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PROGRESSION OF INTRAVAGINAL INFECTION BY HERPES SIMPLEX-2 IN GENETICALLY ATHYMIC MICE

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Abstract The purpose of this paper was to study the pathogenesis of wild-type Herpes simplex-2 (HSV-2) primary intravaginal (IVAG) infection in genetically athymic (nude) mice. Nude (nu/nu) N: NIH(S) and Balb/c mice, as well as their euthymic counterparts were IVAG infected with 5 x 10^5 pfu of HSV-2. The progression of the infection was followed by HSV-2 immunolabeling using the peroxidase-antiperoxidase technique in tissue sections of the whole body, electron microscopy, and viremia titration at two different timepoints. 70% of athymic NIH mice, 30% of euthymic NIH mice, and 80% of both athymic and euthymic Balb/c mice developed acute vulvovaginitis and died between 8-10 days post-infection (pi). Viremia was not detected in either athymic or euthymic mice. HSV-2 replicated in the vulvovaginal, vesical and perianal epithelia, then progressed towards the central nervous system mainly along autonomic nerves and ganglia. HSV-2 antigens were not detected in liver, spleen, kidney, skin, heart, lung or bone marrow. The conclusion is that the T-cell immune response seems to limit the IVAG infection of NIH mice at the inoculation site, but is not involved in preventing HSV-2 dissemination through the blood.

Resumen ***Progresión de la infección intravaginal por virus Herpes simplex-2 en ratones genéticamente atímicos.*** En este trabajo se estudió la patogénesis de la infección primaria intravaginal (IVAG) por virus Herpes simplex-2 (HSV-2) en ratones genéticamente atímicos (nude). Se emplearon ratones nu/nu de las cepas N:NIH(S) y Balb/c. Cada animal fue infectado IVAG con 5 x 10^5 ufp de una cepa salvaje de HSV-2. El seguimiento de la infección se realizó por cortes seriados de cuerpo entero, que fueron estudiados con peroxidasa-antiperoxidasa contra antígenos de HSV-2, microscopia electrónica de transmisión, y determinación de viremia a los 5 días post-infección (pi) y en estado pre-mortem (8-10 días pi). La mortalidad por infección con HSV-2 fue del 70% en los ratones atímicos NIH, contra el 30% de su contraparte eutímica ($p < 0.02$). En la cepa Balb/c, tanto los animales atímicos como los eutímicos tuvieron 80% de mortalidad. En ningún caso se detectó viremia, ni la presencia de antígenos de HSV-2 en hígado, pulmón, riñón, bazo, corazón, piel o médula ósea. La infección progresó desde el epitelio vulvovaginal hacia el sistema nervioso central, sobre todo a través de nervios y ganglios pertenecientes al sistema nervioso autónomo. Se concluye que en la cepa NIH la respuesta inmune timo-dependiente es capaz de limitar la infección a nivel IVAG. No obstante, la ausencia de respuesta timo-dependiente no facilita la diseminación sistémica de HSV-2.

Key words: HSV-2, intravaginal infection, athymic mice, electron microscopy, viral antigen labeling

Genital herpes is one of the most prevalent human venereal diseases, and Herpes simplex virus type-2 (HSV-2) is its principal cause[1]. After a primary replication in the vulvovaginal epithelium, HSV-2 produces a life-long latent infection in neurons belonging to the dorsal root ganglia of the rachideal nerves. As a consequence of reactivation of the latent virus, recurrences of lesions in the genital tract, systemic dissemination as well as serious illness may occur in neonates born to infected women[1, 2].

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Even though acyclovir and other antiviral chemicals can modify and eventually control the recurrences of genital herpes[3], vaccine development for the prevention of HSV-2 infection is currently the major goal[4]. For this to be achieved, a precise knowledge of the immune response against HSV-2 genital infection is needed. To date, the available information about the roles of cellular and humoral immune responses elicited after the intravaginal (IVAG) infection by HSV-2 is far from clear, and is often contradictory in women and experimental models. Most animal studies focused on the IVAG cellular or humoral immunity against HSV-2 after vaccination with attenuated strains[4-7], or recombinant viruses[8-11], but little is known about the role of T or B cell responses after the IVAG primary infection with wild type HSV-2.

