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Effect of the estrous cycle on susceptibility of female mice to intravaginal inoculation of herpes simplex virus type 2 (HSV-2)

Annette G. Teepe[1], Lois B. Allen[1*], Robert J. Wordinger[2] and Elizabeth F. Harris[1]

[1] *Department of Microbiology and Immunology, Texas College of Osteopathic Medicine, Fort Worth, Texas, U.S.A. and* [2] *Department of Anatomy, Texas College of Osteopathic Medicine, Fort Worth, Texas, U.S.A.*

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Summary

In order to explore the effect of sexual maturity on the susceptibility of mice to genital herpesvirus infections, mice were separated into the four stages of the estrous cycle and inoculated intravaginally with varying doses of HSV-2, strain 186. Deaths were observed as indicators of susceptibility and were recorded as follows: proestrous, 33%; estrous, 16%; metestrous, 9% and diestrous, 75%. To determine the course of infection in animals inoculated at different stages of estrous, cotton swabs were used to collect vaginal specimens at various times post-virus inoculation for virus titration. All mice inoculated during diestrous were positive for virus as early as 6 hours post-virus inoculation and had titers that increased over a 3 day period. Mice inoculated in other stages of estrus were positive only briefly (at 6 h) or had no detectable virus. In order to verify the susceptibility associated with diestrous, mice were ovariectomized to produce a continuous diestrous (pseudodiestrous) and when inoculated ≥66.7% died. In contrast, none of the mice which had been ovariectomized and treated with estrogen to simulate the estrus stage died. We postulate that in stages other than diestrous virus may adsorb to epithelial cells in the lumen of the vagina and/or be expelled from the body by nonspecific resistance functions, thus reducing the likelihood of vaginal infection.

Herpes simplex virus; Estrous cycle; Vaginal inoculation

Correspondence to: E.F. Harris, Dept. of Microbiology and Immunology, Texas College of Osteopathic Medicine, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107, U.S.A.

**Current address:* Southern Research Institute, Birmingham, Alabama, U.S.A.

Introduction

An influence of sex hormones on herpesvirus infections has been suggested by the fact that the frequency of genital herpes increases in women during pregnancy (Ng et al., 1970; Nahmias et al., 1971) and recurrences may be triggered by menstruation (Ng et al., 1970). Observations in mice indicated that pregnant animals (Overall et al. 1975; Baker and Plotkin, 1978) are more susceptible than nonpregnant mice to initiation of genital herpesvirus infections. Susceptibility to intravaginal inoculation was determined by observation of animal deaths due to encephalitis resulting from spread of the virus from the vagina to the brain. In the study performed by Baker and Plotkin (1978) 88% of pregnant mice died following intravaginal inoculation with HSV-2, strain Savage in contrast to 10% deaths in normal nonpregnant mice. The levels of progesterone have been shown to rise early and remain elevated late into the pregnancy of the mouse (Murr, 1974). Therefore, to simulate pregnancy, progesterone was administered to nonpregnant mice and resulted in a death rate of 88% following herpesvirus inoculation. Baker et al. (1980) later demonstrated that it was necessary to pretreat the mice with progesterone for a minimum of three days in order to observe an enhancement of susceptibility. These observations suggest that the hormonal state of the female may affect susceptibility to HSV-2.

McDermott et al. (1984) reported that the resistance of mice to intravaginal inoculation with HSV-2 rose exponentially from 4 to 10 weeks of age and became constant in older animals. Allen (personal communication) observed young mice (prepubescent) to be very susceptible to intravaginal HSV-2 inoculation, while older mice were difficult to infect and the degree of infection and death rate varied considerably. Increased consistency of infection was achieved by vaginal swabbing with dilute (0.1 N) NaOH prior to virus inoculation (Allen et al., 1976). In contrast to these findings, Morahan et al. (1977) found that BALB/c mice were consistently susceptible to HSV-2. There is no clear explanation for these differences, but age of mice (young, possibly prepubescent) or virulence of the HSV-2 strain, which was a recent clinical isolate, may have been influencing factors. Since, in general, the increased resistance and variation in susceptibility seemed to correspond to the onset of sexual maturity, we decided to explore the effect of stage of estrous on susceptibility of mice to HSV-2 inoculated intravaginally.

Materials and Methods

Mice

Female ICR mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) at four to five weeks of age. The mice were housed in a biocontainment facility on a twelve hour light/dark cycle and given food and water ad libitum. All animals were rested for one week after arrival in the facility before being utilized in experiments.

