Effect of Prior Immunization on Induction of Cervical Cancer in Mice by Herpes Simplex Virus Type 2

Abstract. *Previous studies at this laboratory showed that repeated application of inactivated herpes simplex virus type 2 to the mouse cervix produces premalignant and malignant lesions. In the present study mice were inoculated with inactivated herpes simplex virus type 2 or control solution and Freund's adjuvant by intraperitoneal and subcutaneous routes before exposure of the cervix to inactivated virus. It appears that immunization with inactivated virus conferred a protection against the induction of cervical carcinoma.*

Data accumulated over recent years implicate herpes simplex virus type 2 (HSV-2) as an etiological agent in the development of carcinoma of the uterine cervix (*1, 2*). In previous studies at this laboratory it was found that when Formalin and ultraviolet-inactivated HSV-1 and HSV-2 were applied to the cervices of mice three to five times a week, squamous carcinoma, adenocarcinoma, and adenosquamous carcinoma resulted in 35 to 60 percent of the animals. The cytological and histopathological changes in these animals resembled the dysplasia, microinvasion, and frank invasive carcinoma observed in women. The present experiment was performed to determine whether inoculation of mice with inactivated HSV-2 before long-term cervical exposure can modify or prevent the development of these lesions.

Virgin C3H/HeJ mice 5 to 6 weeks of age were maintained in an air-conditioned room and fed a stock diet. The animals' ears were marked so that serial observations of the progression of cervical lesions by cytological examination were possible in each mouse. HSV-2 (strain ATCC VR 734, Roizman) was obtained from the American Type Culture Collection. Monolayer cultures of HEp-2 cells were prepared as previously described (*3, 4*), except that newborn calf serum was used in place of fetal calf serum. Cultures were inoculated with HSV-2 at a multiplicity of infection of 0.01. Cultures of uninoculated cells were maintained in the same manner. When 75 percent of the cells in inoculated cultures showed viral cytopathic effects, cells and fluids were harvested from both virus-inoculated and control cultures. The cell suspensions were centrifuged at 2500 rev/min for 10 minutes. The sedimented cells were frozen, thawed three times, and again centrifuged at 2500 rev/min for 10 minutes. The pellet of cell debris from the freeze-thaw mixture was discarded and the supernatant fluid was added to the saved culture supernatant fluids.

To inactivate HSV-2, 100 ml of virus suspension was dispensed into 24 by 37 cm glass dishes and exposed to ultraviolet light (100 erg/sec-mm^2) for 12 minutes, with the fluid being agitated every 3 minutes. Control fluids were treated in the same way. The virus and control fluids were then divided into 10-ml portions and stored at −70°C.

Virus stocks were assayed for infectious virus before and after inactivation by inoculation of serial tenfold dilutions into tube cultures of African green monkey kidney cells. Virus titers were between 10^5 and 10^6 median tissue culture infectious doses per milliliter before inactivation. Infectious virus was not detected after inactivation by inoculation and passage in HEp-2 and monkey kidney cell cultures.

Vaginal cytology preparations obtained biweekly during a 4-week period showed no preexisting cervical abnormalities in the mice. Each animal in the experimental group then received two intraperitoneal injections and one subcutaneous injection of 0.5 ml of inactivated HSV-2 emulsified with 0.2 ml of Freund's adjuvant. Control animals received injections of 0.5 ml of fluid prepared from uninfected HEp-2 cell cultures and 0.2 ml of Freund's adjuvant in the same manner. The interval between injections was 7 days. A random sample of ten immunized and ten control mice were bled 10 days after the last injection of inactivated HSV-2 or control fluid. Sera were tested at a dilution of 1:100 for antibody against HSV-2 by a microenzyme-linked immunosorbent assay (ELISA) using HSV-2 antigen prepared in African green monkey kidney cell cultures, peroxidase-labeled goat antiserum, and orthophenylenediamine as a substrate. Optical densities were read at 490 nm. Cervical exposure was then initiated in both the experimental and control groups by inserting into the vaginas cotton pledgets saturated with 0.1 ml of inactivated HSV-2 three times a week for 4 weeks and once a week thereafter for a total of 80 weeks.

Cytological preparations were obtained from vaginal aspirates and stained with the Papanicolaou stain (*3, 4*). Cell smears were made for all animals at 10- to 20-week intervals and evaluated by one worker without bias. At the end of the study surviving animals were killed and the entire reproductive tracts were stained with hematoxylin and eosin and reviewed by another worker who was unaware of the cytological interpretations at the time the mice were killed.

A total of 52 mice in the experimental group and 54 mice in the control group were treated by application of inactivated HSV-2 to the uterine cervix. A number of mice from each group were not available for pathological study due to deaths from intercurrent infection and to autolysis of the tissues. At the end of the study there were 20 mice in the experimental group that had been inoculated with inactivated HSV-2 plus Freund's adjuvant and 21 mice in the control group that had been inoculated with uninfected HEp-2 cell fluid plus Freund's adjuvant. Histopathological findings are presented in Table 1. In the control group 12 mice had microinvasive or invasive cancer, 7 had dysplasia, and 2 were normal. In the experimental (immunized) group 16 had a normal cervix and uterus and 4 had cervical dysplasia. The observed difference between the total frequencies of premalignant plus malignant lesions in the two groups was tested for statistical significance by the chi-square test. The P value was < 0.001.

Table 1. Number and frequency of histopathologically confirmed cervical lesions in experimental and control mice after exposure to inactivated HSV-2.

