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Enhanced HSV Recovery from Neuronal Tissues of Latently Infected Rabbit (40785)[1]

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Infection with herpes simplex virus (HSV) is generally divided into acute and latent phases. During the acute phase of infection which usually lasts about 2 weeks in the rabbit, HSV is readily isolated from homogenized or minced tissue (1). When the herpetic infection is in the latent phase, HSV is more difficult to detect. Infectious virus can no longer be recovered from homogenized tissues, and conventional immunofluorescence, radioimmunoassay, and electron microscopic studies have failed to demonstrate HSV antigens or particles in the tissues (1, 2). Although not very sensitive, the most successful method for detecting latent or infectious cell-associated HSV is by cocultivation of minced tissue or by explantation. Recently, Walz *et al.* (3) reported a procedure for detecting intracellular HSV in the mouse nervous system using enzymatic means to separate the cells. We modified this method for use with trigeminal ganglia and other tissue from the rabbit in hopes that enzymatic cell dispersion (ECD) might increase the sensitivity of our search for latent HSV. The purpose of this study was to compare the isolation rate of HSV from rabbit trigeminal ganglionic cells latently infected with HSV using the classical minced tissue method and our ECD method.

Materials and methods. Experimental groups. These experiments were carried out on 53 New Zealand white male rabbits weighing between 3 and 4 kg. Eyes were bilaterally infected with 10^6 PFU/ml of McKrae strain HSV-1 without corneal scarification as previously described (4). Five animals were studied during the acute infection. They were sacrificed 14 days after inoculation and used as positive controls assuming that HSV would be present in most tissues. The remaining 48 animals were sacrificed during latent infection. For this study, a rabbit with a latent infection was defined as one which spontaneously shed HSV in the tear film one or more times beginning at least 30 days after virus inoculation. Daily (6 days/week) conjunctival swab cultures were employed to demonstrate the presence of HSV in the tear film (5).

Previously, we reported that *in vivo* surgical stimulation of the latently infected rabbit trigeminal ganglion induced peripheral release of infectious HSV (4). To test the sensitivity of the minced tissue and ECD methods for detecting infectious HSV in neural tissue, we attempted virus isolation from tissues of stimulated and nonstimulated animals.

An additional eight uninfected rabbits were used as negative controls. Tissues from these rabbits were harvested and treated in the same way as those from latently and acutely infected animals.

Tissue sampling. The animals were sacrificed by intravenous air injection. Trigeminal ganglia, midbrain, lacrimal gland, conjunctiva, and cerebellum were removed aseptically. Each tissue sample was minced with a sterile disposable blade into approximately 0.5-mm pieces. At this point, half of each tissue sample was randomly designated for the standard minced treatment and the other half for ECD treatment.

ECD technique. Enzymatic cell dispersion was carried out by adding 2% collagenase (approximately 10 μl/0.1 g tissue:

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GIBCO, *Clostridium histolyticum*, 160 units/mg at pH 7.4) for 30 min at 37°. Cell dispersion was facilitated by repeated aspiration with a sterile Pasteur pipet. The partially dispersed tissue sample was then treated with trypsin–EDTA (0.25% trypsin with 4×10^{-4} *M* EDTA in normal saline) at 37° for approximately 30 min. Further cell dispersion was carried out by repeated gentle pipetting. Fetal bovine serum (FBS) was added to a final concentration of 25% to stop the action of the trypsin–EDTA mixture. To insure that extracellular HSV was neutralized, we added 0.2 ml of high-titer rabbit McKrae strain HSV antiserum to the mixture for 30 min at 37°. Twice the cell suspension was rinsed with 5 ml of sterile PBS and centrifuged at 900 rpm for 10 min. The enzymatically dispersed cells were resuspended in 1 ml of phosphate-buffered saline (pH 7.2) and consisted of single cells, small aggregates, and cell fragments. This mixture was cocultivated on monolayers of rabbit kidney (RK) cells.

Cell culture technique. Primary and secondary RK monolayers were maintained with minimum essential medium from GIBCO supplemented with 10% FBS and 10% NCTC 135, 2 m*M* L-glutamine, and 10 μg/ml gentamicin and 3 μg/ml Fungizone. This medium is referred to as MEM 10-10. The ECD and minced samples were added to RK monolayers in 75-cm^2 flasks (Falcon Plastics) containing 15 ml of MEM 10-10.

