PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **184**, 50–55 (1987)

# Spread of Herpes Simplex Virus in Lymph Nodes after Experimental Infection of Mice (42445)

RICHARD J. KLEIN AND SHARON M. CZELUSNIAK

*Department of Microbiology, New York University Medical Center, New York, New York 10016*

*Abstract.* Herpes simplex virus was frequently isolated from ipsilateral popliteal lymph nodes after percutaneous inoculation of the dorsal face of the footpad, and from ipsi- and contralateral submandibular lymph nodes after percutaneous inoculation of the cheek or the orofacial area of mice. Virus was detected only on very rare occasion in nondraining lymph nodes (inguinal or axillary) or in contralateral popliteal lymph nodes, but was frequently isolated in contralateral lumbar lymph nodes after footpad inoculation. The presence of virus in lymph nodes paralleled or followed the invasion of ipsilateral sensory ganglia and was associated with dissemination of virus in contralateral sensory ganglia after unilateral inoculation. In older mice virus was detected only occasionally in lymph nodes and dissemination of virus in contralateral sensory ganglia was generally not observed. The results suggest that lymphatic spread may contribute to dissemination of virus in contralateral sensory ganglia after unilateral inoculation of mice. 

The occurence of acute and latent herpes simplex virus (HSV) infections in contralateral sensory ganglia has been repeatedly observed after unilateral inoculation of mice in the cornea, flank, or footpad (1–5). Since there are no direct synaptic connections between the left and the right side sensory ganglia, the spread of virus must be achieved by some unusual pathway. We have shown that contralateral spinal ganglia are colonized by virus 2 to 3 days after the ganglia of the inoculation site (4). The speed of axonal migration of HSV is about 2 to 10 mm/hr (6, 7) and this would permit enough time for the virus to travel along the decussated spino-thalamic tract and return to the contralateral ganglia after reaching the brain stem or the cortex. An alternate to this so called "backdoor" route (8) is the dissemination of virus through leakage into lymphatic or blood vessels.

The regional distribution of lymph nodes offers a good opportunity for the examination of virus dissemination in experimentally infected mice. In this study we show that HSV can be consistently detected in regional lymph nodes and that the pattern of lymphatic dissemination may explain colonization of contralateral ganglia during the acute phase of experimental HSV infections in mice.

**Materials and Methods.** *Virus.* The S strain of HSV type 1 was used in all experiments. The maintenance of the virus, the preparation of stock virus, and the quantification of inocula have been described in a previous publication (9).

*Inoculation of mice.* Female hairless mice of the fully immunocompetent HRS/J strain, and of the Swiss Albino strain were obtained from Jackson Laboratories (Bar Harbor, Maine) and used in the experiments at the age of 8 to 10 weeks, unless otherwise stated. Hairless mice were inoculated percutaneously either in the median area of the snout or unilaterally on the cheek. Swiss Albino mice were inoculated on the dorsal face of the footpad. In all cases inoculation was done by rubbing into the scarified skin a suspension containing $10^6$ PFU/ml of HSV. Approximately $10^4$ PFU were applied in this way on the scarified skin of each mouse.

*Monitoring infectious virus in sensory ganglia and lymph nodes.* At various intervals postinoculation (pi), groups of mice were exsanguinated by heart puncture under sodium pentobarbital anesthesia. From mice inoculated on the footpad the spinal ganglia, the popliteal, lumbar, and inguinal lymph nodes were removed. From mice inoculated in the orofacial area or the cheek, trigeminal ganglia and submandibular and axillary lymph nodes were removed. In all cases specimens ipsi- and contralateral of the inoculation site were collected separately. During the removal of specimens, special precautions were taken to avoid



0037-9727/87 $1.50

cross-contamination of organs harboring infectious virus; i.e., separate surgical instruments were used for each speciman, and the removal order of specimens started with those containing the least amount of virus (inguinal or axillar lymph nodes) and ended with those containing the highest amount of virus (sensory ganglia). All specimens were homogenized immediately by sonication (Branson Sonifer Cell Disruptor 200) and clarified by centrifugation. The supernatants were screened for presence of infectious virus in human fibroblasts (FS7 cells), and virus-containing samples were titrated by a plaque assay on Vero cells.

