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Striated muscle involvement in experimental oral infection by herpes simplex virus type 1

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Herpes simplex virus type 1 is one of the most frequent causes of oral infection in humans, especially during early childhood. Several experimental models have been developed to study the pathogenesis of this virus but all of them employed adult animals. In this work, we developed an experimental model that uses mice younger than 4 days old, to more closely resemble human infection. Mice were infected subcutaneously with the prototype strain McIntyre of Herpes simplex-1, and the progression of infection was studied by immunoperoxidase. All animals died within 24–72 h post-infection, while viral antigens were found in the oral epithelium, nerves and brain. The most striking result was the finding of viral antigens in the nucleus and cytoplasm of cells belonging to striated muscles. Organotypic cultures of striated muscles were performed, and viral replication was observed in them by immunocytochemistry, electron microscopy and viral isolation. We conclude that the infection of striated muscles is present from the onset of oral infection and, eventually, could explain some clinical observations in humans.
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Introduction

Herpes simplex virus type 1 (HSV-1) is one of the most frequent etiological agents associated with oral viral infections (1). It is accepted that the primary infection with this virus occurs early in childhood through the transmission of infective viruses by person-to-person close contact (2, 3). Frequently, clinical signs are not observed during HSV-1 primary infection. Herpes simplex virus has to first replicate in the skin and mucosae before gaining access to the peripheral nerves, but in humans, the nature of this process is still far from clear. After replication in the oral epithelium, the virus establishes a life-long latent infection in the Gasser's trigeminal ganglion, and oral recurrences can be observed from time to time (4). Moreover, clinical observations have associated primary HSV-1 infections or recurrences with transient paralysis of facial muscles (5, 6).

Several experimental models have been developed to study the progression of HSV type 1 and type 2 after oral or genital infections. Adult mice (7–10), rats (11, 12) hamsters (13, 14) and guinea pigs (15) have been used as experimental animals, and different methods have been employed to demonstrate that the virus advances through the nerves and fibers belonging to the peripheral (16) and the autonomic (17) nervous system. However, all those models have employed adult animals, while most natural infections in humans happen very early in life.

Thus, in this work, we developed a new experimental model using newborn mice instead of adult animals to perform a step-by-step description of HSV-1 productive oral infection. The combined use of immunocytochemistry in paraffin-embedded tissues, transmission electron microscopy and classic virological methods allowed us to study in detail the structures infected by the virus at different time-points.

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Materials and methods

Virus

The HSV-1 prototype strain McIntyre (American Type Culture Collection) was used throughout the experiment. Virus stock was produced in Vero cell cultures. When cytopathic effect was complete, cells were harvested, frozen and thawed three times and centrifuged to eliminate the debris. Supernatants were aliquoted and maintained in liquid nitrogen until use. Viral stocks were titrated in Vero cells by plaque assay under methyl cellulose as described previously (18).

Cells

Vero cells were obtained from the Instituto Nacional de Microbiología 'Dr. Carlos G. Malbrán', Buenos Aires,

Argentina and cultured in plastic Petri dishes with Dulbecco Modified Eagle's Medium (DMEM; Gibco BRL, Burlington, VT, USA) and 10% calf serum. Penicillin (100 UI/ml) and Streptomycin (1 μg/ml) were also added to the culture medium.

Animals
Balb/c mice (specific-pathogen-free) were obtained from the bioterium of the University of La Plata, School of Veterinary Medicine (Argentina). Newborn mice were kept with their mothers in the same box. Each box contained only one mother. Mice were fed on pellets *ad libitum*. Animal welfare was accomplished according to the regulations established by the University of Buenos Aires, School of Medicine Guidelines for Laboratory Animal Care.

Experimental design
Thirty two newborn mice (2–4 days old) were inoculated subcutaneously in the upper lip with 6×10^5 plaque-forming units (pfu) of HSV-1 contained in 0.05 ml of cell culture medium under sterile conditions. A control group composed of 15 animals was mock-infected in the upper lip with the supernatant of uninfected Vero cell cultures.

Clinical signs, including those at the inoculation site or neurological, were recorded daily. Infected mice died spontaneously or were sacrificed in a *pre-mortem* state after 24, 48 and 72 h post-infection (pi). The heads were cut off and fixed immediately in Bouin fluid. Some 12-h later, five serial frontal slices were obtained, from the snout to the occipital bone, fixed 12 h more and then kept in 70% ethanol. As the bones were not calcified yet, it was not necessary to treat them with any decalcifying procedure. Tissues were routinely embedded in paraffin and suitable sections were stained with Mayer Hematoxylin and Eosin (H-E). Adjacent sections were taken for viral antigen immunolabeling.