All cultures were incubated at 37° in 5% CO_2 for 3 weeks. They were examined every other day for cytopathic effect (CPE). When CPE involved half of the cells, the tissue monolayer was scraped into the surrounding medium and 0.1 ml of the cell medium suspension was passed to duplicate RK monolayers. All CPE negative cultures were blind passed at the end of 3 weeks by the same method. If no CPE developed in the blind passage after 3 weeks, the sample was recorded as negative. All positive cultures were confirmed as previously described, using a microtest plate neutralization method (5).

Results. Trigeminal ganglia from rabbits with acute HSV infection were tested for virus 14 days after inoculation. In Table I, the results show that 7 out of 10 minced samples were positive while all 10 were positive when tested with the ECD method.

In Table II, results of the two methods testing for presence of latent virus in trigeminal ganglia of 48 rabbits are compared. In these 48 animals (96 eyes), 66 eyes had shown previous episodes of ocular HSV shedding while in the remaining 30 eyes no virus shedding was detected.

In the eyes with the positive shedding record, 65% (42/66) were positive with the ECD method as compared to 17% (11/66) with the minced technique ($P \leq 0.005$). Trigeminal ganglia associated with non-shedding eyes had a lower frequency of virus recovery. Again, there were significantly ($P \leq 0.05$) more positive cultures by the ECD method, 43% positive (13/30), than by the minced method, 13% (4/30). Comparing data from all 96 trigeminal ganglia of latently infected animals, there was 58% HSV isolation rate in the ECD group (56/96) versus 16% from the minced method (15/96) ($P \leq 0.005$).

The ECD and minced methods were both employed in attempts at recovering HSV from 53 rabbits (data not shown). Sixty-two percent (66/106) trigeminal ganglia samples were positive with the ECD method and 21% with the minced method ($P \leq 0.005$). Of the 106 midbrain samples tested (two from each animal), 31% were positive by ECD compared to 10% by the minced

TABLE I. RECOVERY OF HSV FROM TRIGEMINAL GANGLIA OF RABBITS 14 DAYS AFTER ACUTE OCULAR INFECTION

Tissue preparation procedure	No. positive/No. tested	Percentage positive	*P* value
ECD	10/10	100	≤ 0.5
Minced	7/10	70	

TABLE II. RECOVERY OF HSV FROM TRIGEMINAL GANGLIA OF RABBITS WITH LATENT HSV INFECTION

Tissue preparation procedure	TG[a] positive/ total eyes	Percentage positive for HSV isolation	*P* value
HSV shedding eyes			
ECD	42/66	65%	≤ 0.005
Minced	11/66	17%	
Nonshedding eyes			
ECD	13/30	43%	≤ 0.05
Minced	4/30	13%	
Total eyes			
ECD	56/96	58%	≤ 0.005
Minced	15/96	16%	

[a] Trigeminal ganglia.

method ($P \leq 0.005$). The 53 cerebellar samples yielded a 15% HSV isolation rate with the ECD method compared to 6% by the minced method ($P \leq 0.5$). For each tissue type tested (except cerebellum), there was a significantly greater virus isolation rate when the ECD method was employed. In this entire study, there were 40% (107/265) positive cultures by the ECD method and 14% positive by the minced method ($P \leq 0.005$).

Significantly, there were no instances in which the minced method alone was positive. That is, in the 36 samples in which virus was detected by the minced method they were also detected by the ECD method. There were 64 instances where the ECD method detected virus and the minced method did not.

If the ECD method is more sensitive, then one would predict an earlier appearance of CPE with more rapid virus isolation. The time required by the ECD and minced methods for HSV recovery from trigeminal ganglia was compared in 36 instances (data not shown). Of the 36 instances in which both methods were positive, 83% of the time (30/36) CPE was detected earlier in the ECD samples. In 14% (5/36) CPE was discovered at the same time. Therefore, the ECD method was faster or equally fast in virus detection 97% of these samples. In only one instance, the minced sample resulted in more rapid appearance of CPE.

In Fig. 1, a graphic representation of the 107 positive ECD and 36 positive minced samples is presented along with the day on which the CPE was first recognized. By the ECD method, the positive cultures were recognized from Days 2–13 with a mean of 5.14 days. In the minced group, the range was 4–16 days with a mean of 8.75 days. The difference is statistically significant at the $P = 0.01$ level.