**Results.** *Dissemination of HSV after footpad inoculation.* Infectious virus was detected in ipsilateral spinal ganglia by the fourth day pi, and in contralateral ganglia by the sixth day pi. Between Days 5 and 7 pi virus was isolated in 50% of the ipsilateral popliteal lymph nodes, but was present only sporadically in ipsilateral inguinal and lumbar lymph nodes. Virus was not detected in contralateral inguinal lymph nodes, and was isolated only once from contralateral popliteal lymph nodes. However, on Days 6 and 7 pi, infectious virus was detected in 9 out of 15 contralateral lumbar lymph nodes, as opposed to only two isolates from the ipsilateral site. The difference is statistically significant ($P < 0.01$, Fisher's Exact Test). These results are summarized in Table I.

The virus titer in any of the 27 infected lymph nodes was low; in two-thirds of the specimens less than 10 pfu and only in two samples more than 100 pfu were detected. Relative higher titers (10 to 100 pfu) were found among the popliteal lymph nodes of the ipsilateral site and the lumbar nodes of the contralateral site. Virus titers in the ipsilateral spinal ganglia were on the average 10 to 100 times higher than titers in contralateral spinal ganglia (data now shown).

Mice used in the above experiments were between 8 and 10 weeks old. Animals from the same lots were inoculated in the same way at the age of 17 weeks or more. As can be seen in Table II, the frequency of virus isolation from the ipsilateral spinal ganglia was similar in young and older mice. However, the frequency of virus isolation from the contralateral spinal ganglia of older mice was significantly lower ($P < 0.02$). Likewise, in older mice colonization of ipsilateral spinal ganglia was not associated with virus dissemination in ipsilateral popliteal lymph nodes, and the absence of virus in contralateral spinal ganglia was paralled by absence of virus in contralateral lumbar lymph nodes.

*Dissemination of HSV after cheek inoculation.* After unilateral cheek inoculation virus was detected in all ipsilateral trigeminal ganglia from the second through the last day of the 7-day observation period. Infectious virus was isolated on Day 3 pi from the contralateral trigeminal ganglia, and was present in 50 to 75% of the ganglia through Day 7 pi. Infectious virus, in both ipsi- and contralateral lymph nodes, was detected 1 day after the appearance of virus in ipsilateral trigeminal ganglia. The

TABLE I. DISSEMINATION OF HERPES SIMPLEX VIRUS IN SPINAL GANGLIA AND LYMPH NODES AFTER UNILATERAL FOOTPAD INOCULATION OF SWISS ALBINO MICE

| Days after virus inoculation | Frequency of virus isolation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ipsilateral side | | | | Contralateral side | | | |
| | Spinal ganglia | Lymph nodes | | | Spinal ganglia | Lymph nodes | | |
| | | Popliteal | Inguinal | Lumbar | | Popliteal | Inguinal | Lumbar |
| 3 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| 4 | 4/4 | 1/4 | 0/4 | 1/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| 5 | 6/7 | 3/7 | 0/4 | 0/7 | 0/7 | 0/7 | 0/4 | 0/7 |
| 6 | 8/8 | 5/8 | 2/4 | 2/8[a] | 3/8 | 0/8 | 0/4 | 5/8[a] |
| 7 | 7/7 | 3/7 | 0/4 | 0/7[a] | 6/7 | 1/7 | 0/4 | 4/7[a] |

[a] The probability that the difference between the frequency of virus isolation from ipsi- and contralateral lumbar lymph nodes is due to chance is less than 0.01. (Calculated for Days 6 and 7 after virus inoculation.)

TABLE II. ROLE OF AGE IN THE DISSEMINATION OF HERPES SIMPLEX VIRUS IN SPINAL GANGLIA AND LYMPH NODES OF SWISS ALBINO MICE AFTER UNILATERAL FOOTPAD INOCULATION

| Age of mice (weeks) | Frequency of virus isolation (Days 6 and 7 pi) | | | | | |
|---|---|---|---|---|---|---|
| | Ipsilateral side | | | Contralateral side | | |
| | Spinal ganglia | Lymph nodes | | Spinal ganglia | Lymph nodes | |
| | | Popliteal | Lumbar | | Popliteal | Lumbar |
| 10 or less[a] | 15/15 | 8/15 | 2/15 | 9/15 | 1/15 | 9/15 |
| 16 or more | 11/13 | 2/13[b] | 0/13 | 2/13[b] | 0/13 | 0/13[b] |

[a] Data from Table I.

[b] The frequency of virus isolation is statistically significantly lower in ipsilateral popliteal lymph nodes ($P < 0.04$), contralateral spinal ganglia ($P < 0.02$), and contralateral lumbar lymph nodes ($P < 0.001$) of older than younger mice (Fisher's Exact Test).

virus remained detectable in lymph nodes up to Day 6 pi. The highest proportion of virus-positive submandibular lymph nodes was observed between Days 4 and 6 pi (67% on the ipsilateral site, 42% on the contralateral site). In the nondraining axillary lymph nodes, infectious virus was isolated only from a single sample on Day 7 pi. These results are shown in Table III.