In Balb/c mice, primary IVAG infection with wild type strains of HSV-2 produces a lethal disease[12]. In this model,

the specific humoral immune response does not seem to limit replication in the vaginal epithelium[13]. Several attempts to study the role of cellular immunity after HSV-2 IVAG infection in mice have been reported[5, 7]. Some authors used HSV-2 strain deleted in the Thymidine kinase gene (TK-)[5], which is partially responsible for HSV-2 virulence. However, the use of a HSV-2 strain deficient in replication does not reveal what happens with the cellular immune response after the primary IVAG infection with a wild type strain of the virus. Other studies involved mice that were depleted in different T cell subpopulations by inoculation with specific monoclonal antibodies to eliminate each one of the T cell clones[7]. Although this method can elicit some information about the role of T cells during HSV-2 infection, interpretation is difficult because there is no guarantee that depletion is complete. Instead, more reproducible results can be obtained using congenitally athymic mice.

This report describes a straightforward study of the role of the T cell immune response after wild type HSV-2-IVAG infection, using genetically athymic (nude) mice, compared with their euthymic counterpart. In order to study the progression of HSV-2 from the vagina towards other organs in athymic mice, the presence of virus was monitored by the peroxidase-antiperoxidase technique (PAP), transmission electron microscopy and virus isolation.

Materials and methods

Virus. The ATCC VR-734 strain of Herpes simplex-2 was used. Virus stock was prepared by inoculation of Vero cell monolayers grown in plastic flasks, and maintained with Minimal Essential Medium (Gibco) supplemented with 5% calf serum. When 80% of the cells showed cytopathic effect (CPE), cultures were harvested, frozen and thawed 3 times, and spun down at 400 g. The supernatant was aliquoted, and stored at -70°C until used. Virus stock was titrated using the plaque forming unit method in Vero cell monolayers covered with culture medium and methyl-cellulose.

Animals. Euthymic and athymic (nu/nu) N:NIH(S) (NIH) mice, and euthymic and athymic (nu/nu) Balb/c females were obtained from the bioterium of the Comisión Nacional de Energía Atómica, Argentina. Mice were kept 5 to a box, and fed on pellets *ad-libitum.* Water, food and cages were sterile, and the animals were maintained at constant temperature, with natural cycles of light and darkness.

Experimental design. Every experiment included 20 animals of each type. Ten week-old mice were gently swabbed IVAG with sterile, dry cotton wool and inoculated at once with 5 x 10^5 pfu of HSV-2 suspended in 0.05 ml of cell-culture medium. Clinical signs were recorded daily. At two different timepoints [5 days post-inoculation (pi), and in the premortem stage], animals were bled to death after ether anesthesia. Heparinized blood samples were taken from each animal, and kept separately at -70°C until detection of viremia by adsorption on Vero cell monolayers and titration by pfu. A complete necropsy was performed on each animal as follows: 5 consecutive transversal sections of the whole body (each one 5 mm thick) were taken from vulva to the lower kidney tip. The other organs were dissected separately, and included liver, kidney, spleen, lung, heart, skin, brain, and bone marrow. All the samples were immediately fixed in Bouin's fluid for 6 h, then embedded in paraffin. Sections were stained with Hematoxilin-Eosin, and serial adjacent sections were immunolabeled with the PAP technique. This strategy of dissection allowed the detection of HSV-2 antigens in every pelvic and abdominal organ (including the spinal cord in situ) maintaining their normal topographic location, which included the nerves and blood vessels.

Immunocytochemistry. The PAP method was performed as previously described[12], using Dako polyclonal immunosera. The primary was a rabbit immunoserum against HSV-2. Brains of intracerebrally infected or normal uninfected mice embedded in paraffin were used as positive and negative controls respectively.

Electron Microscopy. Suitable samples from different organs were taken immediately after animal sacrifice, minced into 0.5 mm pieces and fixed in 4% paraformaldehyde-1% glutaraldehyde in PBS pH 7.4, post-fixed in osmium tetroxide and embedded in Vestopal. Sections were stained with uranyl acetate and lead citrate, and observed in a Zeiss EM 109-T electron microscope with an acceleration of 80 KV.

Results

Clinical signs and mortality in euthymic and athymic mice after HSV-2-IVAG infection

Fourteen out of 20 (70%) nude NIH mice, 6/20 (30%) euthymic NIH mice, 16/20 (80%) nude Balb/c mice, and 16/20 (80%) euthymic Balb/c mice developed signs of genital and neurological infection. All animals that showed clinical signs of infection died (Fig. 1). Spontaneous regression of infection after the appearance of disease was not seen. At 4-6 days pi mice showed vulvar erythema, edema, congestion and vaginal flux. At 6-7 days pi extensive ulcers were observed in the vulvar mucosa and perivulvar and perianal area. In the case of the euthymic mice, these ulcers also included alopecia. The size and clinical appearance of the lesions were similar in euthymic and athymic mice. At this same time, abdominal distension was also observed in all the mice. At 7-8 days pi hind limb paresia appeared, and death occurred by 8-10 days pi with a terminal picture that included wheezing and lethargy.

