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EXPERIMENTAL STUDIES ON GENITAL HERPETIC INFECTION IN MICE

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SUMMARY Female ICR mice were infected with HSV-1 and HSV-2 by inserting a cotton pellet soaked in viral solution ($10^{7\sim8}$ PFU/ml) into the vagina. The appearance of giant cells and formation of intranuclear inclusions were detected in the epithelial layer of the uterus 24 h after intravaginal inoculation. These histopathological changes were pronounced 3 to 4 days after virus inoculation and then gradually disappeared in the next few days.

Results of fluorescent antibody studies on the appearance of viral antigens in infected uterine tissues and results of viral infectivity titrations of emulsified samples of infected uteri coincided well with the histopathological observations on the general course of virus infection.

The degree of histopathological involvement caused by HSV-1 was somewhat less than that caused by HSV-2, and the laboratory strains of HSV-1 so far examined (HF and Miyama) were found to be especially weakly pathogenic.

INTRODUCTION

On the basis of various criteria, herpes simplex viruses can be divided into two distinct types; type 1 (HSV-1) and type 2 (HSV-2) (Schneweis, 1962). HSV-2 strains are usually isolated from lesions of the genitalia or lower libms, whereas HSV-1 strains are obtained from nongenital lesions; e. g. oral, conjunctival, pharyngeal or upper limb lesions (Dowdle et al., 1967).

The possible association of genital herpetic infection with cervical cancer was first suggested by Naib et al. (1966), who noted that women with cervical cytological changes associated with herpesviruses showed an incre-

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ased incidence of cervical anaplasia. Since then extensive studies to clarify the oncogenic potential of HSV-2 have been performed by numerous investigators. One approach has been to carry out retrospective and prospective epidemiological studies on women with HSV-2 infection (Nahmias et al., 1970, 1973, 1974; Rawls et al., 1973). Another approach has been to establish genital herpetic infection in experimental animals and examine the animals for the development of cervical anaplasia.

Several animals so far have been shown to be susceptible to HSV-2 genital infection. Nahmias et al, (1971) established an excellent experimental model of genital herpes using female cebus monkeys, while Lukáš et al. (1974) reported that vaginally infected guinea pigs were a suitable model.

It is difficult to purchase and handle large numbers of nonhuman primates, so we tried to develop a good model using small animals. A series of experiments in our laboratory showed that mice are useful for obtaining fundamental information on genital herpes infection, although early studies on mice by Nahmias et al. (1967b) did not give satisfactory results.

In this paper we describe kinetic studies of morphological changes, the appearance of viral antigens and virus growth in the uteri of mice infected vaginally with herpes.

MATERIALS AND METHODS

1. *Animals*

Female ICR strain mice were obtained from Clea Japan, Inc. and were used for experiments at six to eight weeks old.

2. *Virus*

The following virus strains were employed: three laboratory strains of HSV-1 (+GC and −GCr Miyama, and HF), two laboratory strains of HSV-2 (Syn^+ and Syn^- UW268), three freshly isolated strains of HSV-1 (Oda, S6 and NI) and two freshly isolated strains of HSV-2 (MK and SM). Among these viruses, the +GC Miyama and HF strains of HSV-1 and the Syn^- UW268 strain of HSV-2 had high cell fusion activity, whereas the other strains of HSV-2 had only low activity.

High-titer virus stocks were prepared from heavily infected FL cells 48 to 72 h after infection with either of the above virus strains, and the supernatant fluid obtained by low speed centrifugation was used for inoculation. The infectious titers of the viral samples used in experiments are shown in Tables 1 and 2.

3. *Virus infection*

Virus was inoculated by inserting a cotton pellet soaked in virus solution into the vagina.

4. *Histological preparations*

Usually in each experiment four animals were

TABLE 1. *Incidence of characteristic pathological changes in uterine tissues of mice after intravaginal infection with HSV-2*

Virus strain of HSV-2	Infectious titer of viral sample PFU/ml	Days after virus inoculation													
		1	2	3	4	5	6	7	8	9	10	11	12	15	20
MK	1.0×10^8	3[a]/4[b]	4/4	4/4	1/4	1/4	0/4								
UW 268 Syn^+	1.0×10^7	2/4	2/4	0/4	3/4	2/4	0/4								
UW 268 Syn^-	1.0×10^7	2/4	1/4	2/4	3/4	2/4	1/4				0/6			0/4	0/4
UW 268 Syn^-	1.0×10^7	1/4	1/4	1/4	2/4	0/4	0/4		0/4			0/2	0/2		
UW 268 Syn^-	1.0×10^7			8/10		1/4	0/4	1/4	0/4	0/4	0/4				
SM Syn^+	3.0×10^6	0/4	2/4	1/4	1/4	2/4	1/4								

[a] Number of mice showing characteristic pathological changes in uterine tissues.
[b] Total number of mice examined.