Determination of the estrous cycle stage

The stage of the estrus cycle was determined by microscopic observation of vaginal smears following lavage with normal saline. The proportion of leukocytes, cornified and non-cornified epithelial cells was used to differentiate the stage of the estrous cycle. Estrous stages were characterized as follows: proestrous, non-cornified epithelial cells and leukocytes in approximately equal numbers; estrus, predominantly cornified epithelial cells; metestrous, cornified epithelial cells and leukocytes in approximately equal numbers; and diestrous, predominantly leukocytes (Snell, 1941).

Ovariectomy

Mice were ovariectomized while under Metofane (methoxyflurane) anesthesia. The skin was retracted using forceps and a one-half inch cut was made perpendicular to the midline. A small incision was made in the abdominal wall above each ovary. The ovary and associated fat were pulled through the incision and the ovary with part of the oviduct excised. The remaining tissue was then placed back into the abdominal cavity and the abdominal wall sutured with sterile, non-adsorbable, silk surgical suture (Ethicon) material. The skin was pulled together and fastened with wound clips. The animals were allowed to rest and recover at least one week before being used in experiments.

Estrogen replacement

In order to simulate estrus in ovariectomized mice, 0.1 μg 17-β-estradiol (Sigma, Saint Louis, MO) in oil was administered subcutaneously per mouse per day for seven days before and 7 days after intravaginal HSV-2 inoculation.

Virus identification, propagation and titration

Herpes simplex virus type 2, strain 186 was inoculated into baby hamster kidney (BHK)-21 cells, fed with minimal essential medium (MEM) plus 10% fetal bovine serum (FBS), and observed until 75–100% cytopathic effect (CPE) developed. Cells were homogenized, supernatant separated from cell debris, and aliquots of supernatants stored at −70°C until used. To titer virus, BHK cells at a concentration of 0.6×10^5 cells/ml in MEM plus 10% FBS were seeded in 0.2 ml volumes into each well of 96-well microtiter plates, covered, wrapped with Saran Wrap and incubated for 18–24 h. Medium was decanted from cells and 0.2 ml volumes of ten-fold virus dilutions in MEM plus 2% FBS were added to four wells per dilution. Medium without virus was placed in six wells to serve as uninfected cell controls. Plates were covered, wrapped, incubated for 3 days at 37°C and observed microscopically for development of virus induced CPE. Titer in tissue culture 50% infectious doses ($TCID_{50}$) was calculated by the technique of Reed and Muench (1938).

Intravaginal inoculation with HSV-2

Virus was diluted in MEM plus 2% FBS and instilled intravaginally (~0.0125 ml) by an automated syringe equipped with an oral feeding needle. Deaths were recorded daily for twenty-one days.

Titration of vaginal virus

Eight-week-old female mice were inoculated intravaginally with 31 400 $TCID_{50}$/mouse and were swabbed with cotton swabs dipped in MEM plus 2% FBS at 6, 24, 48 and 72 h post virus inoculation. The swabs were placed in 1 ml of medium and stored at −70°C until titered in BHK cells.

Results

Effect of stage of estrous cycle on susceptibility of mice to HSV-2 intravaginal inoculation

The percent deaths of six-week-old female mice separated by estrous stage and inoculated with 3 doses of HSV-2 are presented in Table 1. The experiment was performed twice and a total of eight mice (four per experiment) were used per dose and stage of estrous except for metestrous. Too few mice were found to be in metestrous at the time of the first experiment to test the lowest virus concentration, but 6 mice were used in the second. This reflects the fact that metestrous is the shortest stage of estrous and consequently the most difficult stage in which to identify sufficient animals for experiments. Since the data from the two experiments were similar, they were combined to generate Table 1. Mice were found to be highly susceptible to infection when inoculated during diestrous with 75% of the mice dying as compared with proestrous, 33%; estrus, 16%; and metestrous, 9%. In the diestrous group, the animals receiving the highest virus dose (31 400 $TCID_{50}$) died earlier with a mean survival time (MST) of 10.2 days. The animals receiving

TABLE 1
Effect of stage of estrous cycle on susceptibility of ICR mice to herpes simplex virus type 2 following intravaginal inoculation

Concentration of virus inoculum $TCID_{50}$	Stage of estrous at virus inoculation			
	Proestrous	Estrus	Metestrous	Diestrous
No. deaths/no. inoculated				
31 400	2/8	3/8	1/8	6/8
9 800	4/8	1/8	0/8	6/8
3 140	2/8	0/8	1/6	6/8
Summary				
Total	8/24	4/24	2/22	18/24
Percent	33	16	9	75

the middle dose of virus died in 10.7 days and those receiving the lowest dose of virus lived the longest and had a MST of 13.7 days.