From the 48 latently infected animals which had a positive shedding record, 24 conjunctival samples, 44 lacrimal gland samples, and 14 trigeminal nerve samples distal to the ganglion were harvested at the time of sacrifice. These tissues were treated by the minced and ECD methods. No virus was recovered by either method from any of these tissues. Although the ECD method is significantly more sensitive in uncovering virus from the nervous system, it failed to

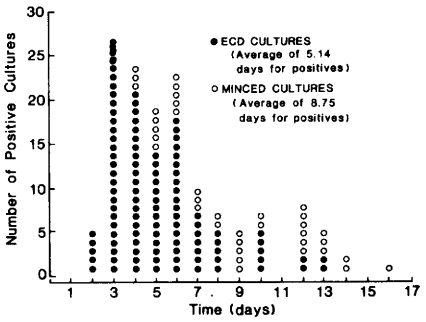

FIG. 1. Comparison of the enzymatic cell dispersion (ECD) and minced methods for detection of herpes simplex virus in neural tissues. The day that typical HSV CPE was first recognized is represented by solid circles (●) for the ECD method and open circles (○) for the minced method.

show virus in these peripheral tissues. This can be considered additional evidence that latent virus is not harbored in the peripheral tissues.

Discussion. Goodpasture was the first to suggest that HSV was probably harbored in the nervous system between episodes of overt herpetic disease (6). Recently, cocultivation and explantation techniques have demonstrated apparent latent HSV infection of sensory and autonomic nerve ganglia of experimental animals (1, 2, 5, 7) and man (8–10). The neuronal theory hypothesizes that after primary infection, the nerve cell acts as a reservoir for latent HSV. Axons of these cells serve as conduits for the virus to reach peripheral sites such as the epithelial cells of the cornea, lip, and skin. The type of virus isolation procedure necessary for detection of HSV depends upon the elapsed time following acute infection. For example, many investigators (1, 3, 5) have isolated infectious virus from homogenized ganglia for 2 weeks following acute infection, but after that time whole cells were required for virus isolation. In the period immediately following inoculation sufficient intracellular infectious virus is present to permit HSV isolation in spite of substantial virus loss caused by tissue homogenization. Later, during latent infection, virus may not be present in an infectious form or may be present in such small amounts as to elude detection by conventional means.

Walz *et al.* (3) found that if virus isolation attempts were delayed 60 days postinfection, then the number of HSV-infected cells in ganglia decreased by at least 90%. If relatively few cells in a piece of tissue are infected, then it would appear to be advantageous to separate the cells composing that tissue. In order to enhance our isolation rates for HSV from latently infected tissues, we employed a method of dispersing cells. We found that the enzymatic cell dispersion not only substantially improves the sensitivity of the virus isolation procedure but also increases the speed of virus recovery.

The action of enzymatic cell dispersion may be only to allow individually infected cells to come into close contact with the indicator monolayer. However, the manipulation may somehow help activate dormant intracellular virus, implement the exit of virus from the cell, or have an effect on the indicator monolayer. While the mechanism of enzymatic cell dispersion on improving isolation of HSV is not known, it clearly has a marked facilitating effect.

Summary. The classical minced tissue method and enzymatic cell dispersion methods were compared for isolating HSV from neural tissues of 48 latently infected and 5 acutely infected rabbits. The ECD method resulted in a 62% HSV isolation rate from 106 trigeminal ganglia samples and the minced tissue method resulted in a 21% HSV isolation rate from the same samples. Thirty-one percent of the 106 midbrain samples were positive for HSV by the ECD method as compared to 6% by the minced method. There were 64 instances when the ECD method detected HSV and the minced method did not. There were no instances in which the minced method alone was positive. The ECD method was faster than or equally as fast as the minced method in HSV detection in 97% of samples tested.

1. Stevens, J. G., and Cook, M. L., Science **173**, 843 (1973).
2. Knotts, F. B., Cook, M. L., and Stevens, J. G., J. Exp. Med. **138**, 740 (1973).
3. Walz, M. A., Yamamoto, H., and Notkins, A. L., Nature (London) **264**, 554 (1976).
4. Nesburn, A. B., Green, M. T., Radnoti, M., and Walker, B., Infect. Immunity **15**, 772 (1977).
5. Nesburn, A. B., Cook, M. L., and Stevens, J. G., Arch. Ophthalmol. **88**, 412 (1972).
6. Goodpasture, E. W., **8**, Medicine (Baltimore) 223 (1929).
7. Martin, R. G., Dawson, C. R., Jones, P., Togni, B., Lyons, C., and Oh, J. O., Arch. Ophthalmol. **95**, 2053 (1977).
8. Baringer, J. R., and Swoveland, P., N. Engl. J. Med. **228**, 248 (1963).
9. Bastain, F. O., Rabson, A. S., Yee, C. L., and Tralka, T. S., Science **178**, 306 (1972).
10. Baringer, J. R., N. Engl. J. Med. **291**, 828 (1974).

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