HSV-2 progression and histologic lesions in IVAG-infected euthymic and athymic mice

Viremia was not detected in athymic or euthymc mice of the NIH and the Balb/c strains, whereas lesions and antigen distribution were similar in the four experimental groups. HSV-2-PAP-positive areas were observed by day 5 pi in the vulvar, vaginal and perianal skin epithelia (Fig. 2A), in neurons belonging to autonomic ganglia located near the vagina and the urinary bladder (Fig. 2B), and in the Auerbach's plexus of the large bowel (Fig. 2C). HSV-2 antigen was also detected in small perivaginal and perivesical nerves. At the premortem stage (8-10 days pi), HSV-2 antigens were still located in the structures and organs described above, but also in the dorsal root ganglia, in Auerbach's plexus of the whole large bowel from rectum to cecum, and in the spinal cord (Fig. 2D). The spinal cord was infected from the lumbar up to the cervical area in the gray and the white matter of lateral and dorsal columns and horns. No virus antigen was present in the brain, but the pons and medulla oblongata were infected throughout.

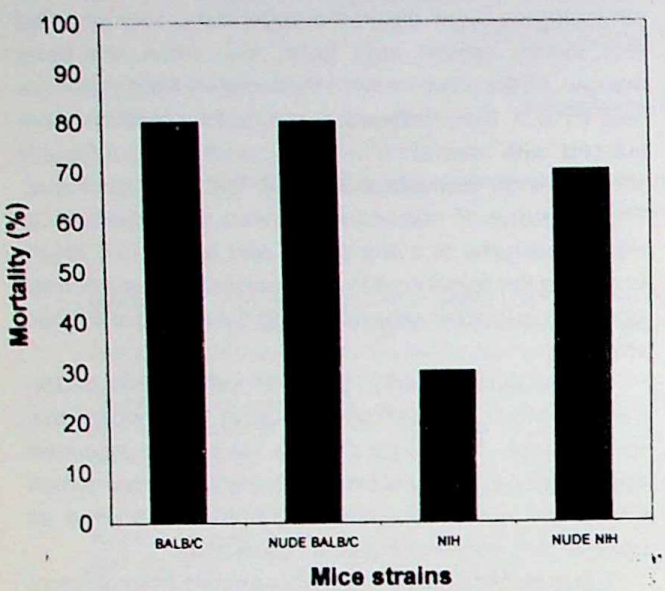

Fig. 1.– Percentage of mortality in athymic and euthymic mice after HSV-2-IVAG infection.

HSV-2 antigens were not detected in the other organs (liver, lung, heart, kidney, skin, spleen and bone marrow) of athymic or euthymic mice.

The histologic lesions coincided with the PAP-positive areas, and were mainly necrotic cells, with scattered intranuclear inclusion bodies. In all the mice (athymic or euthymic) there were mild inflammatory exudates underlying the necrotic epithelia. This exudate was mainly composed of neutrophils.