TABLE 2. *Incidence of characteristic pathological changes in uterine tissues of mice after intravaginal infection with HSV-1*

Virus strain of HSV-1	Infectious titer of viral sample PFU/ml	Days after virus inoculation						
		1	2	3	4	5	6	7
−GCr Miyama	1.6×10^8	0[a]/4[b]	0/4	0/4	0/4	0/4	0/4	
„	1.0×10^8			0/3	0/9	0/3		
„	3.5×10^7	1/4	0/4	0/4	0/4	0/4		
+GC Miyama	3.8×10^7	2/4	1/4	0/4	0/4	0/4	0/4	
„	2.5×10^7	1/4	0/4	0/4	0/4			
HF	2.7×10^7	1/4	0/4	0/4	0/4	0/3		
Oda	6.5×10^7	1/4	0/4	1/4	1/4	0/4	0/2	
S6	2.0×10^8	1/4	3/4	2/4	2/4	1/4	0/4	0/4
NI	2.8×10^8	3/4	2/4	3/4	1/4	1/4	0/4	0/4

[a] Number of mice showing characteristic pathological changes in uterine tissues.
[b] Total number of mice examined.

sacrificed every day after virus inoculation for 6 days and their uteri were excised. Exact sample numbers and durations of observation are shown in Tables 1 and 2. The excised uteri were promptly immersed in Bouin's fixative and then histological preparations were made. More than ten sagittal sections of each uterus were stained with H-E and examined microscopically.

5. *Fluorescent antibody technique*

Infected and control uteri were excised and rapidly frozen and stored at −70°C. Then within a few days, sagittal frozen sections of the uteri were prepared with a cryostat, immersed in acetone fixative, and stained with anti-HSV-2 fluorescent antibody. This antibody was kindly supplied by Dr. Kurata, Institute of Medical Science, Tokyo University. Observations were carried out in a fluorescence microscope (Chiyoda FM200A).

6. *Recovery of infectious viruses from infected uterine tissues*

Mice were infected as described above. Uteri were excised aseptically after 3 h and then every day for 7 days. They were rinsed thoroughly with Hanks' solution and stored at −20°C for examination. Within one week of storage, the frozen samples were thawed and ground with 3 ml of Eagle's minimal essential medium solution and a small amount of sterile sand in a mortar. The resulting emulsion was centrifuged at 2,500 rpm for 10 min, and the supernatant was further centrifuged at 6,000 rpm for 30 min at 4°C. The clarified material was diluted serially and inoculated onto FL cell monolayers. A liquid overlay method was employed and after three days plaques were counted.

7. *Virus inoculation into the cervical canal*

The thread impregnating technique to introduce carcinogenic substances into the cervical canal of mice was also applied for inoculation of viruses. This technique was originally devised by Murphy (1953) and named "the string method". The slight modification of this method by Iijima et al. (1964) was used in the present study. A cotton thread was inserted up to the uterine horn from the vagina through the uterine corpus allowing the viral solution to ascend by infiltration as illustrated in Fig. 1a. Another method tested was to insert a blunt needle into the cervical canal under direct vision with a rhinoscope and inject the viral solution from the syringe at low pressure (Fig. 1b). The virus inoculated into the cervical canal was the Syn⁻ UW268 strain of HSV-2.