Normal		Dysplasia		Microinvasive cancer		Invasive cancer	
Number	Percent	Number	Percent	Number	Percent	Number	Percent
Experimental mice							
16	80	4	20	0	0	0	0
Control mice							
2	9.5	7	33.3	8	38.1	4	19.1

Table 2 illustrates the frequency of cytological alterations occurring in the two groups in relation to the duration of exposure to inactivated HSV-2. After 60 weeks of exposure, at least 70 percent of the mice in the control group exhibited abnormal cellular alterations, and up to 48 percent had cellular changes consist-

ent with microinvasive or invasive cancer. In contrast, 19 percent or less of the mice in the experimental group had cellular abnormalities consistent with dysplasia, and none of the animals had changes consistent with microinvasive or invasive cancer. The accuracy of the cytological interpretations is shown by comparing the cytological and histological findings. In the experimental group (Table 2), 15 percent of the mice had cellular evidence of dysplasia and this lesion was present in 20 percent of the histological specimens (Table 1). In the control group (Table 2), 83 percent of the animals had cellular evidence of dysplasia, microinvasive cancer, or invasive cancer, and these lesions were present in 91 percent of the histological specimens (Table 1). In micro-ELISA tests for HSV-2 antibody, the mean optical densities of sera from ten immunized and ten control mice were 0.554 and 0.070, respectively. The higher optical densities in sera from immunized mice indicate the presence of circulating antibody against HSV-2. The reaction in sera from control animals reflects nonspecific background activity.

In the control mice the yield of preinvasive and invasive lesions of the cervix was similar to that observed in previous studies of carcinogenesis with Formalin or ultraviolet-inactivated HSV-1 or HSV-2 (*3, 4*). The duration of cervical exposure required for development of lesions was also similar to that observed previously. The experimental group developed no invasive lesions, and only 20 percent had dysplasia that persisted to the end of the study. Since an appreciable number of animals in both groups died early in the study from pneumonia or multiple abscesses and were unsuitable for histological analysis, the data in Table 2 are of particular importance. They show that, although many of the deaths in both groups occurred before 40 weeks of exposure had elapsed, the most advanced lesion at that time was microinvasive carcinoma which was present only in the control group. This is not a lethal lesion. In previous studies involving over 600 mice, frank invasive cancer was never observed before the 60th week of cervical exposure to HSV-2. Moreover, throughout the study none of the animals in the experimental group demonstrated cytological changes associated with microinvasive or frank invasive cancer. There was no cytological or gross evidence indicative of acute herpetic infection in any of the animals during the study. It is unlikely, therefore, that any of the early deaths resulted from cancer or acute herpetic infection.

Table 2. Cytological diagnoses in relation to duration of exposure in experimental and control mice. Values are numbers of mice.

Diagnosis	Length of exposure (weeks)				
	0	20	40	60	80
Experimental mice					
Negative	52	48	21	23	17
Dysplasia	0	0	5	3	3
Microinvasion	0	0	0	0	0
Invasion	0	0	0	0	0
Total	52	48	26	26	20
Control mice					
Negative	54	46	9	8	4
Dysplasia	0	0	18	15	8
Microinvasion	0	0	10	6	9
Invasion	0	0	0	0	2
Total	54	46	37	29	23

The detection of circulating antibody against HSV-2 in the inoculated animals and the absence of antibody in the control group indicate that the experimental mice were immunized before cervical exposure. Although the protection against the carcinogenic effects of HSV-2 is presumed to be of immunological origin, the presence of circulating antibody should not be construed as indicating the mechanism involved.

The data suggest that inoculation with ultraviolet-inactivated HSV-2 plus Freund's adjuvant conferred protection from the carcinogenic effect of HSV-2 on the cervical epithelium. Prevention of cervical cancer by this method provides additional support for the concept that HSV-2 has a carcinogenic action in the mouse genital tract. This model may be useful for further investigation of the possibility of vaccinating against cervical cancer.

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Monoclonal Antibodies to a Synthetic Fibrin-Like Peptide Bind to Human Fibrin but Not Fibrinogen

Abstract. *A synthetic heptapeptide from the amino terminus of the β chain in human fibrin was used as an antigen to produce monoclonal antibodies that bind to fibrin even in the presence of human fibrinogen at the concentration found in plasma. As expected, the antifibrin activity was inhibited by the peptide antigen but not by a control heptapeptide. In a chicken ex vivo circulatory model for fibrin detection, intravenously administered monoclonal antibodies bound to human fibrin–coated disks placed in an extracorporeal chamber. These findings may lead to better methods for identifiying deep vein and coronary artery thrombi.*

In man the detection and localization of deep vein and coronary artery thrombi are clinically important problems. The use of antibodies as agents for the immunodetection of fibrin deposits in vivo has been hampered by antifibrin cross-reactivity with fibrinogen, the precursor of fibrin. Day *et al.* (*1*) used ^{131}I-labeled antibodies to fibrin to determine the location of rat sarcomas. However, they and others (*2*) recognized the limitation of using antibodies to fibrin that cross-react with fibrinogen.

Blood clots form when thrombin cleaves two pairs of small peptides from fibrinogen to yield fibrin monomers (*3*). Fibrin monomers spontaneously aggregate into an insoluble gel, which is then covalently stabilized by Factor XIIIa. Despite the dramatic physical change, fibrin retains 98 percent of the original covalent structure of fibrinogen. Thus, antisera to fibrin cross-react strongly with fibrinogen; there has, to our knowledge, been only one isolated report of a fibrin-specific serum (*4*).

Since the fibrin molecule shares many epitopes with fibrinogen, we focused the immune response on a synthetic, fibrin-unique peptide. We anticipated that antibodies which recognized the synthetic fibrin epitope might bind to fibrin exclusive of fibrinogen (*5*). The heptapeptide of the amino terminus of fibrin's β chain was synthesized to serve as a fibrin-unique antigen, since we reasoned that the amino terminus is exposed after thrombin cleavage. Along with the first