	16
	36	—	39	32
	43	+	46	32
	54§	—	53	64
H-140	8	—		
	13	—		
	15	—		
	8–21 mo	—	8–10 mo	32–16
			21 mo	32
H-152	12	—	13	16
	19	—	19	16
H-154			4	<4
	12	—	14	128
	19	—	20§	64
Controls	0–21 mo	—	0–21 mo	<4

* Time is expressed in days except where otherwise indicated.
† Expressed as reciprocals.
‡ Lowest dilution tested.
§ Indicates time animal was sacrificed.

titers decreased from 128 to 32 and then increased to 128 and at times went as high as 256. Antibody titers less than 32 were not observed during the 43-month period. The serum of control animals remained free of detectable levels of neutralizing antibody throughout this investigation.

Discussion. The purpose of the present work was to attempt to establish a persistent inapparent HSV infection in the submaxillary gland ducts of rabbits housed in separate cages. Our histologic data indicate that such an infection was not established in these ducts, since the ducts of rabbits sacrificed more than 8 days after they had been inoculated were free of histological signs of an HSV infection. However, HSV was detectable in the saliva of several animals on the same day that they were sacrificed. Thus, our cultural data suggest that an inapparent HSV infection may have persisted for several weeks in the submaxillary glands proper or in some other oral tissue site.

Recovery of the virus from the saliva of rabbits which had been inoculated intraperitoneally rather than intraorally indicated that the presence of HSV in the saliva was not due to the route of inoculation. Systemic dissemination of the virus after inoculation could have resulted in the establishment of an inapparent HSV infection in a number of sites including the oral cavity. Since the virus was not recovered from the saliva of these two animals with any degree of consistency, two of a number of possible explanations are: 1. the oral cavity may serve as an occasional temporary site for an inapparent HSV infection; or 2. salivation may infrequently serve as a means by which HSV, which has replicated in another site(s), is excreted from the body.

These observations on the cultivability of HSV from the saliva of infected rabbits are similar to those reported by other investigators from their studies of HSV in the saliva of apparently healthy humans. Black(11) isolated this virus from the saliva of one subject 34 days after onset of infection but did not detect it in a specimen taken on the 40th day. Scott *et al*(12) recovered the virus from the saliva of a patient on several occasions up to 5 weeks after onset of infection. Subsequent

attempts made at 10 and 20 weeks were unsuccessful, but they again isolated the virus from a saliva specimen taken during the 22nd week. Buddingh and his associates(13) not only isolated HSV from the saliva of children for varying periods after onset of primary herpetic gingivostomatitis, but also observed fluctuations in HSV neutralizing antibody titers on occasions when there had been no known clinical recurrence of infection.

In human studies there is always the possibility that the asymptomatic individual may become infected as a result of his frequent or periodic contact with other individuals with active herpetic lesions. Since our infected animals were maintained in separate cages, the cultural and serological results obtained in this study were probably not due to adventitious reinfection. This probability is strengthened by the finding that untreated control rabbits tested over long periods under the same conditions showed neither salivary HSV, nor serum neutralizing antibody against HSV. In addition, recent findings that in mice (14) and rabbits (Ashe, W. K., unpublished observations) immunized with UV inactivated HSV, neutralizing anibody does not persist beyond 4 to 5 weeks, indicate that the prolonged presence of HSV neutralizing antibody in our animals is due to continual replication of HSV. Therefore, since we observed no recurrent herpetic lesions and normal laboratory handling of the animals does not lead to adventitious infection, the most likely source of the antigenic stimulation for the persistent levels of HSV neutralizing antibody seen in our animals is a persistent inapparent infection. The exact site(s) of such an infection is yet to be determined.

Summary. Rabbits were inoculated in the oral mucosa adjacent to submaxillary gland ducts with $10^{5.7}$ plaque-forming units of herpes simplex virus (HSV). Gross ulcerations involving the ductal orifices appeared within 4 days and re-epithelialized within 7 days. Histological signs of HSV infection were present in ductal specimens examined on the 8th day but not in specimens examined at subsequent times. HSV was detectable in the saliva of 10 of 13 rabbits for varying periods of time after the surface lesions had healed. Though the virus was most readily isolated during the first 3 to 4 weeks following inoculation, it was not always recovered from a given animal on consecutive attempts. HSV was isolated from the saliva of one animal 169 days after inoculation. In addition, the virus was isolated from the saliva of 2 rabbits 747 and 996 days, respectively, after they had been inoculated intraperitoneally. HSV neutralizing antibody was not manifest in pre-inoculation sera or in sera taken within 5 days after infection. Within 9 days antibody was present in low titer and reached peak levels in most of the rabbits within 2 to 4 weeks. Repeated antibody assays performed at various times over a period of 3 to 21 months revealed that relatively low levels of antibody persisted in the animals tested. The titer of several rabbits, however, appeared to fluctuate during this time.

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