Immunocytochemical method
The peroxidase-antiperoxidase (PAP) technique was employed as described previously (19). Briefly, paraffin-embedded sections were treated with xilene and decreasing concentrations of ethanol and then washed with 0.05 M Tris–HCl pH 7.6. After blocking any possible unspecific reaction with 5% normal goat serum, a rabbit anti-HSV-1 policlonal serum was used, with a dilution of 1:1000. Then, a goat anti-rabbit serum was employed at 1:50, and finally, the unlabeled peroxidase-antiperoxidase complex developed in rabbit was added at a concentration of 1:250. All sera were obtained from Dako® (Carpinteria, CA, USA) and between one and the other, extensive washings with 0.05 M Tris–HCl pH 7.6 were performed. Slides were developed with 3-3′ diaminobenzidyne (DAB) and 2% H_2O_2 in the same buffer under microscopy, then slightly counterstained with Mayer Hematoxylin. The labeling specificity was confirmed using slides of paraffin-embedded brains belonging to mice infected with HSV-1 through intracerebral route, as positive controls. Negative control slides were obtained from uninfected mouse brains and adjacent slides to those used to detect the presence of HSV-1 antigens were treated with normal rabbit serum and used as internal controls.

Organotypic cultures
The tongue of newborn (2 days old) mice were carefully dissected, quickly soaked in iodated ethanol to avoid bacterial infections, washed thoroughly in sterile distilled water and minced into 0.5-mm pieces in DMEM plus 10% fetal calf serum. The small pieces were then cultured in the air–medium interface created by placing the tissues over Spongostan® (Johnson & Johnson, New Brunswick, NJ, USA) strips into plastic Petri dishes with DMEM plus 10% fetal calf serum. Cultures were incubated at 37°C and an atmosphere of 5% CO_2-95% air. Several organotypic cultures were infected with HSV-1 by adding the virus directly on the strips of Spongostan with a final concentration of 10^6 plaque-forming units (pfu)/ml, while others were mock-infected with the supernatant of uninfected Vero cell cultures. The infectious dosage was the highest possible to optimize the viral entrance into the small blocks of tissue.

At 2, 4 and 6 days pi, organotypic cultures were harvested and divided into three groups of samples: one was immediately minced, fixed and processed for electron microscopy studies, other samples were fixed and embedded in paraffin, and the last one was kept frozen. Samples for electron microscopy were immediately cut into 0.2-mm pieces into a drop of cold (4°C) Karnovsky's fixative in a glass Petri dish, then transferred to an eppendorf tube with the same fixative and later on, soaked in 1% osmium tetroxide and routinely embedded (see below). To demonstrate the presence of infectious virus, frozen organotypic cultures were thawed and dounce-homogeneized in DMEM and centrifuged. Supernatants of infected or uninfected striated muscle cultures were adsorbed 1 h onto Vero cell cultures developed on glass coverslips contained in sterile plastic Petri dishes and then washed with DMEM and incubated for further 48 h at 37°C in a 5% CO_2 atmosphere. The presence of HSV-1 antigens was confirmed by indirect immunofluorescence.

Indirect immunofluorescence (IFI)
Coverslips containing Vero cell cultures adsorbed with homogenates obtained from infected or mock-infected striated muscle organotypic cultures were fixed in methanol, washed in phosphate-buffered saline (PBS) and treated with 5% normal goat serum for 1 h. The first serum was employed with a polyclonal rabbit anti-HSV-1 with a 1:500 dilution (the same serum used in the PAP technique) during 1 h. After being washed again with PBS, a TRICT-conjugated goat serum anti-rabbit immunoglobulins was added. Coverslips were washed for a second time, mounted on a glass slide with glycerol and observed in a Zeiss microscope with epi-illumination.

Transmission electron microscopy (TEM)
Tissues were minced into 0.5-mm pieces and immediately fixed with 4% formaldehyde (freshly prepared from para-formaldehyde) and 2% glutaraldehyde in PBS buffer with 5% sucrose (Karnovsky's fixative).