As can be seen in Table IV, the frequency of virus-containing samples is after symmetrical inoculation of in the middle of the orofacial area of the hairless mouse is similar in left and right side trigeminal ganglia and lymph nodes.

Virus titers in the trigeminal ganglia of symmetrically inoculated mice were also similar and of the same order of magnitude as virus titers in ipsilateral trigeminal ganglia of unilaterally inoculated mice, whereas the virus titers in contralateral trigeminal ganglia were 50 to 100 times lower (Table V). Virus titers in submandibular lymph nodes were low, but higher than in lymph nodes colonized by virus after footpad inoculation: 53% of the lymph nodes contained more than 10 pfu of HSV

TABLE III. DISSEMINATION OF HERPES SIMPLEX VIRUS IN TRIGEMINAL GANGLIA AND LYMPH NODES AFTER UNILATERAL CHEEK INOCULATION OF HAIRLESS MICE

| Days after virus inoculation | Frequency of virus isolation | | | | | |
|---|---|---|---|---|---|---|
| | Ipsilateral side | | | Contralateral side | | |
| | Trigeminal ganglia | Lymph nodes | | Trigeminal ganglia | Lymph nodes | |
| | | Submandibular | Axillar | | Submandibular | Axillar |
| 1 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| 2 | 4/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |
| 3 | 4/4 | 1/4 | 0/4 | 1/4 | 1/4 | 0/4 |
| 4 | 4/4 | 3/4 | 0/4 | 2/4 | 1/4 | 0/4 |
| 5 | 4/4 | 2/4 | 0/4 | 3/4 | 2/4 | 0/4 |
| 6 | 4/4 | 3/4 | 0/4 | 3/4 | 2/4 | 0/4 |
| 7 | 3/4 | 0/4 | 1/4 | 2/4 | 0/4 | 0/4 |
| Total days, 3 to 7 | 19/20[a] | 9/20 | 1/20 | 11/20[a] | 6/20 | 0/20 |

[a] The frequency of virus isolation from ipsilateral trigeminal ganglia was significantly higher than in contralateral trigeminal ganglia ($P < 0.025$, $\chi^2$ test).

TABLE IV. DISSEMINATION OF HERPES SIMPLEX VIRUS IN TRIGEMINAL GANGLIA AND SUBMANDIBULAR LYMPH NODES AFTER MEDIAN OROFACIAL INOCULATION OF HAIRLESS MICE

| Days after virus inoculation | Frequency of virus isolation | | | |
|---|---|---|---|---|
| | Left side | | Right side | |
| | Trigeminal ganglia | Submandibular lymph nodes | Trigeminal ganglia | Submandibular lymph nodes |
| 3 | 2/4 | 1/4 | 3/4 | 0/4 |
| 4 | 4/4 | 2/4 | 4/4 | 3/4 |
| 5 | 4/4 | 1/4 | 4/4 | 3/4 |
| 6 | 4/4 | 3/4 | 4/4 | 1/4 |
| 7 | 4/4 | 1/4 | 4/4 | 1/4 |
| Total days, 3 to 7 | 18/20 | 8/20 | 19/20 | 8/20 |

and two submandibular lymph nodes contained over $10^3$ pfu of HSV after orofacial infection. A summary of virus titers in lymph nodes is given in Table VI.

**Discussion.** The results obtained in this series of experiments showed that invasion of regional lymph takes place consistently after percutaneous inoculation of mice with HSV. Nondraining lymph nodes (inguinal after footpad inoculation, and axillary after orofacial or cheek inoculation) are involved only in very rare instances. Colonization of lymph nodes parallels or follows virus invasion, but was not observed before the invasion of sensory ganglia with virus. After subcutaneous inoculation of HSV type 2 in the footpad of guinea pigs virus was detected in all popliteal lymph nodes as soon as 6 hr pi, and before the isolation of virus from spinal ganglia (10). This would suggest a direct invasion of lymph nodes after subcutaneous infection, circumventing the axonal transport of virus.

The highest frequency of virus isolation from lymph nodes was observed 4 to 6 days after virus inoculation. However, only up to 75% of the lymph nodes contained virus on any one day pi, and virus titers were low in the majority of cases: 88% of the specimens contained less than 100 pfu and one-third less than 10 pfu of HSV.