RESULTS

1. *Histopathological changes in herpes infected uterine tissues*

The occurrence of viral infection was confirmed by demonstrating cytologic changes

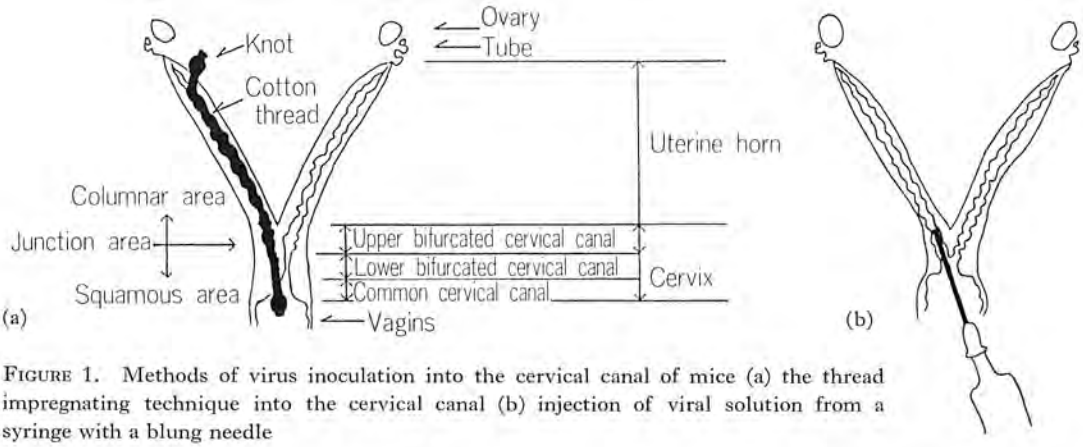

FIGURE 1. Methods of virus inoculation into the cervical canal of mice (a) the thread impregnating technique into the cervical canal (b) injection of viral solution from a syringe with a blung needle

characteristic of herpetic infection, i. e., the appearance of cells with intranuclear inclusions and the formation of giant cells. The latter marker was especially useful in experiments with virus strains that showed cell fusion activity in vitro. Primary viral lesions were recognized as single foci localized on the squamous epithelium of the ectocervix as early as day 1 after inoculation. The time of appearance of primary lesions, however, depended somewhat upon the infectious titer of the viral sample used.

The frequency of detection of cytopathological changes of uterine tissues and the extent of these changes tended to increase until day 3 to 4 after inoculation of HSV-2 (Table 1). Syncytial giant cells with intranuclear inclusions were numerous in the squamous cell area (Fig. 2). The number of degenerating cells, which were probably sloughed off, also increased during this period. The appearance of degenerating cells was associated with gradual increase in the number of inflammatory cells, mainly consisting of polymorphonuclear leucocytes. The numbers of these inflammatory cells also became marked in the

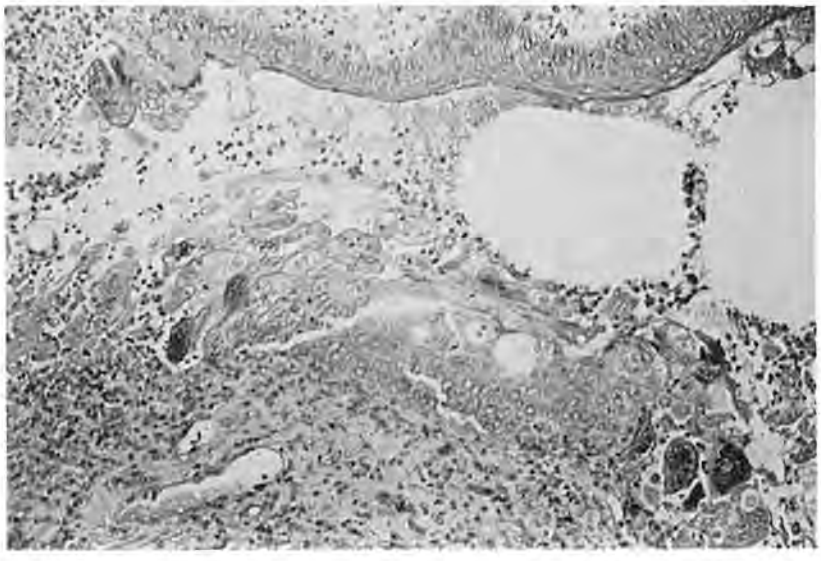

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FIGURE 2. Squamous cell layer of the common cervical canal of a mouse 3 days after intravaginal infection with the Syn⁻ UW268 strain of HSV-2. Syncytical giant cells, hemorrhagic changes, infiltration of inflammatory cells and degenerated cells are seen. ×160