After post-fixation with 1% osmium tetroxide, specimens were embedded routinely in Vestopal® (Electron Microscopy Sciences, Hatfield, PA, USA), cut with glass knives and stained with uranyl acetate and lead citrate as reported previously (20). Grids were observed in a Zeiss EM-109-T transmission electron microscope at 80 kV.

Results

Clinical course of infection

Ten HSV-1 – infected mice were found dead in their boxes and partially eaten by their mothers a few hours after inoculation. Thus, only 22 mice were included in the experiment. HSV-1-infected mice developed overt clinical signs and all of them died or were sacrificed when they were found *pre-mortem* between 24 and 72 h pi, while all the mock-infected mice survived without showing any clinical signs. Six of 22 infected mice developed edema and erythema at 24 h pi at the site of inoculation, although no clinical signs of acute infection could be observed in the oral mucosae. All infected mice showed neurological signs, such as seizures, hindlimb paresia and lethargy, as well as cyanosis and troubles in feeding between 48 and 72 h pi. Deaths occurred at this time-point.

Viral progression

At 24 h pi, HSV antigens were detected in the epithelium of the upper lips at the inoculation site in a patchy fashion (Fig. 1A) in 18 of 22 animals (81.82%) and in small nerves and fibers located around the tongue and the connective tissue surrounding striated muscles. At 48 h pi, several nerves and also ganglia belonging to the central nervous system showed positive labeling for HSV-1 antigens (Fig. 1B,C) in 11 of 22 mice. At 72 h pi, axons belonging to larger nerves and foci of neuronal and glial cells in the brain also showed HSV-1 antigens (Fig. 1D) in seven of 22 mice. Viral antigens were mainly located in the nucleus of the cells, although the cytoplasm also showed antigens. This was particularly evident in the brain neurons (Fig. 1D). No inflammatory infiltrate could be observed by histology in any of the PAP-positive tissues described above. Mock-infected animals showed no positive labeling by PAP. Slides taken from HSV-1-infected animals but treated with normal rabbit serum in the PAP staining were also negative.

Skeletal muscle infection

HSV-1 antigens could be observed by PAP in striated muscles as early as 24 h pi (Fig. 2). Muscles located under the tongue as well as those involved in masking were compromised by HSV-1 infection at the same time as the nerve fibers. This was observed in nine of 22 mice infected with HSV-1 (40.91%) but not in the mock-infected animals. HSV-1 antigens were located in the nucleus and in the cytoplasm of the striated muscle cells. However, muscles were not necrotic and no inflammatory response could be observed.

Organotypic culture infection

HSV-1 antigens were detected by PAP labeling at every single time-point pi. (Fig. 3A), while adjacent slides treated with normal rabbit serum (internal control) showed no immunolabeling at all. The mock-infected tissues were negative, as expected. HSV particles were observed in the nuclei and in the perinuclear cisternae (namely nuclear membrane) by transmission electron microscopy (Fig. 3B). Infectious HSV was also recovered from infected muscle organotypic cultures after infection of Vero cell cultures and viral detection by IFI (Fig. 4).

Discussion

In this work, we produced an acute primary oral infection by HSV-1 in newborn mice, to investigate the early events of viral progression after inoculation in the lips. The first surprising result was that the mortality of the newborn mice infected with HSV-1 was 100%. This is completely different from what has been described in adult mice, where a latent infection establishes after the acute episode of the disease, with minimal mortality. Newborn mice did not develop ulcers in the lips or in the