As expected, after symmetrical inoculation of the orofacial area, HSV was detected at the same frequency in the left and the right side submandibular lymph nodes and trigeminal ganglia. However, after unilateral inoculation of the cheek HSV was detected only slightly less frequently in the contralateral than in the ipsilateral lymph nodes. On the other hand, the frequency of virus isolation in trigeminal ganglia from days 3 to 7 pi is significantly

TABLE V. VIRUS TITERS IN TRIGEMINAL GANGLIA OF HAIRLESS MICE AFTER SYMMETRICAL (MEDIAN) OR UNILATERAL (CHEEK) HERPES SIMPLEX VIRUS INOCULATION

| Days after virus inoculation | Mean virus titers in trigeminal ganglia[a] | | | |
|---|---|---|---|---|
| | Median symmetrical inoculation | | Unilateral inoculation | |
| | Right | Left | Ipsilateral | Contralateral |
| 4 | $3.23 \pm 0.62$ | $3.76 \pm 0.59$ | $3.77 \pm 0.85$ | $2.04 \pm 0.50$ |
| 5 | $3.56 \pm 0.16$ | $3.42 \pm 0.74$ | $3.57 \pm 0.69$ | $1.35 \pm 0.45$ |
| 6 | $2.92 \pm 0.55$ | $2.17 \pm 0.95$ | $2.46 \pm 1.20$ | $1.23 \pm 0.48$ |
| 7 | $1.38 \pm 0.59$ | $1.71 \pm 0.27$ | $1.23 \pm 0.13$ | 0.30 |

[a] PFU per milliliter ($\log_{10}$ units) $\pm$ SD.

TABLE VI. VIRUS TITERS IN LYMPH NODES DURING THE ACUTE PHASE OF HSV INFECTION IN MICE

| Lymph nodes | Number of lymph nodes with virus titers (pfu) of | | | |
|---|---|---|---|---|
| | <10 | $10-10^2$ | $10^2-10^3$ | $>10^3$ |
| Popliteal | 10 (67)[a] | 3 (20) | 2 (13) | 0 |
| Lumbar | 8 (67) | 4 (33) | 0 | 0 |
| Submandibular | 15 (47) | 12 (38) | 3 (9) | 2 (6) |
| All lymph nodes | 33 (56) | 19 (32) | 5 (9) | 2 (3) |

[a] In parentheses frequencies expressed as percentages.

higher in the ipsilateral (19/20) than in the contralateral (11/20) site ($P < 0.025$, $\chi^2$ test).

It is likely that the colonization of the contralateral trigeminal ganglion is the result of natural propagation of the cutaneous skin infection. Grooming of the oral area may have contributed also to the spread of infection to skin areas innervated by the contralateral trigeminal ganglion. These mechanisms are less efficient than the artificial inoculation done on the cheek and resulted therefore in a significantly lower rate of contralateral ganglionic infections. The detection of virus in submandibular lymph nodes is a consequence of the local infection and is not related to colonization of trigeminal ganglia with HSV.

The results obtained after unilateral footpad inoculation suggest several interesting conclusions and observations. In the first place we were able to confirm our previous data, indicating that contralateral spinal ganglia are regularly invaded by HSV after unilateral footpad inoculation (4). Second, we found infectious virus consistently in draining popliteal lymph nodes of the inoculation site, but virus was isolated only occasionally from nondraining inguinal lymph nodes, or from popliteal lymph nodes of the noninoculated footpad. The most interesting observation is the significantly higher frequency of virus isolation from the contralateral than the ipsilateral lumbar lymph nodes. The detection of virus in contralateral lumbar lymph nodes was always associated with isolation of virus from contralateral spinal ganglia.

The draining area of lumbar lymph nodes includes the lumbosacral segment of the spinal cord. Virus detected in contralateral lumbar lymph nodes could originate therefore from virus which has migrated in the spinal cord after the initial colonization of the ipsilateral spinal sensory ganglia. The leakage of HSV in extraneural space might be explained in the following way: virions which migrate axonally from the inoculation site to the neurons of sensory ganglia become established rapidly in a latent form (11). However, continuous virus supply from the inoculation site will lead not only to further virus migration by neural pathways to the spinal cord and brain, but also to leakage of virus in extraneural space of the spinal cord, which might then be drained by lymphatic vessels to the lumbar lymph nodes. The frequent isolation of HSV from contralateral lumbar lymph nodes during the later phase of the acute infection supports the likelihood of such a mechanism. Contralateral spinal ganglia may then become colonized by HSV either through the network of lymphatic vessels, or by migration of virus along efferent sympathetic fibers innervating the lymph nodes (12).