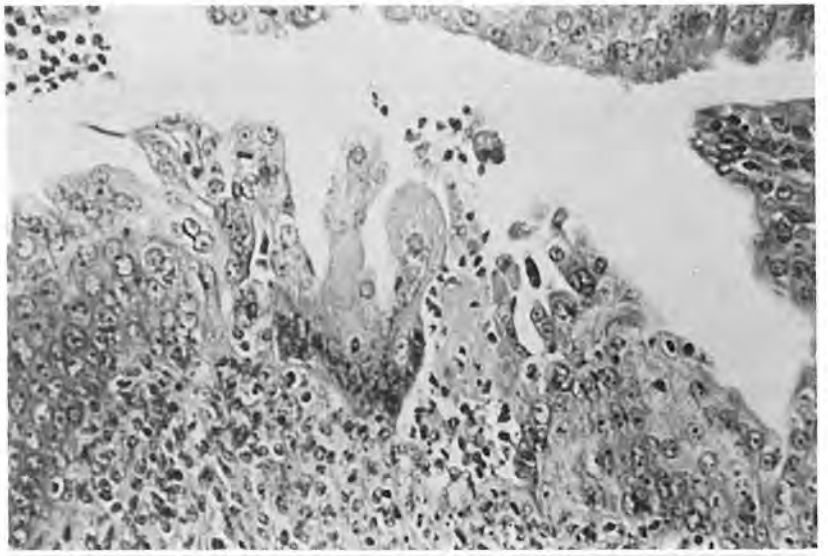

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FIGURE 3. Squamous cell layer of the bifurcated cervical canal near the squamo-columnar junction of a mouse 4 days after intravaginal infection with the Syn⁻ UW268 strain of HSV-2. Syncytial giant cells with intranuclear inclusions are seen. ×160

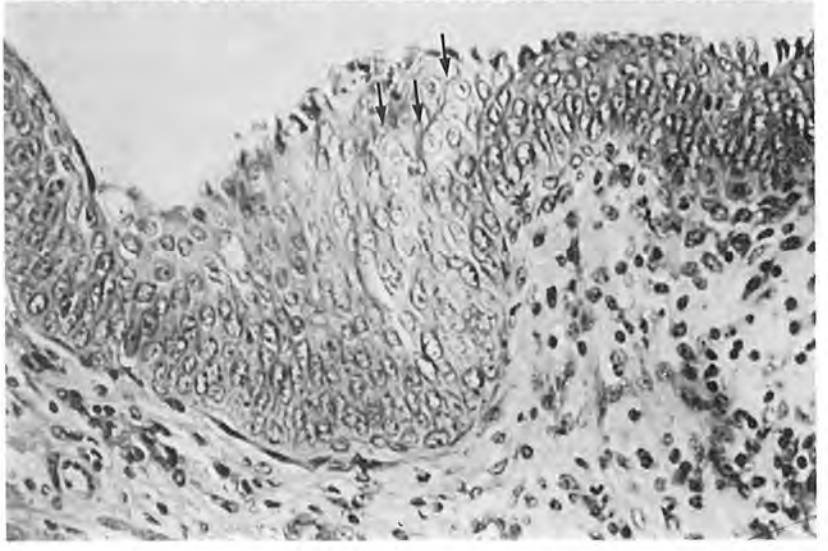

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FIGURE 4. Squamous cell layer of the uterine cervix of a mouse 3 days after intravaginal inoculation with the HF strain of HSV-1. Intranuclear inclusions are seen in some cells (arrows), but in others cytopathological changes are very slight. ×160

submucous layer, where no viral infection was apparent. Syncytial giant cells with intranuclear inclusions were not observed among endometrial columnar cells, but they were seen in the vaginal vault and in the squamous epithelium below the squamo-columnar junction of the bifurcated cervical canal (Fig. 3). Cytopathological changes characteristic of herpetic infection and other histopathological changes disappeared gradually from day 5, and intranuclear inclusion-bearing cells could no longer be seen after day 9.

Although the infectivity titers of the inoculated viral samples of HSV-1 were similar to those of HSV-2 or even more, the frequency and extent of cytopathological changes caused by HSV-1 were generally less than those caused by HSV-2 (Fig. 4). Laboratory strains of HSV-1 (HF and Miyama) seemed to be especially weakly pathogenic.