Effect of ovariectomy and hormonal supplementation on the susceptibility of mice to intravaginal herpesvirus inoculation

In a preliminary experiment, thirty seven fourteen-week-old mice were ovariectomized in order to simulate diestrous, and when inoculated intravaginally with HSV-2, they were found to be highly susceptible to infection (95% deaths). Therefore, a separate experiment, using eight-week-old mice, was set up to compare the susceptibility to HSV-2 infection of ovariectomized mice in continuous diestrous (pseudodiestrous) and ovariectomized animals in which estrus was simulated by estradiol treatment. In this experiment, 36 mice were ovariectomized and the animals separated into groups of 6 animals, three of which were treated with estradiol. A titration of the virus was performed by administering log dilutions of virus to the estradiol treated and untreated groups. As shown in Table 2, 66.7% of the untreated pseudodiestrous mice died from the infections, while none of the animals treated with estradiol died. In the pseudodiestrous group, the 50% lethal dose occurred at 314 $TCID_{50}$ of virus. The percent deaths in the pseudodiestrous group was lower in this experiment than the preliminary one as a result of inoculation of some groups with lower doses of virus.

Detection and titration of virus from vaginal specimens at various times following virus inoculation

When mice were inoculated during diestrous, virus was detected from all 4 animals at 6 h and the titers continued to increase during the 3-day sampling period and all animals died from the infection (Table 3). When mice were inoculated during the estrus stage, virus was only detectable in 2/4 animals at 6 h with no virus being detected after that time. Virus was not detected at any time in specimens from mice inoculated during metestrous. No deaths occurred in the animals from either the estrus or metestrous groups.

TABLE 2
Comparison of HSV-2 susceptibility of ovariectomized mice with and without estrogen supplementation

Virus concentration ($TCID_{50}$)	Ovariectomized	
	(no estrogen)	(estrogen supplemented[a])
No. deaths/no. inoculated		
31 400	4/6	0/6
3 140	5/6	0/6
314	3/6	0/6
Summary of deaths		
Total	12/18	0/18
Percent	66.7	0

[a]These mice received 0.1 μg estradiol for 7 days before and 7 days after virus inoculation.

TABLE 3
Titration of HSV-2 from vaginal swabs at various intervals following intravaginal virus inoculation[a]

Time (h)	Estrous stage at virus inoculation					
	Estrus		Metestrous		Diestrous	
	No. positive/no. inoculated	Mean titer (range)	No. positive/no. inoculated	Mean titer (range)	No. positive/no. inoculated	Mean titer (range)
6	2/4	2.0 (1.6–2.3)	0/4	<1.6	4/4	1.9 (1.6–2.1)
24	0/4	<1.6	0/4	<1.6	4/4	3.0 (2.6–3.1)
48	0/4	<1.6	0/4	<1.6	4/4	4.7 (4.1–5.6)
72	0/4	<1.6	0/4	<1.6	4/4	4.7 (3.9–5.3)
Summary of deaths						
Total	0/4		0/4		4/4	
Percent death	0		0		100	

[a]Mice were inoculated with 31 400 $TCID_{50}$. The mean titer includes only those animals positive for virus and is the $\log_{10}$ $TCID_{50}$/ml. The lowest dilution tested was $10^{-1.6}$. The ranges of titers are shown for those having detectable virus.

Discussion

In the studies reviewed, pregnant mice were found to be more susceptible to HSV-2 than nonpregnant mice but the nonpregnant mice were not distinguished by stage of estrous (Overall et al., 1975; Baker and Plotkin, 1978). In some cases, nonpregnant mice were swabbed with dilute NaOH (Allen et al., 1976) prior to virus inoculation in order to produce a more consistent infection. Further, older mice (McDermott et al., 1984) were found to be more resistant to infection than younger mice suggesting that sexual maturity, hormonal influences or factors such as pH may be involved in determining susceptibility to HSV-2 inoculated intravaginally. From a physiological standpoint, resistance would be expected to be greatest in sexually mature animals during estrus the stage in which mating and the possible introduction of infectious organisms may occur.

The results of our study indicate that variations in susceptibility of cycling female mice to herpesvirus depended upon the route of virus inoculation. In experiments not presented here, we compared the susceptibility of cycling mice to intraperitoneal inoculation and found no differences in susceptibility with stage of estrous. However, with intravaginal inoculation of HSV-2 a major difference in susceptibility was observed between mice in diestrous (75% deaths) and those in other stages (proestrous 33%, estrus 16%, and metestrous 9%) (Table 1). Ovariectomized mice, which have a vaginal mucosa resembling those of mice in diestrous, are highly susceptible (≥66.7% deaths) to HSV-2 inoculated intravaginally (Table 2). In contrast, no deaths occurred in the ovariectomized mice treated with estradiol to simulate the estrus stage.