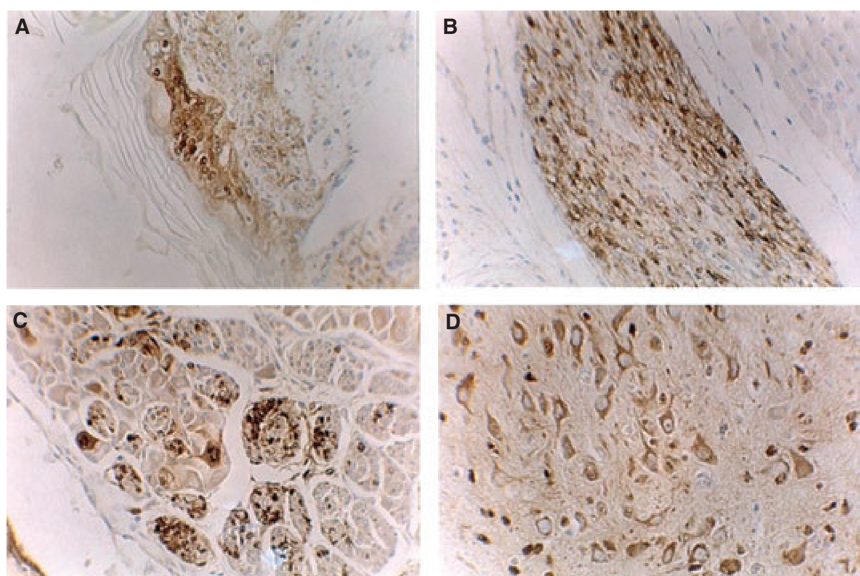

Figure 1 Viral progression. (A) Adjacent skin to the oral mucosae: HSV-1 antigens are present in the nucleus of epidermal cells (dark staining). (B) large nerve with positive-labeled axons. (C) neural ganglion: notice the intranuclear staining of neurons. (D) Small area of brain tissue with HSV-1 antigen. Curiously, labeling is mainly present in cytoplasm. X: 300.

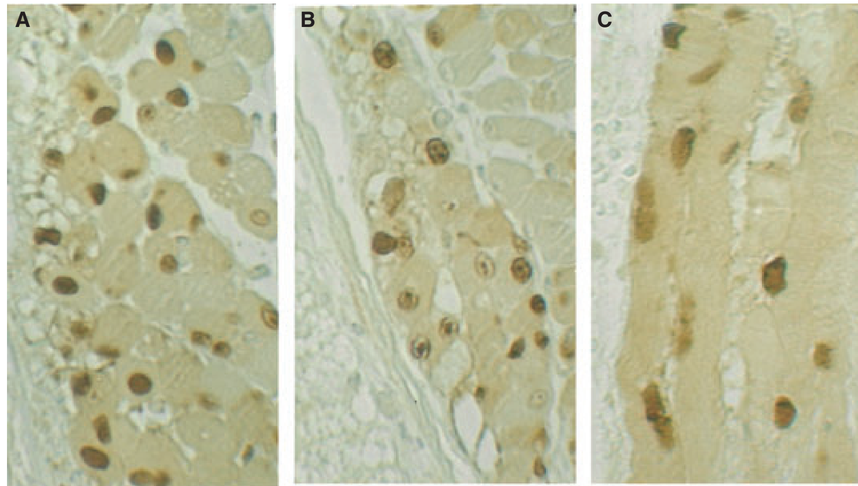

Figure 2 Skeletal muscle infection. Transversal (A,B) and longitudinal (C) cuts of striated muscle cells positive labeled for HSV-1 antigens. Notice the remarkable intranuclear staining (dark). X: 600.

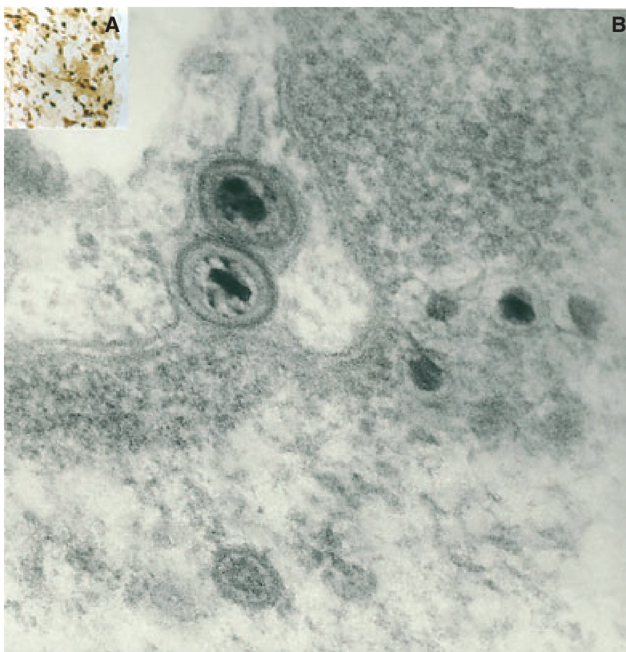

Figure 3 Organotypic culture infection. (A) Striated muscle organotypic culture showing HSV-1 antigens by peroxidase-antiperoxidase (PAP) (dark labeling) after 48 h pi (X: 300). (B) Ultrastructural observation of HSV-1 particles into the perinuclear cisternae. These images demonstrate viral replication in cultures developed *in vitro*. X: 60 000.

oral mucosae and this may be due to the fact that the inoculation was carried out by subcutaneous injection instead of superficial erosion, as it has been proposed as a mechanism for human infection. The observation of HSV-1 antigens in small and large nerves, as well as their location in the cerebrum, confirms findings reported previously in adult animals by other authors.