2. *Site and frequency of appearance of fluorescent antigens in uterine tissues of mice infected with HSV-2*

Mice were infected with the Syn⁻ UW268 strain. Three mice were sacrificed every day for 7 days and also on day 12. Frozen sections of the uteri were prepared and stained with anti-HSV fluorescent antibody. As shown in Table 3 viral antigens were detected in one uterus on day 3 and in all uteri on day 4 after infection. The antigens were located in the squamous cell layer and fluorescent areas were observed as small foci consisting of a few antigen-positive cells, or as macrolesions affecting about a hundred cells (Fig. 5). The degree and extent of the appearance of viral antigens coincided well with the results of histopathological changes on the general course of HSV-2 infection.

3. *Attempts to inoculate viral samples into the uterine endometrium*

Results obtained by direct injection of viral solutions into the endometrium and by infiltration of virus by " the string method " are compared in Table 4. No evidence was obtained that after viral inoculations by these methods herpetic infection had occurred in the epithelial cell layer, since the endometrium did not exhibit either intranuclear inclusions or syncytial giant cells.

4. *Virus recovery from infected mice uteri*

Uteri were extirpated from mice every day after infection for one week. The uteri were emulsified, and the supernatant fluids were titrated as described in the Materials and Methods. Virus was recovered until day 6, although in small amount (Fig. 6) and the max-

TABLE 3. *Incidence of fluorescent antigens in uterine tissues of mice after intravaginal infection with HSV-2*

Virus strain	Infectious titer PFU/ml	Days after virus inoculation							
		1	2	3	4	5	6	7	12
UW 268 Syn⁻	1.0×10^{7}	0[a]/3[b]	0/3	1/3	3/3	1/3	0/3	0/3	0/3

[a] Number of uteri in which fluorescent antigens were detected.
[b] Total number of mice examined.

◀ FIGURE 5. Fluorescent antigen positive cells of the squamous cell layer of a mouse uterus infected with the Syn⁻ UW268 strain. ×400

TABLE 4. *Incidence of characteristic pathological changes in uterine tissues of mice after HSV-2 inoculation into the cervical canal and endometrial cavity*

Method of virus inoculation	Uterine epithelium	Days after virus inoculation					
		1	2	3	4	5	6
Along a thread[a]	Squamous area				1/4		
	Columnar area				0/4		
Injection[b]	Squamous area		2/4	2/4	1/4		
	Columnar area		0/4	0/4	0/4		

[a] Virus inoculation along a thread introduced into the endometrial cavity through the cervical canal.

[b] Virus inoculation by syringe with a blunt-tipped needle into the endometrial cavity and cervical canal.

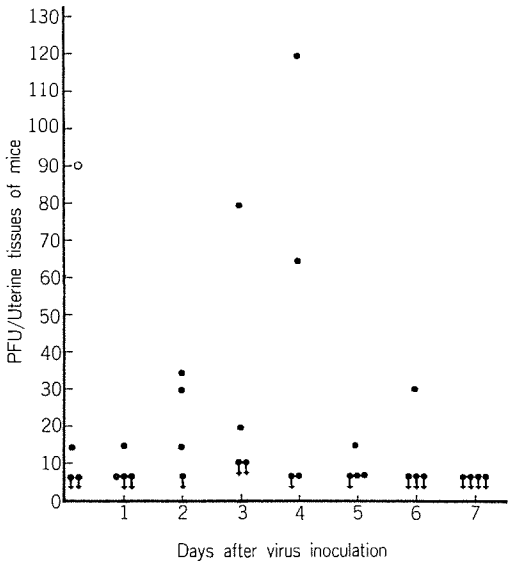

FIGURE 6. Infectivity of uterine tissues of mice after intravaginal inoculation of HSV-2.

imum titer (i. e. 120 PFU/mouse uterus) was obtained with one of four infected uteri on day 4. These results essentially coincide with those of histopathological and immunofluorescent studies on the duration of viral infections.

DISCUSSION

There have been numerous studies on whether or not HSV-2 is a causative agent of squamous cell carcinoma of the cervix, but this problem has not yet been elucidated (Rawls, 1973). To clarify the relation between the virus and carcinoma, many investigators have developed models of genital herpetic infection using various experimental animals; i. e., mice (Nahmias et al., 1967a, b; Muñoz, 1973; Nishiura and Nii, 1976), guinea pigs (Lukáš et al., 1974; Lukáš et al., 1975; Krinke et al., 1975), hamsters (Burnstein, 1965) and monkeys (Nahmias et al., 1971; Kalter et al., 1972).