Further support for a role of lymphatic dissemination in the colonization of contralateral spinal ganglia by virus comes from the results observed in older mice. In these animals, the frequency of colonization of ipsilateral spinal ganglia after footpad inoculation is not different from that observed in younger mice, but contralateral spinal ganglia become colonized by virus at a statistically significant lower rate. The absence of virus in contralateral spinal ganglia was associated with a statistically significant lower rate of virus isolation from ipsilateral popliteal lymph nodes and the absence of virus isolation from contralateral lumbar lymph nodes. Since the dissemination of virus in contralateral spinal ganglia was always associated with a relative high rate of virus isolation from ipsilateral popliteal and contralateral lumbar lymph nodes, it appears that leakage and lymphatic spread play a role in the pathogenetic mechanism of HSV infections in younger mice.

HSV was isolated also from human blood lymphocytes (13, 14), but it appears that virus isolation in the lymphatic system was not systematically investigated. Recently, HSV-like capsids were observed in giant cells of an inguinal lymph node from a patient with lymphadenitis (15). Our data suggest that lym-

phatic dissemination might be more restricted in older than in younger mice. The possibility of lymphatic dissemination is relevant to the treatment of primary HSV infections, especially in the newborn. The presence of virus in regional lymph nodes during the acute phase of infection may elicit and maintain strong local immune responses, able to fend off reinfections occurring at the site of the primary inoculation. Some of these aspects are currently being investigated in our laboratory.

We thank Eileen Brady for her excellent technical assistance. This research was supported in part by funds from the Mellon Foundation.

1. Knotts FB, Cook ML, Stevens JG. Latent herpes simplex in the CNS of rabbits and mice. J Exp Med **138:** 740–744, 1973.
2. McKendall RR, Klassen T, Baringer JR. Host defenses in herpes simplex infections of the nervous system: Effect of antibody on disease and viral spread. Infect Immun **23:**305–311, 1979.
3. Tullo AB, Shimeld C, Blyth WA, Easty DL. Spread of virus and distribution of latent infection following ocular herpes simplex in the non-immune and immune mouse. J Gen Virol **63:**95–101, 1982.
4. Klein RJ, DeStefano E. Dissemination of herpes simplex virus in ganglia after footpad inoculation in neurectomized and non-neurectomized mice. Arch Virol **77:**231–238, 1983.
5. Klein RJ. Initiation and maintenance of latent herpes simplex virus infections: The paradox of perpetual immobility and continuous movement. Rev Infect Dis **7:**21–30, 1985.
6. Cook ML, Stevens JG. Pathogenesis of herpetic neuritis and ganglionitis in mice: Evidence of intra-axonal transport of infection. Infect Immun **7:**272–288, 1973.
7. Kristensson K, Lycke E, Sjostrand J. Spread of herpes simplex virus in peripheral nerves. Acta Neuropathol **17:**44–53, 1971.
8. Tullo AB, Easty DL, Hill JJ, Blyth WA. Ocular herpes simplex and the establishment of latent infections. Trans Opthalmol Soc UK **102:**15–18, 1982.
9. Klein RJ, Friedman-Kien AE, Fondak AA, Buimovici-Klein E. Immune response and latent infection after topical treatment of herpes simplex virus infection in hairless mice. Infect Immun **16:**842–848, 1977.
10. Scriba M, Tatzber F. Pathogenesis of herpes simplex virus infections in guinea pigs. Infect Immun **34:**655–661, 1981.
11. Klein RJ, Friedman-Kien AE. Effect of 9-(1,3-dihydroxy-2-propoxymethyl) guanine on the acute local phase of herpes simplex virus-induced skin infections in mice and the establishment of latency. Antimicrob Agents Chemother 27:763–768, 1985.
12. Felten DL, Livnat S, Felten SY, Carlson SL, Lorton Bellinger D, Yeh P. Sympathetic innervation of lymph nodes in mice. Brain Res Bull **13:**693–699, 1984.
13. Craig CP, Nahmias AJ. Different patterns of neurologic involvement with herpes simplex virus type 1 and 2: Isolation of herpes simplex virus type 2 from the buffy coat of two adults with meningitis. J Infect Dis **127:**365–372, 1973.
14. Naraqui S, Jackson GG, Jonasson OM. Viremia with herpes simplex type 1 in adults: Four nonfatal cases, one with features of chickenpox. Ann Int Med **85:** 165–169, 1976.
15. Taxy JB, Tillawi I, Goldman PM. Herpes simplex lymphadenitis: An unusual presentation with necrosis and viral particles. Arch Pathol Lab Med **109:**1043–1044, 1985.

Received March 17, 1986. P.S.E.B.M. 1987, Vol. 184.
Accepted September 24, 1986.