Variations in susceptibility of groups within an estrous stage were observed. This is not surprising, since screening of stages took several hours (4–8) and the short stages (proestrous, estrous and metestrous) represent ≤20% (≤20 h) each of the

total cycle. Therefore, at the time of staging an animal might be near the end of a stage and progress into the next stage before being inoculated with virus.

Mice in proestrous, estrus or metestrous at the time of intravaginal inoculation with HSV-2 were less susceptible to infection than mice in diestrous. Epithelial cells are present in the vaginal lumen of mice during all stages except diestrous. Therefore, it is possible that the virus inoculum might adsorb to the lumenal epithelial cells which will be sloughed or expelled from the vagina thus preventing the initiation of infection. In diestrous, few epithelial cells are present in the lumen, consequently, virus may penetrate the epithelium and easily initiate infection. Indeed, this might have been a factor contributing to the increased consistency of infection obtained by vaginal swabbing of unstaged mice with dilute NaOH prior to virus inoculation as described by Allen et al. (1976).

To compare the development of infection after virus inoculation of mice in estrus, metestrous or diestrous, vaginal swabs were obtained at 6, 24, 48, and 72 h post-virus inoculation. If virus adsorbed to epithelial cells and was expelled from the vagina in mice inoculated during proestrous, estrus and metestrous, the concentration of virus in vaginal specimens should decrease with time after inoculation since the virus would be expelled and not increased due to replication. In this study, when mice were inoculated during diestrous, virus was detected from all animals and the titers increased from 6 to 48 h and ultimately the animals died of encephalitis (Table 3). Since vaginal specimens from only 2/8 animals inoculated during estrus or metestrous were positive at 6 h and none at 24 h, virus replication does not appear to have occurred in these groups. Absence of virus from vaginal specimens correlated well with the increased survival of mice inoculated in these stages and may suggest that lumen epithelial cells or other factors contribute to this resistance. These data also suggest that factors significant to the control of the infection occur rapidly since virus disappeared between 6 and 24 h.

One factor which varies with stage of estrous cycle is the thickness of vaginal epithelium. The epithelium is thinnest during diestrous, then thickens during proestrous, and is thickest during estrus, the time at which mating may occur. After estrus, the thickness decreases during metestrous. Since we found that animals inoculated during diestrous develop high concentrations of vaginal virus and die due to encephalitis, it is possible that spread of the virus to surrounding tissues and the central nervous system is facilitated by the limited amount of epithelium. In contrast, during stages where the epithelium is thicker, the opportunity for control of the infection by nonspecific resistance mechanisms such as sloughing of epithelial cells might be greater.

Although vaginal pH has not been investigated in mice, studies in rats revealed that pH is lowest during estrus (pH 4.5) and highest during diestrous (pH 7.0) (Beilly, 1940). If the same relationships occur in mice, the pH of the vagina during diestrous (pH 7.0) would be favorable for the attachment and replication of HSV-2 and the pH during estrus (pH 4.5) would be unsuitable for survival and replication of the virus. Therefore, the variations in susceptibility seen in this study may also reflect the pH variations observed in rats. Further, Allen et al. (1976) demonstrated that vaginal swabbing with a basic solution prior to virus inoculation increased

consistency of infection. In addition, intravaginal treatment of mice with a vehicle having a pH of 5.0 following HSV-2 intravaginal inoculation resulted in lower deaths (33%) than a group treated with a vehicle having a higher pH of 6.5 (78% deaths).

Another factor which varies with the stage of estrous is the presence of mucus in the vaginal lumen. As previously noted by Short and Woodnott (1969), we observed the largest quantities of mucus during diestrous. It would seem that mucus might bind virus preventing infection, but this was not observed since the greatest number of deaths occurred during diestrous. In contrast, it may be that mucus could contribute to the contact of virus with the vaginal mucosa thus facilitating virus adsorption. In truth, mucus may not have any effect on the infection.

The presence or concentration of nonspecific and specific immune system cells in the vagina has not been evaluated in the current study. The concentration of phagocytes, natural killer cells and lymphocytes (T and B) probably changes as a function or consequence of the dynamics of other mucosal change such as thickness, cell composition and cell sloughing. Therefore, host response to the infection could vary with changes in local concentration and composition of the cells involved in these processes.

The demonstration by Baker and Plotkin (1978) that the enhanced susceptibility to intravaginal HSV-2 during pregnancy can be mimicked by the administration of progesterone to nonpregnant female mice and our observations concerning the variation of susceptibility to herpesvirus with stage of estrous suggest the importance of sex hormones on the susceptibility/resistance of female mice to virus infections of the genital tract. We feel that further studies concerning the effects of hormonal influences and sexual maturity on the susceptibility of female animals to genital tract infections should be undertaken. Further, these studies may lead to the development of strategies for the artificial alteration of the vaginal mucosa as a means of treatment or prevention of venereal infections.

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