We also established genital infection of mice (the ICR strain) with HSV-1 and HSV-2, and followed the general course of the infection virologically and histopathologically. The cytopathological changes characteristic of herpetic infection found in the squamous epithelial layer of HSV-2 infected uteri were pronounced on day 3 to 4 after infection, and then gradually disappeared in the next few days, being no longer detectable by day 9. Results of fluorescent antibody studies and of infectivity titrations of viruses recovered from infected mouse uteri coincided well with cytopathological findings on the general course of infection.

There are two other reports of establishment of genital herpetic infection of mice. Nahmias et al. (1967a) isolate virus from the vagina of mice (COBS strain) for 12 days after intravaginal infection and also observed positive cytologic changes in genital smears during the same period. Similarly, by observations of vaginal smears of infected mice (BALB/c strain) Muñoz (1973) reported that the frequency of the appearance of multinucleated giant cells typical of herpetic infection reached a

peak on day 6 after virus inoculation and decreased after day 10. Thus, the durations of infection of uterine tissues reported in these two papers are somewhat longer than those shown in our studies. The duration may vary to some extent with the strains of mice and viruses used in experiments.

From the results obtained in this investigation, the general course of herpetic infection of mouse uterus may be summarized as follows. Inoculated viruses is adsorbed to superficial cells of the squamous cell layer of the uterine cervix and then virus growth promptly occurs in some permissive cells. The infection spreads to neighbouring cells and within 24 h after virus inoculation microlesions are formed, which are detectable by light microscopy. In other words, cell masses with intranuclear inclusions characteristic of herpetic infection appear focally in the squamous cell layer. With virus strains having cell fusion activity these microlesions are more obvious as multinucleated syncytial cells with intranuclear inclusions. Virus infection proceeds further chiefly through cell-to-cell transmission, and thus larger lesions are formed. Soon, infected cells are destroyed and infiltration of inflammatory cells, mainly consisting of polymorphonuclear leucocytes, becomes marked in and beneath the squamous cell layer. Sloughing off of degenerative squamous cells into the cervical canal and hemorrhagic changes at and near infected lesions take place. The degree of these pathological changes was usually greatest on day 3 to 4 after infection, gradually decreasing thereafter, and disappearing in a few days.

Although these pathological changes of herpetic infection were observed locally or over a wide area of the squamous cell layer, the existence of inclusion-bearing cells or antigen-positive cells could not be definitely demonstrated at other parts, i. e., the columnar and interstitial cell layers. The inability of HSV to grow in the latter cells might be because these cells were not sufficiently exposured to inoculated viruses or progeny viruses produced in the squamous cell layer. To confirm this possibility, we tried to bring virus in direct contact with the columnar cell layer of the cervical canal and the endometrial epithelia by two methods as shown in Fig. 1. However, we could not detect cytopathological changes caused by HSV in the latter tissues using either of these methods. Therefore, we conclude that columnar cells in the cervical canal and those in the endometrium are not susceptible to infection with HSV.

When mice were infected intravaginally with the UW268 strain and then kept for more than three weeks, 10 to 30% of them suffered from posterior paralysis and finally died. This seemed to be caused by retrograde spread of virus along nerves to the spinal cord. However, no signs of encephalitis were noticed. In contrast, Nahmias et al. (1967a) reported that in their experiments 90% of the mice died of encephalitis. This discrepancy must be due to the differences in strains of mice and/or viruses used.

As shown in this study, the extent of histopathological changes in uterine tissues of mice inoculated with HSV-2 (the UW268 strain) was most remarkable on day 3 to 4. Nevertheless, in most experiments these changes were not always detected in all the uteri extirpated at this time of infection. The most likely explanation for why some mice were not infected is that establishment of herpetic infection depends greatly on cyclic changes of the squamous epithelium of the uterus. Our preliminary experiments support this idea (Nishiura and Nii, 1976).

In one experiment we measured the level of neutralizing antibody in the sera of mice inoculated intravaginally twice, with a three month interval between inoculations, with the UW268 strain of virus. Only a few of the 24 mice examined were found to have antibody at low level. Cell mediated immunity and interferon may play more significant roles than neutralizing antibody in the disappearance of histopathological changes in mouse uterus, but this problem requires further study.

The pathogenicity of HSV-2 was much greater than that of HSV-1 in the present study. This phenomenon needs confirmation using many freshly-isolated strains of HSV-1.

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