However, the most striking result presented here was the involvement of striated muscles as targets of HSV-1 after infection in the lips. Muscles located under the tongue, as well as lateral and inner muscles of the head showed intranuclear and intracytoplasmic HSV-1 antigens as early as 24 h pi and continued to be observed until the end of the experiment. To establish whether the presence of HSV-1 in these structures occurred due to viral amplification or it was just a transitory presence of the virus while spreading, some organotypic cultures of muscular tissue were carried out, and *in vitro* infected. In all cases, HSV-1 was able to replicate in these explants, and the amount of virus increased as the infection progressed, according to the results obtained by PAP. The specificity of the labeling was confirmed, because the treatment of adjacent slides with normal rabbit serum did not show any staining. The ultrastructural study of these infected cultures also demonstrated the presence of viral nucleocapsids inside the nucleus and the existence of viral particles budding or traveling into the perinuclear cisternae. Moreover, HSV-1 could be recovered at different time-points pi from these organotypic cultures and cultured in Vero cell monolayers, where HSV antigens could be demonstrated by IFI. Taken together, the observation of HSV-1 antigens and viral particles in striated muscle cells, as well as the recovery of infectious viruses from the organotypic cultures, clearly demonstrate an active viral replication in theses tissues. It also suggests that this is not an isolated phenomenon, as it was observed in seven of 22 animals. As far as we know, this is the first description of muscle infection by HSV-1 after oral inoculation.

A possible explanation of the results described above is that, after an initial replication of HSV-1 in the lip mucosae, viruses reach the small nerves and advance into their axons toward larger nerves, finally reaching the brain. But it is also possible that, via the nerves or directly from the inoculation site, striated muscles were infected as well, maybe as a mechanism of viral amplification.

Without venturing to extrapolate results obtained in mice to humans, it is hoped that the results described here will lead to further clinical investigations to determine whether the compromise of striated muscles observed during human oral first-infection or reactivation by HSV-1 (such as transient paralysis or swallowing disturbances) are due to

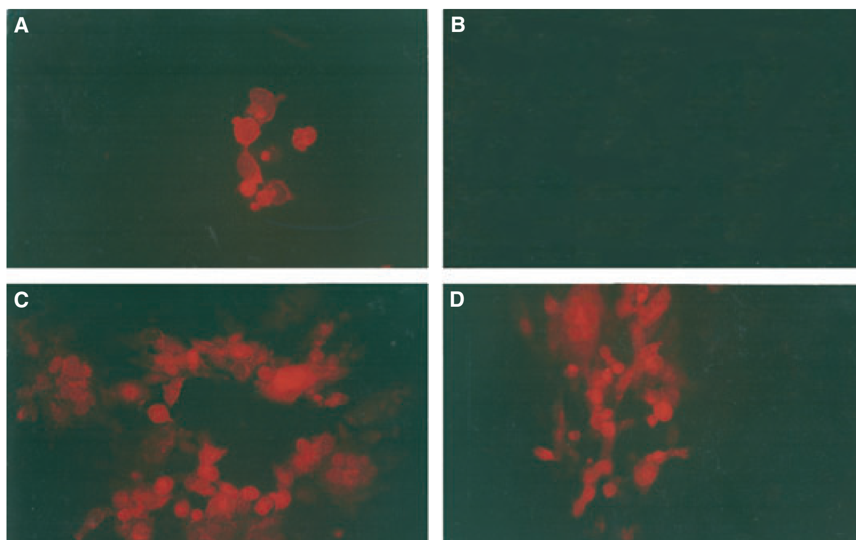

Figure 4 Vero cell cultures inoculated with homogenates of HSV-infected striated muscle organotypic cultures. HSV antigens were detected by IFI. (A) Positive control: cells infected with diluted HSV-1 stock. (B) Negative control: cells inoculated with the supernatant of uninfected Vero cells. (C,D) cells inoculated with two samples of organotypic cultures homogenates, *in vitro* infected with HSV-1. Viral positive-immunolabeling and cytophatic effects are clearly seen. X: 300.

an impairment of muscle innervations or to an active and transient viral replication in the muscle cells.

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Conflict of interests

The authors declare no conflict of interest.