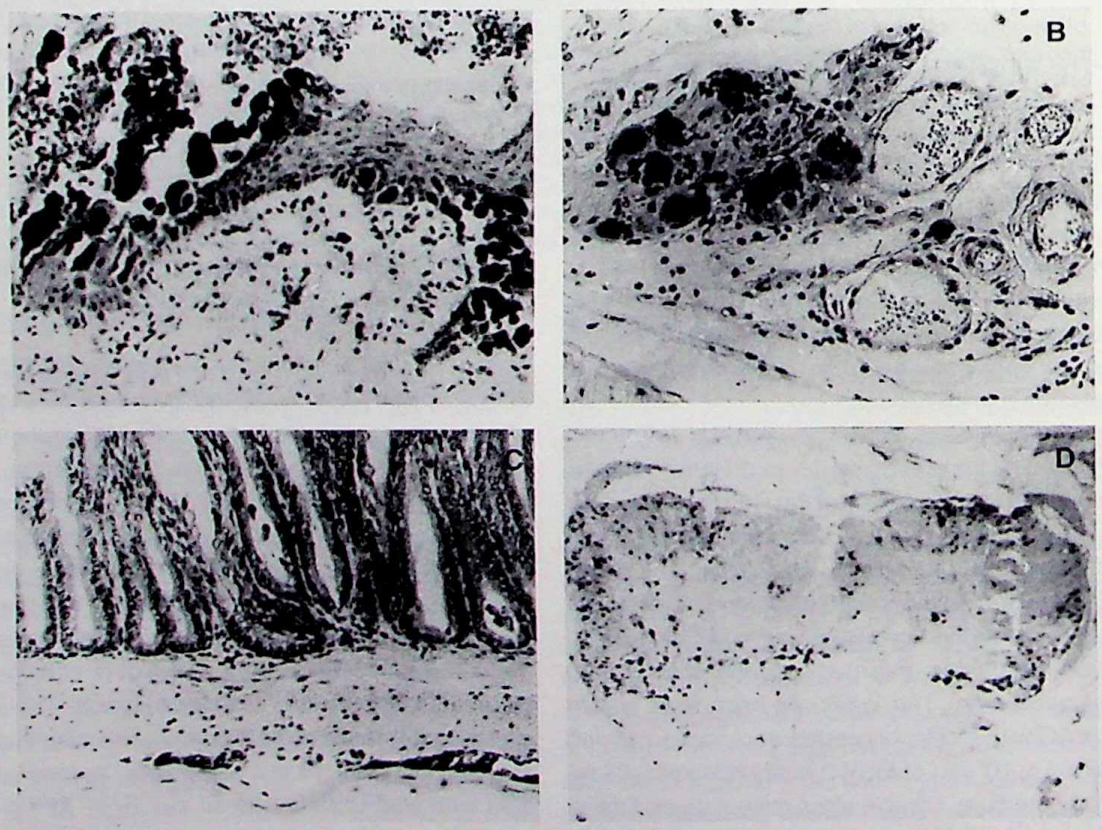

Fig. 2.– HSV-2 antigen detected by PAP method in A: vaginal epithelium, B: neurons of autonomic ganglia, C: Auerbach's plexus of the large bowel, and D: spinal cord. The dark spots are the positive areas. A, B and C are mildly stained with Hematoxilin. A, B and C: X 150; D: X 50.

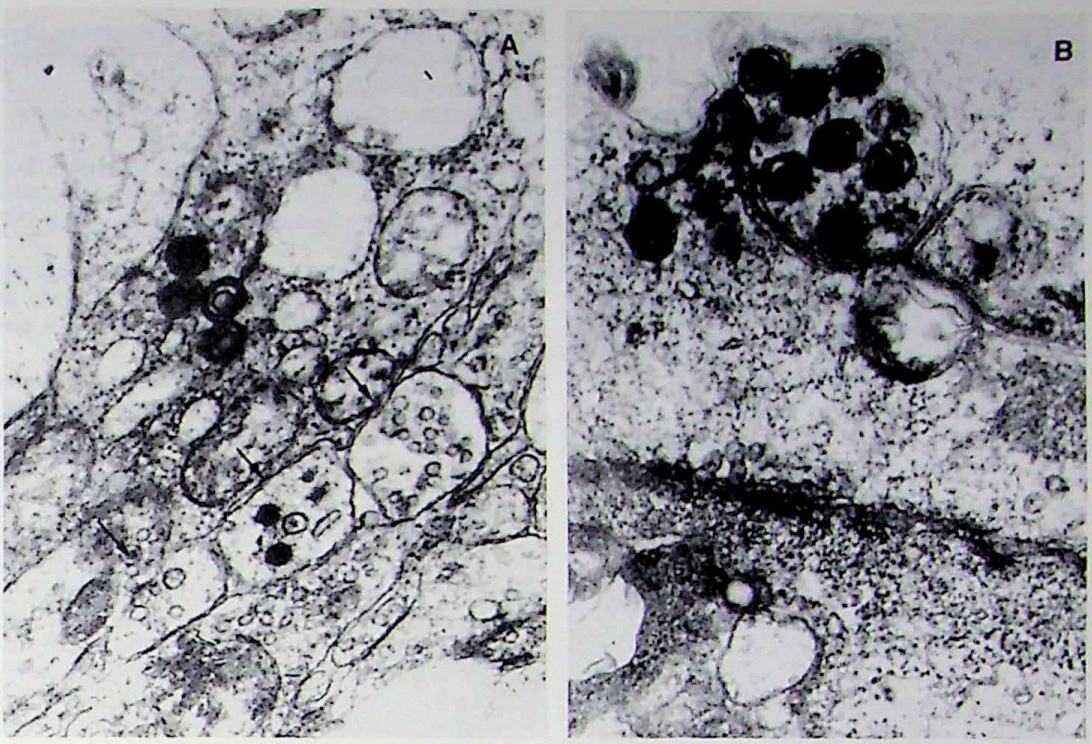

Fig. 3.– A: HSV-2 particles in a neuron of Auerbach's plexus. Amyelinic autonomic axons are indicated by arrows. B: HSV-2 virions in a vaginal epithelial cell. X 40 000.

Electron microscopy showed HSV particles in the nuclei of neurons belonging to Auerbach's plexus (Fig. 3A) and in epithelial cells (Fig. 3B), thus confirming the presence of complete HSV-2 virions and not only HSV-2 antigens in the infected organs.

Discussion

Genetically athymic mice were used to study the pathogenesis of wild-type HSV-2 IVAG infection. The results show a clear difference in the percentage of infection and mortality between the athymic NIH mice and the euthymic animals of the same strain. While 70% of athymic NIH mice IVAG-inoculated with a wild type strain of HSV-2 developed a detectable infection and died, only 30% of NIH euthymic mice showed the same susceptibility. This difference is significant, after analysis with Fisher's exact test ($p < 0.02$). The athymic (nu/nu) NIH mice used in this experiment are reported to lack T helper cell activity, and are unable to generate cytotoxic T cells[14]. Moreover, several histologic and functional criteria such as inability to reject cells and xenografts, failure to mount a graft-vs-host response, negligible response to T cell mitogens, etc, demonstrated that nude mice are severely depleted of thymic derived lymphocytes[14]. Given the fact that the only genetic difference between the NIH nude mice and the euthymic mice of the same strain is the lack of T cell response, it seems clear that the T cell-mediated immunity plays a protective role in the IVAG infection produced by wild type HSV-2 in this mouse strain. Other authors[7] have also described T cell-defence against vaginal HSV-2 challenge in mice after IVAG vaccination with attenuated (thymidine kinase-deleted) HSV-2. This is in agreement with the data shown in this experiment but, furthermore, the results reported herein strongly suggest that T cell immune response can also limit wild-type HSV-2 primary IVAG infection without any previous immunization.

The period between the virus inoculation and the first clinical signs of vulvovaginal infection in mice that developed acute disease was 4-5 days. Thus, the NIH mice that showed no signs of infection and survived HSV-2 IVAG inoculatin should have developed a protective T cell response in less than 4-5 days. It could be argued that this time is not enough for a mouse to activate T lymphocytes. However, specific cytokine-secreting T cells were reported to be present in draining genital lymph nodes of mice 4 days after IVAG infection with HSV-2[15].

According to the results presented herein the T cell-mediated resistance to HSV-2-IVAG infection seems to depend upon the mouse strain since the Balb/c mice, contrarily to what has been described for the NIH mice, showed no difference in infection and mortality between the athymic and the euthymic animals.

In this experimental model, HSV-2 progression was exclusively limited to the nervous system, on the basis of HSV-2 antigen distribution and histology. The strategy of embedding serial transversal slices of whole mouse

bodies in paraffin allowed a detailed observation of organs, nerves, and blood vessels *in situ*, maintaining their normal anatomic location. This method, associated with the use of the PAP technique, and considered together with the chronologic development of clinical signs at two timepoints had previously been used[12, 16], and makes it possible to infer the sequence of HSV-2 infection.

In both athymic and euthymic mice, HSV-2 replicated in the genital epithelium, in Auerbach's plexus, in neurons of sympathetic ganglia, and in the spinal cord, but not in the brain. These results indicate a wide infection of nerves and structures mainly belonging to the autonomic nervous system, as previously reported in the IVAG infection of euthymic Balb/c mice[12]. The infection of the autonomic nervous system can explain the protracted large bowel paralysis with fecal retention as well as the urinary bladder distention with urine retention observed in all the necropsies. The consecutive metabolic disturbances might explain the pathophysiology leading to death rather than encephalitis, given the lack of brain or cerebellar infection.

Viremia was not detected in any animal (athymic or euthymic) either at 5 days pi or at the premortem stage. The complete absence of HSV-2 antigens in liver, lungs, kidneys, bone marrow, skin, heart, spleen, and other organs also suggests that HSV-2 viremia did not occur. The conclusion is that even though the T cell-mediated immune response can limit HSV-2 IVAG infection to the genital tract in NIH mice, it has no role in preventing HSV-2 dissemination through the blood. Otherwise, in the athymic mice, HSV-2 should have infected and produced necrotic lesions in several organs, (especially the liver), as previously described in mice intraperitoneally inoculated[17, 18]. This was not observed in any of the NIH or Balb/c nude mice used in this experiment. Interestingly, other authors have also reported that in nude mice, cell-mediated immune response was essential for eliminating HSV from a site of inoculation other than IVAG, for example the ear pinna, but in no case was viremia detected in T-cell-depleted animals[19, 20].

The employment of genetically athymic mice in this new experimental model permitted a detailed study of HSV-2 progression after IVAG infection, and suggests that viremia and HSV-2 spreading towards organs other than the genital tract is not controlled by the T cell immune response.

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